ADVANCED APPROACHES ON MULTIDISCIPLINARY MANAGEMENT OF RECTAL CANCER

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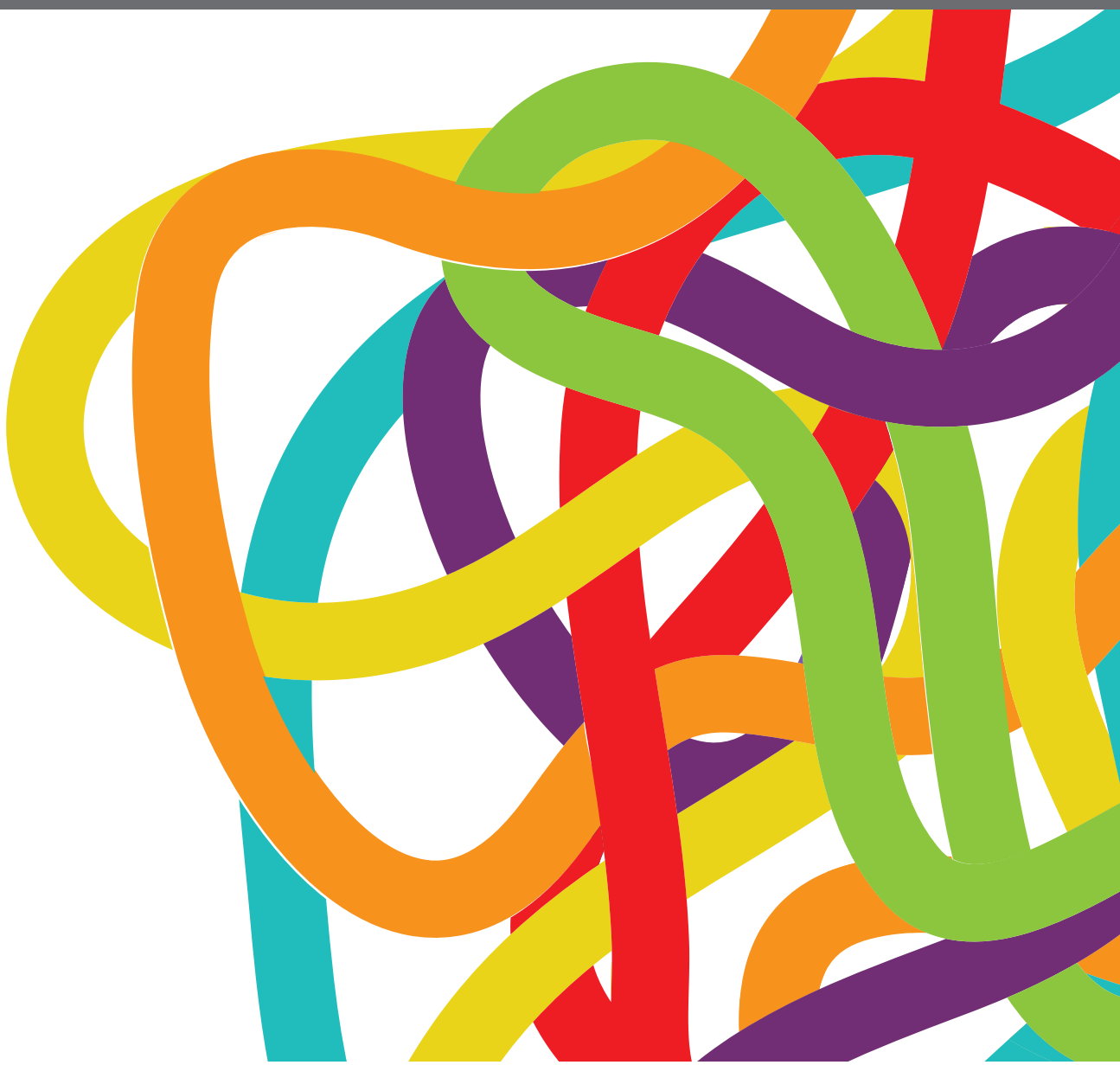

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ADVANCED APPROACHES ON MULTIDISCIPLINARY MANAGEMENT OF RECTAL CANCER

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Table of Contents

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The Distinction of Clinicopathological Characteristics, Treatment Strategy and Outcome in Colorectal Cancer Patients With Synchronous vs. Metachronous Bone Metastasis

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Background: The impact of the timing of bone metastasis (BM) diagnosis on colorectal cancer (CRC) patients is unclear. Our study aimed to explore the differences in clinicopathological characteristics, treatments and prognosis between synchronous BM (SBM) and metachronous BM (MBM) from CRC.

Methods: We retrospectively investigated clinical data of CRC patients with SBM or MBM from 2008 to 2017 at Chinese National Cancer Center. Cancer specific survival (CSS) after BM diagnosis was estimated using the Kaplan-Meier method. The multivariable COX regression model identified the prognostic factors of CSS.

Results: Finally, 63 CRC patients with SBM and 138 CRC patients with MBM were identified. Compared to SBM from CRC, MBM significantly was more involving multiple bone lesions (63.0 vs. 7.9%; $p < 0.001$), and more frequently originated from rectal cancer (60.9 vs. 41.3%; $p = 0.033$). The therapeutic strategies in SBM and MBM group were contrasted including systemic treatment, bisphosphonates, radiotherapy and metastasectomy for BM. 85.5% of patients in MBM group and 25.4% of patients in SBM group underwent primary tumor resection at initial diagnosis ($p < 0.001$). The median CSS was 11 months in both SBM and MBM group ($p = 0.556$), yet MBM patients developed from CRC in early AJCC stage presented obviously longer survival than those from advanced stage. Furthermore, patients could have improved CSS from primary tumor resection while there might be no survival benefit from targeted therapy in both SBM and MBM groups. Bisphosphonates was associated with a better CSS for patients with SBM, while radiotherapy for BM was related to a better CSS for patients with MBM.

Conclusion: The CRC patients in SBM and MBM group represented different clinicopathological characteristics and treatment modalities, which affected the prognosis in different ways. Distinct consideration for CRC patients with SBM and MBM in clinical decision making is required.

Keywords: colorectal cancer, bone metastasis, synchronous, metachronous, cancer specific survival, prognostic factor

INTRODUCTION

Colorectal cancer (CRC) with distant metastasis is one of the main causes of death. About 20% of CRC patients are diagnosed with distant metastasis at initial diagnosis and 50–60% will eventually have metastases (1, 2). The CRC commonly metastasizes to liver, followed by lung, yet seldom to bone (3). Population-based studies have reported the incidence of BM is 3.0–10.4% in CRC patients (4–6), but previous autopsy findings have suggested incidence of up to 23.7% (7). The prognosis after BM detection is generally poor due to the advanced stage and the difficulty in treatment, with 5-year survival rate < 5% (8). Median overall survival of CRC patients after BM diagnosis ranges from 5 to 22 months according to most researches (4, 9), with diverse factors affecting their prognosis such as some clinicopathological characteristics and provision of treatment. However, there is a lack of standard treatment guideline for BM from CRC at present. The possible therapies for BM include systemic therapy, local therapy and supportive treatment, with purpose to prevent skeletal-related events (SREs) like sever bone pain, hypercalcemia, spinal cord compression and pathological fracture and improve the survival of patients.

Synchronous BM (SBM) in CRC patients is relatively rare while most BMs occur metachronously after a length of follow-up time or during palliative treatment for other metastases. Generally, the patients with metachronous BM (MBM) have received systematic clinical intervention before the osseous lesion development, whereas those with SBM are mostly naive. Therefore, SBM and MBM from CRC may represent distinct clinicopathological characteristics, therapeutic sensitivity and outcomes, which require different treatment strategies. Many reports are controversial on the outcomes of synchronous and metachronous metastases from CRC, and most of which agree about the more aggressive clinical and pathological features of synchronous metastases (10–14). However, few studies in specifically exploring the differences between SBM and MBM from CRC have been reported.

Thus, the aims of our study were to (1) compare the clinicopathological characteristics of SBM and MBM from CRC; (2) compare the treatment modalities for SBM and MBM from CRC; (3) explore outcomes and prognostic factors of CRC patients with SBM and MBM, especially the impact of various treatment modalities on their prognosis, which would be helpful in modifying clinical management.

MATERIALS AND METHODS

Data Resources and Study Population

CRC patients who were diagnosed with BM between January 2008 and December 2017 at Chinese National Cancer Center, were retrospectively identified. The primary CRC lesion was confirmed by histopathological examination. The American Joint Committee on Cancer (AJCC) TNM stage and BM were identified by histopathological or imaging examinations such as standard X-rays, whole-body bone scans, computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography-computed tomography (PET-CT). SBM refers to BM found within 3 months after the diagnosis of CRC, while MBM refers to BM found more than 3 months after the diagnosis of CRC (15, 16). For the number of BM, two adjacent vertebral metastases were classified into the solitary bone involvement, while non-consecutive metastases or more than 2 consecutive vertebral metastases were classified as multiple bone involvement. The time of follow-up was calculated from the BM diagnosis to death or January 2020. The cancer specific survival (CSS) was defined as the time from the BM diagnosis until cancer-associated death or the end of follow up. This study was approved by the Ethics Committee of Cancer Hospital, Chinese Academy of Medical Sciences.

Prognostic Factors

Clinicopathological data and treatment methods were collected from medical records or via telephone follow-ups. Common variables were analyzed including age, gender, basic disease, primary tumor location, pathological type of tumor, tumor grade, carcinoembryonic antigen (CEA) levels at BM diagnosis, carbohydrate antigen199 (CA199) levels at BM diagnosis, alkaline phosphatase (ALP) levels at BM diagnosis, bone involvement, Karnofsky performance scores (KPS) at BM diagnosis, extra-osseous metastases, primary tumor resection, systemic treatment for BM, bisphosphonates for BM, radiotherapy for BM and operation for BM. Besides, AJCC TNM stage at initial diagnosis and time until BM were additionally evaluated for MBM. The basic disease was defined as other long-term or chronic coexisting diseases the BM patients from CRC suffered from, which affected basic metabolism or immune function of patients, mainly including hypertension, diabetes, heart disease, hepatitis, tuberculosis, autoimmune diseases, etc.

Statistical Analysis

The comparison of clinicopathological characteristics and treatments between patients with SBM and MBM was done using $\chi 2$-test or Fisher's exact test where appropriate. The CSS was assessed with Kaplan-Meier method, with the log-rank tests used to compare subgroups. In order to reduce the selection bias, variables with $p < 0.10$ by univariate Kaplan-Meier analysis were selected first, then a forward stepwise selection was performed using the selected variables in multivariable COX regression analysis. The independent prognostic factor was defined as the variable with $p < 0.05$ by COX regression. Hazard ratio (HR) and corresponding 95% confidence interval (CI) were also calculated by multivariable COX analysis. All statistical analyses were performed with SPSS version 25.0 for Mac. It is considered as statistically significant when $p < 0.05$.

RESULTS

Finally, in total of 201 patients diagnosed with BM from CRC entered in our final analysis after excluding 31 cases who were not eligible (**Figure 1**). 31.3% of patients (63/201) were identified with SBM at initial diagnosis while additional 68.7% of patients (138/201) developed MBM after diagnosis of CRC.

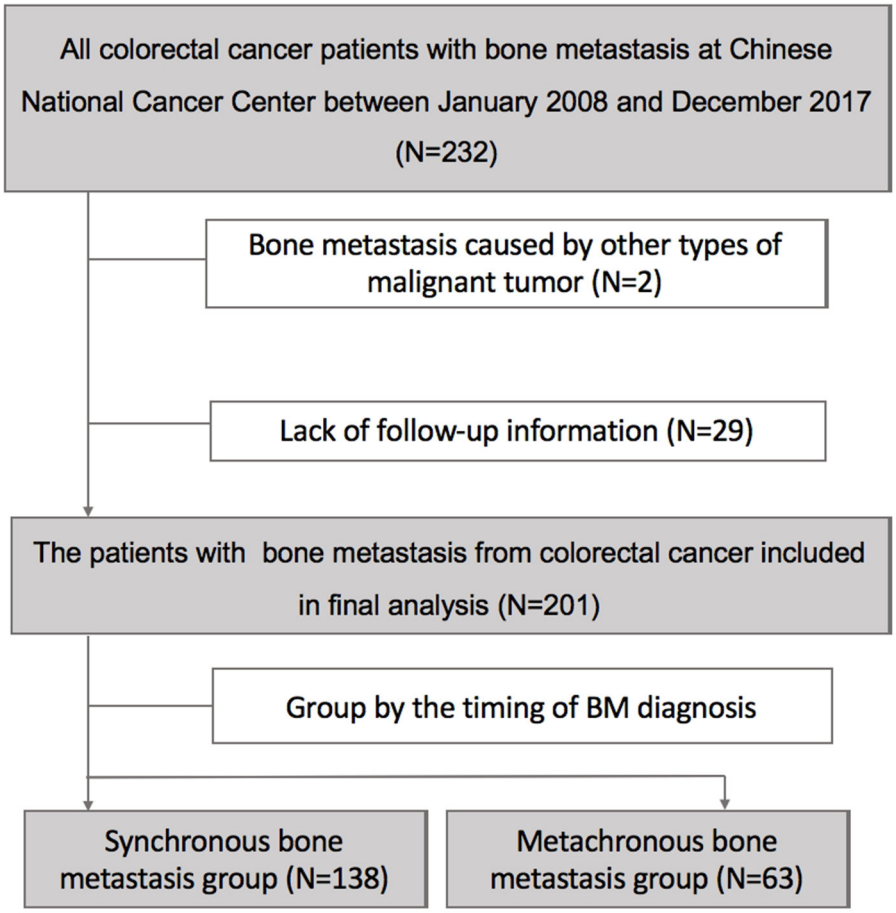

FIGURE 1 | The analytical cohort and exclusion criteria.

Patients Characteristics

Table 1 represented the clinicopathological characteristics of CRC patients with SBM and MBM. The median age of patients was 58 years (range 33–84) in SBM group and 59 years (range 19–79) in MBM group, respectively. CRC patients with SBM and MBM were similar with respect to their age at BM diagnosis ($p = 0.974$), gender ($p = 0.459$) and basic disease ($p = 0.628$). The rectal cancer was more common in MBM group (60.9%) than SBM group (41.3%), with statistical significance ($p = 0.033$). Patients with MBM were diagnosed more often with lower tumor grade (63.8 vs. 46.0%; $p = 0.048$) compared to those with SBM. Performances in CAE levels ($p = 0.511$), CA199 levels ($p = 0.619$), ALP levels ($p = 0.827$) and KPS ($p = 0.631$) at BM diagnosis between two groups were, respectively similar.

Patterns of BM and Extra-Osseous Metastasis

Patients with MBM (63.0%) were significantly more involving multiple bone lesions compared to those with SBM (7.9%; $p < 0.001$). Spine (65.1 vs. 73.2%) was the leading site of BM in SBM and MBM group, followed by pelvis (57.1 vs. 62.0%), long bones (34.9 vs. 22.6%) and ribs (30.2 vs. 21.9%).

There were 88.9% of patients (56/63) in SBM group and 89.1% of patients (123/138) in MBM group having extra-osseous metastases, respectively, with no significant difference ($p = 0.959$). The common extra-osseous sites were liver (61.9%), distant lymph nodes (54.0%) and lung (39.7%) in SBM patients. While lung (57.7%) was the most common extra-osseous metastatic site in MBM patients, followed by liver (45.7%) and lymph nodes (40.9%).

Treatments

There were 85.5% of MBM patients (118/138) receiving primary tumor resection, which had been all performed at initial diagnosis. The proportions of primary tumor resection in MBM patients with AJCC stage I, II, III and IV were 100.0% (6/6), 100.0% (14/14), 97.3% (71/73), and 58.5% (24/41), respectively. Of the four patients with unknown AJCC TNM stage, three received this operation. In SBM group, only 25.4% of patients (16/63) underwent primary tumor resection because of the advanced stage.

All patients received palliative chemotherapy after BM diagnosis. There were 37.9% of cases in SBM group (25/63) and 39.1% of cases in MBM group (54/138; $p = 0.941$) receiving

TABLE 1 | The comparison of clinicopathological characteristics in CRC patients with SBM and MBM.

Variable	Synchronous		Metachronous		*p*-value
	N = 63	%	*N* = 138	%	
Age at BM diagnosis, years					0.974
< 60	35	55.6	77	55.8	
≥ 60	28	44.4	61	44.2	
Gender					0.459
Female	23	36.5	58	42.0	
Male	40	63.5	80	58.0	
Basic disease					0.628
No	37	58.7	86	62.3	
Yes	26	41.3	52	37.7	
Primary tumor location					0.033
Rectum	26	41.3	84	60.9	
Left hemicolon	17	27.0	23	16.6	
Right hemicolon	20	31.7	31	22.5	
Pathological type of tumor					0.054*
Adenocarcinoma	56	88.9	128	92.8	
Signet-ring cell carcinoma	4	6.3	10	7.2	
Others	3	4.8	0	0.0	
Tumor grade					0.048*
Grade I, II	29	46.0	88	63.8	
Grade III, IV	19	30.2	31	22.4	
UK	15	23.8	19	13.8	
AJCC TNM stage at initial diagnosis					<0.001*
I	0	0.0	6	4.3	
II	0	0.0	14	10.2	
III	0	0.0	73	52.9	
IV	63	100.0	41	29.7	
UK	0	0.0	4	2.9	
CEA levels at BM diagnosis					0.511
Negative	16	25.4	28	20.3	
Positive	41	65.1	90	65.2	
UK	6	9.5	20	14.5	
CA199 levels at BM diagnosis					0.619
Negative	27	42.9	50	36.2	
Positive	29	46.0	68	49.3	
UK	7	11.1	20	14.5	
ALP levels at BM diagnosis					0.827
Negative	47	74.6	102	73.9	
Positive	14	22.2	29	21.0	
UK	2	3.2	7	5.1	
Bone involvement					<0.001
Solitary	58	92.1	51	37.0	
Multiple	5	7.9	87	63.0	
KPS at BM diagnosis					0.631
≥ 80	49	77.8	103	74.6	
< 80	14	22.2	35	25.4	
Extra-osseous metastases					0.959
No	7	11.1	15	10.9	
Yes	56	88.9	123	89.1	
Time until BM					-
3 months−1 year	-	-	45	32.6	
1–3 years	-	-	69	50.0	
>3 years	-	-	24	17.4	

No marks indicated the p-value was calculated by Chi-square test and an asterisk (*) indicated the p-value was calculated by Fisher's test. CRC, colorectal cancer; SBM, synchronous bone metastasis; MBM, metachronous bone metastasis; N, number; UK, unknown; CEA, carcinoembryonic antigen; CA199, carbohydrate antigen199; ALP, alkaline phosphatase.

TABLE 2 | The comparison of treatment strategies in CRC patients with SBM and MBM.

Variable	Synchronous		Metachronous		*p*-value
	N = 63	%	*N* = 137	%	
Primary tumor resection					<0.001
No	47	74.6	20	14.5	
Yes	16	25.4	118	85.5	
Systemic treatment after BM diagnosis					0.941
Chemotherapy alone	38	60.3	84	60.9	
Chemotherapy plus targeted therapy	25	39.7	54	39.1	
Bisphosphonates for BM					0.548
No	38	60.3	77	55.8	
Yes	25	39.7	61	44.2	
Radiotherapy for BM					0.192
No	49	77.8	95	68.8	
Yes	14	22.2	43	31.2	
Metastasectomy for BM					0.553*
No	63	100.0	135	97.8	
Yes	0	0.0	3	2.2	

No marks indicated the p-value was calculated by Chi-square test and an asterisk (*) indicated the p-value was calculated by Fisher's test. CRC, colorectal cancer; SBM, synchronous bone metastasis; MBM, metachronous bone metastasis; N, number.

additional targeted therapy, respectively. The proportions of patients who received bisphosphonates treatment (60.3 vs. 55.8%; $p = 0.548$) or radiotherapy (77.8 vs. 68.8%; $p = 0.192$) were, respectively, similar between two groups. Only 2.2% of patients with MBM (3/138) underwent operative treatment for BM due to spinal cord compression while no patient with SBM received metastasectomy for BM ($p = 0.541$). The details were shown in **Table 2**.

Survival

In total of 195 CRC patients (97.0%) died because of cancer during a median follow-up time of 11 (range 1–198) months, with 61 cases in SBM group and 134 cases in MBM group. And only one patient with MBM died due to other disease. Median CSS was both 11 months for patients with SBM and

MBM. The median interval time from CRC diagnosis to MBM was 18.5 months. **Figure 2** displayed the Kaplan-Meier curves of SBM and MBM group according to different situations. The overall CSS of patients with SBM and MBM was similar, with no significant difference ($p = 0.556$; **Figure 2A**). The median CSS in MBM patients with AJCC stage I, II, III and IV at initial CRC diagnosis was 28, 21, 10, and 8 months, respectively, which also showed large differences compared to SBM patients ($p = 0.003$; **Figure 2B**). In addition, patients diagnosed with MBM >3 years after CRC diagnosis had a similar CSS with SBM patients ($p = 0.093$; **Figure 2C**).

To elucidate the outcomes with various treatments in two groups, the Kaplan-Meier curves for SBM and MBM patients were, respectively, represented in **Figure 3**. Patients who had underwent primary tumor resection at initial diagnosis in SBM or MBM group (**Figure 3A**) both had a better survival. The CSS was no significantly different between patients with and without targeted therapy in both two groups (**Figure 3B**). Bisphosphonates therapy was related to a better CSS in synchronous group (**Figure 3C**) while radiotherapy for BM (**Figure 3D**) was related to a better CSS in MBM group. Because only 3 patients took the osseous metastasectomy, the relationship between operation for BM and CSS was unclear.

Prognostic Factors

Table 3 showed the *p*-values obtained by univariate Kaplan-Meier analysis in SBM and MBM group, respectively. And the variables with $p < 0.10$ were selected to be further analyzed. The independent prognostic factors ($p < 0.05$) were finally identified by multivariable COX regression analysis. We found multiple bone involvement (HR: 4.38; 95%CI: 1.61–11.92; $p = 0.002$), KPS scores <80 (HR: 2.74; 95%CI: 1.45–5.20; $p = 0.004$), primary tumor resection (HR: 0.48; 95%CI: 0.24–0.92; $p = 0.028$) and bisphosphonates (HR: 0.23; 95%CI: 0.12–0.43; $p < 0.001$) were independent prognostic factors for SBM patients (**Figure 4A**). While positive CA199 levels (HR: 1.92; 95%CI: 1.30–2.83; $p = 0.001$), primary tumor resection (HR: 0.50; 95%CI: 0.30–0.85; $p = 0.010$) and radiotherapy (HR: 0.53; 95%CI: 0.35–0.80; $p = 0.002$) were independent prognostic factors for MBM patients (**Figure 4B**).

DISCUSSION

To our knowledge, this study is the first to retrospectively analyze the SBM and MBM together from CRC patients. The MBMs were more common, with incidence nearly twice higher than SBMs. The clinicopathological characteristics differed between two groups. The most striking finding was that in total of 63.0% of patients in MBM group had BMs to multiple sites, far more than those (7.9%) in SBM group. That might be because liver metastasis or lung metastasis from CRC in MBM group would have enough time to spread to skeletal systems by systemic circulation or directly invade chest bones such as sternum, rib and clavicle. We found there were similar therapeutic strategies between two groups, except that more MBM patients received the resection of primary tumor at initial diagnosis.

Colloca et al. (14) identified 425 CRC patients with distant metastases, discovering that the survival after metastasis diagnosis was shorter in synchronous group (18.5 vs. 62.8 months, $p < 0.001$). Majority of reports consider synchronous metastases from CRC to be more aggressive than metachronous despite there is a controversy (10–14). In our study, the prognosis of BM was very poor, yet there was no significant difference in CSS between two groups (**Figure 2A**). Several reasons might explain the similar outcomes. First, a significant percentage of patients with MBM had been treated with prior chemotherapy before BM diagnosis, while patients with SBM obviously were not and they were more chemo-naive chemo-sensitive (13, 14). Second, the multiple bone involvement was related to worse prognosis, which was more common in MBM group. Another possibility was that BMs was so aggressive that the timing of BM diagnosis had little impact on the outcome. In addition, patients with different time intervals to MBM diagnosis had similar CSS with SBM patients as **Figure 2C** represented. However, it was

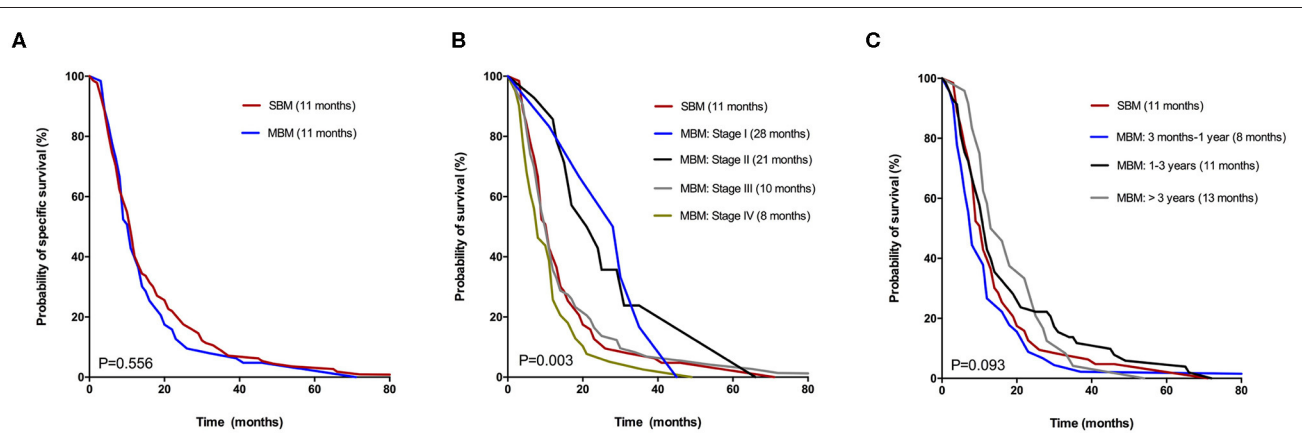

FIGURE 2 | Kaplan-Meier curves for CSS of CRC patients with SBM and MBM according to different situations: **(A)** Overall CSS. **(B)** AJCC TNM stage at initial diagnosis. **(C)** Time until diagnosis of BM.

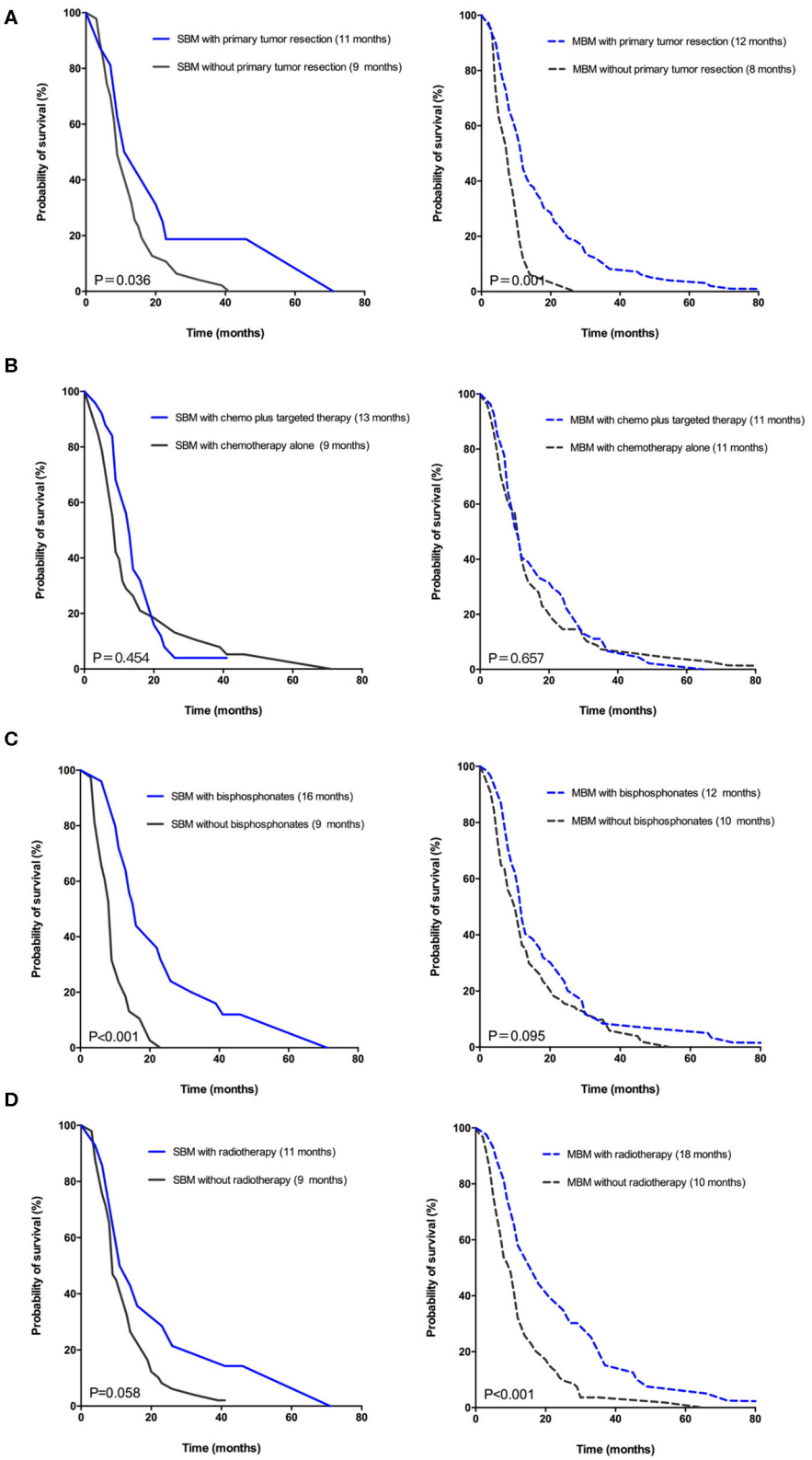

FIGURE 3 | Kaplan-Meier curves for CSS of CRC patients in synchronous group (?) versus metachronous group (—) according to various treatments: **(A)** Primary tumor resection. **(B)** Systemic treatment after BM diagnosis. **(C)** Bisphosphonates for BM. **(D)** Radiotherapy for BM.

TABLE 3 | The univariate Kaplan-Meier analysis in CRC patients with SBM and MBM.

Variable	Synchronous			Metachronous		
	Median CSS (months)	95% CI	*p*-value	Median CSS (months)	95% CI	*p*-value
Age at BM diagnosis, years			0.564			0.521
< 60	9	8.2–9.8		11	9.0–13.0	
≥ 60	11	7.9–14.1		12	10.7–13.4	
Gender			0.617			0.705
Female	10	7.7–12.3		11	8.8–13.2	
Male	11	8.3–13.7		11	9.6–12.4	
Basic disease			0.494			0.209
No	11	9.2–12.8		12	10.5–13.5	
Yes	9	6.0–12.0		11	9.3–12.7	
Primary tumor location			0.990			0.273
Rectum	11	8.0–14.0		12	10.3–13.7	
Left hemicolon	11	6.0–16.0		11	7.9–14.1	
Right hemicolon	9	9.5–12.5		9	6.4–11.6	
Pathological type of tumor			0.766			0.516
Adenocarcinoma	11	8.6–13.4		11	9.8–12.2	
Signet-ring cell carcinoma	4	1.1–6.9		8	5.0–11.0	
Others	8	1.6–14.4		NA	NA	
Tumor grade			0.494			0.539
Grade I, II	13	9.0–17.0		12	10.7–13.3	
Grade III, IV	9	8.2–9.8		8	5.9–10.1	
UK	9	5.2–12.8		9	5.4–12.6	
AJCC TNM stage at initial diagnosis			NA			0.003*
I	NA	NA		28	14.8–41.2	
II	NA	NA		21	8.2–33.8	
III	NA	NA		10	8.2–11.8	
IV	11	9.6–12.4		8	4.3–11.7	
UK	NA	NA		11	0.0–29.6	
CEA levels at BM diagnosis			0.139			0.008*
Negative	11	7.1–14.9		11	8.9–13.1	
Positive	10	8.1–11.9		11	9.2–12.8	
UK	8	0.0–16.4		12	6.2–17.8	
CA199 levels at BM diagnosis			0.367			<0.001*
Negative	11	7.6–14.4		13	9.0–17.0	
Positive	9	8.0–10.0		8	6.5–9.5	
UK	13	0.2–25.8		12	6.2–17.8	
ALP levels at BM diagnosis			0.023*			0.024*
Negative	11	7.6–14.4		12	10.9–13.1	
Positive	8	5.6–10.4		8	2.7–13.3	
UK	4	NA		10	4.9–15.1	
Bone involvement			0.001*			0.034*
Solitary	11	8.9–13.1		15	10.4–19.6	
Multiple	5	3.9–6.1		10	8.0–12.0	
KPS at BM diagnosis			0.001*			0.446
≥ 80	11	8.1–13.9		12	10.7–13.3	
< 80	5	1.3–8.7		7	1.3–12.7	
Extra-osseous metastases			0.603			0.224
No	11	8.7–13.3		12	5.4–18.6	
Yes	10	8.2–11.8		11	9.4–12.6	

(Continued)

TABLE 3 | Continued

Variable	Synchronous			Metachronous		
	Median CSS (months)	95% CI	*p*-value	Median CSS (months)	95% CI	*p*-value
Time until BM			NA			0.063*
3 months−1 year		NA		8	6.7–9.3	
1–3 years		NA		12	10.4–13.6	
>3 years		NA		13	7.0–19.0	
Primary tumor resection			0.036*			0.001*
No	9	7.0–11.0		8	1.2–10.8	
Yes	11	3.2–18.8		12	10.7–13.3	
Systemic treatment after BM diagnosis			0.454			0.657
Chemotherapy alone	9	7.8–10.2		11	9.7–12.3	
Chemotherapy plus targeted therapy	13	11.0–15.0		11	8.8–13.2	
Bisphosphonates for BM			<0.001*			0.095
No	9	8.0–10.0		10	7.4–12.6	
Yes	16	12.8–19.2		12	10.3–13.7	
Radiotherapy for BM			0.058*			<0.001*
No	9	7.6–10.4		10	8.1–11.9	
Yes	11	5.5–16.5		18	11.6–24.4	
Metastasectomy for BM			NA			0.541
No	NA	NA		11	9.6–12.4	
Yes	NA	NA		21	5.0–37.0	

An asterisk (*) *indicated variables with p-value < 0.10, which was selected into multivariable COX regression. CRC, colorectal cancer; SBM, synchronous bone metastasis; MBM, metachronous bone metastasis; CI, confidence interval; NA, not available; CEA, carcinoembryonic antigen; CA199, carbohydrate antigen199; ALP, alkaline phosphatase.*

difficult to interpret this result because the CSS of MBM patients significantly varied by different AJCC stages.

The clinical outcomes of patients with SBM and MBM appeared to be affected by different clinicopathological characteristics. We found CA199 levels was an independent prognostic factor only for MBM patients. But the CSS of patients with positive ALP levels was shorter in both two groups by univariate analysis, which was consistent with previous studies (13). So, careful surveillance in those indicators for patients with BM from CRC is recommended. Most researches have revealed the relationship between multiple BMs and worse prognosis (17, 18), while minority of studies have demonstrated the prognosis of CRC patients has no association with the number of BMs (19). In our study, multiple bone involvement was related to shorter survival. The median CSS of patients with multiple and solitary bone involvement was 5 and 11 months in SBM group ($p < 0.001$) and 10 and 15 months in MBM group ($p = 0.034$), respectively. Therefore, systematic imaging examination is helpful to assess the outcome of BM.

The association between TNM stage and overall survival of CRC is generally confirmed (20). In our study, MBM patients with stage I at initial diagnosis had best prognosis with median CSS of 28 months, while it dramatically decreased to 8 months for those with stage IV ($p = 0.003$). Thus, strengthening early diagnosis of CRC and active treatment might also prolong the CSS even the BM was developed metachronously.

The prognosis of patients with SBM and MBM was also affected by distinct provision of treatment. As the rare metastatic disease, standard treatment guidelines for CRC patients with BM have not been established. Because all cases were treated with palliative chemotherapy after BM diagnosis in our study, the utility of chemotherapy in each group was unclear.

Bisphosphonates therapy can prevent the occurrence of osteolytic lesions and SREs caused by BM, which has become an effective treatment for bone pain and hypercalcemia (21, 22). Commonly used bisphosphonates such as pamidronate, zoledronic acid and ibandronate can be treated for BM patients in combination with conventional anti-tumor drugs. The difference in CSS of patients with and without bisphosphonates is significant only in SBM group, implying the sensitivity to bisphosphonates for SBM and MBM patients might exist difference.

Local treatments of CRC with BM include radiotherapy and surgery, etc. Previous researches have revealed radiotherapy can reduce bone pain and prevent pathological fracture or spinal cord compression (23–25). According to our study, median CSS of patients with palliative radiotherapy was significantly prolonged only in MBM group (18 vs. 10 months, $p < 0.001$), which was also found to be one of independent prognostic factors for MBM patients. A meta-analysis of 1,026 cases from retrospective studies had suggested an improved survival for stage IV CRC patients with primary tumor resection (26). Another recent research enrolled 3,423 patients, reporting a poor prognosis for the patients with synchronous metastases

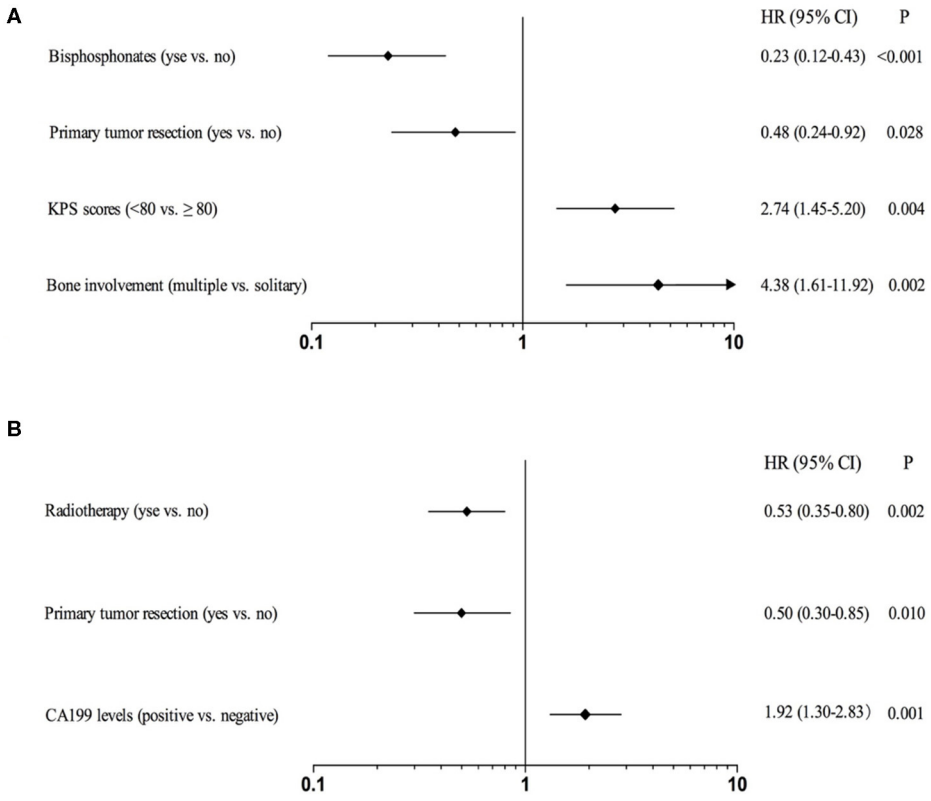

FIGURE 4 | Forest plots for CSS of CRC patients with SBM **(A)** or MBM **(B)** based on multivariable COX proportional hazard model.

who did not receive the resection of primary tumor (27). Despite these evidences, primary tumor resection has not been confirmed as a factor related to prolonged outcome of patients with unresectable synchronous metastases (28, 29). Our study showed that patients with primary tumor resection had significantly longer CSS in SBM group (11 vs. 9 months, $p = 0.036$), which was also an independent prognostic factor. This might be attributable to the reduction of tumor-related complications such as systemic inflammation, bleeding, obstruction and perforation. If advanced patients can tolerate the operation, active treatment for CRC is an alternative method for improving the survival of BM (30). Only 3 patients underwent operative treatment of BM due to spinal cord compression in our study and we could not evaluate its' effect on outcome of patients. When non-operative treatment for pathological fracture, spinal instability or other complications caused by BM is invalid, surgical treatment for BM could be considered.

Our study had some limitations. First, this was a retrospective and single-center study, selection bias might occur. Second, the modest samples and non-randomized design limited the generalizability for the conclusions regarding optimal clinical management. Therefore, further prospective researches with randomized design, large sample and more clinical features are warranted.

CONCLUSION

Our study compared the clinical data and outcomes of SBM and MBM patients from CRC. Meanwhile, we identified favorable clinicopathological characteristics and treatments in SBM and MBM group, respectively. That could potentially guide physicians to treat patients with distinct clinical intervention and therapeutic strategies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

XW: writing-review and editing, supervision. XG: conceptualization, investigation, and supervision. CM: conceptualization, investigation, and writing-original draft. RW: software and formal analysis. SW: validation and data curation. JQ: resources. ZZ: validation. HC: investigation and project administration. ZL: data curation and supervision. ZJ: data curation and revision. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Comparison of NOSES and Conventional Laparoscopic Surgery in Colorectal Cancer: Bacteriological and Oncological Concerns

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Background: Colorectal natural orifice specimen extraction surgery (NOSES) is considered to be a scarless operation that avoids the laparotomy of extraction specimen, but bacteriological and oncological concerns are raised with this technique.

Objective: The purpose of this study was to compare the oncological and bacteriological outcomes of NOSES and conventional laparoscopic (CL) procedures.

Methods: This is a retrospective study of prospectively collected outcomes data. Patients operated with colorectal cancer from January 2016 to December 2019 in Xiangya Hospital were assigned to the group NOSES and the group CL according to the size of the tumor. Prior to dissection, peritoneal lavage fluid was collected for cytological assessment. At the end of the procedure, peritoneal lavage fluid was collected for aerobic culture and cytological assessment. Baseline characteristics and short-term and long-term outcomes for NOSES and CL were compared.

Results: Between January 2016 and December 2019, 212 patients were enrolled from our center and 185 patients were analyzed (96 and 89 in NOSES and CL groups, respectively). The bacterial positive rate of peritoneal lavage fluid was 34.4 vs. 32.6% in NOSES and CL groups, respectively ($P = 0.80$). The positive rate of tumor cells in peritoneal lavage fluid was 7.3 vs. 9.0% in NOSES and CL groups, respectively ($P = 0.67$). Univariate analysis showed that the positive rate of tumor cells in peritoneal lavage fluid was significantly associated with tumor invasion depth and lymph node metastasis ($P < 0.05$). T4 (OR = 20.47, 95%CI = 1.241–337.661; $P = 0.04$), N1 (OR = 5.445, 95%CI = 1.412–20.991; $P = 0.01$), and N2 (OR = 6.315, 95%CI = 1.458–27.348; $P = 0.01$) served as independent predictors of peritoneal lavage fluid positive oncology patients. Local recurrence-free survival was not significantly different between two groups (HR = 0.909, 95%CI = 0.291–2.840; $P = 0.87$).

Conclusions: Compared with conventional laparoscopic procedure, NOSES is in conformity with the principle of asepsis and tumor-free technique and can be worthy of clinical application and promotion.

Keywords: natural orifice specimen extraction surgery, conventional laparoscopy, oncological, bacteriological, colorectal cancer, asepsis and tumor-free technique

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INTRODUCTION

In recent years, NOSES has drawn wide attention in the treatment of colorectal cancer, which has been considered as an alternative approach to conventional laparoscopic surgery and open surgery for selected patients (1–3). NOSES is another stepping stone toward "incisionless" surgery to reduce pain and wound-related complications. Many studies demonstrated that there were lower analgesic requirements and less pain in NOSES compared with conventional laparoscopic colectomy (4–6). Although the recognition of NOSES in the colorectal field is increasing, there are still concerns about its compliance with the principles of bacteriology and oncology.

One potential risk of NOSES is peritoneal contamination secondary to the opening of the colon or rectal stump for extracting the specimen. During the NOSES procedures, enterotomy, and bowel reconstruction are performed in the abdominal cavity, and anvils are inserted into the abdominal cavity through natural orifice, which may cause bacteriological problems (7, 8). In addition to bacteriological issues, another major issue is the oncological safety of NOSES in colorectal cancer. Extraction of specimens through natural orifice may squeeze tumors, causing tumor cells to fall out of the pelvic or abdominal cavity, which is questionable in terms of oncological safety (2, 9).

The issues of bacteriology and oncology are not only NOSES needs to face, conventional laparoscopic surgery also with these problems. Therefore, we collected postoperative peritoneal lavage fluid for oncological and bacteriological examination, and compared the long-term oncological outcomes of NOSES and CL surgery.

MATERIALS AND METHODS

Patients

A total of 212 patients with colorectal cancer were enrolled in the study from January 2016 to December 2019 at our hospital. After strict inclusion and exclusion criteria, a total of 200 patients met the requirements. The orifice selection for specimen extraction is mainly based on the size of the tumor, especially the maximum circumferential diameter (CDmax). Eligible patients were matched into two study groups based on tumor size and signed informed consent: (1) NOSES (CDmax $<$ 3cm); (2) Conventional laparoscopic surgery (CDmax: 3–5cm). All patients were followed up for postoperative abdominal infection and local tumor recurrence. Patients were followed up regularly after discharge, including tumor marker blood tests and enhanced chest/abdominal/pelvic CT. This study was approved by the Ethics Committee of Xiangya Hospital of Central South University, China (No: 201601021), and all patients provided written informed consent.

The inclusion criteria were as follows: (1) patients aged between 18 and 80 years; (2) histopathology confirmed as colorectal adenocarcinoma; (3) preoperative imaging (CT and MR) assessments showed that colorectal cancer did not penetrate the serosa ($\leq$T3); (4) tumor circumference $<$5 cm; (5) enhanced chest and abdominal pelvic CT scans before operation excluded liver metastasis, lung metastasis, and other distant organ metastases.

The preoperative exclusion criteria were as follows: (1) tumors could be resected by endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR); (2) body mass index (BMI) $>$ 30 kg/m^2; (3) patients with severe perforation, bleeding, or obstruction requiring emergency surgery; (4) recurrent cases; (5) patients undergoing neoadjuvant therapy or preoperative radiotherapy; (6) Anesthesiologists (ASA) score $\geq$ IV; (7) active period of infection; (8) blood neutrophils $<3 \times 10^9$/L.

The postoperative exclusion criteria were as follows: (1) combined with other parts of surgical or converted to open surgery; (2) preoperative peritoneal lavage fluid tumor cytology test positive; (3) peritoneal metastasis.

Quality Control of Surgery

To control the quality of the operation, all selected patients' operations were performed by the same group of surgeons in accordance with uniform operating standards. The surgeon and assistants were fixed and had rich experience more than 12 months of practice in NOSES operation. Therefore, it was possible to effectively control the bias caused by different surgical proficiency.

Surgical Procedure

Preoperative Preparation

We took the following bowel preparation for the patients: diet adjustment, semiliquid diet 3 days before surgery, liquid diet 2 days before surgery. From 6 p.m. to 8 p.m. the day before surgery, the bowel preparation was performed with 90 ml of sodium phosphate oral solution mixed well with 1.5 L of water and the patient need to drink the solution in 30 min, and rectal enema with 500 ml normal saline in 10 p.m.

Technique

After successful anesthesia, patients were placed in the modified lithotomy position, and an antibiotic prophylaxis (2 g of ceftazidime) was administered prior to incising abdominal skin. Before exploration of the abdominal cavity and mobilization of the tumor, 100 ml of saline solution was instilled in the area adjacent to the tumor, followed by immediate aspiration this lavage fluid for cytological assessment. Abdominal procedures are the same for both groups, according to the CME and TME principles. The difference between the two groups of surgery is the way of specimen extraction. The specimen of group CL was taken through the assisted abdominal wall incision. Group NOSES specimen was extracted through rectal anus. There were different methods for NOSES depending on the location of the colorectal tumor. According to the methods of specimen extraction, NOSES was divided into three categories (2) (**Figure 1**): (1) Transanal rectal eversion and extra-abdominal resection, this technique was mainly used to lower rectal resection with small tumor. Transected distal stump was retracted by grasping the staple line using a cured clamp and everted out. Rectum was transected distally from the mass by using electrocautery. The proximal closed colonic end in the abdomen was pulled out transanally using a cured clamp

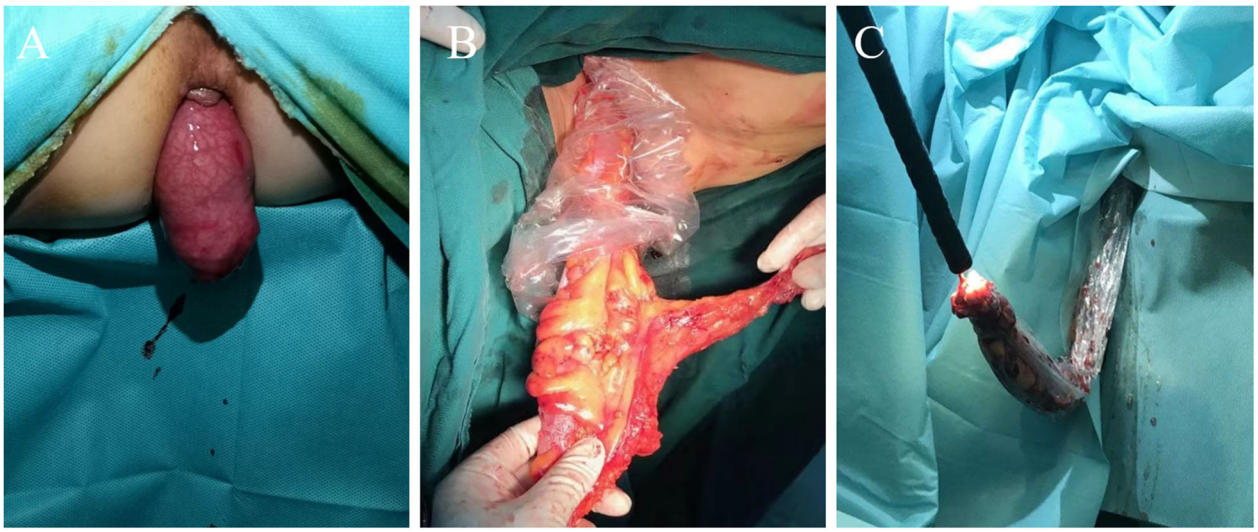

FIGURE 1 | Three methods for extraction specimens. **(A)** Transanal specimen eversion and extra-abdominal resection technique. **(B)** Transluminal specimen extraction and extra-abdominal resection technique. **(C)** Intra-abdominal specimen resection and transluminal extraction technique.

under guidance of the laparoscope. The closed end of the colon was opened. Anvil of end-to-end anastomotic stapler was placed in and fixed with purse-string suture. Proximal colon with anvil was sent back into the abdomen. Rectum was closed using a stapler device. Anastomosis was done intracorporeally under laparoscopic guidance using a transanally placed end-to-end circular stapler. Eversion makes it possible to perform resection and placement of the anvil extracorporeally. (2) Transluminal specimen extraction and extra-abdominal resection, this technique was mainly used for middle rectal resection. The rectal wall is cut off at the distal resection line, and the distal side of specimen is gently pulled outside of the patient body transanally. The proximal rectal resection is performed extra-abdominally. The anvil is introduced into the bowel lumen and closed with purse-string suture, and the sigmoid colon is delivered back to pelvic cavity. The open rectal stump is closed by using linear stapler. The circular stapling device is introduced into the rectum, and an end-to-end anastomosis is performed. (3) Intra-abdominal specimen resection and transluminal extraction, this technique was mainly used for upper rectal resection and colectomy. The distal and proximal bowel division is performed using linear stapler. The specimen is extracted through the anus. The open proximal stump is closed with a linear stapler. In colon cancer, the linear stapling device is introduced into the bowel, and functional side to side anastomosis is performed. When the tumor is located in the colon, we need colonoscopy to assist specimen extraction. In rectal cancer, the circular stapling device is introduced into the rectum, and an end-to-end anastomosis is performed. We used a sterile specimen bag to assist transluminal specimen extraction. The aim was to avoid direct contact between the specimen and the natural opening to ensure sterile and tumor-free operation. After bowel anastomosis, 200 ml of saline solution was instilled around the anastomosis, followed by immediate aspiration this lavage fluid with leaving 50 ml for cytological examination and 5 ml for bacteriological examination. Postoperative intravenous infusion of ceftazidime for 3 days.

Method of Cytological and Bacterial Detecting

We poured the peritoneal lavage fluid sample into a centrifuge tube to centrifuge at 2,500 rpm/min for 10 min and observed the sediment. When there is a lot of sediment, we discarded the supernatant and used a pipette to aspirate the cell layer to make a conventional smear. After drying and fixing with 95% alcohol for 15 min, stain with hamatoxylin and eosin (H &E) was made; When the precipitate is small, we discarded the supernatant, added 30 mL of Cytolyt solution to the remaining sample, shaked on a shaker for 10 min, centrifuged at 1,500 rpm/min for 10 min, discarded the supernatant, and transfered the precipitate to Presevcyt preservation solution vial. After standing for 20 min, we made a liquid-based smear in a Thinprep 5000 instrument, fixed with 95% alcohol for 15 min and stained with HE, and then viewed with an optical microscope to find exfoliated cancer cells. The sample was considered positive if at least one tumor cell was detected. Otherwise, it is negative. The peritoneal lavage smears were checked by two pathologists who would have to agree on the results.

Specimens were inoculated on blood agar plates for incubation, culture, and purification. The drug sensitivity test was performed using the K–B disk method, and the bacteria were identified using the Micro Scan Walk Away 40S system.

Follow-Up

All patients were followed up in an outpatient clinic. Adjuvant chemotherapy with oxaliplatin and capecitabine for 6 months was recommended for patients who had complete tumor resection and pathological stage II with risk factors and all stage

III tumors. Patients were assessed by physical examination and analysis of tumor markers every 3 months for the first 2 years and then every 6 months thereafter until 5 years after surgery. Chest and abdominal pelvic CT was performed every 6 months for the first 3 years after surgery and once a year thereafter.

Statistical Analyses

The results were analyzed with the SPSS (version 25) program. The quantitative data were expressed as the mean ± SD and were compared using an independent samples *t*-test. Qualitative data were compared using the χ^2 test or Fisher's exact test. To determine the risk factors for positive of oncology and positive of bacteriology, a univariate analysis was first performed using χ^2 test or Fisher's exact tests. Subsequently, a multivariate analysis was conducted using a logistic regression model that included all variables at $P < 0.05$ in the univariate analysis. The survival curves were plotted by Kaplan–Meier method. Whether there was a statistically significant difference in the local recurrence-free survival between two groups was detected via Log-rank test. $P < 0.05$ suggested that the difference was statistically significant.

RESULTS

From January 2016 to December 2019, 200 patients underwent colorectal cancer surgery, including NOSES (103 patients) and group CL (97 patients). Excluded from the analysis were five patients with peritoneal metastasis (no resection: two in group NOSES and three in group CL), six patients with conversion to open laparotomy (three in group NOSES and three in group CL), and four patients with positive preoperative cytology (two in group NOSES and two in group CL). As a result, 185 patients were included in the analysis, 96 in group NOSES, and 89 in group CL (**Figure 2**). Patients undergoing NOSES all extract specimen through the anus.

Patient Characteristics

In the present study, 185 patients with colorectal cancer were enrolled, including 93 males (50.3%) and 92 females (49.7%), aged 23–80 years (mean 59.29 ± 11.35). The mean BMI was 22.71 ± 2.79 kg/m^2. Comparison of baseline characteristics between the patients who underwent NOSES and conventional laparoscopic surgery is shown in **Table 1**. There were no statistically significant differences in the age, gender, BMI, ASA score, CEA, cT category (rectum), tumor location, invasion depth, nodal metastasis, TNM stage, venous invasion, and neurological invasion between the two groups ($p > 0.05$).

Comparison of Postoperative Indexes

Postoperative outcomes in **Table 2**. Peritoneal cytology with the Thin-prep method was positive in seven cases (7.3%) in group NOSES, while there were eight cases (9.0%) in group CL. There were no statistically significant differences in the oncological outcomes between the two groups ($p > 0.05$).

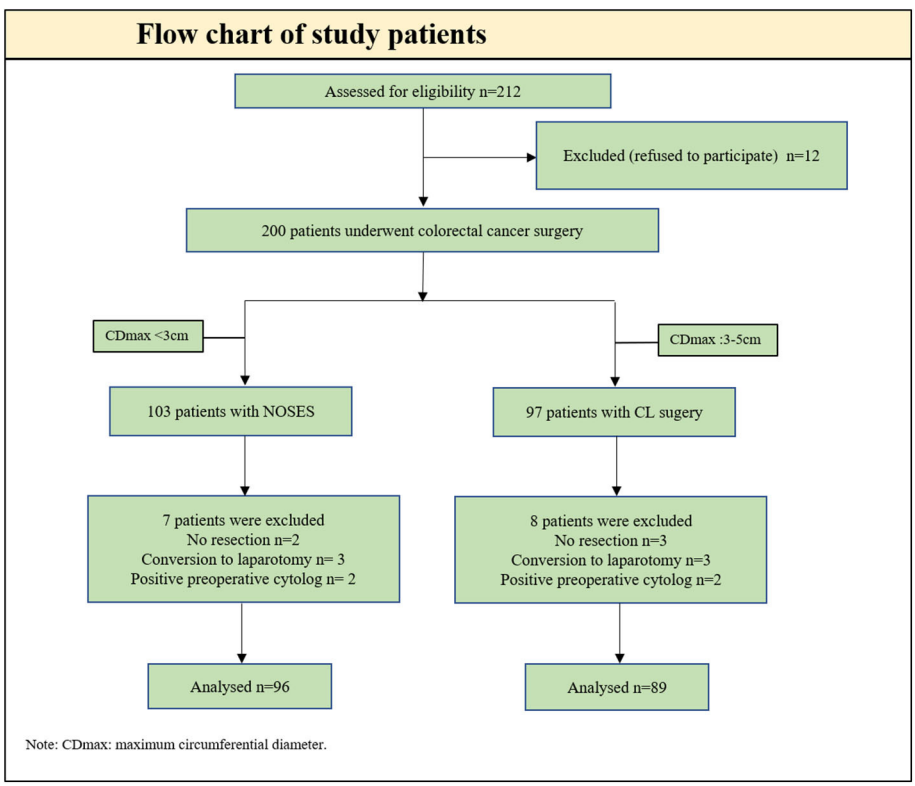

FIGURE 2 | Flow chart of study patients. CD max, maximum circumferential diameter.

TABLE 1 | Comparison of baseline characteristics in patients with colorectal cancer between the group NOSES and Conventional Laparoscopic Surgery (CL) group.

Characteristics	NOSES (*N* = 96)	CL (*N* = 89)	*P*
Age (M ± SD), years	58 ± 11	61 ± 11	0.11
BMI (kg/m^2)	22.5 ± 2.9	22.9 ± 2.6	0.38
Gender			0.21
Male	44 (45.8%)	49 (55.1%)	
Female	52 (54.2%)	40 (44.9%)	
ASA score			0.99
1	13 (13.5%)	12 (13.5%)	
2	25 (26.0%)	24 (26.9%)	
3	58 (60.5%)	53 (59.6%)	
CEA (ng/mL)			0.1
<5	75 (78.1%)	60 (67.4%)	
≥5	21 (21.9%)	29 (32.6%)	
cT category (rectum)			0.79
cT1	11 (13.6%)	7 (11.3%)	
cT2	34 (42.0%)	24 (38.7%)	
cT3	36 (44.4%)	31 (50.0%)	
Tumor location			0.37
Ascending colon	1 (1.0%)	2 (2.2%)	
Transverse colon	1 (1.0%)	3 (3.4%)	
Descending colon	3 (3.1%)	3 (3.4%)	
Sigmoid colon	10 (10.4 %)	19 (21.3%)	
Rectosigmoid colon	24 (25.0%)	17 (19.1%)	
Mid rectum	42 (43.8%)	33 (37.1%)	
Lower rectum	15 (15.6%)	12 (13.5%)	
Invasion depth (T factor)			0.59
T0–T1	8 (8.3%)	4 (4.5%)	
T2	14 (14.6%)	18 (20.2%)	
T3	71 (74.0%)	64 (71.9%)	
T4	3 (3.1%)	3 (3.4%)	
Nodal metastasis (N factor)			0.68
N0	64 (66.7%)	54 (60.7%)	
N1	21 (21.9%)	22 (24.7%)	
N2	11 (11.5%)	13 (14.6%)	
TNM stage			0.49
0–I	16 (16.7%)	10 (11.2%)	
II	48 (50.0%)	44 (49.4%)	
III	32 (33.3%)	35 (39.3%)	
Venous invasion			0.21
Yes	11 (11.5%)	16 (18.0%)	
No	85 (88.5%)	73 (82.0%)	
Neurological invasion			0.18
Yes	8 (8.3%)	13 (14.6%)	
No	88 (91.7%)	76 (85.4%)	

CT category, clinical tumor invasion depth.

TABLE 2 | Comparison of postoperative outcomes in patients with colorectal cancer between the group NOSES and Conventional Laparoscopic Surgery (CL) group.

Variables	NOSES (96)	CL (89)	*P*
Postoperative oncological outcomes			0.67
Negative	89 (92.7%)	81 (91.0%)	
Positive	7 (7.3%)	8 (9.0%)	
Bacteriological Outcomes			0.8
Negative	63 (65.6%)	60 (67.4%)	
Positive	33 (34.4%)	29 (32.6%)	
Postoperative WBC (1d)			0.53
$<10 \times 10^9$ g/L	53 (55.2%)	45 (50.6%)	
$\geq 10 \times 10^9$ g/L	43 (44.8%)	44 (49.4%)	
PTC (1d)			0.35
<0.25 ng/ml	77 (80.2%)	68 (76.4%)	
≥0.25 ng/ml	19 (19.8%)	21 (23.6%)	
CRP (1d)			0.91
<8 mg/L	74 (77.1%)	68 (76.4%)	
≥8 mg/L	22 (22.9%)	21 (23.6%)	
Temperature			0.21
<38.5°C	85 (88.5%)	73 (82.0%)	
≥38.5°C	11 (11.5%)	16 (18.0%)	
VAS score			
Day 1 postoperatively	2.43 ± 0.87	5.34 ± 1.02	<0.001
Day 3 postoperatively	1.43 ± 0.81	3.67 ± 0.84	<0.001
Day 5 postoperatively	0.96 ± 0.75	2.35 ± 0.91	<0.001
Intraperitoneal infection	4 (4.2%)	3 (3.4%)	1
Usage rate of additional analgesics	9 (9.4%)	29 (32.6%)	<0.001
Incision-related complications	0	5 (5.6%)	0.02
Postoperative hospital stay (d)	7.03 ± 1.30	9.37 ± 2.52	<0.001

In terms of bacteriological outcomes, 33 cases (34.4%) were positive in group NOSES, while 29 cases (32.4%) were positive in group CL. There were no statistically significant differences in the bacteriology outcomes between the two groups ($p > 0.05$). Detection of WBC, PTC, CRP, and other infection-related indicators on the first day after operation, no significant difference between the two groups. Moreover, the intraperitoneal infection and postoperative temperature had no statistically significant differences between the two groups ($p > 0.05$). Compared with the CL group, patients in the NOSES group had a lower rate of additional analgesic use (9.4 vs. 32.6%, $p < 0.001$), lower postoperative pain score ($P < 0.001$), shorter hospital stay (7.03 ± 1.30 vs. 9.37 ± 2.52, $p < 0.001$) and incision-related complications rate was lower (0 vs.5.6%, $p = 0.02$).

Analysis of Risk Factors for Oncology Positive

We performed univariate and multivariate analyses of the clinical and pathological variables which could potentially influence the results of oncology (**Table 3**). In the univariate analysis, the invasion depth and nodal metastasis were significantly associated with positive peritoneal lavage fluid oncology. The incidence of oncology positive in T4 with invasion depth was significantly higher than in T3 and T0–T2 (33.3 vs. 8.9 vs. 2.3%, $P < 0.05$). The incidence of oncology positive in N2 and N1 with nodal metastasis was significantly higher than in N0 (20.8 vs. 14.0 vs.

TABLE 3 | Univariate and multivariate analysis of clinical and pathological factors for oncology of peritoneal lavage fluid.

Factors	Number	Univariate analysis		Multivariate analysis	
		Oncology positive rate (%)	*P*	Odds ratio	*P*
Gender			0.77		
Male	93	7.5			
Female	92	8.7			
Age (years)			0.63		
<60	85	7.1			
≥60	100	9.0			
CEA (ng/mL)			0.55		
<5	135	7.4			
≥5	50	10.0			
cT category (rectum)			0.25		
cT1	18	9.0			
cT2	58	5.2			
cT3	67	16.7			
Tumor location			0.79		
Colon	42	7.1			
Rectum	143	8.4			
Invasion depth (T factor)			0.04		
T0–T2	44	2.3		1	
T3	135	8.9		4.699 (0.580–38.067)	0.15
T4	6	33.3		20.470 (1.241–337.661)	0.04
Nodal metastasis (N factor)			0.003		
N0	118	3.4		1	
N1	43	14.0		5.445 (1.412–20.991)	0.01
N2	24	20.8		6.315 (1.458–27.348)	0.01
Venous invasion			0.89		
Yes	27	7.4			
No	158	8.2			
Neurological invasion			0.39		
Yes	21	14.3			
No	164	7.3			

3.4%, $P < 0.05$). In the multivariate analysis, the nodal metastasis (N1, N2) and invasion depth (T4) were an independent risk factor for positive peritoneal lavage fluid oncology.

Follow-Up Results of Patient's Local Recurrence-Free Survival

The patients were followed up for 4–41 months. In group NOSES, the tumor relapsed in five patients at 30, 28, 25, 24, and 19 months after operation, and the recurrence rate was 5.2% (5/96). In group CL, the tumor relapsed in seven patients at 16, 19, 23, 24, 28, 30, and 32 months after operation, and the recurrence rate was 7.9% (7/89). The recurrence rate of tumor had no significant difference between the two groups ($p = 0.56$). The Kaplan–Meier local recurrence-free survival of group NOSES and group CL are shown in **Figure 3**, and the log-rank test revealed that the local recurrence-free survival rate had no statistically significant difference between the two groups ($p = 0.79$). Sixty-six patients were followed for more than 2 years, including NOSES (32 patients) and group CL (34 patients). There was no significant difference in local recurrence rate between the two groups ($p = 0.93$).

Analysis of Bacterial Species

The contamination rate of peritoneal lavage fluid was 34.4 vs. 32.6% in group NOSES and group CL, respectively. Gram-negative bacteria were the main bacteria in two groups of peritoneal lavage fluid including *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter reesei*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Aeromonas caviae*, and *Aeromonas* Vickers. Among them, *E. coli* is the main detected bacteria in line with bowel flora. **Table 4** showed details of the types of bacteria cultured by the two groups of peritoneal lavage fluid.

DISCUSSION

In 1993, Franklin et al. (10) were the first to publish a case of patient who underwent sigmoid resection with transrectal specimen extraction. In recent years, more and more people have noticed that NOSES is more minimally invasive than conventional laparoscopic surgery and has accelerated the postoperative recovery of patients (11–13). It has caused widespread concern in the treatment of colorectal cancer and could be the next step in minimizing minimally invasive surgery (1, 5, 14). However, there is no systematic discussion on whether NOSES operation adds oncological and bacteriological issues. Ngu and Wong (9) reported that five patients with NOSES had no tumor cells found in the peritoneal lavage fluid. Costantino et al. (15) showed the contamination rate of peritoneal fluid was 100 vs. 88.9% in NOSE and non-NOSE procedures. The high contamination rate likely because they did not use a sterile specimen bag to protect the resected specimen during the operation. Our research strengthened the control of aseptic and tumor-free operation and increased the sample size. The use of sterile protective sleeves to reduce tumor cell planting and bacterial contamination of the abdominal cavity.

In this study, our data showed that there were no statistically significant difference in oncology between NOSES and conventional laparoscopic surgery for colorectal cancer. Hence, we think that oncological issue has nothing to do with the surgical approach. We further performed univariate and multivariate analysis on 15 patients with oncology positive. Our study found that the tumor invasion depth and nodal metastasis were independent risk factors for oncology positive. The pT4, pN1, and pN2 increased the risk of postoperative peritoneal lavage fluid oncology positive by 20.47, 5.45, and 6.32 times, respectively. Noura et al. (16) and Temesi et al. (17) showed that the chance of malignant cells being present in the lavage fluid increased as the depth of tumor invasion increased. This showed that the positive rate of cancer cells in the postoperative peritoneal lavage fluid was related to the stage of the tumor itself. Although we used sterile specimen bag during specimen extraction to avoid tumor implantation and peritoneal

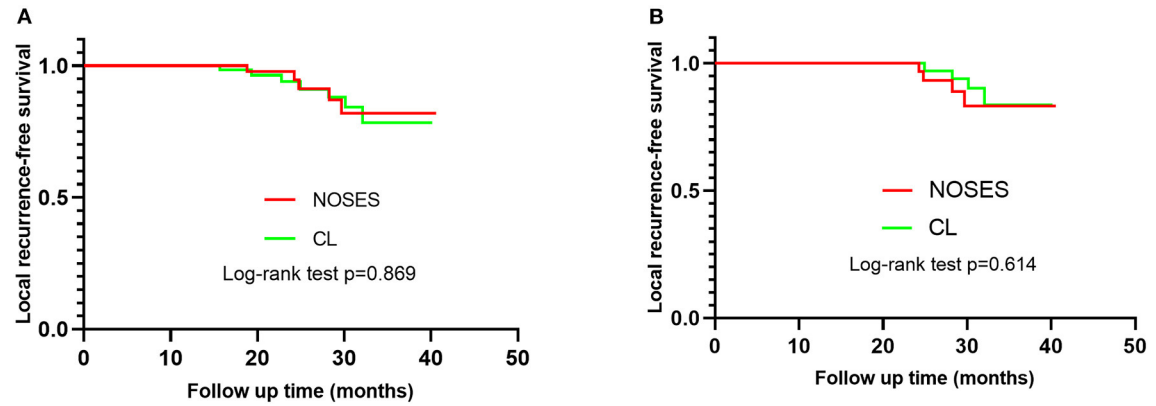

FIGURE 3 | Kaplan–Meier survival curve of patients in NOSE and group CL. **(A)** The difference of the local recurrence-free survival rate of patients in the two group has no statistical significance ($p = 0.87$), Hazard ratio 0.909, (95%CI = 0.291–2.840). **(B)** Sixty-six patients were followed for more than 2 years. The difference of the local recurrence-free survival rate of patients in the two group has no statistical significance ($p = 0.61$).

TABLE 4 | Bacterial types in group NOSES and group CL with bacteriology positive.

Organism	NOSES (33)	CL (29)	No. (62)
Escherichia coli	22	21	43
Enterobacter cloacae	1	1	2
Pseudomonas aeruginosa	1	0	1
Enterococcus faecium	3	1	4
Corynebacterium	1	0	1
Streptococcus oralis	2	1	3
Acinetobacter reesei	1	0	1
Klebsiella pneumoniae	1	1	2
Enterobacter aerogenes	1	1	2
Citrobacter freundii	0	1	1
Aeromonas Vickers	0	1	1
Aeromonas caviae	0	1	1

contamination, there are still tumor cells in the peritoneal lavage fluid of NOSES. Probably because live tumor cells that have the potential to proliferate and possibly metastasize have shed from the primary site before or during surgical resection. Other studies showed the positive rate of postoperative peritoneal lavage fluid in colorectal cancer patients is between 0 and 52% (17–20), the worse the tumor stage, the higher positive rate of tumor cells in peritoneal lavage fluid. The low rate of positive samples in our study may be due to our having only included early stage patients undergoing scheduled curative surgery. This also explains why our patients with NOSES had a lower positive rate of cancer cells in the peritoneal lavage fluid, and did not increase the probability of pelvic and abdominal implantation of cancer cells.

Bacterial contamination of the peritoneal cavity is frequent in colorectal laparoscopic procedures (21), but it is unclear whether NOSES causes increased levels of contamination. We found that the bacterial culture results of the peritoneal lavage fluid collected during NOSES showed a 34.4% positive rate of bacteriology, which was no significantly different from the results of the group CL. There was also no significant difference in the incidence of intraoperative abdominal infection between the two groups after surgery. The higher bacteriological positive rate after surgery is mainly due to the large intestinal flora in the colorectum (22). The bacterial culture results of our peritoneal lavage fluid showed that they were mainly Gram-negative bacteria such as *Escherichia coli*. No epidermal colony was found, which proves that we did not bring in external bacteria when we placed an anvil. The international consensus of NOSES suggests that prophylactic antibiotics should be used before surgery, perfect bowel preparation, intraperitoneal irrigation during operation, anal lavage with a large amount of povidone iodine and normal saline, use of transluminal wound protector and placement of pelvic or abdominal drainage tube to reduce the bacterial load of NOSES (1, 23). We perform NOSES procedures in accordance with specifications, which will not increase the incidence of bacterial contamination and abdominal infections.

We compared the tumor cytology and bacterial aerobic culture results of peritoneal lavage fluid in patients with NOSES and patients with conventional laparoscopic surgery, and the results of local recurrence of tumor in two groups were followed up for a long time. The most important finding of our research is that NOSES will not increase tumor implantation and abdominal contamination. Liu et al. (24) analyzed 14 studies through meta-analysis and demonstrated that compared with CL surgery, NOSES may be a safe operation and can achieve similar oncology results. Our conclusions further provide reliable evidence that NOSES meets the expectations of tumor safety. We consider that NOSES is feasible and safe for colorectal cancer surgery, and that it can achieve satisfactory clinical outcomes without noticeable scars in carefully selected patients.

The present study has several limitations. Firstly, the preoperative evaluation of tumor invasion mainly depends on the imaging data. To a certain extent, it depends on the judgment of the imaging doctor and the chief surgeon, which sometimes

deviates from the pathological results. Our 185 patients were studied according to the inclusion criteria. The depth of postoperative pathological invasion in six patients was T4, which was different from the selection criteria and increased the positive rate of oncology of the abdominal lavage fluid to a certain extent. Second, the number of patients was not large enough, only 185 patients being ultimately enrolled. A larger population study is need to further confirm our results. Third, some patients have a shorter follow-up time, which may ultimately reduce the relapse rate. Forth, several papers are pointing at size as a prognostic indicator, grouping by tumor size may affect research results, but there is no definite conclusion about whether the size of the tumor diameter affects the prognosis. Finally, oncological and bacteriological problems are caused by many factors, our research can only show that there is no difference between NOSES and conventional laparoscopic surgery in this regard.

In our opinion, we screen patients strictly according to the scope of application of NOSES, employing specimen bags and wound protectors to reduce the possibility of bacterial contamination and tumor cell metastasis (1). NOSES has no significant differences in bacteriology and oncology compared with conventional laparoscopic surgery. Therefore, NOSES is safe and feasible, and can be carried out safely for the right patient. In the next step, we can continue to expand the NOSES sample size and follow up its 5-year overall survival (OS) and disease-free survival (DFS).

CONCLUSION

There were no significant differences of bacteriological and oncological results in peritoneal lavage fluid between NOSES and conventional laparoscopic surgery, as well as in long-term oncological outcomes. NOSES did not increase postoperative pelvic and abdominal infections or promote tumor cell planting and metastasis. It is conformed to the principle of asepsis and tumor-free and worthy of clinical application and promotion.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JP and QO designed the study. JP, QO, WW, and SX collected and analyzed data. QO wrote the paper. JP, SX, and JC revised the paper. All authors contributed to the article and approved the submitted version.

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Predictive Risk Factors and Online Nomograms for Synchronous Colon Cancer With Liver Metastasis

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Objectives: To develop and validate predictive nomograms of cancer specific survival (CSS) and overall survival (OS) for synchronous colon cancer with liver metastasis (SCLM) patients.

Methods: Patients with pathologically diagnosed colon cancer with liver metastasis were retrieved from the SEER database between 2010 and 2015. Only SCLM patients were included. Univariate and multivariate cox regression analyses were conducted to identify the potential predictors of patients' survival outcomes. The selected variables were integrated to create predictive nomograms via R tools. Furthermore, the concordance index Harrell's C statistic (C-index) was calculated to describe the discrimination of nomograms. Calibration (1000 bootstrap resamples) curves were plotted to compare the predictions of nomograms with the observed outcomes. Decision curve analysis (DCA) and clinical impact curves were performed to evaluate the clinical effects of nomograms.

Results: A total of 22,378 SCLM patients were included. The median time of OS and CSS was 13 and 17 months, respectively. The 1-, 2-, and 3-year rate of OS was 50.6, 28.1, and 14.8%, respectively. While the 1-, 2-, and 3-year rate of CSS was 58.7, 36.8, and 22.5%, respectively. SCLM patients with increased age, left primary tumor location, AJCC IVb stage, and no chemotherapy were associated with an obviously reduced OS and CSS. Variables including age, histological grade, T/N/M stage, tumor size, bone/lung metastasis, CEA, surgery of primary site, and chemotherapy were closely related to the prognoses of SCLM patients. Nomograms of OS and CSS were built and displayed online for convenient utilization. The C-index of OS and CSS monograms were 0.74 and 0.73, respectively, indicating relatively good discrimination of the nomograms. The calibration curves suggested a good agreement between the actual observation and the nomogram prediction. DCAs and clinical impact curves reflected favorable potential clinical effects of predictive nomograms.

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Conclusion: Chemotherapy, surgery of primary site, and age were important independent risk factors for the CSS and OS of SCLM patients. We built and validated two reliable nomograms of OS and CSS to predict the prognoses of SCLM patients, which can be accessed online at (https://predictive-tool.shinyapps.io/CSS-DynNomapp/; https://predictive-tool.shinyapps.io/OS-DynNomapp/).

Keywords: colon cancer, liver metastasis, SEER, prognosis, nomogram

INTRODUCTION

Colorectal cancer (CRC) is a commonly diagnosed malignant digestive tract cancer both in men and women worldwide. CRC is responsible for 10% of cancer-specific deaths in the United States, ranking as the second leading cause (1). Like other solid tumors, the distant metastasis is an essential prognostic factor of poor cancer survival. Most distantly metastatic CRC patients have only approximately a 13.5% chance of 5-year survival, while locally advanced patients have a favorable survival rate of 71% (2). Despite the difference in primary site and histology subtypes, generally, the most frequently metastatic organ of CRC is the liver, followed by the lungs, bone, and the brain (3). Specifically, liver metastases were observed in more than 25% of CRC patients when initially diagnosed. Liver metastases occurred in up to 25% of patients after the resection of a primary tumor. A total of 50% of CRC patients may develop liver metastases during the whole disease course (4, 5).

Of note, colon cancer patients have a higher metastatic potential for liver rather than rectal cancer. The most well-known mechanism is that the metastatic pattern is different due to the direction of hematogenous metastasis of colon cancer and rectal cancer. In colon cancer, the majority of the intestinal mesenteric drainage enters the hepatic portal venous system. Therefore, the liver is the primary organ involved. Whereas, the most common metastatic site of rectal cancer is the lungs since the rectum venous-collected blood flows into the systemic circulation (6).

The surveillance, epidemiology, and end results (SEER) database covers most of the cancer population from 18 American registries, thus providing opportunities to estimate the sociodemographic and clinical predictors of cancer prognosis in a large population (7). Nomograms are useful tools that can assist in quantitatively predicting the prognosis for each patient (8). Previous retrospective studies based on the SEER database has assessed the risk factors of poor survival for CRC patients with lung and bone metastasis and established a nomogram to estimate the cancer survival, respectively (9, 10). Synchronous colon cancer with liver metastasis (SCLM), a subtype of colon cancer with liver metastasis, is characterized with poor prognosis. The treatment for SCLM patients is also controversial. However, the patients' characteristics and survival pattern of SCLM is still not clear.

In this study, we aimed to perform a retrospective analysis to investigate the pathological characteristics and treatment experience of SCLM patients using data from the SEER database. Furthermore, we intended to identify potential prognostic factors and build original predictive models for evaluating 1-, 3-, and 5-year cancer-specific survival (CSS) and overall survival (OS).

PATIENTS AND METHODS

Study Populations

Based on the SEER database, patients diagnosed with primary colon cancer from 2010 to 2015 were retrospectively identified with the SEER*Stat software version 8.3.6[1]. Patients with liver metastasis were selected. Individuals with the following information were excluded: unclear M stage, T0 stage, unclear survival time, or status of OS and CSS at the end of follow-ups. Variables including age, gender, race, histological grade, AJCC 7th TNM stage, tumor size, bone/lung/brain metastasis, CEA, surgery of primary site, surgery of liver metastasis, and chemotherapy were sorted. OS and CSS were defined as the primary outcomes. The follow-up time was defined as the time from diagnosis to death or to the last follow-up (December 31, 2015).

Statistical Methods

The basic characteristics of the included patients were described with different variables. Univariate and multivariate cox analysis were performed to test each variable's contribution in predicting survival outcomes and the hazard ratio (HR) was calculated with a corresponding 95% confidence interval (CI). Statistically significant risk factors were used to establish predictive nomograms of the 1-, 3-, and 5-year survival rate of individuals. The discrimination and calibration of nomograms were measured to evaluate the predicted probabilities of the nomogram. Calibration (1000 bootstrap resamples) curves were plotted to compare the predictions of the nomogram with observed outcomes. Decision curve analysis and clinical impact curves were performed to evaluate clinical effects of the nomogram (8, 11). The Kaplan-Meier method and groups were compared using the log-rank test when applicable. Statistical analyses were conducted via the SPSS version 25.0 (IBM Corporation, Armonk, NY, United States) and R software version 3.6.1[2]. P-value < 0.05 was considered as statistically significant.

RESULTS

Patient Characteristics and Survival Outcomes

A total of 179,426 patients diagnosed with colon cancer were extracted from the SEER database. Of these, 22,697 patients were

[1]www.seer.cancer.gov/seerstat

[2]http://www.r-project.org

TABLE 1 | Baseline characteristics of colon cancer patients with synchronous liver metastasis.

Variables	Patients
N	22378
Median age (year)	66 ± 14
Age	
≤50	3097 (13.8%)
51–60	4658 (20.8%)
61–70	5666 (25.4%)
71–80	4742 (21.2%)
>80	4215 (18.8%)
Gender	
Female	10459 (46.7%)
Male	11919 (53.3%)
Race	
White	16760 (74.9%)
Black	3763 (16.8%)
Other	1855 (8.3%)
Tumor primary site	
Ascending colon	3680 (16.4%)
Transverse colon	1764 (7.9%)
Descending colon	1240 (5.5%)
Sigmoid colon	6394 (28.6%)
Other	9300 (41.6%)
Grade	
I	833 (3.7%)
II	10306 (46.1%)
III	3748 (16.7%)
IV	781 (3.5%)
Unknown	6710 (30%)
AJCC stage	
IVa	11631 (52%)
IVb	9312 (41.6%)
IVnos	1435 (6.4%)
T	
T1	2134 (9.5%)
T2	403 (1.8%)
T3	6818 (30.5%)
T4	5675 (25.4%)
Tx	7348 (32.8%)
N	
N0	7013 (31.3%)
N1	6835 (30.6%)
N2	5132 (22.9%)
Nx	3398 (15.2%)
M	
M1a	11639 (52.0%)
M1b	9306 (41.6%)
M1nos	1433 (6.4%)
Surgery of primary site	
No surgery	10933 (48.9%)
Tumor lesion	204 (0.9%)
Partial colectomy	4260 (19.0%)
Total/subtotal colectomy	6891 (30.8%)
Unknown	90 (0.4%)
Surgery of liver metastasis	
Yes	196 (0.9%)
No	22182 (99.1%)
Bone metastasis	
Yes	1157 (5.2%)
No	20460 (91.4%)
Unknown	761 (3.4%)
Brain metastasis	
Yes	209 (0.9%)
No	21336 (95.4%)
Unknown	833 (3.7%)
Lung metastasis	
Yes	4720 (21.1%)
No	16843 (75.3%)
Unknown	815 (3.6%)
Tumor size	
≤2 cm	370 (1.7%)
2–5 cm	7226 (32.3%)
5–10 cm	6108 (27.3%)
>10 cm	640 (2.8%)
Unknown	8034 (35.9%)
CEA	
Positive	12915 (57.7%)
Negative	1970 (8.8%)
Boardline	31 (0.2%)
Other	7462 (33.3%)
Radiotherapy	
Yes	754 (3.4%)
No	21624 (96.6%)
Chemotherapy	
Yes	13098 (58.5%)
No	9280 (41.5%)

(Continued)

TABLE 1 | Continued

CEA, carcinoembryonic antigen; Grade I, well differentiated; Grade II, moderately differentiated; Grade III, poorly differentiated; Grade IV, undifferentiated.

identified who had synchronous liver metastasis. After removing 253 patients with unavailable necessary information, 22,378 individuals were included and analyzed. The basic characteristics of the included patients are presented in **Table 1**.

Survival Outcomes

During the follow-up period, 64.8% (14500/22378) of patients died from SCLM. The survival outcomes showed that the 1-, 2-, and 3-year rate of OS was 50.6, 28.1, and 14.8%, respectively. The 1-, 2-, and 3-year rate of CSS was 58.7, 36.8, and 22.5%, respectively. The median time of OS and CSS was 13 months and 17 months, respectively.

When stratified by different variables, the OS and CSS of SCLM patients decreased significantly with an increase in age (**Figures 1A1,A2**). The survival outcomes of both OS and CSS were also influenced by different primary tumor locations. The prognosis of patients with a primary site of the ascending or transverse colon was significantly worse than those within

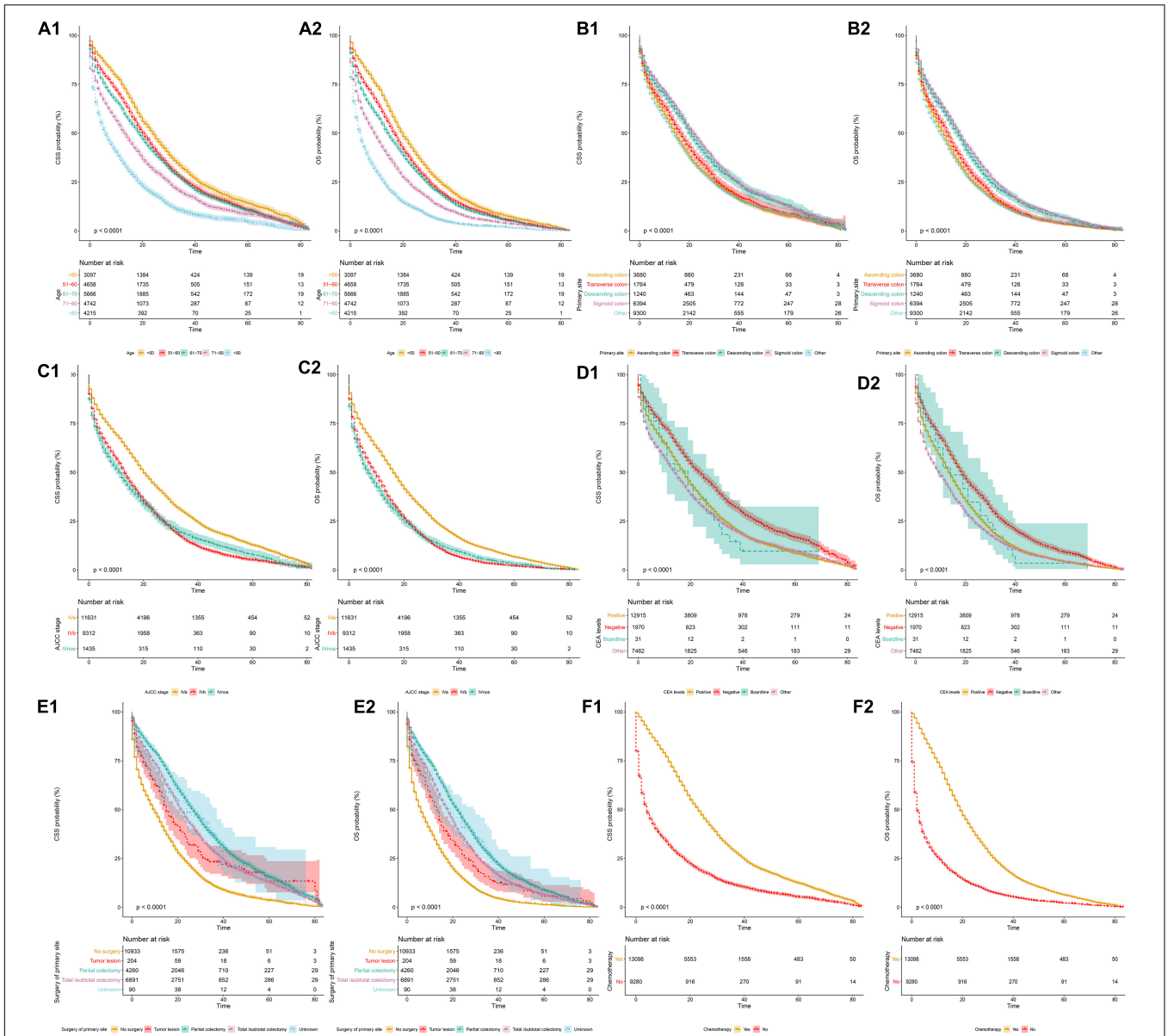

FIGURE 1 | Cancer-specific and overall survival curves stratified by patient characteristics: **(A1,A2)** age; **(B1,B2)** primary tumor location; **(C1,C2)** AJCC stage; **(D1,D2)** CEA levels; **(E1,E2)** surgery of primary site; **(F1,F2)** chemotherapy.

the descending and sigmoid colon (**Figures 1B1,B2**). SCLM patients in the AJCC IVb stage were associated with obviously worse OS and CSS than those in IVa stage. The median OS of stage IVb patients was 9 months, while for stage IVa patients it was 16 months (**Figures 1C1,C2**). Patients with a negative CEA level had better CSS and OS prognosis than those with a positive CEA level [HR (95.0% CI) CSS 0.75 (0.70∼0.80), OS 0.75 (0.72∼0.80)] (**Figures 1D1,D2**). The SCLM patients that underwent partial colectomy and total/subtotal colectomy showed a relatively better prognosis that those without surgery (**Figures 1E1,E2**). The SCLM patients that received chemotherapy treatment had obviously better survival outcomes of both OS and CSS than those without chemotherapy (**Figures 1F1,F2**). The median CSS in SCLM patients with chemotherapy was 23 months compared to 4 months in patients without chemotherapy ($P < 0.0001$).

Univariate and Multivariate Analyses

Variables that might possibly predict the CSS and OS of SCLM patients were analyzed. The results revealed that age, histological grade, T/N/M stage, tumor size, bone/lung metastasis, CEA, surgery of primary site, and chemotherapy were independent risk factors for the CSS and OS of SCLM patients. The factors of gender, brain metastasis, and radiotherapy seemed to have no significant relationship with the outcomes of OS and CSS. The detailed outcomes are presented in **Table 2**.

TABLE 2 | Univariate and multivariatecox analyses of prognostic factors associated with CSS and OS in the studied cohort.

Variables	CSS Univariate		CSS Multivariate		OS Univariate		OS Multivariate	
	HR (95.0% CI)	*P* value	HR (95.0% CI)	*P* value	HR (95.0% CI)	*P* value	HR (95.0% CI)	*P* value
Age at diagnosis, years								
≤50	Reference		Reference		Reference		Reference	
51-60	1.18 (1.12∼1.25)	<0.01	1.11 (1.05∼1.18)	<0.01	1.17 (1.11∼1.23)	<0.01	1.10 (1.05∼1.16)	<0.01
61-70	1.27 (1.20∼1.34)	<0.01	1.15 (1.09∼1.22)	<0.01	1.27 (1.21∼1.33)	<0.01	1.15 (1.10∼1.21)	<0.01
71-80	1.61 (1.52∼1.70)	<0.01	1.35 (1.27∼1.43)	<0.01	1.63 (1.55∼1.71)	<0.01	1.36 (1.30∼1.43)	<0.01
>80	2.53 (2.38∼2.68)	<0.01	1.58 (1.48∼1.68)	<0.01	2.55 (2.42∼2.69)	<0.01	1.60 (1.51∼1.69)	<0.01
Gender								
Female	Reference				Reference			
Male	0.98 (0.94∼1.00)	0.140	–	–	0.971 (0.94∼1.00)	0.060	–	–
Race								
White	Reference		Reference		Reference		Reference	
Black	0.98 (0.95∼1.03)	0.620	1.00 (0.95∼1.05)	0.950	1.01 (0.97∼1.05)	0.630	1.02 (0.98∼1.06)	0.280
Tumor primary site								
Ascending colon	Reference		Reference		Reference		Reference	
Transverse colon	0.92 (0.86∼0.99)	0.020	0.95 (0.88∼1.02)	0.140	0.93 (0.87∼0.99)	0.020	0.95 (0.90∼1.02)	0.141
Descending colon	0.77 (0.71∼0.84)	<0.01	0.89 (0.82∼0.96)	0.003	0.77 (0.72∼0.83)	<0.01	0.88 (0.82∼0.95)	<0.01
Sigmoid colon	0.74 (0.70∼0.77)	<0.01	0.81 (0.77∼0.86)	<0.01	0.73 (0.69∼0.76)	<0.01	0.80 (0.76∼0.84)	<0.01
Grade								
I	Reference		Reference		Reference		Reference	
II	0.99 (0.91∼1.08)	0.860	1.22 (1.11∼1.33)	<0.01	1.02 (0.94∼1.10)	0.711	1.25 (1.16∼1.35)	<0.01
III	1.40 (1.27∼1.53)	<0.01	1.61 (1.46∼1.77)	<0.01	1.44 (1.32∼1.56)	<0.01	1.67 (1.54∼1.82)	<0.01
IV	1.63 (1.45∼1.84)	<0.01	2.01 (1.78∼2.27)	<0.01	1.63 (1.47∼1.82)	<0.01	2.03 (1.82∼2.26)	<0.01
T								
T1	Reference		Reference		Reference		Reference	
T2	0.51 (0.45∼0.58)	<0.01	0.79 (0.69∼0.91)	0.001	0.51 (0.45∼0.57)	<0.01	0.78 (0.69∼0.89)	<0.01
T3	0.60 (0.57∼0.64)	<0.01	0.94 (0.87∼1.01)	0.094	0.60 (0.57∼0.64)	<0.01	0.94 (0.88∼1.00)	0.060
T4	0.80 (0.75∼0.85)	<0.01	1.08 (1.00∼1.17)	0.042	0.79 (0.75∼0.84)	<0.01	1.08 (1.09∼1.15)	0.029
N								
N0	Reference		Reference		Reference		Reference	
N1	0.85 (0.81∼0.88)	<0.01	1.09 (1.04∼1.14)	<0.01	0.84 (0.81∼0.87)	<0.01	1.07 (1.03∼1.12)	0.001
N2	0.85 (0.81∼0.89)	<0.01	1.35 (1.279∼1.43)	<0.01	0.84 (0.81∼0.88)	<0.01	1.33 (1.26∼1.39)	<0.01
M								
M1a	Reference		Reference		Reference		Reference	
M1b	1.47 (1.42∼1.52)	<0.01	1.22 (1.17∼1.28)	<0.01	1.48 (1.44∼1.53)	<0.01	1.23 (1.19∼1.28)	<0.01
Bone metastasis								
Yes	Reference		Reference		Reference		Reference	
No	0.57 (0.53∼0.62)	<0.01	0.78 (0.72∼0.84)	<0.01	0.58 (0.54∼0.61)	<0.01	0.79 (0.73∼0.84)	<0.01
Tumor size								
≤2 cm	Reference		Reference		Reference		Reference	
2–5 cm	1.12 (0.99∼1.28)	0.082	1.06 (0.93∼1.21)	0.385	1.14 (1.01∼1.28)	0.030	1.07 (0.95∼1.21)	0.244
5–10 cm	1.32 (1.16∼1.51)	<0.01	1.23 (1.071∼1.4)	0.003	1.29 (1.15∼1.45)	<0.01	1.20 (1.06∼1.35)	0.003
>10 cm	1.50 (1.28∼1.76)	<0.01	1.28 (1.091∼1.51)	0.003	1.45 (1.26∼1.67)	<0.01	1.24 (1.07∼1.43)	0.004
CEA								
Positive	Reference		Reference		Reference		Reference	
Negative	0.75 (0.70∼0.79)	<0.01	0.79 (0.74∼0.83)	<0.01	0.75 (0.72∼0.79)	<0.01	0.79 (0.75∼0.84)	<0.01
Boardline	1.08 (0.73∼1.60)	0.697	1.14 (0.77∼1.69)	0.510	0.99 (0.69∼1.43)	0.960	1.04 (0.72∼1.50)	0.823
Brain metastasis								
Yes	Reference		Reference		Reference		Reference	
No	0.58 (0.48∼0.69)	<0.01	0.89 (0.74∼1.06)	0.181	0.54 (0.46∼0.62)	<0.01	0.83 (0.71∼0.96)	0.055

(Continued)

TABLE 2 | Continued

Variables	CSS Univariate		CSS Multivariate		OS Univariate		OS Multivariate	
	HR (95.0% CI)	*P* value	HR (95.0% CI)	*P* value	HR (95.0% CI)	*P* value	HR (95.0% CI)	*P* value
Lung metastasis								
Yes	Reference		Reference		Reference		Reference	
No	0.71 (0.68∼0.74)	<0.01	0.95 (0.90∼0.99)	0.039	0.70 (0.67∼0.72)	<0.01	0.93 (0.89∼0.98)	<0.01
Surgery of primary site								
No surgery	Reference		Reference		Reference		Reference	
Tumor lesion	0.60 (0.50∼0.70)	<0.01	0.66 (0.55∼0.78)	<0.01	0.60 (0.51∼0.69)	<0.01	0.66 (0.57∼0.77)	<0.01
Partial colectomy	0.42 (0.40∼0.44)	<0.01	0.50 (0.47∼0.56)	<0.01	0.42 (0.41∼0.44)	<0.01	0.51 (0.48∼0.54)	<0.01
Total /subtotal colectomy	0.51 (0.49∼0.52)	<0.01	0.50 (0.47∼0.54)	<0.01	0.51 (0.49∼0.53)	<0.01	0.51 (0.48∼0.54)	<0.01
Surgery of liver metastasis								
Yes	Reference		Reference		Reference		Reference	
No	1.63 (1.37∼1.95)	<0.01	1.13 (0.95∼1.35)	0.181	1.55 (1.33∼1.81)	<0.01	1.08 (0.92∼1.26)	0.339
Radiotherapy								
Yes	Reference				Reference			
No	1.07 (0.97∼1.17)	0.165	–	–	1.06 (0.98∼1.15)	0.131	–	–
Chemotherapy								
Yes	Reference		Reference		Reference		Reference	
No	2.69 (2.60∼2.79)	<0.01	2.53 (2.44∼2.62)	<0.01	2.69 (2.61∼2.77)	<0.01	2.51 (2.42∼2.59)	<0.01

CEA, carcino-embryonic antigen; Grade I, well differentiated; Grade II, moderately differentiated; Grade III, poorly differentiated; Grade IV, undifferentiated; CSS, cancer-specific survival; OS, overall survival; HR, hazard ratio; CI, confidence interval.

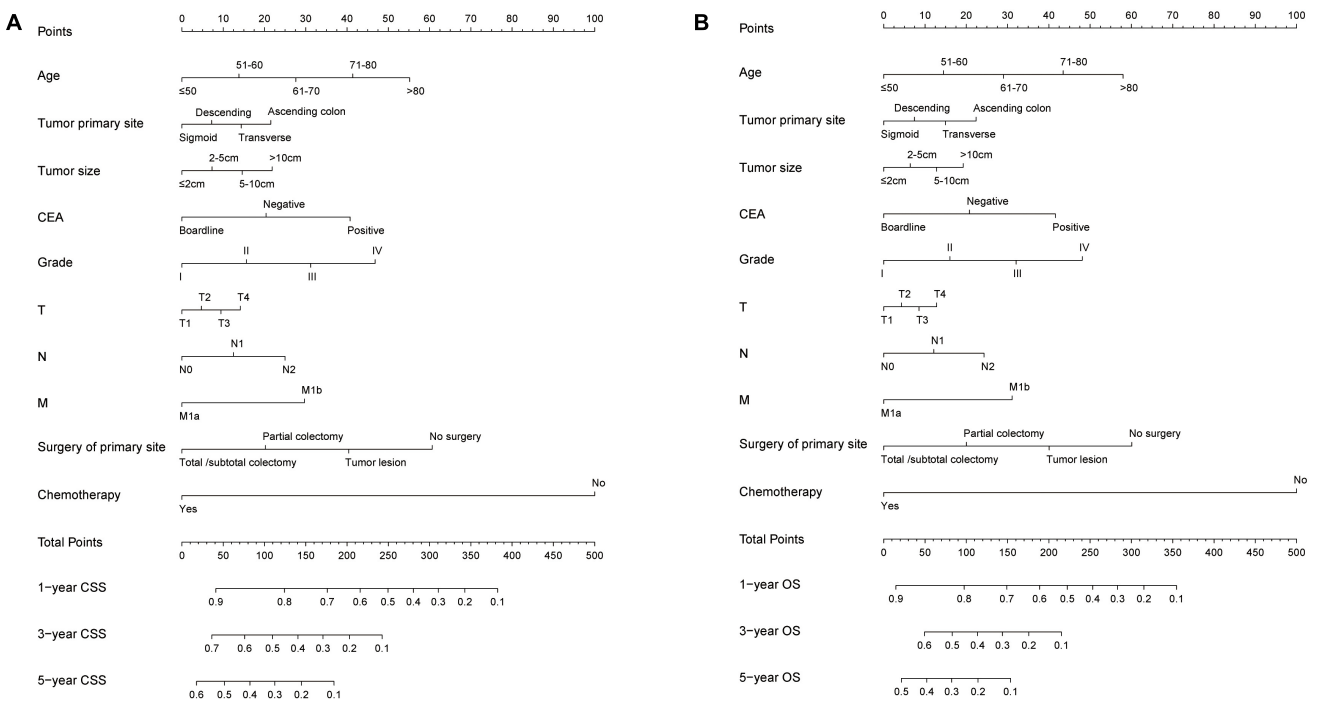

FIGURE 2 | The predicting nomograms for the 1-, 3-, and 5-year CSS and OS of SCLM patients: **(A)** The nomogram for CSS; **(B)** The nomogram for OS.

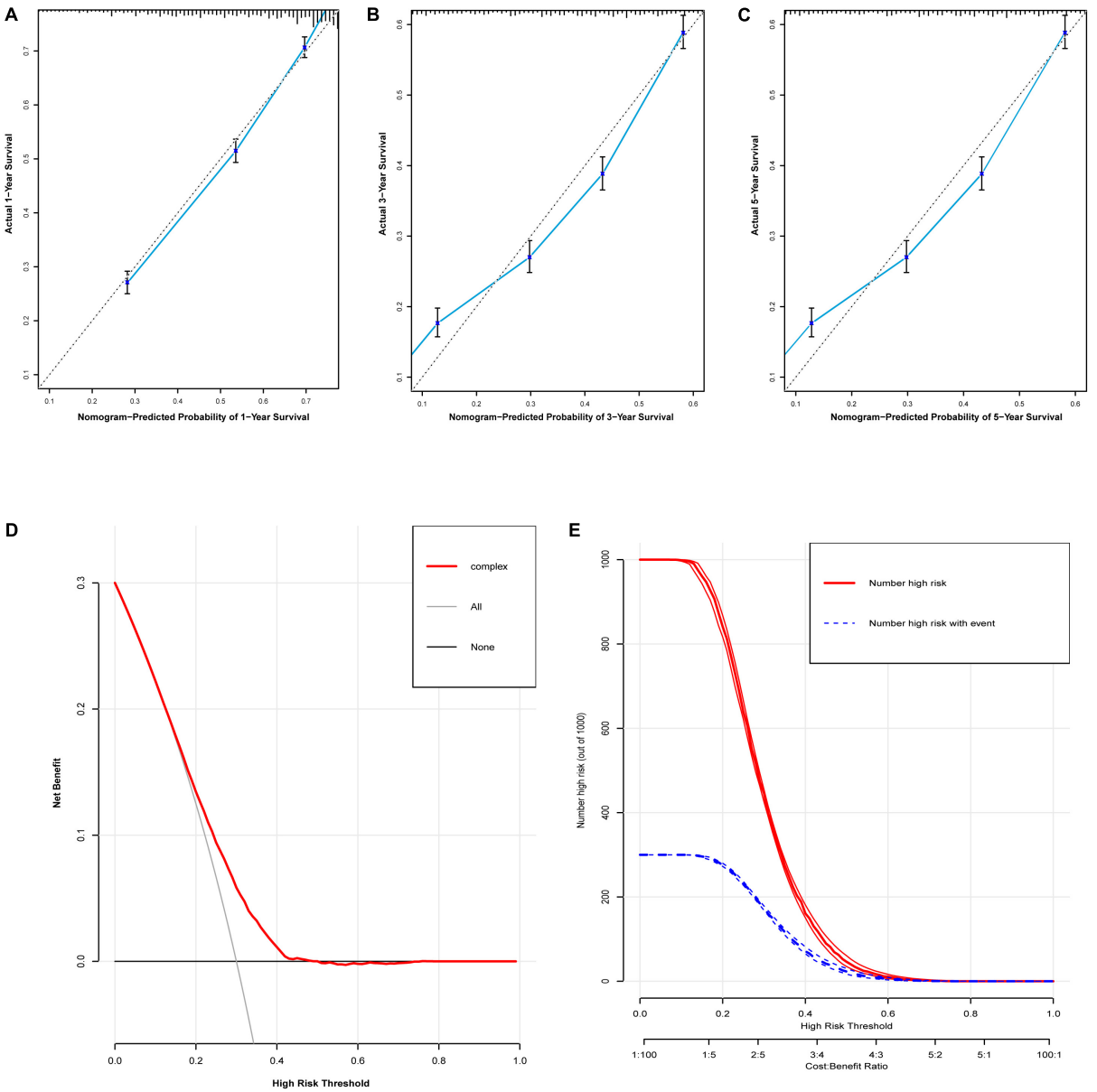

FIGURE 3 | Evaluation of the nomogram for predicting CSS in the studied patients. **(A)** The calibration curves of 1-year CSS; **(B)** the calibration curves of 3-year CSS; **(C)** the calibration curves of 5-year CSS; **(D)** decision curves of CSS; **(E)** clinical impact curve of the predicted nomogram.

Nomograms and Calibrations

Based on the predictive factors in the multivariable analysis, two nomograms were constructed to predict probabilities of CSS and OS (**Figure 2**). The C-index of two nomograms in predicting CSS and OS was 0.73 and 0.74, respectively, indicating good discrimination. The calibration curves of CSS and OS suggested a good agreement between the actual observed probabilities and predicted rates (**Figures 3A–C**, **4A–C**). In addition, decision curve analysis (DCA) is a novel method to evaluate the net clinical benefit of a predictive model. DCAs reflected positive net benefits with a wide clinically reasonable risk threshold probability (**Figures 3D**, **4D**). The clinical impact curves also represented acceptable potential clinical effects of the predictive nomograms (**Figures 3E**, **4E**).

The Webserver for Easy Access to Our Nomograms

We made an online version of our nomograms on the webserver[3,4]. After inputting the predictive variables on the webserver, the dynamic nomograms can easily display

[3]https://predictive-tool.shinyapps.io/CSS-DynNomapp/

[4]https://predictive-tool.shinyapps.io/OS-DynNomapp/

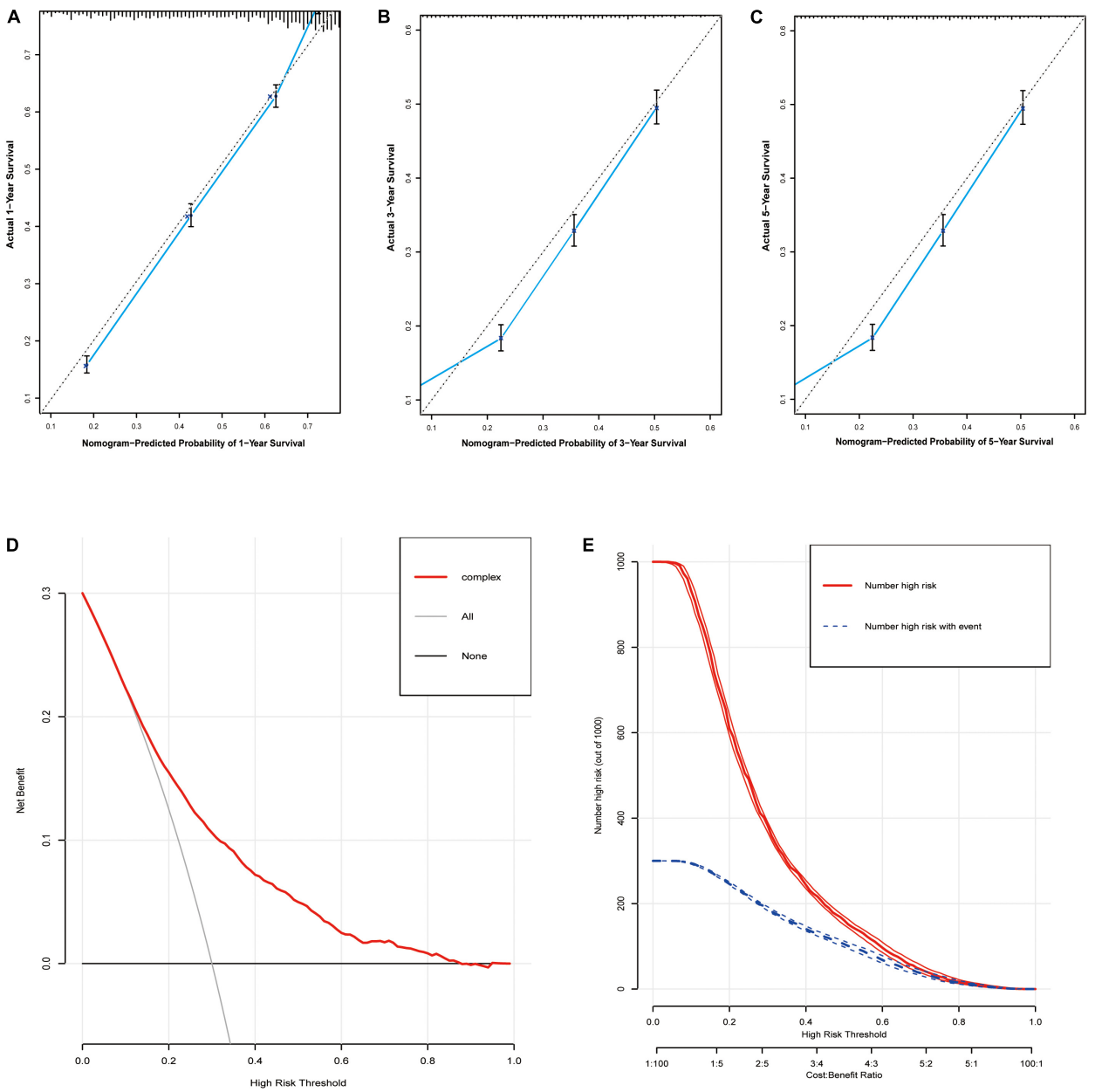

FIGURE 4 | Evaluation of the nomogram for predicting OS in the studied patients. **(A)** The calibration curves of 1-year OS; **(B)** the calibration curves of 3-year OS; **(C)** the calibration curves of 5-year OS; **(D)** decision curves of OS; **(E)** clinical impact curve of the predicted nomogram.

the calculated survival probabilities and generate relevant figures and tables.

DISCUSSION

Metastasis is closely related to the poor prognosis of patients with colon cancer. The liver is the most common organ of distant metastasis in advanced colon cancer (12). Based on the time of occurrence of liver metastasis, there are two types of synchronous and metachronous metastases of colon cancer. SCLM patients are commonly associated with obviously poorer prognoses. It is important to evaluate and predict the survival outcomes of SCLM patients. However, to the best of our knowledge, no nomogram has ever predicted the prognosis of SCLM patients. We extracted SCLM patients from the SEER database and built a predicting model of nomograms.

In our study, we included 22,378 SCLM patients. The low median time and survival rates of CSS and OS indicated that SCLM patients had poor prognoses in both OS and CSS. The lower median survival time is consistent with the reported overall survival time of synchronous colorectal cancer with liver

metastasis patients (18.5 months) (13, 14). Similarly, another population-based study also revealed that the median overall survival time of SCLM patients is 7 months (15).

We analyzed the survival outcomes of included patients stratified by the factors of age, primary tumor site, AJCC stage, and chemotherapy. Our results found that the prognoses of SCLM patients were significantly reduced with the increase of age. Considering the primary tumor location of SCLM patients, SCLM patients with right-sided tumor location were associated with obviously poorer prognosis than those with other tumor sites. Similar results had been reported by some previous studies (13, 14, 16, 17). Compared with left-sided colorectal tumors, the liver metastatic area of right-sided tumors seemed to be more extensive, indicating that these patients had significantly worse prognoses (18). Our results indicated that SCLM patients with positive CEA levels had poorer prognoses than those with negative CEA levels. Previous studies have also demonstrated that CEA levels played an important role in the prognoses of SCLM patients (19–21). The SCLM patients with IVb of the AJCC stage showed obviously worse prognoses than those within the IVa stage. It suggested that SCLM patients combined with another distant organ or peritoneal metastasis had obviously poor prognoses. Other distant metastases, including bone and lung metastases, were important independent risk factors for the prognoses of SCLM patients. The metastasis of CRC to the brain is rare (3). Our results did not detect that brain metastases were significantly related with the prognosis of SCLM patients. It might be affected by the small number of patients with brain metastasis (209, 0.9%). The SCLM patients benefited from partial colectomy and total/subtotal colectomy comparing with those without surgery. Chemotherapy remarkably prolonged the survival time of SCLMs. In the clinical practice, chemotherapy and surgery are the most common effective treatments for SCLM patients due to significantly improved survival time of patients (22, 23).

In our study, the factors, including tumor primary site, tumor size, histological grade, T/N/M stage, surgery of primary site, and chemotherapy showed an association with the prognoses of SCLM patients. Our nomograms of both OS and CSS were built based on these factors. The C-index, calibration curves, and DCAs showed the excellent accuracy and consistency of the prediction models. In order to show the predicted results of our nomograms accurately, we established a user-friendly tool on an online webserver. The tool is available any time any place anywhere on mobile devices. It is convenient to detect the precise prognosis prediction for individual patients. A nomogram of a previous study also indicated that primary tumor location, lung metastasis, and CEA level were independent risk factors for the bone metastasis of colorectal patients (24). In another study, a nomogram was created to predict the probability of liver metastasis in patients with colon cancer (6). Some factors, including age, sex, race, tumor primary site, grade, and T/N stage were integrated in this nomogram. It calibrated well and had a high C-index (0.95). It could be an alternative to predict liver metastasis as a supplement to imaging tests.

There are some limitations in the present study. Firstly, even though we analyzed 22,378 patients, our study is still a retrospective study. There are some inevitable risks of bias and confounding factors in our study, which might influence the accuracy of our results. Secondly, we analyzed the patients from 2010 to 2015. However, the treatments for colon cancer and SCLM have been greatly updated in the past 5–10 years. New strategies, including percutaneous ablations, tumor embolization, and the introduction of new chemotherapeutic regimens as well as immune check point inhibitors are not mentioned. Therefore, the reference value of our results may be limited. High-quality studies with comprehensive and time-updated information are expected in the future. Thirdly, our nomograms are only tested by internal validations. Our results still need to be validated by data from the real world.

CONCLUSION

In conclusion, the SCLM patients had poor prognoses. Variables including age, histological grade, T/N/M stage, tumor size, bone metastasis, lung metastasis, CEA, surgery of primary site, and chemotherapy were independent risk factors for SCLM patients. Nomograms of predicting the prognoses of SCLM patients were established and made available online. The nomograms were validated to be reliable and accurate for predicting the 1-, 3-, and 5-year OS and CSS rates of SCLM patients.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Surveillance, Epidemiology, and End Results (SEER) database (https://seer.cancer.gov/).

AUTHOR CONTRIBUTIONS

Y-JZ collected the data, performed the statistical analysis, and drafted the manuscript. YC substantially revised the manuscript and gave some meaningful suggestions on the modification. H-YH re-revised the manuscript. Y-WZ and Y-TZ supervised the data collection and analysis. J-YL designed the main study. All authors have read and approved the final submitted manuscript.

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Potential of miR-21 to Predict Incomplete Response to Chemoradiotherapy in Rectal Adenocarcinoma

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Background: Patients with locally advanced rectal adenocarcinoma (LARC) are treated with neoadjuvant chemoradiotherapy (CRT). However, biomarkers for patient selection are lacking, and the association between miRNA expression and treatment response and oncological outcomes is unclear.

Objectives: To investigate miRNAs as predictors of response to neoadjuvant CRT and its association with oncological outcomes.

Methods: This retrospective study analyzed miRNA expression (miR-16, miR-21, miR-135b, miR-145, and miR-335) in pre- and post-chemoradiation rectal adenocarcinoma tissue and non-neoplastic mucosa in 91 patients treated with neoadjuvant CRT (50.4 Gy) and proctectomy. Two groups were defined: a pathological complete responders group (tumor regression grade—TRG 0) and a pathological incomplete responders group (TRG 1, 2, and 3).

Results: miR-21 and miR-135b were upregulated in tumor tissue of incomplete responders comparing with non-neoplastic tissue ($p = 0.008$ and $p < 0.0001$, respectively). Multivariate analysis showed significant association between miR-21 in pre-CRT tumor tissue and response, with a 3.67 odds ratio (OR) of incomplete response in patients with higher miR-21 levels ($p = 0.04$). Although with no significance, patients treated with 5-fluorouracil (5-FU) presented reduced odds of incomplete response compared with those treated with capecitabine (OR = 0.19; 95% confidence interval (CI) 0.03–1.12, $p = 0.05$). Moreover, significant differences were seen in overall survival (OS) in relation to clinical TNM stage ($p = 0.0004$), cT ($p = 0.0001$), presence of distant disease ($p = 0.002$), mesorectal tumor deposits ($p = 0.003$), and tumor regression grade ($p = 0.04$).

Conclusion: miR-21 may predict response to CRT in rectal cancer (RC).

Keywords: rectal cancer, chemoradiotherapy response, tumor regression grade, miR-21, biomarkers

INTRODUCTION

Colorectal cancer (CRC) is the third most prevalent neoplasia in the world, and rectal cancer (RC) corresponded to 30% of all colorectal malignancies in 2019 (1). The current treatment for patients with locally advanced rectal adenocarcinoma (LARC) is neoadjuvant chemoradiotherapy (CRT) in order to achieve downstaging, increase R0 resections, allow sphincter-sparing surgery, and decrease local recurrence (LR) (2). After neoadjuvant treatment, patients are restaged and almost 30% develop clinical complete response (cCR) with no residual tumor identified, 46–60% achieve some degree of tumor downstaging, while 30% exhibit resistance to CRT (3). Non-responders are at increased risk of disease progression and unnecessary toxicity caused by CRT.

Recent data suggest that clinical complete responders can safely undergo a conservative approach without surgery (4). By contrast, the European Society for Medical Oncology (ESMO) guidelines recommend upfront surgery in T3a-bN1 tumors if there is no evidence of involvement of the mesorectal fascia (2). Thus, pretreatment prediction of good and bad responders could be important in deciding whether the patient should or not undergo neoadjuvant CRT. Currently, although molecular heterogeneity is a well-recognized feature of most tumors, CRC patients are still treated based solely on clinical stage. The inclusion of molecular markers in a treatment algorithm could potentially stratify patients and thus allow a better choice of candidates. No biomarkers are yet validated for selection of patients for CRT.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs that act as post-transcriptional regulators binding a variety of messenger RNA targets, inhibiting its translation. Although the precise biological role of many miRNAs is yet to be entirely elucidated, up to 30% of the human genome is regulated by these molecules through influence in relevant cellular functions, including stress responses, angiogenesis, metastasis, and programmed cell death (5). Carcinogenic pathways are regulated by miRNAs and their potential role in oncogenesis raised the possibility of being used as biomarkers in cancer treatment response or prediction of prognosis (6).

Although most published data is on colon cancer, some studies have addressed RC differentiating the miRNAome between these two malignancies. Moreover, specific miRNAs have been proposed as predictors of response to CRT in RC although with some inconsistent findings (7–11). These results need to be validated and are mostly related to 5-fluorouracil (5-FU)-based therapies, not much being known about miRNAs as biomarkers of response to capecitabine.

This study aimed to investigate miRNAs as predictors of pathological response to CRT in RC. Based on literature review including our own previously published data (12), five miRNAs were chosen by virtue of having been demonstrated to be potential biomarkers for CRC. Thus, miR-16, miR-21, miR-135b, miR-145, and miR-335 expression was determined and correlated with pathological response and oncological outcomes.

MATERIALS AND METHODS

Patients and Tissue Samples

This was a retrospective study of prospectively analyzed data and samples. Patients with RC (stages I–IV, American Joint Committee on Cancer, AJCC) diagnosed between March 2013 and September 2017 in the Surgical Department of Hospital Beatriz Ângelo (Loures, Portugal) treated with long course CRT and proctectomy were eligible.

Patients had a preoperative staging with pelvic magnetic resonance (MR), thoraco-abdomino-pelvic computed tomography (CT), and endoanal ultrasound when pelvic MR was not clinically possible. Histopathological features were confirmed by pathological analysis and patients were staged according to TNM staging system (8th edition, 2017). Patients with other histological types of rectal malignancy, not submitted to CRT or surgical resection, pregnant, or under the age of 18 were excluded.

Written and signed informed consent for collection and use of biological samples was obtained from all volunteer study participants prior to sample collection. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approval by the institution's Human Research Committee and Ethical Committee on March 13, 2017. The study was registered in the Portuguese Data Protection Agency.

Neoadjuvant Treatment

All patients underwent neoadjuvant CRT consisting of a 2-Gy daily fraction of pelvic irradiation, 5 times a week, in a total of 50.4 Gy. Radiation was delivered with capecitabine (825 mg/m^2/day) or 5-FU (1,000 mg/m^2/day on days 1–5 and days 29–33). All patients except for one received more than 80% of the planned radiotherapy with a curative intent. Surgery was performed 10–12 weeks after CRT.

Assessment of Pathological Response

Pathology specimens were graded by tumor regression grade (TRG) according to the College of American Pathologists guidelines (CAP, TNM 7th edition). TRG was assessed by two pathologists, blinded to patients clinical data, and categorized as TRG 0 (no viable tumor cells or complete response), TRG 1 (single cells or little groups of cancer cells), TRG 2 (residual cancer outgrown by fibrosis), and TRG 3 (minimal or no tumor kill with extensive residual cancer). Tissue was retrieved from formalin-fixed paraffin embedded (FFPE) samples. Histological confirmation of the biopsy samples was done by pathologist review, and neoplastic and adjacent non-neoplastic rectal tissues were differentiated based on hematoxylin and eosin (H&E) stain. A fixed amount of tissue (80 μm) across the samples was extracted for RNA isolation. Pre-CRT RC biopsies (colonoscopy) were obtained from complete and incomplete responders as well as post-CRT tumor tissues (protectomy specimen) from incomplete responders. To allow a direct comparison of RC to matched non-neoplastic rectal mucosa, we collected adjacent (>1 cm distant) non-tumor tissue in both biopsies and

TABLE 1 | Patient clinical parameters.

Clinical parameters		Patients ($n = 91$)
Gender, *n* (%)	Male	60 (66)
	Female	31 (34)
Age, median		68 (45–83)
BMI, median		26 (15–45)
ASA score, *n* (%)	Not discriminated	11 (12)
	I	2 (2)
	II	56 (62)
	III	21 (23)
	IV	1 (1)
Grade	G1/G2	85 (93)
	G3/G4	6 (7)
Location (%)	1/3 superior	19 (21)
	1/3 medium	28 (31)
	1/3 inferior	44 (48)
Tumor extension (mm), median		58 (5–120)
Distance to anal verge (mm), median		60 (0–130)
cT	1	1 (1)
	2	10 (11)
	3	64 (70)
	4	16 (18)
cN	0	9 (10)
	+	82 (90)
cM	0	78 (86)
	1	13 (14)
CRM, *n* (%)	Free	67 (74)
	Threatened or invaded	24 (26)
EMVI, *n* (%)	Negative	86 (95)
	Present	5 (5)
c Stage, *n* (%)	I	3 (3)
	II	8 (9)
	III	68 (75)
	IV	12 (13)
CEA (mg/mL)		1.9 (0.5–163)
Chemotherapy	Capecitabine based	83 (91)
	5-FU based	8 (9)
TRG (CAP), *n* (%)	0	15 (17)
	1	24 (26)
	2	33 (36)
	3	19 (21)

BMI, Body Mass Index; ASA, American Society of Anaesthesiologists; CRM, circumferential resection margin; EMVI, extramural vascular invasion; CEA, carcinoembrinonary antigen; TRG, tumor regression grade; CAP, College of American Pathologists.

protectomy specimens. Two groups of patients were defined, including a pathological complete responders group (TRG 0) and a pathological incomplete responders group (TRG 1, 2, and 3).

RNA Isolation

For total RNA isolation, pre- and post-CRT FFPE non-neoplastic and tumor rectal tissue samples were first deparaffinized with xylene (VWR International, Radnor, PA, USA) in two washing steps at 50°C. The samples were then fully homogenized into fine particles in 100% ethanol using a motor-driven grinder and centrifuged at maximum speed for 5 min. The collected pellet was rehydrated with 95% ethanol for 10 min following a new centrifugation step at maximum speed for 5 min. Then, samples were lysed with 500 μg/mL proteinase K in 100 μL of protease digestion buffer (20 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$ 0.5% SDS) at 55°C. Total RNA was isolated using Ribozol™ reagent (VWR International, Radnor, PA, USA) according to the manufacturer's instructions and eluted into 20 μL RNase-free water. For a better evaluation of miRNAs quantity in total RNA, the miRNA concentration was determined using Qubit™ miRNA Assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

Expression Analysis by Real-Time PCR (RT-PCR)

cDNA synthesis was performed using TaqMan® Advanced miRNA cDNA synthesis kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For a uniform quantification of the quantity of miRNA to be used in cDNA, 2 μL of total RNA (corresponding to 2 ng of RNA) was extended by a 3′ poly-A tailing reaction and a 5′ adaptor ligation to the mature miRNAs. miRNAs were reverse transcribed into cDNA by reverse transcription using Universal RT primers. In order to improve detection of low-expressing miRNA targets, a pre-amplification of the cDNA was performed using the Universal miR-Amp Primers and miR-Amp Master Mix to uniformly increase the amount of cDNA for each target, maintaining the relative differential expression levels. cDNA samples were stored at −20°C. Real-time polymerase chain reaction (PCR) was performed on a Quantstudio™ 7 Flex real-time PCR instrument (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with TaqMan™ Advanced microRNA Assays (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) to assess the expression profile of hsa-miR-16-5p (Assay ID 477860_mir), hsa-miR-135b-5p (Assay ID 478582_mir), hsa-miR-145-5p (Assay ID 477916_mir), hsa-miR-335-5p (Assay ID 478324_mir), and hsa-miR-21-5p (Assay ID 477975_mir). All reactions were performed in duplicate.

Due to the fact that a consensual endogenous control for miR expression in rectal tissue has still not been determined, initial preliminary analyses were performed to test several miRNAs as controls. Normalization was then performed with hsa-miR-484 (Assay ID 478308_mir), identified as the most stably expressed miRNA with the lowest expression variability between samples in these patient data set when compared with mir-1228-5p, miR-345-5p, and miR-103a-3p and the small nuclear (snRNA) U6 and RNU6B, some considered controls for CRC tissues. Expression levels were calculated by the threshold cycle ($2^{-\Delta\Delta Ct}$ method) where $\Delta\Delta Ct$ = (Ct target miR − Ct control) sample − (Ct target miR − Ct control) median, when amplification values were detected in the real-time PCR. Due to lack of amplification values detected by the real-time PCR in all patient tissues,

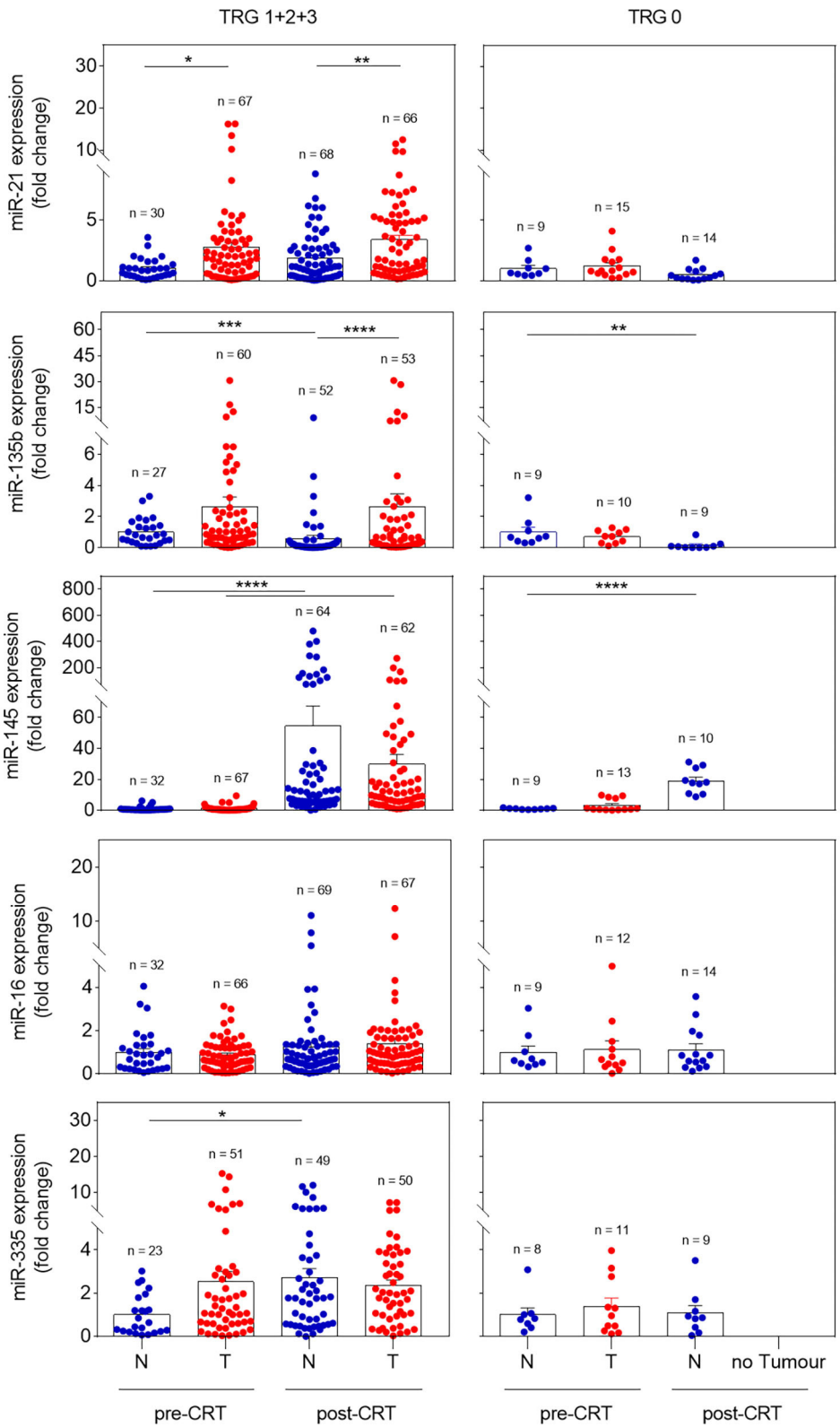

FIGURE 1 | Expression profile of miR-21, miR-135b, miR-145, miR-16, and miR-335 in pre- and post-CRT non-neoplastic and tumor tissues in incomplete (TRG 1 + 2 + 3) and complete responders (TRG 0). Pre-CRT non-neoplastic tissue samples used in this study were derived from a maximum of 37 and 10 patients in TRG 1 + 2 + 3 and TRG 0 groups, respectively. Pre-CRT tumor tissue and post-CRT tissue samples were analyzed from a maximum of 76 patients (TRG 1 + 2 + 3) and 15 patients (TRG 0). Data are mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$), in which N corresponds to non-neoplastic tissue and T to tumor tissue.

TABLE 2 | Association between miRNA expression and TRG.

Variables		OR	95% CI	*p*-Value
miR-21	≤0.66	1.00		
Pre-CRT non-neoplastic	>0.66	1.428	0.32–6.79	0.6407
miR-21	≤1.18	1.00		
Pre-CRT tumor	>1.18	3.58	1.13–12.65	0.0346
miR-135b	≤0.8	1.00		
Pre-CRT non-neoplastic	>0.8	1.85	0.40–10.27	0.4420
miR-135b	≤1.01	1.00		
Pre-CRT tumor	>1.01	2.33	0.58–11.62	0.25
miR-145	≤1.28	1.00		
Pre-CRT non-neoplastic	>1.28	0.65	0.11–5.18	0.643
miR-145	≤0.73	1.00		
Pre-CRT tumor	>0.73	0.88	0.26–3.02	0.838
miR-16	≤0.77	1.00		
Pre-CRT non-neoplastic	>0.77	2.00	0.44–10.80	0.3806
miR-16	≤0.54	1.00		
Pre-CRT tumor	>0.54	1.75	0.49–6.19	0.375
miR-335	≤1.16	1.00		
Pre-CRT non-neoplastic	>1.16	4.5	0.64–91.58	0.191
miR-335	≤1.01	1.00		
Pre-CRT tumor	>1.01	1.86	0.49–7.24	0.354

Simple logistic regression using miRNA dichotomized according to cut-offs determined with ROC curve analysis. OR, odds ratio of incomplete/non-response; CI, confidence interval.

a variable number of samples were included in each miRNA expression profile.

Statistical Analysis

The estimated sample size was 86 patients (43 patients per group of low and high miR expression). Sample size was calculated with an estimated proportion of patients TRG 0 with high and low miR-21 expression of 0.067 and 0.35, respectively. Type I and II errors were set at $\alpha = 0.05$ and $\beta = 0.2$, respectively. miRNA expression was analyzed using the GraphPad Prism software package, version 7.0 (GraphPad software Inc., San Diego, CA, USA). Normal distribution was determined using the D'Agostino and Pearson omnibus test. Data was analyzed according to normality of values distribution using the one-way analysis of variance (ANOVA) followed by Kruskal–Wallis non-parametric Dunn's multiple comparison test or ANOVA Tukey's multiple comparisons test according to Gaussian distribution.

Receiver operating characteristic curve (ROC) analysis was then conducted, establishing the optimal cutoffs for each miRNA before CRT in non-neoplastic and tumor tissue, determined as the point closest to the top left part of the plot with perfect sensibility and sensitivity. All miRNAs were dichotomized according to these cutoffs. Further analysis was also performed to explore the best discriminative cutoff point for miR-21 by comparing the cutoff determined in this study (1.18) with the previously reported miR-21 cutoff (2.8) (13). Both cutoffs presented a similar area under the curve (AUC), with our cutoff having an AUC value of 0.65 (95% CI = 0.518–0.790), a higher specificity (66 vs. 60%), a lower sensitivity (64 vs. 87%), a similar positive predictive value (PPV) (92 vs. 90%) and a lower negative predictive value (NPV) (29 vs. 43%) (**Supplementary Figure 1** and **Supplementary Table 1**). Although both dichotomizations presented similar performance, we chose the cutoff determined in this study that yielded a better-distributed categorization of miR-21.

Simple and multiple logistic regressions were used to correlate each variable with the outcome response after CRT: "pathological complete response (TRG 0)" or "pathological incomplete response (TRG 1, 2, and 3)." For continuous variables, linearity of the logit in the predictor was assessed using a cubic spline and Wald test of linearity.

The association between high and low miR-21 expression and clinical characteristics was tested with chi-square test. Only variables with $p \leq 0.25$ in simple logistic regression or considered clinically relevant were selected to multiple logistic regression. Multicollinearity was also analyzed through the observation of variance inflation factors. A stepwise both-selection technique was used to create the multiple regression model. ROC curve was computed and the respective AUC was calculated to assess discriminatory ability of the model.

RESULTS

Patient Clinical Parameters

Demographic and clinical parameters of the 91 patients are summarized in **Table 1**. With 4 patients lost (4.4%), median follow up was 4.2 years.

miRNA Expression in Complete and Incomplete Responders

miRNA expression profiles were analyzed in non-neoplastic and tumor rectal tissue before and after CRT in all 91 patients. Significant changes were observed when comparing incomplete and complete responders (**Figure 1**). In incomplete responders, miR-21 revealed higher expression in pre-CRT tumor tissue in comparison with non-neoplastic tissue ($p = 0.03$). Post-CRT samples also presented higher levels of miR-21 in tumor tissue ($p = 0.008$). In contrast, in complete responders, miR-21 showed similar levels in pre-CRT tumor and non-neoplastic tissue.

miR-135b presented a profile equivalent to miR-21. In incomplete responders, miR-135b upregulation was detected in tumor tissue, either pre- or post-CRT ($p < 0.0001$), whereas in complete responders equal levels were found in pre-CRT tumor samples and non-neoplastic tissue. Although miR-145 expression showed significant differences among pre- and post-CRT non-neoplastic and tumor tissues ($p < 0.0001$) in incomplete responders, similar results were detected in complete responders, suggesting a lack of discriminative value of this miRNA.

Moreover, there were no significant differences in miR-16 and miR-335 expression between groups. Thus, these results suggest that miR-21 and miR-135b might be useful biomarkers to predict treatment response.

Identification of miRNAs Involved in TRG

The significantly different expression of miRNAs between incomplete (TRG 1, 2, and 3) and complete responders (TRG

TABLE 3 | Clinical parameters and TRG in miR-21 expressing patients.

Simple logistic regression		TRG 0 $n = 15$	TRG 1 + 2 + 3 $n = 67$	OR	95% CI	p-Value
Continuous variables		**Median (Max–Min)**	**Median (Max–Min)**			
Age		67.0 (53–81)	68 (45.0–83)	1.00	0.94–1.06	0.976
Weight		70.0 (45–113)	68 (44.0–119)	0.99	0.96–1.03	0.645
BMI		25.0 (19–41)	26 (15.0–45)	1.00	0.91–1.13	0.921
Tumor extension (mm)		54.5 (21–110)	56 (5–120)	0.99	0.97–1.03	0.901
CEA		2.8 (0.5–8.3)	1.9 (0.5–163)	1.07	0.99–1.29	0.299
Weeks post-chemo		11 (7.0–28)	10 (2.0–21)	0.87	0.73–1.01	0.081
Categorical variables		**Number**	**Number**			
Gender	Male	11	45	1.00		
	Female	4	22	1.34	0.41–5.29	0.643
Tumor location	0	3	14	1.00		
	1	8	16	0.43	0.08–1.81	0.271
	2	4	37	1.98	0.35–10.13	0.407
ASA	1 + 2	9	54			
	3 + 4	6	13	0.36	0.11–1.24	0.0955
CRM MR	Free	11	50		1.00	
	Threatened	1	4	0.88	0.12–18.11	0.913
	Invaded	3	13	0.95	0.25–4.66	0.947
Extramesorectal nodes	Negative	12	43	1.00		
	Positive	3	24	2.23	0.63–10.50	0.247
cT	1 + 2	1	8	1.00		
	3 + 4	14	59	0.53	0.03–3.23	0.561
cN	0	2	6	1.00		
	1	13	61	1.56	0.21–7.721	0.608
cM	0	14	57	1.00		
	1	1	10	2.46	0.42–46.96	0.41
Stage	I	1	2	1.00		
	II	2	5	1.25	0.04–23.53	0.880
	III	11	51	2.32	0.10–26.38	0.508
	IV	1	9	4.50	0.14–156.82	0.352
Stage	I + II	3	7	1.00		
	III + IV	12	60	2.14	0.42–8.99	0.315
Chemotherapy	Capecitabine	12	64	1.00		
	5-FU	3	3	0.188	0.03–1.12	0.05

Simple logistic regression analysis using TRG as dependent variable and clinical/molecular variables as independent variables. From the initial group of 91 patients, 82 expressed miR-21.

TRG, Tumor regression grade; OR, odds ratio of incomplete response; CI, confidence interval; BMI, body mass index; CEA, carcinoembrionary antigen; ASA, American Society of Anaesthesiologists; CRM, circumferential resection margin; MR, magnetic resonance.

0) suggested a possible association between miRNA expression and treatment response. The relation between miRNA in pre-CRT samples and response was analyzed with logistic regression (**Table 2**). A significant association was found between miR-21 in pre-CRT tumor tissue and TRG. Patients with expression higher than 1.18 (fold change) were 3.58 more likely to obtain an incomplete response than those with expression lower than 1.18 ($p = 0.03$). However, there was no association between pre-CRT non-neoplastic or tumor tissue expression of miR-135b and TRG. The same was found for miR-16, miR-145, and miR-335. Given the association of miR-21 and response, we proceeded with the study of this miRNA.

Clinical Parameters and TRG in miR-21 Expressing Patients

From the initial group of 91 patients, only 82 patients expressed miR-21 due to lack of amplification. Although with no significant association between type of radio-sensitizing agent and TRG, patients treated with 5-FU presented reduced odds ratio (OR) of incomplete response compared with patients treated with capecitabine [OR = 0.19; 95% confidence interval (CI) 0.03–1.12, $p = 0.05$]. It was also recognized a definitive trend toward reduced odds of incomplete response with longer waiting times (OR = 0.87; 95% CI 0.73–1.01, $p = 0.08$). However, there was no association between patient gender, age, weigh, American Society of Anaesthesiologists (ASA) score, body mass index

TABLE 4 | Clinical parameters and levels of miR-21 expression.

Variables		Number (%)	High miR-21	Low miR-21	*p*-Value
miR-21 pre-CRT tumor		82 (100)	48 (58.5)	34 (41.5)	
Age	<60	15 (18.3)	7 (14.6)	8 (23.5)	0.302
	≥60	67 (81.7)	41 (85.4)	26 (76.5)	
Sex	Male	56 (68.3)	32 (66.7)	24 (70.6)	0.707
	Female	26 (31.7)	16 (33.3)	10 (29.4)	
BMI	Low weight	1 (1.2)	0 (0)	1 (2.9)	0.236
	Normal	27 (32.9)	17 (35.4)	10 (29.4)	
	Pre-obesity	39 (47.6)	25 (52.1)	14 (41.2)	
	Obesity	15 (18.3)	6 (12.5)	9 (26.5)	
ASA score	1	2 (2.4)	1 (2.1)	1 (2.9)	0.330
	2	53 (64.6)	29 (60.4)	24 (70.6)	
	3	18 (22)	11 (22.9)	7 (20.6)	
	4	1 (1.2)	1 (2.9)	0 (0)	
	ND	8 (9.8)	7 (14.6)	1 (2.9)	
Stage pre-CRT	I	3 (3.7)	1 (2.1)	2 (5.9)	0.720
	II	7 (8.5)	4 (8.3)	3 (8.8)	
	III	62 (75.6)	36 (75.0)	26 (76.5)	
	IV	10 (12.2)	7 (14.6)	3 (8.8)	
Stage post-CRT	0	12 (14.6)	6 (12.5)	6 (17.6)	0.607
	I	6 (7.3)	4 (8.3)	2 (5.9)	
	II	6 (7.3)	5 (10.4)	1 (2.9)	
	III	9 (11.0)	4 (8.3)	5 (14.7)	
	IV	3 (3.7)	1 (2.1)	2 (5.9)	
	NA	5 (6.1)	4 (8.3)	1 (2.9)	
	ND	41 (50)	24 (50.0)	17 (50.0)	
Grade pre-CRT	Low	77 (93.9)	45 (93.8)	32 (94.1)	1.00
	High	5 (6.1)	3 (6.2)	2 (5.9)	
cT	1	1 (1.2)	1 (2.1)	0 (0.0)	0.852
	2	8 (9.8)	5 (10.4)	3 (8.8)	
	3	59 (72.0)	34 (70.8)	25 (73.5)	
	4	14 (17.1)	8 (16.7)	6 (17.6)	
cN	0	8 (9.8)	4 (8.3)	4 (11.8)	0.606
	1	74 (90.2)	44 (91.7)	30 (88.2)	
cM	0	71 (86.6)	41 (85.4)	30 (88.2)	0.712
	1	11 (13.4)	7 (14.6)	4 (11.8)	
pTRG	TRG 0	15 (18.3)	5 (10.4)	10 (29.4)	0.064
	TRG 1	21 (25.6)	16 (33.3)	5 (14.7)	
	TRG 2	32 (39.0)	20 (41.7)	12 (35.3)	
	TRG 3	14 (17.1)	7 (14.6)	7 (20.6)	
Distant recurrence	No	60 (73.2)	33 (68.8)	27 (79.4)	0.283
	Yes	22 (26.8)	15 (31.2)	7 (20.6)	
Local recurrence	No	75 (91.5)	43 (89.6)	32 (94.1)	0.694
	Yes	7 (8.5)	5 (10.4)	2 (5.9)	
Death	No	61 (74.4)	33 (68.8)	28 (82.4)	0.164
	Yes	21 (25.6)	15 (31.2)	6 (17.6)	

From the initial group of 91 patients, 82 expressed miR-21.

ASA, American Society of Anaesthesiologists; BMI, body mass index; CRT, chemoradiotherapy; pTRG, pathological tumor regression grade.

(BMI), tumor location, tumor extension, histological grade, pre-therapeutic carcinoembrionary antigen (CEA), radiological involvement of the circumferential resection margin (CRM), presence of extramural vascular invasion (EMVI), mesorectal deposits (N1c), extramesorectal nodes, cT, cN, cM, stage (TNM, AJCC), and TRG (**Table 3**).

TABLE 5 | Association between clinical parameters and TRG.

Variables		OR	95% CI	*p*-Value
Stage	1 + 2	1.00		
	3 + 4	2.16	0.388–10.16	0.341
miR-21	≤1.18	1.00		
	>1.18	3.67	1.126–13.49	0.036
ASA score	1 + 2	1.00		
	3 + 4	0.33	0.090–1.185	0.082

Multiple logistic regression analysis using TRG as dependent variable and disease stage, miR-21 and ASA score as independent variables.
OR, odds ratio; CI, confidence interval; ASA, American Society of Anaesthesiologists.

Clinical Parameters and Levels of miR-21 Expression

Although no statistically significant association between clinical parameters and expression of miR-21 was observed, a near significant association was established between this miRNA and TRG, with higher proportion of incomplete response in patients with higher miR-21 levels ($p = 0.06$) (**Table 4**). In multivariate analysis, after adjustment for clinically and statistically relevant variables (disease stage and ASA score), this association was again demonstrated with odds of incomplete response 3.67 times greater in individuals with a miR-21 overexpression (>1.18-fold change) when compared with those with lower miR-21 levels (≤1.18-fold change) (95% CI 1.13–13.5; $p = 0.04$) (**Table 5**).

Oncological Outcomes

Overall survival (OS) at 2 and 5 years was 90% (95% CI 83.4–96.9) and 72% (95% CI 61.6–85.1), respectively. Overall disease-free survival (DFS) at 2 and 5 years was 74.1% (95% CI 64.4–84.8) and 66% (95% CI 55–80), respectively (**Figure 2**).

Overall survival was not influenced by age, gender, tumor location, grade, mesorectal nodes, extramesorectal nodes, type of radio-sensitizing agent, post-operative complications, and levels of miR-21 ($p = 0.36$) (**Figure 3** and **Supplementary Figure 2**). As expected, there was an impact in OS in relation to T ($p < 0.0001$) mesorectal tumor deposits, N1c ($p = 0.003$), distant metastasis M ($p = 0.002$), stage ($p = 0.0004$), and TRG ($p = 0.04$) with a borderline significance for threatened circumferential resection margin, CRM ($p = 0.05$) (**Figure 3**). Also, there was increase death risk in individuals with higher cT (HR = 4.78; 95% CI 1.96–11.66, $p = 0.0006$), higher stage (HR = 11.1; 95% CI 1.34–91.88, $p = 0.03$), threatened mesorectal fascia (HR = 4.24; 95% CI 1.19–15.08, $p = 0.03$), positive N1c (HR = 5.47; 95% CI 1.56–19.14, $p = 0.008$), distant metastasis (HR = 3.78; 95% CI 1.52–9.4, $p = 0.004$), and TRG 3 (HR = 3.25; 95% CI 0.83–12.71, $p = 0.08$). No association was, however, established between miR-21 expression and risk of death (**Table 6**).

Finally, the utility of miR-21 as a predictor of survival was investigated. The model of prediction, in multivariate analysis, adjusted to the most relevant clinical variables, did not show a significant association between risk of death and higher miR-21 expression (HR = 2.68; 95% CI 0.86–8.36, $p = 0.09$) (**Table 7**).

DISCUSSION

Rectal cancer (RC) patients treated with CRT urgently need biomarkers to distinguish responders from non-responders and allow individualized treatment, with non-responders avoiding neoadjuvant therapy and complete responders eluding mutilating resections. In this work, we investigated five miRNAs as biomarkers to predict response to CRT in RC.

miR-145 and miR-335 are acknowledged to act as tumor suppressor genes (14, 15) and miR-145 is overexpressed in post-CRT tumor tissue in comparison with pre-CRT with significant correlation with tumor regression (7). In our work, no differences were detected in these miRNAs before and after CRT and no correlation was found with response. In addition, miR-16 has been described as a tumor suppressor with downregulation predicting poor prognosis in CRC (16). In our study, miR-16 was not a predictor of response either. miR-135b is an oncomiR that often mediates CRC genes whose overexpression has been correlated with tumor stage and poor clinical outcome (17). We have further analyzed its potential as predictor of response to CRT and found significant differences in expression. In incomplete responders, higher miR-135b levels were found in both pre- and post-CRT tumor tissues comparing with non-neoplastic tissues, whereas in complete responders similar expression was obtained in all samples. We could not, however, correlate miR-135b expression with clinical parameters or TRG.

Finally, in our study we found that incomplete responders had higher miR-21 expression in tumor tissue in comparison with non-neoplastic tissue in both pre- and post-CRT samples. In contrast, complete responders had similar levels in all samples. Moreover, an association was discovered between pre-CRT tumor miR-21 levels and TRG, with a 3.67 odds of non-response in patients with expression higher than 1.18 ($p = 0.04$). Higher miR-21 expression in the tumor prior to treatment was indicative of a worst response. As expected, OS was influenced by cT, cM, N1c, TRG, and threatened CRM but no association was noted between risk of death and miR-21 expression. Thus, in this study, we showed that miR-21 expression levels before neoadjuvant therapy had the potential to predict response and that patients with miR-21 overexpression exhibited less response to standard CRT dose. This did not, however, translate in a change in survival.

miR-21 is often upregulated in solid tumors influencing cell proliferation, invasion, and apoptosis (18). Considered to be an oncomiR, multiple studies report its role in CRC biology as a screening, diagnostic, and prognostic biomarker (6, 19–23). Also, miR-21 upregulation has been related to advanced stage, presence of positive lymph nodes, venous invasion, and metastatic behavior (24, 25).

In contrast to colonic cancer, very limited data is available on miRNA expression and response to CRT in RC (26–28) with most patients treated with 5-FU-based therapies and not capecitabine. So far, miR-21 has been described to induce resistance to 5-FU when overexpressed in colon cancer cells (13, 29), which could eventually explain its effect regarding 5-FU-based CRT response.

Literature is controversial regarding the use of miR-21 as biomarker of response in RC. In one study with 76 RC

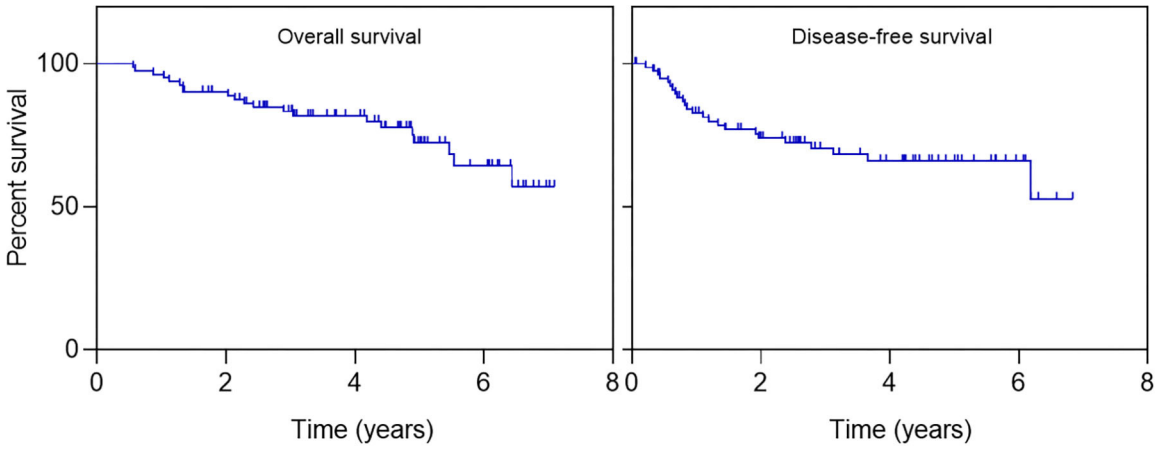

FIGURE 2 | Patient outcomes in miR-21-expressing patients. Kaplan–Meier curves for overall survival and disease-free survival.

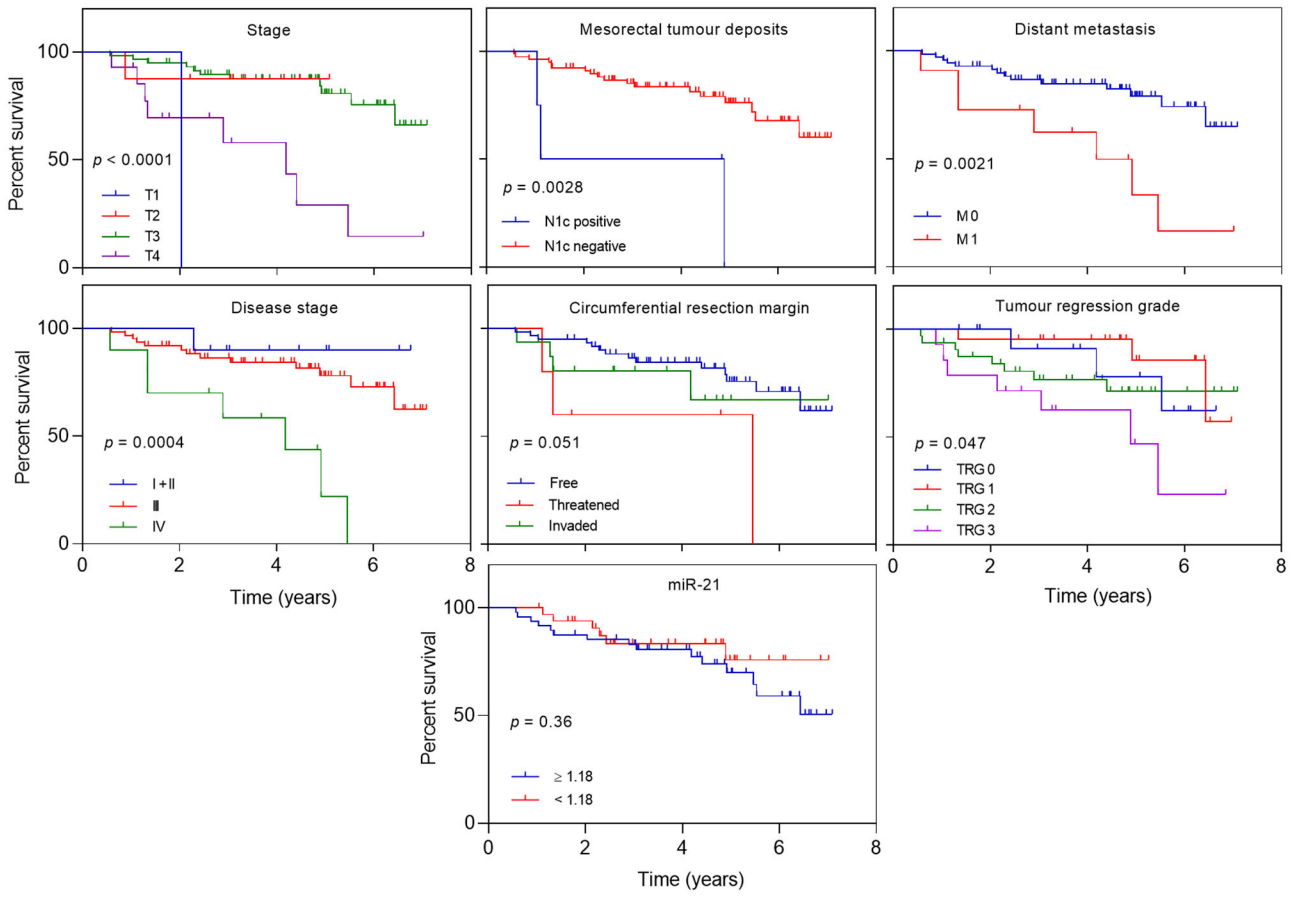

FIGURE 3 | Overall survival according to clinical and oncological parameters. Kaplan–Meier curves estimating overall survival according to stage, mesorectal tumor deposits (cN1c), M, stage, circumferential resection margin (CRM) involvement, tumor regression grade and levels of miR-21.

biopsies, high pre-CRT miR-21 could discriminate responders from non-responders with an OR of 9.75 (95% CI 2.24–42) (30). Recently, 96 complete responders had significantly inferior miR-21 expression comparing with patients with incomplete response ($p = 0.01$), with an AUC of 0.669 (95% CI 0.55–0.79, $p = 0.01$) (31). These observations are in accordance with our own results and with the well-reported miR-21 oncomiR function. Contrarily, in another study, 40 RC patients

TABLE 6 | Patient survival according to miR-21 expression and clinical parameters.

		Patients $n = 82$	Deaths $n = 21$	Survival		Simple cox proportional hazards models			
				Mean	*p*-Value	Coef	HR	95% CI	*p*-Value
miR-21	<1.18	34	6	6.04			1.00		0.36
	≥1.18	48	15	5.50	0.36	0.44	1.56	0.60–4.03	
Age	<60	17	3	5.81	0.58		1.00		0.57
	>60	65	18	5.51		0.35	1.42	0.41–4.8	
Sex	Male	56	16	5.56	0.57		1.00		0.57
	Female	26	5	5.82		−0.29	0.75	0.27–2.04	
Tumor location	1/3 upper	17	3	6.09	0.14		1.00		
	1/3 middle	24	5	6.13		0.05	1.045	0.25–4.40	0.94
	1/3 lower	41	13	5.16		0.91	2.49	0.70–8.85	0.158
ASA score	1 + 2	55	14	5.71	0.97		1.00		
	3 + 4	19	5	5.44		0.10	1.11	0.39–3.094	0.879
	ND	8	2	5.10		0.12	1.12	0.25–4.99	0.986
Stage	I+II	10	1	6.32	0.0004		1.00		
	III	61	13	5.74		0.83	2.31	0.30–17.65	0.4218
	IV	11	7	3.54		2.41	11.10	1.34–91.88	0.0256
Grade	Low	77	19	5.74	0.41		1.00		
	High	5	2	4.87		0.60	1.83	0.42–7.88	0.42
CRM	Free	61	14	5.91	0.051		1.00		
	Threatened	5	3	3.77		1.45	4.24	1.19–15.08	0.025
	Invaded	16	4	5.47		0.51	1.67	0.54–5.142	0.37
EMVI	Negative	77	20	4.45	0.77		1.00		0.768
	Positive	5	1	4.20		0.31	1.36	0.17–10.41	
N1c	Negative	78	18	5.15	0.0028		1.00		0.00788
	Positive	4	3	2.98		1.69	5.47	1.56–19.14	
Extramesorectal nodes	Negative	55	13	5.77	0.26		1.00		
	Positive	27	8	5.15		0.51	1.67	0.68–4.07	0.263
cT	T1-3	68	13	6.05	0.0001		1.00		
	T4	14	8	3.73		1.56	4.78	1.96–11.66	0.0006
cN	0	8	1	6.25	0.42		1.00		
	1	74	20	4.48		0.81	2.24	0.29–16.7	0.432
cM	0	71	14	5.98	0.0021		1.00		
	1	11	7	4.02		1.33	3.78	1.52–9.4	0.00416
TRG	0	15	3	5.94	0.047		1.00		
	1	21	3	6.32		0.49	0.61	0.12–3.05	0.5504
	2	32	8	5.54		0.34	1.41	0.37–5.35	0.6130
	3	14	7	4.31		1.18	3.25	0.83–12.71	0.0897
Chemotherapy	Capecitabine	76	19	5.24	0.47		1.00		
	5-FU	6	2	4.83		0.54	1.71	0.39–7.43	0.476
Post-op complications	Negative	38	9	5.85	0.6		1.00		
	Positive	44	12	5.55		0.23	1.26	0.53–0.98	0.604

Kaplan–Meier estimates, simple cox proportional hazards model. From the initial group of 91 patients, 82 expressed miR-21.
HR, hazard ratios; CI, confidence interval; ASA, American Society of Anaesthesiologists; CEA, carcinoembrionary antigen; CRM, circumferential resection margin; EMVI, extramural vascular invasion; TRG, tumor regression grade.

treated with 5-FU-based CRT had higher miR-21 in post-CRT tumor tissue than in pre-CRT tumor and post-CRT normal tissues (7). It has also been reported overexpression of miR-21 in patients with complete response (32, 33). It is important to note, however, that in one of these studies, the responder group involved a different set of patients, including individuals submitted to surgery with pathological complete response (pCR) and patients with complete clinical response (cCR) not treated with surgery but only observed by follow up (33). The latest might have had undetectable residual disease and not be a real pCR. This different response assessment invalidates an accurate comparison of results and

TABLE 7 | Association between patients survival and miR-21 expression.

		Multiple cox proportional hazards models				Multiple cox proportional hazards models			
		Coef	**HR**	**95% CI**	***p*-Value**	**Coef**	**HR**	**95% CI**	***p*-Value**
miR-21	<1.18			Not included			1.00		
	≥1.18					0.99	2.68	0.86–8.36	0.089
Mesorectal deposits	Negative		1.00				1.00		
	Positive	1.84	6.26	1.74–22.48	0.005	2.49	12.17	2.61–56.70	0.001
cT	T1-3		1.00				1.00		
	T4	1.63	5.09	2.06–12.61	0.0004	1.69	5.45	2.17–13.63	0.0003
C-statistics				0.671				0.674	

Multiple Cox Proportional Hazards Models obtained with stepwise variable selection.
HR, hazard ratios; CI, confidence interval.

may explain the distinct observations when compared with our work.

Overall, the heterogeneity of results is related to the fact that most published studies included patients with colon and RC, 2 distinct entities with different treatment strategies that previous contributions failed to separate. Patient variability, nature of biological samples (blood, tissue, serum, or feces), miRNA extraction, array platforms, bioinformatics analysis, and different TRG grading systems also contribute to these discrepancies. Likewise, it is possible that population may have different miRNA signatures and transcriptome vary according to tumor site.

In this study, we recognized the significance of miR-21 expression in RC in response to neoadjuvant CRT. Although including a sizeable cohort with uniform sampling and treatment, there is a potential for intratumoral heterogeneity and results are currently being validated in a prospective series. If confirmed as a biomarker, translation to clinical practice with miR-21 inclusion in treatment algorithms may allow a stratification of responders and better selection of candidates for CRT.

Of note, in addition to possible markers of response and prognosis at the time of diagnosis, miRNAs may be potential therapeutic targets *via* reintroducing miRNAs absent in carcinogenic pathways or by inhibiting oncomiRs (34–36). Likewise, affecting miRNAs implicated in the mechanism of resistance to CRT may improve the therapeutic outcome. The biggest challenge will continue to be the identification of miRNA targets that shed light on our understanding of downstream cellular mechanisms of resistance to CRT.

In conclusion, the present study suggests miR-21 as a potential biomarker of pathological response in RC. The results provide an association between a miRNA in the neoadjuvant therapy setting and tumor regression with significant implications that strengthen the role of miRNAs as predictors of response. This work further emphasizes the need for prospectively conducted trials of miRNA as biomarkers in RC patients treated with CRT.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institution's Ethical Committee (Comissão de Ética para a Saúde do Hospital Beatriz Ângelo) on 13th March 2017. The study was registered in the Portuguese Data Protection Agency (Comissão Nacional de Protecção de Dados) on 27th January 2017. Written and signed informed consent for collection and use of biological samples was obtained from all volunteer study participants prior to sample collection.

AUTHOR CONTRIBUTIONS

SO: study conception and design, funding, sample collection, sample treatment, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and final approval of the article. CM: miRNA isolation, expression analysis, interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and final approval of the article. SV: statistical analysis of the data and final approval of the article. AC: miRNA isolation, expression analysis, interpretation of the data, and final approval of the article. MF and DA: sample collection and critical revision of the article for important intellectual content. RC and RM: critical revision of the article for important intellectual content and final approval of the article. CR: study design, funding, critical revision of the article for important intellectual content, and final approval of the article. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.577653/full#supplementary-material

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Simultaneous Integrated Boost Intensity-Modulated Radiotherapy for Locally Advanced Drug-Resistant Gastrointestinal Stromal Tumors: A Feasibility Study

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Background: As an emerging clinical problem, locally advanced drug-resistant gastrointestinal stromal tumors (LADRGISTs) has relatively few therapeutic schemes. Although radiotherapy is not often considered for GISTs, it could be a valuable contributing modality. The aim of our study is to explore a safe and effective radiation regimen for LADR-GISTs.

Methods: Three patients with LADR-GISTs were treated with simultaneous integrated boost intensity-modulated radiation therapy (SIB-IMRT) plans. In the SIB-IMRT plans, gross target volume (GTV) was divided into GTV-outer, GTV-mid, and GTV-center. And the prescribed dose of planning gross target volume (PGTV) and GTV-outer were both set to 50.4 Gy in 28 fractions. GTV-mid and GTV-center were simultaneously boosted to 60–62 Gy and 62–64 Gy respectively. For comparison purposes, conventional IMRT (Con-IMRT) plans with uniform dose distribution were generated for same optimization objectives without a dose boost to GTV-mid and GTV-center. All plans were optimized to make sure that deliver at least 95% of the prescription dose was delivered to PGTV. Isodose distribution, dose profiles, conformity indexes (CIs), monitor units (MUs), and dose volume histogram (DVH) was evaluated for each individual patient. After the three patients were treated with SIB-IMRT plans, the relative changes in the tumor size and CT values by CT scanning were also tracked.

Results: Compared with Con-IMRT plans, SIB-IMRT plans saw a significant increase from D_{95} to D_2 of the GTV. With steeper dose gradients in the dose profiles, SIB-IMRT plans had GTV-mid and GTV-center accumulated with higher dose mainly by delivering extra 93 MUs in average. However, there was no significant difference in CIs and organs at risks (OARs) DVH. The relative changes in tumor size and CT values of the three patients in follow up were up to the Choi criteria and the three patients were all assessed as partial response.

Conclusions: The proposed SIB-IMRT may be a potential technique for achieving objective response and prolonging survival of selected GISTs patients.

Keywords: gastrointestinal stromal tumors, locally advanced, drug-resistant, simultaneous integrated boost, intensity-modulated radiation therapy

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasm of the gastrointestinal tract, arising from the interstitial cells of Cajal. The relevant researches reported the pathogenesis of GISTs are mainly related to mutations in the tyrosine kinase receptor (KIT) and/or platelet-derived growth factor receptor alpha (PDGFRA) gene (1–3). Currently, GISTs are typically treated with resection and adjuvant therapy with tyrosine kinase inhibitors (TKIs) at high risk for recurrence (4, 5). Unresectable or metastatic tumors are treated primarily by TKIs therapy (6, 7). Under the current treatment guidelines, radiotherapy is not a recommended option, or is only used for palliative intent of bone metastases (8).

Historically, GISTs have been considered to be relatively insensitive to radiotherapy, just as most other soft issue sarcomas. So far, a significant number of publications have demonstrated that GISTs are not uniformly radioresistant and radiotherapy could be beneficial to the management of GISTs. Pollock et al. (9) presented that a patient who underwent 50.4 Gy postoperative radiation after a R1 resection of a 7-cm rectal GIST, did not relapse with two years. Ciresra et al. (10) reported that radiotherapy combined with TKIs therapy resulted in a lesion reduction in a case of rectal GIST. They concluded that a pathologic complete response can be achieved with a dose of 50.4 Gy. Subsequently, a number of case reports provided insight into the efficacy of radiotherapy (11–13). In a retrospective series of 15 patients, Cuaron et al. (14) suggested that GISTs were more sensitive to a higher radiation dose. After reviewing the literature of radiotherapy in rectal GISTs, Ozkan (15) demonstrated that GISTs were radiosensitive in long-term local control and most patients could benefit from radiotherapy with palliative, adjuvant or definitive intent. According to the above, in certain circumstances, GISTs are radiosensitive and radiotherapy can be a valuable alternative in GISTs management.

According to the previously mentioned, radiotherapy could be regarded as a promising and viable option for GISTs. However, radiotherapy for GISTs was still limited to dose-limiting toxicity of the adjacent small bowel. In recent years, intensity-modulated radiation therapy (IMRT), image-guided radiation therapy (IGRT) and other technological advances has realized dose escalation in target volume and potential reduction in acute and delayed toxicity by facilitating treatment delivery and normal tissue protection (6). Moreover, simultaneous integrated boost intensity-modulated radiation therapy (SIB-IMRT) can deliver the highest possible dose to target volume and increase tumor response without significant increase of healthy tissue irradiation (16, 17). This technique has been successfully applied to the several types of bulky tumors, such as esophageal cancer, head and neck tumors, lung cancers, pelvic tumors, and soft tissue sarcomas (18–22). A better biochemical control can be achieved in SIB-IMRT by increasing dose (23). Therefore, SIB-IMRT may offer a valuable alternative option for patients of moderately radiosensitive GISTs.

In current clinic practice, locally advanced drug-resistant GISTs (LADR-GISTs) that are technically unresectable and failed in systemic TKIs therapies have emerged as a common clinical problem, with relatively few therapeutic schemes. To the best of our knowledge, there is no report about SIB-IMRT for LADR-GISTs. In this study, we designed a novel SIB-IMRT plan and the dose was gradually escalated from the peripheral region of GTV to the center region. The focus of this study was to compared efficacy and toxicity between conventional IMRT (Con-IMRT) plans and SIB-IMRT plans and explore a safe and practical radiation regimen for LADR-GISTs.

METHODS AND MATERIALS

Patient, Tumor, and Treatment Characteristics

From 2016 to 2019, three patients with LADR-GISTs were treated with SIB-IMRT. The enrolled patients are 62, 50, and 56 years old at diagnosis. They underwent R_0 resection of the primary tumor as soon as the disease was detected, and then started on systemic TKIs therapies. After a period of time (median time: 3 years), their tumors recurred due to drug resistance. Moreover, the progression of lesions were detected in all enrolled patients. Due to the resistance to TKIs therapy and lack of surgical options, they received radiation therapies to relieve symptoms (such as poor appetite, bloating, abdominal pain, frequent urination and constipation). **Table 1** illustrates the summary of patients, tumors, and treatment characteristics in detail.

Clinical CT Data and Volume Definition

Each patient underwent computed tomography simulation in the supine position using GE CT scanner (GE Medical Systems, Milwaukee, WI). The CT scan covered the total abdomen and pelvic cavity. Moreover, all patients were instructed to drink

TABLE 1 | Patient, tumor, and treatment characteristics.

No.	Age (diagnosis/RT)	Primary tumor site	Initial tumor size	Type of resection	TKIs therapy	Indication for RT	Tumor size before RT	RT site
1	62/67	Small intestine	10cm	R_0	Imatinib/ sunitinib	Progression on TKIs resistance and unresectable	18.0cm	Abdomen and pelvic
2	50/55	Ileum	4.3cm	R_0	Imatinib/ sunitinib	Progression on TKIs resistance and unresectable	17.2cm	Abdomen and pelvic
3	56/60	Jejunum	15cm	R_0	Imatinib/ sunitinib	Progression on TKIs resistance and unresectable	20.0cm	Abdomen and pelvic

**No.: patient number; initial tumor size: the maximum diameter of tumor in CT imaging; R0: no residue under the microscope after surgical. Tumor size before RT: the maximum diameter of tumor in CT imaging; abdomen and pelvic: abdomen and peritoneal seeding mass in pelvic.*

400 ml of water and empty the rectum in one hour prior to the CT scan, and they were advised to follow the same instructions in daily radiotherapy. The gross tumor was defined as GTV. Due to a rare (1-2%) lymph-node metastases in GISTs, the lymphatic drainage of the gross tumor were not irradiated as the clinical target volume (CTV) (24–26). The planning gross target volume (PGTV) was obtained by applying an isocentric margin of 5mm to the GTV. OARs mainly contained the rectum, bladder, and intestines. The rectum ranged from the anus to the junction of the rectal sigmoid colon. Due to the squeezing action of large tumor, it was difficult to distinguish the small bowel and the large intestine. Thus, both of them were included in the intestines in our research. The normal tissue (NT) structure was Body minus PGTV. In addition, GTV was divided into GTV-outer, GTV-mid and GTV-center as required by SIB-IMRT. As shown in **Figure 1**, these structures were detailed in the three standard orthogonal planes. GTV-center was created with an isocentric contraction of 2–3 cm in GTV. GTV-mid was defined as the GTV minus 1-2 cm excluding GTV-center. The rest of GTV was defined as GTV-outer. The contraction in GTV-mid and GTV-center was determined by the relative location between OARs and GTV in order to avoid hotspots in the overlap regions of OARs and GTV. All these structures were contoured with Eclipse v. 13.5 (Varian Medical Systems, Palo Alto, CA) by an experienced oncologist, and were reviewed by another senior oncologist.

Treatment Planning

In this study, Con-IMRT plan and SIB-IMRT plan were offered to each patient. In SIB-IMRT plan, GTV was divided into three parts (GTV-outer, GTV-mid, and GTV-center) to obtain inhomogeneity dose in target volume. Therefore, the prescribed dose of PGTV and GTV-outer were both set to 50.4 Gy in 28 fractions. However, the presibribed dose of GTV-mid and GTV-center were boosted to 60–62 and 62–64Gy respectively. The detailed dose objectives of different patients were listed in **Table 2**. For comparison purposes, the Con-IMRT plans with PGTV and GTV being only set to 50.4 Gy were generated in the same beam arrangement. The optimization objectives of specific structure in Con-IMRT plan for each patient were same with that in SIB-IMRT plan except for GTV. All plans were calculated on a 2.5mm isotropic dose grid with anisotropic analytical algorithm (AAA) through Eclipse v.13.5 (Varian Medical Systems, Palo Alto. CA. USA). They were performed with six MV photon beams from a Varian-21EX linear accelerator. Dynamic MLC delivery (sliding window) was selected as the delivery method. In addition, all plans were made by an experienced medical physicist and reviewed by a senior medical physicist.

Plan Analysis and Evaluation

To perform a better analysis and evaluation, both Con-IMRT plans and SIB-IMRT plans were normalized by having 95% of the PGTV receive 100% of the prescribed dose. Dose-volume histograms (DVH) was applied for calculation and evaluation of GTV, PTV and OARs. The dose profile (along the dashed line drawn in **Figure 2A**, PGTV's conformality indexes (CIs: ratio of total volume receiving 95% of prescription dose to planning target volume receiving 95% of prescription dose) and moniter units (MUs) were obtained for comparison (27). In addition, DVH information of OARs, such as V_{20}, V_{30}, V_{40}, V_{45}, V_{50}, D_{1cc}, and D_{2cc}, was also compared.

Treatment and Follow-Up

All patients received the treatment of SIB-IMRT plans. In order to minimize the influence of structure movement, they were

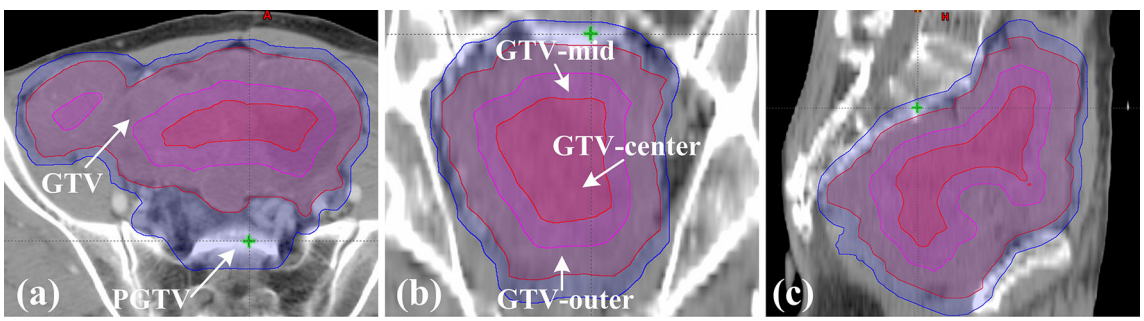

FIGURE 1 | Definition of target volume in patient 1. **(A)** The axial plane; **(B)** the coronal plane; **(C)** the sagittal plane.

TABLE 2 | Dose objectives of gross target volume (GTV) for simultaneous integrated boost intensity-modulated radiation therapy (SIB-IMRT) plans and conventional IMRT (Con-IMRT) plans in three patients.

Category	Structure	Dose objectives (Gy)		
		Patient 1	Patient 2	Patient 3
SIB-IMRT	GTV-outer	D_{100}≥50.4, D1cc ≤ 56	D_{100}≥50.4, D_{1cc} ≤ 56	D_{100}≥50.4, D_{1cc} ≤ 56
	GTV-mid	D_{100}≥60, D1cc ≤ 62	D_{100}≥60, D_{1cc} ≤ 62	D_{100}≥60, D_{1cc} ≤ 62
	GTV-center	D_{100}≥62	D_{100}≥64.4	D_{100}≥64.4
Con-IMRT	GTV	D_{100}≥50.4, D_{1cc} ≤ 56	D_{100}≥50.4, D_{1cc} ≤ 56	D_{100}≥50.4, D_{1cc} ≤ 56

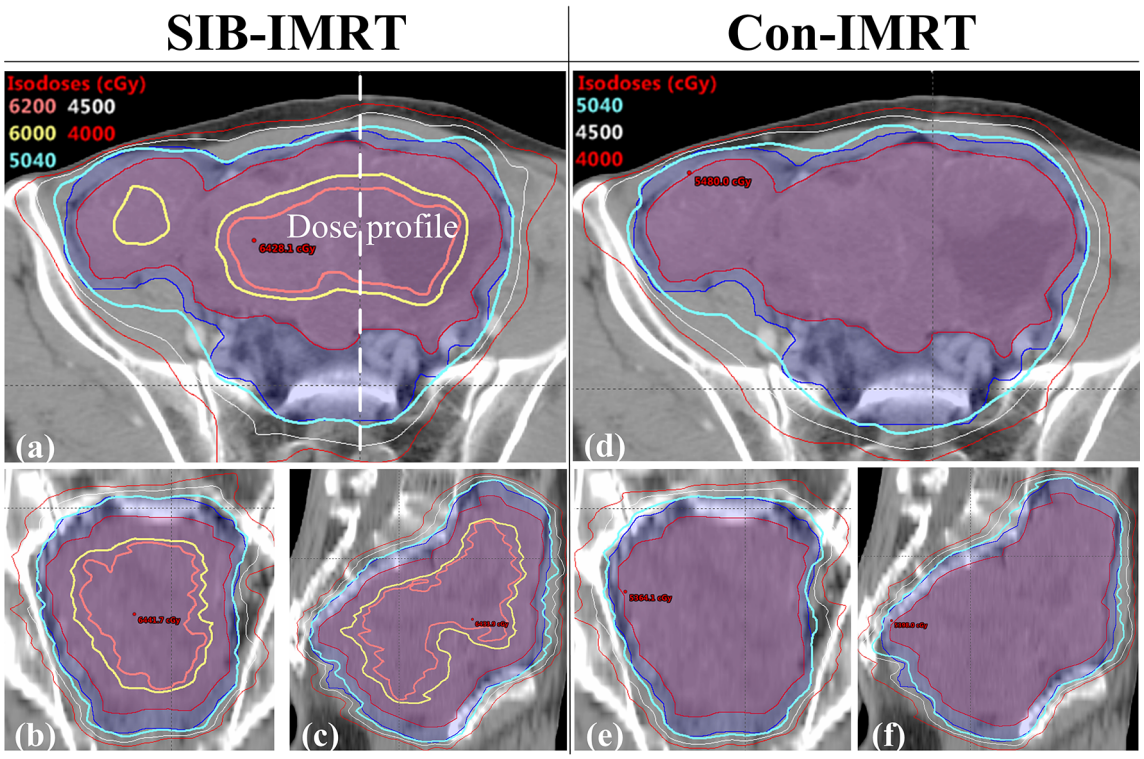

FIGURE 2 | Comparison of the isodose distribution in patient 1. **(A)** the axial plane in the SIB-IMRT plan; **(B)** the coronal plane in the SIB-IMRT plan; **(C)** the sagittal plane in the SIB-IMRT plan; **(D)** the axial plane in the Con-IMRT plan; **(E)** the coronal plane in the Con-IMRT plan; **(F)** the sagittal plane in the SIB-IMRT plan.

advised to they were advised to keep their bladder full and rectum empty during every radiation therapy. Daily cone-beam CT imaging was carried out before daily radiotherapy. CT scanning were provided for all patients in 3 months after final treatment and every 6 months thereafter. In order to perform quantitative evaluation to the response of irradiated lesions, the tumor size of three patients was measured by the maximum diameter in three planes (the axial, coronal, and sagittal plane). Meantime, the corresponding CT values was extracted from the same area with abundant blood supply at the arterial phase. Tumor response to radiotherapy was assessed by Choi criteria. Choi criteria includes the following four response categories: complete response (CR: Disappearance of all target lesions), partial response (PR: Decrease in tumor size ≥10% or decrease in tumor density ≥15% on CT), stable disease (SD: Does not meet the criteria for CR, PR or PD) and progressive disease (PD: Increase in tumor size ≥10% and does not meet PR criteria by tumor density).

RESULTS

Results of Plan Evaluation

Figure 2 shows the isodose distribution comparison of patient 1. The coverage of 4,000 cGy isodose in SIB-IMRT plan was largely consistent with that in Con-IMRT plan. However, the escalating isodose of SIB-IMRT plans in GTV was clearly identifiable in three orthogonal planes. And the GTV-center received a dose in excess of 62Gy (123% of the prescribed dose in PGTV). As shown in **Figure 3**, the dose profile comparison of three patients clearly demonstrated the steeper dose gradients within the GTV for SIB-IMRT plans. It was worth noting that a higher dose was mainly concentrated in GTV-mid and GTV-center. In addition, **Figure 3** shows that the profiles of SIB-IMRT plans excluding PGTV are nearly consistent with that of Con-IMRT plans. The CIs and MUs are shown in **Table 3**. Along with higher boost dose in GTV, the total MUs of SIB-IMRT plans are 93 MUs higher than that of Con-IMRT plans in average. Nonetheless, there is little difference in the CIs between the two plans of each patient.

As shown in **Figure 4**, the dose received in PGTV and GTV from D95 to D2 are significantly increased in SIB-IMRT plans. But the OARs DVH of SIB-IMRT plans was roughly in line with that of Con-IMRT plans. There is also no significant difference in NT structure between two types of plan for each patient.

DVH indexes of each OAR are listed in **Tables 4**–**6** for further analysis. The rectum and bladder had slightly lower volumes at higher dose levels in SIB-IMRT plans. For the bladder of the three cases, V_{30}, V_{50}, D_{1cc}, and D_{2cc} of SIB-IMRT plans are better than Con-IMRT plans. Although other bladder DVH indexes of Con-IMRT plans is a little bit better than that of SIB-IMRT plans, the

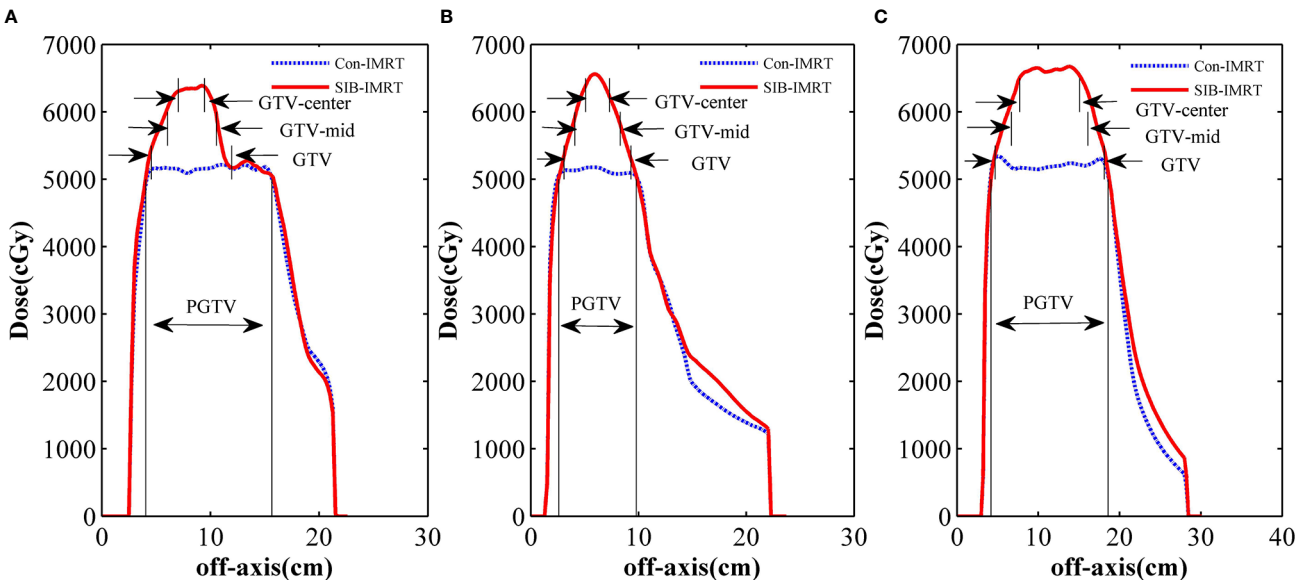

FIGURE 3 | Profile comparisons in three patients. **(A)** Patient 1; **(B)** patient 2; **(C)** patient 3.

TABLE 3 | Comparisons in conformity indexes (CIs) and monitor units (MUs) between conventional intensity-modulated radiation therapy (Con-IMRT) plans and simultaneous integrated boost-IMRT (SIB-IMRT) plans.

Patient No.	Group	CIs	MUs
1	Con-IMRT	0.899	645
	SIB-IMRT	0.901	763
2	Con-IMRT	0.927	326
	SIB-IMRT	0.932	370
3	Con-IMRT	0.911	724
	SIB-IMRT	0.912	843

difference is too small to be clinically significant. Similar results are seen in the rectum and the intestines. On the whole, most of OARs DVH parameters in SIB-IMRT plans were superior to that in Con-IMRT plans.

Follow-Up

During applying SIB-IMRT plans, three patients were well tolerated and their symptoms caused by abdominal mass compression were gradually alleviated. Their abdominal discomfort and deleterious effect disappeared after the end of treatment. As shown in **Figure 5A**, tumor lesion of patient 1 diminished obviously in follow-up CT examination. The gradually decreased size of tumor and CT values were observed in the CT imaging. The relative changes in tumor size and CT values of three patients was tracked in **Figure 5B**. Patient 2 and patient 3 also saw their irradiated lesions continuously shrinked within one year after treatment. More importantly, patient 1 had no tumor progression for nearly 2 years after radiotherapy. Based on the Choi criteria (27), the three patients were assessed as partial response (PR).

DISCUSSION

Rare intra-abdominal tumors, localized GISTs are typically treated with surgical resection. So, TKIs therapy is a recommended option for recurrent, metastatic or unresectable patients. However, it is well-known that about 40–50% of GISTs recurs after surgery. In addition, resistance to TKIs therapy is also a known clinical problem (28). The post-resistance treatment presents a huge challenge for the management of LADR-GISTs. Under this circumstance, radiotherapy may be a valuable alternative in LADR-GISTs with curative intent. In our study, three patients with LADR-GISTs were treated with SIB-IMRT plans respectively. Their irradiated lesions were generally in good control through the subsequent radiological examination. The results demonstrate that SIB-IMRT technique is feasible in LADR-GISTs and the role of radiotherapy in GISTs may have been underestimated (29, 30).

Historically, radiotherapy has been less commonly considered in GISTs due to two reasons: the moderate radiosensitivity in GISTs and the dose-limiting toxicity to adjacent intra-abdominal organs.

First of all, radiotherapy is mainly used for local control of abdominal metastases and relief of symptoms (25). Conventional fractionation and modest cumulative dose were recommended for GISTs. The total bioequivalent dose that was frequently used ranged from 30 to 50 Gy (31). In addition, a uniform dose distribution was commonly recommended within target volume and the maximum dose was limited within 110–115% to the prescription dose. However, in the prospective study of Joensuu et al. (32), only 2 out of 25 GIST patients achieved partial response under conventional radiotherapy. Moreover, the tumor was usually under control only for a few months (12, 33). In fact, GISTs are the commonest sarcoma in the gastrointestinal tract and relatively

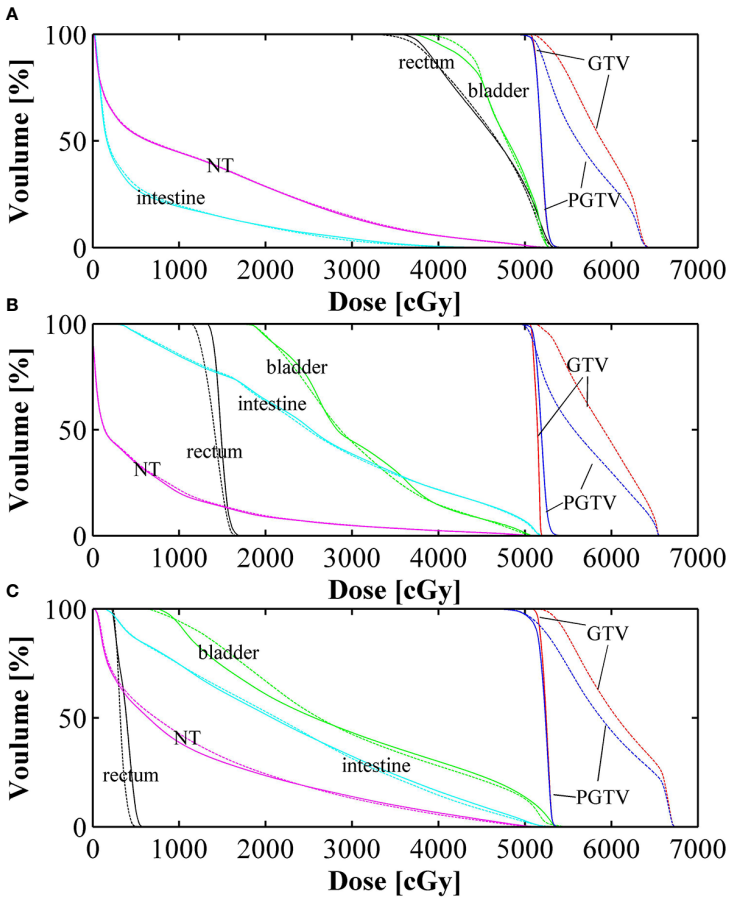

FIGURE 4 | Dose volume histogram (DVH) comparisons between conventional intensity-modulated radiation therapy (Con-IMRT) (solid line) and simultaneous integrated boost-IMRT (SIB-IMRT) (dashed line) for three patients. **(A)** Dose volume histogram (DVH) comparison in patient 1; **(B)** DVH comparison in patient 2; **(C)** DVH comparison in patient 3.

TABLE 4 | Summary of dose volume histogram (DVH)-based analysis for the bladder of the three patients.

No.	Category	V_{20} (%)	V_{30} (%)	V_{40} (%)	V_{50} (%)	D_{1cc} (cGy)	D_{2cc} (cGy)
1	Con-IMRT	100	100	**96**	33	5,210	5,176
	SIB-IMRT	100	100	99	**31**	**5,192**	**5,152**
2	Con-IMRT	**61**	43	30	14	5,322	5,307
	SIB-IMRT	67	**42**	**28**	**13**	**5,314**	**5,246**
3	Con-IMRT	**94**	45	**14**	1	5,038	4,999
	SIB-IMRT	95	**44**	15	1	**5,021**	**4,966**

The better results are bolded.

TABLE 5 | Summary of dose volume histogram (DVH)-based analysis for the rectum of the three patients.

No.	Category	V_{20} (%)	V_{30} (%)	V_{40} (%)	V_{50} (%)	D_{1cc} (cGy)	D_{2cc} (cGy)
1	Con-IMRT	100	100	85	29	5,243	5,193
	SIB-IMRT	100	100	**85**	**27**	**5,221**	**5,166**
2	Con-IMRT	0	0	0	0	1,613	1,585
	SIB-IMRT	0	0	0	0	**1,582**	**1,559**
3	Con-IMRT	0	0	0	0	543	532
	SIB-IMRT	0	0	0	0	**462**	**449**

TABLE 6 | Summary of dose volume histogram (DVH)-based analysis for the intestines of the three patients.

No.	Category	V_{20} (%)	V_{30} (%)	V_{40} (%)	V_{50} (%)	D_{1cc} (cGy)	D_{2cc} (cGy)
1	Con-IMRT	10	4	1	0	4,590	4,449
	SIB-IMRT	10	**3**	1	0	**4,539**	**4,397**
2	Con-IMRT	64	39	22	7	**5,167**	**5,176**
	SIB-IMRT	**63**	**38**	**22**	7	5,192	5,206
3	Con-IMRT	**52**	33	17	3	5,130	5,051
	SIB-IMRT	53	**31**	**14**	**2**	**5,128**	**5,020**

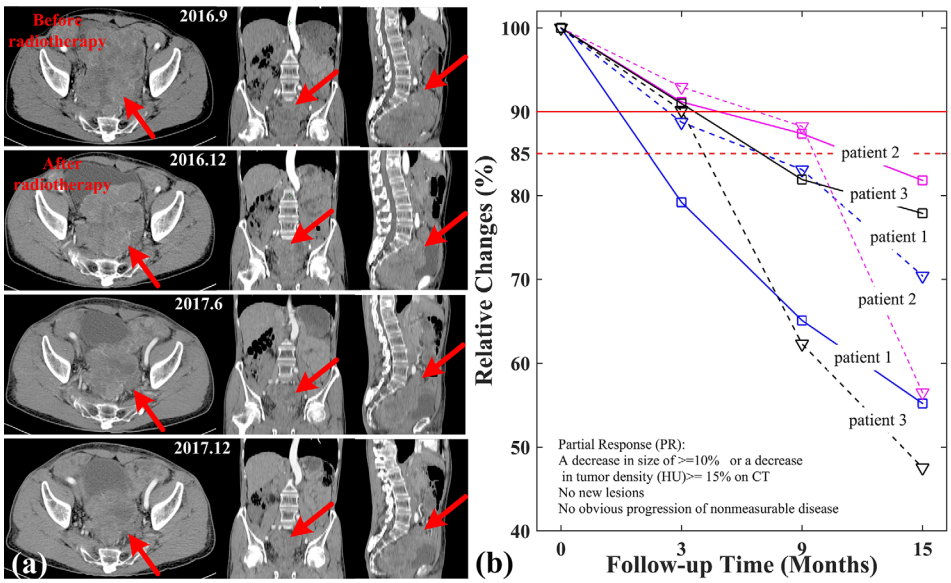

FIGURE 5 | Relative changes of tumor size and CT values in three patients. **(A)** CT imaging of patient 1; **(B)** Relative changes of tumor size (solid line) and CT (dashed line) values in three patients.

resistant to conventional dose schemes (26). Therefore, a higher biological equivalent dose are needed, especially in hypoxic area of tumor central region. Furthermore, an ablative does escalated to the subvolume of tumor has been proven to be more effective such as prostate, liver, or sarcoma as early as 1986 (34). Nomiya et al. (20) and Cilla et al. (17) demonstrated a heterogeneous dose distribution by SIB-IMRT technique could induce a higher rate of tumor cell apoptosis in bulky and hypoxic tumors, that were not controlled using. That cannot be achieved by conventional radiotherapy. In our study, the prescription dose of PGTV and GTV-outer was set to 50.4 Gy for a pathologic complete response (10). Meanwhile, the prescription dose of GTV-mid and GTV-center was boosted up to 60–62 Gy and 62–64 Gy respectively. Therefore, an ablative-like dose distribution was generated by SIB-IMRT and a steeper dose gradient within GTV was observed in **Figure 3**. Although the maximum dose in three patients were respectively escalated up to 129, 135, 131% of the prescribed dose in PGTV, only a slight increase of dose to NT structure were seen from profiles comparison. More importantly, the three patient were well-tolerated during the radiotherapy and continuous reduction in tumor size and CT values were found in the follow-up. The results about an ablative-like dose distribution by SIB-IMRT is feasible for large tumors. That was confirmed again in LADR-GISTs, which were historically considered to be relatively radio-resistant. Of course, the lesion reduction in patient 2 seemed to be less obvious than that in other patients. The reason may be that the area of patient 1 which received higher radiation dose was smallest of the three patients in **Figure 3**. It also implied that the proportion of area which received higher radiation dose had an impact on the tumor response. In addition, the SIB-IMRT plans required averagely 93 more MUs to be delivered compared with the Con-IMRT plans, under approximately identical practical treatment time. Above all, the results of our study imply that our SIB-IMRT plans has the potential to obtain an effective high tumor control with negligible treatment toxicities in the management of GISTs.

Secondly, the radiotoxicity of healthy tissue is another factor of concern in GISTs. For one thing, the gastrointestinal location, patterns spread and tumor size would potentially require large abdominal fields (6). For another, it is difficult to target tumor in a mobile segment of the gastrointestinal (31). So, radiotherapy may raise the risk in toxicities of the small bowel and visceral

structures. Although IMRT has a significant reduction in acute and delayed toxicity of abdominal RT in recent years, large abdominal fields mean that it is still difficult to deliver too high radiation dose by Con-IMRT in bulky GISTs. SIB-IMRT may provide a means to deal with the dilemma of GISTs between increasing the radiation dose of target volume and alleviating radiotoxicity of OARs. In our study, GTV-mid and GTV-center was built on the contraction of GTV. Meanwhile, a higher radiation dose was delivered to the two parts to improve tumor's response. A relaxed upper dose constraint was assigned for the GTV-outer and GTV-mid during optimization process in order to avoid higher radiation dose in the overlap region of OARs and GTV. As shown in **Tables 4–6**, a dose boost to target volume had no risk of overdosing the OARs. Some of OARs DVH indexes in SIB-IMRT plans were even lower than that in Con-IMRT plans. The reason may be that the relaxation of GTV upper dose constraint in SIB-IMRT plans increased the relative weight of all other constraints. Sun et al. (35) already involved in similar study. He concluded that removing the upper dose constraints in target volume may theoretically improve the OARs sparing and tumor control probability. In addition, the large tumor size of three patients also created a good condition for radiotherapy in our study. It is because that the relatively fixed tumor in the abdominal cavity is easily to be targeted. Nevertheless, rigorous IGRT is essential to the efficacy and safety of radiotherapy.

There are also some limitations in our study. Firstly, our sample size was small, only three patients, and the follow-up was short. That is why we reached the conclusion through observation and comparative analysis rather than statistical analysis. Secondly, despite the fact that some patients with LADR-GISTs have acquired efficacy through radiotherapy, further research is needed to make certain the optimal radiation dose schedule. Finally, although three patients did not take TKIs therapy after radiotherapy for personal reasons, radiotherapy influenced by TKIs therapy is a noticeable problem and will be an attractive topic. To sum up, it was just our preliminary study, and we will expand the sample size to continue our exploration in the future.

CONCLUSION

A novel SIB-IMRT technique was designed for locally advanced drug-resistant GISTs and a heterogeneous dose distribution was escalated from the peripheral region of GTV to the center region. Compared to the Con-IMRT plans, the SIB-IMRT plans had the potential to improve the tumor response without significant increase in the radiotoxicity of the adjacent normal tissue in LADR-GISTs. Radiotherapy may be underutilized for GISTs, and SIB-IMRT technique may provide a new method for achieving objective response and prolonging survival in selected GISTs patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was reviewed and approved by the Ethical and Scientific Committees of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). The patient gave written informed consent in accordance with the Declaration of Helsinki. Written, informed consent was obtained from the patient individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YL, LL, and XY: drafting of work, analysis and interpretation of trials and literature, drafting of manuscript, and manuscript review. HC and XZ collected the data, reviewed the literature, and wrote the paper. JD and QJ prepared the figure and contributed in the revision of the literature. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Which Definition of Upper Rectal Cancer Is Optimal in Selecting Stage II or III Rectal Cancer Patients to Avoid Postoperative Adjuvant Radiation?

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Background: In most guidelines, upper rectal cancers (URC) are not recommended to take neoadjuvant or adjuvant radiation. However, the definitions of URC vary greatly. Five definitions had been commonly used to define URC: 1) >10 cm from the anal verge by MRI; 2) >12 cm from the anal verge by MRI; 3) >10 cm from the anal verge by colonoscopy; 4) >12 cm from the anal verge by colonoscopy; 5) above the anterior peritoneal reflection (APR). We hypothesized that the fifth definition is optimal to identify patients with rectal cancer to avoid adjuvant radiation.

Methods: The data of stage II/III rectal cancer patients who underwent radical surgery without preoperative chemoradiotherapy were retrospectively reviewed. The height of the APR was measured, and compared with the tumor height measured by digital rectal examination (DRE), MRI and colonoscopy. The five definitions were compared in terms of prediction of local recurrence, survival, and percentages of patients requiring radiation.

Results: A total of 576 patients were included, with the intraoperative location of 222 and 354 tumors being above and straddle/below the APR, respectively. The median distance of the APR from anal verge (height of APR) as measured by MRI was 8.7 (range: 4.5–14.3) cm. The height of APR positively correlated with body height (r=0.862, P<0.001). The accuracy of the MRI in determining the tumor location with respect to the APR was 92.1%. Rectal cancer above the APR had a significantly lower incidence of local recurrence than those straddle/below the APR (P=0.042). For those above the APR, there was no significant difference in local recurrence between the radiation and no-radiation group. Multivariate analyses showed that tumor location regarding APR was an independent risk factor for LRFS. Tumor height as measured by DRE, MRI and colonoscopy were not related with survival outcomes. Fewer rectal cancer patients required adjuvant radiation

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using the definition by the APR, compared with other four definitions based on a numerical tumor height measured by MRI and colonoscopy.

Conclusions: The definition of URC as rectal tumor above the APR, might be the optimal definition to select patients with stage II/III rectal cancer to avoid postoperative adjuvant radiation.

Keywords: upper rectal cancer, anterior peritoneal reflection, intraoperative finding, MRI, radiotherapy

INTRODUCTION

Preoperative chemoradiotherapy (CRT) has become an integral part of the multimodal treatment for stage II and III rectal cancer. Preoperative CRT has been shown to improve the local control and sphincter preservation rates, without significant effect on the overall survival (OS) and disease-free survival (DFS) (1–3). However, the benefit of radiation for upper rectal cancer (URC) is not clear. The Dutch TME trial (4) and Swedish rectal cancer trial (5) demonstrated that, although local recurrence in the middle and lower rectum was significantly reduced by preoperative radiation, no significant reduction in local recurrence was found in patients with URC. Prior to the widespread use of total mesorectal excision (TME), postoperative adjuvant radiation was believed to potentially compensate for suboptimal surgical resection (6). However, with the advances in systemic chemotherapy and the quality of surgical excision, especially the increased use of TME, local recurrence of rectal cancer has decreased dramatically in the last three decades (6, 7). Moreover, considering the significant long-term side effects of radiation and a lack of clear benefit for URC, most current guidelines don't recommend preoperative or postoperative radiation for URC (2, 3, 8–10). However, the definitions of URC vary greatly across these guidelines. In the 2020 NCCN guidelines, URC was defined as a rectal tumor with inferior margin located between the anterior peritoneal reflection (APR) and the sacral promontory, as determined by MRI (1). According to the 2017 ESMO guidelines, URC was defined as a tumor with inferior margin located at 10–15 cm from the anal margin, as measured by rigid sigmoidoscopy (2). In the German Guideline Program in Oncology (GGPO) 2019 guidelines, URC was defined as a tumor located at 12–16 cm from the anal verge as measured by rigid rectoscopy (9). In the Chinese Society of Clinical Oncology (CSCO) 2018 guidelines, URC was defined as a tumor located 10 cm above the anal verge, as observed on the MRI (10).

Although the tumor height-related definitions provide a reproducible method for defining URC, the body habitus and sex must be considered during the assessment of tumor location as, for instance, the rectum is longer in taller patients (3). The decision to administer radiation solely based on the numerical tumor height involves anatomical pitfalls. The distance between the anal margin and the APR varies from 3.5 to 16 cm, depending on the height, sex and age of the patient (11–14). Rectal cancers located 3.5–16 cm from the anal verge can also be intraperitoneal, which is often too large of a range to be reliably targeted with radiation; or it can be extraperitoneal, which should be amenable to receive radiation. Therefore, some surgeons propose that the APR could be a suitable landmark for identifying patients with rectal cancer for radiation (15). Furthermore, the 2020 NCCN guidelines also suggest that rectal tumor above the APR should be defined as URC (1). The overall reported 5-year local recurrence rate for intraperitoneal and extraperitoneal rectal cancer is 4.2% and 13.3%, respectively (15). There is also growing evidence that radiation may not be useful for intraperitoneal cancers (16–18). More importantly, blood-borne metastases or disseminated disease is predominant among intraperitoneal rectal tumors, whereas local failure is more frequent among extraperitoneal tumors. In addition, there is evidence that the rectum above the APR is quite distinct from that below the APR in terms of embryology, morphology, function and lymphatic drainage (11). The APR is a distinct anatomical landmark which could be easily identified by intraoperative examination and preoperative MRI (17, 19). Based on these reasons, we hypothesized that the definition of URC as a rectal tumor above the APR is the optimal definition to identify patients with stage II/III rectal cancer that should avoid radiation (11).

Five definitions of URC were used in this study and are as follows: 1) tumor >10 cm from the anal verge by MRI; 2) tumor >12 cm from the anal verge by MRI; 3) tumor >10 cm from the anal verge by flexible colonoscopy; 4) tumor >12 cm from the anal verge by flexible colonoscopy; 5) rectal tumor above the APR (1). In this study, we aimed to compare the five different definitions of URC for predicting OS, DFS and local recurrence free survival (LRFS), radiation effect and percentages of patients requiring radiation.

METHODS

Patients

In this retrospective study, all consecutive patients with rectal cancer who underwent radical resection of the primary tumor between July 2017 and October 2018 at Changhai Hospital were included. This study was approved by the Ethics Committee of Changhai Hospital, which waived the requirement for informed consent as it was a retrospective study. The study was conducted in accordance with the principles of the Declaration of Helsinki. Perioperative clinicopathological parameters, tumor height, and tumor location relative to the APR were recorded and maintained in our colorectal cancer database.

Selection Criteria

Inclusion criteria: 1) adult patients (> 18 years) of either sex with histopathologically confirmed rectal adenocarcinoma; 2) tumor within 15cm from the anal verge by flexible colonoscopy; 3) pathological stage II (T3-4N0M0) or stage III (T1-4N1-2 M0) rectal cancer; 4) underwent curative resection of primary rectal cancer; 5) without preoperative CRT.

Exclusion criteria: 1) patients who underwent palliative resection; 2) positive resection margin (including proximal, distal and circumferential); 3) synchronous or metachronous multiple primary colorectal cancer; 4) hereditary colorectal cancer syndrome; 5) previous history of pelvic radiation; 6) preoperative concomitant intestinal obstruction or perforation; 7) patients without recurrence who did not complete at least 24 months of follow-up after primary surgery.

Surgery and Histopathological Assessment

All surgeries were performed by seven chief surgeons, each with the experience of performing at least 100 operations for colorectal cancer per year, following a standardized operation protocol (including standard TME and high vascular ligation of the inferior mesenteric artery and vein). All resected specimens were examined using a standardized protocol that included TNM classification according to the American Joint Committee on Cancer-International Union Against Cancer (8^{th} edition). Resection margins, including circumferential, proximal, and distal margins, were considered positive if tumor cells were identified within 1 mm of the surgical resection margin.

Postoperative CRT

For middle and lower rectal cancer, preoperative CRT was recommended to all patients with stage II/III tumor, and some patients refused. For upper rectal cancer, preoperative CRT was not recommended to patients with stage II/III tumor (excluding T4b). All patients with stage II/III rectal cancer who did not receive preoperative CRT were recommended to undergo postoperative CRT, and some patients refused to take postoperative CRT or chemotherapy. Postoperative adjuvant CRT was initiated 4 weeks after surgery and continued for 6 months. The dose of postoperative adjuvant radiation was 1.8 to 2.0 Gy daily for a total of 23 to 28 fractions over 5–6 weeks and resulted in a total dose of 46.0 to 50.4 Gy. Postoperative adjuvant radiation was delivered by the three-field or four-field box technique to the original area of tumor and mesorectum, presacral region, and the internal iliac lymph nodes. Postoperative adjuvant concurrent chemotherapy [capecitabine (1000 mg/m^2, twice daily)] was administered orally throughout the period of radiation treatment. Postoperative adjuvant chemotherapy (CapeOX or mFOLFOX6) was administered 3 weeks after the completion of radiation and continued for 4–6 months.

Follow Up

Clinical follow-up consisted of physical examination, DRE, chest CT scan, liver contrast-enhanced MRI, rectal contrast-enhanced MRI and the serum level measurement of CEA and CA19-9. These examinations were performed every 3 months for the first 2 years after surgery, every 6 months for another 3 years and annually thereafter. A flexible colonoscopy was performed annually for 5 years. The local recurrence and distant metastasis were confirmed by biopsy when appropriate or based on the progressive increase in the size of the lesions or the appearance of new lesions.

Definition of Parameters

Rectal cancer was defined as a large intestine tumor with its lower margin located within 15cm from the anal verge by flexible colonoscopy. *Local recurrence* was defined as evidence of recurrent disease within the pelvis after radical resection, including recurrence at the site of anastomosis, the pelvic cavity and the perineal wound. *LRFS* was defined as the period between the date of surgery for primary rectal tumor and the date of local recurrence, or death from any cause. *DFS* was defined as the time between the date of surgery for primary rectal tumor and the date of local recurrence, distant metastasis or death from any cause. ***OS*** was defined as the time interval between the date of surgery for primary rectal tumor and the date of death or last follow-up, with no restriction on the cause of death.

Measurements of Tumor Height and Tumor Location Relative to the APR

Preoperatively, tumor height was measured by DRE, flexible colonoscopy and rectal contrast-enhanced MRI. The relationship between the APR and inferior tumor margin was determined by preoperative MRI. Both DRE and flexible colonoscopy were performed by experienced colorectal surgeons. All rectal MRI images were reviewed by experienced radiologists on the PACS system. Sagittal and axial T2-weighted images were used for the identification of the APR. In the midsagittal plane, the APR was identified as a thin hypointense line extending from the superior aspect of the urinary bladder (men) or uterus (women) to the anterior rectal wall (**Figure 1A**). The height of the tumor was defined on the sagittal images as the distance from the anal verge to the inferior tumor margin (**Figure 1B**). In some cases, it was necessary to interconnect two or more angulated lines, sometimes on two or more adjacent sagittal slices for an approximate total length (13). On axial imaging, the APR attached to the anterior rectal wall in a V-shaped hypointense configuration (**Figure 1C**). MRI was also used to identify the relationship between the tumor and the APR preoperatively (**Figures 2A–C**, **Supplemental Figures 1A–C**). The relationship between tumor location and the APR was also determined intraoperatively by palpation and visualization **Figures 2D–F**). All patients had an intraoperative assessment of the APR. Based on intraoperative findings, rectal cancer patients were classified into the "above the APR" group, or the "straddle/below the APR" group. The accuracy of the MRI in determining tumor location relative to the APR, was calculated using the intraoperative finding as the gold standard.

Statistical Analysis

Statistical analysis was performed with the SPSS 22.0 software (Chicago, IL). The "t" test or Wilcoxon test was used to compare continuous variables. The Chi-square test or Fisher exact test was

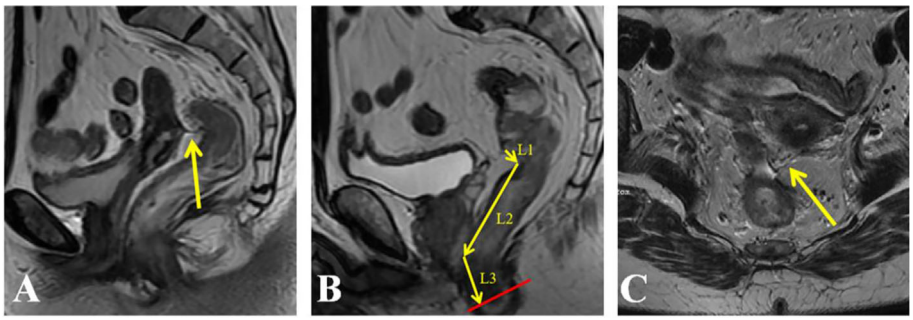

FIGURE 1 | Identification of the anterior peritoneal reflection (APR) and measurement of tumor height on an MRI. **(A)** APR (arrow) in the sagittal plane; **(B)** the height of the tumor defined as the distance from the anal verge to the inferior tumor margin was measured in sagittal images; **(C)** APR (arrow) in the axial plane.

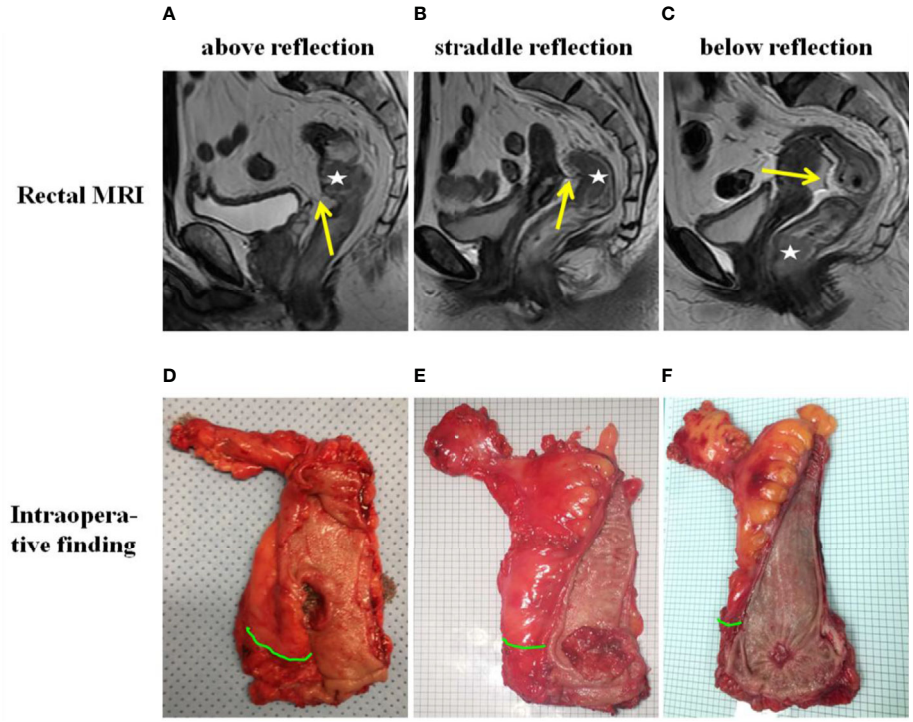

FIGURE 2 | Tumor location relative to the anterior peritoneal reflection (APR) as determined by MRI **(A–C)** and intraoperative palpation and visualization **(D–F).** The "☆" in the MRI indicates the tumor. The yellow arrow in the MRI indicates the APR. The green curve in intraoperative finding indicates the APR.

used to compare categorical variables. The Pearson correlation coefficient was used to characterize the agreement between the measurements by DRE, flexible colonoscopy, and MRI. The agreement between each pair of measurements was compared using the Bland and Altman plot. The Kaplan-Meier analysis and log-rank tests were used to compare survival differences between two groups. Multivariable Cox proportional hazard analyses were used to explore the factors affecting OS, DFS, and LRFS. All parameters which showed statistical significance in the univariate analysis or had potential clinical significance were included into the multivariate analysis. The multivariate Cox proportional hazard analysis was employed using the stepwise method (forward: likelihood ratio) with an entry criterion of $P<0.05$ and a removal criterion of $P>0.10$. For all analyses, $P<0.05$ (two-sided) was considered statistically significant.

RESULTS

Patient Characteristics

A total of 576 patients with rectal cancers were included in this study, with 222 tumors above the APR and 354 tumors straddle/

below the APR as determined by intraoperative findings. The flowchart of patient selection is shown in **Figure 3**. The median age of patients was 63 years (interquartile range, 54 to 69 years), and the median follow up period was 22 months (interquartile range, 18 to 28 months). The demographic, clinicopathological and treatment data are presented in **Table 1**.

Of the included 576 patients with rectal cancer, 384 had preoperative rectal MRI imaging and 102 patients had only preoperative rectal CT scan in our picture archiving and communication (PACS) system. The remaining 90 had preoperative assessment at other hospitals. The APR was visible in 330 cases (85.9%) on the rectal MRI. The median distance between the APR and the anal verge was 8.7 (range: 4.5–14.3) cm (**Supplemental Table 1**). The median distance between the APR and the anal verge was significantly higher in the males compared to females [8.9 (range: 5.3–14.3) cm vs. 8.4 (range: 4.5–12.9) cm, P = 0.001]. The distance of the APR from the anal verge showed a positive correlation with body height (r = 0.862, P < 0.001), and could be calculated with the following formula: distance (cm) = [0.1 × height (cm)] - 8.0. The accuracy of the MRI in determining tumor location relative to the APR was 92.1%. The accuracy of MRI to identify the tumors above, straddle and below the APR was 89.1%, 95.3%, and 93.1%, respectively (**Supplemental Table 2**). The Kappa value of tumor location with respect to the APR, as determined by MRI and intraoperative findings, was 0.881 (P < 0.001).

The Relationship Between Tumor Location Relative to the APR, Postoperative Radiation and Survival Related Parameters (OS, DFS, and LRFS)

During the follow-up period, a total of 39 deaths occurred, including 32 (82.1%) from rectal cancer, 5 (12.8%) from cardiovascular diseases and 2 (5.1%) from unknown causes. Eight patients (1.4%) developed local recurrence [1 (0.5%) patient with tumor above the APR and 7 (2.0%) patients with tumor straddle/below the APR]. Local recurrence and distant metastasis occurred in 1.4% and 12.0% of patients at 2 years, respectively. The actual 2-year rate of OS, DFS and LRFS were 95.0%, 86.8% and 91.5%, respectively.

Rectal cancer above the APR exhibited a significantly lower incidence of local recurrence than those straddle/below the APR (**P=0.042, Figure 4A**). No significant difference was identified for OS and DFS between the two groups (**Supplemental Figures 2A** and **3A**). No significant difference was identified for OS (**Supplemental Figure 2B**), DFS (**Supplemental Figure 3B**) and LRFS (**Figure 4B**) between the radiation group and the no-radiation group. Subgroup analyses revealed that, for patients with rectal cancer above the APR, there was no significant difference in LRFS between the radiation group and the no-radiation group (**Figure 4C**). For patients with rectal cancer straddle/below the APR, the radiation group had significant longer LRFS than the no-radiation group (**Figure 4D**).

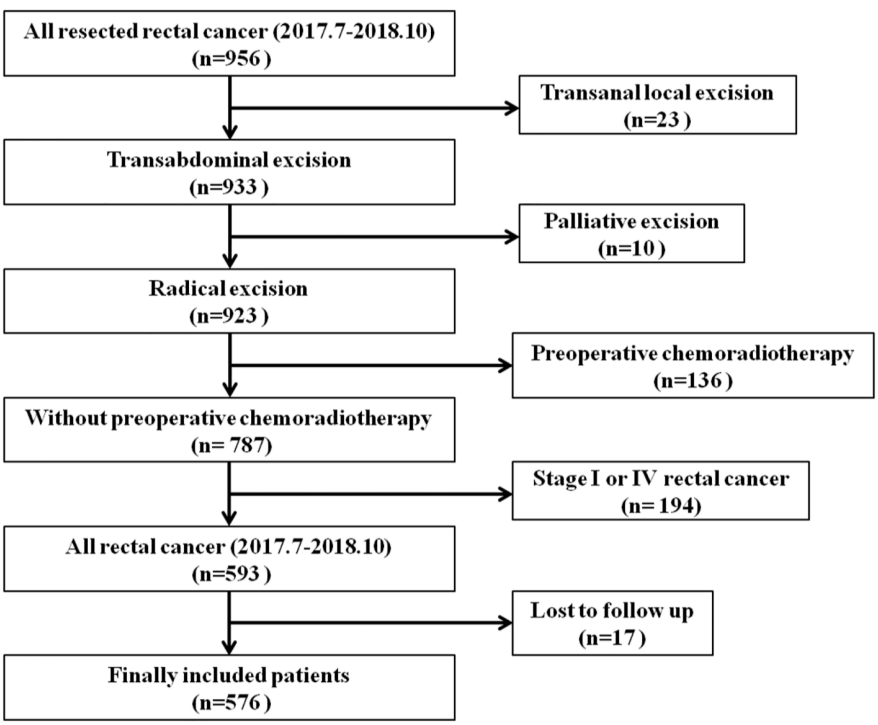

FIGURE 3 | Flow chart of patient selection.

TABLE 1 | The demographic, clinicopathological and treatment details of the study patients.

Parameters		Tumor location in relation to the APR		P value
		Above (n=222)	Straddle or below (n=354)	
Sex (Male/Female)		154/68	241/113	0.745
Age (year)		61.35±10.87	61.34±10.74	0.987
Body height (cm)		166.39±8.24	166.17±7.80	0.750
Body weight (kg)		66.74±11.90	64.70±10.65	**0.038**
BMI (kg/m^2)		23.99±3.24	23.36±3.05	**0.019**
Tumor diameter (cm)*		4.17±1.40	4.18±1.45	0.969
Tumor height by DRE (cm)*		6(4–8); n=74	4(0–8); n=325	**<0.001**
Tumor height by colonoscopy (cm)*		10(4–15); n=215	4(0–15); n=346	**<0.001**
Tumor height by MRI (cm)*		9(4.4–15); n=127	5(0–13); n=257	**<0.001**
Preoperative T stage	1	6(33.3%)	12(66.7%)	0.610
	2	59(38.3%)	95(61.7%)	
	3	147(38.2%)	238(61.8%)	
	4	10(52.6%)	9(47.4%)	
Preoperative N stage	0	97(40.6%)	142(59.4%)	0.587
	1	83(38.2%)	134(61.8%)	
	2	42(35.0%)	78(65.0%)	
Preoperative TNM stage	I	21(43.8%)	27(56.3%)	0.614
	II	76(39.8%)	115(60.2%)	
	III	125(37.1%)	212(62.9%)	
Postoperative pathological TNM stage	II	107(42.5%)	145(57.5%)	0.088
	III	115(35.5%)	209(64.5%)	
Differentiation	Well	2(40.0%)	3(60.0%)	0.624
	Moderate	196(39.3%)	303(60.7%)	
	Poor	24(33.3%)	48(66.7%)	
Tumor deposit	No	186(38.8%)	294(61.3%)	0.818
	Yes	36(37.5%)	60(62.5%)	
Lymphovascular invasion	No	181(40.0%)	272(60.0%)	0.181
	Yes	41(33.3%)	82(66.7%)	
Perineural invasion	No	159(38.7%)	252(61.3%)	0.910
	Yes	63(38.2%)	102(61.8%)	
Tumor budding	No	164(37.4%)	275(62.6%)	0.296
	Yes	58(42.3%)	79(57.7%)	
dMMR status	pMMR	210(38.2%)	340(61.8%)	0.414
	dMMR	12(46.2%)	14(53.8%)	
KRAS	Wild type	131(40.1%)	196(59.9%)	0.391
	Mutant type	91(36.5%)	158(63.5%)	
NRAS	Wild type	211(38.2%)	341(61.8%)	0.453
	Mutant type	11(45.8%)	13(54.2%)	
BRAF	Wild type	217(38.5%)	347(61.5%)	0.822
	Mutant type	5(41.7%)	7(58.3%)	
Postoperative radiation	Yes	186(43.3%)	244(56.7%)	**<0.001**
	No	36(24.7%)	110(75.3%)	
Postoperative chemotherapy	Yes	37(34.3%)	71(65.7%)	0.310
	No	185(39.5%)	283(60.5%)	
CEA	<5 ng/ml	146(37.9%)	239(62.1%)	0.664
	>= 5 ng/ml	76(39.8%)	115(60.2%)	
CA199	< 37 U/ml	198(38.6%)	315(61.4%)	0.939
	>= 37 U/ml	24(38.1%)	39(61.9%)	

**Median (range). BMI, body mass index; DRE, digital rectal examination; APR, anterior peritoneal reflection; dMMR, deficient mismatch repair; pMMR, proficient mismatch repair. In bold: p < 0.05.*

Univariate and Multivariate Analyses of Risk Factors Affecting OS, DFS, and LRFS

Univariate and multivariate analyses demonstrated that the degree of tumor differentiation, tumor deposits, lymphovascular invasion and perineural invasion were independent risk factors affecting OS (**Supplemental Table 3**). Postoperative pathological TNM stage, tumor deposit and lymphovascular invasion were independent risk factors affecting DFS (**Supplemental Table 3**). Postoperative pathological TNM stage, differentiation, tumor deposit, perineural invasion, tumor budding, postoperative radiation [0.20(0.08-0.46), P < 0.001], postoperative chemotherapy [0.44 (0.24–0.81), P = 0.008] and tumor location with regards to the APR [1.97(1.04–3.72), P=0.038] were independent predictors of LRFS (**Table 2**).

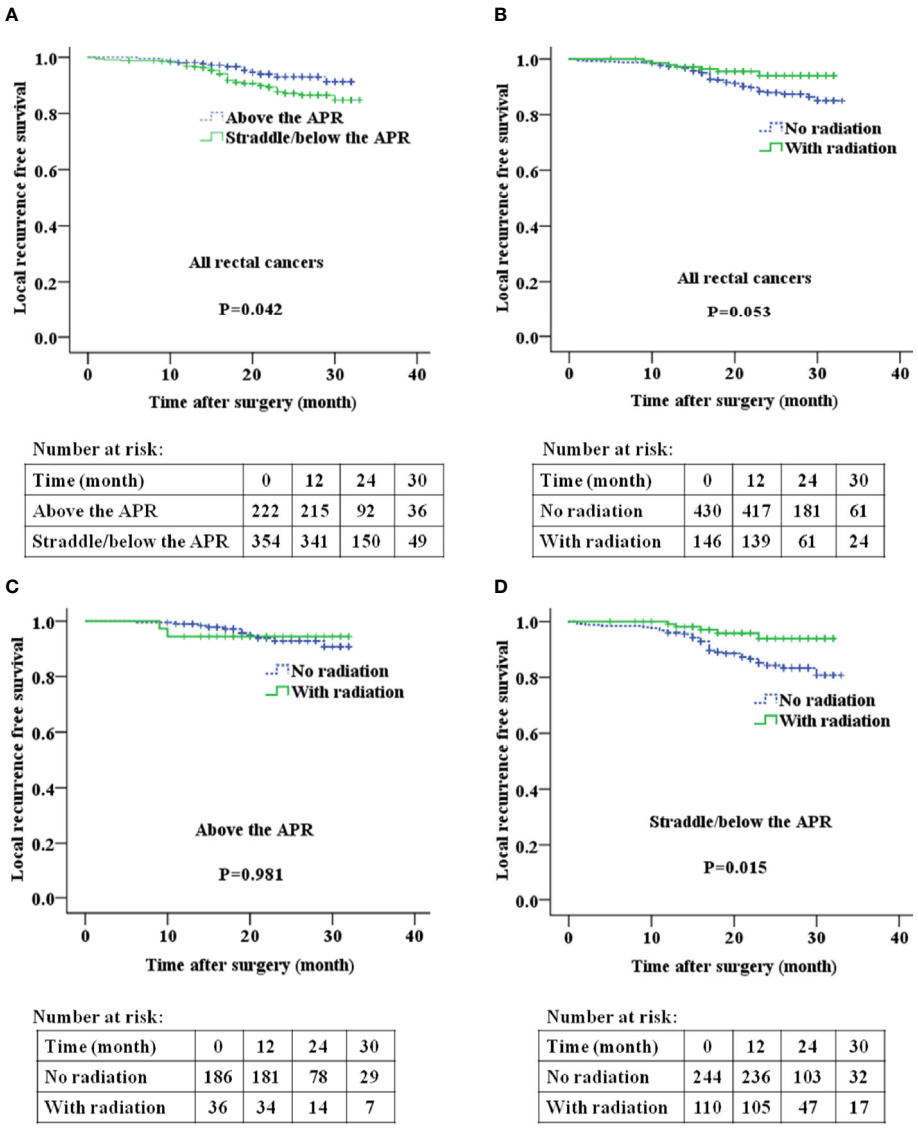

FIGURE 4 | The relationship between tumor location relative to the APR and local recurrence free survival **(A, B)**, postoperative radiation and local recurrence free survival **(C, D)** in patients with rectal cancer. APR: anterior peritoneal reflection.

Consistency of Tumor Height Measured by DRE, MRI, and Flexible Colonoscopy

All patients underwent DRE and the inferior tumor margin could be reached by an examiner's finger in 399 cases. In addition, 576 patients had undergone a flexible colonoscopy, and 384 received rectal contrast MRI preoperatively. The tumor height in 290 cases, as measured by DRE and MRI, were correlated with each other (Pearson correlation coefficient R = 0.723, **Supplemental Figure 4A**), as indicated by the regression equation ($y = 0.79x + 1.93$). The tumor height measured by DRE was correlated with that measured by colonoscopy in 394 cases (R = 0.785, $y = 1.11x$ -0.30, **Supplemental Figure 4B**). The tumor height measured by colonoscopy was correlated with that measured by MRI in 377 cases (R = 0.822, $y = 0.67x + 2.58$, **Supplemental Figure 4C**).

To estimate the degree of measurement difference in each individual, we used the Bland and Altman plot. In the scatter plot between DRE and MRI (**Supplemental Figure 4D**), the mean difference was –1.1 cm (95% CI: –4.2 to 2.1 cm). Similarly, the mean difference between DRE and colonoscopy was –0.2 cm (95% CI: –3.7 to 3.3 cm) (**Supplemental Figure 4E**), and the mean difference between colonoscopy and MRI was –0.7 cm (95% CI: –4.8 to 3.4 cm) (**Supplemental Figure 4F**).

The Relationship Between Survival Related Parameters and Tumor Height as Measured by DRE, MRI, and Flexible Colonoscopy

Kaplan-Meier analyses found no significant difference in OS, DFS, or LRFS between these two groups divided by a fixed tumor

TABLE 2 | Univariate and multivariate analyses of risk factors of LRFS using a Cox regression model (n = 576).

Parameters	LRFS			
	Univariate		Multivariate	
	HR(95%CI)	P	HR(95%CI)	P
Gender (male vs. female)	0.96(0.53–1.73)	0.894		
BMI (>=23.59 vs. <23.59kg/m^2)	1.05(0.61–1.82)	0.850		
Diameter (>=4 vs. <4cm)	1.09(0.62–1.92)	0.756		
Postoperative pathological TNM stage (III vs. II)	3.10(1.60–6.04)	**0.001**	2.38(1.13–5.04)	**0.023**
Differentiation (Poor vs. Well/moderate)	2.29(1.17–4.46)	**0.015**	2.39(1.19–4.78)	**0.014**
Tumor deposit (Yes vs. No)	3.47(2.00–6.05)	**<0.001**	2.70(1.45–5.02)	**0.002**
Lymphovascular invasion (Yes vs. No)	2.43(1.39–4.25)	**0.002**	1.18(0.62–2.24)	0.608
Perineural invasion (Yes vs. No)	3.39(1.96–5.86)	**<0.001**	2.72(1.55–4.79)	**0.001**
Tumor budding (Yes vs. No)	2.59(1.44–4.66)	**0.002**	2.62(1.45–4.76)	**0.002**
dMMR status (dMMR vs. pMMR)	0.46(0.06–3.32)	0.440		
KRAS (Mutant vs. Wild)	1.43(0.83–2.46)	0.198		
NRAS (Mutant vs. Wild)	0.05(0.00–28.92)	0.351		
BRAF (Mutant vs. Wild)	0.05(0–1697.75)	0.571		
Postoperative radiation (Yes vs. No)	0.47(0.21–1.03)	**0.060**	0.20(0.08–0.46)	**<0.001**
Postoperative chemotherapy (Yes vs. No)	0.41(0.23–0.72)	**0.002**	0.44(0.24–0.81)	**0.008**
CEA (>= 5 vs. <5 ng/ml)	1.45(0.84–2.52)	0.184		
CA19-9 (>= 37 vs. <37U/ml)	2.44(1.26–4.75)	**0.009**	1.79(0.88–3.65)	0.107
Tumor location relative to the APR (straddle/below vs. above)	1.89(1.01–3.55)	**0.046**	1.97(1.04–3.72)	**0.038**

BMI, body mass index; APR, anterior peritoneal reflection; dMMR, deficient mismatch repair; pMMR, proficient mismatch repair.
In bold: p < 0.05.

height [colonoscopy and MRI (> 10 vs. <= 10 cm, > 12 vs. <= 12 cm)] (**Table 3**). Patients with rectal cancer above the APR had significantly longer LRFS than those straddle/below the APR (P = 0.046), but not for OS or DFS (**Table 3**).

Different Percentages of rectal Cancer Patients Requiring Radiation Based on the Five Commonly Used Definitions of URC

Most guidelines do not recommend patients with stage II/III URC to receive neoadjuvant or adjuvant radiation. However, the definition of URC varied greatly in different guidelines. Here we compared the percentages of rectal cancer patients requiring radiation based on the five commonly used definitions of URC. The results demonstrated that fewer patients required radiation using the definition based on the APR (61.5%) compared with the other four definitions using a numerical tumor height measured by MRI and colonoscopy (64.2%–100.0%, **Table 4**).

DISCUSSION

Although most current guidelines do not recommend radiation for URC, the definitions of URC vary greatly across these different guidelines. The present study showed that the height of the APR, which correlates with sex and body height, is a distinct and individualized landmark. Rectal cancer above the APR had a significantly lower incidence of local recurrence than those that straddle/below the APR. Univariate and multivariate COX analyses demonstrated that tumor location relative to the APR was an independent risk factor of LRFS, while other tumor height related parameters measured by DRE, colonoscopy and MRI were not related to OS, DFS, or LRFS. Subgroup analyses showed that, only in patients with rectal cancer straddle/below the APR, the radiation group had significant longer LRFS than the no-radiation group. Moreover, fewer rectal cancer patients required radiation when URC was defined by the APR compared with those defined by the other four definitions. Hence, we suggest that the definition of URC as a rectal tumor above the

TABLE 3 | Kaplan-Meier analysis of the relationship between tumor height-related parameters and survival outcomes.

Parameters	OS		DFS		LRFS	
	HR(95%CI)	P	HR(95%CI)	P	HR(95%CI)	P
Tumor height by MRI (<=10 vs. >10 cm)	0.91(0.26–3.16)	0.887	0.67(0.30–1.48)	0.324	0.41(0.13–1.35)	0.143
Tumor height by MRI (<=12 vs. >12 cm)	1.41(0.32–6.16)	0.648	0.21(0.03–1.53)	0.124	0.33(0.05–2.41)	0.274
Tumor height by colonoscopy (<=10 vs. >10 cm)	1.54(0.76–3.14)	0.230	1.35(0.86–2.10)	0.187	1.05(0.57–1.94)	0.880
Tumor height by colonoscopy (<=12 vs. >12 cm)	2.02(0.87–4.65)	0.100	1.58(0.91–2.76)	0.106	1.91(0.96–3.83)	0.067
Tumor location in relation to the APR (straddle/below vs. above)	1.01(0.50–2.01)	0.983	1.20(0.78–1.85)	0.416	**1.89(1.01–3.55)**	**0.046**

In bold: p < 0.05.

TABLE 4 | The percentages of rectal cancer patients requiring postoperative radiation based on 5 commonly used definitions of URC.

No.	Definitions of URC	Requiring postoperative radiation		Total
		Yes	No	
1	>10 cm from the anal verge by MRI	319(83.1%)	65(16.9%)	384
2	>12 cm from the anal verge by MRI	357(93.0%)	27(7.0%)	384
3	>10 cm from the anal verge by colonoscopy	410(71.2%)	166(28.8%)	576
4	>12 cm from the anal verge by colonoscopy	496(86.1%)	80(13.9%)	576
5	Above the APR	354(61.5%)	222(38.5%)	576

URC, upper rectal cancer; APR, anterior peritoneal reflection.

APR might be the optimal definition in selecting patients with stage II/III rectal cancer to avoid radiation.

To the best of our knowledge, this is the first study focusing on the definition of URC in identifying patients with stage II/III rectal cancer that should avoid radiation. The 2020 NCCN guidelines defined URC as a rectal tumor above the APR, but it recommended that all patients with URC should receive neoadjuvant or adjuvant radiation (1). In this study, we found that the optimal definition of URC was rectal tumors above the APR, and for these patients there was no significant difference between the radiation group and the no-radiation group in terms of OS, DFS and LRFS. The tumor location relative to the APR can be determined by intraoperative findings and preoperative rectal MRI, and the APR can be easily identified during open or laparoscopic surgery (12, 14). For the selection of postoperative radiation, intraoperative determination of the tumor location relative to the APR is more direct and accurate. When it comes to the selection of preoperative radiation, preoperative rectal MRI is the most useful test to identify the APR. The APR was visible in 85.9% cases in this study, which is similar to other previous studies (13, 20). Our results showed that the accuracy of using the MRI for determining tumor location relative to the APR was 89.1%, 95.3%, and 93.1% for tumors above, straddle and below the APR, respectively. Corresponding percentages in other studies were 70%, 50%, and 98.2% (20), and 93.5%, 90.0%, and 84.6% (17), respectively, which are similar to our results (17, 20).

The height of APR varies greatly in patients of different sex and body height. Several studies have measured the distance of the APR from the anal verge by intraoperative rigid sigmoidoscopy. In an American study of 50 patients, the mean height of the APR was 9 cm (range: 5.5–13.5 cm) for females, and 9.7 cm (7–16 cm) for males (14). In a Korean study of 46 patients, the mean height of the APR was 8.8 ± 2.2 cm for males and 8.1 ± 1.7 cm for females (12). The position of the APR can also be assessed by rectal MRI. A large study (n=319) using MRI to measure the APR showed that there was a significant difference in the height of the APR between females and males (10.4 ± 1.1 cm vs. 10.0 ± 1.2 cm, P=0.014) (20). Our results showed that the median height of APR measured by MRI was 8.7 cm (4.5–14.3 cm) and positively related with body height, which are consistent with published results (12, 14).

The APR divided rectal cancer into two subtypes: intraperitoneal and extraperitoneal. The local recurrence rate has been found to be much lower in intraperitoneal compared to the extraperitoneal rectal cancer patients (15) and is consistent with our results. The univariate and multivariate analyses of this study showed that only in patients with rectal tumors straddle/below the APR, the radiation group had significant longer LRFS than the no-radiation group, which was consistent with the results of previous works. The Dutch TME trial (4) and Swedish rectal cancer trial (5) demonstrated that local recurrence was reduced significantly in middle and lower rectal cancer, but not in URC. Some studies also suggested that omission of radiation may not jeopardize oncologic outcomes in stage II/III URC (21). In a retrospective study of 547 URC cases, only in high-risk patients (positive lymph node > 6, or tumor deposit) the radiation group had significant longer cancer-specific survival than the no-radiation group (22). Our large retrospective study showed that, for URC with all resection margins negative, there was no significant difference between the radiation group and the no-radiation group in terms of OS, DFS and LRFS.

Rigid sigmoidoscopy is recommended for measuring the height of rectal cancer, but it is performed in only a minority of patients (23) and is not frequently used in China. Instead, flexible colonoscopy, DRE and MRI are generally used instead. The current gold standard for the detection of colorectal cancer is flexible colonoscopy (24). The ESMO guidelines indicate that the difference in measurements obtained by rigid versus flexible colonoscopy is small (25). MRI-based measurements of the distance between inferior tumor margin and the anal verge is a reproducible alternative to rigid sigmoidoscopy (23). However, during rigid sigmoidoscopy, the curve of the rectum is straightened and may lead to an underestimation of the tumor height. During MRI evaluation, however, this distance is measured using the sum of multiple straight lines. In the case of high cancers, several straight lines are often combined to follow the curved line of the rectum, which can result in a longer distance than the actual distance. Our results showed that measurements by different methods highly correlated with each other, although significant differences still existed in many cases (26). Therefore, flexible colonoscopy is an acceptable alternative for rigid sigmoidoscopy if the latter is unavailable.

Our results demonstrated that the APR is a distinct and individualized landmark that can be easily identified by preoperative MRI and intraoperative finding. Patients with rectal cancer above the APR exhibited a lower incidence of local recurrence, and there was no significant difference between the radiation group and the no-radiation group in terms of OS, DFS and LRFS. Tumor location with respect to the APR is an independent predictor of LRFS, while other subdivisions based on a fixed distance measured by DRE, MRI or colonoscopy were not associated with survival outcomes.

Therefore, the definition of URC as a rectal tumor above the APR is superior to other definitions, based on a fixed tumor height as measured by MRI and colonoscopy, for selecting patients with stage II/III rectal cancer that should avoid radiation. This definition will not only help us to select suitable cases that should undergo radiation, but also to reduce the incidence of radiation-related toxicity and medical expenses.

There are several limitations in our study. First, this was a retrospective study. In clinical practice, physicians may have preferred to recommend postoperative radiation to high risk patients. Therefore, selection bias is unavoidable. Second, patients with positive resection margin were excluded in this study. Adjuvant radiation might also be needed for some URC patients receiving R1/R2 resection. Third, the included patients did not receive preoperative CRT as recommended by the NCCN and ESMO guidelines due to various reasons. In Asian countries (China, Japan, Korean), postoperative adjuvant CRT or adjuvant chemotherapy is considered the treatment of choice for stage II or III rectal cancer, especially for low risk cases and those with URC (22). Fourth, we do not have the data of tumor location measured by rigid sigmoidoscopy, which is not frequently used in China. The ESMO guidelines indicate that the difference in measurements obtained by rigid versus flexible colonoscopy is small (25). Therefore, the tumor height measured by flexible colonoscopy can, to some extent, replace the measurement by rigid colonoscopy.

CONCLUSION

The definition of URC as a rectal tumor above the APR might be better than other definitions based on a numerical tumor height measured by MRI and colonoscopy in selecting patients with stage II/III rectal cancer to avoid adjuvant radiation. The tumor location relative to the APR could be recorded in the preoperative rectal MRI imaging, intraoperative surgical records and postoperative histopathology reports for prognostication and treatment planning (especially adjuvant radiation). However, further prospective RCTs with a larger sample size are required to validate the findings of this study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical committee at Changhai Hospital.

AUTHOR CONTRIBUTIONS

FS, LL, and WZ conceptualized the study. XG, BZ, and JL conducted the data curation and wrote the original draft. XG and JL performed the formal analysis. XG acquired the funding. JK, HG, CB, ML, and SZ conducted the investigation. XG developed the methodology. FS and WZ provided the resources. FS, LL, and WZ supervised the study. XG, BZ, JL, JK, HG, CB, ML, SZ, FS, LL, and WZ wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.625459/full#supplementary-material

Supplementary Table 1 | The distance between the anal verge and the APR (cm) as measured by MRI.

Supplementary Table 2 | Tumor location relative to the APR as determined by MRI and intraoperative findings.

Supplementary Table 3 | Univariate and multivariate analyses of risk factors of OS and DFS using a Cox regression model (n = 576).

Supplementary Figure 1 | Tumor location relative to the anterior peritoneal reflection (APR) as determined by MRI **(A–C)** and intraoperative palpation and visualization **(D–F).** The "☆" in the MRI indicates the tumor. The yellow arrow in the MRI indicates the APR. The green curve in intraoperative finding indicates the APR.

Supplementary Figure 2 | The relationship between tumor location relative to the APR and overall survival **(A, B)**, postoperative radiation and overall survival **(C, D)** in patients with rectal cancer. APR, anterior peritoneal reflection.

Supplementary Figure 3 | The relationship between tumor location relative to the APR and disease free survival **(A, B)**, postoperative radiation and disease free survival **(C, D)** in patients with rectal cancer. APR, anterior peritoneal reflection.

Supplementary Figure 4 | Comparison of tumor height measured by digital rectal examination (DRE), MRI and flexible colonoscopy. Scatter plots of discrepancies between the DRE and MRI **(A)**, the DRE and colonoscopy **(B)**, the colonoscopy and MRI **(C)**; Bland-Altman graphs **(D–F)** illustrate the variability between two measurements: mean (central blue line) and 95% confidence intervals (upper and lower red lines).

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Primary Tumor Resection for Rectal Cancer With Unresectable Liver Metastases: A Chance to Cut Is a Chance for Improved Survival

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Background: About half of the patients with rectal cancer will develop liver metastasis during the course of their illness. Unfortunately, a large proportion of these metastases are unresectable. Surgical resection of the primary tumor vs. palliative treatment in patients with unresectable synchronous liver metastases remains controversial.

Methods: Patients with rectal cancer with surgically unresectable liver metastases were identified from the Surveillance, Epidemiology, and End Results (SEER) database from January 1, 2010, to December 31, 2015. According to different treatment modalities, patients were divided into a primary tumor resection group and a non-resection group. Rates of primary tumor resection and survival were calculated for each year. Kaplan–Meier methods and Cox regression models were used to assess long-term survival. Multivariable logistic regression models were used to evaluate factors potentially associated with primary tumor resection.

Results: Among 1,957 patients, 494 (25.2%) had undergone primary tumor resection. Patients with primary tumor resection had significantly better 5-year survival rate (27.2 vs. 5.6%, $P < 0.001$) compared to the non-resection group. Chemoradiotherapy with primary site resection was associated with the longest mean and 5-year OS (44.7 months, 32.4%). The Cox regression analyses of the subgroup indicated that patients who underwent primary tumor resection had improved survival compared with those who did not undergo resection in all 25 subgroups. Factors associated with primary tumor resection were well or moderately differentiated tumor grade, undergoing radiation, and primary tumor size <5 cm.

Conclusions: The majority of patients with rectal cancer with unresectable liver metastases did not undergo primary tumor resection. Our results indicate that resection of the primary tumor appears to offer the greatest chance of survival. Prospective studies are needed to confirm these results.

Keywords: rectal cancer, SEER (Surveillance Epidemiology and End Results) database, metastasis, liver, resection

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and is associated with a high mortality rate (1). Distant metastasis is the leading cause of cancer-related mortality with the liver constituting the most common site of distant metastases. In fact, ∼20% of patients suffer from liver metastases at the time of diagnosis, whereas about 50% of patients develop liver metastases during the course of their illness (2, 3). Liver resection combined with chemotherapy is the only treatment offering the possibility of long-term survival in patients with metastatic colorectal cancer (mCRC) and can lead to a 5-year survival rate of 40–50% and 10-year survival rate of 20% (4, 5). Unfortunately, up to 80% of mCRC patients have an unresectable tumor and undergo palliative treatment as a standard of therapy (6).

In clinical practice, surgical resection of the primary tumor site in patients with unresectable liver metastases is recommended as a palliative approach. Initial resection of the primary tumor has been advocated to prevent malignancy-related complications such as bowel obstruction or perforation (7). Some studies have reported that resection of the rectal tumor at the primary site was independently associated with a better overall survival (7, 8). Conversely, other researchers reported that the benefits of primary tumor resection on survival are unclear since surgical resection of the primary tumor cannot eradicate the tumor completely (9, 10). Furthermore, surgery may delay the start of systemic chemotherapy, which may have a negative impact on survival (9, 10).

According to the National Comprehensive Cancer Network (NCCN) guidelines, the treatment of metastatic colon and rectal cancer is not uniform. This reflects the difference in anatomical, functional and metastatic patterns of the two entities (11). Although it has been established that the application of radiotherapy in metastatic rectal cancer can lead to better local control of disease prior to surgery, no role for radiation in metastatic colon cancer has been identified (12).

Despite the NCCN recommendation of the use of systemic chemotherapy or palliative care for mCRC patients with an asymptomatic primary tumor, previous study analysis of the SEER database showed that 67.4% of patients with stage IV CRC had undergone primary tumor resection (13). The study included mCRC patients diagnosed between 1988 and 2010, and their results showed that the resection rate was decreasing but survival rate improved. This serves to show that the role of surgery in the course of treatment for patients with advanced stage disease is an evolving field of study. A recent study by Concors et al. (14) evaluated the role of combined proctectomy and hepatectomy in patients with stage IV rectal adenocarcinoma. A stratified analysis was able to identify the role of combined therapy in offering improved survival in a specific cohort of patients with metastatic rectal adenocarcinoma. Although colon and rectal cancer have different treatment strategies, no multicenter, prospective clinical trial has evaluated the value of resection of the primary tumor for patients presenting with unresectable metastatic rectal cancer. The primary goal of this study was to explore the primary tumor resection rate in patients with unresectable metastatic rectal cancer and to assess the effect of resection on OS.

METHODS

Data Resources

We obtained the rectal cancer data from the National Cancer Institute (NCI) linked Surveillance, Epidemiology, and End Results (SEER) database. The SEER database contains demographic information and data regarding cancer incidence and survival from 18 population-based registries that represent ∼30% of the US population. SEER is an open public database. Data related to patients are de-identified, therefore, there was no need for written informed consent for this study. The Institutional Review Board of the National Cancer Center, Chinese Academy of Medical Sciences approved this study.

Study Population

Patients with rectal cancer with unresectable liver metastases diagnosed between January 1, 2010, and December 31, 2015 were eligible to be included in the study. We included only patients with tumor sequence numbers labeled "one primary only," patients Mets at diagnoses-Liver labeled "yes," and patients with Collaborative Stage (CS) Mets at Diagnoses labeled "metastases limited to a single distant organ" or "staged as M1a." Systemic chemotherapy is the standard treatment approach for patients with stage IV rectal cancer, therefore only patients who received chemotherapy were included in the study. We restricted the Surgery Primary Site to (1) no surgery of primary site; (2) partial proctectomy, such as low anterior resection, Hartmann's operation, total mesorectal excision; (3) total proctectomy (abdominoperineal resection). We excluded patients who underwent local excision of their tumor or local tumor destruction. Patients with unknown radiation therapy or radioactive implants were excluded. Patients who had surgery to the metastatic site were also excluded from our study. After excluding 49,221 patients who were not eligible, 1,957 cases were included in the final cohort. Patients were divided into the following two groups according to the treatment strategy of the primary site: (1) Patients with primary tumor resection; (2) Patients without primary tumor resection. Each group comprised two subgroups based on whether they received radiation (**Figure 1**). Other relevant clinical characteristics including age, race, gender, marital status, tumor size, tumor grade, year of diagnosis were also collected.

Statistical Analysis

Baseline characteristics of patients with unresectable metastatic rectal cancer who had or had not undergone primary tumor resection were compared using the Chi-squared test. The primary tumor resection rate was calculated for each year from 2010 to 2015. Our primary outcome was the OS. OS was defined as the time in months from diagnosis to either death or the last follow-up date. Survival analysis was performed by year of diagnosis and treatment modalities. The survival probability was estimated by the Kaplan-Meier methods, and the differences in survival of different groups of patients were compared by using Log-rank tests. Univariate and multivariate Cox's proportional hazard regression models were performed to estimate the independent prognostic factors. We also used a multivariate

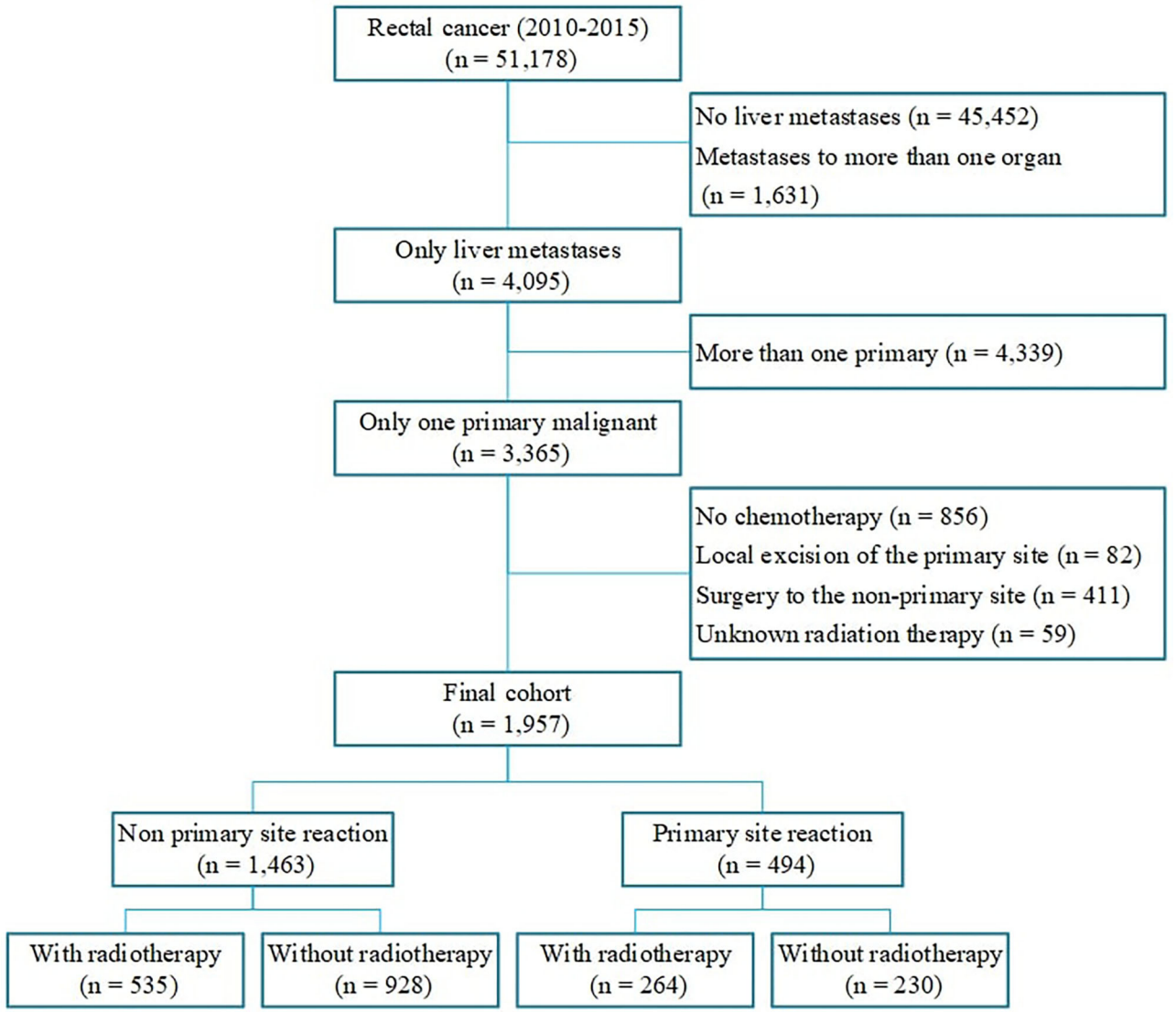

FIGURE 1 | Flowchart of the inclusion and exclusion criteria.

logistic regression model to identify factors associated with primary tumor resection. To better evaluate the impact of primary tumor resection on the survival of patients, we then divided the patients into 25 subgroups, the subgroup analyses of OS were separately performed using Cox's regression model. All statistical tests were two-sided and statistical significance was defined as $P < 0.05$. All statistical analyses were performed using the SPSS statistical software package (version 21.0; Chicago, IL) and R software (version 3.6.3; www.r-project.org).

RESULTS

Patient Characteristics

A total of 1,957 patients met our inclusion criteria (**Figure 1**), with a mean age of 58.87 ± 12.33 years. Overall, 25.2% of patients with unresectable metastatic rectal cancer had undergone primary tumor resection. At the time of presentation, patients were more likely to have been male, with an age of 50–75 years. Furthermore, patients who had undergone primary site resection were more likely to have been younger, white, and married compared with patients who had not undergone primary tumor resection. The current study also showed that patients with well-differentiated or moderately differentiated tumors, tumor size <5 cm and had undergone radiation were more likely to undergo primary tumor resection (**Table 1**).

Primary Tumor Resection Rate by Year

Figure 2 shows the primary tumor resection rates, 1-year OS, and 2-year OS by year. The highest resection rate was seen in 2010 (32.5%) and the lowest in 2014 (16.7%). The highest 1-year OS rate was seen in 2013 (71.4%) with a resection rate of 27.6%. 2010 had the highest 2-year OS rate (45.6%). Additionally, 2011

TABLE 1 | Baseline characteristics of unresectable metastatic rectal cancer patients between January 1, 2010 and December 31, 2015.

Characteristics	All patients (n = 1,957)	Primary tumor resection (n = 494)	Non-resection (n = 1,463)	*P*-value
Age at diagnosis, year, No. (%)	58.87 ± 12.33			0.012
21–49	428 (21.9%)	121 (24.5%)	307 (21.0%)	
50–75	1,327 (67.8%)	338 (68.4%)	989 (67.6%)	
76–96	202 (10.3%)	35 (7.1%)	167 (11.4%)	
Sex, No. (%)				0.119
Female	638 (32.6%)	147 (29.8%)	491 (33.6%)	
Male	1,319 (67.4%)	347 (70.2%)	972 (66.4%)	
Race, No. (%)				0.013
White	1,543 (78.8%)	399 (80.8%)	1,144 (78.2%)	
Black	221 (11.3%)	39 (7.9%)	182 (12.4%)	
Asian or Pacific Islander	188 (9.6%)	56 (11.3%)	132 (9.0%)	
Unknown	5 (0.3%)	0 (0.0%)	5 (0.3%)	
Marital status, No. (%)				0.001
Married	1,004 (51.3%)	288 (58.3%)	716 (48.9%)	
Single	452 (23.1%)	96 (19.4%)	356 (24.3%)	
Separated, divorced, or widowed	501 (25.6%)	110 (22.3%)	391 (26.7%)	
Radiation, No. (%)				< 0.001
Yes	799 (40.8%)	264 (53.4%)	535 (36.6%)	
No	1,588 (81.1%)	230 (46.6%)	928 (63.4%)	
Tumor grade, No. (%)				< 0.001
Well + Moderate	1,157 (59.1%)	369 (74.7%)	788 (53.9%)	
Poor + Undifferentiated	363 (18.5%)	84 (17.0%)	279 (19.1%)	
Unknown	437 (22.3%)	41 (8.3%)	396 (27.1%)	
Tumor size, cm, No. (%)				< 0.001
0–5	648 (33.1%)	271 (54.9%)	377 (25.8%)	
>5	562 (28.7%)	155 (31.4%)	407 (27.8%)	
Unknown	747 (38.2%)	68 (13.8%)	679 (46.4%)	
Year of diagnosis, No. (%)				< 0.001
2010	308 (15.7%)	100 (20.2%)	208 (14.2%)	
2011	309 (15.8%)	71 (14.4%)	238 (16.3%)	
2012	325 (16.6%)	84 (17.0%)	241 (16.5%)	
2013	316 (16.1%)	90 (18.2%)	226 (15.4%)	
2014	336 (17.2%)	56 (11.3%)	280 (19.1%)	
2015	363 (18.5%)	93 (18.8%)	270 (18.5%)	

exhibited the lowest 1-year OS (65.5%) and 2-year OS (39.6%) with a primary tumor resection rate of 23.0%. As can be seen in the line chart, the 2-year OS change trend is basically consistent with that year of primary tumor resection rates.

Survival Analysis

The OS of the patients with unresectable metastatic rectal cancer were analyzed by using Kaplan-Meier survival curves, and the results are shown in **Figure 3**, **Supplementary Table 1**. Patients with primary tumor resection had significantly better 5-year OS compared to patients without primary tumor resection ($p < 0.0001$) (5-year OS: 27.2 and 5.6%, respectively) (**Figure 3A**). The mean survival in the two groups were 41.1 and 21.7 months, respectively. We further conducted a stratified analysis by whether patients underwent radiotherapy or not (**Figure 3B**). The results showed that patients who receive neither primary tumor resection nor radiotherapy had the worst 5-year OS rate (3.6%). Moreover, we analyzed the OS of different radiation sequences with surgery in the primary tumor resection group (**Figure 3C**), and the *P*-value of the log-rank test was 0.0196. However, when we further included this variable into multivariate Cox's regression analyses (**Table 2**), this difference was not significant ($P = 0.055$).

Univariate and multivariate Cox's regressions were used to analyze the factors that may influence the OS (**Table 2**). Variables with $P < 0.10$ in the univariate analysis, including age at diagnosis, race, marital status, tumor size, tumor grade, treatment modality, were taken forward to multivariate Cox's regression analysis. Consequently, age older than 75 years at diagnosis (Hazard Ratio [HR] = 1.658; 95% confidence interval [CI]:1.366–2.013; $P < 0.001$), single (HR = 1.281, 95% CI: 1.126–1.458; $P < 0.001$), separated, divorced, or widowed (HR =

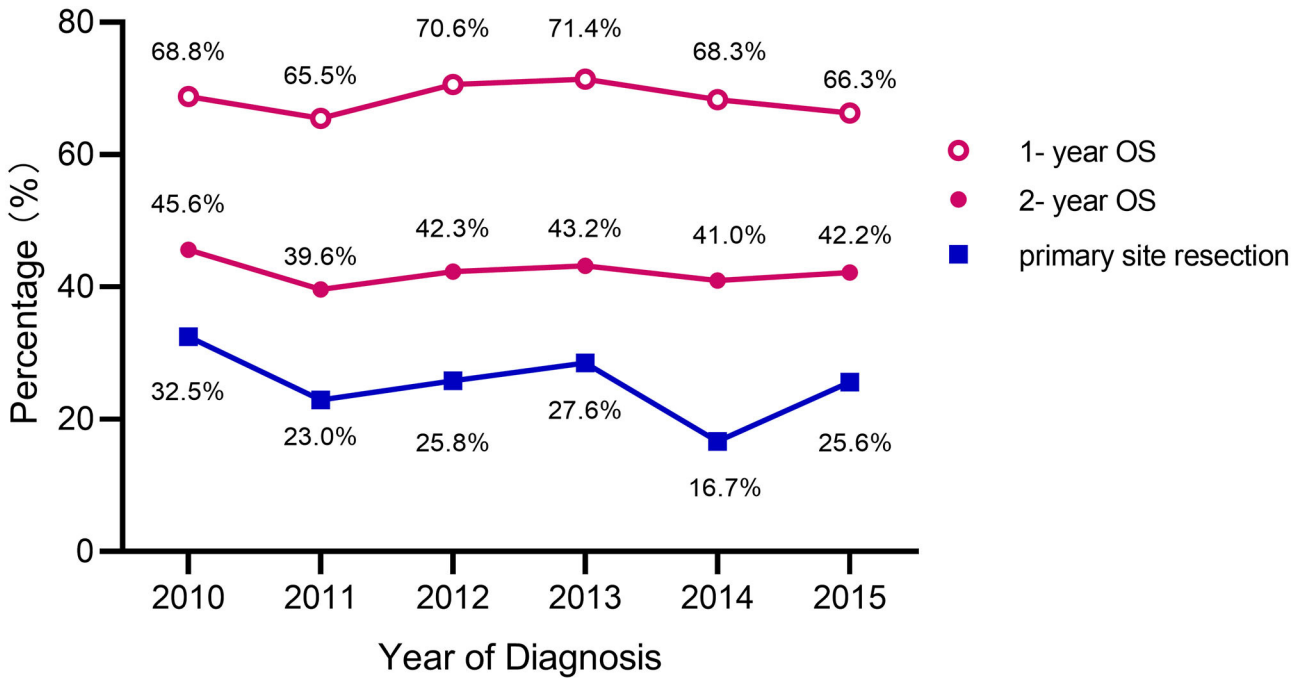

FIGURE 2 | Primary tumor resection rates and OS for rectal cancer patients with unresectable liver metastases.

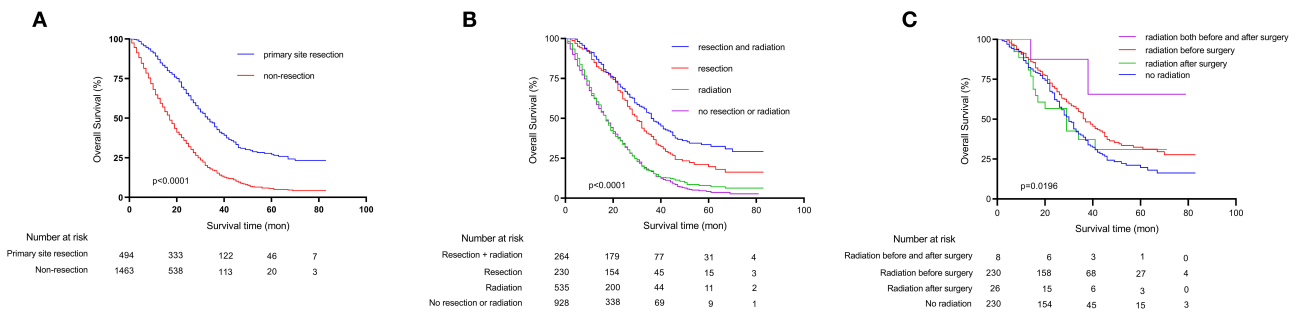

FIGURE 3 | Kaplan-Meier survival curves depicting survival in unresectable metastatic rectal cancer patients. **(A)** Patients OS based on whether primary tumor was surgically resected or not. **(B)** Patients OS based on detailed treatment modality. **(C)** Patients OS based on radiation sequences.

1.155, 95% CI: 1.019–1.309; $P < 0.001$), poorly differentiated or undifferentiated tumor (HR = 1.835, 95% CI: 1.624–2.113; $P < 0.001$), no primary site resection (radiation only or no radiation) (HR = 2.397, 95% CI: 1.969–2.918, $P < 0.001$; HR = 2.619, 95% CI: 2.168–3.162, $P < 0.001$, respectively) were confirmed to be independent risk factors for poor prognosis.

To better elucidate the effect of different treatment modalities on the prognosis of patients with unresectable metastatic rectal cancer, we divided patients into 25 subgroups according to demographic data and clinicopathological characteristics, Cox's regression model was used in each subgroup to estimate hazard rate and 95% confidence interval. The results indicated that patients who received primary tumor resection had a better prognosis than those who did not in all subgroups ($P < 0.05$) (**Figure 4**).

Multivariable Analysis

A multivariable analysis was performed using logistic regression to determine factors associated with primary tumor resection at diagnosis. The results showed that having a well-differentiated or moderately differentiated tumor, receiving radiation, and tumor size $\leq$5 cm were significantly associated with primary tumor resection (all $P < 0.001$). On the other hand, patients who were diagnosed in 2012 and 2014 were less likely to have undergone surgical resection (**Table 3**).

DISCUSSION

In this nationwide population-based study, we found that in patients with rectal cancer diagnosed with unresectable liver metastases, primary tumor resection was one of the strongest

TABLE 2 | Univariate and Multivariate analyses for OS of all patients ($n = 1,957$).

Characteristics	Univariate analysis		Multivariate analysis	
	HR [95% CI]	*P* value	HR [95% CI]	*P*-value
Age at diagnosis, year				
21–49	1		1	
50–75	1.174 (1.031-1.337)	0.016	1.129 (0.990–1.288)	0.069
76–96	1.771 (1.465–2.141)	<0.001	1.658 (1.366–2.013)	< 0.001
Sex				
Female	1			
Male	0.947 (0.848–1.056)	0.328		
Race				
White	1		1	
Black	1.245 (1.065–1.455)	0.006	1.164 (0.993–1.363)	0.061
Asian or Pacific Islander	0.994 (0.834–1.185)	0.945	0.984 (0.824–1.174)	0.854
Unknown	0.498 (0.124–1.994)	0.325	0.431 (0.107–1.732)	0.236
Marital status				
Married	1		1	
Single	1.328 (1.170–1.508)	<0.001	1.281 (1.126–1.458)	< 0.001
Separated, divorced, or widowed	1.278 (1.129–1.446)	<0.001	1.155 (1.019–1.309)	< 0.001
Tumor grade				
Well + Moderate	1		1	
Poor + Undifferentiated	1.881 (1.651–2.144)	<0.001	1.853 (1.624–2.113)	< 0.001
Unknown	1.393 (1.228–1.580)	<0.001	1.162 (1.022–1.322)	0.022
Tumor size, cm				
0–5	1		1	
>5	1.276 (1.117–1.458)	<0.001	1.115 (0.974–1.277)	0.115
Unknown	1.440 (1.273–1.628)	<0.001	1.039 (0.913–1.183)	0.562
Year of diagnosis				
2010	1			
2011	1.114 (0.943–1.315)	0.206		
2012	0.954 (0.806–1.129)	0.581		
2013	0.976 (0.821–1.160)	0.781		
2014	1.098 (0.919–1.312)	0.304		
2015	1.114 (0.912–1.361)	0.288		
Treatment modality				
Surgery + radiation	1		1	
Surgery	1.348 (1.069–1.700)	0.012	1.257 (0.995–1.588)	0.055
Radiation	2.647 (2.184–3.207)	<0.001	2.397 (1.969–2.918)	< 0.001
None	2.819 (2.349–3.383)	<0.001	2.619 (2.168–3.162)	< 0.001

predictors of a better OS. The mean OS of patients receiving primary tumor resection was 41.1 months, which was almost 20 months longer than those without resection. Nevertheless, only 25.4% of the patients in our study underwent primary tumor resection between 2010 and 2015. Well-differentiated or moderately differentiated tumor grade, tumor size $\leq$5 cm, and having radiation are associated with an increased likelihood of having undergone primary tumor resection. Our findings also indicate that resection of the primary tumor was beneficial for patients with certain clinical and pathological characteristics namely those <75 years of age with well or moderately differentiated tumors. Although this is subject to the confounding effect of better tumor differentiation as the reason of the improved survival; however, this does not exclude the likely benefit of primary tumor resection in this specific population.

Whether resection of the primary tumor in patients with unresectable liver metastases affords a survival advantage is still controversial, and research in this area has remained rather limited. One previous study reported that the use of primary tumor resection in patients with stage IV CRC had been decreasing over time, the resection rates were 74.5% in 1988 and 57.4% in 2010, however, with the improvement of systemic chemotherapy, patient survival rates improved (13). Furthermore, the newly updated NCCN guidelines recommend against routine resection of the primary tumor (12). In our study, only patients with metastatic rectal cancer were enrolled.

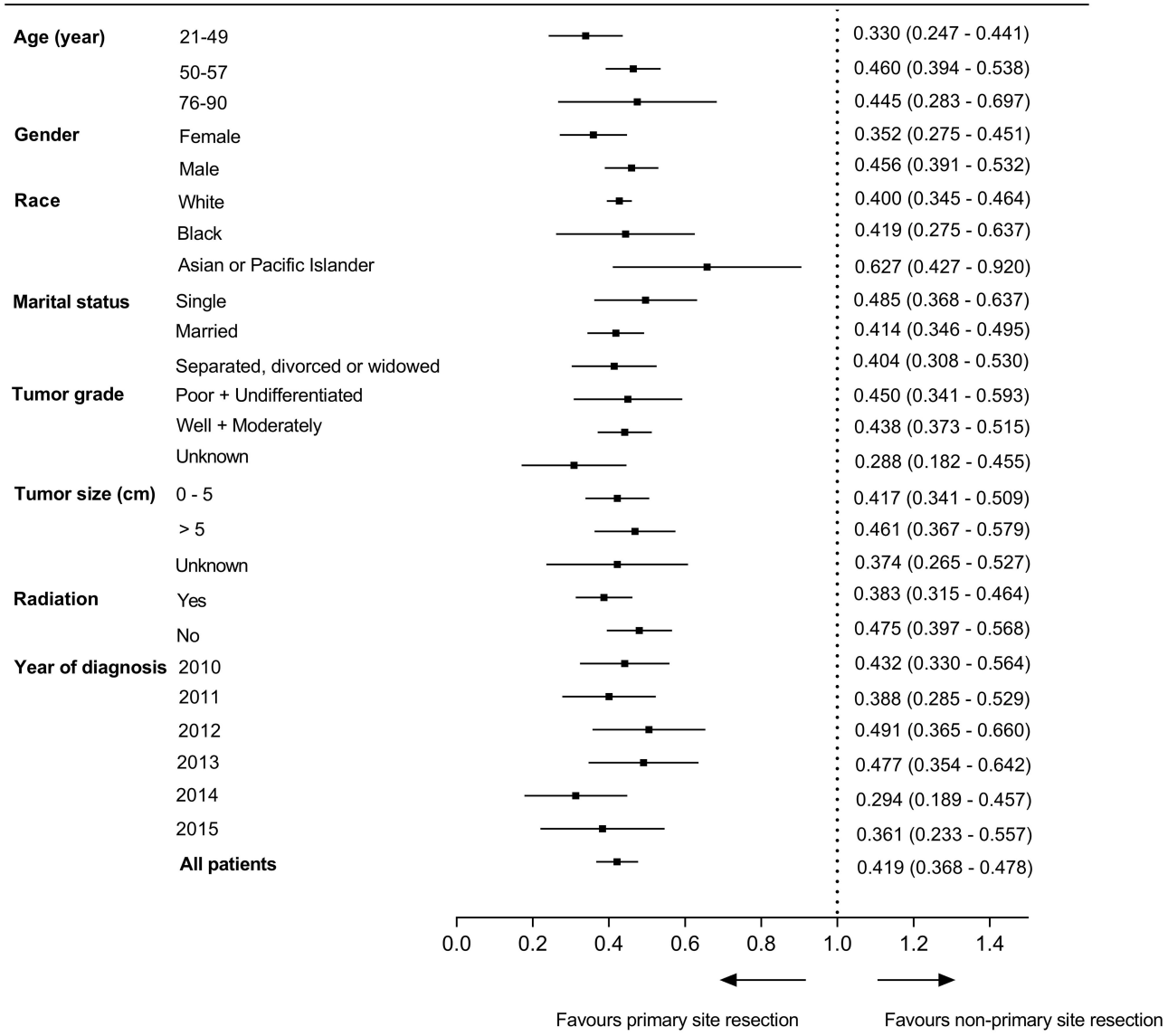

FIGURE 4 | Survival comparisons between primary tumor resection group and non-resection group in subgroup analysis.

According to the year of diagnosis, the largest proportion of primary tumor resection occurred in 2010, where only 32.5% of patients underwent resection. The differences in the resection rates are most likely due to the fact that rectal surgery has a greater postoperative complication rate and frequently requires a diverting stoma, furthermore, abdominoperineal resection (APR) must be performed for the patients with low rectal cancer, making neither surgeons nor patients willing to receive surgery in a metastatic context. Although the study mentioned above suggested that patients' survival rates improved with a decreasing resection rate (13). However, the important limitations of this study are that the conclusion did not draw from the rigorous statistical method (no multivariate Cox's regression was performed) and they had no information about whether chemotherapy was received by patients, which makes it difficult to assess the relative contribution of resection and chemotherapy on outcomes.

There are some studies with findings that are consistent with ours. Venderbosch et al. performed a retrospective analysis of two phase III studies (CAIRO and CAIRO2) investigating the prognostic value of resection of the primary tumor in in patients with unresectable stage IV CRC. Their results indicated that resection of the primary tumor is a prognostic factor for median survival and progression-free survival in mCRC patients (8). They also reviewed the literature regarding this topic and identified 22 non-randomized, single-center studies, 14 of 24 studies demonstrated an improved median OS in the resection compared with the non-resection group. Matthieu

TABLE 3 | Multivariable analysis of factors associated with receiving primary tumor resection at diagnosis.

Characteristics	OR (95% CI)	*P*-value
Age at diagnosis, year		
21–49	1 [Reference]	
50–75	0.935 (0.715–1.224)	0.627
76–96	0.636 (0.399–1.014)	0.057
Sex		
Female	1 [Reference]	
Male	1.116 (0.872–1.428)	0.383
Race		
White	1 [Reference]	
Black	0.732 (0.486–1.076)	0.11
Asian or Pacific Islander	1.220 (0.843–1.765)	0.291
Unknown	0 (0.000–)	0.999
Marital status		
Married	1 [Reference]	
Single	0.777 (0.581–1.040)	0.09
Separated, divorced or widowed	0.850 (0.642–1.125)	0.255
Tumor grade		
Poor + Undifferentiated	1 [Reference]	
Well + Moderate	1.502 (1.121–2.013)	0.006
Unknown	0.413 (0.271–0.631)	<0.001
Radiation		
No	1 [Reference]	
Yes	1.802 (1.438–2.257)	<0.001
Tumor size, cm		
>5	1 [Reference]	
0–5	1.750 (1.358–2.256)	<0.001
Unknown	0.283 (0.205–0.390)	<0.001
Year of diagnosis		
2010	1 [Reference]	
2011	0.707 (0.477–1.050)	0.086
2012	0.672 (0.459–0.983)	0.041
2013	0.821 (0.562–1.199)	0.307
2014	0.397 (0.264–0.596)	< 0.001
2015	0.697 (0.481–1.010)	0.056

et al. performed a study on the outcomes of 810 patients with CRC with unresectable synchronous metastases of which 59% underwent resection of the primary tumor. A lower baseline carcinoembryonic antigen (CEA), alkaline phosphatase levels, and normal white-blood-cell count ($P < 0.001$ each) was noted in the resection group when compared to the non-resection group. Primary tumor resection was independently associated with better OS (HR = 0.63, 95%CI: [0.53–0.75]; $P < 0.001$) (7).

The most important argument against an initial resection of the primary tumor is that surgery can delay the start of chemotherapy and patients are also subject to possible postoperative complications, both may have a negative effect on survival (8, 15). Scheer et al. reported that the overall postoperative morbidity in the patients with primary tumor resection ranged from 18.8 to 47.0%, which potentially delays beneficial systemic chemotherapy (16). Our results proved this partly true. While we analyzed survival based on the year of diagnosis, we found that the trend of 2-year OS was basically consistent with the resection rate, as the highest value of resection rate and 2-year OS both in 2010 (32.5 and 45.6%, respectively). However, the 1-year OS may be affected by surgery-related complications, and the trend was not as good as the former one (**Figure 2**). We speculated that postoperative complications have an impact on the 1-year OS, however, survival changes over time, primary tumor resection played a leading role in the 2-year OS.

The noted treatment difference between stage IV colon and rectal cancer is that radiotherapy is applied in metastatic rectal cancer for better local control of disease (17). Afshari et al. conducted a Swedish nationwide study to explore the prognostic factors that affect survival and their results showed that preoperative radiotherapy ($P = 0.001$), metastasectomy ($P < 0.001$) and radical resection of the primary tumor ($P = 0.014$) were better prognostic factors (18). From our results, we can see from the multivariate Cox's regression analysis that radiotherapy (HR = 1.257, 95%CI: [0.995–1.588]; $P = 0.055$) had no significant survival benefit for patients with metastatic rectal cancer, but patients who received radiotherapy were more likely to undergo primary tumor resection (OR = 1.802, 95%CI: [1.438–2.257]; $P < 0.001$). Furthermore, primary tumor resection is beneficial for survival in all subgroups of patients (25 subgroups, all $P < 0.05$). Therefore, radiotherapy might affect the OS indirectly.

Our study had several limitations. First, although we used multivariable analysis to adjust for clinical confounders in view of the difference between the primary tumor resection group and the non-resection group, it remains probable that primary tumor resection had been preferably performed in patients with better functional status, a selection bias cannot be excluded due to its retrospective nature and the lack of data on patient-specific comorbidities in the database. Second, the tumor size in the non-primary tumor resection group may go from endoscopic examination or computerized tomography (CT), so the values may not be as accurate as of the resection group, also key information like number and size of liver metastases were not recorded in the database; and the SEER database is short of detailed information about chemoradiotherapy regimen and biological targeted therapy, which could also influence the prognosis. Additionally, we only included patients diagnosed between 2010 and 2015, long-term survival data in those patients are still lacking, we observed only 2-year OS based on year of diagnosis, the survival trend might be more convincing if the follow-up time was longer.

CONCLUSION

Our study demonstrates that primary tumor resection in patients with unresectable metastatic rectal cancer is associated with significant improvements in survival. However, only a quarter of the patients with metastatic rectal cancer received surgical resection of the primary site. Prospective, randomized trials are necessary to determine the role of primary tumor resection in patients with unresectable metastatic rectal cancer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

J-nC, SS, and ZW: acquisition of data. J-nC, JH, and F-qZ: analysis and interpretation of data. J-nC and SS: drafting of the manuscript. JY, ZL, and QL: critical revision of the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.628715/full#supplementary-material

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Presacral Tumor: Insights From a Decade's Experience of This Rare and Diverse Disease

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Background: Presacral tumors are a group of rare and heterogeneous tumors that arise from the potential presacral space between the rectum and sacrum. The low occurrence and diverse origins make the diagnosis and treatment of these tumors a challenge. The aim of the study was to retrospectively review patient demographics and to identify advantages and disadvantages in the diagnosis and treatment of these tumors.

Methods: Retrospectively collected and reviewed data from patients who received treatment of presacral tumors at the First Affiliated Hospital of China Medical University between August 2009 and June 2019.

Results: The data from forty-four patients (33 females) with a median age of 50 years who were diagnosed with a presacral/retrorectal tumor were analyzed. The majority of tumors were congenital (61.4%) and benign tumors are more common (59.1%). The median age of patients with benign tumor was significantly higher than that of malignant tumor. The most common symptoms were sacrococcygeal/perianal pain (56.8%) and mass (36.4%), and 8 out of 9 patients having lower limb symptoms diagnosed with malignant tumor. The tumor detection rate of digital rectal examination was 75% and more than 90% of all patients underwent one or more radiology imaging exams for tumor diagnosis. Every patient had a biopsy result. The most common type of tumor was presacral cyst (40.9%) with overall tumor median size of 5.6 cm. Thirty-one (70.5%) patients underwent surgery, most often *via* the posterior route (83.9%). Posterior route surgery had significantly shorter operation time and tumors operated *via* posterior route were significantly smaller. The survival rate after surgery was 100%. The median course of disease was 6 months and median follow-up was 25 months.

Conclusions: Presacral tumors have low occurrence and are more frequently observed in females in their 30s and 50s indicating a possible link between tumor occurrence and hormonal changes. Patients with lower limb symptoms were more likely to have a malignant presacral tumor. Posterior route was the most commonly utilized surgical approach. Supplementary iodine tincture treatment of cysts ruptured in operation could potentially be helpful in reducing the chance of recurrence.

Keywords: presacral tumor, retrospective cohort study, clinical presentation, diagnosis, surgical resection

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INTRODUCTION

Presacral tumors refer to a group of rare and heterogeneous tumors that occur in the potential space between the rectum and the sacrum (1, 2) (**Figure 1**). Most of the published papers on these tumors are single case reports or studies on a limited number of cases. A few retrospective cohort studies were conducted mostly from large treatment centers with patients collected over a period of 1-5 decades (3–9). Tumor incidence was reported as 1.4–6.3 patients per year (1, 3–9). These heterogeneous tumors are difficult to classify because the tissues surrounding the presacral space are originated from the embryologic stem cells that later differentiated into three germinal layers which further develop into connective, osseous and neural tissues. The traditional Lovelady and Dockerty (10) classification and the more recent Uhlig and Johnson (11) classification system are currently utilized by surgeons.

Presacral tumors occur in both sexes at the median age of 45–50 years and more frequently in female (3, 12, 13). The fact that majority of patients are asymptomatic or show only non-specific symptoms such as pain in the perianal area or lower back, constipation or lower limb numbness (14–16) makes diagnosis a challenge. Misdiagnoses often happen and lead to delay of proper treatment and sometimes undesired consequences (1, 16).

Biopsy remains the gold standard for diagnosis of these tumors, but many advanced imaging modalities including MRI, CT scans have become as effective in making diagnosis and treatment plans. Complete surgical excision is still the best choice for these tumors because of the possibility of infection or the malignant tendency (5, 15).

The current study collected 44 cases of presacral tumors in one of the largest tertiary institutions in Liaoning province of China in the past decade. The aim of this study was to evaluate patient information from this institution and compare it to what has been published in other centers. The goal was to identify patient demographics as well as some of the advantages and disadvantages in diagnosing and treating presacral tumors.

MATERIALS AND METHODS

This study was conducted by retrospectively reviewing the surgical pathology and tumor registries at the First Affiliated Hospital of China Medical University. The research protocol was approved by the Medical Science Research Ethic Committee of the First Affiliated Hospital of China Medical University. Written informed consent was obtained from patients prior to their treatment. All patients who had a confirmed diagnosis of a benign or malignant presacral/retrorectal tumor between August 2009 and June 2019 were selected and all available data on each case were collected. Patients whose diagnose could not be confirmed by pathology or have no biopsy records were excluded from the study. In most cases, data collected include the demographics, clinical presentation, pre-surgery diagnosis, surgical approach, surgical margins, tumor pathology, adjuvant therapy, biopsy results, radiologic imaging, mortality, and local

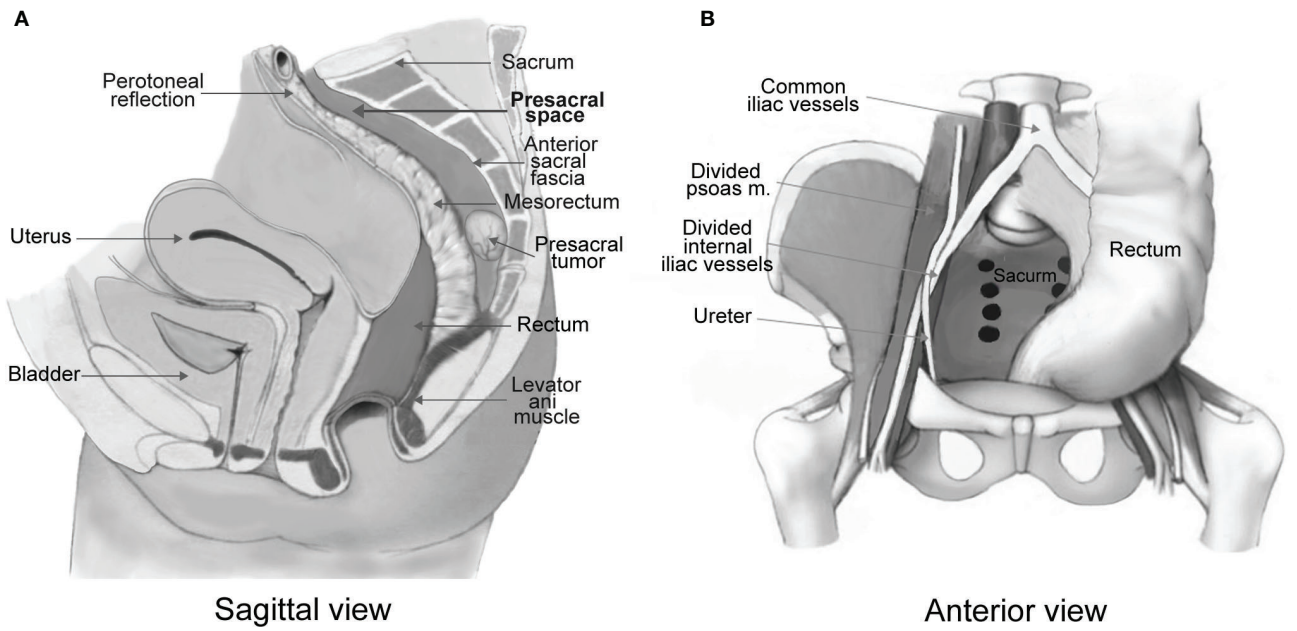

FIGURE 1 | Anatomical illustration of a presacral tumor in relation with the presacral space **(A)**. The presacral space is formed with the rectum and mesorectum in the anterior, the anterior sacral fascia in the posterior, the levator ani muscle in the inferior, the peritoneal reflection in the superior **(A)**, and the iliac vessels and ureters laterally **(B)**. Illustrations are modified from Dozois and Marcos (2).

recurrence. We also conducted follow-up on available patients after patient identifications were uncovered.

Complications are reported based on the Clavien-Dindo classification (16). Literature review was conducted by searching English or Chinese peer reviewed articles including some of the Chinese articles with English abstracts.

Statistical Analysis

Non-normally distributed quantitative variables were reported as median and range. Associations with quantitative variables between groups with different sample sizes were analyzed with the non-parametric Kruskal-Wallis one-way analysis of variance test by ranks, categorical variables were analyzed with Pearson's Chi-Square test and survival differences were analyzed with Kaplan-Meier estimates. Statistical significance was defined as $p < 0.05$.

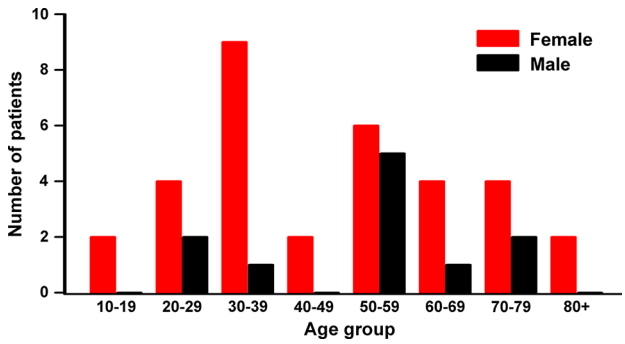

FIGURE 2 | Patient distribution by gender and age groups. High occurrence age is the 50s in both male and female, while the highest in female is the 30s. Nonparametric Kruskal-Wallis ANOVA test showed significant differences between genders by age group ($p = 0.0136$), but not the median age between genders (p = 0.65452).

RESULTS

General Patient Information

Forty-four patients were identified including 33 (75%) females and 11 (25%) males. The median age of all patients was 50 years (range 13–87 years) with the median age of female at 49 (range 13–87 years) and that of male at 55 (range 24–77 years). The average course of disease at presentation was 6 months (range 0.1–720 months). Twenty six patients were diagnosed with benign tumors whereas 18 were diagnosed with malignant tumors (**Table 1**). There were 31 patients who underwent surgery, eight patients received adjuvant therapy, and five patients were simply followed up and observed.

Patients with malignant tumors were significantly younger ($p = 0.01214$) with a median age of 38 years (range 13–87 years) while patients with benign tumors had a median age of 59 years (range 14–80 years).

When patients were grouped according to age by decades, we found that the highest number of female patients is in the 30s (30–39 years; 9/33, 27.3%) followed by the 50s (6/33, 18.2%), while five out of 11 male patients are in their 50s (45.6%; **Figure 2**). Non-parametric Kruskal-Wallis analysis indicated that the number of female patients in each age group was significantly higher than the number of males ($p = 0.01356$), while there was no difference in the median age between genders ($p = 0.65452$).

The female patients in their 30s were mostly diagnosed with congenital tumors (8/9, 88.9%) which included six developmental cysts and two teratomas. The female patients in the 50s have a similar pattern with four congenital tumors (4/6, 66.7%) with three developmental cysts and one chordoma. Interestingly, these higher occurrences of the tumors in female patients in their 30s and 50s coincide with periods of dramatic changes in female hormones. Better understating of the contribution of female hormones to the development and growth of these congenital tumors could lead to novel and improved methods for the management of the presacral tumors in female patients.

TABLE 1 | Initial symptoms (patient may have more than one symptom).

Symptom	Benign		Malignant		No. of Patient
	Male	Female	Male	Female	
Sacrococcygeal/perianal pain	0	12	4	9	25 (56.8%)
Sacrococcygeal/perianal mass	2	10	2	2	16 (36.4%)
Bowel or urinary complaints	2	7	2	2	13 (29.5%)
Lower limb symptoms	1	0	3	5	9 (20.5%)
Physical exam or others	0	11	2	2	15 (34.1%)
Total Number	4	22	7	11	44

Clinical Presentations

The most common initial symptoms were sacrococcygeal and perianal pain (25, 56.8%) followed by sacrococcygeal and perianal mass (16, 36.4%) and dysfunction of bowel or urinary systems (13, 29.5%; **Table 1**). In addition, some lower limb symptoms (20.5%) such as numbness, radiating pain or movement problems have been reported. The overall tumor size of our cohort of patients is 5.6 cm (range 1.2–20 cm), the median tumor size for the surgical group is 5.6 cm (range 2.2–20 cm); that of the adjuvant therapy group is 5.36 cm (range 3.1–16.7 cm) while that of the untreated group is 6.4 cm (range 3.2–16 cm).

When compare presenting symptoms by malignancy, patients with benign tumors have pain less frequently (12/26, 46.2%) than the ones with malignant tumors (13/18, 72.2%), Pearson's Chi-Square test showed a trend towards significance ($p = 0.08609$). It is worth noting that majority of patients (eight of nine cases, 88.9%) who had lower limb symptoms have malignant tumors ($p = 0.00103$, **Table 2**), and all female patients presenting these symptoms were diagnosed with malignant tumors (**Table 1**). There was no difference in tumor sizes between benign and malignant cases ($p = 0.51606$, **Table 2**).

TABLE 2 | Comparison between patients with benign and malignant tumors.

Tumor type	Benign	Malignant	*p* Value
Age (year)	59 (14–80)	38 (13–87)	**0.01214**
Tumor size (cm)	4.805 (1.2–20)	6.3 (3.1–20)	0.51606
Follow-up (mon)	27 (3–69)	25 (6–93)	0.91708
Surgical case	23 (88.5%)	8 (44.4%)	**0.00114**
Sacrococcygeal/perianal pain	12 (46.2%)	13 (72.2%)	0.08609
Sacrococcygeal/perianal mass	12 (46.2%)	4 (22.2%)	0.10470
Bowel or urinary complains	9 (34.6%)	4 (22.2%)	0.37568
Lower limb symptoms	1 (3.8%)	8 (44.4%)	**0.00103**
Recurrence rate*	23.5%(4/17)	71.4%(5/7)	**0.02746**
Total cases	26	18	

**Follow-up patients; bold numbers represent p < 0.05.*

Diagnostic Modalities and Tumor Characteristics

There were 20 patients who had digital rectal examination (DRE), 15 (75%) of which had positive tumor palpation. There were 37 (84.1%) patients who had preoperative or postoperative biopsies in our hospital to confirm the tumor pathology, and seven (15.9%) were confirmed by other institutions. Most patients (41, 93.2%) had preoperative diagnostic imaging of MRI/CT/B-mode ultrasound/PET-CT (one or multiple), and the other three (6.8%) were diagnosed by DRE combined with intra-operative findings (examples shown in **Figure 3**). Among the 41 patients with diagnostic imaging, CT scan along was performed in 20 (58.5%), MRI along in 12 (46.3%), and ultrasound along in two (4.9%) patients; a combination of CT and MRI in four (9.6%), MRI and ultrasound in three (7.3%) patients. We found that the accuracy of the MRI is at 89.5%, a little bit higher than the CT scan (83.3%), though not statistically significant.

In our cohort of patients, the most common presacral tumors were congenital ones (28, 63.6%) including developmental cysts (18, 40.9%) with 14 (77.8%) tailgut cysts, two epidermoid and two dermoid cysts followed by chordomas (6, 13.6%) and teratomas (4, 9.1%; **Table 3**). Majority of the tumors were benign (26, 59.1%); among the 18 cases of malignant tumors (40.9%), 13 were originated in the presacral space which included six chordomas and two teratomas, and the other five were metastasized ones (**Table 3**).

Clinical Managements

Thirty-one patients (70.5%) received surgery, among which two had previously received rectal cyst excision and perianal abscess removal from other institutions. The 13 non-surgical patients included eight patients with malignant tumors, four of whom (9.1%) received adjuvant therapy (previously underwent

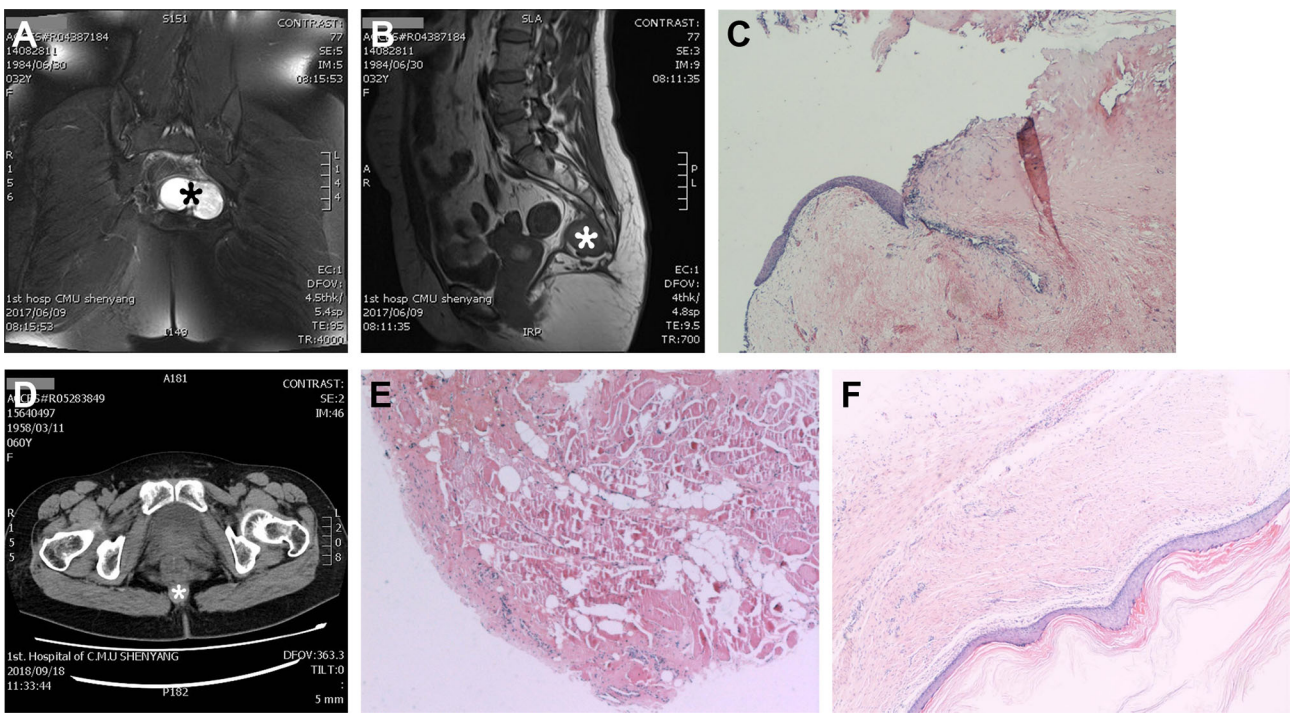

FIGURE 3 | Representative cases of the use of diagnostic imaging and pathology. MRI pelvis with axial view **(A)** showing a 6.2 cm × 4.4 cm × 4.1 cm tumor (*), and sagittal view **(B)** showing the relationship between tumor (*) and the walls of the presacral space. **(C)** Biopsy showing that the tumor contains variety of tissues and surrounded by a cystic wall, which is consistent with a presacral teratoma **(A–C)**. **(D)** CT scan image showing a size 2.0 cm × 2.0 cm round tumor (*) with smooth edge and biopsy **(E)** confirms that it is a benign epidermoid presacral tumor. **(F)** Patient who was diagnosed with a perianal tumor on digital rectal examination and confirmed with biopsy, the Hematoxylin-Eosin stain of the cyst wall and content indicating a presacral cyst.

TABLE 3 | Tumor classification according to Uhlig and Johnson system (3).

Types of presacral tumor	N = 17
Congenital (28)	
Developmental (18)	
Epidermoid	2
Dermoid	2
Tailgut cyst	14
Teratomas (2*)	4
Chordomas*	6
Inflammatory: abscesses	1
Neurogenic	
Chordal meninginoma*	1
Miscellaneous (14)	
Plasmocytoma*	2
Mucinous adenocarcinoma*	1
Epithelial neoplasms	1
Smooth muscle tumor	1
Giant cell tumor of sacrum	1
Clear cell sarcoma of soft tissue*	1
Retrorectal benign nodule	1
Vascular tumor with abscess	1
Secondary malignant tumor**	1
Metastatic adenocarcinoma**	4

*Original malignant tumor. **metastatic tumor.

surgery), and four (9.1%) received radiation/chemotherapy only; and the presacral tumors of the other five patients (11.4%) were not treated by patients' requests, four of which discovered the presacral tumors incidentally when treating the original diseases.

Complete tumor resection was the goal of the presacral tumor operation. Among the operations, 21/31 (67.7%) were surgical resections alone and 10/31 (32.3%) were presacral cysts resections combined with supplementary iodine tincture cauterization that resulted in similar overall cure rate (87.5% *vs.* 88.9%, respectively). There were more patients with benign tumor who underwent surgeries (23/26, 88.5%) than the ones with malignant tumor (8/18, 44.4%; $p = 0.00114$; **Table 2**).

The most common surgical approach was the posterior approach *via* sacrococcyx or perineal (26/31, 83.9%), which had the shortest median operation time of 45 min (range 20–315 min; $p = 0.00702$) when compared with an anterior approach *via* abdomen (3/31, 9.7%) and a combined anteroposterior approach for those with larger tumors (2/31, 6.4%; **Table 4**). The median tumor size of the posterior surgery group was 5.3 cm (range 2.2–9.1 cm) which was also significantly smaller than the other two groups ($p = 0.03479$; **Table 4**). There was no difference in tumor size between male and female patients who underwent surgery ($p = 0.57217$).

Follow-Up and Complications

There were 34 follow-up consultations conducted (77.3%) and the median follow-up time was 25 months (range 3–93 months). Among the 24 follow-ups of the 31 patients in the surgery group, nine recurred (37.5%) and all survived (**Table 4**). Majority of the recurred patients (6/9, 66.7%) underwent a second surgery, and only one recurred after two operations. The remaining patients (3/9, 33.3%) chose non-operational treatment because they were asymptomatic. Of the 10 follow-up patients in the non-surgical groups, two patients were in the radiation/chemotherapy group one of which recurred with no symptom and the other one died after 18 months from the malignant tumor; four follow-up patients were in the post-operative radiation/chemotherapy group including one recurrence, two deaths from the malignant tumors after four and 66 months of survival (respectively), and one remained in treatment; the other four were untreated patients of which three died with one due to the presacral malignant tumor and two from other pre-existing diseases.

There were no significant differences between the three surgical approaches in recurrence rate ($p = 0.25281$) and survival rate (**Table 4**). When divided patients with tumor malignancy, malignant tumors have significantly higher recurrence rate ($p = 0.02746$, **Table 2**), and there was a trend that patients with benign tumor have higher survival rate than the ones with malignant tumor ($p = 0.0629$; **Figure 4A**), but there was no gender difference in overall survival (**Figure 4B**).

Seven patients developed postoperative complications, five of which had poorly healed incisions (Grade I) after a posterior surgical approach, one had rectal fistula (Grade IIIa) following an anterior approach and another one who underwent a combined approach surgery plus coccygectomy had lower limb movement dysfunctions (Grade Id).

DISCUSSION

Cases of presacral tumors diagnosed and treated at a large regional hospital for the past 10 years were reviewed to gain insights in the occurrence rate, diagnosis, and treatment of this rare tumor. The data indicate that these tumors are most

TABLE 4 | Comparison of three surgical approaches.

Surgical approach	Posterior	Anterior	Combined	Total	*p* Value
No. of Patient	26	3	2	31	
Course of disease (mon)	12 (0.1–720)	24 (8–240)	19 (2–36)	6 (0.1–720)	0.46199
Operation time (min)	45 (20–315)	195 (120–210)	429.5 (420–439)	60 (20–439)	**0.00702**
Hospital stay (day)	7 (2–177)	12 (9–19)	14.5 (14–15)	8.5 (2–177)	0.31613
Tumor size (cm)	5.3 (2.2–9.1)	7.6 (7.5–20)	12.85 (5.7–20)	5.6 (2.2–20)	**0.03479**
Follow-up (mon)	30 (3–93)	20.5 (16–25)	17 (17–17)	25 (3–93)	0.51029
Recurrence rate*	8 (21*;38.1%)	0 (2*;0%)	1 (1*;100%)	9 (24*;37.5%)	0.25281
Survival rate*	100%	100%	100%	100%	0.99999

*Follow-up patients only; bold numbers represent $p < 0.05$.

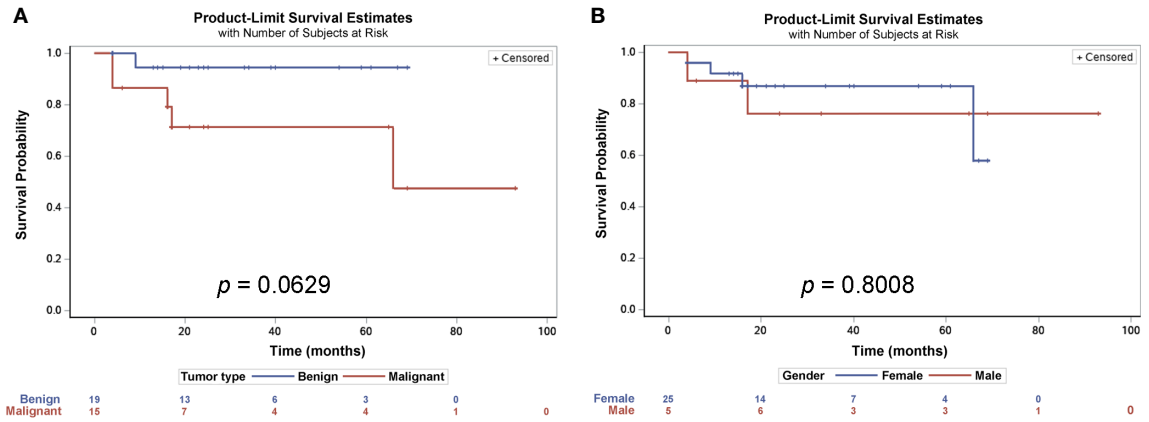

FIGURE 4 | Survival probability estimates of patients with presacral tumors. Kaplan-Meier estimates analysis showed that there was a trend for patients with benign tumors having a longer survival rate, though not statistically significant **(A)**, but there were no differences between male and female patients **(B)**.

commonly seen in female patients in their 3rd and 5th decade of age and that the majority of these tumors can be characterized as congenital presacral tumors. The high incidence coincides with the ages when female hormonal changes are the most pronounced. Lower limb symptoms were predictive of malignant tumors and we reported for the first time that surgical resection of presacral cysts supplemented with iodine tincture treatment could have helped to reduce the risk of recurrence.

Presacral tumors, also referred as retrorectal tumors, are very rare with reported cases in the literature of only 1.4–6.3/year (1, 3–9). The occurrence in this patient cohort was 4.4 cases/year, and the number of female patients is significantly higher than that of male patients in every age group especially in the 3rd and 5th decades. Female fertility starts decreasing significantly in the third decade (17) and menopause happens around the beginning of the fifth decade of age (18). It is notable from our data pool that females in the 30s and 50s age groups had the highest incidence of these tumors coinciding with the periods of drastic hormonal fluctuations. Due to the rarity and diversity of these tumors, this phenomenon has apparently not been documented before. In support of our findings, the Buchs et al. (4) study of 16 cases included 13 female patients, among which the highest numbers were in their 30s (6, 46.2%) and 50s (3, 23.1%). Other evidence shows that a presacral benign cyst had strong estrogen receptor immunohistochemical staining in both the cyst-lining cells and the tumor cells in humans (19) and that age-related difference of teratoma growth rate in female mice was due to the changes in the levels of the estrogen and progesterone (20). Based on the evidence above, we postulate that dramatic changes in female hormones may contribute to the development of congenital presacral tumors. This may represent a potential avenue for discovering new treatment strategies. For example, when a female patient presenting non-specific sacral/anal pain that is in her 30s or 50s, a presacral congenital tumor should be considered; estrogen or progesterone receptor inhibitors may be of benefit to prevent tumor growth providing a potential non-invasive treatment option for these tumors especially for patients who are asymptomatic. Such endocrine inhibitors have been the primary systemic treatment for estrogen or progesterone receptor positive breast cancer (21).

It is not unusual for patients with presacral tumor to be asymptomatic. Most of the initial symptoms are related to tumor compression or invasion of the surrounding tissues and organs (22). In our study, five patients (11.4%) showed no initial symptoms as similarly reported in some studies (12, 23, 24), while other studies showed a higher percentage of patients with no symptoms (up to 50%) (8). Besides the pain and mass found in majority of patients, we found 8 out of 9 patients who have lower limb symptoms are diagnosed with malignant tumors. Evidence indicates that the lower limb symptoms such as lumber radiculopathy were caused by malignant tumors invading the spine (25), and it is important when presenting with lower limb symptoms patients should be carefully examined for evidence of a malignant presacral tumor.

The frequency of malignant tumors in our cohort of patients is 40.9% which is similar to previous studies (3, 26). The malignant tumor develops differently between genders, and our cohort has higher frequency in males (1) while others reported more in females (3). When compared to the benign tumors, the median age of our patients with malignant tumors is much younger (p = 0.01214), whereas the recurrence rate of the malignant tumor is much higher (p = 0.02746, **Table 2**). The survival rate between benign and malignant tumors was trending towards being statistically different (p = 0.0629, **Figure 4A**), but may not have reached significance levels on the account of the fact that many of the patients had the low-grade malignant tumor chrodoma which is believed to have longer survival rate after tumor resection (27). In addition, the follow-up period may not have been long enough for the differences to be manifested. The fact that the median age of the patients with a malignant tumor in the present study is much younger and the recurrence rate is much higher underscores the need for early detection and early treatment of any presacral tumors despite the malignancy.

Surgery is and has been the pillar of the presacral tumor management. Ideally, surgery should remove the entire presacral tumor. In our institution, the posterior approach usually was performed using transsacrococcygeal or transperineal technique. Most (83.9%) of the tumors in our study were removed through the less invasive posterior approach which had the shortest operation time and short hospital stays (**Table 4**) in addition to an overall post-operational survival of 100%. It is also worth noting that in our study, 10 of the 18 patients with cystic presacral tumor received surgical resection followed by iodine tincture cauterization treatment. Iodine tincture is an alcohol-based solution used as an antiseptic and disinfectant. The iodine in the solution causes protein denaturation and necrosis of the cyst wall (28). Our study was the first to report the use of iodine tincture for treatment of presacral cysts even though it has been used for treating other types of cyst (29–32). This procedure was used in patients with cysts that had intra-operation capsule ruptures or when the cyst decompression was necessary during surgery. This is especially of consequence since capsule rupture is a common occurrence during surgical resection of presacral cysts (33, 34). The use of 2% iodine tincture solution to rinse the anterior sacral space can prevent seeding caused by residue cystic contents and in turn reduce the chance for tumor to recur. As a result, the overall cure rate with the supplementary iodine tincture treatment has reached similar level to that of the surgical resection alone (88.9% *vs*. 87.5%, respectively). A limited number of patients and follow-up in our study suggest a need for more research regarding the use of iodine tincture solution.

It is difficult to identify critical factors in retrospective study of a rare disease. While previous studies on presacral tumors were similarly based on a relatively small number of patients with median follow-up periods ranging from several months to 2 years (3–5, 9, 35), the present study is able to report new findings related to this rarely encountered tumor. In summary, presacral tumors are more common in females of 30 and 50 years of age when dramatic hormonal changes occur. Patients are often asymptomatic with palpable mass. Patients with lower limb symptoms are highly suspicious of a malignant presacral tumor. Posterior route is the most utilized approach because of the shorter operation time and hospital stays. Supplementary iodine tincture treatment could be of help in preventing cyst content seeding especially when the cyst was ruptured during surgery, which in turn lower the chance of these tumors to recur.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Science Research Ethic Committee of the First Affiliated Hospital of China Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZL conceived and designed the study, collected patients' data, analyzed the data, and wrote the manuscript. ML provided critical comments for the manuscript. All authors contributed to the article and approved the submitted version.

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Oncologic Nomogram for Stage I Rectal Cancer to Assist Patient Selection for Adjuvant (Chemo)Radiotherapy Following Local Excision

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Background: Because of the low rate of lymph node metastasis in stage I rectal cancer (RC), local resection (LR) can achieve high survival benefits and quality of life. However, the indications for postoperative adjuvant therapy (AT) remain controversial.

Methods: A retrospective analysis was performed in 6,486 patients with RC (pT1/T2) using the Surveillance, Epidemiology, and End Results (SEER) database. Patients were initially diagnosed from 2004 to 2016; following LR, 967 received AT and 5,519 did not. Propensity score matching (PSM) was used to balance the confounding factors of the two groups; the Kaplan–Meier method and the log-rank test were used for survival analysis. Cox proportional hazards regression analysis was used to screen independent prognostic factors and build a nomogram on this basis. X-tile software was used to divide the patients into low-, moderate-, and high-risk groups based on the nomogram risk score.

Results: Multivariate analysis found that age, sex, race, marital status, tumor size, T stage, and carcinoembryonic antigen (CEA) in the non-AT group were independent prognostic factors for stage I RC and were included in the nomogram prediction model. The C-index of the model was 0.726 (95% CI, 0.689–0.763). We divided the patients into three risk groups according to the nomogram prediction score and found that patients with low and moderate risks did not show an improved prognosis after AT. However, high-risk patients did benefit from AT.

Conclusion: The nomogram of this study can effectively predict the prognosis of patients with stage I RC undergoing LR. Our results indicate that high-risk patients should receive AT after LR; AT is not recommended for low-risk patients.

Keywords: stage I, rectal cancer, nomogram, prognosis, postoperative adjuvant therapy

INTRODUCTION

Colorectal cancer is the third most common cancer in the world and the second leading cause of cancer death. While rectal cancer (RC) accounts for one-third of the colorectal cancer cases, most are distal RC (1, 2). In recent years, due to progress with imaging and endoscopy, RC can be detected in the early stage. In the early stage of RC, tumor cells are mostly well-differentiated, the rate of lymph node metastasis is < 10%, complete cure can be achieved through local resection (LR), and LR reduces the perioperative complication rate and mortality (3, 4). LR primarily includes transanal resection (TAE) and transanal endoscopic microsurgery (TEM). In 1977, Professor Morson (5) of St. Mark's Hospital in the United Kingdom first published the results of the application of local excision in the treatment of early RC. Only 10 of 119 patients were reported to have a recurrence, and the recurrence rate was 8.4%. Since then, the application of LR in stage I RC has become increasingly widespread.

Studies have shown that risk factors for local recurrence include tumor size > 3 cm, stage > T1, tumor invasion depth of submucosal invasion 3 (SM3) and above, poor differentiation of adenocarcinoma, lymphovascular invasion, and positive margins. However, there is no agreement on risk factors for evaluating recurrence and prognosis, and some studies have shown that age and gender are also high-risk factors for recurrence (6–8). In patients with high-risk factors, the local recurrence rate can reach ∼20%, which then requires remedial radical surgery or adjuvant therapy (AT). AT (radiotherapy, chemotherapy, or chemoradiotherapy) can be used as an alternative to remedial radical surgery because it has the potential to not only reduce the recurrence rate and organ-preservation after LR, but also has the same effect on prognosis compared with remedial surgery (9–14). Therefore, this paper also focuses on the clinical effect of AT in patients with RC with a high risk of recurrence after LR.

This study evaluated the prognosis of patients with stage I RC by analyzing various clinical case factors in the Surveillance, Epidemiology, and End Results (SEER) database. The nomogram was used to select candidates for AT.

MATERIALS AND METHODS

Patient Cohort

The SEER*Stat (version 8.3.6) software was used to analyze data from 6,486 patients with stage I (pT1/2N0M0) RC diagnosed between 2004 and 2016. Inclusion criteria were: (1) RC confirmed

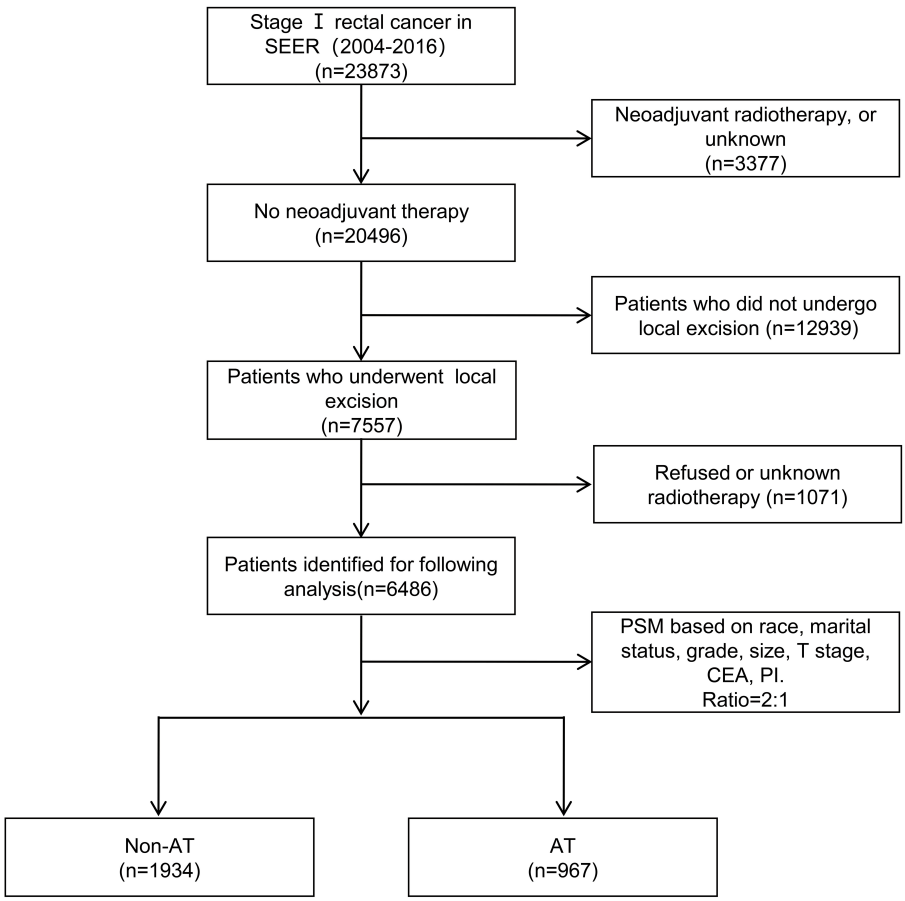

FIGURE 1 | A flowchart of the selection process of included patients.

by pathology (ICD-O-3: C20.9); (2) complete follow-up and survival data; (3) adenocarcinoma histology type (ICD-O-3: M-8140); (4) no neoadjuvant radiotherapy received; and (5) completion of LR. The following variables were evaluated: age, sex, race, marital status, histology, tumor grade, tumor size, T stage, carcinoembryonic antigen (CEA), perineural invasion (PI), AT information, and survival information. Cases with unknown information related to these variables were excluded.

Statistical Analysis

A chi-square test was used to analyze the relationship between the non-AT and AT groups. In order to balance the confounding bias of the included cases, the meaningful clinical pathological factors of the chi-square test were included in propensity score matching (PSM). The nearest neighbor matching was performed at 2:1 in the non-AT and AT groups (15). Then, the Kaplan–Meier method and the log-rank test were used for survival analysis.

In the non-AT group, the prediction model was established by following a series of steps. First, Cox univariate analysis was used to analyze the correlation between variables and overall survival (OS). Second, variables with statistical differences in univariate analysis ($p < 0.05$) were included in the Cox multivariate analysis. Third, on the basis of the Cox multivariate analysis, the nomogram survival prediction model was established. The effectiveness of the prediction model was tested and the degree of discrimination was measured by the concordance index (C-index) (16). The calibration curve intuitively showed the consistency between the predicted survival rate and the actual survival rate, and decision curve analysis (DCA) was used to evaluate the clinical net benefit compared with T stage. Fourth,

TABLE 1 | Characteristics of patients.

Variable	Unmatched cohort			P-value	Matched cohort			*P*-value
	Total [n (%)]	Non-AT [n (%)]	AT [n (%)]		Total [n (%)]	Non-AT [n (%)]	AT [n (%)]	
Age				0.227				0.404
<65	2,724 (42.0)	2,335 (42.3)	389 (40.2)		1,136 (39.2)	747 (38.6)	389 (40.2)	
≥65	3,762 (58.0)	3,184 (57.7)	578 (59.8)		1,765 (60.8)	1,187 (61.4)	578 (59.8)	
Sex				0.186				0.137
Male	3,741 (57.7)	3,202 (58.0)	539 (55.7)		1,673 (57.7)	1,134 (58.6)	539 (55.7)	
Female	2,745 (42.3)	2,317 (42.0)	428 (44.3)		1,228 (42.3)	800 (41.4)	428 (44.3)	
Race				0.003				0.987
White	5,286 (81.5)	4,480 (81.2)	806 (83.4)		2,421 (83.5)	1,615 (83.5)	806 (83.4)	
Black	550 (8.5)	458 (8.3)	92 (9.5)		272 (9.4)	180 (9.3)	92 (9.5)	
API	506 (7.8)	446 (8.1)	60 (6.2)		183 (6.3)	123 (6.4)	60 (6.2)	
Other	144 (2.2)	135 (2.4)	9 (0.9)		25 (0.8)	16 (0.8)	9 (0.9)	
Marital status				0.001				0.780
Married	4,056 (62.5)	3,404 (61.7)	652 (67.4)		1,970 (67.9)	1,318 (68.1)	652 (67.4)	
Unmarried	769 (11.9)	656 (11.9)	113 (11.7)		346 (11.9)	233 (12.0)	113 (11.7)	
Unknown	1,661 (25.6)	1,459 (26.4)	202 (20.9)		585 (20.2)	383 (19.8)	202 (20.9)	
Grade				<0.001				0.002
Well/moderately	5,023 (77.4)	4,242 (76.9)	781 (80.8)		2,406 (82.9)	1,625 (84.0)	781 (80.8)	
Poorly/undifferentiated	418 (6.4)	302 (5.5)	116 (12.0)		271 (9.3)	155 (8.0)	116 (12.0)	
Unknown	1,045 (16.2)	975 (17.6)	70 (7.2)		224 (7.8)	154 (8.0)	70 (7.2)	
Size (cm)				<0.001				0.006
<3	2,890 (44.6)	2,394 (43.4)	496 (51.3)		1,587 (54.7)	1,091 (56.4)	496 (51.3)	
≥3	764 (11.8)	552 (10.0)	212 (21.9)		549 (18.9)	337 (17.4)	212 (21.9)	
Unknown	2,832 (43.6)	2,573 (46.6)	259 (26.8)		765 (26.4)	506 (26.2)	259 (26.8)	
T stage				<0.001				<0.001
T1	5,451 (84.1)	4,921 (89.2)	530 (54.8)		1,866 (64.3)	1,336 (69.1)	530 (54.8)	
T2	1,035 (15.9)	598 (10.8)	437 (45.2)		1,035 (35.7)	598 (30.9)	437 (45.2)	
CEA (ng/ml)				<0.001				0.312
≤5	1,653 (25.5)	1,336 (24.2)	317 (32.8)		953 (32.9)	636 (32.9)	317 (32.8)	
>5	403 (6.2)	303 (5.5)	100 (10.3)		267 (9.2)	167 (8.6)	100 (10.3)	
Unknown	4,430 (68.3)	3,880 (70.3)	550 (56.9)		1,681 (57.9)	1,131 (58.5)	550 (56.9)	
PI				<0.001				0.280
Negative	2,391 (36.9)	2,082 (37.7)	309 (32.0)		881 (30.4)	572 (29.6)	309 (932.0)	
Positive	34 (0.5)	23 (0.4)	11 (1.1)		27 (0.9)	16 (0.8)	11 (1.1)	
Unknown	4,061 (62.6)	3,414 (61.9)	647 (66.9)		1,993 (68.7)	1,346 (69.6)	647 (66.9)	

AT, adjuvant therapy; API, Asian/Pacific Islander; CEA, carcinoembryonic antigen; PI, perineural invasion.

according to the risk score of the nomogram, X-tile software was used to artificially divide the cases into low-, moderate-, and high-risk groups (17). All statistical analyses in this study were performed using SPSS 24.0 and R language (version 3.6.3), and $p < 0.05$ was considered to be statistically significant.

RESULTS

Patient Demographics

According to inclusion and exclusion criteria (**Figure 1**), a total of 6,486 patients were included with LR of stage I RC before the PSM, including 5,519 in the non-AT group and 967 in the AT group. The median survival was 55 months (0–155) and the number of deaths was 2,107 (32.5%). The clinicopathological data showed that AT was significantly correlated with race, marital status, tumor grade, tumor size, T stage, CEA, and PI ($p < 0.05$). After including these variables related to AT for PSM, the final patient number was 2,901, including 1,934 in the non-AT group and 967 in the AT group (**Table 1**). The median survival in this final cohort was 57 months (0–155) and the number of deaths was 1,098 (37.8%).

Before PSM, the prognosis of the group without AT was better than that of the group with AT (5-year survival rate: 73.7 vs. 68.5%; $p < 0.05$; **Figure 2A**). After PSM, there was no difference in prognosis between the non-AT group and the AT group (5-year survival rate 69.3 vs. 68.5%; $p > 0.05$; **Figure 2B**).

Construction of the Nomogram

The data of patients who did not receive AT were included in the Cox proportional hazards regression analysis (**Table 2**). Univariate analysis showed that age, sex, race, maritime status, tumor grade, tumor size, T stage, and CEA were related to OS ($p < 0.05$). Furthermore, these variables were included in the multivariate analysis, which found that age, sex, race, marital status, tumor size, T stage, and CEA were independent prognostic factors ($p < 0.05$). Based on this, a nomogram was constructed to predict 3-year and 5-year survival after LR of stage I RC (**Figure 3**).

Testing the Effectiveness of Predictive Models

We used seven variables that were significant upon multivariate analysis to build a nomogram for predicting prognosis. The C-index of the nomogram model was 0.726 (95% CI, 0.689–0.763), which was significantly higher than that of the T stage model 0.594 (95% CI, 0.557–0.631). The nomogram calibration curves

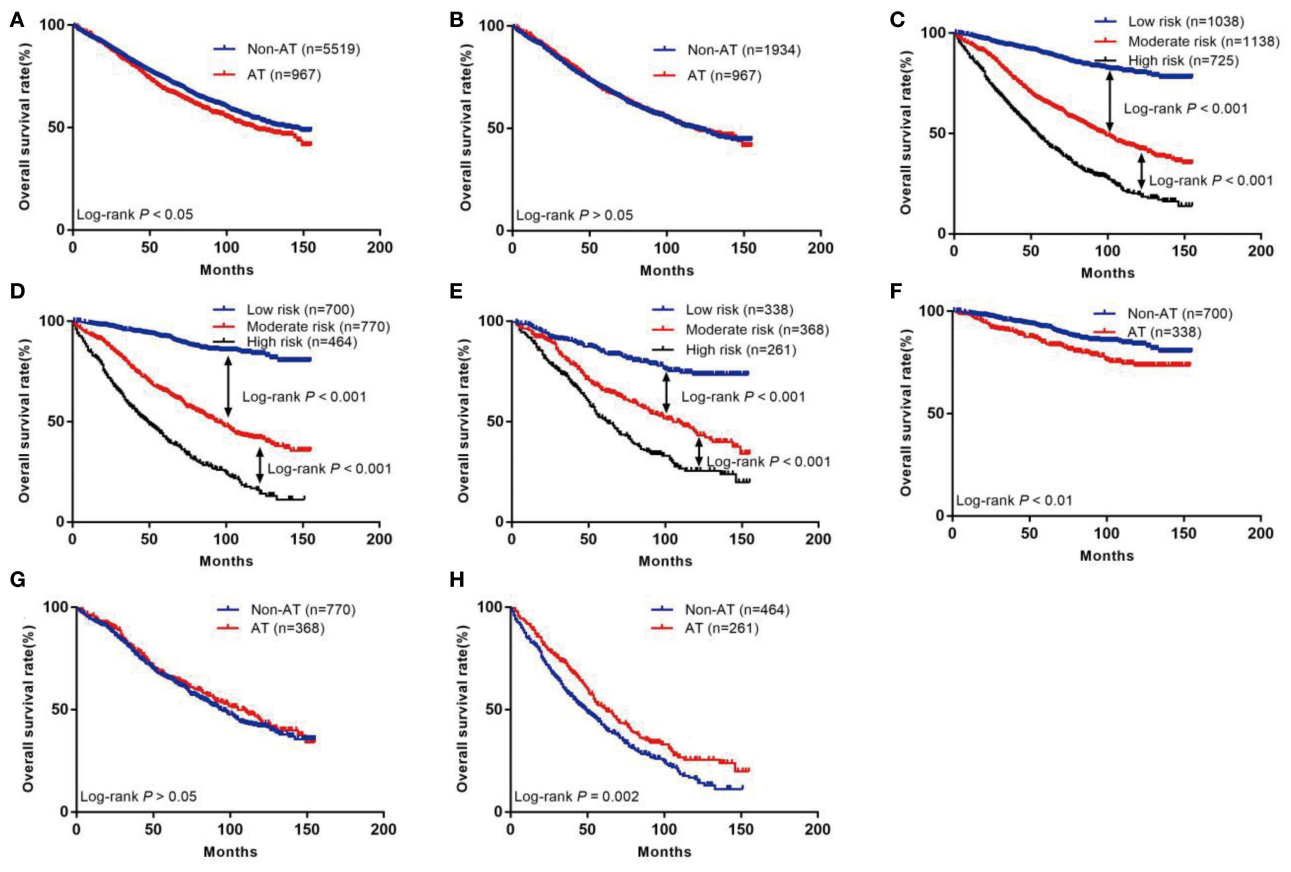

FIGURE 2 | The Kaplan–Meier curves of OS for patients in our study. **(A)** All patients; **(B)** Patients after PSM; **(C)** OS in different subgroups of all patients; **(D)** OS in different subgroups of non-AT group; **(E)** OS in different subgroups of AT group; **(F)** OS for patients with or without AT in low-risk group; **(G)** OS for patients with or without AT in moderate-risk group; **(H)** OS for patients with or without AT in high-risk group.

TABLE 2 | The univariate and multivariate analyses of factors associated with overall survival.

Variable	Univariate cox regression		Multivariate cox regression	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age				
<65	1			
≥65	5.295 (4.274–6.560)	<0.001	4.446 (3.565–5.545)	<0.001
Sex				
Male	1			
Female	0.854 (0.736–0.992)	0.039	0.747 (0.637–0.876)	<0.001
Race				
White	1			
Black	1.053 (0.817–1.357)	0.691	1.293 (0.998–1.674)	0.052
API	0.672 (0.476–0.950)	0.024	0.690 (0.488–0.976)	0.036
Other	0.136 (0.019–0.970)	0.047	0.180 (0.025–1.281)	0.087
Marital status				
Married	1			
Unmarried	1.122 (0.878–1.434)	0.357	1.342 (1.044–1.724)	0.022
Unknown	2.048 (1.737–2.415)	<0.001	1.434 (1.194–1.721)	<0.001
Grade				
Well/moderately	1			
Poorly/undifferentiated	1.340 (1.047–1.713)	0.020		
Unknown	1.002 (0.758–1.323)	0.991		
Size (cm)				
<3	1			
≥3	1.974 (1.651–2.360)	<0.001	1.568 (1.306–1.881)	<0.001
Unknown	1.070 (0.896–1.278)	0.453	1.069 (0.892–1.281)	0.468
T stage				
T1	1			
T2	2.218 (1.914–2.569)	<0.001	1.572 (1.343–1.840)	<0.001
CEA (ng/ml)				
≤5	1			
>5	2.268 (1.768–2.909)	<0.001	1.816 (1.414–2.333)	<0.001
Unknown	1.284 (1.085–1.520)	0.004	1.243 (1.049–1.474)	0.012
PI				
Negative	1			
Positive	1.200 (0.492–2.929)	0.689		
Unknown	1.001 (0.826–1.213)	0.995		

API, Asian/Pacific Islander; CEA, carcinoembryonic antigen; PI, perineural invasion.

of the 3- and 5-year OS indicate that the predicted survival probability was in good agreement with the actual survival probability. DCA was used to determine that the nomogram prognostic model net income for different decision thresholds was higher than the prediction ability of the T stage system (**Figure 4**).

Risk Stratification System

The risk scores of all patients were calculated using the nomogram (**Table 3**), and patients were then divided into three risk groups using X-tile software (**Figure 5**): a low-risk group (score ≤ 149, $n = 1{,}038$), a moderate-risk group (score 150–218, $n = 1{,}138$), or a high-risk group (score ≥ 219, $n = 725$). The 5-year survival rates of low-, moderate-, and high-risk groups were 89.7, 65.6, and 46.1%, respectively. The differences were statistically significant ($p < 0.001$, **Figure 2C**).

Through the existing scoring system, we divided the non-AT group into three subgroups: low ($n = 700$), moderate ($n = 770$), or high ($n = 464$). The 5-year OS rates of the low-, moderate-, and high-risk subgroups were 92.3, 65.5, and 42.8%, respectively, with statistical significance ($p < 0.001$, **Figure 2D**). In the AT group, the 5-year OS rate of the low-, moderate-, and high-risk subgroups was 84.7, 65.8, and 51.8%, respectively, with statistically significant differences ($p < 0.001$, **Figure 2E**).

Evaluating the Efficiency of AT for Patients in Different Groups

We further compared the outcomes of low-, moderate-, and high-risk patients receiving AT (**Table 4**). The results showed that the low-risk group had a poor prognosis after receiving AT (HR = 1.72; 95% CI: 1.21–2.44; $p < 0.01$; **Figure 2F**), the prognosis of patients in the moderate-risk group receiving AT was similar to that without AT (HR = 0.92; 95% CI: 0.76–1.11; $p > 0.05$; **Figure 2G**), and patients in the high-risk group benefited from AT (HR = 0.74; 95% CI: 0.61–0.89; $P = 0.002$; **Figure 2H**).

DISCUSSION

For surgeons, the goal of RC surgery should be to not only radically resect the tumor, but also to maintain the integrity of intestinal and anal functions as much as possible. LR of RC is a surgical method allowing for minimal damage, good oncological effect, and retention of the rectum, and is receiving more attention from clinicians. For patients with cT1N0 rectal cancer without risk factors, the guidelines recommend LR. If found pT > 1, SM3 invasion, poor differentiation, tumor budding, and lymphovascular or perineural invasion, the guidelines recommend follow-up radical resection or AT (18). Borstlap et al. (19) found that patients with pT1/T2 RC who went on to receive AT ($n = 405$) were compared to those who underwent radical resection ($n = 130$) after LR. pT1 RC local recurrence rates for AT and radical resection were 10% (95% CI: 4–21) vs. 6% (95% CI: 3–15), and 15% (95% CI: 11–21) vs. 10% (95% CI: 4–22) for patients with pT2. However, it is important to note that oncology safety is an important factor that restricts the application of this surgical approach. Willett

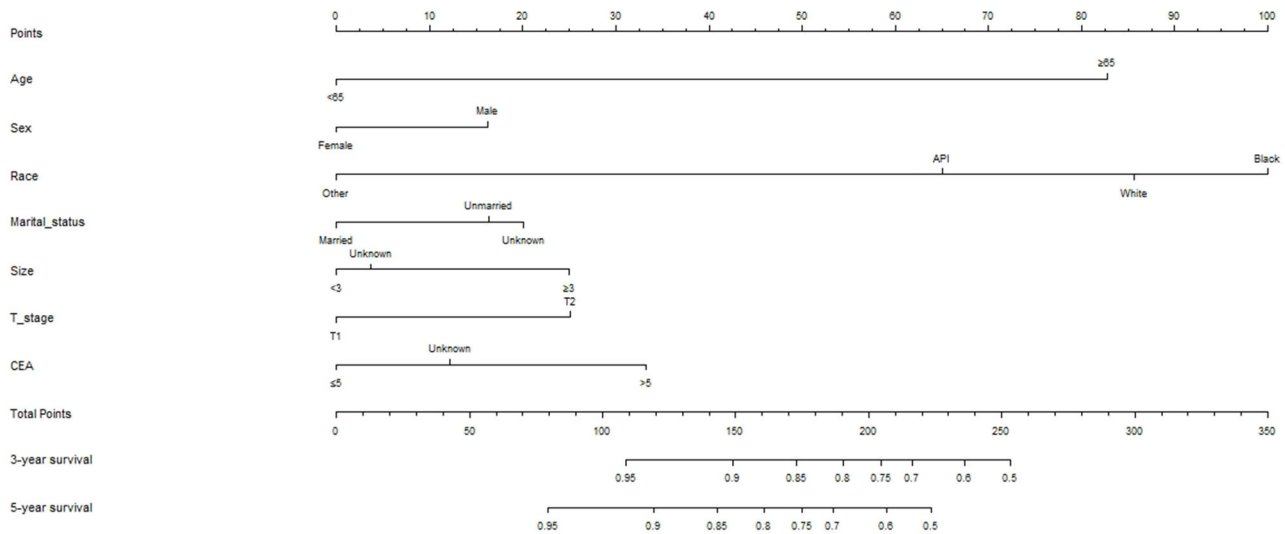

FIGURE 3 | Oncologic nomogram for patients with stage I rectal cancer after local excision.

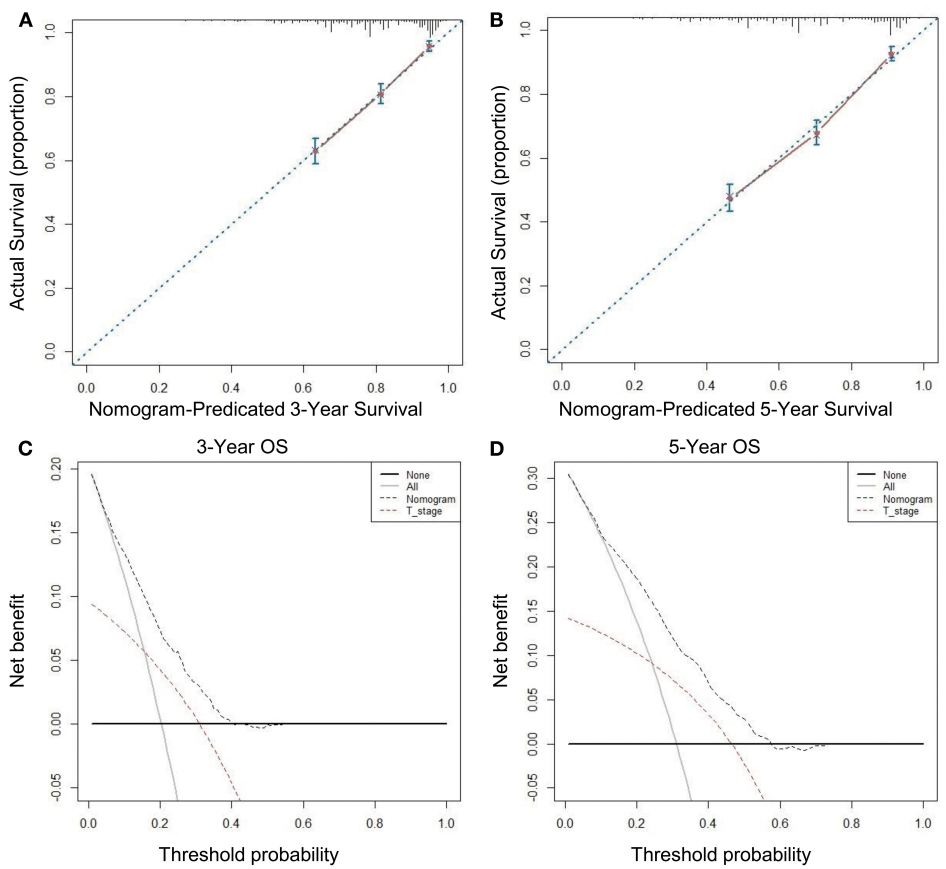

FIGURE 4 | Calibration curves and decision curve for OS prediction: **(A)** 3-year OS calibration curve in our cohort; **(B)** 5-year OS calibration curve in our cohort; **(C)** Nomogram was compared to the T stage in terms of 3-year OS in our decision curve analysis; **(D)** Nomogram was compared to the T stage in terms of 5-year OS in our decision curve analysis.

TABLE 3 | Point assignment of each component and prognostic score for stage I rectal cancer.

Group	Score	Estimated 3-y OS (%)	Estimated 5-y OS (%)
Age			
<65	0		
≥65	83		
Sex			
Male	16		
Female	0		
Race			
White	86		
Black	100		
API	65		
Other	0		
Marital status			
Married	0		
Unmarried	16		
Unknown	20		
Size (cm)			
<3	0		
≥3	25		
Unknown	4		
T stage			
T1	0		
T2	25		
CEA (ng/ml)			
≤5	0		
>5	33		
Unknown	12		
Total score			
	109	95	
	149	90	
	173	85	
	190	80	
	204	75	
	216	70	
	236	60	
	253	50	
	79		95
	119		90
	143		85
	161		80
	175		75
	187		70
	207		60
	225		50

API, Asian/Pacific Islander; CEA, carcinoembryonic antigen.

et al. (20) found that the following risk factors contribute to an LR failure rate of more than 20%: tumor size > 3 cm, poor differentiation of adenocarcinoma, lymphovascular invasion, and positive margins. This leads to poor postoperative oncological effects because the presence of these high-risk factors increases the risk of lymph node metastasis. The guidelines for patients with postoperative recurrence risk support recommendation of remedial surgery or AT. However, after LR failure, the highest 5-year survival rate of patients receiving remedial surgery is only 58% (21–23). The latest research shows that AT can achieve the same long-term prognosis as remedial surgery (24). Compared with remedial surgery, AT has advantages in trauma and postoperative complications and can eliminate subclinical lesions so as to improve the local control rate. For patients at high risk for recurrence after LR, AT and follow-up should be given (25). At present, controversies remain about the prognostic factors of stage I RC after LR and the influence of AT on prognosis (26–28). The purpose of our study was to select patients who would benefit from AT after LR.

A better understanding of the high-risk factors for recurrence after LR is of great significance for guiding AT. The incidence of RC among young patients is increasing each year (29, 30). Meyer et al. (9) found that young patients aged 20–39 with T1 stage disease had a worse prognosis than those aged 60–69 years (HR = 1.97; 95% CI: 1.36–2.86; $p < 0.001$). Younger patients aged 20–39 years with T2 stage disease had a worse prognosis than those aged 60–69 years (HR = 1.48; 95% CI: 1.13–1.95; $p < 0.001$). Younger patients with RC were associated with poor tumor cell differentiation, lymphovascular invasion, and a higher rate of distant metastasis than older patients (45 vs. 25%) (31). A study by Patel found that the prognosis of patients with stage I RC aged over 65 years was poor (HR = 2.30; $p = 0.04$) (32). As a result, it is controversial whether old age is a high-risk factor in colorectal cancer. Interestingly, our study found that patients ≥ 65 had a worse prognosis (HR = 5.30; 95% CI: 4.27–6.56; $p < 0.001$). The possible reasons are that the elderly patients in our study had a high proportion of T2 stage disease (39.8 vs. 16.7%) and a high proportion of tumor size ≥ 3 cm (20.8 vs. 12.0%). Furthermore, patients of older age are likely to be in relatively poor physical condition, have more basic diseases, and have a high proportion of postoperative complications (33).

Our study found that female patients had a better prognosis than male patients (HR = 0.75; 95% CI: 0.64–0.88; $p < 0.001$). Yang et al. (34) found that OS (HR = 0.87; 95% CI: 0.85–0.89; $p < 0.001$) and cancer specific survival (CSS) (HR = 0.92; 95% CI: 0.89–0.95; $p < 0.001$) were better in women than in men, which is consistent with our results. Moreover, estrogen in female patients has a positive effect in reducing the incidence rate and mortality of colorectal cancer (35).

Our study also found that blacks had a worse prognosis than whites (HR = 1.29; 95% CI: 1.00–1.67; $P = 0.052$), and the API prognosis was better than that of whites (HR = 0.69; 95% CI: 0.49–0.98; $P = 0.036$), which is consistent with previously published results from Pulte (36). Our research also found that divorced patients have a worse prognosis, which may be related to hormone levels and living conditions. Our study found that tumor size ≥ 3 cm was correlated with a worse prognosis (HR = 1.57; 95% CI: 1.31–1.67; $p < 0.001$), and this is an undisputed high-risk factor for a poor prognosis (20, 37).

It has been reported that the recurrence rate after LR is slightly higher than that after traditional radical resection. The high recurrence rate is mainly concentrated in RC at pT2 stage,

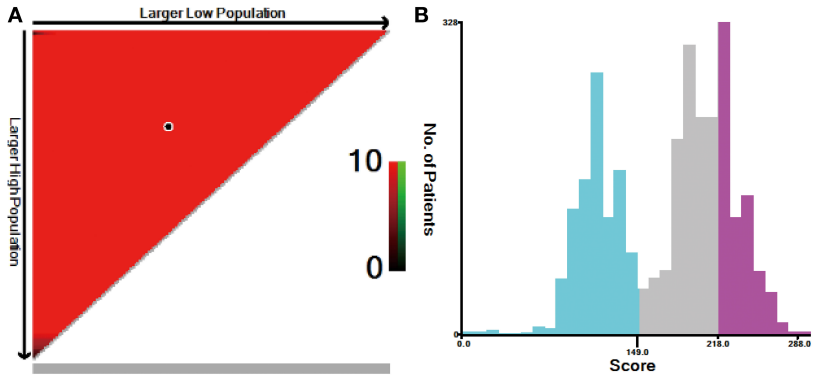

FIGURE 5 | X-tile analysis for risk stratification: **(A)** The optimal cutoff value; **(B)** Numbers of patients in low-, moderate-, and high-risk subgroups.

TABLE 4 | Risk stratification in non-AT and AT group.

Survival status	Non-AT Group				AT Group			
	Low risk [n (%)]	Moderate risk [n (%)]	High risk [n (%)]	*P*-value	Low risk [n (%)]	Moderate risk [n (%)]	High risk [n (%)]	*P*-value
Live	624 (89.1)	429 (55.7)	156 (33.6)	<0.001	272 (80.5)	215 (84.2)	107 (41.0)	<0.001
Death	76 (10.9)	341 (44.3)	308 (66.4)		66 (19.5)	153 (15.8)	154 (59.0)	

AT, adjuvant therapy.

while the recurrence rate of RC at pTl stage is not significantly different from that of traditional radical resection (13, 38). The characteristics of lymph drainage vary in different layers of the colon and rectum. There is almost no lymph drainage in the mucosa layer; there is some drainage in the submucosa layer; and most lymph drainage occurs in the muscular layer. Thus, the risk of lymph node metastasis in RC is different depending on the level of invasion of the intestinal wall. The risk of lymph node metastasis is the highest with invasion of the muscular layer. This is the reason for the high recurrence rate and poor prognosis of pT2 RC (39, 40). Indeed, our study also found that patients with pT2 stage RC had a poor prognosis (HR = 1.57; 95% CI: 1.34–1.84; $p < 0.001$).

We know that elevated CEA means that colorectal cancer has a high degree of malignancy and is more likely to have lymphatic or distant metastasis (41). CEA is not considered to be a high-risk factor for recurrence of stage I RC in the National Comprehensive Cancer Network (NCCN) guidelines (42), although our study did find positive CEA to be a high-risk factor (HR = 1.82; 95% CI: 1.41–2.33; $p < 0.001$). With this finding, we further expand the range of risk factors, which is of great significance for a more comprehensive evaluation of patient prognosis.

Moreover, the nomogram that we developed based on these prognostic factors shows good discrimination and repeatability. The C-index of our nomogram is 0.726 (95% CI, 0.689–0.763), which is significantly higher than that of T stage at 0.594 (95% CI, 0.557–0.631), indicating that our nomogram has a stronger predictive ability than the traditional tumor/nodes/metastases (TNM) staging system. We used DCA to further confirm that the nomogram is superior to traditional T staging in predicting the OS of patients with stage I RC.

We introduce this concept in the face of controversy surrounding the influence of AT on the prognosis of stage I RC after LR. The latest review results show that AT is beneficial for high-risk patients in pT1 stage, but has no survival benefit for patients in pT2 stage (26). A study by Jae-Uk found no significant difference in OS between AT and non-AT groups in patients with stage I RC after LR (43), while a study by Wang reported that AT improved OS of pT2 patients (44). The purpose of this portion of our study was to improve the selection of patients who could truly benefit from AT. Our study showed that AT did not bring survival benefits to all patients before and after PSM. This is mainly because AT is often used in clinical patients with already poor prognosis, and therefore beneficial effects are minimal. Therefore, we scored each patient according to their risk factors for recurrence and divided the patients into low-, moderate-, and high-risk groups, so as to accurately treat the target patients. Between the non-AT group and AT group, there were significant survival differences across the three risk levels, which show that our risk stratification is reasonable and effective. In order to investigate which group of patients may benefit from AT, we found that the 5-year survival rate of low-risk patients receiving AT was lower than that of the group not receiving AT (84.7 vs. 92.3%, $p < 0.01$). Therefore, we do not recommend AT for low-risk patients, because our findings suggest that the harm caused by AT outweighed the benefit. The 5-year survival rate of patients at moderate risk who received AT was similar to that of those who did not receive AT (65.8 vs. 65.3%, $p > 0.05$). Therefore, for these patients, consideration to perform AT must take into account all relevant factors. The 5-year survival rate of patients at high risk who received AT was higher than those who did not receive AT (51.8 vs. 42.8%, p

< 0.01), indicating that high-risk patients are likely to benefit from AT.

This paper comprehensively analyzes the prognostic factors of patients with stage I RC after LR based on the latest large sample data from the SEER database and establishes an accurate and convenient nomogram prognosis model. However, the study is not without limitations. First, the lack of external verification by other populations may reduce the universality of our model. Second, our study is a retrospective study, and the exclusion of some patients with stage I RC due to missing data, or missing risk factors not present in this database could all introduce bias. Third, we do not know the AT regimen and compliance of each patient and the rate of patients with high-risk factors receiving AT and non-AT is different, which will lead to heterogeneity. There is no survival prognostic model incorporated into these clinical pathologic factors for stage I RC after LR. It is most important to stratify patients into different groups, as this has great significance to guide clinical AT. Thus far, there is no conclusion as to whether stage I RC after LR should be observed, AT, or radical surgery, this further highlights the importance of our study. This study analyzed and constructed the nomogram prognostic model based on the SEER large-sample multicenter data, which ensured the robustness of the model.

CONCLUSIONS

Our nomogram effectively predicts the prognosis of stage I RC after LR. AT is recommended for high-risk patients, while AT is not recommended for patients at low or moderate risk.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XW designed the research. SZ took part in designing the research. DW collected the data. XC analyzed the data and wrote the manuscript. CZ collected the data and analyzed the data. All authors approved the final manuscript.

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REVIEW
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Perspectives on Immunotherapy of Metastatic Colorectal Cancer

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Colorectal cancer, especially liver metastasis, is still a challenge worldwide. Traditional treatment such as surgery, chemotherapy and radiotherapy have been difficult to be further advanced. We need to develop new treatment methods to further improve the poor prognosis of these patients. The emergence of immunotherapy has brought light to mCRC patients, especially those with dMMR. Based on several large trials, some drugs (pembrolizumab, nivolumab) have been approved by US Food and Drug Administration to treat the patients diagnosed with dMMR tumors. However, immunotherapy has reached a bottleneck for other MSS tumors, with low response rate and poor PFS and OS. Therefore, more clinical trials are underway toward mCRC patients, especially those with MSS. This review is intended to summarize the existing clinical trials to illustrate the development of immunotherapy in mCRC patients, and to provide a new thinking for the direction and experimental design of immunotherapy in the future.

Keywords: colorectal cancer, liver metastasis, deficient DNA mismatch repair, immunotherapy immune checkpoint inhibitors, vaccine, adoptive cellular immunotherapy

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INTRODUCTION

Colorectal cancer (CRC) has received a lot of attention and research due to its high incidence (10.2%) as well as high fatality rate (9.2%) among tumors worldwide (1). Because its early clinical symptoms are atypical and not obvious, CRC is often ignored, leading to delayed diagnosis and treatment. To make the matter worse, approximately 15% of patients had already developed liver metastases at the time of the diagnosis and nearly half of patients progressed to liver metastasis later (2). CRC patients with limited liver metastases lesions may be cured by surgical resection (3). However, a majority of patients are not suitable for surgery due to the following reasons, such as bone or brain metastasis, coexisting systemic diseases, or insufficient residual liver volume (4).

This leads to the need for other novel therapies to improve the poor clinical outcomes of mCRC patients who are not eligible for surgical excision. Chemotherapy, radiotherapy, emerging molecular targeted therapies and combination therapy have demonstrated efficacy for some patients in numerous clinical trials and some of them have been approved for clinical use (5). Among them, the more noteworthy is the emergence of various immunotherapies. Immunotherapies mainly consist of immune checkpoint inhibitors (ICIs), adoptive cellular immunotherapy (ACI) and cancer vaccines. The principle of immunotherapy is to enhance or weaken the function of various immune cells (T cells, NK cells, macrophages, myeloid-derived suppressor cells) to achieve anti-tumor effect (6). These therapies, especially ICIs (anti-PD-1; anti-PD-L1; anti-CTLA-4), have been shown to be

effective in patients with CRC that are mismatch repair deficient (dMMR). In other words, immunotherapies including ICIs have a limited effect on those patients with pMMR tumors. More than that, immunotherapy has also been challenged by the increasing discovery of resistance due to mutations and other causes, and the suboptimal stratification of patients by MMR status. This makes immunotherapy combined with chemotherapy and radiotherapy especially molecular targeted therapy get more and more attention and research.

The review aims to expound the rationality and feasibility of the use of immunotherapy in clinical practice by summarizing the existing evidence. Based on an updated analysis of the existing literature, as well as expected results from ongoing and planned clinical trials, we discuss practical strategies for future research targeting novel potential immunotherapies and discuss current barriers.

RATIONALE FOR IMMUNOTHERAPY IN mCRC

Immune Checkpoint Molecules

Immune checkpoints were originally essential molecules for preventing autoimmunity, but their existence has become a mechanism by which tumors escape the surveillance of the immune system (7). Common immune checkpoint molecules include programmed death cell protein 1 (PD-1), programmed death-ligand 1(PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). PD-1 is a transmembrane protein, mainly expressed on the surface of a variety of immune cells (e.g., T cells, B cells, dendritic cells, and NK cells) and the corresponding receptor PD-L1 expressed on the surface of tumor cells. The PD-1 signaling pathway can negatively regulate the human immune system, thereby inhibiting the Th1 cytotoxic activity and damaging the host, as did PD-L1 and CTLA-4 (8). Specifically, when PD1 interacts with PD-L1, downstream signaling pathways are induced to directly inhibit tumor cell apoptosis and stimulate the conversion of effector T cells to regulatory T cells (Tregs). In a similar manner, CTLA-4 on the surface of T cells can preferentially bind to the receptors (B7-1; B7-2) on the surface of antigen-presenting cells (APC) due to their higher affinity, so that the activity of T cells is reduced, their proliferation is inhibited, and their anti-tumor effect is weakened (9) (**Figure 1**). These molecules have been found to be overexpressed in solid tumors and in their microenvironment. Wei et al. found that the levels of PD-L1 in liver metastases were higher compared with primary tumors (10). The immune escape of the tumors was reversed by immune checkpoint inhibitors, novel drugs developed to block these negative feedback pathways by binding to PD-1 (nivolumab, pembrolizumab), PD-L1 (atezolizumab), CTLA-4 (ipilimumab). Many existing clinical trials have demonstrated encouraging results in a variety of solid tumors, directly leading to FDA approval of some of these drugs for clinical use (11).

mCRC With MSI/dMMR or MSS/pMMR

With the recognition of the potential of immunotherapy to improve some patients with advanced solid tumors, it is apparent that we need new biomarkers that can distinguish between tumors that respond to immunotherapy and those that do not. Some studies showed that there is a strong connection between mutation prevalence and immunotherapy response (12). After that, CRC can be divided into two discrete groups according to the MMR mutation status: MSI/dMMR tumors mainly with high overall mutation burden and MSS/pMMR tumors mostly with relatively much lower mutation burden (13). Sad to say, only about 2–4% of mCRC was diagnosed as MSI/dMMR (14). DNA mismatch repair (MMR) is to ensure the integrity and stability of genetic material by correcting mismatched bases during DNA replication. When the mismatch repair system is defective in the main MMR proteins MLH1, MSH2, MSH6, and PMS2 or microsatellites, multiple mutations accumulate, eventually leading to the development of tumors called mismatch-repair deficiency/microsatellite instability (MSI/dMMR) tumors (15). Immunohistochemistry and PCR are commonly used to diagnose patients with MSI/dMMR or MSS/pMMR. One of the mechanism by which dMMR tumors are sensitive to immunotherapy is the production of multiple neoantigens induced by genomic mutations (16). More importantly, immune cells (CD8+ infiltrating lymphocytes; CD4+ TILS; macrophages; NK cells) are abundant in MSI-H/dMMR tumors and cell surface inhibitory checkpoint molecules of lymphocytes and tumor cells (PD-1, PD-L1, respectively) are increased correspondingly (17, 18) (**Figure 2**). This also means that the corresponding MSS tumor is less likely to respond to immunotherapy, which is showed in multiple studies (19). This is a barrier to immunotherapy that needs to be addressed.

IMMUNE CHECKPOINT INHIBITORS THERAPY

Since mCRC patients' response to ICIs can vary significantly depending on MMR status, we will focus on mCRC patients with MSI/dMMR or MSS/pMMR here (**Table 1**).

MSI/dMMR mCRC

The efficacy of ICIs was studied in mCRC patients before the patients were stratified with MSS status. In a phase I study of nivolumab (anti-PD-1) in the 39 patients with treatment-refractory solid tumors, only one mCRC patient (7%, 1/14) achieved a lasting complete response for 6 months (27). Similarly, in another phase I study of nivolumab (n = 296), objective responses were observed

Abbreviations: mCRC, metastatic colorectal cancer; MSI/dMMR, microsatellite instability/mismatch-repair-deficiency; MSS/pMMR, microsatellite stability/mismatch-repair-proficiency; ICIs, immune checkpoint inhibitors; ACI, adoptive cellular immunotherapy; CT, chemotherapy; RFA, radiofrequency ablation; RT, radiotherapy; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death 1; PD-L1, PD-1 ligand; Treg, regulatory T cell; DC, dendritic cell; NK cell, natural killer cell; APC, antigen-presenting cell; TLR, toll-like receptor; OS, overall survival; PFS, progression-free survival; ORR, objective response rate; irORR, immune-related objective response rate; DCR, disease control rate; BSC, best supportive care; 5-FU, 5-flourouracil; 5-FU/LV, 5-flourouracil, leucovorin; FOLFOX, 5-flourouracil, leucovorin, oxaliplatin; FOLFIRI, 5-flourouracil, leucovorin, irinotecan; FOLFOXIRI, 5-flourouracil, leucovorin, oxaliplatin, irinotecan.

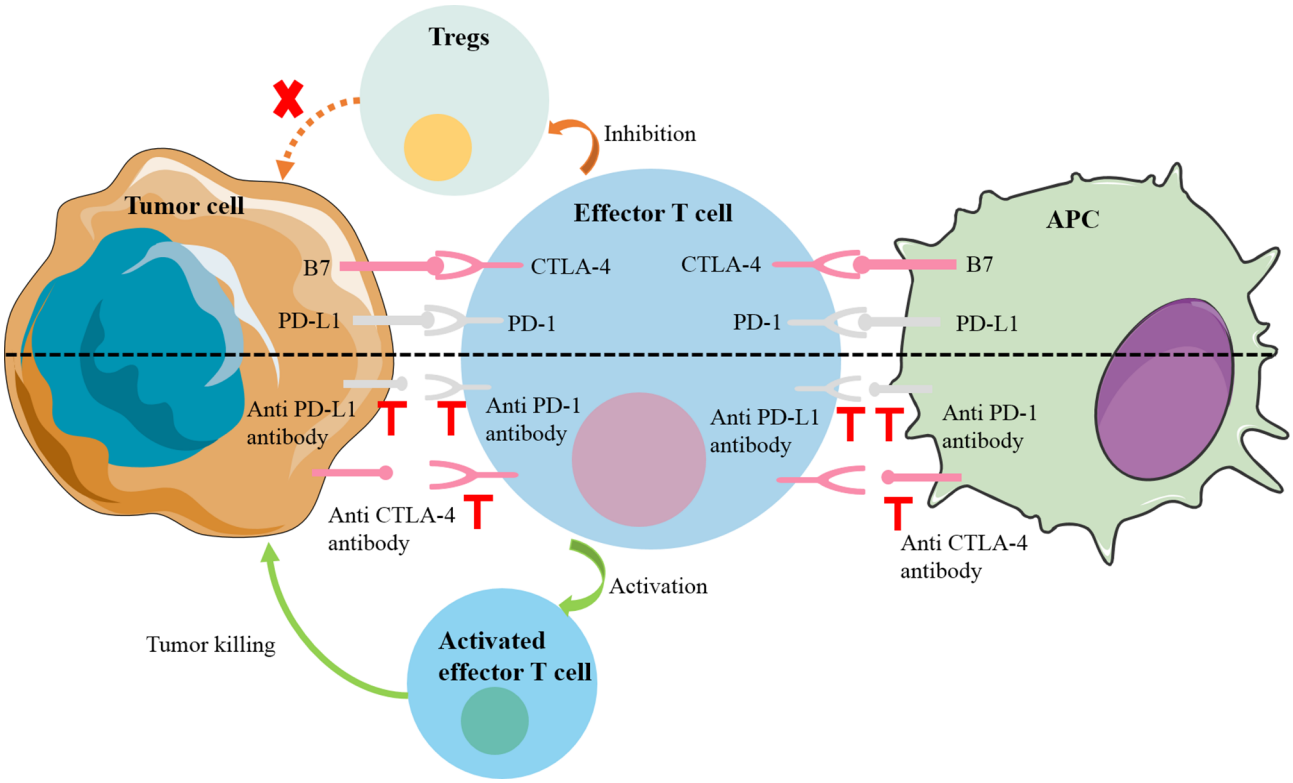

FIGURE 1 | Mechanisms of common immune checkpoint inhibitors. PD1 on the surface of effector T cells interacts with PD-L1 on the surface of tumor cells, downstream signaling pathways are induced to directly inhibit tumor cell apoptosis and stimulate the conversion of effector T cells to Tregs. In a similar manner, CTLA-4 on the surface of T cells can preferentially bind to the receptors (B7-1; B7-2) on the surface of APC to inhibit the activity and proliferation of T cells. APC, antigen-presenting cell; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death 1; PD-L1, PD-1 ligand; Treg, regulatory T cell.

only in patients with non-small cell lung cancer, melanoma, or renal cell carcinoma, and not in the mCRC population (0 of 19, 0%) (20). A phase II study of tremelimumab (CTLA4) in the 47 patients with refractory metastatic colorectal cancer also failed, only one patient (2%) achieved partial response (28). These studies suggested that single-agent immune checkpoint therapy is not effective in unselected mCRC. This has led to a shift to research into population-specific immunotherapies in mCRC, such as MSI-H/DMMR mCRC and MSS/PMMR mCRC. In a population-based cohort of 798 mCRC patients, Aasebø et al. reported that the proportion with MSI-H among mCRC patients is nearly twice as high as most previous reports of mCRC (4, 3.5, 4.2, 5%) (21, 22, 32, 33), with 40/583 (7%) tumor samples of MSI-H (23). Wang et al. evaluated the status of MMR and MSI in 40 pairs of situ tumors and liver metastases by immunohistochemistry (IHC) and Polymerase Chain Reaction (PCR) respectively. inconsistent MMR and MSI status were observed in`15% patients (six of 40 patients). There was no significant difference between primary and metastatic tumors in the expression status of MMR (P = 0.1405) (24). Although the proportion of patients with dMMR in metastatic colorectal cancer is not high, the poor prognosis in mCRC patients makes any treatment that can improve survival significant.

Le et al. conducted a phase 2 study that evaluated the clinical efficacy of pembrolizumab(anti-PD1) in the 32 patients with advanced metastatic cancer with and without dMMR. For dMMR mCRC patients, 40% (four of 10 patients; 95% CI, 12 to 74) achieved immune-related objective response and 78% (seven of nine patients; 95% CI, 40 to 97) survive without progression for 20 weeks, compared with 0% (0 of 18 patients; 95% CI, 0 to 20) and 11% (two of 18 patients; 95% CI, 1 to 35) in pMMR CRC. A disease control rate (DCR) of >12 weeks was achieved in 90% dMMR mCRC and 11% pMMR mCRC (8). The efficacy of another anti-PD1 drug nivolumab on MSI/AdMMR mCRC was confirmed in a phase 2 study (CheckMate 142). At a median follow-up of 12.0 months, investigator-assessed objective response (OR) was 31.1% (23 of 74 patients, 95% CI, 20.8–42.9) and DCR for 12 weeks or longer was 69% (51 of 74 patients; 95% CI, 57 to 79%). Two patients (2.7%) had complete responses (CRs) and 22 patients (29.7%) had partial responses (PRs) (25). And on this basis the study further evaluated the role of nivolumab plus ipilimumab(anti-CTLA-4) on MSI/dMMR mCRC patients. At median follow-up of 13.4 months, 55% patients achieved investigator-assessed objective response, and DCR for ≥12 weeks was 80%. Progression-free survival (PFS) rate and overall survival (OS) rate at one year was 71 and 85%, respectively. Surprisingly, in 16 patients (13%) who did not complete the treatment cycle due to immune-mediated toxicity, 63% of these achieved the OR, comparable to the total

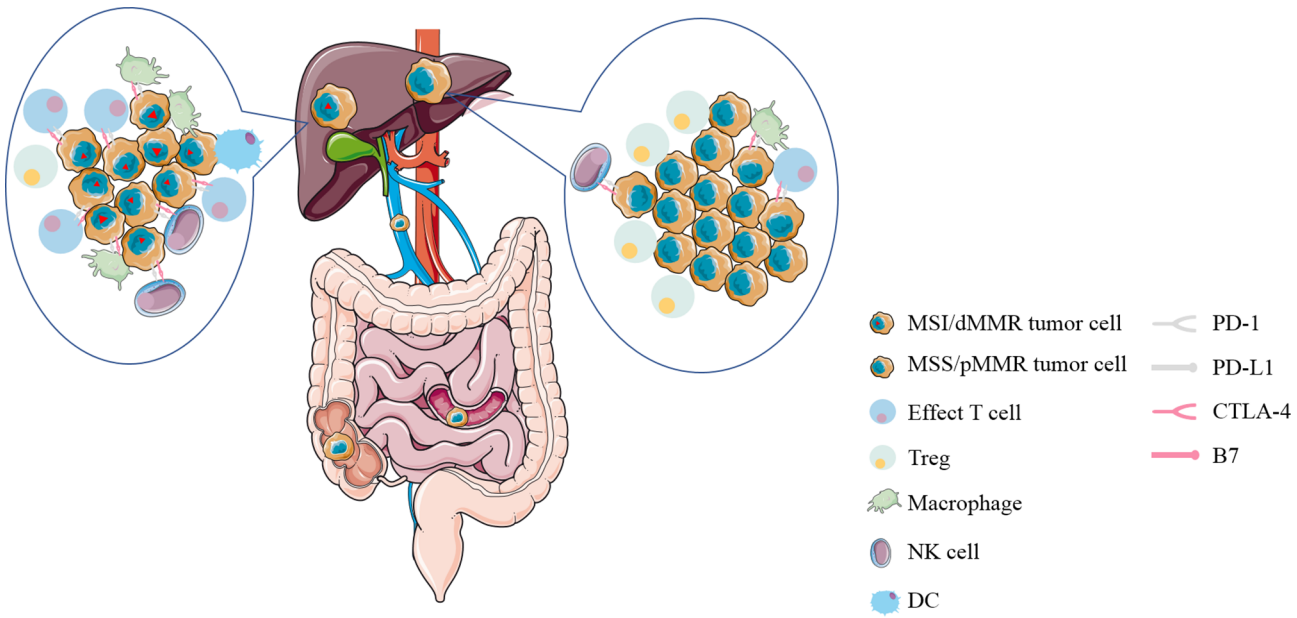

FIGURE 2 | The immune microenvironment of liver metastases from colorectal cancer with MSI/dMMR or MSS/pMMR. Immune cells (CD8+ infiltrating lymphocytes; CD4+ TILS; macrophages; NK cells) are abundant in MSI-H/dMMR tumors and inhibitory checkpoint molecules on the surface of lymphocytes and tumor cells (PD-1, PD-L1, respectively) are increased correspondingly. MSI/dMMR, microsatellite instability/mismatch-repair-deficiency; MSS/pMMR, microsatellite stability/mismatch-repair-proficiency; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death 1; PD-L1, PD-1 ligand; Treg, regulatory T cell; DC, dendritic cell; NK cell, natural killer cell.

population (34). A couple of studies above led to FDA approval of pembrolizumab and nivolumab for dMMR CRC previously treated by conventional chemotherapy.

Recently, a phase 3 study comparing the clinical effect of PD-1 blockade and chemotherapy as first-line treatment in MSI-H-DMMR mCRC was reported. Some 307 previously untreated mCRC patients with MSI-H-dMMR were randomly assigned to two groups at a ratio of 1:1, and received 200 mg of pembrolizumab every 3 weeks or chemotherapy every 2 weeks, respectively. At a median follow-up of 32.4 months, median PFS was 16.5 months for the pembrolizumab group, compared with 8.2 months for the chemotherapy group, respectively (P = 0.0002). About 43.8% patients in the pembrolizumab group had OR and 33.1% in the chemotherapy group. In addition, 22% of patients in the pembrolizumab group experienced treatment-related adverse events of grade 3 or higher, while 66% in the chemotherapy group (including one death). As a first-line treatment for MSI-H-DMMR metastatic colorectal cancer, pembrolizumab can remarkably improve PFS and reduced treatment-related adverse events, compared with chemotherapy (35). This further suggests that for MSI-H-DMMR metastatic colorectal cancer, chemotherapy is not recommended and immunotherapy should be accepted. But the remarkable thing is that 153 (50%) had synchronous liver metastases, and 77 (25%) had BRAFV600E mutant tumors. This means that more accurate stratification is needed to further study the efficacy of immunotherapy and chemotherapy on MSI-H-DMMR mCRC patients.

Additionally, the anti-PD-L1 therapy of patients has also been increasingly studied. In a phase II study of Avelumab in the 21 patients with dMMR/MSI-H mCRC, complete response rate (CRR) and partial response (PRR) were both 14.3% (three patients), with ORR and DCR of 28.6 and 90.5%, respectively. At a median follow-up of 16.3 months, median PFS was 8.1 months (95% CI, 1.1 to 15.1 months) (36). Chen et al. conducted a phase 2 study to assess whether combination therapy with anti-PD-L1 and anti-CTLA-4 is effective in patients with intractable mCRC. With a median follow-up of 15.2 months, the median OS was 6.6 months for durvalumab and tremelimumab, compared with 4.1 months for best supportive care (BSC) alone (P = .07). However, PFS was 1.8 and 1.9 months respectively. There was no CR. It is worth noting that durvalumab plus tremelimumab significantly improved OS in MSS patients (HR, 0.66; 90% CI, 0.49–0.89; P = .02). This underlines the possibility of combining immunotherapy in unselected advanced mCRC (37).

What is the effect of immunotherapy as neoadjuvant therapy in perioperative period? In a retrospective study of eight patients with advanced MSI-H CRC, pathologic complete response was observed in five of the seven resected patients, and clinical complete response was observed in an unoperated patient (26). In another retrospective analysis of 121 advanced dMMR mCRC patients treated with ICIs, 13 patients achieved pathologic complete response as is shown in the resected specimens. Preoperative imaging in 12 of those patients, however, still showed residual tumor. The result indicates that patients with residual radiographic tumors may not need surgery based on anti-PD1 response (38). In general, the possibilities of ICIs for mCRC continue to expand.

TABLE 1 | Summary of Immune Checkpoint Inhibitors Therapies for MCRC.

Study	Phase	Agent	Population	MSI status	Endpoint	Reference
NCT00730639	1	anti-PD-1 (MDX-1106)	296 advanced solid tumors, including19 CRC	-	-	(20)
NCT01876511	2	anti-PD-1 (pembrolizumab)	41 advanced tumors, including 32 mCRC	dMMR (n = 11) pMMR (n = 21)	The primary endpoints: immune-related objective response rate and the 20-week immune-related progression-free survival rate	(8)
NCT02060188	2	anti-PD-1 (nivolumab)	74 recurrent or metastatic CRC	dMMR	The primary endpoints: investigator-assessed ORR	(21)
NCT02060188	2	anti-PD-1 (nivolumab) + anti-CTLA4 (ipilimumab)	119 recurrent or metastatic CRC	dMMR	The primary endpoints: investigator-assessed ORR; The secondary endpoints: ORR per blinded independent central review (BICR) and DCR	(22)
NCT02563002	3	anti-PD-1 (pembrolizumab) or chemotherapy (5-fluorouracil–based therapy with or without bevacizumab or cetuximab)	307 mCRC	dMMR	The primary endpoints: PFS and OS; The secondary endpoints: OS and safety	(23)
NCT03150706	2	anti-PD-L1 (avelumab)	33 mCRC	dMMR (n = 30)	The primary endpoint: ORR	(24)
NCT02870920	2	Anti-PD- L1 (durvalumab) + anti-CTLA4 (ipilimumab) + best supportive care (BSC); or BSC alone	180 mCRC	dMMR	The primary endpoint: OS	(25)
NCT03350126	2	Anti-PD-1 (nivolumab) plus anti-CTLA4 (ipilimumab)	57 mCRC	dMMR	the frequency of pseudoprogressions (DCR by RECIST and iRECIST at 12 weeks)	(26)
-	1	Anti-PD-1 (MDX-1106)	14 advanced mCRC	-	The primary objectives: safety; tolerability; maximum-tolerated dose; pharmacokinetics. The secondary objectives: assessing antitumor activity, pharmacodynamics, immunologic end point	(27)
-	2	anti-CTLA4 (tremelimumab)	47 refractory or metastatic CRC	-	The primary endpoints: objective response; The secondary endpoints : safety, duration of response, PFS, and OS	(28)
NCT02788279	3	atezolizumab + cobimetinib or atezolizumab monotherapy versus regorafenib	383 advanced or metastatic CRC	MSS	The primary endpoints: OS; The secondary endpoints: investigator-assessed OR, duration of response, and PFS	(19)
NCT03912857	2	anti-PD-1(SHR-1210) + apatinib	10 mCRC	MSS	The primary endpoints: ORR; The secondary endpoints: PFS, OS, DCR and safety.	(29)
NCT02851004	2	anti-PD-1 (pembrolizumab) + STAT3 inhibitor (napabucasin)	50 mCRC	MSS (n = 40)	The primary endpoints: irORR	(30)
NCT03406871	1b	anti-PD-1 (nivolumab) + regorafenib	50 patients, including 25 mCRC	MSS (n = 24)	Secondary objectives: assessing incidences of adverse events, ORR, DCR, PFS, and OS.	(31)

OS, overall survival; PFS, progression-free survival; ORR, objective response rate; irORR, immune-related objective response rate; DCR, disease control rate; BSC, best supportive care; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand-1; CTLA-4, cytotoxic T-lymphocyte–associated protein 4; 5-FU, 5-flourouracil; FOLFOX, 5-flourouracil, leucovorin, oxaliplatin; FOLFIRI, 5-flourouracil, leucovorin, irinotecan; mCRC, metastatic colorectal cancer; MSI, microsatellite instability-(high); MSS, microsatellite stability.

Although ICIs has made significant progress in dMMR mCRC, the objective response rates shown in various studies were still unsatisfactory. This may be due to the following reasons. The first reason is misdiagnosis. In a *post hoc* analysis of 38 patients with mCRC diagnosed as MSI/dMMR, five individuals (13%) were resistant to immune checkpoint inhibitors. After reassessment of the status, three of these patients (60%) were confirmed as MSS/pMMR. Misdiagnosis of their MSI/dMMR status is the main cause of the resistance to ICIs in mCRC shown as MSI/dMMR. Therefore, Cohen et al. advocated that immunohistochemistry and polymerase chain reaction should be combined routinely to detect of MSI/MMR status prior to ICIs. But this increases the cost of the tests, which may be bad for future adoption (39). The second reason is tumor

heterogeneity. Although MSI is considered to be an early event of CRC, there is the possibility of heterogeneity in MSI/dMMR tumors. In a case report, a mCRC patient was found to possess immunohistochemical and molecular heterogeneity in MSI/dMMR status in the primary tumor. Significantly, treatment with nivolumab plus ipilimumab showed clear clinical benefit for the patients, with a deep and lasting response. This conclusion needs to be further confirmed by large sample studies (40). The presence of pseudoprogression (PSPD) is also a possible cause. After treatment, the phenomenon of enlargement of the original lesion or the appearance of new lesion, which is similar to the recurrence of tumor, called pseudoprogression. Pseudoprogression could be misjudged as unresponsive status, resulting in the difficulties with the following treatment choices. Colle et al. retrospectively analysis the data of 123 patients with MSI/dMMR mCRC treated with ICIs. About 10% of the population (12/123) experienced PSPD. The median time to PSPD was 5.7 weeks (95% CI, 4.1–11.4), however, after 3 months, no one experienced PSPD. Some nine of 61 patients (14.8%) had PSPD in the anti-PD1 alone group, compared with three of 62 patients (4.8%) in the anti-PD1 plus anti-CTLA-4 group. These results suggest that iRECIST criteria should be questioned after 3 months of immunotherapy (41). In a phase II study of 57 patients with MSI/dMMR mCRC treated with nivolumab and ipilimumab, only 3.5% (2/57) patients experienced PSPD. This result is consistent with the previous study (42). Parseghian et al. found that PSPD was not seen in 59 MSS mCRC treated with immunotherapy, which may be related to its poor efficacy (29).There are many other mechanisms of drug resistance in tumors. It is well known that CD8+ cytotoxic T lymphocytes (CTLs) are the main immune cells that kill target tumor cells in cancer immune surveillance. In terms of mechanism, Fas-FasL apoptosis pathway plays an important part (43). Fas is a cell surface receptor of the tumor necrosis factor receptor superfamily, which is expressed multiple kinds of cells including tumor cells. FasL is also a member of the tumor necrosis factor superfamily, but it is selectively expressed in activated T cells and NK cells. The binding of Fas and its ligand FasL induces the trimerization of Fas receptors, and then forms the death-inducing signal complex (DISC) in the cytoplasmic region of Fas receptors, and cleaves procaspase-8 at DISC, resulting in Fas-mediated cells apoptosis (44). *In vivo* and *in vitro*, Xiao et al. found that Fas expression was decreased in a subset of CD133+CD24lo colon cancer cells, leading to immune evade (31). In addition, the neoantigen is likely to form a complex with human leukocyte antigen class I (HLA class I) on the surface of tumor cells and presented by antigen-presenting cells in dMMR tumors. However, it has been reported that HLA class I expression defects occur in most dMMR CRC, which will prevent the antigen presentation of these tumors (30, 45). Likewise, Ijsselsteijn et al. determined that majority (73–78%) of dMMR cases in two independent cohorts of CRC had loss of HLA class I expression, which may cause immune escape (46).

MSS/pMMR mCRC

Unfortunately, MSS/pMMR mCRC patients in the ICIs trials failed to gain any clinically significant response or survival benefit from either monotherapy or dual therapy. Eng et al. reported a phase III study of 363 patients with MSS mCRC treated with atezolizumab plus cobimetinib or atezolizumab monotherapy or regorafenib in the third-line setting.After a median follow-up of 7.3 months, Median overall survival was 8.87 months (95% CI 7.00–10.61) in the atezolizumab plus cobimetinib group, 7.10 months (6.05–10.05) in the atezolizumab group, and 8.51 months (6.41–10.71) in the regorafenib group. None of the three groups achieved complete response. Partial response rate was 3% (five of 183) in the combination group, 2% (two of 90) in the atezolizumab group, 2% (two of 90) in the regorafenib group. In general, there is no significant difference across all three groups in OS, PFS, OR, and duration of response (19). In a phase II study of 10 patients with MSS mCRC treated with SHR-1210 (anti-PD-1) plus apatinib, no one (0%) achieved OR and two (22.2%) patients achieved disease control. The median PFS and the median OS was 1.83 months (95% CI, 1.80–1.86 months) and 7.80 months (95% CI, 0–17.07). In conclusion, MSS mCRC failed to benefit from SHR-1210 combined with apatinib (47). In a retrospective study of 23 MSS or pMMR mCRC treated with regorafenib plus antiPD-1 antibody, ORR was 0% and DCR was 78.3% (18/23), with the median PFS of 3.1 months (95% CI, 2.32–3.89). The results are consistent with clinical trials above (48). In another retrospective study, Wang et al. found that MSS CRC patients with no history of liver metastasis are more likely to benefit from this combination regorafenib plus antiPD-1 antibody (49).

However, the recent results of a phase Ib trial suggest the encouraging antitumor activity of regorafenib plus nivolumab in MSS mCRC, with ORR of 36% (9/25) and median PFS of 7.9 months in mCRC (50). In a recent phase II clinical trial, mCRC patients with MSS seemed to benefit from napabucasin (STAT3 inhibitor) plus pembrolizumab, with irORR of 10.0% (four of 40 patients; 95% CI, 2.8–23.7) (51). These conflicting results indicate that the combination of molecular targeted therapy with immunotherapy remains controversial for MSS patients and there is no conclusive evidence to validate its efficacy. With clinical trials of multiple molecular targeted therapies under way, this remains a promising therapeutic strategy for MSS patients. Before the era of immunotherapy, the efficacy of chemotherapy alone in CRC patients with MSS is also limited. In an ACCENT pooled analysis of seven studies, survival time after recurrence in stage III CRC patients with MSS/pMMR treated with adjuvant chemotherapy was shorter compared with those MSI/dMMR patients (52). Martin-Romano et al. reported that pts with refractory MSS mCRC might benefit from chemotherapy after ICI. In the retrospective study of 29 pts with mCRC received chemotherapy after ICI failure [MSS tumors, 27 pts (86%)], four patients (19%) achieved partial response and 9 pts (43%) achieved stable disease, with disease control rate of 62%. The median PFS and OS were 3.8 months (95% CI = 1.5–5.4) and 8.0 months (95% CI = 4.2–14.0), respectively. Since single chemotherapy or single immunotherapy is not effective, this also suggests the potential efficacy of chemotherapy combined with immunotherapy in MSS patients (53).

Biomarkers of Immune Response

Microsatellite instability (MSI) is recognized as a biomarker to predict the response to ICIs in solid tumors. The KEYNOTE-016

trial underlined the utility of MSI-H:dMMR as a predictive biomarker to antiPD-1 therapy (pembrolizumab) in mCRC (8). High DCR and beneficial PFS were observed in in mCRC patients with MSI-H treated by PD-1 inhibitors; however, less than half of the patients had clinical response, suggesting that patients needed additional predictive biomarkers. MSI-H tumors tend to have high tumor mutational burden (TMB), and studies have demonstrated that TMB is commonly increased in MSI-H mCRC, but still unclear (54). In mCRC patients treated with durvalumab and tremelimumab, OS is the greatest in MSS Patients with more plasma TMB of 28 variants per megabase or more (HR, 0.34; 90% CI, 0.18-0.63; P = .004) (37). Schrock et al. analyzed TMB in 22 patients treated with PD-1/L1 inhibitors, TMB was strongly associated with objective response (OR; P <0.001) and PFS, by univariate (P <0.001) and multivariate analysis (P <0.01). At a median follow up of 18 months, patients with high TMB has not reached the median PFS while patients with low TMB had median PFS of only 2 months. In MSI-H mCRC, TMB appears to be a crucial independent biomarker, which can stratify patients who may respond to ICIs (55). By analyzing CRC tissue sections, 164 of 5,702 (2.9%) MSS cases were assessed as TMB-high. It means that more people may benefit from ICI When TMB was used as a prognostic marker (56). However, based on the clinical response data collected from six patients with metastatic MSIH/DMMR GI cancers treated by ICIS, Hirsch et al. found that TMB wasn't associated with extent and duration of response (57). By comparing the expression of 44 selected immune-related genes in the primary colon tumor between responders (n = 13) and nonresponders (n = 6) after anti-PD-1 therapy, Llosa et al. concluded that preexisting antitumor immune response has little predictive value for immunoreactive pMMR CRC (58). A growing body of evidence suggests that infiltrating lymphocytes are inextricably associated with TMB, infiltrating lymphocytes is also an important prognostic marker for CRC patients after ICIs. High infiltrating lymphocytes densities (CD3, CD8, FoxP3, and CD45RO) had significant correlation with improved overall survival for primary colorectal cancer (all p <0.001). Moreover, the densities of CD8 cells predicted the good tumor regression grade well in locally advanced rectal cancer after chemoradiotherapy (59). In a study, Loupakis et al. collected data from 85 patients with MSI-H mCRC treated with ICIs. RR in patients with high number of TILs (TILs-H) and those with low number of TILs was 70.6 and 42.9%, respectively (odds ratio = 3.20, p = .0291). Patients with TILs-H had better survival outcomes than those with TILs-L (PFS: not reached *vs* 27.8 months, HR = 0.42, p = .0278; OS: HR = 0.41, p = .0463) (60).

In addition to these now routinely studied, some new biomarkers are increasingly being studied. The levels of B7-H3, B7-H4, and PD-L1 protein in tissues from 805 primary tumors and matched metastases were evaluated by microarrays. Detectable rate of B7-H3, B7-H4 and PD-L were 50.9, 29.1 and 29.2%, and elevated B7-H3 expression had an association with advanced overall stage. B7-H3 overexpression in primary tumors predicted poor DFS, while B7-H4 and PD-L1 had no significant relationships with survival. Overall, B7-H3 had a higher expression rate than B7-H4 and PD-L1, and was significantly associated with poor prognosis (61). Lu et al. found mCRC patients with early decrease in serum interleukin 1 receptor antagonist had longer PFS (not reached *vs* 2.1 months; HR = 0.06; 95% CI, 0.01 to 0.38; P <.001). Compared with MSI status or PD-L1 expression, an early decrease in serum interleukin 1 receptor antagonist can better determine who will respond to ICIs in patients with metastatic CRC (62). A case was reported that a mCRC patient who carried the rare 9p24.1 CNG achieved a lasting partial response after immunotherapy, which may support the use of ICIs in solid tumors carrying the rare 9p24.1 CNG (63). The evaluation of TMB and TILs should be incorporated into future trials of ICIs in mCRC to confirm our results and to explore methods and threshold issues for routine clinical use.

ADOPTIVE CELLULAR IMMUNOTHERAPY

Generally, autologous T cells were targeted to tumor specific antigens by gene editing, then were injected back into the patient to stimulate the host antitumor immune response. As significant efficacy was reported in a large amount of hematologic malignancies and solid tumors, adoptive T-cell therapy is recently another novel immunotherapy option for mCRC patients. In gastrointestinal tumors, cancer embryonic antigen (CEA) is a sensitive tumor biomarker, which can be detected in CRC tissues and serum with increased levels. In one of the earliest clinical trials, three refractory mCRC patients were administered autologous T lymphocytes genetically engineered to express a murine T cell receptor (TCR) against human carcinoembryonic antigen (CEA). Levels of CEA in serum were profoundly decreased in all patients (74–99%), and objective shrinkage of liver and lung metastatic lesions was observed in one patient, although a severe transient inflammatory colitis was observed in all three patients (64). In a phase I study of CEA CAR-T cell in 10 CEA+ mCRC patients, seven progressive patients had stable disease after CAR-T therapy. Among them, two patients maintained more than 30 weeks, and two patients showed tumor regression. In conclusion, most treated patients achieved some efficacy (65). Here Hege et al. report results of trials of CAR-T cells targeting tumor-associated glycoprotein (TAG)-72 (CART72 cells) in the treatment of metastatic colorectal cancer. CART72 cells in blood last for a short time (≤14 weeks), and CART72 cells in tumor tissues can be detected in tumor biopsy from one of three patients. CART72 cells had limited efficacy in mCRC, suggesting that incorporation of co-stimulatory domains in the CAR design was needed (66). The study showed that postoperative CRC patients may benefit from adjuvant sentinel lymph nodes lymphocyte (SLN-T) immunotherapy. 1-year survival rate in SLN-T lymphocyte group was 55.6%, compared with 17.5% in the control group (p = 0.02). The median OS of the SLN-T lymphocyte was 28 months, compared with 14 months of the control group (67). In addition, specific T cells targeting other neoantigens detected in tumor tissue can also be used for treatment. In a case report, objective regression of all seven lung metastases was observed after the transfusion of tumor-

infiltrating lymphocytes specifically targeted KRAS G12D, which was identified in tumor-infiltrating lymphocytes obtained from a patient with metastatic colorectal cancer (68). In another case report, tumor-infiltrating lymphocytes with HLA-A*0201-restricted recognition of mutated p53 p.R175H were identified, which can mediated recognition of multiple epithelial cancers that expresses both HLA-A*0201 and the p53 p.R175H mutation (69). CD4+ and CD8+ memory T cells targeting the mutated KRASG12D and KRASG12V variants respectively in the peripheral blood of cancer patients were conformed and isolated, suggesting that we can detect memory T cells targeting distinct or common somatic mutations in the peripheral blood of epithelial cancer patients and can hopefully use them to develop efficient individualized T cell-based cancer immunotherapy among a variety of patients (70).

Because NK cells can induce antitumor activity, independent of antigen and major histocompatibility complex (MHC), increasing clinical trials are testing the efficacy of adoptive cancer therapy with NK cells. NK cells treatment effectively extend the lives of leukemia patients. Due to good therapeutic effect and safety, NK cell therapy is considered to be superior to adoptive therapy of autologous T cells, however, many clinical trials of NK cells in solid tumors failed to achieve end points. Veluchamy et al. confirmed the antitumor efficacy *in vivo* and *in vitro* where umbilical cord blood stem cell-derived NK cells (UCB-NK) showed enhanced antitumor cytotoxicity against colon cancer cells independent of EGFR and RAS status (71). Xiao et al. used CAR-NK cells fusing the extracellular domain of the natural killer (NK) cell receptor NKG2D to DAP12 to treat three mCRC patients. Ascites and number of tumor cells in ascites samples were decreased in the first two patients after treatment with intraperitoneal injection of the CAR-NK cells. The third patient with liver metastatic experienced tumor regression in the liver region after treatment with intraperitoneal infusion of the CAR-NK cells following percutaneous injection (72).

In addition, the synergistic anti-tumor immunity of T cells and NK cells can also be achieved by targeting NKG2D for T cells. In an animal experiment, tumor burden was significantly reduced in established peritoneal colorectal xenografts after treatment with CAR-T cells specific for NKG2D ligands (73).

VACCINE THERAPY

Colorectal cancer overexpressed some common tumor associated antigens, which can serve as a target for vaccine in immunotherapy. Multiple types of vaccines studied in mCRC include autologous, peptide, and dendritic cell vaccines (**Table 2**). A phase I/II trial of p53 synthetic long peptide (p53-SLP) vaccine was performed in ten mCRC patients. p53-specific T-cell reactivity (≥6 months) was observed in 67% patients (six of nine), however, polarized p53-specific CD4 + T cells accounted for only a small proportion. How to improve the polarization of the p53-SLP vaccine-induced T-cell response should be focused in future trials (75). Balint et al. observed the decreased Treg to Teff cell ratio in samples from three of five patients and increased cytolytic T cell responses after immunizations in a phase 1/2 clinical trial of advanced-generation Ad5 [E1-, E2b-]-CEA(6D) vaccine in mCRC patients. After a long-term follow-up, 20% of patients were still alive, with median survival of 11 months (79). Morse et al. demonstrated that patients produced less neutralizing antibodies and more CEA-specific T cell responses when using VRP as vectors in a phase I/II study. In a further study, the 5-year RFS was 75% in patients with stage III cancer (95%CI 40 to 91%) and no one died. CD8+T_{EM} increased and FOXP3 + Tregs decreased in 83% patients (10/12) after vaccination treatment. The results suggested that VRP-CEA may prolong the OS in stage III CRC patients (76, 80). A Randomized Clinical Trial reported that PFS and OS of mCRC patients in the modified vaccinia Ankara-5T4 (MVA-5T4) treatment group was significantly prolonged, compared with those in the no treatment group (5.6 months *vs* 2.4 months,P <.001; 20.0 *vs* 10.3 months; HR, 0.32; 95% CI, 0.14–0.74; P = .008).In addition, baseline anti-5T4 responses was doubled in 16 of 35 mCRC patients treated with MVA-5T4 (81). It is worth mentioning that the vaccine with poxvirus vectors highlights a critical component of vaccine therapy. Poxvirus vectors can be used to incorporate multiple transgenes and its safety has been increasingly proven. In a pilot study of 25 patients treated with a poxviral vaccine regimen targeting CEA and MUC-1, along with a triad of costimulatory molecules engineered into vaccinia (PANVAC-V) as a prime vaccination and into fowlpox (PANVAC-F) as a booster vaccination, the vaccine was tolerable and nine of 16 patients achieved immune responses to MUC-1/CEA (77). A randomized phase II study further study the therapeutic effect of vaccines based on dendritic cells (DCs) and poxvectors targeting CEA and MUC1 (PANVAC) in resected mCRC patients. Patients (n = 74) were randomized to injections of autologous DCs modified with PANVAC (DC/PANVAC) or PANVAC with per injection GM-CSF. Two-year recurrence-free survival in DC/PANVAC and PANVAC/GM-CSF group was 47 and 55% respectively (P = 0.48). In addition, the vaccinated patients have better survival than the unvaccinated group (78). An open-label, 3 + 3 design, dose-escalation trial proved the safety and potential clinical activity of a new poxviral-based vaccine (BN-CV301), comprised of recombinant (rec.) modified vaccinia Ankara (MVA-BN-CV301; prime) and rec. fowlpox (FPV-CV301; boost) (74).

There are also new tumor-associated antigens being developed for use in vaccine therapy. A phase 2 study was performed to test the efficacy of tecemotide (an antigen-specific cancer vaccine inducing immunity against mucin-1). There is no significant difference in RFS and 3-year OS rate between mCRC patients after resection of CRLM treated with tecemotide and those treated with placebo (82). Accumulated abundant insertion/deletion mutations in dMMR cancer cells at microsatellites resulted in the production of immunogenic frameshift peptide (FSP) neoantigens. Kloor et al. performed a clinical phase I/IIa trial of FSP-based vaccine in dMMR CRC. All patients achieved humoral and cellular immune responses induced by the vaccine. However, only two patients (9%, two of 22 patients) achieved stable disease as best overall response. Among them, stable disease and stable CEA levels (≥7 months)

TABLE 2 | Summary of Vaccine Treatment for mCRC.

Study	Phase	Agent	Population	MSI status	Endpoint	Reference
NCT01147965	1/2	AD5-CEA Vaccine	32 mCRC	-	The primary purpose: determine the safety The secondary objectives: evaluate CEA-specific immune responses and clinical response rate	(68)
NCT00529984	2	AVX701 (VRP-CEA Vaccine)	28 metastatic tumors; including 21 mCRC	-	the primary objectives: determine the safety The secondary objectives: evaluate CEA-specific immune responses and clinical response rate	(69)
NCT01890213	2	AVX701 (VRP-CEA Vaccine)	12 Stage III CRC	-	-	(70)
NCT00154713	1	CEA-pulsed DC	12 mCRC	-	The primary endpoint: safety	(74)
NCT01462513	2	Tecemotide (L-BLP25) or placebo	121 mCRC with R0/ R1 resection		The primary endpoints: RFS and 3-year overall survival (OS) rate; The secondary endpoints: RFS and OS in subgroups with different MUC1 expression and safety	(72)
NCT01461148	1/2a	FSP-based vaccine	22 CRC	MSI	The primary endpoints: safety (phase I) and immunogenicity (phase IIa); The secondary endpoints: tumor response (both phases) and immunogenicity (phase I) and safety (phase IIa)	(73)
NCT00027833	2	ALVAC-CEA-B7.1 vaccine + FOLFIRI; FOLFIRI + ALVAC-CEA-B7.1 vaccine; ALVAC-CEA-B7.1 vaccine + tetanus toxoid + FOLFIRI	180 mCRC	-	The primary endpoints: Immune response to the vaccine.	(75)
NCT00676949	1	5 peptide vaccines of KOC1, TTK, CO16, DEPDC1, MPHOSPH1	18 metastatic Tumors, including nine mCRC	-	The primary end point: safety and tolerability. The secondary endpoints: MTD and immune response	(76)
NCT01413295	2	DC vaccine + BSC or BSC alone	52 mCRC	-	The primary endpoints: PFS; The secondary endpoints: PFS, OS, toxic effects, and ORR.	(77)
NCT01348256	2	DC vaccine	19 mCRC	-	-	(78)
-	1/2	p53-SLP	10 mCRC	-	-	(67)
-		MVA-5T4, metronomic low-dose cyclophosphamide, or a combination of both treatments	55 mCRC	-	The primary endpoints: magnitude of 5T4-specific responses at treatment day 43; The secondary end points: the kinetics of anti-5T4 immune responses overtime, PFS, OS	(71)
-	2	TroVax(MVA-5T4)	19 mCRC	-	-	(79)
-	2	a peptide vaccine combined with UFT/LV	46 stage III CRC	-	The primary end point: RFS; The secondary endpoints: OS, safety, tolerability and peptide-specific activities	(80)
-		DC vaccine	46 mCRC	-	-	(81)

OS, overall survival; PFS, progression-free survival; RFS, recurrence-free survival; ORR, objective response rate; irORR, immune-related objective response rate; DCR, disease control rate; BSC, best supportive care; FOLFIRI, 5-flourouracil, leucovorin, irinotecan; CEA, carcinoembryonic antigen; FSP, frameshift peptide; DC, dendritic cell; mCRC, metastatic colorectal cancer; MSI-(H), microsatellite instability-(high); MSS, microsatellite stable.

was observed in a severely metastatic patient received extensive treatment (83).

What is the effect of a combination of vaccine and chemotherapy? Kaufman et al. conducted a study to assess whether systemic chemotherapy can affect the on T-cell immunity induced by ALVAC-CEA/B7.1 vaccine in mCRC patients. The vaccine was injected before and after treatment with 5-fluorouracil, leukovorin and irinotecan. The generation of CEA-specific T-cell responses following vaccination was not affected by systemic chemotherapy, with no differences in clinical or immune response across the treatment groups (84). Similar results were found in another study of MVA-5T4 (TroVax) and systemic chemotherapy in 19 mCRC patients (85). The benefits of the vaccine combined with chemotherapy for patients have been further confirmed in subsequent studies. HLA-A2402+ patients with advanced solid tumors (nine colorectal cancer) were treated with vaccine composed of five HLA-A2402-restricted, tumor-associated antigen (TAA) epitope

peptides, following by escalating doses of cyclophosphamide. After treatment of cyclophosphamide, regulatory T cells baseline was decreased. TAA-specific T cell responses were significantly collected to longer overall survival (86). A similar phase II clinical trial of a peptide vaccine combined with UFT/LV as adjuvant treatment was performed in patients with stage III CRC. Three-year RFS rate was 85.7% in patients with positive CTL responses in the HLA-A*2402 matched group, compared with 33.3% in those without (HR = 0.159, 95% CI: 0.023–0.697; P = 0.011), although there was no significant difference in three-year RFS between HLA-A*2402 matched and unmatched groups (67.8 *vs.* 73.6%, respectively; HR = 1.254, 95% CI: 0.48–4.63; P = 0.706) (87).

Because dendritic cell cells (DCs) are the most effective antigen-presenting cells, it is possible to exploit their diversity to produce improved therapeutic vaccines. Many therapeutic vaccination routes against cancer are being developed clinically. Twenty-six colorectal patients received DCs treatment after resection of the metastatic lesion. 5-year RFS rate was 63% in patients with evidence of a vaccine-induced immune response 1 week after vaccination, compared with 18% in nonresponders (P = 0.037) (88). In another phase II trial, pre-treated mCRC patients were randomly assigned to receive autologous tumor lysate dendritic cell vaccine (ADC) + best supportive care (BSC) (experimental arm [EA]) or BSC (control arm [CA] alone. No one in EA achieved objective radiological response. Median PFS was 2.7 months (95% CI, 2.3–3.2 months) and 2.3 months (95% CI, 2.1–2.5 months) (p = 0.628),median OS was 6.2 months (95% CI, 4.4–7.9 months) and 4.7 months (95% CI, 2.3–7 months) in the CA *vs.* EA group (p = 0.41), respectively. OS in responders was 7.3 months (95% CI, 5.2–9.4 months), compared with 3.8 months (95% CI, 0.6–6.9 months) in non-responders (p = 0.026).The results mean that patients don't benefit from ADC, although ADC-induced tumor-specific immune response was observed in patients (89). However, Rodriguez et al. reported that mCRC patients who received the DC vaccine as postoperative adjuvant therapy were less likely to relapse, with median DFS of 25.26 months in the vaccine arm versus 9.53 months of months in the observation arm (90). Dendritic cell vaccines that target specific tumor-associated antigens may further enhance the effectiveness of immunotherapy. In twelve patients treated with CEA-pulsed DCs mixed with tetanus toxoid and subsequent interleukin-2, two patients had stable disease and 10 patients showed disease progression, suggesting that a small proportion of patients had clinical benefit (91). In another phase I study of DC vaccination targeting WT1 for resectable advanced CRC patients, patients achieved lasting immunity from DC vaccination (≥2 years) and prolonged survival (92).

In addition to developing a variety of vaccines, the corresponding vector is also under continuous research to better enhance the immune response. Adenovirus serotype 5 (Ad5), as a common viral vector, is often used to prepare vaccines against pathogens and tumor antigens. However, AD5-induced virus-specific neutralizing antibodies appear after exposure to an AD5-based vaccine, limiting transgenic transmission and target-specific immunity. Flickinger et al. reported that more patients (≥90%) with Ad5.F35-based vaccines targeting tumor antigens achieved clinically relevant immune responses, compared with approximately 50% patients with Ad5-based vaccines (93).

Attempts to confirm the efficacy of the vaccine for mCRC through multiple ways (e.g., dendritic cells, autologous tumor cells, recombinant viral vectors, and peptides) appear to have failed, with limited clinical efficacy and outcomes, despite improved specific immune responses.

The Combination of Immunotherapy and Targeted Therapy

In recent years, a variety of targeted therapies led by anti-angiogenic drugs have been increasingly used in the clinical treatment of various tumors. In addition to their excellent anti-angiogenic effects, they also have immune-enhancing effects. Manzoni et al. found that patients responded to bevacizumab showed a trend of increasing CD3 (p = 0.07) and CD4 (p = 0.05) (94). By analyzing immune cell infiltration in the liver metastatic sites of 53 colorectal cancer patients treated with chemotherapy plus cetuximab, chemotherapy without cetuximab, and no chemotherapy before operation, Inoue et al. reported that the chemotherapy with cetuximab group had a higher infiltration of CD3+ (P = 0.003), CD8+ (P = 0.003) and CD56+ (P = 0.001) cells, compared with other groups (95). This opens up new possibilities to further improve clinical outcomes in combination with immunotherapy, especially for immunotreatment-resistant MSS tumors. A single arm, multi-center phase II study (CAVE Colon) was conducted to study the efficacy of avelumab and cetuximab in RAS WT mCRC patients treated with a first-line CT in combination with an anti-EGFR agent with a major response achieved (complete or partial). We are looking forward to its clinical trial results (96). In another single arm phase II AVETUX trial, 43 RAS/BRAF wildtype mCRC pts (40 MSS) received the treatment of mFOLFOX6 and cetuximab combined with avelumab. The ORR and DFS were 79.5 and 92.3% respectively. Among them, 6 pts had CR and 25 pts had PR.; 2 pts had progression and 1 was not evaluable. In addition, 79.5% patients achieved early tumor shrinkage (ETS) (≥20% after 8 weeks). In short, The AVETUX regimen is feasible and produces a high response rate in MSS patients, mainly occurring in the first 8 weeks (97). However, 445 BRAFwt mCRC pts in MODUL study who received 16 weeks of induction treatment with FOLFOX + BEV were randomized to take medication of FP/BEV + atezolizumab (297 pts) or FP/BEV (148 pts). At a median follow-up of 10.5/18.7 months there was no significant difference in PFS and OS. Adding atezolizumab to FP/BEV as a first-line treatment did not benefit BRAFwt mCRC patients (98). Recently, MEK inhibitors have received increasing attention, particularly in the area of combined immunotherapy, whose efficacy has been evaluated in multiple clinical trials. In a phase I/Ib study of MEK inhibitor (cobimetinib) and PD-L1 inhibitor (atezolizumab) in patients with solid tumors (mCRC; n = 84), 8% mCRC patients (seven of 84 patients) achieved confirmed responses, independent of KRAS/BRAF status across diseases. However, potential collaborative activity observed in mCRC disappeared in a further phase III study (99). Due to the potential to induce antibody-dependent cell-mediated cytotoxicity (ADCC), stimulation of NK cells represents another ideal target for this molecular approach. In

LOVO xenograft tumor models with positive EGFR expression, the combination of cetuximab and NK cells showed great antitumor effect (100). Similarly, cetuximab enhanced the cytotoxic activity of NK cells on EGFR+ tumor cells independent of RAS status (101).

The Combination of Immunotherapy and Chemotherapy

Tumor associated antigens, such as CEA and other specific molecules, tend to be overexpressed as chemotherapeutic drugs kill tumor cells. Meanwhile, death signaling induced by tumor antigen-specific cytotoxic T lymphocytes during chemotherapy moderates tumor cell resistance. These provide a theoretical basis for the combination of chemotherapy and immunotherapy. In a phase II trial, CRC patients were administered subcutaneously granulocyte macrophage colony-stimulating factor and low-dose interleukin-2, following gemcitabine + FOLFOX-4 (oxaliplatin, fluorouracil, and folinic acid) polychemotherapy. At a median follow up of 12.5 months, the ORR and DCR were as high as 68.9 and 96.5%, respectively. Analysis of peripheral blood mononuclear cells (PBMCs) in 20 patients showed that immune response to colon carcinoma antigen increased and suppressive regulatory T lymphocytes (CD4+CD25T-reg+) decreased significantly (102). Subsequent multicenter phase II and phase III clinical trials were conducted to further assess the combination (GOLFIG) in mCRC patients. In the phase II trial (GOLFIG-1 trial), including 46 mCRC patients who have had previous chemotherapy, RR and DCR were 56.5 and 91.3%, respectively, with a mean PFS of 12.3 months (103). In the phase III trial (GOLFIG-2), 124 mCRC patients were randomly assigned in a 1:1 ratio to receive the GOLFIG regimen or FOLFOX-4 regimen for the 1st line setting. Significant difference in RR (66.1% *vs.* 37·0%, P = 0.002), DCR, and PFS (9·23 *vs.* 5.70 months; P = 0·002) indicated that GOLFIG chemo-immunotherapy is markedly better than FOLFOX regimen for first-line treatment of mCRC (104). Caraglia et al. then retrospectively analyzed 179 mCRC patients in these two trials and followed them up for 15 years. Median PFS and OS were 15.28 (95% CI: 10.36–20.20) and 24.6 (95% CI: 19.07–30.14) months, respectively, To note, 14 patients survived for 10 years without disease progression (105). In their latest investigation of the GOLFIG-2 trial, patients in the GOLFIG group tend to achieve longer OS and PFS than those in the FOLFOX group (HR = 0.69, P = 0.06; HR = 0.58, p = 0.006).Their analysis also confirmed that pretreated patients had significant antitumor response, with a mean PFS of 12.55 (95% CI: 7.19–17.9) and OS of 20.28 (95% CI: 14.4–26.13) months, respectively. The GOLFIG regimen may be a reliable therapeutic option for pre-treated mCRC patients.

The above sequential clinical trials initially demonstrated the efficacy of chemotherapy combined with immunotherapy. The effect of chemotherapy on immune cells has been studied more and more. Roselli et al. analyzed mononuclear cell subsets from peripheral blood in mCRC patients (n = 23) before and during treatment with FOLFIRI plus bevacizumab. Despite differences among patients, most patients experienced small changes in the ratio of CD4(+) T cells to regulatory T cells (Treg) or small changes in Treg inhibitory activity during treatment. Tregs in responders to the chemotherapy was significantly decreased during therapy *vs.* pre-therapy compared with non-responders (106). In the same way, Scurr et al. observed a reduction in the percentage and absolute number of Treg in peripheral blood-derived lymphocytes from cyclophosphamide-treated mCRC patients. Cyclophosphamide significantly enhanced IFNγ+ tumor-specific T-cell responses and markedly delayed tumor growth in mCRC patients. [HR = 0.29; 95% CI, 0.12–0.69; P = 0.0047), compared with nonresponders and no-treatment controls (107). However, Dagenborg et al. reported that intratumoral T-cell densities was not associated with neoadjuvant chemotherapy therapy (NACT) before surgery in 45 mCRC patients. What is noteworthy is that intratumoral T-cell densities increased significantly in a short period of time aftertreatment, <9.5 weeks *vs* >9.5 weeks (medians 491, 236 cells/mm^2, respectively; P <.0001).The results indicated that intratumoral T-cells may increase only for a short time after NACT administration, and the best combination of chemotherapy and immunotherapy should be further investigated (108). The relationship between chemotherapy and tumor PDL1 expression has also received more and more attention. Huang et al. found that expression of tumor PD-L1 and other immune-related genes were enhanced by decitabine (DAC)-induced DNA hypomethylation and intratumoral T cell infiltration increase *in vitro* and *in vivo* (109). Further, tumor samples from mCRC patients received Folfox regimen showed induction of PD-L1 expression and high CD8 T cell infiltration (110).

Other studies have shown that chemotherapy has a negative effect on immunotherapy. Bruni et al. reported that chemotherapy accelerates the aging of Vδ2pos T cells in CLM patients,which is non-classical lymphocytes possessing a wide range of anti-tumor activities (111). In 15 refractory mCRC patients treated with AMP-224 in combination with SBRT and low-dose cyclophosphamide, no one achieved objective response and three patients (20%) had stable disease. Patients did not benefit from the combination, with median PFS of 2.8 months (95%CI, 1.2–2.8 months) and OS of 6.0 months (95% CI, 2.8–9.6 months), respectively (112). Standard-of-care treatment seems to be harmful to early-stage CRC patients with high PD-L1 expression (HR = 4.95; CI, 1.10–22.35), suggesting that standard chemotherapy should not be used in stage II/III colorectal carcinoma patients with PD-L1 (high)/MSI/immune (high) (113).

The Combination of Immunotherapy and Ablation

Ablation, as one of the established effective methods for resectable liver metastases from colorectal cancer, can also elicit tumor antigen-specific T cell responses and enhance the efficacy of immunotherapy. It can lead to tumor regression in untreated lesions, known as abscopal effect. This occurs because a variety of harmful molecules are released during ablation, including tumor-associated antigens, inflammatory cytokines, etc. In mouse models, we observed that incomplete radiofrequency ablation (iRFA) promotes tumor growth and impedes the efficacy of anti-PD-1 therapy. Mechanistically, more myeloid suppressor cells infiltrated into the local persistent inflammatory areas caused by iRFA, resulting in the inhibition of T cell function in tumors (114). Lemdani et al. demonstrated that TIL in metastatic lesion of patients and in mice model did not increase after RFA and RFA

could not prevent recurrence. By adding systemic PD-1 blockade, immune deficiency in large secondary lesions can be reversed. In the situation of large lesions that do not respond to single RFA, the use of ICIs in metastatic MSS CRC may be reconsidered (115). Consistent with the above results, immunohistochemistry showed immune cells in metastatic lesions did not increase in six patients after RFA treatment, although induced immune responses and/or pre-existing T cell immunity against the specific targets was observed (116). Shi et al. reported that PD-L1-PD-1 axis plays a key role in inhibiting the antitumor immune responses induced by RFA. Not only T-cell infiltration, but also PD-L1 expression in primary human colorectal tumors increased after RFA treatment of liver metastases. Significantly enhanced T-cell immune responses, stronger antitumor immunity and prolonged survival were observed in mice model after the combined therapy of RFA and anti-PD-1 antibodies. This indicates the rationality and feasibility of ablation combined with immune checkpoint therapy for mCRC patients (117).

The Combination of Immunotherapy and Radiotherapy

Another hot area of tumor immunotherapy is the use of monoclonal antibodies to deliver cytotoxic substances directly to the tumor site, known as radioimmunotherapy (RIT), which can increase the toxic dose of the tumor site and reduce the damage to the surrounding normal tissue. In a phase II study, 23 patients received RAT with radiolabeled anti-CEA antibodies after surgery for LM of CRC. At a median follow-up of 64 months, median OS and median DFS from initial hepatectomy for RAT patients was 68.0 months (95% CI, 46.0 months to infinity) and 18.0 months (95% CI, 11.0 to 31.0 months), with 5-year survival rate of 51.3%. Historical and contemporaneous controls without RAT were analyzed, adjuvant RAT seem to improve survival for CRC patients undergoing complete LM resection (118). Over a longer period of follow-up, Liersch et al. found 3- and 5-year survival rate of 68.4 and 42.1% for patients with RAT, compared with 36.8 and 15.8% for the controls (119). Some 13 patients are receiving the same type of RIT after complete resection of liver metastases (LM) from colorectal cancer. At a median follow-up of 127 months, median DFS and OS are 12 and 50 months, respectively (120). RIT targeting other antigen also showed safety and feasibility for 19 patients, with one patient of partial response, and 10 patients of stable disease (121). Studies have also evaluated the efficacy of RIT in combination with other treatments. The combination of cetuximab and RIT targeting CEA significantly reduced tumor growth and prolonged survival of mice than RIT monotherapy (122). Chen et al. demonstrated that RIT significantly increased PD-L1 expression on T cells. RIT plus PD-L1 blockade improved local tumor control, overall survival and avoid relapse, with expanded infiltration of CD8+ T cells (123).

OTHER TYPES OF IMMUNOTHERAPY

Many experiments are also investigating the possibility of other molecules as future immunotherapies.

TGF-β is a kind of can influence a variety of cellular events and therefore has a dual role. On the one hand, this cytokine can block the ability of tumor cells to multiply by interfering with key molecules (CDK4, CDKI) in the cell cycle during the initial stages of cancer. On the other hand, TGF-β promotes tumor growth and metastasis as the tumors progress to an advanced stage (124). Many studies have demonstrated that blocking TGF-b signaling reduced metastasis in CRC and other solid tumors (125). Tauriello et al. discovered the significant role of TGF-β in the immune system for metastasis CRC. Increased TGFβ in the tumor microenvironment promoted immune evasion by decreasing T-cell infiltration and inhibiting acquisition of the TH1-effector phenotype. In the quadruple-mutant mice model bearing metastatic intestinal tumors with TGFβ-activated stroma, inhibition of TGFβ prevented metastasis by enhancing cytotoxic T-cell response against tumor cells, while the use of anti-PD1 drug drew finite efficacy. Furthermore, combination of TGFβ inhibitor and anti-PD1 drug had excellent effect in mice with severely hepatic metastases (126). Immunotherapies targeting TGFβ signaling and the combination with ICIs may therefore be potential and promising options for advanced CRC patients.

CC chemokines consist of 28 chemotactic cytokines crucial to all kinds of immune system cells, including CD4+ and CD8+ lymphocytes, dendritic cells, eosinophils, macrophages, monocytes, and NK cells. At the same time, they are essential in the development of tumors (127).

Halama et al. found that tumor-infiltrating lymphocytes delivering CCL5 are abundant in the invasive margin of hepatic metastatic samples from CRC patients, which instead promotes the growth and dissemination of tumor by polarizing macrophages to pro-tumoral phenotype *via* CCR5. Blocking CCR5 repolarized the macrophages to exert the anti-tumor efficiency *in vitro* organoid models, which was further confirmed in a phase I trial of CCR5 antagonist in refractory mCRC patients (128). Zhang et al. demonstrated that the lack of CCL5 inhibited tumor growth and metastasis by enhancing CD8+ T cells infiltration into tumor areas in CRC mouse models. Meanwhile, the absence of CCL5 could increase the PD-1 and PD-L1 expression and alleviate the resistance to ICIs in CRC mouse model. Clinical specimen from CRC patients also confirms the results (129). Same changes of immune-related molecules (CCR5, CCL5, PD1, PD-L1) in the microenvironment of hepatic metastases were also showed by Suarez-Carmona et al. By further analyzing two available cohorts, the data showed that patients with low gene expression of CCR5 in metastases had prolonged DFS (130). The study above suggests that targeting CCL5–CCL5 axis monotherapy or in combination with ICIs may be a possible therapeutic strategy for CRC and need to be tested in future trials.

Many studies have been looking at Toll-like receptors (TLRs) due to the ability of stimulating antitumor immunity by initiating innate and adaptive immune responses (131). 28 patients with metastatic solid tumors received a novel synthetic DNA-based toll-like receptor 9 (TLR9)-immunomodulator. In 15 patients completing the treatment cycle, six (40%) had stable disease (SD). NK cells, DCs and B cells were transiently increased, although there were no changes in the composition and activation status of various kinds of T cells (helper T cells, cytotoxic T cells, naive and memory T cells) (132). Sorski et al.

found that the percentage of NK cells in marginating-hepatic (MH) cells was high in BALB/c mice, but the cytotoxicity was weak. However, TLR-9 agonist (CpG-C) treatment increased MH-NK cell numbers and activities in the mice model with mCRC, with increased maturation markers (NKp46, CD11b) and decreased the inhibitory NKG2D (133). In the murine colon metastatic cancer models, entolimod (TLR5 agonist) induces a large number of NK cells to migrate from blood and bone marrow to the liver (134). Then, we observed CD8(+) T-cell response following the activation of DCs depending on NK cells. Therefore, entolimod provoked tumor specific and persistent immune memory. TLR5 agonists can be used as efficient antitumor vaccine without the need to identify tumor-specific antigens. However, Zheng et al. found TLR ligands (TLR4, TLR5) released by nonvirulent tumor-targeting bacteria played a prominent part in tumor suppression in mouse models (135).

CONCLUSION

In general, we are developing a variety of immunotherapies and achieved some successes in the field of immunotherapy, especially for mCRC patients with dMMR (**Figure 3**). However, for one thing, the response rate of these patients is still not high enough, and for another, there is no effective treatments including immunotherapy for other mCRC patients with MSS yet. First, ICIs are well researched, and there are already drugs approved for clinical use. Now, due to the relatively good advantages of molecular targeted therapies, more consideration should be given to the combination of ICIs and molecular targeted therapies (136). A small number of clinical trials have shown its potential with increased response rate and better prognosis, and more trials are underway and planned (**Table 3**). The results are worth waiting for. Secondly, few studies of ACT have been

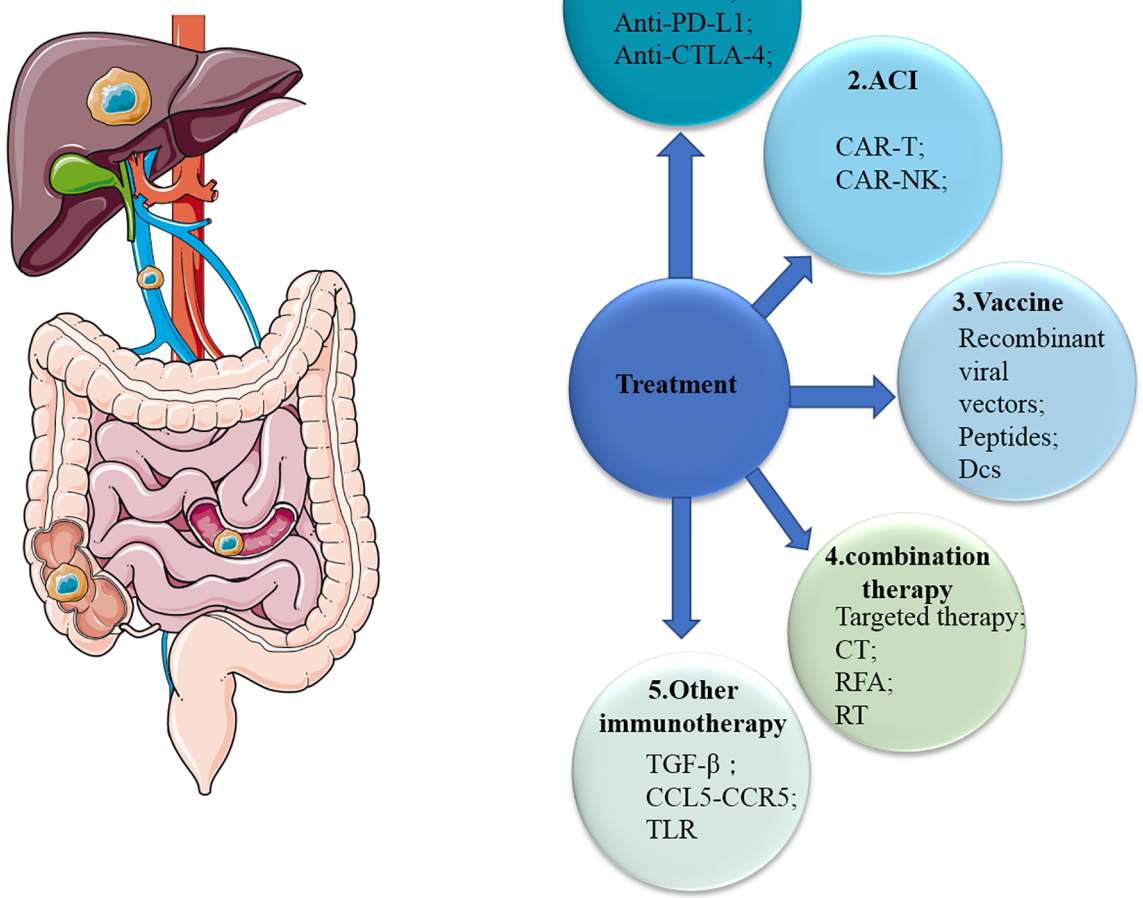

FIGURE 3 | Overview of therapies for mCRC. mCRC, metastatic colorectal cancer; ICIs, immune checkpoint inhibitors; ACI, adoptive cellular immunotherapy; CT, chemotherapy; RFA, radiofrequency ablation; RT, radiotherapy; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death 1; PD-L1, PD-1 ligand; Treg, regulatory T cell; DC, dendritic cell; NK cell, natural killer cell; TLR, toll-like receptor.

TABLE 3 | Ongoing or Future Clinical Trials of Immunotherapy for mCRC.

Study	Phase	Agent	Population	Status	Endpoint
NCT03721653	2	FOLFOXIRI + Bevacizumab + atezolizumab *vs* FOLFOXIRI + Bevacizumab	201 mCRC	-	The primary end point: DCR; The secondary endpoints: PFS, ORR, OS
NCT03202758	1/2	Durvalumab + Tremelimumab + FOLFOX	48 mCRC	-	The primary end point: safety
NCT04072198	2	Nivolumab + FOLFOXIRI/Bevacizumab	70 advanced CRC	RASm/BRAFm	The primary end point: ORR; The secondary endpoints: OS
NCT03186326	2	Avelumab versus a standard second-line chemotherapy plus a targeted agent according to tumor RAS status	132 mCRC	MSI/dMMR	The primary end point: median PFS; The secondary endpoints: ORR, OS, quality of life and toxicity
NCT03827044	3	Avelumab + 5-FU Based Chemotherapy	402 stage 3 CRC	MSI-High or POLE Mutant	The primary end point: DFS
NCT04062721	1	Local Immunomodulation (TLR agonist and GM-CSF) + Radiofrequency Ablation	50 mCRC	unresectable	The primary endpoints: PFS rate at 12 months; The secondary endpoints: median PFS; response rate; OS
NCT04513431	1	Anti-CEA-CAR T	18 mCRC	-	The primary endpoints: adverse effects including cytokine storm response and any other adverse effects
NCT03698461	2	Atezolizumab versus Atezolizumab + Bevacizumab + FOLFOX	20 mCRC	-	
NCT04030260	2	Regorafenib + Nivolumab + Radiotherapy	43 mCRC	pMMR/MSS	The primary endpoints: PFS rate at 6 months; The secondary endpoints: objective response; DCR; OS
NCT04599140	1/2	SX-682 + Nivolumab	53 mCRC	RAS Mutated; MSS	The primary end point: safety; The secondary endpoints: ORR
NCT03202758	1/2	Durvalumab + Tremelimumab + FOLFOX	48 mCRC	-	The primary end point: PFS; The secondary endpoints: OS
NCT02754856	1	Durvalumab + Tremelimumab	26 mCRC	-	The primary end point: safety and feasibility; The secondary endpoints: RFS

OS, overall survival; PFS, progression-free survival; DFS, disease-free survival; RFS, recurrence-free survival; ORR, objective response rate; irORR, immune-related objective response rate; DCR, disease control rate; BSC, best supportive care; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand-1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; 5-FU/LV, 5-flourouracil, leucovorin; FOLFOX, 5-flourouracil, leucovorin, oxaliplatin; FOLFIRI, 5-flourouracil, leucovorin, irinotecan; mCRC, metastatic colorectal cancer; FOLFOXIRI, 5-flourouracil, leucovorin, oxaliplatin, irinotecan; MSI-(H), microsatellite instability-(high); MSS, microsatellite stable; TLR, toll-like receptor; CEA, carcinoembryonic antigen.

conducted in mCRC patients, although ACT has long been used in patients with hematological malignancies due to its excellent efficacy. We should explore more of its possibilities in mCRC patients, especially with regard to NK cell therapy. Thirdly, despite many studies, cancer vaccines have not made major breakthroughs in mCRC patients because of its limited role and possible safety issues. The cancer vaccines may be used more as an adjunct to other treatments to boost the immune response. Other molecules (TGF-β, CCL5, CCR5, toll receptor) found to affect the immune system are also promising. In addition, the development of High-Tech has made some progress in the application of nanotechnology in immunotherapy, mainly as a drug carrier (137, 138). In conclusion, although there are many challenges and problems, the possibilities of immunotherapy are endless.

AUTHOR CONTRIBUTIONS

YD, WZ and LY were responsible for gathering information of the related research and designing the review. XD, DR and FW were responsible for language editing. XQ and JG have contributed to informatio0n interpretation, editing and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Implications of Habitual Alcohol Intake With the Prognostic Significance of Mean Corpuscular Volume in Stage II-III Colorectal Cancer

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Objective: To elucidate the prognostic significance of mean corpuscular volume (MCV), with implications of habitual alcohol intake in stage II-III colorectal cancer (CRC).

Background: MCV had the potential to become an ideal prognostic biomarker and be put into clinical application. Few studies, however, have explored whether habitual alcohol intake which greatly increased the value of MCV would affect the prognostic role of MCV.

Methods: Eligible patients were identified from the CRC database of Fudan University Shanghai Cancer Center (FUSCC) between January 2012 and December 2013. Survival analyses were constructed using the Kaplan–Meier method to evaluate the survival time distribution, and the log-rank test was used to determine the survival differences. Univariate and multivariate Cox proportional hazard models were built to calculate the hazard ratios of different prognostic factors.

Results: A total of 694 patients diagnosed with stage II-III CRC between January 2012 and December 2013 were identified from FUSCC. Low pretreatment MCV was independently associated with 72.0% increased risk of overall mortality compared with normal MCV (HR = 1.720, 95%CI =1.028-2.876, P =0.039, using normal MCV as the reference). In patients with habitual alcohol intake, however, pretreatment MCV positively correlated with the mortality (P = 0.02) and tumor recurrence (P = 0.002) after adjusting for other known prognostic factors.

Conclusions: In CRC patients without habitual alcohol intake, low (<80 fL) level of pretreatment MCV was a predictor of poor prognosis. In patients with habitual alcohol intake, however, pretreatment MCV showed the opposite prognostic role, which would elicit many fundamental studies to elucidate the mechanisms behind.

Keywords: alcohol intake, mean corpuscular volume, prognostic, stage II-III, colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) was one of the most commonly diagnosed malignances worldwide (1). Among them, stage II (T3-4N0M0) and stage III (TanyN1-2M0) diseases accounted for a vast majority (2, 3). Despite the significant improvements of oncologic outcomes in stage II-III CRC due to the development of surgery techniques and adjuvant therapy over the past decades, 30% of stage II and 50-60% of stage III CRC patients were reported to experience a recurrence within 5 years after the operations (4).

Over the past decades, researchers were looking for new biomarkers related to cancer incidence, mortality and oncologic outcomes (5, 6). However, reliable, low-cost and easily accessible biomarkers that can be optimally put into a real clinical application were still rare.

As a measure of the average volume of a red blood cell, mean corpuscular volume (MCV) was related to the prognosis of liver cancer (7), esophageal cancer (8) and adenocarcinomas of the gastroesophageal junction (9). Interestingly enough, MCV was also reported to be associated with the risk of colorectal adenoma (10), advanced CRC (11) and response to chemotherapy in CRC (12, 13), suggesting MCV had the potential to be an ideal biomarker and be put into clinical application. In particular, previous study revealed that high MCV value may be used as an index of the risk of colorectal adenomas (10), but a recent research reported decreased MCV was an independent predictor for the detection of advanced colorectal cancer (11), indicating that the clinical role of MCV in colorectal cancer was still uncertain.

Alcohol drinking, which was an important health and social problem worldwide, was a significant cause of higher MCV (9, 14, 15). Alcohol drinking was one of the global health priorities, however, to the best of our knowledge, no previous studies have investigated the prognostic value of MCV in CRC patients with habitual alcohol intake (16). Therefore, we conducted this study to elucidate the prognostic significance of MCV with implications of habitual alcohol intake in CRC.

METHODS

Patient Selection

In the present study, we identified patients meeting the following criteria from the CRC database of Fudan University Shanghai Cancer Center (FUSCC) between January 2012 and December 2013: (1) diagnosed with stages II or III CRC by histopathology; (2) without neoadjuvant treatment; (3) underwent curative surgery without positive surgical margin; (4) adenocarcinoma; (5) with the information of pretreatment MCV and carcinoembryonic antigen (CEA); (6) without history of gastrectomy, upper aerodigestive tract cancer, recent bleeding or anemia; (7) with complete relevant demographic and clinicopathologic data. Nine patients (1.3%) with high pretreatment MCV (>100fL) were also excluded from the cohort because of the small sample size (**Figure S1**). Eligible patients were divided into two groups according to the standard value of pretreatment MCV: normal-MCV group (80–100fL) and low-MCV (<80fL) group. We then extracted the demographic and clinicopathological characteristics of patients from FUSCC database including the information of pretreatment MCV and CEA from blood routine examination (all the blood samples were obtained from patients within 3 days prior to the radical resection). In our center, 5-Fu-based adjuvant chemotherapy was recommended for both high-risk pathological stage II diseases and stage III diseases. The information of alcohol intake was extracted from of personal history, those with habitual alcohol intake recently were identified. This study was approved by the Ethical Committee and Institutional Review Board of FUSCC.

Statistical Analyses

In this study, Pearson's chi-squared test was used to compare clinicopathological and demographic characteristics according to the levels of pretreatment MCV. Survival analyses were conducted using the Kaplan–Meier method to evaluate the survival time distribution, and the log-rank test was used to determine the univariate survival difference. Univariate and multivariate Cox proportional hazard models were constructed to calculate the hazard ratios of prognostic factors, including tumor grade (high/moderate or low), habitual alcohol intake (yes or no), vascular invasion (yes or no), nerve invasion (yes or no), serum CEA levels (high or low), gender (male or female), age at diagnosis (years), tumor location (rectum or colon), postoperative complications (yes or no), stage (II or III), adjuvant treatment (yes or no), and No. of lymph nodes retrieved (<12 or ≥12). Only the clinicopathological characteristics that showed prognostic significance (log-rank, $P < 0.20$) in the univariate Cox analyses were included into the multivariate Cox analyses. A variable with two-sided P <0.05 was considered statistically significant. Statistical analyses in the present study were carried out using the SPSS version 22 (IBM Corporation, Armonk, NY, USA).

RESULTS

Clinical Characteristics of Patients From FUSCC

A total of 694 patients diagnosed with stage II-III CRC between January 2012 and December 2013 were identified from FUSCC. The median follow-up time among the whole cohort was 68 months. Among them, 409 (58.9%) patients were men and 285 (41.1%) patients were women; 81 (11.7%) patients were associated with low levels of pretreatment MCV and 613 (88.3%) patients were associated with normal levels of pretreatment MCV; 122 (17.6%) patients had habitual alcohol intake and 572 (82.4%) patients not; the median age at diagnosis was 60 years; 313 (45.1%) patients were diagnosed with colon cancer and 381 (54.9%) patients were diagnosed with rectal cancer; 331 (47.7%) patients were with stage II disease and 363 (52.3%) patients were with stage III disease. The baseline characteristics according to the pretreatment MCV levels were shown in **Table 1**. Low MCV was significantly associated with low tumor grade, female and colon cancer ($P < 0.05$).

Low MCV Was Associated With Worse Overall Survival in CRC

Figure S2 showed the result of Kaplan-Meier OS analysis according to the pretreatment MCV levels. Compared with normal MCV, low MCV was significantly associated with reduced 5-year OS rate (87.3% *vs*. 76.4%, P < 0.0077). In addition, we also conducted univariate and multivariate Cox regression analyses to evaluate the prognostic value of clinicopathologic factors including MCV status (**Table 2**). In univariate analysis, low MCV was associated with 94.7% increased risk of overall mortality compared with normal MCV (HR = 1.947, 95%CI =1.181-3.211, P =0.009, using normal MCV as the reference; **Table 2**). The univariate analysis produced nine prognostic characteristics including MCV status, habitual alcohol intake, tumor grade, vascular invasion, nerve invasion, pretreatment CEA levels, age at diagnosis, tumor stage and the receipt of adjuvant treatment, which were included into multivariate analyses. It was shown that pretreatment MCV was also an independent prognostic factor and low pretreatment MCV was independently associated with 72.0% increased risk of overall mortality compared with normal level of MCV (HR = 1.720, 95%CI =1.028-2.876, P =0.039, using normal level of MCV as the reference; **Table 2**). In addition, it was also found that patients with habitual alcohol were independently associated with 75.4% increased risk of overall mortality compared with patients not (HR = 1.754, 95%CI =1.093-2.816, P =0.020, using without habitual alcohol intake as the reference; **Table 2**).

After adjusting for other prognostic factors, we also used restricted cubic splines to show the preoperative MCV levels and the corresponding HRs of OS and recurrence-free survival (RFS) on a continuous scale (**Figures 1A, B**). Similarly, it was clear that low level of MCV negatively correlated with the mortality and tumor recurrence after adjusting for other prognostic factors.

Prognostic Role of Pretreatment MCV With the Complications of Habitual Alcohol Intake

The results of Kaplan-Meier OS analysis according to pretreatment MCV levels with the implications of habitual

TABLE 1 | Baseline characteristics of the of the overall cohort by the levels of pretreatment MCV.

Characteristics	No. of Patients (%)		P
	Low MCV (fl) (n=81)	Normal MCV (fl) (n=613)	
Tumor grade			0.004
High/Moderate	48 (59.3)	456 (74.4)	
Low	33 (40.7)	157 (25.6)	
Habitual alcohol intake			0.700
No	68 (84.0)	504 (82.2)	
Yes	13 (16.0)	109 (17.8)	
Vascular invasion			0.549
No	63 (77.8)	458 (74.7)	
Yes	18 (22.2)	155 (25.3)	
Nerve invasion			0.802
No	65 (80.2)	499 (81.4)	
Yes	16 (19.8)	114 (18.6)	
Pretreatment CEA levels			0.066
Normal	39 (48.1)	361 (58.9)	
High	42 (51.9)	252 (41.1)	
Gender			0.036
Male	39 (48.1)	370 (60.4)	
Female	42 (51.9)	243 (39.6)	
Age at diagnosis (years)			0.920
<65	52 (64.2)	397 (64.8)	
≥65	29 (35.8)	216 (35.2)	
Tumor location			<0.001
Rectum	14 (17.3)	299 (48.8)	
Colon	67 (82.7)	314 (51.2)	
Postoperative complications			0.523
No	76 (93.8)	585 (95.4)	
Yes	5 (6.2)	28 (4.6)	
Stage			0.204
II	44 (54.3)	287 (46.8)	
III	37 (45.7)	326 (53.2)	
Adjuvant treatment			0.184
No	22 (27.2)	127 (20.7)	
Yes	59 (72.8)	486 (79.3)	
No. of lymph nodes retrieved			0.153
<12	5 (6.2)	70 (11.4)	
≥12	76 (93.8)	543 (88.6)	

TABLE 2 | Univariate and multivariate Cox regression analyses for OS in the whole cohort.

Characteristics	Univariate analyses		Multivariate analyses	
	HR (95%CI)	P value	HR (95%CI)	P value
Pretreatment MCV (fl)		0.009		0.039
80-100	1		1	
<80	1.947 (1.181-3.211)		1.720 (1.028-2.876)	
Habitual alcohol intake		0.104		0.020
No	1		1	
Yes	1.472 (0.923-2.347)		1.754 (1.093-2.816)	
Tumor grade		<0.001		0.002
High/Moderate	1		1	
Low	2.372 (1.594-3.529)		1.940 (1.270-2.963)	
Vascular invasion		0.002		0.750
No	1		1	
Yes	1.895 (1.261-2.847)		0.928 (0.586-1.470)	
Nerve invasion		<0.001		0.001
No	1		1	
Yes	2.536 (1.675-3.840)		2.084 (1.351-3.216)	
Pretreatment CEA levels		0.001		0.005
Normal	1		1	
High	2.032 (1.362-3.032)		1.772 (1.186-2.647)	
Gender		0.603		
Male	1			
Female	1.111 (0.747-1.653)			
Age at diagnosis (years)		0.002		0.003
<65	1		1	
≥65	1.893 (1.276-2.807)		1.890 (1.237-2.887)	
Tumor location		0.433		
Rectum	1			
Colon	1.174 (0.787-1.751)			
Postoperative complications		0.221		
No	1			
Yes	1.617 (0.749-3.488)			
Stage		<0.001		<0.001
II	1		1	
III	3.234 (2.028-5.157)		3.619 (2.132-6.145)	
Adjuvant treatment		0.112		0.008
No	1		1	
Yes	0.698 (0.449-1.087)		0.511 (0.311-0.839)	
No. of lymph nodes retrieved		0.629		
<12	1			
≥12	0.862 (0.471-1.576)			

alcohol intake were shown in **Figure 2**. In patients without habitual alcohol intake, compared with normal level of MCV, low level of MCV was significantly associated with worse reduced 5-year OS rate (88.5% VS. 73.3%, P < 0.001); in patients with habitual alcohol intake, however, low level of MCV (92.3%) had better 5-year OS rate compared with normal level of MCV (81.6%), while the survival difference did not achieve statistical significance (P = 0.342). Using restricted cubic splines, we then showed preoperative MCV levels and the corresponding HRs of OS and RFS on a continuous scale with the complications of habitual alcohol intake (**Figures 3A–D**). In patients without habitual alcohol intake, pretreatment MCV still negatively correlated with the mortality (**Figure 3A**, P < 0.001) and tumor recurrence (**Figure 3B**, P < 0.001) after adjusting for other prognostic factors; in patients with habitual alcohol intake, however, pretreatment MCV positively correlated with the mortality (**Figure 3C**, P = 0.02) and tumor recurrence (**Figure 3D**, P = 0.002) after adjusting for other prognostic factors, showing the opposite prognostic role of pretreatment MCV compared with patients without habitual alcohol intake.

The results of multivariate Cox regression analyses of OS in CRC patients without habitual alcohol intake also showed that pretreatment MCV was an independent prognostic factor and low pretreatment MCV was independently associated with 133.0% increased risk of overall mortality compared with normal level of MCV (HR = 2.330, 95%CI =1.350-4.020, P = 0.002, using normal level of MCV as the reference; **Table 3**), meaning that the poor prognostic role was even more pronounced in CRC patients without habitual alcohol intake than in the whole cohort.

DISCUSSION

In the present study, 81 (11.7%) patients were associated with low levels of MCV, and 613 (88.3%) patients were associated with

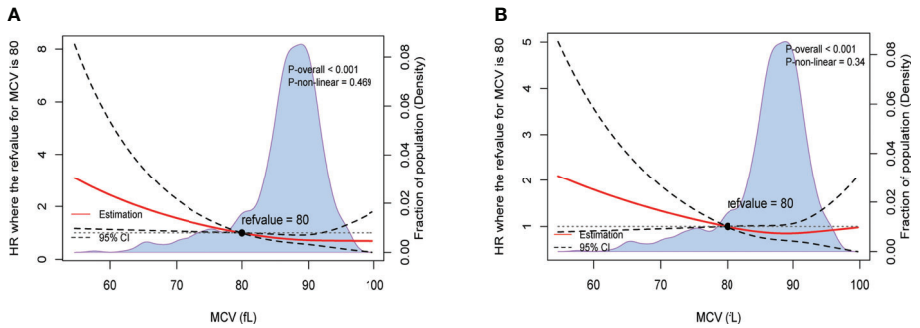

FIGURE 1 | Preoperative MCV and the corresponding hazard ratios on a continuous scale, including **(A)**. OS after adjusting for other prognostic factors; **(B)**. RFS after adjusting for other prognostic factors. Analyses were carried out using restricted cubic splines, with hazard ratios and 95% confidence intervals from multivariate Cox proportional hazards regression. The pretreatment MCV of 80fL was chosen as the reference. The purple area indicated the distribution of concentration of the pretreatment MCV.

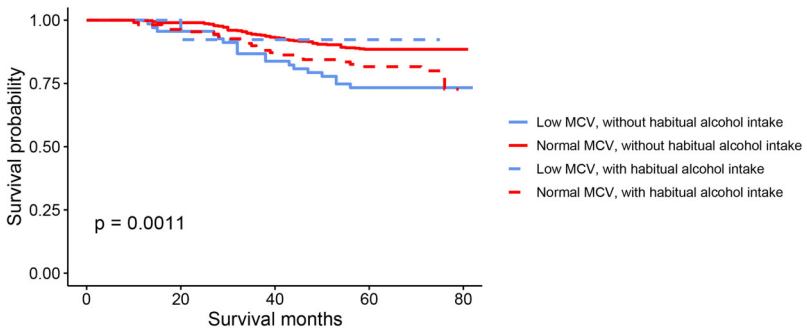

FIGURE 2 | Kaplan–Meier OS curves according to the levels of pretreatment MCV with the implications of habitual alcohol intake.

normal levels of MCV. It was found that low (<80 fL) level of pretreatment MCV was a poor prognostic feature in CRC, both in univariate and multivariate analyses. And low pretreatment MCV was independently associated with 72.0% increased risk of overall mortality compared with normal level of MCV; in CRC patients without habitual alcohol intake, furtherly, results of multivariate Cox analyses showed that this number increased to 133.0% compared with CRC patients with normal level of MCV, meaning that the poor prognostic role of low pretreatment MCV was even more pronounced than in the whole cohort. In patients with habitual alcohol intake, however, pretreatment MCV showed the opposite prognostic role and pretreatment MCV positively correlated with the mortality and tumor recurrence after adjusting for other prognostic factors.

Previously, there were several studies focusing on the clinical role of MCV in CRC, showing MCV was associated with the risk of colorectal adenoma (10), advanced CRC (11) and response to chemotherapy in CRC (12, 13) with even conflicting results and only one study was available investigating the prognostic role of pretreatment MCV in CRC patients (16). In this study, Hidemasa and his colleagues carried out a retrospective analysis in 1174 patients with stage I, II, and III CRC, and it was found that MCV of <80 fL was a favorable prognostic factor in CRC. The opposite prognostic role in this study might result from the different patient populations included into the two studies, that early stage CRC were excluded from our analyses and the proportion of patients with habitual alcohol intake in our study might be different from it, with the finding that pretreatment MCV positively correlated with the mortality and tumor recurrence in patients with habitual alcohol intake.

Shown as **Figure 4**, reasonable mechanisms behind our findings were summarized. The decrease of pretreatment MCV might result from a lack of globin product (thalassemia), restricted iron delivery to the heme group of hemoglobin (anemia of inflammation) and a lack of iron delivery to the heme group (iron-deficiency anemia) (17). In 2015, Chung et al. (18) conducted a nationwide study of 2655 patients diagnosed with thalassemia between 1998 and 2010 by using data from the Taiwan Longitudinal Health Insurance Database with comparison to 10620 people without thalassemia from the general population and found that patients with thalassemia exhibited a 1.54-fold greater overall risk of cancer than the general population, meaning that lack of globin product would increase the risk of multiple primary cancers in addition to CRC.

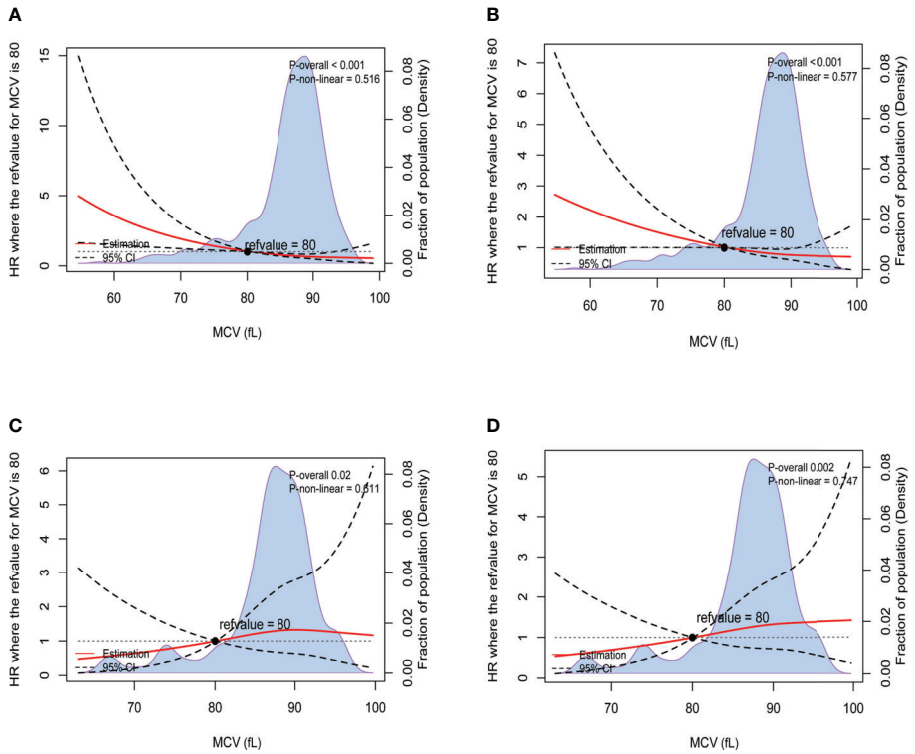

FIGURE 3 | Preoperative MCV and the corresponding hazard ratios on a continuous scale with the implications of habitual alcohol intake, including **(A)**. OS after adjusting for other prognostic factors, without habitual alcohol intake; **(B)**. RFS after adjusting for other prognostic factors, without habitual alcohol intake; **(C)**. OS after adjusting for other prognostic factors, with habitual alcohol intake; **(D)**. RFS after adjusting for other prognostic factors, with habitual alcohol intake. Analyses were conducted using restricted cubic splines, with hazard ratios and 95% confidence intervals from multivariate Cox proportional hazards regression. The pretreatment MCV of 80fL was selected as the reference. The purple area indicated the distribution of concentration of the pretreatment MCV.

Hypoxia, caused by iron deficiency anemia, would activate multicellular signaling pathways for cell survival, tumor progression, angiogenesis and metastasis. For example, hypoxia-hypoxiainducible-miR-210 would promote cell proliferation, vascular Endothelial Growth Factor (VEGF) expression and cell survival in hypoxic regions of tumors (19, 20). Moreover, immune system could also be affected by iron deficiency anemia, which decreased the proliferation and cytotoxic as well as phagocytic activities of the immune cells against tumor cells through downregulation of different immunological pathways, making patients with iron deficiency anemia more susceptible to development of cancer (21).

Inflammatory states were often associated microcytic anemia (17). As a response that an organism used to resolve infection, tissue injury or other cellular stress, and to restore tissue function through repair mechanism, inflammation also played an important role in cancers (22). Tumor associated inflammation was a source of survival, growth and pro-angiogenic factors, as well as extracellular matrix (ECM)-modifying enzymes that facilitate angiogenesis, invasion and metastasis of tumor cells (23, 24). Inflammation induced angiogenesis not only provided the necessary nutrients for tumor growth, but also provided a 'highway' for the tumor to escape from the primary tumor site to promote the distal metastasis of tumor cells. Inflammation could also suppress the anti-tumor immune responses, resulting into the escape of tumor cells from host immune surveillance, which was critical for almost all steps of metastatic tumor progression (25, 26).

In addition, it was found that higher MCV was associated with an elevated oxygen pressure (27) and an increased oxygen affinity in red blood cells (28). Then higher MCV could result in enhanced oxygen saturation in red blood cells. Therefore, higher MCV may facilitate oxygen delivery. Compared with normal MCV, decreased oxygen delivery in CRC with low MCV would result in decreased physical functions and hypoxia which played a main role in tumor progression and treatment resistance, then leading to worse oncologic outcomes (29, 30).

Alcoholism was a devastating disease which occurred in approximately 8% of the general population, and approximately 20% of hospitalized patients (31–35). Many previous researches supported a positive association between alcohol consumption and CRC risk (36–38). Our study also demonstrated that CRC patients with habitual alcohol intake was independently associated with 75.4% increased risk of overall mortality.

MCV had been reported to be increased in chronic alcoholism (39–42). We therefore investigated the prognostic

TABLE 3 | Multivariate Cox regression analyses for OS in patients without habitual alcohol intake.

Characteristics	Multivariate analyses	
	HR (95%CI)	P value
Pretreatment MCV (fl)		0.002
80-100	1	
<80	2.330 (1.350-4.020)	
Tumor grade		0.001
High/Moderate	1	
Low	2.321 (1.441-3.739)	
Vascular invasion		0.574
No	1	
Yes	0.860 (0.507-1.458)	
Nerve invasion		0.017
No	1	
Yes	1.842 (1.118-3.035)	
Pretreatment CEA levels		0.006
Normal	1	
High	1.935 (1.214-3.083)	
Age at diagnosis (years)		0.003
<65	1	
≥65	2.062 (1.273-3.342)	
Stage		<0.001
II	1	
III	3.627 (1.992-6.603)	
Adjuvant treatment		0.054
No	1	
Yes	0.568 (0.319-1.010)	

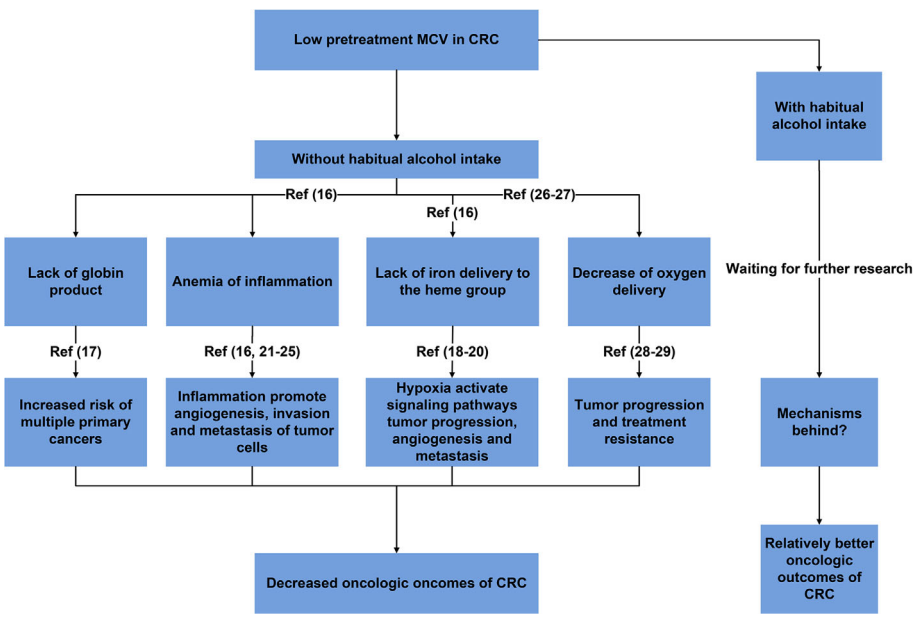

FIGURE 4 | Summary of reasonable mechanisms behind the prognostic role of MCV with the implications of habitual alcohol intake.

role of pretreatment MCV in CRC patients with implications of habitual alcohol intake. To the best of our knowledge, it was the first study that showed pretreatment MCV positively correlated with the mortality and tumor recurrence in patients with habitual alcohol intake, indicating that researches focused on the clinical value of pretreatment MCV should take alcohol consumption status into consideration and earlier studies were thoughtless. However, mechanisms behind the opposite prognostic role of pretreatment MCV in CRC patients with habitual alcohol intake was still uncertain, and we believed

our findings would elicit many fundamental studies to elucidate them.

There were a few limitations in the present study. First, this study was only a single-institution one, and the sample size was required to be enlarged. Second, some factors (including hypothyroidism, blood disease, liver disease and so on) which might affect the levels of pretreatment MCV were not taken into account in our analyses. Finally, the present study was only a retrospective one, and more evidence need to be provided by randomized controlled clinical trials to support our findings in the future.

CONCLUSIONS

In CRC patients without habitual alcohol intake, low (<80 fL) level of pretreatment MCV was a predictor of poor prognosis. In patients with habitual alcohol intake, however, pretreatment MCV showed the opposite prognostic role and pretreatment MCV positively correlated with the mortality and tumor recurrence. We believed our findings would elicit many fundamental studies to elucidate the mechanisms behind.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

XL and SZ conceptualized and designed the study. QL conducted the analyses of the study. QL and YY interpreted the data. QL drafted the manuscript. QL, XL, and SZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.681406/full#supplementary-material

Supplementary Figure 1 | Flow chart of patient inclusion.

Supplementary Figure 2 | Kaplan–Meier OS curves according to the levels of pretreatment MCV.

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Clinical Implications of Nonbiological Factors With Colorectal Cancer Patients Younger Than 45 Years

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Background: To evaluate the clinical implications of non-biological factors (NBFs) with colorectal cancer (CRC) patients younger than 45 years.

Methods: In the present study, we have conducted Cox proportional hazard regression analyses to evaluate the prognosis of different prognostic factors, the hazard ratios (HRs) were shown with 95% confidence intervals (CIs). Kaplan–Meier method was utilized to compare the prognostic value of different factors with the log-rank test. NBF score was established according to the result of multivariate Cox analyses.

Results: In total, 15129 patients before 45 years with known NBFs were identified from the SEER database. Only county-level median household income, marital status and insurance status were NBFs that significantly corelated with the cause specifical survival in CRC patients aged less than 45 years old ($P < 0.05$). Stage NBF 1 showed 50.5% increased risk of CRC-specific mortality (HR = 1.505, 95% CI = 1.411-1.606, $P < 0.001$). Stage NBF 0 patients were associated with significantly increased CRC-specific survival (CCSS) when compared with the stage NBF 1 patients in different AJCC TNM stages.

Conclusions: NBF stage (defined by county-level median household income, marital status and insurance status) was strongly related to the prognosis of CRC patients. NBFs should arouse enough attention of us in clinical practice of patients younger than 45 years.

Keywords: non-biological factors, colorectal cancer, young, screening, prognosis

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors. The vast majority of patients with CRCs are > 50 years of age. 75% of CRC patients present with rectal cancer and 80% with colon cancer at an age higher than 60 years at the time of diagnosis (1). However, the incidence rate of CRC is increasing in young persons, the American Cancer Society (ACS) therefore recommends average-risk CRC screening at 45 years old (2).

CRC incidence rates have risen by 1.3% and 2.3% per year in patients at the age of 40–49 years in the United States over the last two decades, respectively. On the contrast, incidence rates of patients over the age of 55 years have decreased by 2- to 3-fold, which is largely attributed to the screening of this disease (3).

Recently, the ACS recommended average-risk CRC screening in adults aged ≥ 45 years with stool-based test or a visual examination (4). It is worth noting that CRC screening before the age of 45 is still somewhat neglected, which may cause the increasing percentage of CRC patients aged less than 45 years.

The oncological outcomes of cancer patients would be affected by biological factors and non-biological factors (NBFs). The prognostic effects of different biological factors on CRC patients have been widely studied, including patient age, race, histological type, lymph node invasion, tumor grade, tumor size, gender and so on. The associations of NBFs with tumors, such as CRC, breast cancer and testicular cancer, have been reported (5–11). However, their prognostic significance was neglected to some extent (12–15).

Moreover, the widely utilized AJCC staging system is only based on the biological factors, and it is sometimes unable to accurately predict the prognosis of CRC patients. We therefore conducted this study to evaluate the implications of NBFs with staging, prognosis and clinical management of CRC patients younger than 45 years.

METHODS

Patients

The SEER-Stat software (SEER*Stat 8.3.8, https://seer.cancer.gov/seerstat/) was used in the present study, patients meeting the strict criteria were identified from the Surveillance, Epidemiology and End Results (SEER) database, which is a comprehensive source of population-based information on clinicopathological features and survival of cancer patients in the USA. Initially, CRC patients aged less than 45 years old were selected from SEER 18 registries between January 1, 2007 and December 31, 2015. Subsequently, only CRC patients with known NBFs were included in the present study according to the following criteria: ①. Marital status (married or unmarried), ②. insurance status (insured, medicaid or uninsured), ③. median household income, ④. county % with bachelor degree (N=17189), ⑤unemployment status (N=17189), ⑥. year of diagnosis (N=17189). In addition, patients with incomplete surgery history data, non-adenocarcinomatous histologies, non-specified AJCC stage and not specified or AJCC stage = 0 were excluded from our analyses (**Figure 1**). The primary endpoint of this study was CRC-specific survival (CCSS). The death of CRC patients was categorized as CRC-specific or non-CRC-related. CCSS of CRC-specific death was calculated from the date of diagnosis to the date of CRC death, whereas non-CRC related deaths were censored at the date of death.

NBF Score, NBF Stage, and Statistical Analysis

Initially, univariate Cox analysis was conducted to identify all the independent prognostic variables. Subsequently, the prognostic factors with P value < 0.2 in the univariate analysis were entered

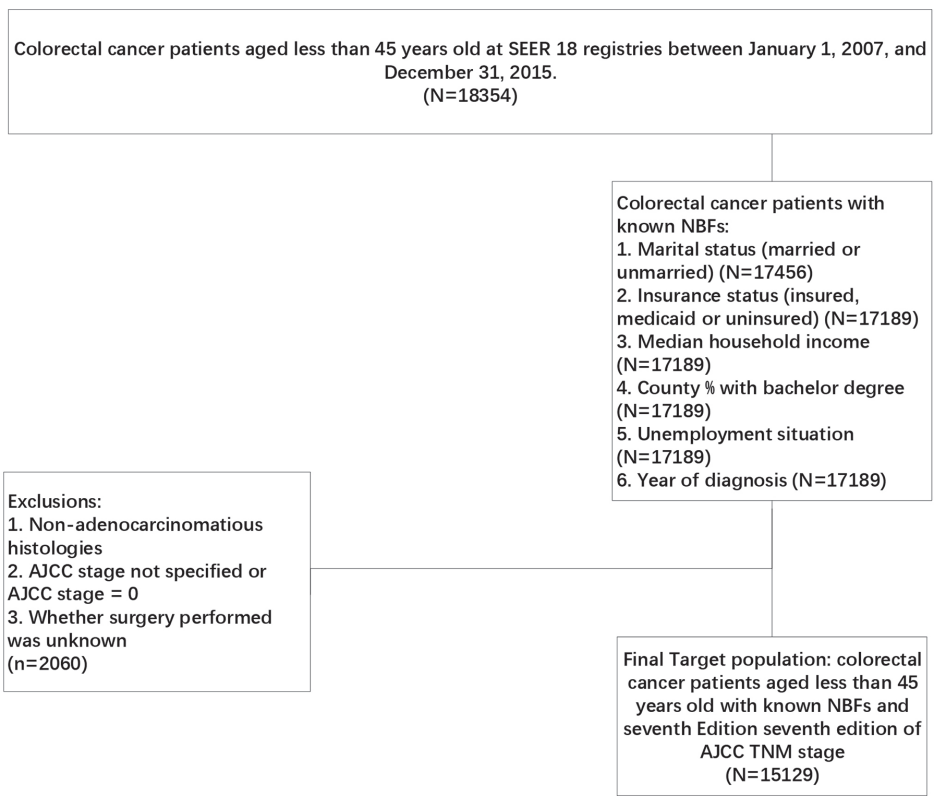

FIGURE 1 | Flow diagram of patient selection.

into the multivariate Cox analyses, including gender, tumor grade, AJCC stage, surgery status, histology, the receipt of chemotherapy and all the NBFs (insurance status, county-level median household income, county % were unemployed, year of diagnosis, county % with bachelor degree and marital status), which indicated that only the variables county-level median household income, marital status and insurance status were significantly associated with the cause specific survival in patients before 45 years.

The NBF score was determined according to the results of the multivariate Cox analysis. As shown in **Figure 2**, we considered the point of each group of each NBF equivalent to the value of the hazard ratios which were generated in multivariate Cox analysis. Subsequently, we assigned each patient a NBF score that was the total of the hazard ratio points in the three NBFs. For instance, a married and insured patient whose county-level median household income was 42.20–51.48 K (dollars) had a calculated score of the sum of "1.000", "1.000", and "1.164" which was equivalent to "3.164". The NBF stage of each patient was subsequently stratified according to the NBF score. It was shown that the total score ranged from 3.000–3.864, which was divided into two groups with the median NBF score of all the CRC patients aged less than 45 years old as the cut-off value (3.227). Patients with lower NBF score were assigned to stage NBF 0 and others with higher NBF score were assigned to NBF 1 (14). The distribution and associations of county-level median household income, marital status and insurance status are presented in **Figure 3**.

In the present study, Cox proportional hazard regression models were constructed to evaluate the prognosis of different prognostic factors. The hazard ratios (HRs) were shown with 95% confidence intervals (CIs). Kaplan–Meier method was utilized to compare the prognostic value of different factors with the log-rank test. Only P-values lower than 0.05 were considered to reach statistical significance. Statistical analyses in the present were performed with the Statistical Package for Social Science (SPSS version 23; IBM Corp, Armonk, NY, USA).

RESULTS

In total, 15,129 patients were identified from the SEER database before 45 years with known NBFs. The median follow-up time was 41 (range, 0–119) months. A total of 3,730 (24.7%) patients succumbed to CRC at the end of the follow-up time. The baseline characteristics of the total cohort were summarized, as shown in **Table 1**.

NBFs Are Significant Prognostic Factors of Patients Before 45 years

As shown in **Table 2**, univariate Cox analyses resulted in the identification of the patient characteristics with P values less than 0.20. These data were introduced in multivariate Cox analyses. Only county-level median household income, marital status and insurance status were NBFs that were significantly associated with cause-specific survival in CRC patients aged less than 45 years old ($P < 0.05$). In addition, gender, tumor grade, AJCC stage, surgical status, histology and the receipt of chemotherapy were also found to be independent prognostic factors in CRC patients aged less than 45 years old. The variables including lower county-level median household income, Medicaid, uninsured and unmarried were found to be associated with higher risk of CRC-specific mortality ($P < 0.01$).

The NBF Stage Was Strongly Associated With the Prognosis of Patients Before 45 Years

A total of 8,830 (58.4%) patients were assigned to stage NBF 0 and 6,299 (41.6%) patients were assigned to stage NBF 1. Both univariate and multivariate Cox analyses indicated that NBF

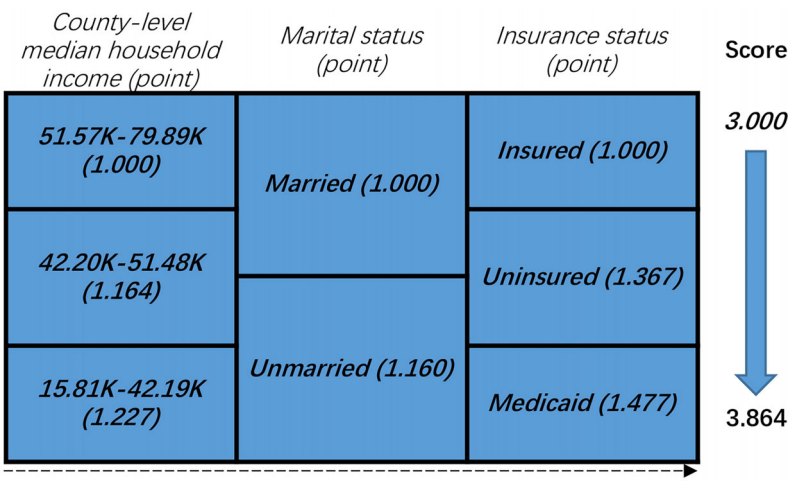

FIGURE 2 | Non-biological factor (NBF) score in colorectal cancer patients younger than 45 years: risk-stratifications.

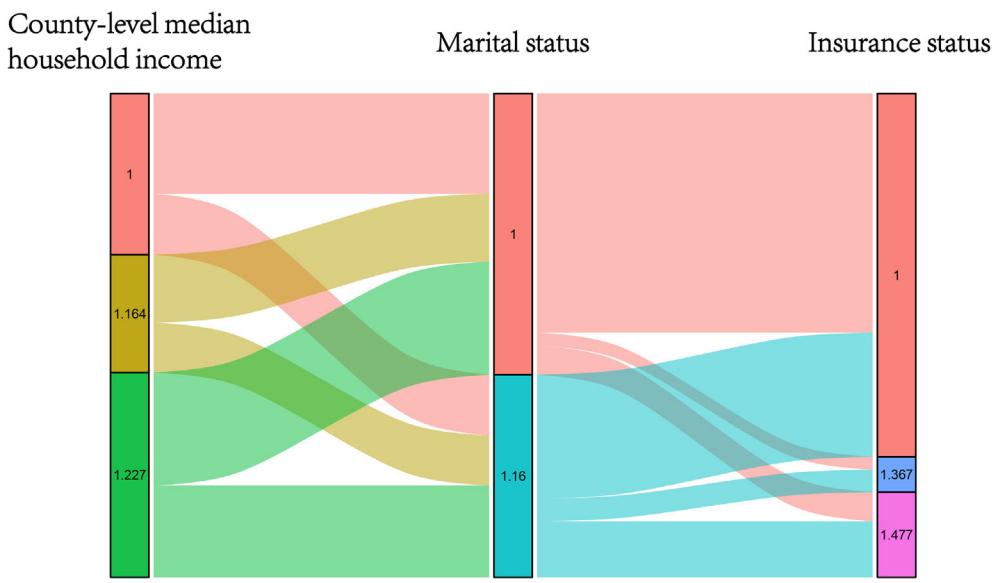

FIGURE 3 | Graphical summary of the distribution and associations of different score subgroups in county-level median household income, insurance status and marital status, respectively.

stage was a strong prognostic factor in CRC patients aged less than 45 years old, whereas stage NBF 1 was independently associated with 50.5% increased risk of CRC specific mortality (HR = 1.505, 95% CI = 1.411-1.606, P < 0.001; **Table 3**).

Prognostic Significance of NBF Stage Following the Combination With TNM Stage

After the combination with NBF stage, each AJCC TNM stage was assigned to stage NBF 0 or stage NBF 1, including I NBF0, I NBF1, IIA NBF0, IIA NBF1, IIB NBF0, IIB NBF1, IIC NBF0, IIC NBF1, IIIA NBF0, IIIA NBF1, IIIB NBF0, IIIB NBF1, IIIC NBF0, IIIC NBF1, IVA NBF0, IVA NBF1, IVB NBF0 and IVB NBF1.

Kaplan-Meier survival analyses indicated that all stage NBF 0 patients were associated with a statistically significant increased CCSS compared to stage NBF 1 patients in different AJCC TNM stages (**Figures 4A–C**). Moreover, these results were also validated in multivariate Cox analyses as follows: All the stage NBF 0 patients indicated lower HRs compared with the respective stage NBF 1 patients, which was in agreement with the results of the Kaplan-Meier survival analyses.

It should also be noted that several stage NBF 1-TNM patients exceeded stage NBF 0 with higher conventional AJCC TNM stage. For example, the risk of CRC-specific mortality of stage IIB NBF 1 (HR = 4.264, 95%CI = 2.843-6.396, using stage NBF 1 as the reference, P < 0.001) was significantly higher than that of stage IIC NBF 0 (HR = 2.988, 95%CI = 1.611-5.541, using stage NBF 1 as the reference, P = 0.001). The risk of CRC-specific mortality of stage IIC NBF 1 (HR = 5.095, 95%CI = 3.034-8.556, using stage NBF 1 as the reference, P < 0.001) was significantly higher than that of stage IIIA NBF 0 (HR = 0.967, 95%CI = 0.580-1.612, using stage NBF 1 as the reference, P = 0.898) and the risk of CRC-specific mortality of stage IIIA NBF 1 (HR = 2.556, 95%CI = 1.586-4.121, using stage NBF 1 as the reference, P < 0.001) was significantly higher than that of stage IIIB NBF 0 (HR = 2.191, 95%CI = 1.584-3.030, using stage NBF 1 as the reference, P < 0.001). Finally, the risk of CRC-specific mortality of stage IIIB NBF 1 (HR = 4.327, 95%CI = 3.146-5.952, using stage NBF 1 as the reference, P < 0.001) was significantly higher than that of stage IIIC NBF 0 (HR = 4.119, 95%CI = 3.001-5.651, using stage NBF 1 as the reference, P < 0.001), which indicated that stage NBF 1 could increase the diagnostic value of conventional TNM stage (**Table 4**). In other words, the NBF stage could significantly affect the prognosis of patients younger than 45 years.

DISCUSSION

The present study demonstrated that NBF stage was strongly related to the prognosis of patients before 45 years, whereas stage NBF 1 was independently associated with 50.5% increased risk of CRC specific mortality. Following combination with the TNM stage, the results demonstrated that NBF 0 patients were associated with a statistically significant increased CCSS compared to the stage NBF 1 patients in all the respective AJCC TNM stages. It should also be noted that several stages of NBF 1-TNM patients exceeded stage NBF 0 with higher conventional AJCC TNM stage. For example, the risk of CRC-specific mortality of stage IIB NBF 1 patients was significantly higher than that of stage IIC NBF 0 subjects, whereas the risk of CRC-specific mortality of stage IIC NBF 1 patients was

significantly higher than that of stage IIIA NBF 0. The risk of CRC-specific mortality of stage IIIA NBF 1 subjects was significantly higher than that of stage IIIB NBF 0 subjects, whereas the risk of CRC-specific mortality of stage IIIB NBF 1 patients was significantly higher than that of stage IIIC NBF 0 patients, indicating that stage NBF 1 could increase the diagnostic value of the conventional TNM stage. In other words, the NBF stage could significantly affect the prognosis of patients younger than 45 years. Therefore, the present findings indicated that the combination of the NBF stage could increase the prognostic value of the TNM stage system. 45 years.

Incidence rates of patients over the age of 55 years have shown a decline during the last several decades. This trend was accelerated in 2000, and this phenomenon would be even more pronounced in adults aged 65 years or older (16). In contrast to these subjects, CRC incidence rates have increased by 1.3% and 2.3% per year in patients at the age of 40–49 years in the United States over the last two decades (3). The vast majority of CRCs occurred following the age of 50. Therefore, the ACS recommended average-risk CRC screening in adults aged ≥ 45 years with stool-based test or a visual examination. These findings indicate that CRC patients under the age of 45 are still somewhat ignored (4).

NBFs have been demonstrated to contribute to tumor development by previous studies. NBFs may act directly or indirectly to facilitate the consequences of different biological changes, thus affecting the prognostic effect of the biological factors in cancer patients (12).

The present study indicated that three NBFs were significantly associated with the oncological outcomes of CRC prior to 45 years, including county-level median household income, marital status and insurance status. The lower the income, the worse the prognosis of CRC (the income of 51.57K-79.89K was used as reference and the income of 15.81k-42.19K increased the risk of death by 22.7% compared with the income of 51.57K-79.89K). The prognostic effect of income on survival in the present study was in agreement with a previous study in ovarian cancer (17). This may be attributed to the fact that low-income patients were less likely to prefer active treatment owing to the fragile financial support network in CRC treatment.

As shown in our previous analyses, in the United States, Medicaid increased the risk of CRC-specific mortality death by 47.7% compared with that noted in insured patients. We held the view that late initiating treatment, inadequate treatment and poor physical conditions might contribute to the poor prognosis of young CRC patients with Medicaid. Previous studies have reported the prognostic effect of insurance status in many cancers, and Medicaid or uninsured patients would have worse survival compared with insured ones (18–21).

It has been reported in several previous studies that marital status had a prognostic effect on survival of several cancer types including rectal cancers (11, 15, 22–25). The improved prognosis noted in married CRC patients can be attributed to the improved endocrine, cardiovascular and immune function as well as treatment compliance in married patients (26).

TABLE 1 | Baseline characteristics of colon cancer patients included in our study.

Characteristic	No. (%)
Gender	
Male	7947 (52.5)
Female	7182 (47.5)
Tumor grade	
Grade I	1001 (6.6)
Grade II	9915 (65.5)
Grade III	2692 (17.8)
Grade IV	425 (2.8)
Unknown	1096 (7.2)
AJCC stage	
I	2471 (16.3)
IIA	2854 (18.9)
IIB	400 (2.6)
IIC	208 (1.4)
IIIA	614 (4.1)
IIIB	2916 (19.3)
IIIC	2181 (14.4)
IVA	2313 (15.3)
IVB	1172 (7.7)
Surgery	
Surgery not performed	1125 (7.4)
Surgery performed	14004 (92.6)
Histology	
Adenocarcinoma	13483 (89.1)
Mucinous adenocarcinoma	1306 (8.6)
Signet-ring cell carcinoma	340 (2.2)
Chemotherapy	
No/unknown	4865 (32.2)
Yes	10264 (67.8)
County % with bachelor degree	
6.83%-26.58%	5068 (33.5)
26.62%-35.68%	5039 (33.3)
35.83%-54.45%	5022 (33.2)
County-level median household income[#]	
15.81K-42.19K	6406 (42.3)
42.20K-51.48K	3685 (24.4)
51.57K-79.89K	5038 (33.3)
County % were unemployed	
1.29%-5.97%	5075 (33.5)
5.98%-7.80%	6201 (41.0)
7.84%-17.16%	3853 (25.5)
Year of diagnosis	
2007	1624 (10.7)
2008	1656 (10.9)
2009	1707 (11.3)
2010	1665 (11.0)
2011	1651 (10.9)
2012	1607 (10.6)
2013	1629 (10.8)
2014	1858 (12.3)
2015	1732 (11.4)
Insurance status	
Insured	11356 (75.1)
Medicaid	2664 (17.6)
Uninsured	1109 (7.3)
Marital status	
Married	8972 (58.1)
Unmarried	6337 (41.9)

[#]*Shown in US dollars.*

Previous studies have proposed the inadequate prognostication of the present AJCC TNM staging system in CRC (27–29). Therefore, in the present study, the implications of

TABLE 2 | Univariate and multivariable Cox regression analyses of all independent prognostic factors in patients before the recommended initiating colorectal cancer screening age.

Groups	Variable	Univariate analyses		Multivariate analyses	
		HR (95%CI)	*P*	HR (95%CI)	*P*
Gender			<0.001		<0.001
	Male	Reference		Reference	
	Female	0.864 (0.810-0.922)		0.845 (0.792-0.902)	
Tumor grade			<0.001		<0.001
	Grade I	Reference		Reference	
	Grade II	1.344 (1.141-1.582)		1.306 (1.106-1.542)	
	Grade III	3.133 (2.647-3.708)		2.255 (1.898-2.680)	
	Grade IV	3.222 (2.579-4.026)		2.158 (1.720-2.709)	
	Unknown	2.608 (2.160-3.148)		1.550 (1.279-1.879)	
AJCC stage			<0.001		<0.001
	I	Reference		Reference	
	IIA	1.761 (1.389-2.232)		1.674 (1.317-2.128)	
	IIB	5.186 (3.876-6.938)		4.485 (3.334-6.034)	
	IIC	5.966 (4.048-8.794)		5.252 (3.543-7.784)	
	IIIA	1.939 (1.380-2.724)		2.076 (1.468-2.935)	
	IIIB	4.279 (3.455-5.300)		4.116 (3.286-5.155)	
	IIIC	8.031 (6.516-9.897)		7.098 (5.687-8.859)	
	IVA	26.618 (21.784-32.525)		22.369 (18.059-27.707)	
	IVB	41.338 (33.597-50.862)		29.702 (23.749-37.148)	
Surgery			<0.001		<0.001
	Surgery not performed	Reference		Reference	
	Surgery performed	0.167 (0.154-0.182)		0.385 (0.351-0.423)	
Histology			<0.001		<0.001
	Adenocarcinoma	Reference		Reference	
	Mucinous adenocarcinoma	1.371 (1.233-1.524)		1.101 (0.987-1.227)	
	Signet-ring cell carcinoma	4.183 (3.632-4.818)		1.819 (1.567-2.112)	
Chemotherapy			<0.001		0.015
	No/unknown	Reference		Reference	
	Yes	2.677 (2.453-2.921)		0.888 (0.807-0.978)	
County % with bachelor degree			<0.001		0.072
	6.83%-26.58%	Reference		Reference	
	26.62%-35.68%	0.968 (0.897-1.045)		1.010 (0.924-1.103)	
	35.83%-54.45%	0.798 (0.737-0.864)		0.909 (0.815-1.015)	
County-level median household income			<0.001		0.001
	51.57K-79.89K	Reference		Reference	
	42.20K-51.48K	1.267 (1.161-1.382)		1.164 (1.052-1.288)	
	15.81K-42.19K	1.367 (1.267-1.476)		1.227 (1.103-1.365)	
County % were unemployed			<0.001		0.912
	1.29%-5.97%	Reference		Reference	
	5.98%-7.80%	1.160 (1.074-1.251)		0.996 (0.914-1.085)	
	7.84%-17.16%	1.267 (1.165-1.378)		0.979 (0.881-1.087)	
Year of diagnosis			0.458		
	2007	Reference			
	2008	1.049 (0.929-1.184)			
	2009	1,082 (0.960-1.221)			
	2010	0.985 (0.868-1.116)			
	2011	1.006 (0.885-1.143)			
	2012	1.021 (0.894-1.165)			
	2013	1.099 (0.958-1.260)			
	2014	0.957 (0.824-1.111)			
	2015	0.921 (0.762-1.113)			
Insurance status			<0.001		<0.001
	Insured	Reference		Reference	
	Medicaid	1.988 (1.842-2.146)		1.477 (1.363-1.600)	
	Uninsured	1.670 (1.493-1.869)		1.367 (1.219-1.534)	
Marital status			<0.001		<0.001
	Married	Reference		Reference	
	Unmarried	1.440 (1.350-1.536)		1.160 (1.084-1.241)	

TABLE 3 | Univariate and multivariable Cox regression analyses of NBF stage and other prognostic factors.

Groups	Variable	Univariate analyses		Multivariate analyses	
		HR (95%CI)	*P*	HR (95%CI)	*P*
NBF-stage			<0.001		<0.001
	NBF-stage 0	Reference		Reference	
	NBF-stage 1	1.725 (1.618-1.840)		1.505 (1.411-1.606)	
Gender			<0.001		<0.001
	Male	Reference		Reference	
	Female	0.864 (0.810-0.922)		0.851 (0.798-0.908)	
Tumor grade			<0.001		<0.001
	Grade I	Reference		Reference	
	Grade II	1.344 (1.141-1.582)		1.320 (1.118-1.559)	
	Grade III	3.133 (2.647-3.708)		2.271 (1.912-2.698)	
	Grade IV	3.222 (2.579-4.026)		2.171 (1.730-2.724)	
	Unknown	2.608 (2.160-3.148)		1.570 (1.296-1.901)	
AJCC stage			<0.001		<0.001
	I	Reference		Reference	
	IIA	1.761 (1.389-2.232)		1.689 (1.329-2.148)	
	IIB	5.186 (3.876-6.938)		4.591 (3.413-6.175)	
	IIC	5.966 (4.048-8.794)		5.331 (3.597-7.900)	
	IIIA	1.939 (1.380-2.724)		2.101 (1.486-2.972)	
	IIIB	4.279 (3.455-5.300)		4.167 (3.327-5.219)	
	IIIC	8.031 (6.516-9.897)		7.168 (5.743-8.946)	
	IVA	26.618 (21.784-32.525)		22.745 (18.364-28.170)	
	IVB	41.338 (33.597-50.862)		30.075 (24.050-37.611)	
Surgery			<0.001		<0.001
	Surgery not performed	Reference		Reference	
	Surgery performed	0.167 (0.154-0.182)		0.383 (0.349-0.420)	
Histology			<0.001		<0.001
	Adenocarcinoma	Reference		Reference	
	Mucinous adenocarcinoma	1.371 (1.233-1.524)		1.109 (0.994-1.236)	
	Signet-ring cell carcinoma	4.183 (3.632-4.818)		1.886 (1.625-2.189)	
Chemotherapy			<0.001		0.011
	No/unknown	Reference		Reference	
	Yes	2.677 (2.453-2.921)		0.884 (0.803-0.973)	

NBFs with staging, prognosis and clinical management were assessed in CRC patients before 45 years.

The current study demonstrated that county-level median household income, marital status and insurance status were significantly associated with cause-specific survival in CRC patients younger than 45 years. NBFs were often neglected in clinical practice and patients with poor NBFs deserved more attention and a more intense treatment. The present study indicated that NBF stage was strongly related to the prognosis of patients. Following combination with the TNM stages, NBF 0 patients were associated with a statistically significant increased CCSS compared to the stage NBF 1 patients in all the respective AJCC TNM stages. Therefore, the present study findings indicated that the combination of the NBF stage could increase the prognostic value of the TNM stage.

The present study aimed to increase the information regarding CRC patients younger than 45 years, as well as analyze the NBFs that significantly affect the prognosis of CRC patients, including only county-level median household income, marital status and insurance status. NBFs should arouse sufficient attention of us in clinical practice of patients younger than 45 years. In such way, the research focus on these aspects will be enhanced by the scientific community.

Several limitations of this study should be addressed. Firstly, our analyses were only based on a US population. In the future, therefore, a validation study should be carried out. In the validation study, patients could be from countries beyond the United States. In addition, the validation study could investigate the prognostic value of NBFs in older CRC patients who were not included in the present study, which might lead to other interesting conclusions. And analyses could also be conducted based on different stratification factors such as gender, race and tumor stage. Above all, recruited patients should have complete information of NBFs including individual income, education, insurance, marital status and employment status. Secondly, some prognostic factors were not available in the SEER database and they were not included in our analyses, such as serum biomarkers, family history, microsatellite instability status, ras mutation and braf v600e status (30–32). Finally, the analyses were merely based on retrospective data, which would cause inevitable bias.

CONCLUSION

County-level median household income, marital status and insurance status were significantly associated with cause-specific

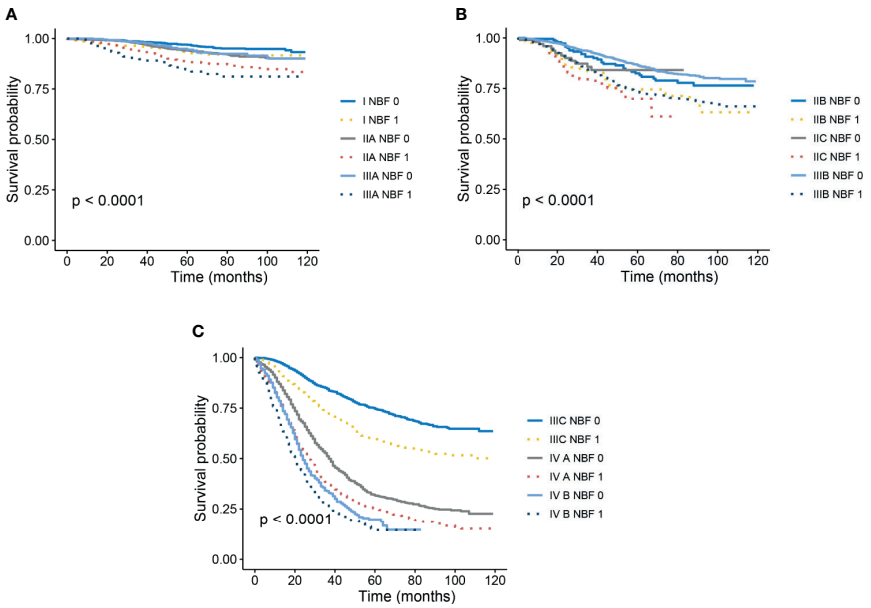

FIGURE 4 | Kaplan–Meier survival curves of NBF-TNM staging system. **(A)** Cancer-specific survival (CSS) of I-NBF 0 stage, I- NBF 1 stage, IIA-NBF 0 stage, IIA-NBF 1 stage, IIIA- NBF 0 stage, and IIIA- NBF 1 stage. **(B)** CSS of IIB- NBF 0 stage, IIB- NBF 1 stage, IIC- NBF 0 stage, IIC- NBF 1 stage, IIIB- NBF 0 stage, and IIIB- NBF 1 stage. **(C)** CSS of IIIC- NBF 0 stage, IIIC- NBF 1 stage, IVA- NBF 0 stage, IVA- NBF 1 stage, IVB- NBF 0 stage, and IVB- NBF 1 stage.

TABLE 4 | Prognosis of NBF-stage and TNM stage in patients before the recommended initiating colorectal cancer screening age.

AJCC TNM staging system					TNM-C staging system				
Stage	**Number of the patients**	**Cancer-specific survival**			**Stage**	**Number of the patients**	**Cancer-specific survival**		
		HR (95% CI)	**SE**	***P* value**			**HR (95% CI)**	**SE**	***P* value**
I	2471	Reference	\	\	**I NBF0**	1647	0.562 (0.381-0.829)	0.198	0.004
					I NBF1	824	Reference	\	\
IIA	2854	1.689 (1.329-2.148)	0.122	<0.001	**IIA NBF0**	1702	0.941 (0.663-1.335)	0.178	0.733
					IIA NBF1	1152	1.668 (1.181-2.357)	0.176	0.004
IIB	400	4.591 (3.413-6.175)	0.151	<0.001	**IIB NBF0**	202	2.691 (1.718-4.215)	0.229	<0.001
					IIB NBF1	198	4.264 (2.843-6.396)	0.207	<0.001
IIC	208	5.331 (3.597-7.900)	0.201	<0.001	**IIC NBF0**	101	2.988 (1.611-5.541)	0.315	0.001
					IIC NBF1	107	5.095 (3.034-8.556)	0.264	<0.001
IIIA	614	2.101 (1.486-2.972)	0.177	<0.001	**IIIA NBF0**	415	0.967 (0.580-1.612)	0.261	0.898
					IIIA NBF1	199	2.556 (1.586-4.121)	0.244	<0.001
IIIB	2916	4.167 (3.327-5.219)	0.115	<0.001	**IIIB NBF0**	1713	2.191 (1.584-3.030)	0.165	<0.001
					IIIB NBF1	1203	4.327 (3.146-5.952)	0.163	<0.001
IIIC	2181	7.168 (5.743-8.946)	0.113	<0.001	**IIIC NBF0**	1223	4.119 (3.001-5.651)	0.161	<0.001
					IIIC NBF1	958	6.829 (4.985-9.356)	0.161	<0.001
IVA	2313	22.745 (18.364-28.170)	0.109	<0.001	**IVA NBF0**	1241	14.300 (10.554-19.374)	0.155	<0.001
					IVA NBF1	1072	19.819 (14.615-26.878)	0.155	<0.001
IVB	1172	30.075 (24.050-37.611)	0.114	<0.001	**IVB NBF0**	586	20.162 (14.712-27.630)	0.161	<0.001
					IVB NBF1	586	24.939 (18.215-34.146)	0.160	<0.001

survival in CRC patients younger than 45 years. The present study showed that NBF stage was strongly related to the prognosis of patients. Following combination with the TNM stages, NBF 0 patients were associated with a statistically significant increased CCSS compared to the stage NBF 1 patients in all the respective AJCC TNM stages, indicating the combination of the NBF stage could increase the prognostic value of the TNM stage. Staging45 years NBFs should not be neglected in clinical practice and patients with poor NBFs deserved more attention and more intense treatment of CRC patients before 45 years.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://seer.cancer.gov/data/access.html.

ETHICS STATEMENT

The study was approved by the Ethical Committee and Institutional Review Board of the Fudan University Shanghai Cancer Center. The data did not include the use of human subjects or personal identifying information and no informed consent was required for this study.

AUTHOR CONTRIBUTIONS

QGL and XL conceptualized and designed the study. QL and RZ conducted the analyses of the study. QL interpreted the data. QL and RZ drafted the manuscript. QL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Magnetic Resonance Imaging Evaluation of the Accuracy of Various Lymph Node Staging Criteria in Rectal Cancer: A Systematic Review and Meta-Analysis

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Background: Magnetic resonance imaging (MRI)-based lymph node staging remains a significant challenge in the treatment of rectal cancer. Pretreatment evaluation of lymph node metastasis guides the formulation of treatment plans. This systematic review aimed to evaluate the diagnostic performance of MRI in lymph node staging using various morphological criteria.

Methods: A systematic search of the EMBASE, Medline, and Cochrane databases was performed. Original articles published between 2000 and January 2021 that used MRI for lymph node staging in rectal cancer were eligible. The included studies were assessed using the QUADAS-2 tool. A bivariate random-effects model was used to conduct a meta-analysis of diagnostic test accuracy.

Results: Thirty-seven studies were eligible for this meta-analysis. The pooled sensitivity, specificity, and diagnostic odds ratio of preoperative MRI for the lymph node stage were 0.73 (95% confidence interval [CI], 0.68–0.77), 0.74 (95% CI, 0.68–0.80), and 7.85 (95% CI, 5.78–10.66), respectively. Criteria for positive mesorectal lymph node metastasis included (A) a short-axis diameter of 5 mm, (B) morphological standard, including an irregular border and mixed-signal intensity within the lymph node, (C) a short-axis diameter of 5 mm with the morphological standard, (D) a short-axis diameter of 8 mm with the morphological standard, and (E) a short-axis diameter of 10 mm with the morphological standard. The pooled sensitivity/specificity for these criteria were 75%/64%, 81%/67%, 74%/79%, 72%/66%, and 62%/91%, respectively. There was no significant difference among the criteria in sensitivity/specificity. The area under the receiver operating characteristic (ROC) curve values of the fitted summary ROC indicated a diagnostic accuracy rate of 0.75–0.81.

Conclusion: MRI scans have minimal accuracy as a reference index for pretreatment staging of various lymph node staging criteria in rectal cancer. Multiple types of evidence should be used in clinical decision-making.

Keywords: rectal cancer, magnetic resonance imaging, metastasis, lymph node, lymph node staging, node-by-node

INTRODUCTION

Rectal cancer has become the leading cause of cancer-related deaths in China and worldwide. By 2030, it is estimated that there will be approximately 2.2 million cases (1, 2). The determination of lymph node staging remains a significant challenge in rectal cancer treatment. Lymph nodes at a risk of metastasis in rectal cancer are mainly located in the mesentery and usually range in size from 1 to 10 mm. Lymph node status is the most important determinant of local recurrence and overall survival (3).

According to the National Comprehensive Cancer Network (NCCN) and American Joint Committee on Cancer (AJCC) staging standards (4, 5), lymph node invasion should be evaluated before treatment to guide the formulation of treatment plans. Patients with lymph node involvement can benefit from preoperative neoadjuvant therapy, considerably reducing the local recurrence rate. However, over-treatment of the lymph node stage may lead to genitourinary system damage and other consequences (6, 7). Therefore, accurate preoperative staging is essential for providing patients with the optimal treatment.

The diagnostic methods currently used for preoperative lymph node staging include magnetic resonance imaging (MRI), computed tomography (CT), and endoscopic ultrasound (EUS). MRI can accurately display the mesorectal fascia, the depth of tumor invasion, circumferential resection margin (CRM), and extramural venous invasion (EMVI), and it has now become the gold standard for preoperative staging and re-staging in local areas (8).

Unfortunately, the results of previous studies have shown that MRI has a poor performance in detecting metastatic lymph nodes (9, 10). At present, there are various diagnostic criteria for metastatic lymph nodes, including size, shape, and boundaries, that have been widely discussed. However, there is no consensus on the accurate diagnosis of metastatic lymph nodes (11–13).

Four previous meta-analyses assessed the accuracy of MRI for lymph node staging of rectal cancer but did not differentiate the lymph nodes defined by different morphological standards (14–17). Additionally, the included studies only used histological results to assess the lymph node status indirectly and did not directly assess lymph nodes on MRI scans. The studies did not perform a histological examination of each lymph node in the specimen so that the position of each lymph node was accurately matched with its corresponding MRI scan, allowing for the node-by-node comparison of MRI scans and histological results to accurately analyze the status of each lymph node.

To the best of our knowledge, this study is the first systematic review and meta-analysis of the accuracy of various lymph node staging criteria in rectal cancer with MRI and includes the literature that contained the node-by-node correspondence between MRI scans and histopathologic results for analysis. To more accurately evaluate the accuracy of MRI in the pretreatment staging of rectal cancer lymph nodes, we hope to obtain more detailed results by synthesizing a large number of published studies.

Abbreviations: MRI, magnetic resonance imaging; RC, rectal cancer; LN, lymph nodes; CRM, circumferential resection margin; EMVI, extramural venous invasion; AJCC, American Joint Commission on Cancer; NCCN, National Comprehensive Cancer Network; QUADAS-2, Quality assessment of Diagnostic accuracy studies-2; CI, confidence interval; AUC, area under the ROC curve; SROC, summary receiver operating characteristics; USPIO, ultrasmall particles of iron oxide; DCE-MRI, dynamic contrast-enhanced MRI.

METHODS

Search Strategy

A comprehensive search of Medline (January 2000–January 2021), Embase (January 2000–January 2021), and the Cochrane Database (2000–January 2021) was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (18) by two investigators (ZZX and ZY), using index terms "(((((((((N-stage) OR (Nodal staging)) OR (Lymph node)) OR (Diagnostic imaging)) OR (mesorectal lymph nodes)) OR (Neoplasm Staging)) OR (Lymphatic Metastasis))) AND (((“Magnetic Resonance Imaging”[Mesh]) AND (“Rectal Neoplasms”[Mesh])) AND (sensitiv*[Title/Abstract] OR sensitivity and specificity[Mesh Terms] OR (predictive[Title/Abstract] AND value*[Title/Abstract]) OR predictive value of tests[Mesh Term] OR accuracy*[Title/Abstract])) as text words. The last search was on January 10, 2021.

Study Selection

Studies were included based on the following criteria: 1) original articles on the diagnostic performance of MRI in the staging of rectal cancer, 2) a phased-array MRI coil was used for imaging, 3) histopathologic findings were used as reference standards, 4) the reference criteria for assessing metastatic lymph nodes were clearly mentioned, and 5) sufficient data were available to calculate true-positive, false-positive, false-negative, and true-negative values.

The exclusion criteria were as follows: 1) inclusion of patients with non-rectal cancer, 2) research using other less common MRI types, 3) assessment of staging according to a non-Tumor–Node–Metastasis (TNM) staging system, 4) inclusion of patients who received preoperative chemoradiotherapy, 5) articles that

were not original research articles, such as reviews, letters, or case reports, 6) repeated publications.

Titles and abstracts identified by the search strategy were independently reviewed by two reviewers. For all abstracts that met the inclusion criteria or were potentially eligible, full articles were retrieved and independently reviewed by two reviewers. Disagreements were resolved by consensus or by discussion with a third reviewer. All included studies followed the PICOS criteria.

Data Extraction and Quality Assessment

Two reviewers independently extracted the data. The following data was collected: (year of publication, sample size, country), study design (prospective or retrospective), MRI protocol (field strength and resolution parameters), reference criteria for assessing metastatic lymph nodes, and blinding procedure.

The diagnostic results were calculated on a lesion level for each outcome: Patients/lymph nodes with histologically confirmed lymph node metastasis are classified as node-positive (pN+), regardless of the number of metastatic lymph nodes. Patients/lymph nodes without any metastatic lymph nodes are classified as node-negative (pN-).

The QUADAS-2 evaluation tool was used to evaluate the quality of all studies in the systematic review.

Statistical Analysis

Meta-analysis and the associated I^2 statistic were evaluated with Meta-Disc 1.4(Ramón y Cajal Hospital, Madrid, Spain) and Stata 16.0(STATA Corporation, College Station, TX, USA) (19).

The threshold effect was evaluated using Spearman's correlation coefficient of the logit of sensitivity and logit of 1-specificity.

A bivariate random-effects model was used to summarize diagnostic statistics and displayed using summary receiver operating characteristics (SROC) plots.

Meta-regression and subgroup analyses were performed to detect heterogeneity. Additionally, a sensitivity analysis was conducted (20).

Publication bias was evaluated with an asymmetry test and a Deek's funnel plot assessment using Stata 16.0 (21).

RESULTS

Description of Included Studies

A preliminary database search yielded 1,970 articles, of which 163 were considered relevant for a full test assessment. After screening and data extraction to evaluate whether the articles were suitable for inclusion, 37 eligible items were included in this meta-analysis (9, 11, 22–56). The research selection flowchart is presented in **Figure 1**. The characteristics of the studies are presented in **Table 1**. The reference standards were divided into the following five categories according to different morphological criteria: (A) a short-axis diameter of 5 mm (22–34), (B) morphological standard, including an irregular border and mixed-signal intensity within the lymph node (35–40), (C) a short-axis diameter of 5 mm with the morphological standard (11, 41–48), (D) a short-axis diameter of 8 mm with the morphological standard (49–52), and (E) a short-axis diameter of 10 mm with the morphological standard (11, 45, 53, 54). In all of the included articles, 36 indirectly evaluated the lymph node stage of patients through histopathology and 5 (9, 41, 42, 55, 56) identified the node-by-node correspondence between lymph node MRI scans and histopathologic results. Across all studies analyzed, 2,875 patients and 983 lymph nodes were included. **Table 2** shows the details of the quality assessment. **Figure 2** gives a graphical display for QUADAS-2 results regarding the distribution of the risk of bias.

Diagnostic Performance

The pooled sensitivity and specificity of MRI in the comprehensive diagnosis of metastatic lymph nodes were 0.73 (95% confidence interval [CI], 0.68–0.77) and 0.74 (95% CI, 0.68–0.80), respectively. The pooled sensitivity, specificity, diagnostic odds ratio, positive likelihood ratio, and negative likelihood ratio with corresponding 95% CIs are listed in **Table 3**. The area under the ROC curve (AUC) value of the fitted summary ROC was 0.7877 (**Figure 3**).

Among the different morphological criteria, "a short-axis diameter of 5 mm with the morphological standard" revealed the highest sensitivity of 0.81 (95% CI, 0.74–0.87), and "a short-axis diameter of 10 mm with the morphological standard" revealed the highest specificity of 0.91 (95% CI, 0.51–0.99) (**Table 3**). The AUCs indicated a diagnostic accuracy rate of 0.75–0.81. The morphological standards with the highest accuracy were "a short-axis diameter of 5 mm with the morphological standard" and "a short-axis diameter of 10 mm with the morphological standard" (**Figure 4**).

Test of Heterogeneity and Metaregression Analysis

The heterogeneity tests showed that the Spearman's correlation coefficient was 0.446 ($p = 0.004$), indicating the presence of a threshold effect. This means that different evaluation criteria have led to a significant heterogeneity. Under different morphological standards, there is considerable heterogeneity among 1) the morphological standard, 2) a short-axis diameter of 8 mm with the morphological standard, and 3) a short-axis diameter of 10 mm with the morphological standard (all $p < 0.05$, $i2 > 50\%$). Therefore, in addition to the threshold effect, there must be other factors that cause significant heterogeneity. A single-factor meta-regression analysis was performed on all the elements. The results showed that the blinding procedure had a particular impact on the heterogeneity of the research (**Table 4**).

Subgroup Analysis

Subgroup analyses were performed for the different study characteristics. By comparing references with or without node-by-node correspondence, we found that a lower sensitivity of 0.55 (95% CI, 0.40–0.69) and higher specificity of 0.89 (95% CI, 0.79–0.95) were yielded. When considering different MRI types, both 3.0T and high-resolution MRI yielded a higher sensitivity and specificity. Through a subgroup analysis of the study design, read approach, and blinding procedure, studies that used double blinding yielded a higher

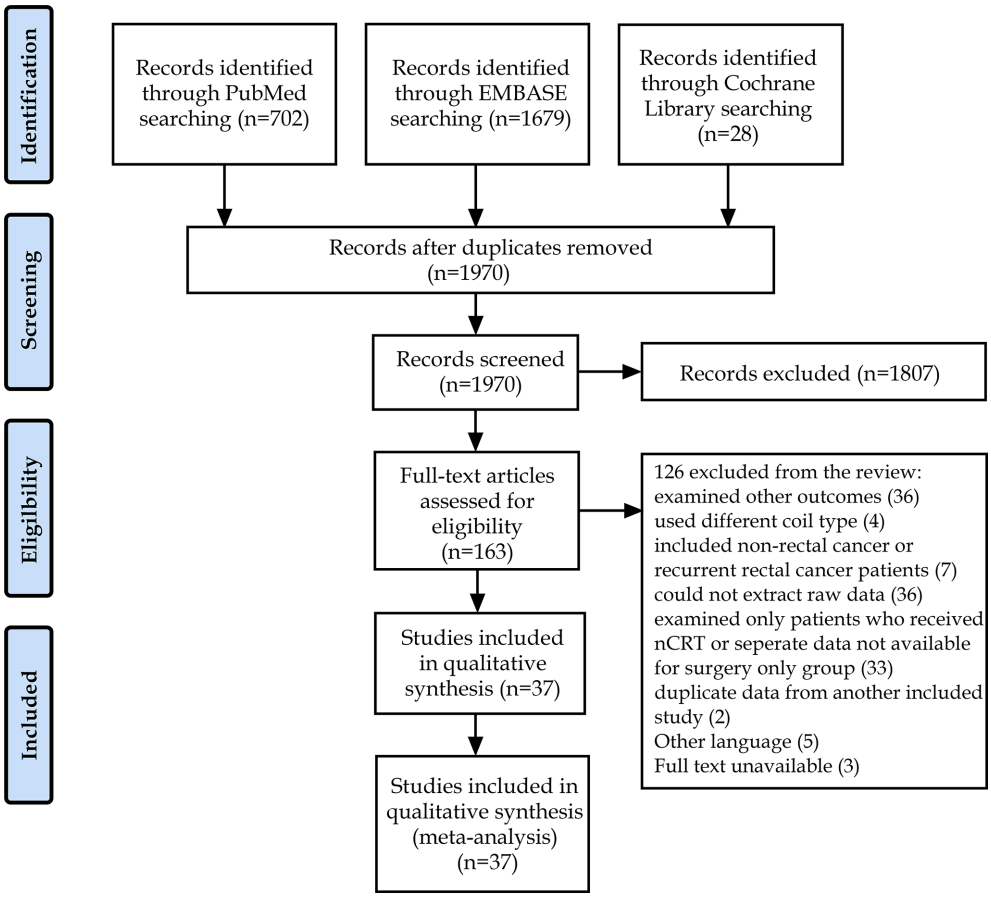

FIGURE 1 | Flow chart according to PRISMA.

sensitivity of 73% (95% CI, 0.67–0.78) and specificity of 78% (95% CI, 0.70–0.84), whereas prospective studies yielded a higher specificity of 77% (95% CI, 0.75–0.79). The results of the subgroup analysis are shown in **Table 4**.

Sensitivity Analysis

A sensitivity analysis of all the studies revealed that five original studies had a strong sensitivity (**Figure 5**), whereas the other original studies did not strongly affect the calculation results. After excluding the literature mentioned above, the other 36 sub-datasets still had threshold effects. The pooled sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, and diagnostic odds ratio were 0.74 (95% CI, 0.71–0.78), 0.70 (95% CI, 0.64–0.75), 2.45 (95% CI, 2.08–2.89), 0.37 (95% CI, 0.32–0.42), and 6.67 (95% CI, 5.23–8.48), respectively. Further, the AUC was 0.7750.

Publication Bias

For all studies, the p-value of the bias on the Deek's funnel plot asymmetry test was 0.55, indicating that these studies did not have significant publication bias (**Figure 6**).

DISCUSSION

Lymph node status plays a vital role in selecting treatment strategies for colorectal cancer, with the presence or absence of regional lymph node metastasis being the key to treatment selection. The advantage of MRI is that it can identify the mesorectal fascia, enabling accurate preoperative identification of patients with lymph nodes that cannot be entirely surgically removed. Therefore, in the context of neoadjuvant therapy, preoperative MRI must provide an accurate diagnosis of regional lymph nodes, avoid overestimation and underestimation before treatment, and provide the optimal treatment decision for individual patients. In this study, we evaluated the ability of MRI to determine the lymph node stage of rectal cancer. The results showed that the value of MRI in diagnosing metastatic lymph nodes was low (57–59).

These findings are similar to those reported by Al-Sukhni et al. (15–17), who concluded that MRI only moderates the diagnostic ability for lymph node metastasis. It is worth noting that the previous meta-analysis found significant heterogeneity in the assessment of lymph node metastasis and speculated that

TABLE 1 | Characteristics of studies included in the analysis.

Study	Year	Country	Design	Assessment approach, No. of readers for each MRI	Field strength	High resolution	Blinding	Reference standard	IC	PN/LN
Xu et al. (22)	2020	China	P	Consensus	3.0	Y	D	H, S	5mm,short-axis	354
Xu HS et al. (49)	2021	China	P	Consensus	3.0	Y	D	H, S	8mm,short-axis+MS	120
Tersteeg et al. (23)	2020	Netherlands	R	Consensus	1.5	N	Y	H, S	5mm,short-axis	324
Iannicelli et al. (24)	2014	Italy	P	Consensus	1.5	Y	D	H, S	5mm,short-axis	73
White et al. (25)	2013	Australia	R	Consensus	1.5	N	Y	H, S	5mm,short-axis	58
Park et al. (41)	2014	Korea	P	Consensus	3.0	Y	D	H, S	5mm,short-axis+MS	40/205LN
Lambregts et al. (42)	2011	Netherlands	R	Consensus	1.5	N	D	H, S	5mm,short-axis+MS	26/111LN
Kim et al. (50)	2011	Korea	R	Independent	3.0	Y	D	H, S	8mm,short-axis+MS	30
Fernández-Esparrach et al. (35)	2011	Spain	P	Consensus	3.0	Y	D	H, S	MS	90
Koh et al. (55)	2010	United Kingdom	P	Consensus	1.5	N	D	H, S	MS	126LN
Jao et al. (36)	2010	Taiwan	P	Consensus	1.5	N	D	H, S	MS	37
Zhang et al. (51)	2007	China	P	Consensus	1.5	N	D	H, S	8mm,short-axis+MS	53
Winter et al. (26)	2007	Germany	P	Consensus	3.0	Y	D	H, S	5mm,short-axis	21
Tatli et al. (27)	2006	USA	R	Independent	1.5	N	D	H, S	5mm,short-axis	25
Song et al. (53)	2018	China	R	Consensus	1.5	N	D	H, S	10mm,short-axis+MS	84
Rafaelsen et al. (28)	2008	Danish	R	Consensus	1.5	N	D	H, S	5mm,short-axis	134
Matsuoka et al. (29)	2003	Japan	P	Independent	1.5	N	D	H, S	5mm,short-axis	21
Kocaman et al. (30)	2014	Turkey	R	Consensus	1.5	N	D	H, S	5mm,short-axis	50
Kim MJ et al. (52)	2008	Korea	R	Consensus	3.0	Y	D	H, S	8mm,short-axis+MS	42
Kim et al. (54)	2000	Korea	R	Independent	1.5	N	Y	H, S	10mm,short-axis+MS	217
Kim JH et al. (11)	2004	Netherlands	P	Independent	1.5	Y	D	H, S	5mm+MS/10mm+MS	75
Kim et al. (56)	2006	Korea	P	Consensus	3.0	N	D	H, S	MS	257LN
Jiang et al. (43)	2006	China	P	Consensus	3.0	Y	D	H, S	5mm,short-axis+MS	53
Halefoglu et al. (31)	2008	Turkey	P	Independent	1.5	N	D	H, S	5mm,short-axis	34
Gagliardi et al. (32)	2002	England	R	Independent	1.5	N	Y	H, S	5mm,short-axis	28
Chun et al. (37)	2006	Korea	P	Consensus	3.0	Y	D	H, S	MS	24
Algebally et al. (44)	2015	Egypt	P	Independent	1.5	Y	Y	H, S	5mm,short-axis+MS	56
Armbruster et al. (45)	2018	Germany	P	Consensus	1.5	N	D	H, S	5mm+MS/10mm+MS	22
Halefoglu et al. (46)	2013	Turkey	P	Independent	1.5	N	D	H, S	5mm,short-axis+MS	93
Kim et al. (38)	2007	Korea	P	Consensus	3.0	Y	D	H, S	MS	26
Bogach et al. (39)	2017	Canada	R	Consensus	3.0	N	Y	H, S	MS	109
Akasu et al. (47)	2009	Japan	P	Consensus	1.5	Y	D	H, S	5mm,short-axis+MS	104
Gröne et al. (33)	2017	Germany	R	Consensus	1.5	Y	D	H, S	5mm,short-axis	60
Brown et al. (9)	2003	England	P	Consensus	1.5	N	D	H, S	5mm,short-axis+MS	284LN
Ferri et al. (34)	2005	Italy	R	Consensus	1.5	N	D	H, S	5mm,short-axis	29
Kim MJ et al. (48)	2004	Korea	P	Independent	1.5	N	D	H, S	5mm,short-axis+MS	62
Kim JH et al. (11)	2009	Korea	P	Independent	1.5	N	D	H, S	MS	66

P, prospective; R, retrospective; Y, yes; N, no; D, double blinding; H, histologic diagnosis; S, surgery; IC, interpretation criteria; MS, morphological standards; PN, patient number; LN, lymph nodes.

TABLE 2 | Quality assessment of the 37 included diagnostic studies.

Study Authors	Year	Risk of bias			Flow and timing	Applicability concerns		
		Patient selection	Index test	Reference standard		Patient selection	Index test	Reference standard
Xu et al. (22)	2020	+	+	+	+	+	+	+
Xu HS et al. (49)	2021	+	+	+	?	+	+	+
Tersteeg et al. (23)	2020	?	–	?	?	?	–	+
Iannicelli et al. (24)	2014	+	+	+	+	+	+	+
White et al. (25)	2013	?	+	?	+	?	+	+
Park et al. (41)	2014	?	+	+	+	?	–	+
Lambregts et al. (42)	2011	+	+	+	+	+	+	+
Kim et al. (50)	2011	?	+	+	+	?	+	+
Fernández-Esparrach et al. (35)	2011	+	+	?	–	?	+	?
Koh et al. (55)	2010	+	+	+	+	+	+	+
Jao et al. (36)	2010	+	+	+	+	+	+	+
Zhang et al. (51)	2007	–	+	+	?	–	+	+
Winter et al. (26)	2007	?	+	+	?	?	+	+
Tatli et al. (27)	2006	?	+	+	+	?	+	+
Song et al. (53)	2018	+	+	+	+	+	+	+
Rafaelsen et al. (28)	2008	+	+	?	+	+	+	+
Matsuoka et al. (29)	2003	?	+	+	+	?	+	+
Kocaman et al. (30)	2014	–	+	+	+	–	+	+
Kim et al. (52)	2008	+	+	+	+	+	+	+
Kim et al. (54)	2000	?	+	+	+	?	+	+
Kim JH et al. (11)	2004	?	+	+	+	?	+	+
Kim et al. (56)	2006	+	+	+	+	+	+	+
Jiang et al. (43)	2006	+	+	+	+	+	+	+
Halefoglu et al. (31)	2008	+	+	+	+	+	+	+
Gagliardi et al. (32)	2002	+	?	?	+	+	?	+
Chun et al. (37)	2006	+	+	+	+	+	+	+
Algebally et al. (44)	2015	+	?	?	+	+	?	+
Armbruster et al. (45)	2018	–	?	+	+	–	?	+
Halefoglu et al. (46)	2013	+	+	+	+	+	?	+
Kim et al. (38)	2007	+	+	+	+	+	+	+
Bogach et al. (39)	2017	?	–	?	+	?	?	+
Akasu et al. (47)	2009	+	+	+	+	+	+	+
Gröne et al. (33)	2017	+	+	+	+	+	+	+
Brown et al. (9)	2003	+	+	+	?	+	+	+
Ferri et al. (34)	2005	?	+	+	+	?	+	+
Kim MJ et al. (48)	2004	+	?	?	+	+	?	+
Kim JH et al. (11)	2009	+	+	?	+	+	+	+

+, low risk; -, high risk; ?, unclear risk.

the threshold effect is the primary source of heterogeneity. Therefore, we corrected for some of the limitations recognized by previous studies by including more original articles and classifying lymph nodes for statistical analysis based on different morphological standards.

Most MRI studies on colorectal cancer published have used lymph node size as a standard criterion for predicting lymph node involvement. However, previous studies demonstrated that using only the size of lymph nodes as a criterion does not improve the accuracy of lymph node staging of colorectal cancer (9–11), which is consistent with our results. We found that there was no significant difference in the accuracy of MRI diagnosis when using different standards. It is worth mentioning that under the same morphological standard, as the shorter diameter of the lymph node increases, the sensitivity gradually decreases and the specificity gradually increases (**Table 3**). This may be because although malignant lymph nodes usually have a larger short-axis diameter than benign lymph nodes, there is a considerable size overlap between benign and malignant lymph nodes, with approximately 30% of metastatic lymph nodes having a diameter of ≤4 mm (12). In addition, benign lymph nodes may appear to increase in size with the development of fibrosis (60).

Compared with the size standard alone, different morphological features have been previously considered as good criteria for judging metastatic lymph nodes. Brown et al. first described the use of MRI to improve the correct diagnosis of lymph node involvement in rectal cancer when boundary contours and signal intensity features were used instead of size standards alone (9). Kim et al. demonstrated that in addition to size, new criteria, such as burr-like or inconspicuous borders and uneven appearance, can be used to predict regional lymph node involvement (11). Their results were better than our findings. We found that after adding morphological features, the pooled

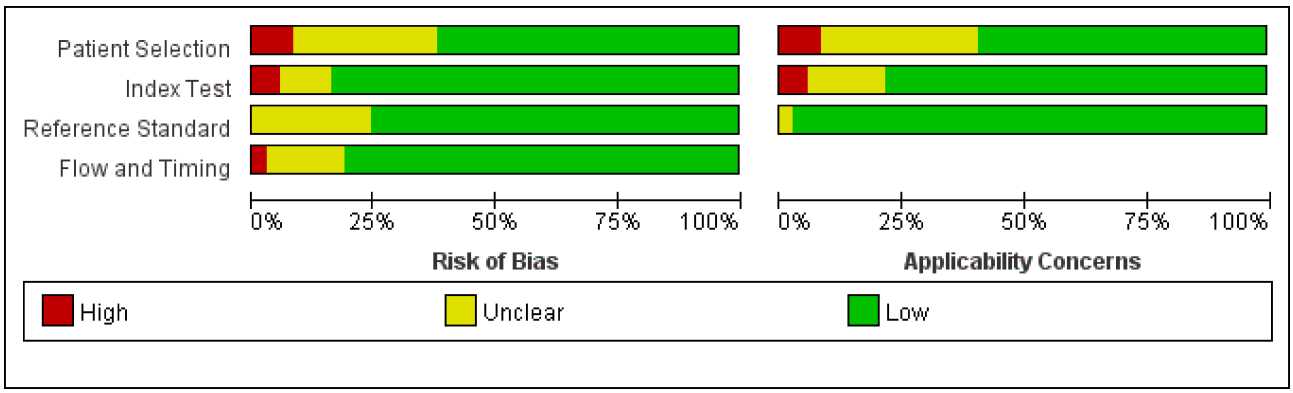

FIGURE 2 | Graphical display for Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) results regarding the proportion of studies with low, high, or unclear risk of bias.

TABLE 3 | The pooled sensitivity, specificity, PLR, and NLR with corresponding 95% CIs for each included study under different morphological standards.

Index test	SEN (95% CI)	SPE (95% CI)	DOR (95% CI)	PLR (95% CI)	NLR (95% CI)	AUC
Total	0.73 (0.68-0.77)	0.74 (0.68-0.80)	7.85 (5.78-10.66)	2.85 (2.27-3.58)	0.36 (0.31-0.42)	0.79 (0.76-0.83)
5MM	0.75 (0.67-0.81)	0.64 (0.57-0.71)	5.20 (3.76-7.18)	2.07 (1.76-2.43)	0.40 (0.31-0.50)	0.75 (0.71-0.78)
MS	0.74 (0.67-0.80)	0.79 (0.58-0.91)	10.86 (4.19-28.13)	3.57 (1.65-7.74)	0.33 (0.25-0.43)	0.77 (0.73-0.81)
5MM+MS	0.81 (0.74-0.87)	0.67 (0.58-0.74)	8.53 (5.59-13.01)	2.42 (1.94-3.03)	0.28 (0.21-0.39)	0.81 (0.78-0.85)
8MM+MS	0.72 (0.60-0.82)	0.66 (0.47-0.81)	5.18 (1.60-16.80)	2.15 (1.16-3.99)	0.42 (0.23-0.75)	0.76 (0.72-0.79)
10MM+MS	0.62 (0.34-0.83)	0.91 (0.51-0.99)	16.21 (3.74-70.21)	6.80 (1.22-37.81)	0.42 (0.24-0.72)	0.81 (0.77-0.84)

MS, morphological standards; DOR, diagnostic odds ratio; PLR, positive likelihood ratio; NLR, negative likelihood ratio; AUC, area under the curve.

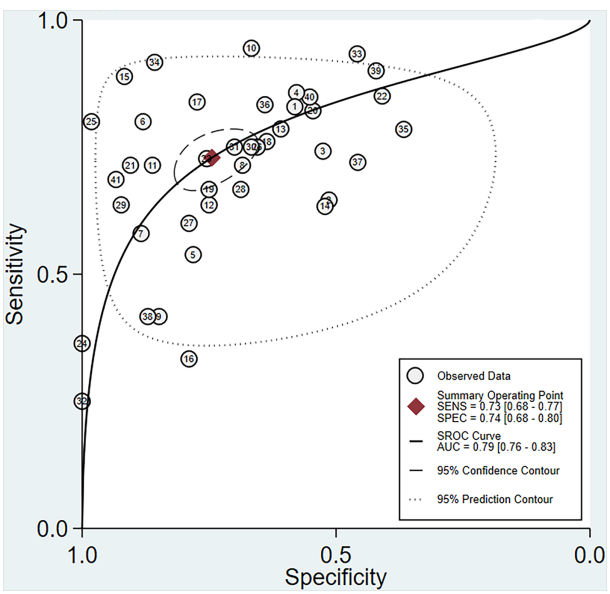

FIGURE 3 | Summary receiver operating characteristic (SROC) curve for MRI assessment of lymph node metastasis in rectal cancer.

sensitivity and specificity of lymph node diagnosis improved. However, the diagnostic performance did not improve significantly (**Table 3**), possibly because the morphological characteristics are more subjective among different observers.

We found that both high-field strength (3.0 Tesla) and high-resolution MRI yielded a higher sensitivity and specificity than low-field strength (1.5 Tesla) according to a subgroup analysis (**Table 4**). Due to the retrospective design of the research, patient selection, and MRI plan, the diagnostic performance of prospectively designed research was slightly better in the subgroup analysis. In addition, double-blind studies had a higher specificity than single-blind studies (0.78, 95% CI 0.70–0.84). As with other diagnostic meta-analyses, heterogeneity is a vital limitation among studies, including study design, MRI protocols, blinding procedures, and reference standards. In the regression analysis, we found that the blinding procedure (single-blind/double-blind) helps assess heterogeneity, leading to differences among research conclusions.

In most previous studies, the assessment of the lymph node staging of patients mainly relied on the number of positive lymph nodes found in the mesorectum after the overall sampling of rectal specimens, which does not have a high accuracy and reliability. Thus far, few studies have reported that the individual lymph nodes seen on MRI scans match the exact pathological

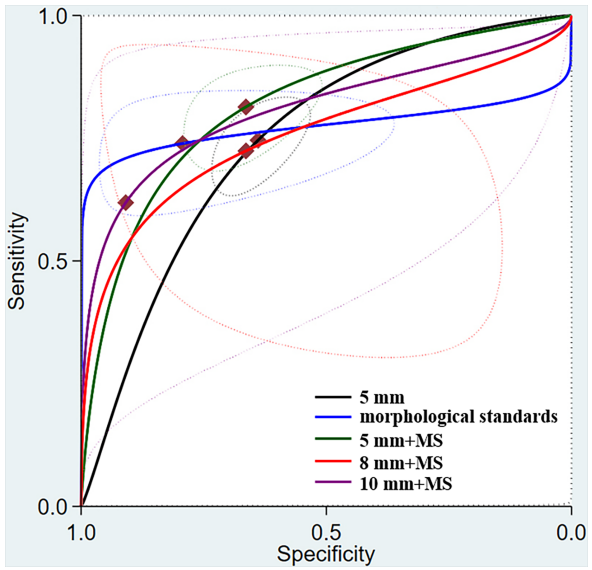

FIGURE 4 | Summary receiver operating characteristic (SROC) curve for MRI assessment of lymph node metastasis under different morphological standards.

correspondence after rectal resection. We included five references as subgroups. Our analysis found that the sensitivity of MRI for the diagnosis of a single lymph node decreased, the specificity significantly improved, and the accuracy of the assessment was lower than expected. The possible reasons for the inconsistent diagnostic accuracy could be due to small number of references, a lack of consistency in the threshold, and the difference in the realization of node-by-node correspondence.

Currently, new technologies are being explored to improve preoperative staging. The chemical shift effect is a reliable indicator for identifying benign and malignant lymph nodes (61), and Farshchian first proposed that it has the potential to diagnose benign lymph nodes (62). Grovik et al. showed that a low K^{trans} of the primary tumor can predict the presence of nodal metastasis (63), which can be achieved by dynamic contrast-enhanced MRI (DCE-MRI) (64, 65). In addition to DCE-MRI, special diffusion-weighted MRI parameters are helpful in differentiating metastatic lymph nodes (45, 66).

The use of lymphatic contrast agents is considered a method for improving the staging of lymph nodes. USPIO is the most widely used contrast agent (67, 68). This technology allows for the differentiation of malignant and benign lymph nodes according to the contrast-enhanced pattern. Although MRI with USPIO has achieved some success in characterizing small lymph nodes, further research is needed regarding its clinical applicability (55, 69–71).

Radiomics is a rapidly developing discipline that uses computer algorithms to extract quantitative features from MRI scans (72–74). These algorithms capture the image texture and morphology of tumors based on their gray values. Since 2018, many reports on radiological methods for rectal cancer lymph node assessment have been published (75–78). However, when analyzing imaging information and building predictive models, all these parameters require time-consuming calculations. In the future, artificial intelligence is expected to become the optimal option for determining lymph node staging and treatments options for patients with locally advanced rectal cancer.

Recently, the importance of lymph node metastasis in the process of tumor recurrence has begun to be questioned, i.e., the indications of neoadjuvant therapy are not based on clinical

TABLE 4 | Results of subgroup analysis for evaluation of all studies.

Study characteristics	No.	Pooled sensitivity (95% CI)	Pooled specificity (95% CI)	Positive LR (95% CI)	Negative LR (95% CI)	AUC	p
Total	41	0.73 (0.68-0.77)	0.74 (0.68-0.80)	2.85 (2.27-3.58)	0.36 (0.31-0.42)	0.7877	
Field strength, Tesla							
1.5	28	0.72 (0.69-0.75)	0.70 (0.67-0.72)	2.04 (1.78-2.33)	0.46 (0.39-0.55)	0.7559	0.0524
3.0	13	0.77 (0.73-0.80)	0.78 (0.75-0.81)	3.92 (2.49-6.18)	0.35 (0.27-0.46)	0.8412	
High resolution							
Yes	17	0.74 (0.67-0.80)	0.78 (0.67-0.86)	3.30 (2.27-4.80)	0.34 (0.28-0.41)	0.8125	0.2513
No/Not specified	24	0.72 (0.65-0.81)	0.73 (0.64-0.81)	2.73 (2.03-3.66)	0.37 (0.30-0.47)	0.7936	
Design							
Retrospective	15	0.77 (0.73-0.81)	0.62 (0.58-0.66)	1.85 (1.53-2.22)	0.46 (0.37-0.58)	0.7421	0.1358
Prospective	26	0.72 (0.69-0.75)	0.77 (0.75-0.79)	2.87 (2.28-3.62)	0.40 (0.33-0.49)	0.8056	
Node by node							
Yes	5	0.55 (0.40-0.69)	0.89 (0.79-0.95)	5.21 (2.03-13.46)	0.51 (0.34-0.76)	0.7813	0.9405
No	36	0.74 (0.70-0.79)	0.71 (0.64-0.77)	2.59 (2.12-3.10)	0.36 (0.31-0.42)	0.7937	
Read approach							
Independent	12	0.77 (0.72-0.81)	0.64 (0.60-0.69)	2.14 (1.65-2.77)	0.42 (0.31-0.55)	0.7853	0.6774
Consensus	29	0.73 (0.70-0.76)	0.75 (0.73-0.77)	2.53 (2.06-3.10)	0.42 (0.35-0.51)	0.7894	
Blinding							
Single	7	0.72 (0.63-0.80)	0.57 (0.46-0.67)	1.70 (1.40-2.03)	0.49 (0.38-0.63)	0.7008	0.0281
Double	34	0.73 (0.67-0.78)	0.78 (0.70-0.84)	3.31 (2.54-4.28)	0.34 (0.29-0.41)	0.8082	

No., number of data subsets; AUC, area under the curve; p, p value of meta-regression analysis.

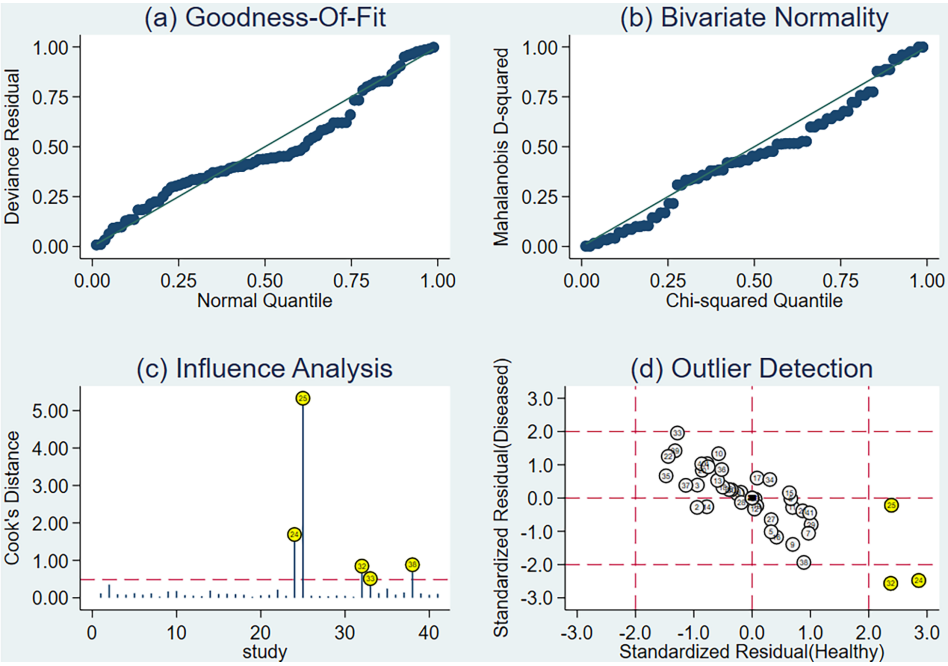

FIGURE 5 | Sensitivity analysis results of all studies: **(A)** goodness of fit, **(B)** bivariate normality, **(C)** influence analysis, and **(D)** outlier detection.

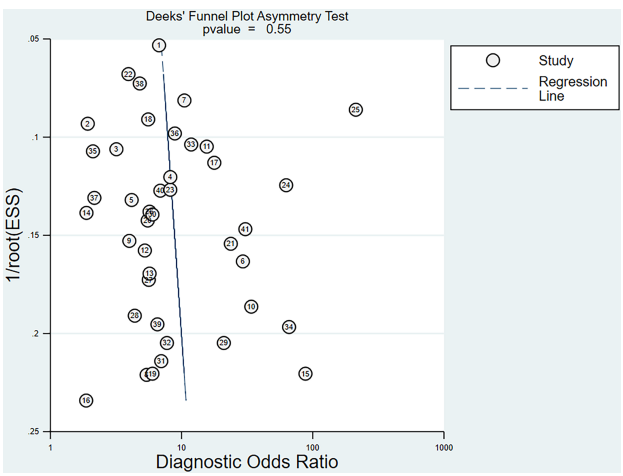

FIGURE 6 | Funnel plot of the reciprocal of effective sample size (ESS) plotted on the y-axis against the diagnostic odds ratio plotted on the x-axis. The regression line is used as a measure of asymmetry. The circles represent included studies.

TNM staging. Additionally, determining whether there are other prognostic markers detected by MRI, such as extra-mural venous invasion (EMVI) and circumferential resection margin (CRM), is more important (79–81). The MERCURY study showed that lymph node involvement is not an independent predictor of local recurrence, and using CRM was recommended for evaluating neoadjuvant therapy (82). In this case, clinical lymph node assessment for rectal cancer may only play a secondary role in guiding future treatment decisions (83, 84).

This study has some limitations. First, our meta-analysis included 37 studies and 2,875 patients. Although this is a comprehensive literature search, more studies may provide more accurate estimates and comparisons of results. Second, the content of some reports is insufficient, limiting our quality assessment and individual analysis of more subgroups. Finally, heterogeneity is still an essential issue in meta-analyses. In future studies, the definition of critical staging elements and MRI protocols should be standardized to reduce heterogeneity. Therefore, considering the limitations of diagnostic meta-analysis, the results should be interpreted prudently.

CONCLUSION

In summary, the performance of MRI in the detection of lymph node metastasis is inadequate, and either through using more morphological characteristics or shorter diameter, is not significantly improved. At present, when making preoperative neoadjuvant treatment decisions, evidence from a variety of imaging methods should be combined to determine the optimal treatment strategy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

ZZ contributed the most to this article. ZZ designed the project, developed the search strategy and wrote the manuscript. ZZ and YZ checked the search, and reviewed the manuscript. MW performed literature screening and data extraction, conduct the quality assessment of the included studies. XY carried out the data analysis. ZW reviewed the manuscript and finally approved the version to be published. All authors contributed to the article and approved the submitted version.

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Management of Clinically Involved Lateral Lymph Node Metastasis in Locally Advanced Rectal Cancer: A Radiation Dose Escalation Study

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Background: Patients with lateral lymph nodes (LLNs) metastasis are not effectively treated with neoadjuvant chemoradiotherapy. This study aimed to compare the efficacy of three neoadjuvant therapeutic regimens, namely, chemotherapy, chemoradiotherapy, and chemoradiotherapy with a dose boost of LLNs, and to identify the optimal approach for treating LLNs metastasis of locally advanced rectal cancer.

Methods: A total of 202 patients with baseline LLNs metastasis (short axis ≥5 mm) and treated with neoadjuvant treatment, followed by radical surgery from 2011 to 2019, were enrolled. The short axis of the LLNs on baseline and restaging MRI were recorded. Survival outcomes were compared.

Results: In the booster subgroup, shrinkage of LLNs was significantly greater than in the neoadjuvant chemotherapy and chemoradiotherapy subgroups (P <0.001), without increasing radiation related side effects (P = 0.121). For patients with baseline LLNs of short axis ≥5 mm in the booster subgroup, the response rate (short axis <5 mm on restaging MRI) was 72.9%, significantly higher than patients in the neoadjuvant chemotherapy subgroup (48.9%, P = 0.007) and higher than for patients in the neoadjuvant chemoradiotherapy group (65.0%), but there was no statistical difference (P = 0.411). The 3-year local recurrence and lateral local recurrence rates were both 2.3% in the dose booster group, which were lower than those of the other two subgroups (local recurrence: P <0.001; lateral local recurrence: P <0.001). The short axis of lateral lymph nodes (≥5 and <5 mm) on restaging MRI was an independent risk factor for prognosis (P <0.05).

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Conclusion: Radiation dose boost is an effective way of increasing the response rate and decreasing recurrence rates. The restaging LLNs with short axis ≥5 mm is a predictor of poor prognosis.

Keywords: locally advanced rectal cancer, lateral lymph node, lateral lymph node dissection, dose escalation, MRI

INTRODUCTION

Neoadjuvant chemoradiotherapy (nCRT), followed by total mesorectal excision (TME), has become the standard treatment for locally advanced rectal cancer (LARC) (1). However, 5–8% of patients continue to experience local recurrence after 3 years (1–3). Previous studies have confirmed that lateral lymph node (LLN) metastasis is one of the most important factors influencing recurrence in middle and low rectal cancer (4, 5). In some Asian countries, lateral lymph node dissection (LLND) is recommended for patients with LARC (6, 7). However, LLND is associated with various complications, including longer operation time, larger blood loss, and severe sexual and urinary dysfunction (7, 8). In contrast, in western countries, LLND is not performed regularly, and nCRT before TME surgery is considered standard treatment (9).

Numerous studies have suggested that preoperative nCRT does not eradicate LLN metastasis, especially when the short axis (SA) is persistently greater than 5 mm after standard nCRT, and the pathologically positive rate was observed in 60–75% of cases (10–12). In contrast, when the LLNs show a favorable response (SA <5mm) to nCRT, the positive rate is reduced to <20% (12). Therefore, to avoid overtreatment and morbidity, it is strongly recommended that LLND should be delivered to patients with LLNs who do not respond well to nCRT (restaging SA ≥5 mm) (10, 13, 14). However, it has been reported that after standard 45 Gy radiation of LLNs, the response rate of the LLNs was in the range of 45.5–56.1% (4, 12, 15).

Considering the development of TME surgery and the side effect of radiotherapy, a strategy of removing neoadjuvant radiotherapy by intensified neoadjuvant chemotherapy was proposed for LARC. Prospective trials, such as a phase II study and FORWAC, revealed that the intensified nCT treatment without radiotherapy might be a promising way to improve oncological outcomes for LARC (2, 16). However, for LARC patients with LLN metastasis, the effect of omitting neoadjuvant radiotherapy by intensifying the neoadjuvant chemotherapy was unclear.

Radiation dose escalation studies have shown that increasing radiation doses could improve local control (17, 18). In recent years, the simultaneous integrated boost intensity-modulated radiotherapy treatment (SIB-IMRT) strategy has been implemented, allowing the simultaneous delivery of various dose prescriptions and target volumes in the same fraction, thus avoiding a delay in total treatment time (18). In a prospective study, SIB-IMRT has been shown to improve the pathological complete response rate with acceptable toxicity effects for patients with LARC (17). However, the findings of studies on the escalation of LLNs in rectal cancer are unclear. There was only one single-center study (12 cases) that tried to boost the LLN doses to 60 Gy, while long-term follow-up was not reported (19).

In this large-scale retrospective cohort study, we compared three different treatment regimens: neoadjuvant chemotherapy (nCT), nCRT, and neoadjuvant chemoradiotherapy with a radiation dose boost (nCRT-boost) of LLNs. The aim was to identify the optimal neoadjuvant treatment regimen of LLNs metastasis in patients with LARC. This study was approved by the central ethics committee of the Sixth Affiliated Hospital, Sun Yat-sen University (No. 2020ZSLYEC-274).

METHODS

Study Population

This was a retrospective study that included patients from January 2011 to October 2019 at a gastrointestinal specialist hospital. Patients who were staged clinically T3–T4 and N-positive for rectal adenocarcinoma were included. Other inclusion criteria were as follows: adenocarcinoma was located within 10 cm of the anal verge; the patient had undergone neoadjuvant chemoradiotherapy or chemotherapy, followed by TME surgery; the baseline and restaging MRI scans were available; there was at least one baseline LLN (SA ≥5 mm) at the internal iliac, obturator, and external iliac region. The exclusion criteria included the presence of distant metastases at diagnosis or before TME surgery, the absence of MRI scans, and/or poor scan quality.

Radiotherapy

Patients received 5-field SIB-IMRT with an Elekta Synergy accelerator. From July 2015, an attempt at radiation dose escalation of baseline LLNs (SA ≥5 mm) was made. The gross tumor volume (GTV) was defined as gross disease determined from MRI scans. The lymph nodes (SA ≥5 mm) at the internal iliac, obturator, and external iliac regions were delineated, named GTVnd, and were given a radiation dose boost. The clinical

Abbreviations: LLN, lateral lymph node; nCRT, neoadjuvant chemoradiotherapy; TME, total mesorectal excision; LARC, locally advanced rectal cancer; LLND, lateral lymph node dissection; SIB-IMRT, simultaneous integrated boost intensity-modulated radiotherapy treatment; nCT, neoadjuvant chemotherapy; nCRT-boost, neoadjuvant chemoradiotherapy with a radiation dose boost; LLR, lateral local recurrence; LR, local recurrence; DR, distant recurrence; CSS, cancer-specific survival; AJCC/CAP TRG, American Joint Commission on Cancer and College of American Pathologists Tumor Regression Grade; GTV, gross tumor volume; CTV, clinical target volume; PTV, planning target volume; CRM, circumferential resection margin; HR, hazard ratio; CI, confidence interval; NCCN, National Comprehensive Cancer Network.

target volume (CTV) was defined as the GTV and GTVnd plus areas considered at significant risk of harboring microscopic area. The planning target volume (PTV) was generated by adding an 8-mm margin around the GTV, GTVnd, and CTV in all directions. Doses of 56–58, 50, and 45 Gy were delivered to PTV-GTVnd, PTV-GTV, and PTV-CTV at 25 fractions, respectively. The dose of the normal organs at risk was based on the following criteria: bowel bag, V50 ≤5%; bladder, V50 ≤50%; femoral heads, V50 ≤5% (20).

Chemotherapy

During radiotherapy treatment, intravenous fluoropyrimidine-based chemotherapy was concurrently administered. Patients were given fluoropyrimidine-based consolidation chemotherapy during the waiting time before TME surgery. The regimens were fluorouracil-based, consisting of fluorouracil; folinic acid, fluorouracil, and oxaliplatin (FOLFOX); or irinotecan and fluorouracil (FOLFIRI). A subgroup of patients enrolled in a prospective study did not receive neoadjuvant radiotherapy (2). After TME surgery, fluoropyrimidine-based chemotherapy was administered. Four to six cycles of chemotherapy were administered before surgery, and six to eight cycles were administered as postoperative adjuvant chemotherapy (2).

Surgical Procedure

Surgery with curative intent was performed according to TME principles at six to eight weeks following the completion of neoadjuvant radiotherapy or two weeks after completion of neoadjuvant chemotherapy (2, 21). Surgery was performed by the experienced director, who had been trained for at least 10 years in a third-grade class A hospital.

Evaluation of MRIs

Two experienced physicians reviewed the baseline and restaging MRI scans. The baseline MRI examination was performed within two weeks before the beginning of treatment; LLNs in the internal iliac, obturator, or external iliac regions were recorded when SA was ≥5 mm with or without morphological changes. After neoadjuvant treatment, the reduction in SA size on the restaging MRI scans before the TME surgery was also recorded. The examination time of the restaging MRI was within two weeks after the consolidation chemotherapy and one week before the TME surgery. When the size of SA was <5 mm on the restaging MRI scan, it was defined as LLN-responsive. When the two experts came to different conclusions, a third physician would make the final decision.

Follow-up

After the TME surgery, all patients were followed up at three-month intervals during the first three years and thereafter at six-month intervals. Physical examinations, chest and abdomen CT, and contrast-enhanced pelvic MRI would be monitored. Lateral local recurrence (LLR) was defined as recurrence at internal iliac, obturator, and external iliac lymph nodes sites, and local recurrence (LR) was defined as recurrence at one of five sites—lateral, presacral, anastomotic site, anterior, or perineal. Distant recurrence (DR) was defined as distant recurrence, when censored, at the latest time. Cancer-specific survival (CSS) was defined as time from the date of surgery to death caused by tumor progression or, when censored, at the latest date if the patient was still alive.

Statistical Analyses

Statistical analyses were performed using IBM SPSS v26 (Chicago, IL, USA). Individual variables were compared by *t*-tests. Survival curves for LR, LLR, DR, and CSS were calculated using the Kaplan–Meier method. To compare the degrees of the reduction of SA of LLN in different treatments, ANOVA was used. To identify risk factors, a univariate Cox regression model was employed, and for patients with multiple LLNs on primary MRI scan, only the largest LLN was analyzed. Differences with a *p*-value of 0.05 were considered statistically significant.

RESULTS

Patient Characteristics

In this study, at least one LLN with SA ≥5 mm was detected in the baseline MRI in 202 cases (**Figure 1**). The median time interval between restaging MRI and surgery was five days (IQR, 0–7 days). Among the 202 patients with clinically positive LLNs on baseline MRI, 94 cases (46.5%) were treated by nCT, 60 cases (29.7%) were treated by nCRT, and 48 cases (23.8%) were given nCRT-boost therapy. Except for the distribution of the LLNs (unilateral or bilateral), there were no statistically significant differences in other variables among the three subgroups (all P >0.05). In the nCRT-boost subgroup, there were more patients with bilateral metastasis LLNs than those in the nCT and nCRT subgroups (P = 0.012) (**Table 1**).

All of the patients underwent TME surgery without LLND, and no patients were surgically margin positive. After the radical surgery, there were no statistically significant differences in the pathologic N stage after neoadjuvant therapy and TME surgery (ypN), vascular and neural invasion, circumferential resection margin (CRM), and adjuvant chemotherapy treatment among the three subgroups (all P >0.05). However, patients in the nCT subgroup had more advanced pathologic T stage after neoadjuvant therapy and TME surgery (ypT) (P <0.001) and the American Joint Committee on Cancer and College of American Pathologists Tumor Regression Grade (AJCC/CAP TRG) stages (P <0.001) (**Supplementary Table 1**).

Primary and Restaged MRI Scans

Based on the largest SA of LLNs on the baseline MRI scans, the mean SA of the LLNs was 8.0 mm (IQR, 5.0–20.3 mm), 8.2 mm (IQR, 5.0–58.0 mm), and 9.6 mm (IQR, 5.2–41.0 mm) in nCT, nCRT, and nCRT-boost subgroups, respectively. After the neoadjuvant treatment, size shrinkage in the nCRT-boost subgroup was greater than that in the nCT and nCRT subgroups (nCRT-boost vs. nCT, P <0.001; nCRT-boost vs. nCRT, P = 0.030). On the restaging MRI, 120 cases (59.4%) with reduced LLNs were smaller than 5 mm. In the nCRT-boost

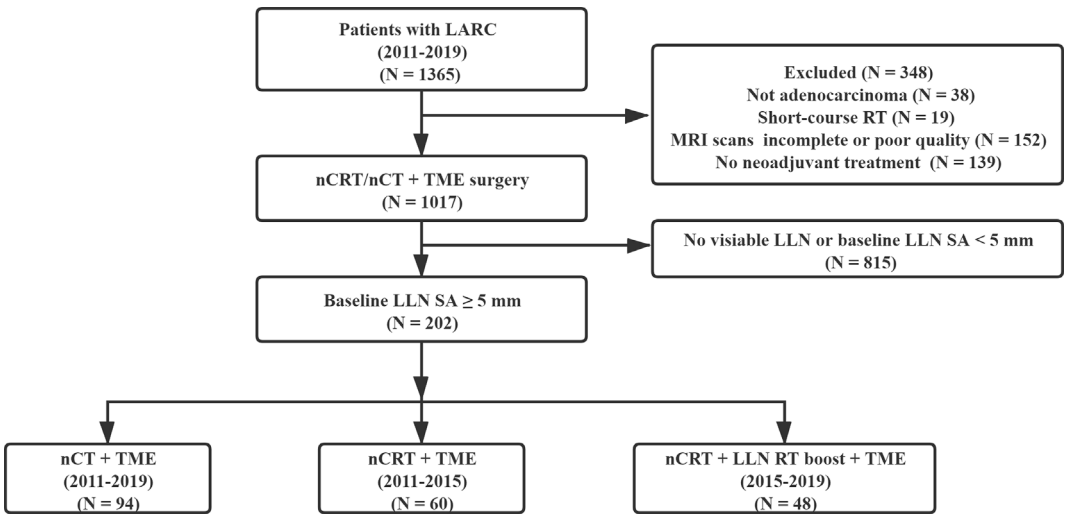

FIGURE 1 | Patient flowchart.

TABLE 1 | Clinicopathological characteristics of 202 patients with LLN metastasis.

Variables	nCT No. (%) n = 94	nCRT No. (%) n = 60	nCRT-boost No. (%) n = 48	*P*-value
Age, median 55 years				0.897
<55	45 (47.9)	31 (51.7)	24 (50.0)	
≥55	49 (52.1)	29 (48.3)	24 (50.0)	
Gender				0.382
Male	64 (68.1)	47 (78.3)	34 (70.8)	
Female	30 (31.9)	13 (21.7)	14 (29.2)	
Clinical T stage				0.320
cT2	1 (1.1)	3 (5.0)	2 (4.2)	
cT3–4	93 (98.9)	57 (95.0)	46 (95.8)	
Clinical N stage				0.287
cN1	32 (34.0)	28 (46.7)	18 (37.5)	
cN2	62 (66.0)	32 (53.3)	30 (62.5)	
Location from anal verge (cm)				0.058
0–5	50 (53.2)	41 (68.3)	34 (70.8)	
5–10	44 (46.8)	19 (31.7)	14 (29.2)	
Tumor differentiation				0.864
Highly differentiated	26 (27.7)	19 (31.7)	16 (33.3)	
Moderately differentiated	54 (57.4)	31 (51.7)	23 (47.9)	
Poorly differentiated	14 (14.9)	10 (16.6)	9 (18.8)	
LLN metastasis				**0.012**
Unilateral	89 (94.7)	55 (91.7)	38 (79.2)	
bilateral	5 (5.3)	5 (8.3)	10 (20.8)	
Chemotherapy regimen				0.947
5-Fu	30 (31.9)	20 (33.3)	15 (31.3)	
FOLFOX	55 (58.5)	36 (60.0)	30 (62.5)	
FOLFIRI	9 (9.6)	4 (6.7)	3 (6.2)	
Location				0.207
Inter iliac	45 (48.4)	40 (66.7)	24 (50.0)	
Obturator	37 (39.8)	15 (25.0)	20 (41.7)	
External iliac	12 (11.8)	5 (8.3)	4 (8.3)	
Surgery type				0.760
Sphincter-preserving operation	85 (90.4)	52 (86.7)	43 (89.6)	
Abdominoperineal resection	9 (9.6)	8 (13.3)	5 (10.4)	

The bold type indicates that the P value is statistically significant.

subgroup, the response rate of the internal iliac nodes, obertuotor nodes, and external iliac nodes were 62.5% (15/24), 95.0% (19/20), and 25.0% (1/4), respectively (internal iliac nodes vs. obertuotor nodes, P = 0.013; internal iliac nodes vs. external iliac nodes, P = 0.285). The entire response rate of the patients in the nCRT-boost subgroup was 72.9% (35/48), which was significantly higher than those of the patients in the nCT (46/94, 48.9%, P = 0.007). The entire response rate of the patients in the nCRT-boost group was also higher than those in the nCRT group (39/60, 65.0%), but there was no statistical difference (P = 0.411). (**Table 2** and **Supplementary Table 2**, **Supplementary Figure 1**).

For further analysis of the pathological characters of patients with restaging LLNs in SA ≥5 mm or SA <5 mm, there were significantly more advanced ypT, ypN, and AJCC/CAP TRG scales in patients with restaging LLN in SA ≥5 mm (all P <0.05). Especially, a higher number of patients in the subgroup with SA ≥5 mm received adjuvant treatment than those with SA <5 mm (P = 0.003) (**Supplementary Table 3**).

Side Effects of Escalation Radiotherapy of LLNs

In the nCRT and nCRT-boost groups, all patients completed radiotherapy. In the nCRT-boost group, a median of 58 Gy (IQR, 56–58 Gy) was boosted in 58 lateral lymph nodes of 48 patients (56 Gy: 23 LLNs; 58 Gy: 35 LLNs). Using the criteria of CTCAE v4.0 (22), the occurrence rate of radiation-related grades 3–4 complications was 29.1% in the nCRT-boost group, and a comparison of the occurrence of toxicity of the patients in the nCRT group did not reveal any significant differences (P = 0.121) (**Supplementary Table 4**).

Survival Analysis

For the 202 patients, the median follow-up time was 35 months (IQR: 12.0–82.0 months). LR was observed in 44 patients, including 30 cases (68.2%) in nCT subgroup, 13 cases (29.5%) in the nCRT subgroup, and only one case (2.3%) in the nCRT-boost subgroup. Out of the 44 patients with LR, 30 had primary internal iliac nodes metastasis (68.2%, 30/44), 14 had primary obturator nodes metastasis (31.8%, 14/44), but none had primary external iliac nodes metastasis (0%, 0/44). Furthermore, 41 local recurrence cases out of the total 44 (93.2%) developed LLR, and 20 patients (45.5%) developed distant metastasis. The LR rates for different cut-off values in SA in baseline LLN-positive clinical patients who received neoadjuvant treatment are listed in **Table 3**. For patients with baseline LLNs SA ≥5 mm, the 3-year LR rate reached 25.1%. We then compared three different neoadjuvant treatment regimens on those patients; the 3-year LR and LLR rates in the nCRT-boost subgroup were lower than the nCT and nCRT subgroups (LR, 2.3% *vs*. 35.6% *vs*.20.4%, P <0.001; LLR, 2.3% *vs*. 31.6% *vs*. 20.4%, P <0.001) (**Figure 2**).

On restaging MRI scans, 120 patients with LLNs disappeared or with SA <5 mm; however, 82 patients were persistently with LLNs ≥5 mm. As summarized in **Figure 3**, the SA of LLNs (≥5 mm vs. <5 mm) was a significant influencing factor for 3-year LR, LLR, DR, and OS. Patients with LLNs ≥5 mm in SA had a significantly high LR (51.3% *vs*. 5.3%, P <0.001), LLR (48.6% *vs*. 4.4%, P <0.001), DR (29.2% *vs*.11.1%, P = 0.001), and poor CSS (85.5% *vs*. 98.6%, P <0.001), compared with those who had LLNs <5 mm in SA.

Furthermore, the associations between the restaging SA size, LR, and LLR rates were analyzed. The SA cut-off value of the LLNs on restaging MRI was 5 mm. The areas under the ROC curve (AUC) for LR and LLR were 0.903 and 0.906, respectively (**Supplementary Figure 2**).

Univariable and Multivariable Analyses

As summarized in **Supplementary Tables 5** and **6**, patients with restaging LLNs ≥5 mm in SA had a significantly higher risk of LR (HR, 8.880; 95% CI, 3.660 to 21.544; P <0.001), LLR (HR, 11.992; 95% CI, 4.679 to 30.731; P <0.001), DR (HR, 2.118; 95% CI, 1.006 to 4.460; P = 0.048), and poor CSS (HR, 8.456; 95% CI, 1.766 to 40.495; P = 0.008) than patients with LLNs <5 mm in SA. In addition, compared with patients in the nCT and nCRT groups, patients treated with nCRT-boost showed independent prognosticators of 3-year LR (HR, 0.075; 95% CI, 0.010 to 0.552; P = 0.011).

DISCUSSION

In our study, we evaluated the survival outcomes in patients with LLN clinical metastasis who underwent three different neoadjuvant treatments. Our study showed that for patients with baseline LLNs SA ≥5 mm, the nCRT-boost to LLNs decreased the LR and LLR rates and reduced the SA of LLNs compared to nCT and nCRT treatments. In addition, the response rate of LLNs in the nCRT-boost group was significantly higher than the response rate of LLNs in the nCT group and tended to be higher than that in the nCRT group. After neoadjuvant treatment, restaging LLNs SA ≥5 mm was associated with inferior LR, LLR, DR, and poor CSS.

According to previous studies, baseline LLNs SA cut-off from 5 to 10 mm were adopted as the clinically positive standard before nCRT (13, 23–25). In a recent large-scale study conducted

TABLE 2 | Baseline and restaging MRI values of LLN SA for patients treated by three different treatment regimens (n = 202).

Variable	nCT n = 94	nCRT n = 60	nCRT-boost n = 48	*P*-value
Baseline SA mean, (mm)	8.0 (5.0–20.3)	8.2 (5.0–58.0)	9.6 (5.2–41.0)	0.208
Shrinkage SA mean, (mm)	3.2 (–1.9–10.7)	4.9 (–0.4–46.0)	6.6 (0.2–16.6)	**<0.001**
Restaging SA mean, (mm)	4.7 (0–18.0)	3.3 (0–14.0)	3.0 (0–24.4)	**0.016**
Response rate (%) (Restaging SA <5 mm)	48.9% (46/94)	65.0% (39/60)	72.9% (35/48)	**0.013**

The bold type indicates that the P value is statistically significant.

TABLE 3 | Three-year local recurrence rates for different cutoff values in SA on baseline MRI in patients with lateral node metastasis.

SA, mm	No. (%)	3-year LR (%)	*P*-value
SA 5			/
<5	/	/	
≥5	202 (100.0)	25.1	
SA 6			**0.002**
<6	58 (28.7)	9.3	
≥6	144 (71.3)	32.1	
SA 7			**<0.001**
<7	79 (39.1)	8.6	
≥7	123 (60.9)	34.1	
SA 8			**0.001**
<8	117 (57.9)	14.3	
≥8	85 (42.1)	36.2	
SA 9			**<0.001**
<9	141 (69.8)	14.5	
≥9	61 (30.2)	45.4	
SA 10			**<0.001**
<10	153 (75.7)	14.2	
≥10	49 (24.3)	52.6	

The bold type indicates that the P value is statistically significant.

by the Lateral Node Study Consortium, a baseline SA of 7 mm was adopted by the cut-off value, and 20% LLR rate was observed in those patients. However, for the patients with baseline LLN SA >5 mm in that study, the LLR rate was also approximately 16% (23). Indeed, LLN SA >5 mm on MRI scans has been proposed as the best cut-off standard in several studies (10, 13, 14). A multicenter MRI study showed that when 5 mm was used as the cut-off value, the LR rate was 21.7% (10). A cut-off value of 5 mm was selected for our study, and the LR rate was 25.1%.

There was a need for a consensus in selecting LLND for the patients based on the restaging imaging findings of LLNs (10, 12, 13, 25, 26). A study conducted at the MD Anderson Cancer Center found that after nCRT, none of the patients with LLN <5 mm had pathologically positive LLNs (13). Oh et al. analyzed 66 patients with suspected LLN involvement who underwent nCRT and LLND, and none of the LLNs were pathologically positive for SA <5 mm; especially for the restaging LLN SA ≥5 mm, the LR rate reached 45.4% (12). Thus, several studies have confirmed that LLN SA ≥5 mm is the optimal criterion for selecting patients for LLND, which might reduce the LR rate, and

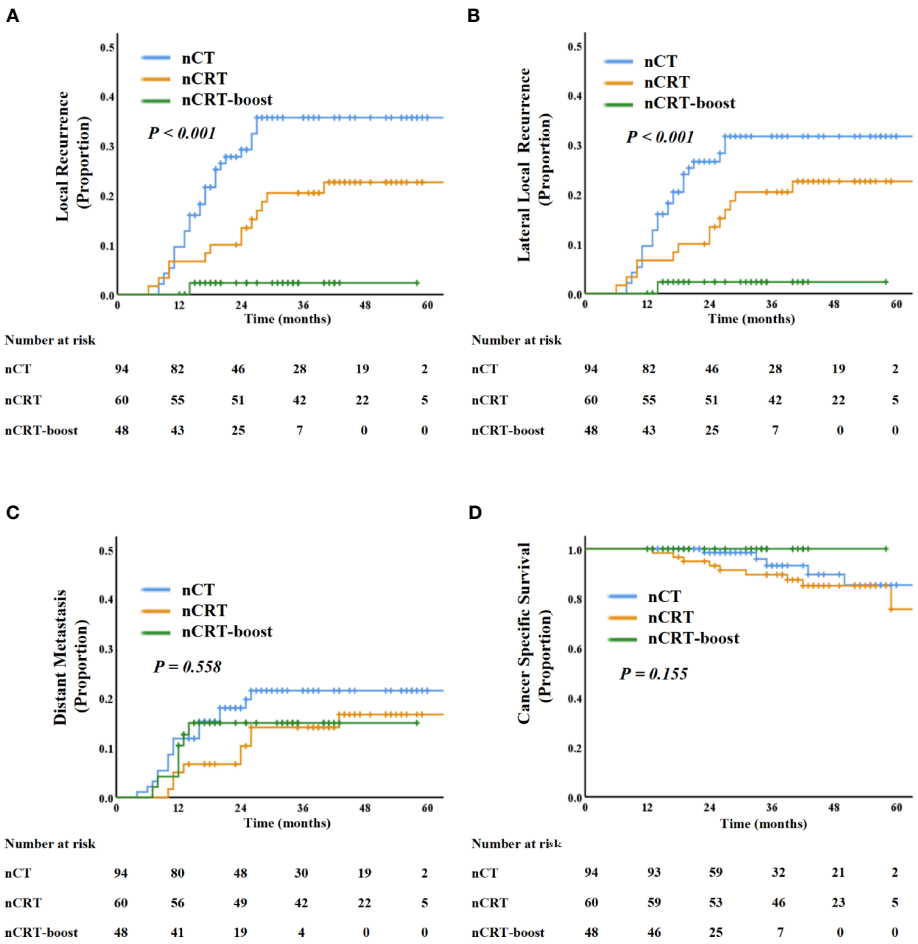

FIGURE 2 | Kaplan–Meier curve analysis of local recurrence **(A)**, lateral local recurrence **(B)**, distant recurrence **(C)**, and cancer-specific survival **(D)** comparing the patients with LLNs metastasis underwent three different treatment regimens: nCT, nCRT, and nCRT-boost treatment.

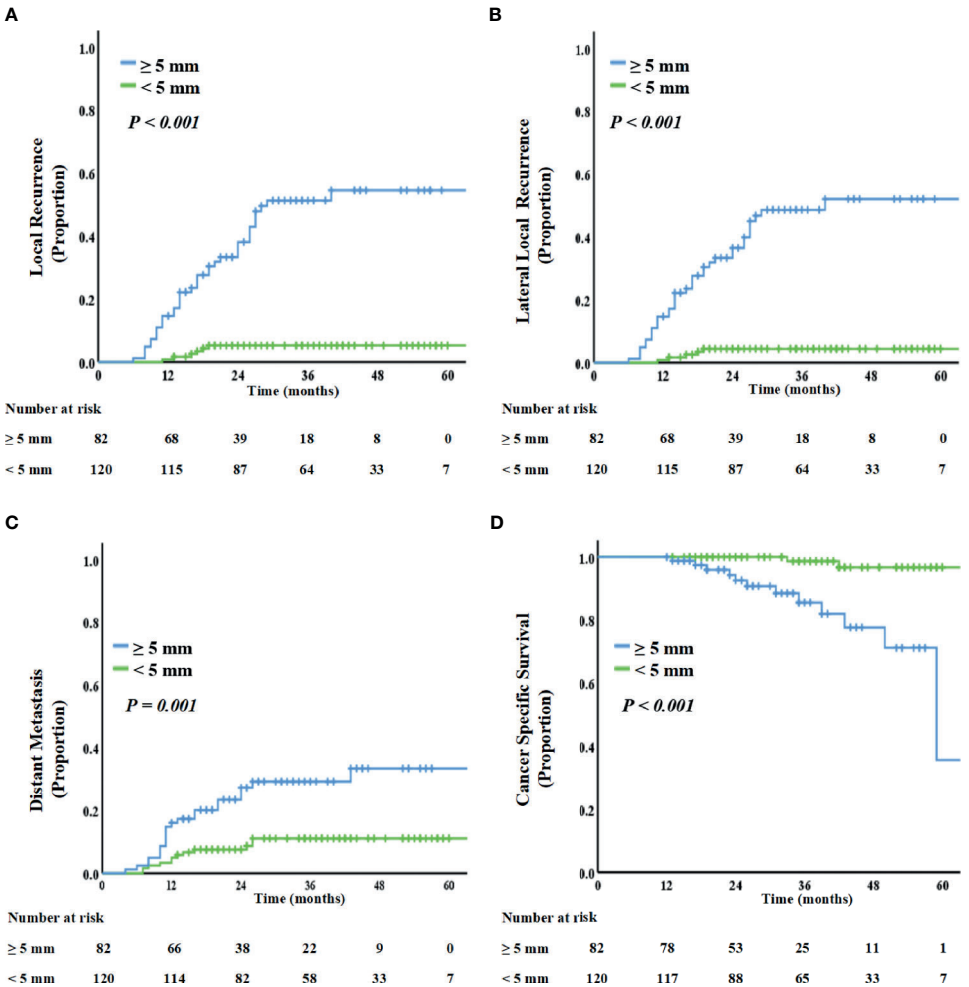

FIGURE 3 | Kaplan–Meier curve analysis of local recurrence **(A)**, lateral local recurrence **(B)**, distant recurrence **(C)**, and cancer-specific survival **(D)**, comparing patients with LLN SA ≥5 mm or <5 mm on restaging MRI.

a recent study has also shown that LLND is also an effective way to decrease the relapse-free survival rate (10, 12, 25). Similarly, in our study, the 3-year LR was 51.3% for patients with restaging LLN SA ≥5 mm, although a greater proportion of patients received adjuvant chemotherapy than those with restaging LLN SA <5 mm. Moreover, our study showed that restaging LLNs with SA ≥5 mm was a significant and independent predictor of LR, LLR, DR, and CSS. The reason might be that the patients with poor reduction in LLN also showed poor tumor response to neoadjuvant treatment. This was demonstrated in our study, i.e., patients with restaging LLN SA ≥5 mm had more advanced ypT stages, ypN stages, and AJCC/CAP TRG stages. Thus, although none of the patients in our study underwent LLND, in light of the high LR rates for the patients with restaging LLN SA ≥5 mm, LLND might be employed.

Although minimally invasive surgery methods such as laparoscopy and robotics cause less bleeding and offer good nerve protection than open surgery (27), it is highly dependent on the experience of the surgeon and is thus generally difficult to perform. Therefore, how to increase the response rate of baseline LLN during the nCRT period is a major challenge. Here, an attempt at an escalation radiation dose based on the regular dose of 56–58 Gy was prescribed to a subgroup of patients with baseline LLN SA ≥5 mm from 2015. In line with our study, a radiation dose boost for the clinical suspicious LLNs was reported in several studies (19, 28, 29). In gynecologic cancers, the radiation dose of LLN was boosted to 60 Gy and did not result in higher morbidity rates (28). In the REG001-09 trial, a median dose of 66.5 Gy was given to the clinically involved prostate cancer lymph node, and the side effect of radiation was acceptable (29). To date, reports on LLN dose escalation in rectal cancer are limited. Only a small-scale study (involving 12 patients) with short-term LR was reported (19). In our study, for patients with LLN metastasis, the LR and LLR rates in the dose escalation subgroup were significantly lower, and size reduction was significantly better than in the nCT and nCRT subgroups. Especially, for patients with LLN SA ≥5 mm on baseline MRI, the response rate in the nCT subgroup was only

48.9%, indicating that the omitting radiotherapy was unfeasible. However, the response rate in the nCRT-boost subgroup was 72.9%, meaning that more patients would avoid LLND. Especially, in line with the studies from the Lateral Node Study Consortium, patients with obturator nodes metastasis achieved a much higher response rate and a lower recurrence rate than those with internal iliac nodes metastasis (30). Significantly, compared with the nCRT subgroup, the rates of enteritis and dermatitis in the nCRT-boost subgroup were similar. These findings suggest that radiation dose escalation might be an effective and acceptable treatment selection for LARC patients with LLN metastasis.

Our study has some limitations. First, it was a retrospective single-hospital study and thus may suffer from selection bias. Some patients with clinically LLN metastasis could not have been included due to unavailable or poor-quality MRI scans. Second, although the patients' baseline clinicopathological characters were not significantly different among the three neoadjuvant treatment schemes, the number of patients included in each subgroup was relatively small. Third, none of the 202 patients enrolled in our study underwent LLND surgery, thus the pathology of the LLNs was missing. Finally, for the patients in the nCRT-boost group, the median follow-up was 30.5 months (IQR: 12.0–58.0 months). Thus, the findings of this study need to be warranted by long-term follow-up and other prospective clinical trials. These limitations were unavoidable given the retrospective nature of this study. A prospective study on the escalation of LLNs is currently being conducted at our hospital.

CONCLUSIONS

For patients with LARC with baseline LLNs SA ≥5 mm, dose escalation of LLNs may lead to a significantly lower rate of LR and LLR. In addition, SA of restaging LLNs was an independent influence factor for prognosis. Especially, for patients with LLN metastasis, dose escalation of LLNs is an effective and acceptable way to reduce the size of LLNs, and LR and LLR rates, and increase the response rate of LLNs, thus allowing more patients to avoid LLND.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JZ and SL were the principal investigators and designed the study. XP performed contouring, treatment planning, and statistical analysis. YM, LH, and ZL provided patient data. XP, HL, XW, and PX checked the data. YM, LH, ZL, JZ, and SL reviewed all data. All authors discussed the data. JZ and XP wrote the draft manuscript and performed subsequent revisions, which were reviewed by all other authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.674253/full#supplementary-material

Supplementary Figure 1 | The baseline and restaging LLNs SA underwent nCT, nCRT, and nCRT-boost treatment.

Supplementary Figure 2 | ROC curve analysis of LLNs SA on restaging MRI to predict individual risk to local recurrence (LR) **(A)** and lateral local recurrence (LLR) **(B)**.

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A Pipeline for Predicting the Treatment Response of Neoadjuvant Chemoradiotherapy for Locally Advanced Rectal Cancer Using Single MRI Modality: Combining Deep Segmentation Network and Radiomics Analysis Based on "Suspicious Region"

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Patients with locally advanced rectal cancer (LARC) who achieve a pathologic complete response (pCR) after neoadjuvant chemoradiotherapy (nCRT) typically have a good prognosis. An early and accurate prediction of the treatment response, i.e., whether a patient achieves pCR, could significantly help doctors make tailored plans for LARC patients. This study proposes a pipeline of pCR prediction using a combination of deep learning and radiomics analysis. Taking into consideration missing pre-nCRT magnetic resonance imaging (MRI), as well as aiming to improve the efficiency for clinical application, the pipeline only included a post-nCRT T2-weighted (T2-w) MRI. Unlike other studies that attempted to carefully find the region of interest (ROI) using a pre-nCRT MRI as a reference, we placed the ROI on a "suspicious region", which is a continuous area that has a high possibility to contain a tumor or fibrosis as assessed by radiologists. A deep segmentation network, termed the two-stage rectum-aware U-Net (tsraU-Net), is designed to segment the ROI to substitute for a time-consuming manual delineation. This is followed by a radiomics analysis model based on the ROI to extract the hidden information and predict the pCR status. The data from a total of 275 patients were collected from two hospitals and partitioned into four datasets: Seg-T (N = 88) for training the tsraUNet, Rad-T (N = 107) for building the radiomics model, In-V (N = 46) for internal validation, and Ex-V (N = 34) for external validation. The proposed method achieved an area under the curve (AUC) of 0.829 (95% confidence interval [CI]: 0.821, 0.837) on In-V and 0.815 (95% CI, 0.801, 0.830) on Ex-V. The performance of the method was

considerable and stable in two validation sets, indicating that the well-designed pipeline has the potential to be used in real clinical procedures.

Keywords: LARC, nCRT, MRI, radiomics analysis, deep learning

INTRODUCTION

Colorectal cancer is currently still the third most common cancer and the second most fatal cancer in the world (1). Nearly 30% sufferers are rectal cancer patients (2), great numbers of which are in the locally advanced stage at initial diagnosis (3).

To date, for patients with locally advanced rectal cancer (LARC), neoadjuvant chemoradiotherapy (nCRT) followed by total mesorectal excision (TME) has been the standard clinical treatment (4–6). The purpose of nCRT is to improve the feasibility of surgical procedures for LARC and reduce the incidence of complications, as it not only improves the local tumor control rate but also exhibits less toxicity to the human body (7). Clinically, the pathological response of LARC patients after nCRT treatment has demonstrated obvious heterogeneity (8). For a large percentage of patients (approximately 70–80%), the tumor will have been found to be shrunken or down-staged, and some patients may even have complete regression. It has been reported that approximately 20% of patients, defined as pathologic complete response (pCR) patients, contain no residual surviving tumor cells after nCRT and surgery (9, 10). These patients have a favorable long-term prognosis with superb local control and disease-free survival (11). Therefore, for pCR patients, the option of organ-saving treatment could be developed to replace surgery. However, currently, the only way to accurately diagnose pCR is to utilize a pathological diagnosis after TME surgery, which presents an insoluble dilemma (12–14). As a result, a prediction method before surgery would greatly assist doctors in evaluating the treatment effects of nCRT and construct a tailored plan for each patient.

In recent years, magnetic resonance imaging (MRI) has been widely used as a non-genetic and non-invasive diagnostic method to assess the tumor condition due to its superior soft-tissue contrast and high spatial resolution. The T2-weighted (T2-w) MRI is recognized as the most important modality for rectal cancer assessments (15). Some previous works have attempted to evaluate the response of nCRT on T2-w MRI using assessments of post-treatment T staging (ymrT), tumor regression grading (mrTRG), volume reduction post-treatment, and other characteristics (16). However, the results were dependent on the experience of doctors, thus introducing subjectivity. The development of more objective ways to extract information from the MRI and guide clinical diagnosis is required.

Radiomics is a mathematical technique that utilizes high-throughput extraction of shape, intensity, and texture features from images, and transforms this visual information into high dimensional features for quantitative analysis (17). Radiomics analysis can help obtain additional image information with reliability and objectivity that may be invisible in human assessments (18). Applying a radiomics analysis to predict the pCR on an MRI has drawn increasing attention. In many studies (19–21), researchers have used a pre-nCRT MRI to analyze the relationship of the radiomics features and the pCR status. The use of a pre-nCRT MRI could provide a clear tumor region for analysis. However, considering that the nCRT could affect the tumor and change its properties, the use of a pre-nCRT MRI is indirect and may not reflect the true condition of the patient after nCRT treatment.

Analysis of a post-nCRT MRI might be a more direct method. However, the problem still remains that the region of interest (ROI) delineation on a post-nCRT is much more difficult due to tumor recession and the appearance of the fibrosis region. In previous studies (22, 23), researchers applied a pre-nCRT MRI to provide a reference of the primary tumor region or the treated region for a post-nCRT MRI. However, a pre-nCRT MRI is not always available in real clinical practice, as some patients may be diagnosed with LARC using proctoscopy, and some patients may be transferred from other hospitals without access to the two previously scanned MRIs. Currently, only a few of studies have utilized a post-nCRT MRI to predict the pCR status. The work of Horvat et al. (15) obtained considerable results by applying a radiomics analysis on a post-nCRT T2 and diffusion-weighted imaging (DWI) MRI; however, the ROI was still obtained due to a careful discussion by at least two experienced physicians. An accurate delineation might be difficult to obtain for less experienced physicians without the reference of a pre-nCRT MRI. Additionally, it is time-consuming and resource-wasting if each delineation requires at least two physicians in clinical practice. Inspired by this, the aim of this study is to explore a pipeline that only uses the information from a single post-nCRT T2 MRI combined with a new method to provide a fast and reliable ROI. Deep learning uses multiple layers as a portion of a broader family of machine learning methods and has been successfully applied to various medical tasks (24–28).

In this study, we introduce a deep learning model for ROI delineation. A novel two stage model, termed the two-stage rectum-aware U-Net (tsraU-Net), is proposed to replace human evaluation. The ROI should be feasible for a deep learning model to find and contain sufficient information relating to the pCR status; hence, it is defined on a continuous region having abnormal intensity signals on a T2-w MRI assessed by radiologists that has a high possibility to contain a tumor or fibrosis. It is considered a rougher region, as the further identification of a tumor, fibrosis, or other tissues like edema is not defined. The following analysis extracts a great number of radiomics features, including texture, first-order statistics, and shape, on the ROI and its wavelet decompositions to represent certain properties. Machine learning and statistical techniques are later applied to select the most representative features and construct a final model to predict the pCR status.

MATERIALS AND METHODS

Patients

We included a total of 496 patients from multiple institutions who received nCRT treatment diagnosed with LARC. Patients were retrospectively enrolled from July 2011 to December 2018 from two hospitals (Guangdong Institute of Gastroenterology, Sixth Affiliated Hospital of Sun Yat-sen University and Yunnan Cancer Center). The inclusion criteria were as follows: (1) adenocarcinoma confirmed by pathologists (excluding mucinous adenocarcinoma); (2) tumor located within 15 cm from the edge of the anus; (3) received nCRT treatment and TME; (4) in the clinical stage of T3–4 or N-positive; (5) completed the restaging MRI; and (6) the restaging MRI was performed no more than 1 week before the TME. This clinical trial was approved by the Clinical Ethics Review Committee of Sixth Affiliated Hospital of Sun Yat-sen University (2020ZSLYEC-010).

Patients from the Sixth Affiliated Hospital of Sun Yat-sen University were separated into three groups. Dataset Seg-T consisted of patients from July 2011 to June 2013 to train deep networks for ROI segmentation; dataset Rad-T consisted of patients from June 2013 to May 2017 to build the radiomics model for predicting the pCR status; and dataset In-V consisted of patients from June 2017 to December 2018 for internal validation. In addition, patients from Yunnan Cancer Center (dataset Ex-V) were used as an external validation set. Patients were further selected according to the following exclusion criteria: (1) poor MRI quality caused by severe inflammatory effusion, intestinal adhesions, or bowel movements, and (2) (for dataset Rad-T, Val, E-Val) the absence of a postoperative pathological diagnosis. **Figure 1** shows the flowchart of the patient selection process.

All of the patients' treatments were discussed by the multidisciplinary team (MDT). Patients were delivered the intensity-modulated radiotherapy treatment (IMRT), and a dose of 45 Gy for 25 fractions was delivered to the clinical target volume. Then, a boost dose of 5.4 Gy was delivered to the

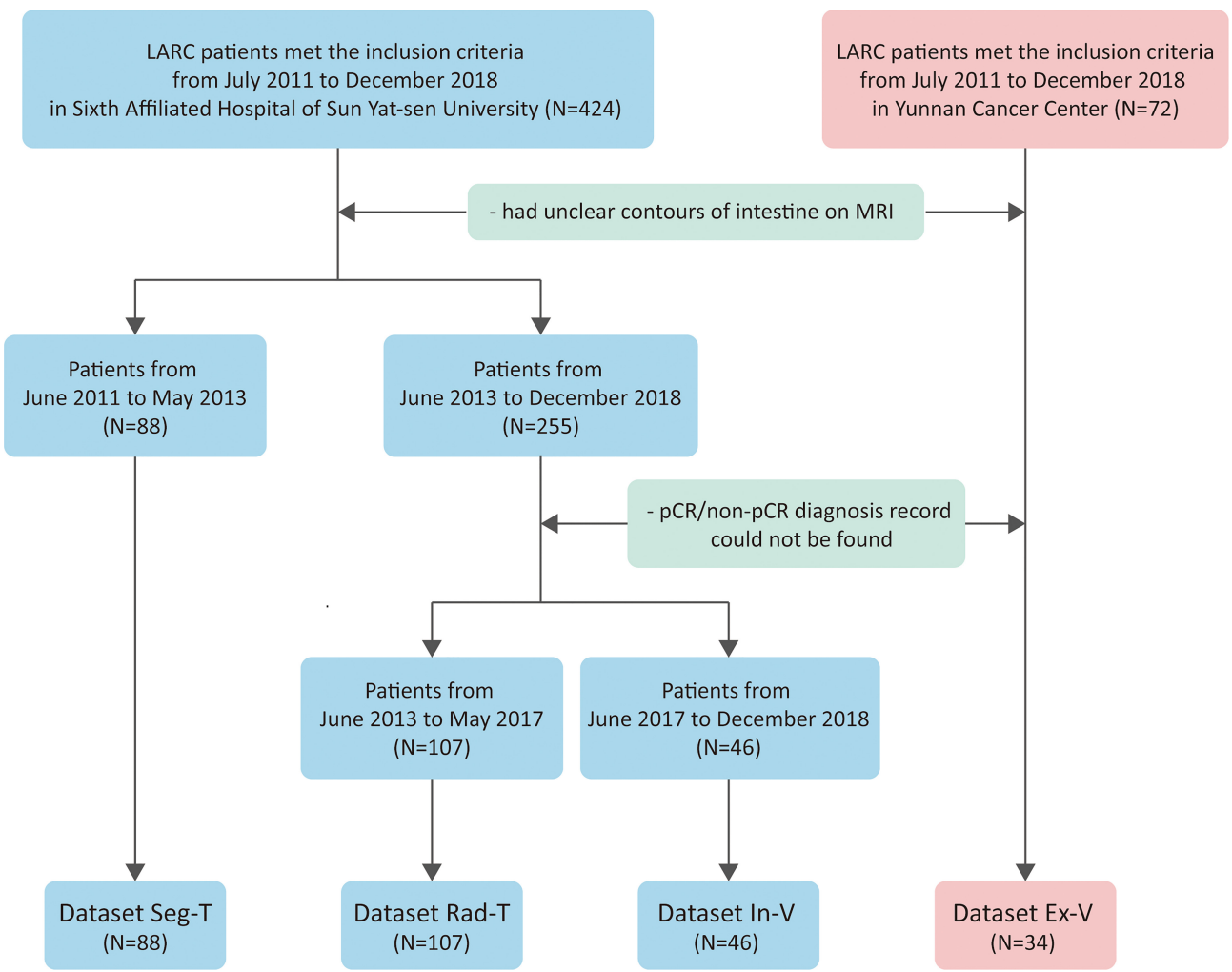

FIGURE 1 | Flowchart showing how patients from two hospitals were collected and partitioned.

gross tumor. The concurrent chemotherapy treatment was based on oral or intravenous 5-fluorouracil. Following the completion of neoadjuvant treatment, all of the patients received TME surgery. The majority of patients received adjuvant chemotherapy based on FOLFOX or CAPOX based on the decision of the members of the MDT.

Pathology Assessment of Response

In this study, the pCR diagnosis was confirmed by two pathologists with more than 12 years of experience. Following the recommendation of the NCCN Guidelines for rectal cancer (29), patients with no surviving tumor cells in the surgical pathological specimens were judged as pCR; otherwise, they were judged as non-pCR.

MRI Data Acquisition

All of the MRI images were scanned under a 1.5-Tesla MRI unit (30). Bowel preparation was not routinely used for most cases prior to the examination. However, some specific patients with relatively small tumors in the sagittal view were filled with some rectal gel, making it easier to identify tumors on the oblique axis. The MRIs in the datasets Seg-T, Rad-T, and In-V (Sixth Affiliated Hospital of Sun Yat-sen University) were acquired using GE OPTIMA MR360 with a 100 ms echo time, a 4000 ms repetition time, a 100 field of view, a 512 × 512 matrix, 0.4–0.5 pixel spacing, and 5 mm slice thickness. The MRIs in the dataset Ex-V (Yunnan Cancer Hospital) were acquired using Philips Ingenia with a 100 ms echo time, a 4000 ms repetition time, a 100 field of view, a 432 × 432 matrix, 0.4–0.5 pixel spacing, and 5 mm slice thickness.

Data Pre-Processing

As suggested by some researchers (31), we applied complex methods to the pre-process MRI to both improve the image quality and unify the geometric and intensity patterns with the aim to assure the success of our analysis. The steps included (1) all of the MRIs were resampled into 0.4 mm × 0.4 mm pixel spacing using bilinear interpolation; (2) the size of each image matrix was unified into 544 × 544 by cutting or padding the background; (3) the intensity of each patient was adjusted using BiasCorrection to remove any inhomogeneity; and (4) the intensity histogram of each patient was matched to one selected patient (as template) who was from Seg-T. All of the procedures were implemented using the open-source python package "SimpleITK" (32).

Suspicious Region Definition

The "suspicious region" in our study was defined as a continuous region containing 129 abnormal intensity signals compared to a normal rectal wall, which are highly suspected to be cancer or fibrosis according to clinical experience. Following the guidance (33), the abnormal signals may have presented as slightly high, low, or mixed intensities. By such definition, the exact cancer and fibrosis region was not further distinguished. Instead, we relied on the radiomics analysis to elicit the hidden properties of the cancer or fibrosis and predict the pCR. In particular, the "suspicious region" was delineated by radiologists on only a post-nCRT T2-w MRI. As the region was visible to human vision, we assumed it could be captured by deep learning as well. Therefore, the use of "suspicious region" was both sufficient and proper in the pipeline that combined a radiomics analysis and deep learning. **Figure 2** provides some examples of the suspicious region.

Deep Learning-Based Segmentation

The tsraU-Net Model

To provide a reliable ROI using deep learning for the radiomics analysis, the most important consideration was rectum localization. If the deep segmentation network misrecognized the rectum with other organs such as the colon, uterus, bladder, or prostate due to a morphology change or location shift of the rectum, it could still find a "suspicious region," but not related to the pCR at all, thereby making the radiomics analysis totally meaningless. To address such a problem, we proposed a two-stage model, named the two-stage rectum-aware U-Net (tsraU-Net), which would first find the rectum region and then segment the ROI using the awareness of the rectum location. The overall framework of the tsraU-Net model is shown in **Figure 3**.

U-Net, a deep segmentation network expressly designed for biomedical segmentation tasks (24), was applied as a base model in both of the two stages of the tsraU-Net. Further improvements were made in each stage according to the task. The detailed descriptions were organized as follows. First, we briefly introduced the base model, U-Net. Next, we provided comprehensive explanations of the improvements in the two stages of tsraU-Net. Finally, we described other adjustments of the base model.

The Original U-Net Model

The original U-Net is a fully convolution network (FCN) containing an encoder to extract features and a decoder to reassemble features. Typically, both of them have five convolution blocks, s.t. each block consists of two 3 × 3 convolutions and a rectified linear unit (ReLU) for activation. After going through the convolution block, the number of features (more specifically, channels) would double in the encoder part and halve in the decoder part symmetrically. Between each convolution block in the encoder, the max pooling operation is applied to reduce the image resolution. Oppositely, an up-sample operation is inserted into the neighboring convolution blocks in the decoder to increase the image resolution. To fully utilize high resolution information, high resolution features in the encoder are concatenated to the corresponding convolution blocks in the decoder. This is named a "skip connection." In addition, a 3 × 3 convolution is applied after the last convolution block in the decoder to combine the rest of the features and obtain the final segmentation result.

The First Stage

In the first stage, we designed a four-channel 2D U-Net aiming to guide the segmentation with a plentiful amount of information. Knowing that rectal regions would maintain certain continuity between neighboring MRI slices, the input of our model contains not only the currently input MRI slice but also its previous slice and the next slice to help detect the contours of the rectum. If the rectal wall is unclear compared with the neighboring region on

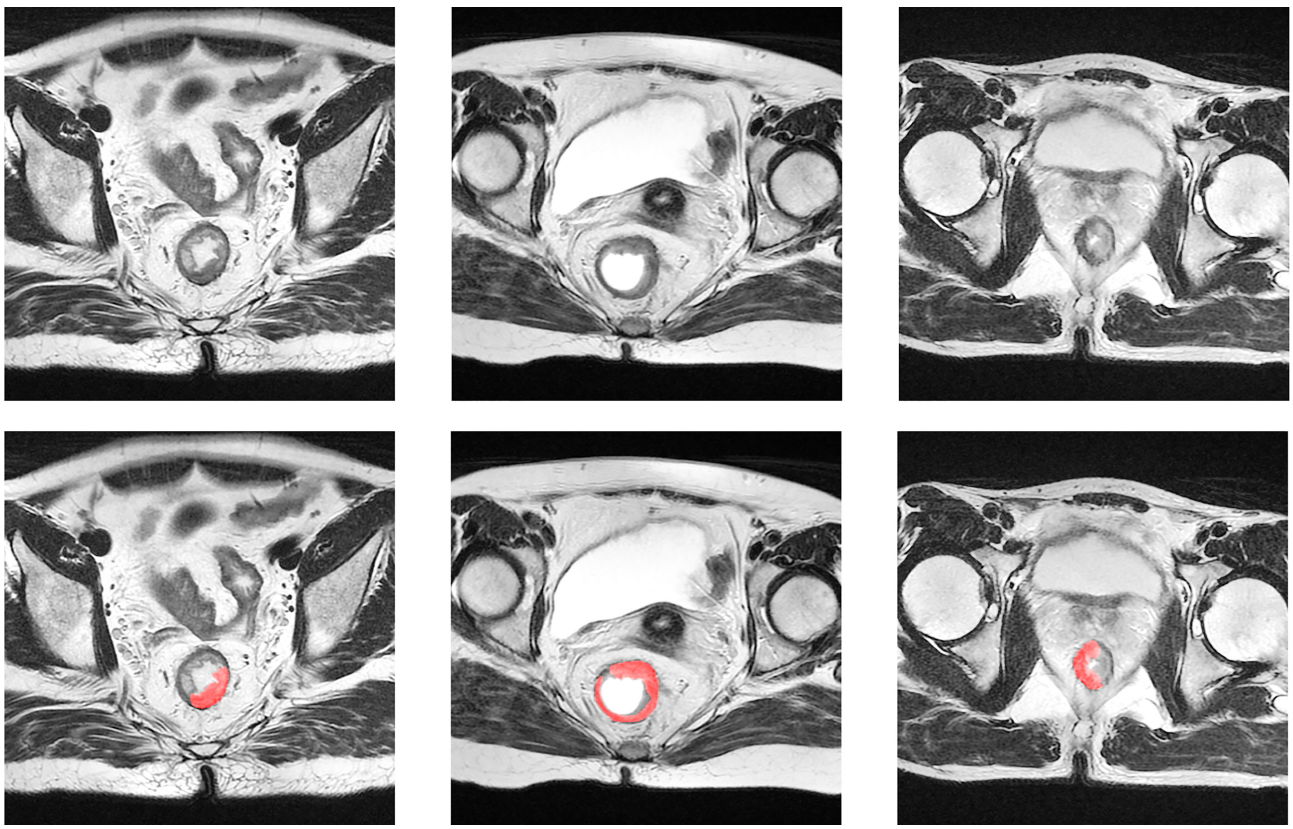

FIGURE 2 | Examples of suspicious region. The first row is the original T2-w MRI, and the second row displays the delineation of the suspicious region in red color.

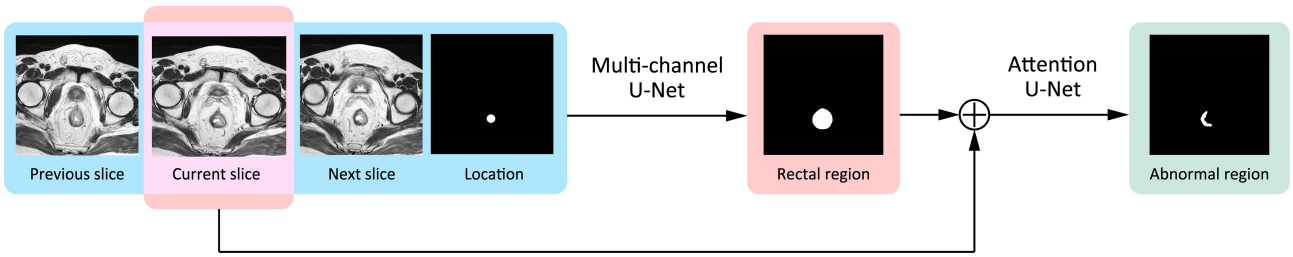

FIGURE 3 | The framework of tsraU-Net model.

the current slice, the other two slices may provide extra information. Furthermore, for each patient, we roughly marked two to four points inside the rectum for localization. Initially, the first and last MRI slices were given localization points, with bilinear interpolation applied between them to give localization information to the middle layers. If the shape of the rectum was not regular, another one to two points will be given in the middle layers. The previous slice, the current slice, the next slice, and the position information were combined into four channels as the input of our first stage model. The output of the first stage was the region of the rectum.

The Second Stage

The second stage would use the currently inputted slice and the predicted rectal region in the first stage to find the "suspicious region." In this stage, we focused on strengthening the model awareness of the abnormal intensity signals and, hence, applied an "attention" mechanism. "Attention" was first introduced in the natural language process (NLP) tasks to encourage models to pay more attention to efficacious information and suppress irrelevant information. There are two types of attention, i.e., soft attention and hard attention. In this task, we used the soft attention mechanism for the model (34). This method would

update the propagated features from "skip connection" by point-wise multiplication with a weight matrix given by an attention gate. An attention gate is a block containing several 1 × 1 convolutions and activation functions. It uses both the propagated features and the features from the corresponding former decoder block as input. **Figure 4** provides a detailed explanation of an attention gate. Specifically, we inserted four attention gates into the original U-Net model s.t. every "skip connection" was followed by an attention gate.

Adjustments on Base Model

In addition to the above methods, we also made some small adjustments to the original U-Net model: (1) the addition of image padding during convolution so that the image size would not change; (2) the addition of instance normalization after each convolution block to accelerate the convergence; and (3) the replacement of the ReLU activation function with the Leaky Rectified Linear Unit (Leaky ReLU) to prevent the vanishing gradient problem.

Loss Function

The loss function in both two stages is the Dice Loss, which is widely used in medical image segmentation. It is defined from the dice coefficient, which essentially measures the overlap of two sets. The dice coefficient has a range of 0–1, where 1 means complete overlap. It is defined as Equation 1:

$$\text{Dice} = \frac{2|A \cap B|}{|A| + |B|} \tag{1}$$

where $A \cap B$ is the intersection of sets A and B, $|\,|$ represents the number of elements in the set.

As for Dice Loss, it is simply defined by the following Equation 2.

$$\text{Dice Loss} = 1 - \textit{Dice} \tag{2}$$

Experiment Setup

To provide the gold standard of segmentation, two radiologists (one had 6 years of experience and one had 9 years of experience) reviewed the post-nCRT T2-w MRI of the Dataset Seg-T and InV (for validating the performance), and they jointly provide delineations of the rectum and the "suspicious region." When delineating the "suspicious region," the normal rectal wall should be avoided, and for some patients with rectal gel filling, the gel should also be avoided. Both radiologists were completely blinded to the histopathology information, as well as the pre-nCRT MRI of patients. Following the instructions in the guidance (33), they used the rectal wall as a reference to find abnormal signals. This work was performed *via* ITK-snap version 3.4.0 software (http://itk-snap.org).

While training, the initial hyper-parameters were established identically in two stages. The optimizer we used was the Adaptive Moment Estimation (Adam) (35) with an initial learning rate $\alpha = 2 \times 10^{-4}$, $\beta = (0.9,0.999)$ and would decay 30% every 20 epochs. The maximum training epoch was set to 120, the early stopping method was applied to prevent overfitting, and the training would stop if performance on the minority set did not improve over 30 epochs. The model was implemented with PyTorch 1.8.1 (36) on a Nvidia Titan X GPU with 12 G of memory.

Radiomics Analysis

After the segmentation model was well-trained, it was directly applied to the Rad-T, In-V, and Ex-V to obtain the ROI. Then, the following procedures of the radiomics analysis were applied to build the pCR prediction model.

Feature Extraction

In order to extract useful information related to the pCR status, a large feature space was generated, which included features not only from the original image but also from its wavelet decomposition images. A total of 93 types of features were calculated on each original image and its four Harr wavelet subbands, i.e., HH, HL, LH, and LL. The 93 features include 18 intensity features and 75 texture features. In addition, nine shape features were extracted on the original image. Together, 474 features were generated from each MRI slice.

Concerning that each patient had a different number of MRI slices, we used the arithmetic mean, three quartiles (Q1, Q2, and Q3 points), and the standard deviation of the features extracted from all of the slices as representations. Therefore, each patient had 2370 features in total.

All of the features were extracted using the Python package "PyRadiomics" (37). As announced in its document, most

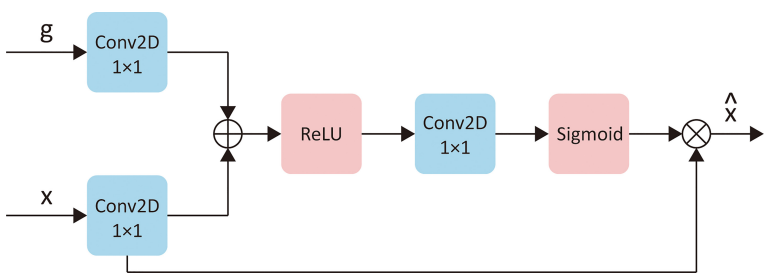

FIGURE 4 | The detailed explanation of attention gates. Let x represents the "skip connect" features from encoder, g represents the features from corresponding former decoder layer, then $\hat{x}$ is the updated of x. "Conv2D 1×1" represents a 2D convention with kernel size of 1×1. "ReLU" and "Sigmoid" are two different activation functions.

features meet the Introduction Intellidyne Business Systems (IBSI) standard, which would increase the reliability to our experiments. More details are shown on the "PyRadiomics documentation" website (http://pyradiomics.readthedocs.io).

Feature Selection

Two feature selection steps were applied in our study to increase the robustness and avoid overfitting. First, we evaluated the discriminative power of each single feature by calculating the Harrell's concordance index (C-index) (38) between the features and the pCR status. The univariate Cox analysis is a commonly used procedure in survival analysis, and we adopted this method because it does not require the features to follow a normal distribution compared with a *t*-test. Let $A = \{\alpha_1, \alpha_2, \ldots, \alpha_n\}$ denote the features of patients, β present the pCR status of patients, for each feature $\alpha_f \in A$, the C-index can be computed with the Equation 3.

$$C - \text{index}(\alpha_f, \beta) = \frac{\Sigma_{i,j} U(\alpha_{fj} < \alpha_{fi}) \cdot U(\beta_j < \beta_i)}{\Sigma_{i,j} U(\beta_j < \beta_i)}, \quad (3)$$

where α_{fi} is the i-th value of α_f, β_i is the i-th value of β, and $U(a < b) = 1$ if $a < b$ else 0.

By definition, C-index equals to 1 means the best discriminative power and C-index equals to 0.5 represents a theoretically result of random prediction. We calculate the maximum of C-index(α_f, β) and C-index($-\alpha_f$, β) as the predictive score of feature α_f. After calculation, scores are sorted and features with lower score are excluded.

In the second step, the remaining features were put into the least absolute shrinkage and selection operator (LASSO) (39) for further selection. LASSO is a logistic regression model with L1 regularization as a penalty of the coefficients. It will encourage the regression use of sparse features. The objective function of LASSO is:

$$\min \frac{1}{2} ||Ax - \beta||_2^2 + \lambda ||x||_1, \quad (4)$$

where A is the matrix of radiomics features, x is the coefficient of each feature, β is the pCR status, and λ is the regularization penalty coefficient.

Due to the L1 regularization, the LASSO forces the sum of the absolute value of the regression coefficients to be less than a fixed value, minimizing the residual sum of the squares. Such an operation forces the certain coefficient to zero. After the LASSO regression, features with a coefficient of non-zero are retained. Here, λ was determined using a grid search and 5-fold cross validation on 100 iterations between 0.01 and 0.2.

The pCR Status Prediction

In our study, the support vector machine (SVM) (40) was applied to predict the pCR status. As suggested in study (41), the radial basis function (RBF) kernel was used. The RBF kernel is defined as Equation 5:

$$K(a, b) = e^{(-\gamma ||a-b||^2)} \quad (5)$$

where a, b are two samples from dataset and γ is a hyper-parameter. γ and the regularization coefficient C of SVM were determined also by grid search on 5-fold cross validation within set {1/16, 1/8, 1/4, 1/2, 1}. After choosing the best γ and C, the SVM model was trained for the pCR prediction.

Performance Evaluation

Various evaluation metrics of suspicious region segmentation and pCR prediction are listed below.

Three metrics—dice coefficient, sensitivity, and specificity—were applied to evaluate the performance of segmentation. The dice coefficient's definition has been given in Equation 1. Sensitivity (SEN) and specificity (SPC) are defined as Equation 6 and Equation 7, where TP, FP, TN, and FN denoted true positive, false positive, true negative, and false negative, respectively.

$$\text{SEN} = \frac{TP}{TP + FN} \quad (6)$$

$$\text{SPC} = \frac{TN}{TN + FP} \quad (7)$$

As for the pCR status prediction, five metrics—the area under receiver operating characteristic (ROC) curve (AUC), accuracy, sensitivity, specificity, and the F-score—were applied for the evaluation. The F-score is a weighted harmonic mean that comprehensively considers sensitivity and specificity, which can be calculated as Equation 8.

$$F_\beta - score = (1 + \beta^2) \cdot \frac{SEN \times SPC}{\beta^2 \cdot SPC + SEN} \quad (8)$$

Here, we included $F_{0.5}$, F_1, and $F_{1.5}$ in order to provide a multiple trade-off between specificity and sensitivity under different situations.

RESULTS

Clinical Characteristics

In our study, 241 of 424 patients from Guangdong Institute of Gastroenterology, Sixth Affiliated Hospital of Sun Yat-sen University and 34 of 72 patients from Yunnan Cancer Center met the inclusion criteria and did not meet the exclusion criteria. After selection, the number of patients in SegT, Rad-T, In-V, and Ex-V were 88, 107, 46, and 34, respectively. More clinical information of the Rad-T, In-V, and Ex-V groups is provided in **Table 1**. Statistical comparisons were performed for each clinical characteristic between the two response groups (pCR vs. non-pCR). There were no statistical differences between the pCR and non-pCR in sex, age, and the pre-CRT N stage in all three sets.

Segmentation Performance

When training the segmentation model, Seg-T was randomly separated into two sets with percentages of 70% and 30%. The s.t. of the majority set was used for updating the model, and the minority set was used for selecting the best network parameters.

TABLE 1 | The clinical characteristics of patients in dataset Rad-T, In-V, and Ex-V.

Characteristics	Dataset Rad-T		*P-value*	Dataset In-V		*p-value*	Dataset Ex-V		*p-value*
	pCR[a] (n = 36)	NonpCR (n = 71)		pCR (n = 8)	NonpCR (n = 38)		pCR (n = 6)	NonpCR (n = 28)	
Sex			0.72			0.24			0.18
Male	8 (22.2%)	18 (25.4%)		5 (62.5%)	31 (81.6%)		2 (33.3%)	19 (67.9%)	
Female	28 (77.8%)	53 (74.6%)		3 (37.5%)	7 (18.4%)		4 (66.7%)	9 (32.1%)	
Age (mean ± SD[b], year)	53.4 ± 12.1	56.9 ± 9.9	0.11	57.9 ± 8.4	55.0 ± 11.1	0.49	60.0 ± 10.8	59.2 ± 8.2	0.83
Pre-CRT[c] T stage			0.51			<0.01			0.31
T0	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
T1	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
T2	1 (2.8%)	3 (4.2%)		0 (0%)	0 (0%)		1 (16.7%)	0 (0%)	
T3	31 (86.1%)	55 (77.5%)		8 (100%)	30 (78.9%)		2 (33.3%)	8 (28.6%)	
T4	4 (11.1%)	13 (18.3%)		0 (0%)	8 (21.1%)		3 (50.0%)	20 (71.4%)	
Pre-CRT N stage			0.59			0.2			0.33
N0	4 (11.1%)	13 (18.4%)		0 (0%)	13 (34.2%)		1 (16.7%)	1 (3.6%)	
N1	17 (47.2%)	29 (40.8%)		4 (50.0%)	9 (23.7%)		1 (16.7%)	1 (3.6%)	
N2	15 (41.7%)	29 (40.8%)		4 (50.0%)	16 (42.1%)		4 (66.6%)	26 (92.8%)	
Post-CRT T stage			< 0.01			<0.01			< 0.01
T0	36 (100%)	0 (0%)		8 (100%)	0 (0%)		6 (100%)	0 (0%)	
T1	0 (0%)	7 (9.9%)		0 (0%)	2 (5.3%)		0 (0%)	4 (14.4%)	
T2	0 (0%)	18 (25.4%)		0 (0%)	11 (28.9%)		0 (0%)	6 (21.4%)	
T3	0 (0%)	43 (60.6)		0 (0%)	22 (57.9%)		0 (0%)	9 (32.1%)	
T4	0 (0%)	3 (4.2%)		0 (0%)	3 (7.9%)		0 (0%)	9 (32.1%)	
Post-CRT N stage			0.01			<0.01			0.17
N0	36 (100%)	50 (70.4%)		8 (100%)	30 (78.9%)		6 (100%)	21 (75.0%)	
N1	0 (0%)	20 (28.2%)		0 (0%)	8 (21.1%)		0 (0%)	6 (21.4%)	
N2	0 (0%)	1 (1.4%)		0 (0%)	0 (0%)		0 (0%)	1 (3.6%)	
Post-CRT CRM[d]			0.48			/			/
Negative	36 (100%)	70 (98.6%)		8 (100%)	38 (100%)		6 (100%)	28 (100%)	
Positive	0 (0%)	1 (1.4%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	

[a]pCR, pathologic complete response. [a]SD, standard deviation. [c]CRT, chemoradiotherapy, [d]CRM, circumferential resection margin.

After training, the model was validated on In-V, and the results are listed below.

The First Stage

The numerical results of stage one, segmentation of the rectum, are presented in **Table 2**. The results of the original U-Net are also provided for comparison. It can be seen that the 4-channel U-Net had a significant improvement compared to the original U-Net on the rectum segmentation. Moreover, as the 4-channel U-Net achieved (0.942, 0.965) 95% CI of the dice coefficient, it could be inferred that this network could provide a stable 300 and accurate rectum segmentation.

The visual results of the 4-channel U-Net and the original U-Net are also presented. **Figure 5** shows eight typical cases of rectum segmentation. The 4-channel U-Net had successfully segmented the rectum regions in all of the cases, while the U-Net showed different defects. In case (A), the U-Net was able to find the rectum, but the morphology lacked accuracy. In case (B), because the U-Net had no position information, the prostate was mistakenly judged as the rectum. In case (C), the U-Net outputted a continuous region containing both the rectum and the uterus. In case (D), due to the unclear rectal wall, the U-Net produced a bad result with an undesirable shape. In case (E), the prediction of the U-Net was less regular compared with the 4-channel U-Net. In case (G), the U-Net seriously under-segmented the rectum region. In case (H), the U-Net found two separated regions with similar sizes. Among those defects, (A) and (E) might be improved by post-processing methods, but for the rest, even postprocessing methods such as image dilation or the removal of the smaller region seems useless to obtain the correct rectum region. Thus, our design in the first stage has great importance for guaranteeing the success of the following analysis.

The Second Stage

Numerical results of stage two, segmentation of the "suspicious region," are presented in **Table 3**. For a fair comparison, the U-Net model in this stage was also given the rectum localization information from the first stage, and we intended to evaluate the use of the attention mechanism. From the results, the attention slightly improved the result. The dice coefficient and specificity may not be considerably high, but the sensitivity achieved nearly 0.8, indicating that the network could find the major portion of the "suspicious region."

Figure 6 shows a visual display of the tsraU-Net, including the last attention maps, the final segmentation results, and the overlapping region compared with the gold standard. In some cases, such as (B), (D), and (F), the segmentation results are oversized. However, the morphology between them is still similar, and the segmentation results do not neglect most of the gold standard. Consequently, we believe the model is capable of providing the ROI with enough information for radiomics analysis.

TABLE 2 | Comparison between U-Net and 4-channael U-Net in the dice coefficient, sensitivity and specificity between the gold standard and the results from stage one, that is, 4-channel U-Net, in tsraU-Net as well as the baseline, original U-Net.

Model	Dice	Sensitivity	Specificity
U-Net	0.861 (95% CI[a]: 0.850, 0.873)	0.876 (95% CI: 0.864, 0.888)	0.867 (95% CI: 0.852, 0.882)
4-channel U-Net	0.954 (95% CI: 0.942, 0.965)	0.967 (95% CI: 0.955, 0.980)	0.96 (95% CI: 0.945, 0.976)

[a]*CI, confidence intervals.*

Treatment Response Prediction

A total of 2370 features representing certain properties of the "suspicious region" predicted by tsraUNet were extracted from each patient. These features were progressively selected in the initial univariate analysis, and only approximately the top 2.5% features, which was 63 features, remained according to their predictive scores. The histogram of the predicted scores with the number of features is illustrated in **Figure 7A**. A distinct gap was found between the remaining features and the excluded features with a corresponding threshold of 0.622.

The remaining features were then put into LASSO. After a grid search and cross-validation, the best λ was 0.293. The grid search of λ in the LASSO regression to minimize the residual mean square error (MSE) is visually provided in **Figures 7B, C**, which provides the coefficients of the features during the grid search. Ten features were finally chosen, and they are presented in **Table 4**. The detailed descriptions of these features can be found in the "PyRadiomics documentation."

The remaining 10 features was used to build a SVM classifier. The hyperparameters were decided after grid search and cross-validation: $C = 1$ and $\gamma = 0.125$. After training, the SVM achieved 0.924 of the AUC on Rad-T, 0.829 on In-V, and 0.815 on Ex-V. More numerical results are displayed in **Table 5**. In addition, **Figure 8** gives a visual display of the AUC and SVM scores [provided by the Python package "scikit-learn" (42)].

We further applied *t*-test on the three datasets to test the distribution of SVM score between real pCR and non-pCR patients. The *p*-values on Rad-T, In-V, and Ex-V are 1.26×10^{-10}, 1.41×10^{-3}, and 3.26×10^{-3}, respectively, indicating that the SVM score between real pCR and non-pCR is from different distribution under significance level $\alpha = 0.05$.

From the above results, we conclude that the radiomics model could extract information related to the pCR and predict its status with certain reliability. Furthermore, the results indicated that the "suspicious region" is capable to be the ROI in this research, which means a single post-nCRT T2-w MRI has the ability to predict the pCR status without the help of a pre-nCRT MRI or other post-nCRT modalities. The overall pipeline is provided in **Figure 9**.

DISCUSSION

In this study, we proposed a method for predicting the pCR status of LARC patients after nCRT that only requires the post-nCRT T2-w MRI and a few manual operations. We provided a

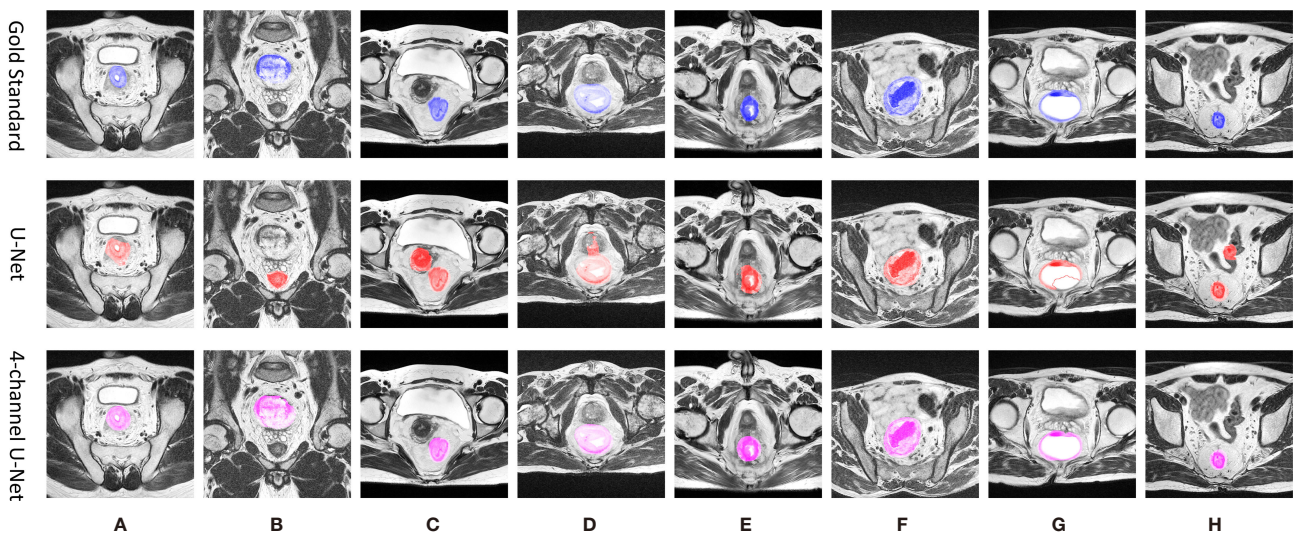

FIGURE 5 | Eight typical cases of ructum segmentation **(A–H)**. The first row provide the gold standard, the second is the prediction of original U-Net, and the third row is the results of the first stage of tsraU-Net.

TABLE 3 | Comparison between U-Net and Attention U-Net in dice, sensitivity and specificity.

Model	Dice	Sensitivity	Specificity
U-Net	0.656 (95% CI[a]: 0.630, 0.683)	0.781 (95% CI: 0.750, 0.812)	0.624 (95% CI: 0.590, 0.659)
4-channel U-Net	0.66 (95% CI: 0.628, 0.691)	0.785 (95% CI: 0.752, 0.817)	0.632 (95% CI: 0.594, 0.668)

[a]*CI, confidence intervals.*

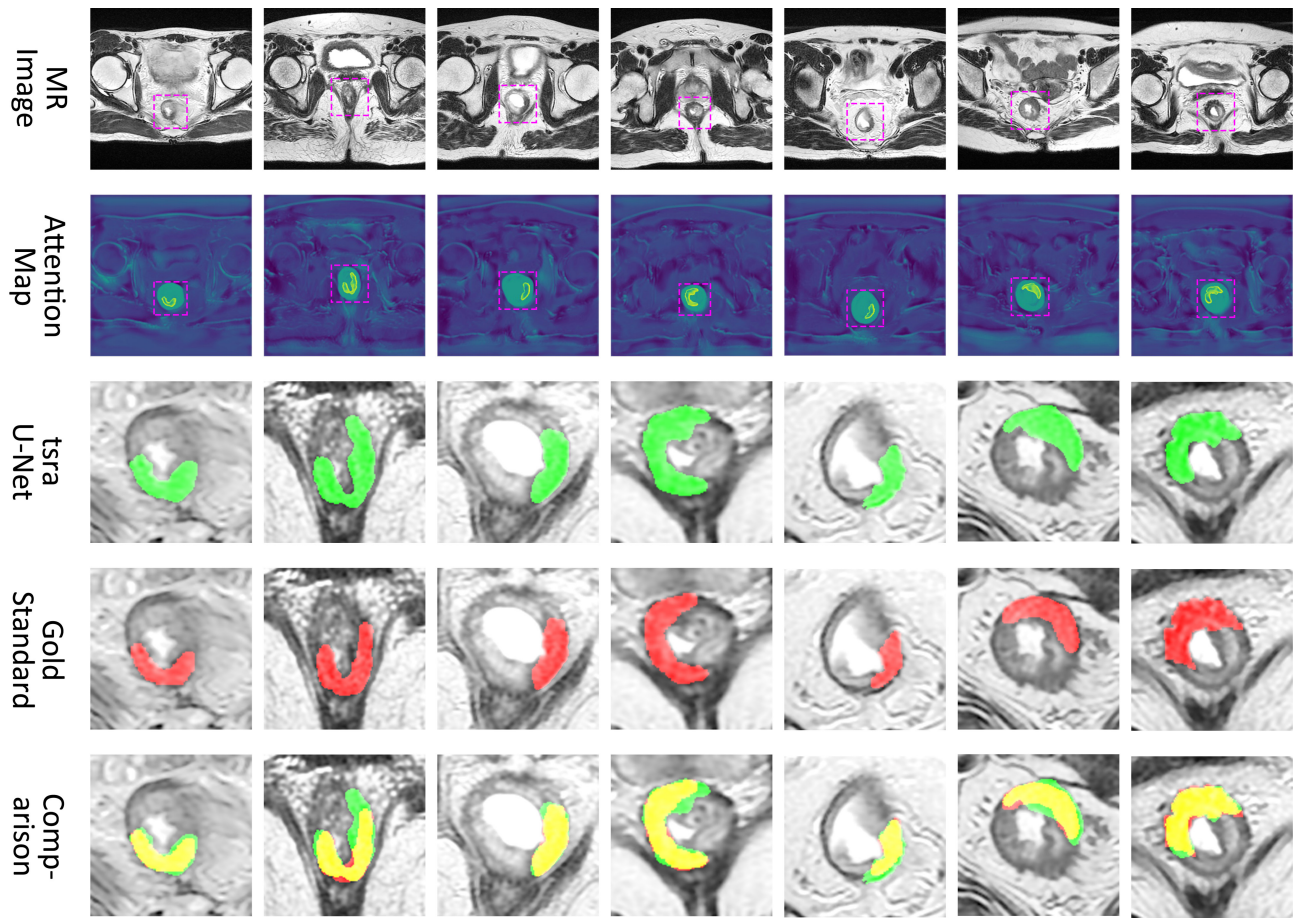

FIGURE 6 | Attention map and comparison of suspicious region segmentation between gold standard and tsraU-Net. The first row is the input of the network, the second row is the attention map provided by attention U-Net, the third row is the prediction of tsraU-Net, the fourth row is the gold standard, and the last row is the overlapped region between prediction and gold standard.

novel definition of the suspicious region and used it as the ROI to build the radiomics analysis. Furthermore, we designed a deep learning model for suspicious region segmentation that could greatly reduce the workload of radiologists. Our experimental results, 0.829/0.815 AUC on the internal/external validation set, prove the feasibility and stability of our method in pCR prediction, indicating that our method has great potential for providing assistance to doctors in clinical diagnosis.

Some information was obtained from the 10 selected features. First of all, no shape feature remained after selection. The reason might come from the fact that all of the ROIs were provided by the deep networks, which had a homogeneous shape regardless of the pCR status. Furthermore, half of the selected features were wavelet features, which might imply that some valuable information was hidden in the frequency domain. Many recent studies have also highlighted the importance of wavelet features in radiomics analysis (43–46). Finally, 8 of the 10 features were quantiles, and 1 was the standard deviation. This suggested that simply averaging the features from all of the slices per patient was insufficient compared with using multiple statistics.

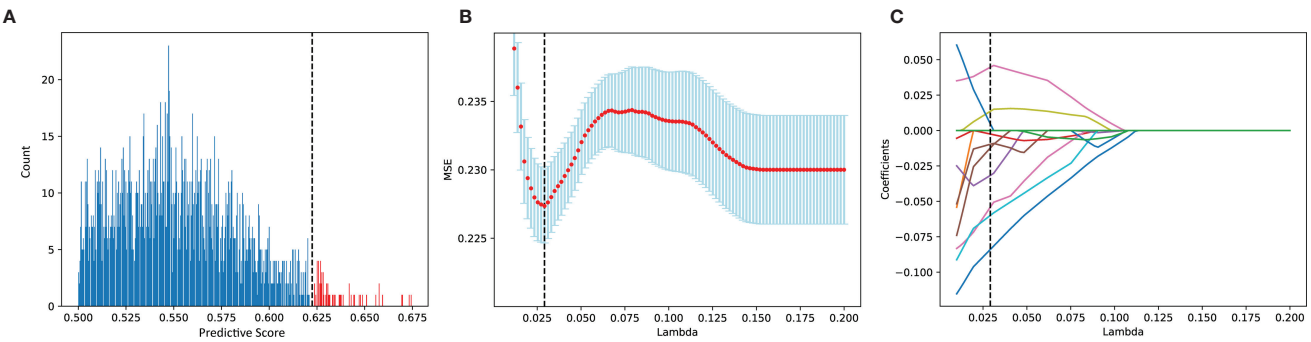

FIGURE 7 | **(A)** Histogram of predicted score. Features in red were remained and in blue were excluded. **(B)** MSE of each λ in LASSO while grid search. **(C)** The coefficient of each feature in lasso while grid search.

TABLE 4 | The features finally remained, and their coefficients.

Features Group	Abbreviation	Attribute	Coefficient
original firstorder	Maximum	Q2	-0.03166
original firstorder	Maximum	Q3	-0.009714
original glcm	MCC	SD	-0.083918
original gldm	LargeDependenceHighGrayLevelEmphasis	Q2	-0.002476
original gldm	LowGrayLevelEmphasis	Q1	0.044713
wavelet-LH ngtdm	Busyness	Q1	-0.012269
wavelet-LH ngtdm	Busyness	Q2	-0.054026
wavelet-LH ngtdm	Strength	Q2	0.013605
wavelet-HL firstorder	Median	AVG	-0.059893
wavelet-LL glszm	LargeAreaLowGrayLevelEmphasis	Q3	0.004648

TABLE 5 | The pCR[a] status predicted performance on datasets Rad-T, In-V and Ex-V, in terms of AUC, accuracy, sensitivity, specificity, $F_{0.5}$-score, F_1-score, $F_{1.5}$-score.

Dataset	AUC[b]	Accuracy	Sensitivity	Specificity	F0.5-score	F_1-score	$F_{1.5}$-score
Rad-T	0.924 (95% CI[c]: 0.923, 0.926)	0.860 (95% CI: 0.856, 0.863)	0.861 (95% CI: 0.855, 0.867)	0.859 (95% CI: 0.855, 0.863)	0.860 (95% CI: 0.856, 0.863)	0.860 (95% CI: 0.820, 0.880)	0.860 (95% CI: 0.825, 0.901)
In-V	0.829 (95% CI: 0.821, 0.837)	0.804 (95% CI: 0.794, 0.815)	0.750 (95% CI: 0.720, 0.780)	0.816 (95% CI: 0.805, 0.827)	0.802 (95% CI: 0.789, 0.811)	0.782 (95% CI: 0.689, 0.793)	0.769 (95% CI: 0.722, 0.794)
Ex-V	0.815 (95% CI: 0.801, 0.830)	0.853 (95% CI: 0.841, 0.865)	0.500 (95% CI: 0.453, 0.548)	0.929 (95% CI: 0.919, 0.938)	0.793 (95% CI: 0.746, 0.813)	0.650 (95% CI: 0.634, 0.678)	0.583 (95% CI: 0.555, 0.615)

[a]pCR, pathologic complete response. [b]AUC, area under the curve; [c]CI, confidence intervals.

To further address the thoughtful design of our study, we wanted to highlight the importance of independent training of the segmentation and radiomics model. As a model always tends to overfit more or less while training, if we use the same set to train the segmentation as well as the radiomics, the predicted ROI on the training set and validation set might have different distributions, and consequently affect the stability of the radiomics analysis. Due to the above consideration, we used a particular dataset, Seg-T, to train the segmentation model and choose the network parameters. We then applied the best model on Rad-T, In-V, and Ex-V to assure the independence between segmentation and radiomics.

Unlike other studies (47, 48) that have utilized a two-sample *t*-test as the first step to select the features, we used univariate Cox analysis. In fact, prior to the feature selection, we examined whether each feature was normally distributed by calculating the skewness and kurtosis (49). A total of 34 features did not pass this simple normality test under a significance level $\alpha = 0.05$. Therefore, *t*-test could not be applied to all features in our study. Consequently, we utilized the concordance index as a replacement because it did not limit the distribution of data.

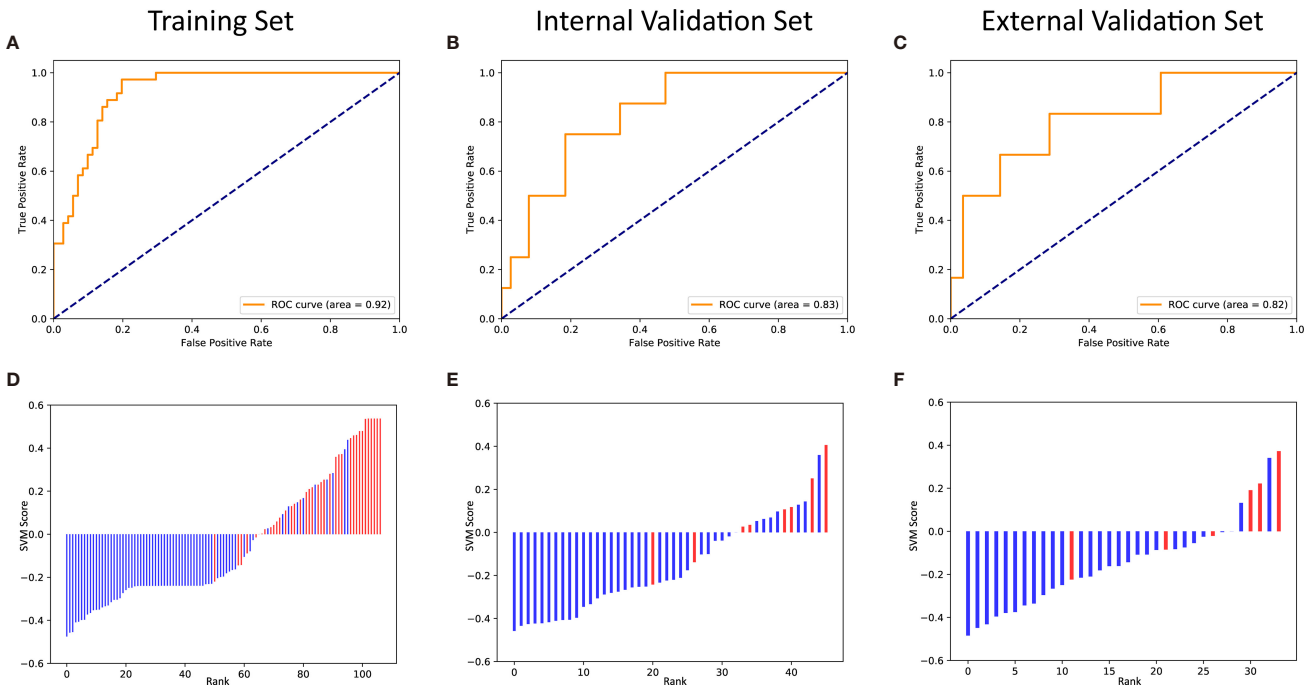

FIGURE 8 | The ROC curves and scores of predicting pCR in the training and validation sets. The ROC curves of the training set **(A)**, internal validation set **(B)** and external validation set **(C)**. The scores of the training set **(D)**, internal validation set **(E)** and external validation set **(F)**.

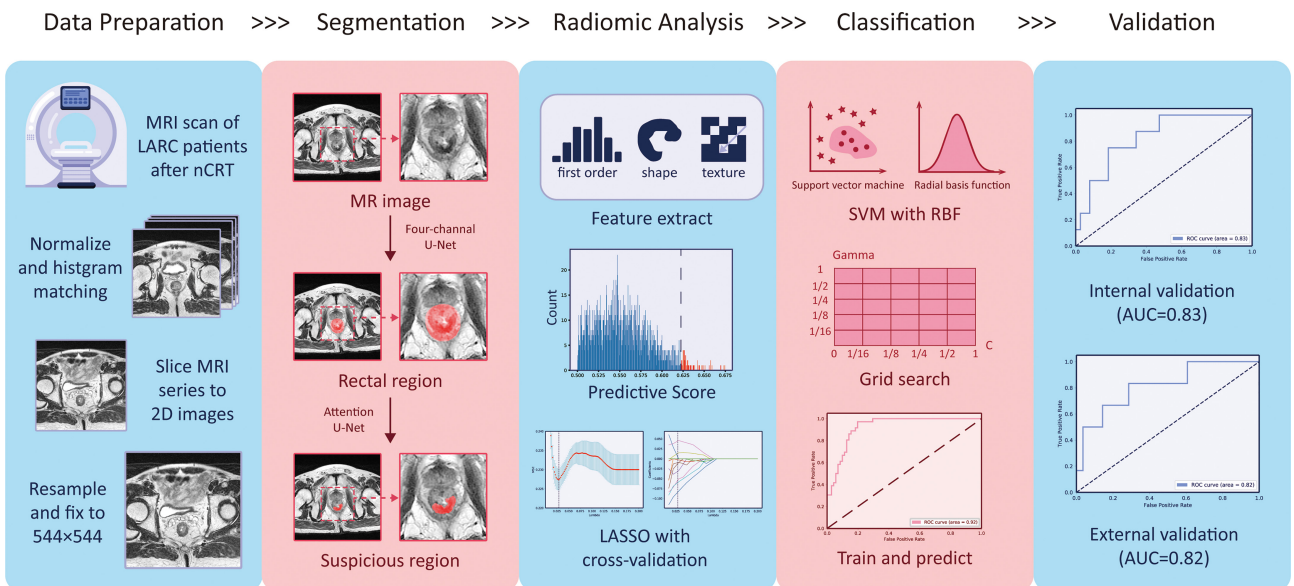

FIGURE 9 | The overall workflow in this study.

There were some limitations in our study. However, this was only a preliminary exploration. In the future, we will attempt further improvements. For instance, we could collect more unified standard data for analysis, and at the same time carefully choose the year of patient recruitment to avoid data mixing. In addition, we could encourage radiologists to delineate more precisely so that our segmentation network could better learn the characteristics of the suspicious regions. Additionally,

our segmentation network could be adjusted and improved using techniques, such as combining different loss functions following previous works (50–53) or adding clinical characteristics for a joint analysis. In addition, if we could obtain MRI with smaller slice thickness ($\leq 1mm$), we could consider building a 3D model and studying the 3D radiomics features that may contain richer information of the suspicious region.

Finally, the motivation of this study was different than other related works. We wanted to explore the possibility of using a single post-nCRT T2 MRI for patients missing a pre-nCRT MRI or other modalities. In addition, we intended to improve the efficiency of the model and reduce the workload of doctors for clinical use. Our method has great potential to guide less-experienced doctors, as it does not require manual delineation of the ROI. Moreover, as our model is less restricted regarding data requirements and the prediction of the pCR was easily obtained, it can be combined with other studies for joint decision-making.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by This clinical trial was approved by the Clinical Ethics Review Committee of the Sixth Affiliated Hospital of Sun Yat-sen University (2020ZSLYEC-010).

AUTHOR CONTRIBUTIONS

XP, FW, QZ, and YL jointly wrote the manuscript. XP, RH, and XY collected all the input sources data. XP and FW annotated the images data. FW, QZ, and YL designed the model and implemented the main algorithm and other computational analysis. XF performed a review of the manuscript on the clinical aspects. All authors contributed to the article and approved the submitted version.

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Surgical Outcomes of Robotic Resection for Sigmoid and Rectal Cancer: Analysis of 109 Patients From a Single Center in China

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Background: Robotic colorectal surgery has been increasingly performed in recent years. The safety and feasibility of its application has also been demonstrated worldwide.However, limited studies have presented clinical data for patients with colorectal cancer (CRC) receiving robotic surgery in China. The aim of this study is to present short-term clinical outcomes of robotic surgery and further confirm its safety and feasibility in Chinese CRC patients.

Methods: The clinical data of 109 consecutive CRC patients who received robotic surgery at Sun Yat-sen University Cancer Center between June 2016 and May 2019 were retrospectively reviewed. Patient characteristics,tumor traits, treatment details, complications, pathological details, and survival status were evaluated.

Results: Among the 109 patients, 35 (32.1%) had sigmoid cancer, and 74 (67.9%) had rectal cancer. Thirty-seven (33.9%) patients underwent neoadjuvant chemoradiotherapy. Ten (9.2%) patients underwent sigmoidectomy, 38 (34.9%) underwent high anterior resection (HAR), 45 (41.3%) underwent low anterior resection (LAR), and 16 (14.7%) underwent abdominoperineal resection (APR). The median surgical procedure time was 270 min (range 120–465 min). Pathologically complete resection was achieved in all patients. There was no postoperative mortality. Complications occurred in 11 (10.1%) patients, including 3 (2.8%) anastomotic leakage, 1 (0.9%) anastomotic bleeding, 1 (0.9%) pelvic hemorrhage, 4 (3.7%) intestinal obstruction, 2 (1.8%) chylous leakage, and 1 (0.9%) delayed wound union. At a median follow-up of 17 months (range 1–37 months), 1 (0.9%) patient developed local recurrence and 5 (4.6%) developed distant metastasis, with one death due to disease progression.

Conclusions: Our results suggest that robotic surgery is technically feasible and safe for Chinese CRC patients, especially for rectal cancer patients who received neoadjuvant treatment. A robotic laparoscope with large magnification showed a clear surgical space for pelvic autonomic nerve preservation in cases of mesorectal edema.

Keywords: sigmoid cancer, rectal cancer, robotic surgery, surgery outcome, oncological outcome, neoadjuvant chemoradiotherapy

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BACKGROUND

Colorectal cancer (CRC) is the third most common cancer and a leading cause of cancer death worldwide (1), which is an increasingly important obstacle to gains in life expectancy in China (2–4). Despite improvements in the comprehensive treatment and management of CRC patients in recent years, surgery remains the most effective treatment and offers the possibility of a cure for CRC. The quality of surgery is closely associated with oncological outcome. Therefore, a suitable technique for CRC surgery is urgently needed in clinical practice.

Increasing evidence supported by randomized controlled trials demonstrated that laparoscopic surgery was not inferior to open surgery with respect to short-term surgical outcomes and long-term oncological outcomes (5–9), which is becoming the new standard for colorectal cancer treatment. Many advantages of laparoscopic surgery have been reported, including shorter length of stay, smaller scars, and reduced recovery time (10). However, laparoscopic surgery may present some technical drawbacks, such as loss of three-dimensional (3D) view, long instruments that can increase physiological hand tremor, and loss of dexterity. Recently, robot-assisted laparoscopic surgery (RALS) using the Intuitive Surgical® da Vinci™ surgical system (Intuitive Surgical®, Sunnyvale, CA) was developed to facilitate minimally invasive surgery, and this technique provides a stable 3D view and intuitively transfers movements from the handle to the tip of the instrument with tremor filtering to offer enhanced dexterity (11).

Robotic colorectal surgery has been increasingly performed in recent years, and the safety and feasibility have also been confirmed in previous studies (12–14). Limited studies have presented clinical data for patients with CRC receiving robotic surgery in China. The aim of this study is to present short-term surgical and oncological outcomes of robotic surgery and further confirm its safety and feasibility in Chinese patients with sigmoid and rectal cancer.

PATIENTS AND METHODS

Patient Selection

The medical records of 109 consecutive patients were reviewed. All patients were diagnosed with sigmoid colon or rectal cancer and underwent robotic surgery between June 2016 and May 2019 at Sun Yat-sen University Cancer Center (Guangzhou, China). All cases were staged according to the 8th edition American Joint Committee on Cancer (AJCC) staging system. The patients were excluded from robotic approach according to contraindications for robot-assisted colorectal surgery described by expert consensus on robotic surgery for colorectal cancer (2015 edition) (15). In addition, if patients were unwilling to receive robot surgery, we also excluded the cases. The selected case met the following inclusion criteria: (1) histologically confirmed sigmoid colon or rectal adenocarcinoma; (2) underwent robotic curative resection of tumor using the da Vinci Surgical System (Intuitive Surgical Inc., Sunnyvale, CA, USA); and (3) had a complete record of the whole treatment. The patient demographics, tumor characteristics, type of procedure performed, comorbid conditions, operative variables, including operative time, conversion to open, lymph nodes retrieved, estimated blood loss, and blood transfusion, and postoperative variables, including length of stay, and 30-day mortality were carefully reviewed, and oncological outcomes were assessed. The present study was performed according to the ethical standards of the World Medical Association Declaration of Helsinki and was approved by the Institutional Review Board and Independent Ethics Committees of Sun Yat-sen University Cancer Center. The informed consent requirement was waived based on the nature of this retrospective study, in which patient data were kept confidential.

Surgical Techniques

In this study, five surgeons performed the all series. The exact trocar placement is shown in **Figure 1A**. There are 4 trocars placed for the surgery: 1 for the camera, 2 for the robotic arms, and 1 for the assistant. A camera port (12 mm) was placed 3–4 cm above and to the right of the umbilicus. Robotic arm 1 (8 mm) was placed right of the iliac fossa along a line drawn from the umbilicus to the anterior superior iliac spine, one third of the way from the anterior superior iliac spine. Robotic arm 2 (8 mm) was placed 3–4 cm below the xiphoid process. An assistant port was placed (12 mm) at the intersection of the vertical line through McBurney's point and the horizontal line through the camera port.

Total mesorectal excision (TME) and tumor-specific mesorectal excision (TSME) were performed as previously described (15). The procedure of pelvic autonomic nerves preservation (PANP) was performed at the same time (**Figures 1B–D**). The sigmoid mesocolon was cut along the right pararectal sulcus using the middle approach, and the inferior mesenteric artery was fully exposed. Spleen flexure were released if intestine segment or mesentery is not long enough for anastomosis steps. The inferior mesenteric artery was clamped and cut off approximately 1 cm from the root of the blood vessel in order to protect the superior hypogastric plexus. The "cavity effect" of electric heating equipment was quickly exposed, and Toldt's plane was subsequently entered. The white filamentous connective tissue in Toldt's space was cut sharply using an electric knife and kept in the neurosurgical plane of the white filamentous connective tissue at all times. We separated the posterior wall of the rectum closely behind the fascia propria of the rectum under direct vision in order to protect the inferior hypogastric nerve and the anterior sacral vessel. Similarly, sharp separation of the rectal lateral walls was performed near the outer edge of the rectal ligament and the inside edge of the pelvic plexus to protect the pelvic plexus. The anterior rectal space between the anterior and posterior Denonvilliers' fascia was separated to protect the branches of the pelvic plexus. When the intestine segment or mesentery is not long enough for anastomosis steps, we would conduct splenic flexure taking down.

Follow-Up

Patients were scheduled for subsequent visits every 3 months for 2 years then semiannually until 3 years after surgery. Physical

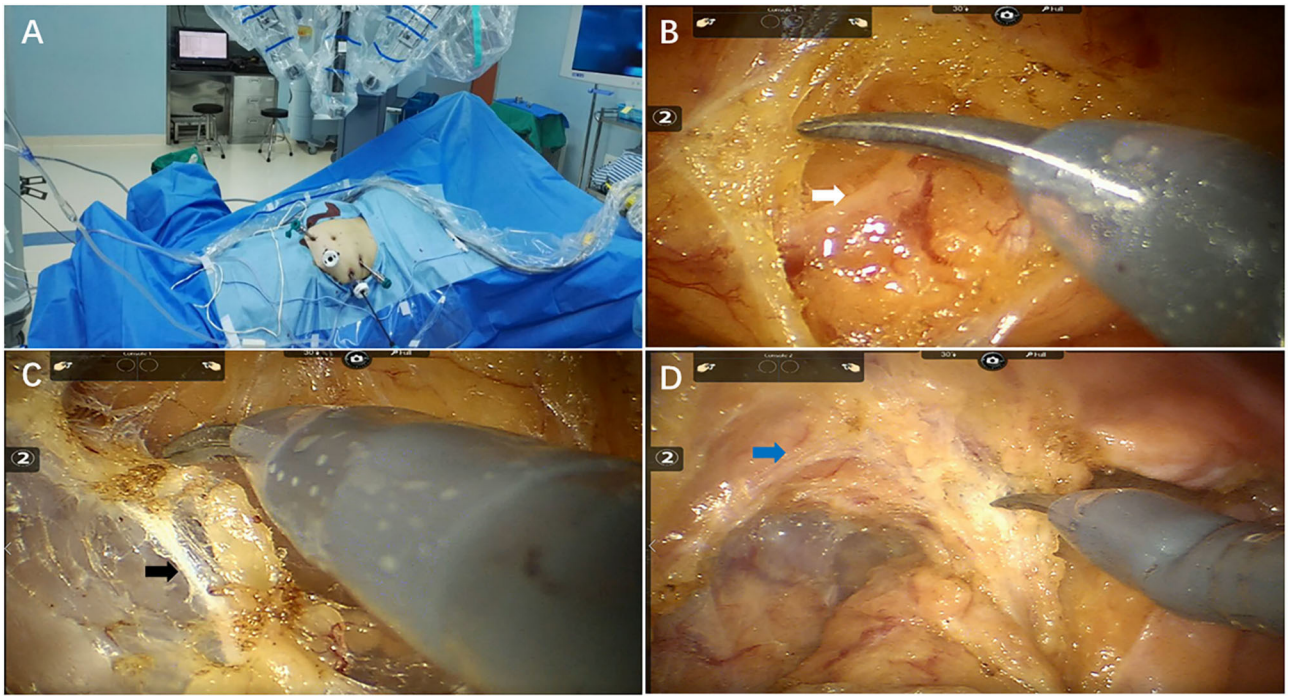

FIGURE 1 | Key techniques of total mesorectal excision after chemoradiotherapy for pelvic autonomic nerve preservation. **(A)** Operation room setup **(B)** Inferior mesenteric nerve (white arrow) preservation **(C)** Hypogastric nerves (black arrow) preservation **(D)** Pelvic plexus (blue arrow) preservation.

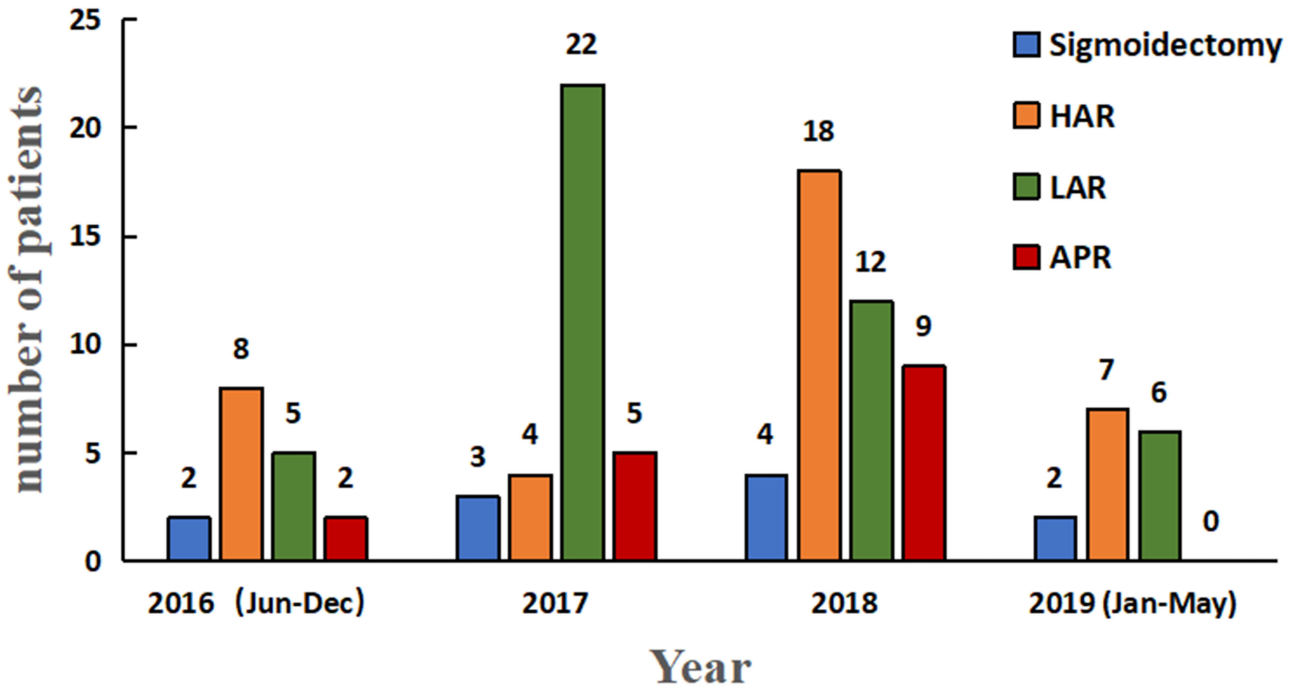

FIGURE 2 | Histogram depicting year-wise distribution of robotic sigmoidectomy. HAR, high anterior resection; LAR, low anterior resection; APR, abdominoperineal resection for colorectal cancer.

examination, blood tests for carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) levels, abdominal ultrasonography, and chest X-rays were performed every 3 months postoperatively. Chest/abdominal/pelvic computed tomography (CT) and colonoscopy were performed annually. Disease-free survival (DFS) was defined as the interval from surgery to disease recurrence, death, or the last follow-up.Overall survival (OS) was defined as the interval from the date of surgery until death of any cause or the last follow-up. Patients without any event (metastasis or death) at the last follow-up date were regarded as random censoring. The last follow-up visit was in July 2019.

Statistical Analysis

All statistical analyses were performed using IBM SPSS statistics software, version 21.0 (IBM Corp., Armonk, NY, USA). All of the continuous data are expressed as the means with standard deviation and range. All of the categorical data were calculated as numbers and percentages. The 2-year OS rate and 2-year DFS rate were calculated using the Kaplan-Meier method.

RESULTS

Patient Characteristics

Over a 3-year period, 10 (9.2%) patients underwent sigmoidectomy, 38 (34.9%) underwent high anterior resection (HAR), 45 (41.3%) underwent low anterior resection (LAR), and 16 (14.7%) underwent abdominoperineal resection (APR) (**Figure 2**). Their demographic features and clinicopathological characteristics are summarized in **Table 1**. Of the total 109 patients, 35 (32.1%) patients presented with sigmoid colon cancer and 74 (67.9%) patients had rectal cancer. Seventy-five patients (68.8%) were males, and 34 (31.2%) were females, with a median age of 59 years (range, 31–82 years). The mean body mass index (BMI) was 22.8 ± 3.0 and comparable between the patients with sigmoid cancer and rectal cancer. Preoperative clinical stage included 17 (15.6%) stage I, 43 (39.4%) stage II, 45 (41.3%) stage III, and 4 (3.7%) stage IV. Ten (9.2%) patients underwent sigmoidectomy. Thirty-eight (34.9%) of the 74 patients with rectal cancer, 37 (50%) received neoadjuvant chemoradiotherapy (CRT) and 4 (5.4%) received neoadjuvant chemotherapy.

Intraoperative Outcomes

The intraoperative outcomes are presented in **Table 2**. The median operative time for robotic surgery was 270 min, with a range of 120 min to 465 min. Median intraoperative transfusion volume for the total cohort was 2,000 ml (range 1,000–4,500 ml). Median intraoperative urine volume for the cohort was 400 ml (range 100–2,100 ml). Median estimated blood loss for the cohort was 50 ml (range 20–400 ml). Three patients had blood transfusion, including two patients in the APR group (12.5%) and one patient in the sigmoidectomy and HAR group (2.1%). None of the cases was converted to an open or laparoscopic procedure, and no intraoperative ureteral injury occurred. Twenty-two patients underwent preventive ileostomy, including four patients in the sigmoidectomy and HAR group (8.3%) and 18 patients in the LAR group (40.0%). Among total patients in this study, there was no case receiving splenic flexure taking down.

Pathological Outcomes

The pathological outcomes are presented in **Table 3**. Pathological stages were stage 0 in 12 patient, stage I in 27 patients, stage II in 38 patients, stage III in 28 patients and stage IV in 4 patients. There were 41 patients with rectal cancer who had received neoadjuvant treatment, and 11 of these patients exhibited a pathologically complete response (pCR). Another one patient who achieved pCR was a sigmoid colon cancer patient who received neoadjuvant chemotherapy. All case received a radical resection and achieved a status of no evidence of disease after surgery.

Postoperative Outcomes

As shown in **Table 4**, the median length of stay (LOS) was 7 (range, 4–30) days. There was no postoperative mortality within 30 days. Eleven (10.1%) patients suffered complications after surgery, including 3 (2.8%) patients with anastomotic leakage, 1 (0.9%) patient with anastomotic bleeding, 1 (0.9%) patient with pelvic hemorrhage, 4 (3.7%) patients with intestinal obstruction, 2 (1.8%) patients with chylous leakage, and 1 (0.9%) patient with delayed wound healing. Only 5 (4.6%) and 8 (7.3%) patients developed urinary and sexual dysfunction, respectively. Details about the complication events are presented in **Table 5**. Among the patients who suffered postoperative complications, two patients required surgery, and nine patients received conservative treatment. All of these patients achieved recovery after invention.

Survival Analysis

The median follow-up period for all patients was 17 months (range 1–37 months). One hundred and two patients (93.6%) in our study cohort were alive with no evidence of disease. One (0.9%) patient developed local recurrence, and 5 (4.6) patients developed distant metastasis. One patient died due to disease progression. The 2-year OS rate of all patients ($n = 109$) was 97.2% (**Figure 3A**), and the 2-year DFS rate of non-metastatic patients ($n = 104$) was 92.9% (**Figure 3B**). The 2-year DFS rate of patients in stages 0, I, II, and III were 100, 95.5, 90.5, and 88.8%, respectively (**Figure 3C**).

DISCUSSION

In this retrospective study, we investigated the surgical and oncological outcomes of robotic resection for sigmoid and rectal cancer in Chinese patients. Our data found that robotic surgery had a low conversion rate, low morbidity rate, and remarkable oncological outcomes, which confirms its safety and feasibility in Chinese patients with sigmoid and rectal cancer.

Rectal cancer resection is very difficult to perform using traditional laparotomy, but laparoscopic surgery has an advantage for rectal surgery under a clearer view despite the narrow and deep pelvic space. Several studies (12–14, 16, 17) confirmed that laparoscopic surgery presented better short-term outcomes and comparable long-term outcomes compared to traditional laparotomy. The surgical advantages and comparable

TABLE 1 | Clinical characteristics of study population.

Variables	Total ($n = 109$)	Sigmoid cancer ($n = 35$)	Rectal cancer ($n = 74$)
Age [median (range), years]	59 (31–82)	60 (34–82)	57 (31–73)
Age > 65 years (*n*,%)	30 (27.5)	10 (28.6)	20 (27.0)
Male gender (*n*,%)	75 (68.8)	25 (71.4)	50 (67.6)
BMI (mean ± SD, kg/m^2)	22.8 ± 3.0	22.7 ± 2.8	22.9 ± 3.2
< 18.5	5 (4.6)	1 (2.9)	4 (5.4)
18.5–23.9	63 (57.8)	23 (65.7)	40 (54.1)
24–27.9	36 (33.0)	10 (28.6)	26 (35.1)
≥ 28	5 (4.6)	1 (2.9)	4 (5.4)
ASA classification (*n*,%)			
1	18 (16.5)	5 (14.3)	13 (17.6)
2	84 (77.1)	26 (74.3)	58 (78.4)
3	7 (6.4)	4 (11.4)	3 (4.1)
Smoking history (*n*,%)	23 (21.1)	7 (20.0)	16 (21.6)
Hypertension (*n*,%)	29 (26.6)	11 (31.4)	18 (24.3)
Diabetes mellitus (*n*,%)	14 (12.8)	4 (11.4)	10 (13.5)
Bowel obstruction (*n*,%)	3 (2.8)	2 (5.7)	1 (1.4)
Weight loss within 6 months (*n*,%)	27 (24.8)	10 (28.6)	17 (23.0)
Hemoglobin (mean ± SD, g/dl)	132.2 ± 17.6	133.2 ± 20.2	131.7 ± 16.4
Severe anemia (*n*,%)	2 (1.8)	1 (2.9)	1 (1.4)
Albumin (mean ± SD, g/dl)	41.8 ± 3.6	41.8 ± 4.2	41.9 ± 3.4
Median DAV [median (range), cm]	10 (1–30)	20 (16–30)	7 (1–15)
>15	35 (32.1)	35 (100)	0
11–15	13 (11.9)	0	13 (17.6)
6–10	35 (32.1)	0	35 (47.3)
≤5	26 (23.9)	0	26 (35.1)
Preoperative TNM stage (*n*,%)			
I	17 (15.6)	4 (11.4)	13 (17.6)
II	43 (39.4)	15 (42.9)	28 (37.8)
III	45 (41.3)	15 (42.9)	30 (40.5)
IV	4 (3.7)	1 (2.9)	3 (4.1)
Neoadjuvant CRT (*n*,%)	37 (33.9)	0	37 (50.0)
Neoadjuvant chemotherapy (*n*,%)	4 (3.7)	0	4 (5.4)

BMI, body mass index; ASA, American Society of anesthesiologists; SD, standard deviation; DAV, inferior tumor margin from the anal verge; TNM stage, tumor-node-metastasis classification; CRT, chemoradiotherapy.

TABLE 2 | Intraoperative outcomes of total patients.

Variables	Total ($n = 109$)	Sigmoidectomy + HA ($n = 48$)	LAR ($n = 45$)	APR ($n = 16$)
Procedure time [median (range), minutes]	270 (120–465)	240 (120–435)	300 (165–450)	295 (170–465)
Intraoperative transfusion volume [median (range), ml]	2,000 (1,000–4,500)	2,000 (1,000–3,500)	2,000 (1,000–4,500)	2,500 (1,500–3,300)
Intraoperative urine volume [median (range), ml]	400 (100–2,100)	400 (100–1,600)	350 (100–2,100)	500 (200–2,000)
Estimated blood loss [median (range), ml]	50 (20–400)	50 (20–300)	50 (50–400)	100 (30–300)
Blood transfusion, *n* (%)	3 (2.8)	1 (2.1)	0	2 (12.5)
Conversion, *n* (%)	0	0	0	0
Ureteral injury, *n* (%)	0	0	0	0
Preventive ileostomy, *n* (%)	22 (20.2)	4 (8.3)	18 (40.0)	0

HAR, high anterior resection; LAR, low anterior resection; APR, abdominoperineal resection.

TABLE 3 | Pathologic outcomes.

Variables	Total ($n = 109$)	Sigmoid colon cancer ($n = 35$)	Rectal cancer	
			Without neoadjuvant treatment ($n = 33$)	With neoadjuvant treatment ($n = 41$)
Tumor size [median (range), cm]	2.5 (0.5–13.0)	4 (10.0–13.0)	2.7 (1.5–6.5)	1.5 (0.5–5.5)
Tumor differentiation, *n* (%)	18 (16.5)	4 (11.4)	1 (3.0)	13 (31.7)
No tumor cells				
Well-differentiated carcinoma	0	0	0	0
Moderate carcinoma	76 (69.7)	28 (80.0)	25 (75.8)	23 (56.1)
Poor carcinoma	14 (12.8)	3 (8.6)	7 (21.2)	4 (9.8)
Mucous carcinoma	1 (0.9)	0	0	1 (2.4)
Pathological T stage, *n* (%)	1 (0.9)	1 (2.9)	0	0
Tis				
T0	12 (11.0)	0	0	12 (29.3)
T1	12 (11.0)	4 (11.4)	8 (24.2)	0
T2	22 (20.2)	2 (5.7)	9 (27.3)	11 (26.8)
T3	52 (47.7)	24 (68.6)	13 (39.4)	15 (36.6)
T4a	8 (7.3)	3 (8.6)	3 (9.1)	2 (4.9)
T4b	2 (1.8)	1 (2.9)	0	1 (2.4)
Pathological *N* stage, *n* (%)	79 (72.5)	22 (62.9)	21 (63.6)	36 (87.8)
N0				
N1a	13 (11.9)	5 (14.3)	6 (18.2)	2 (4.9)
N1b	5 (4.6)	1 (2.9)	2 (6.1)	2 (4.9)
N1c	6 (5.5)	4 (11.4)	1 (3.0)	1 (2.4)
N2a	4 (3.7)	2 (5.7)	2 (6.1)	0
N2b	2 (1.8)	1 (2.9)	1 (3.0)	0
Pathological TNM stage, *n* (%)	12 (11.0)	1 (2.9)	0	11 (26.8)
0				
I	27 (24.8)	5 (14.3)	12 (36.4)	10 (24.4)
II	38 (34.9)	16 (45.7)	9 (27.3)	13 (31.7)
III	28 (25.7)	12 (34.3)	12 (36.4)	4 (9.8)
IV	4 (3.7)	1 (2.9)	0	3 (7.3)
Positive/total harvested lymph nodes [median (range), *n*]	0 (0–22)/12 (1–51)	0 (0–14)/16 (3–33)	0 (0–22)/15 (5–51)	0 (0–2)/5 (1–23)
Positive/total central harvested lymph nodes [median (range), *n*]	0 (0–3)/3 (0–26)	0 (0–3)/4 (0–12)	0 (0)/4 (0–26)	0 (0)/2 (0–8)
Positive/total intermediate harvested lymph nodes [median (range), *n*]	0 (0–3)/3 (0–16)	0 (0–3)/4 (0–9)	0 (0–2)/4 (0–16)	0 (0)/2 (0–9)
Positive/total paraintestinal harvested lymph nodes [median (range), *n*]	0 (0–22)/4 (0–23)	0 (0–8)/7 (1–15)	0 (0–22)/6 (1–23)	0 (0–2)/2 (0–12)
Distance of distal resection margin [median (range), cm]	5 (1.0–10.0)	8 (5.0–10.0)	5 (1.0–10.0)	2.5 (1.0–10.0)
Positive resection distal margin, *n*	0	0	0	0

TNM stage, tumor-node-metastasis classification; T stage, clinical tumor stage; N stage, clinical node stage.

oncological outcomes of laparoscopic surgery were clearly demonstrated in patients with locally advanced rectal cancer after preoperative chemoradiotherapy in the COREAN trial (18). Because of the features of robotic technology, robotic surgery is much more advantageous, especially for patients with locally advanced rectal cancer after treatment with preoperative chemoradiotherapy. In our study, 37.6% patients presented with a BMI $\geq$ 24 kg/m^2, and 55.4% patients with rectal cancer received neoadjuvant treatment. No conversion occurred with a median procedure time of 270 min, a median estimated blood loss of 50 ml and a median length of stay of 7 days. Only 11 patients (10.1%) experienced postoperative complications, which shows the remarkable surgical advantages of robotic surgery in patients with rectal cancer who received neoadjuvant treatment.

As previously reported, the most commonly encountered complication was anastomotic leakage, and its average

TABLE 4 | Postoperative and oncologic outcomes.

Variables	Total (n = 109 ,%)	Sigmoidectomy + HAR (n = 48,%)	LAR (n = 45 ,%)	APR (n = 16 ,%)
LOS after surgery [median (range), days]	7 (4–30)	7 (4–12)	7 (4–24)	8 (6–30)
30 day mortality	0	0	0	0
Postoperative complication	11 (10.1)	2 (4.2)	7 (15.6)	2 (12.5)
Anastomotic leakage	3 (2.8)	0	3 (6.7)	0
Anastomotic bleeding	1 (0.9)	1 (2.1)	0	0
Pelvic hemorrhage	1 (0.9)	0	1 (2.2)	0
Intestinal obstruction	4 (3.7)	0	3 (6.7)	1 (6.3)
Chylous leakage	2 (1.8)	1 (2.1)	1 (2.2)	0
Delay wound healing	1 (0.9)	0	0	1 (6.3)
Defecated dysfunction	38 (34.9)	9 (18.8)	29 (64.4)	0
Urinary dysfunction	5 (4.6)	0	1 (2.2)	4 (25.0)
Sexual dysfunction	8 (7.3)	0	4 (8.9)	4 (25.0)
Alive (NED)	102 (93.6)	45 (93.8)	41 (91.1)	16 (100)
Alive with tumor	6 (5.5)	2 (4.2)	4 (8.9)	0
Death due to tumor	1 (0.9)	1 (2.1)	0	0
Local recurrence	1 (0.9)	1 (2.1)	0	0
Distant metastasis	5 (4.6)	2 (4.2)	3 (6.7)	0

HAR, high anterior resection; LAR, low anterior resection; APR, abdominoperineal resection; LOS, length of stay; NED, no evidence of disease.

TABLE 5 | Summary of postoperative complication events.

Order	Gender	Age (years)	Tumor location	DAV (cm)	NACRT	Pathological stage	Types of operation	Complication	Complication detected on POD (days)	Invention	Invention outcome	LOS after surgery (day)	Survial status
1	Female	37	Rectum	4	Yes	T2N0M0	LAR	Anastomotic leakage, Intestinal obstruction	2	Conservative treatment	Recovery	12	Alive (NED)
2	Male	59	Rectum	10	No	T3N1M0	LAR	Pelvic hemorrhage	5	Conservative treatment	Recovery	14	Alive (NED)
3	Male	47	Rectum	6	Yes	pCR	LAR	Intestinal obstruction	5	Conservative treatment	Recovery	12	Alive (NED)
4	Female	54	Rectum	7	No	T3N0M0	LAR	Intestinal obstruction	3	Conservative treatment	Recovery	12	Alive (NED)
5	Female	37	Sigmoid colon	25	No	T3N0M0	HAR	Chylous leakage	3	Conservative treatment	Recovery	10	Alive (NED)
6	Male	69	Rectum	5	Yes	T3N0M0	LAR	Chylous leakage	6	Conservative treatment	Recovery	9	Alive (NED)
7	Male	43	Rectum	3	Yes	pCR	APR	Intestinal obstruction	3	Operation	Recovery	24	Alive (NED)
8	Male	50	Rectum	1	Yes	T4N0M0	APR	Delay wound healing	8	Conservative treatment	Recovery	30	Alive (NED)
9	Male	68	Sigmoid colon	28	No	T3N2M0	Sigmoidectomy	Anastomotic bleeding	1	Conservative treatment	Recovery	10	Alive (NED)
10	Male	49	Rectum	10	No	T3N1M0	LAR	Anastomotic leakage	6	Conservative treatment	Recovery	24	Alive (NED)
11	Female	59	Rectum	6	Yes	T3N0M1	LAR	Anastomotic leakage	5	Operation	Recovery	11	Alive with tumor

DAV, inferior tumor margin from the anal verge; NACRT, neoadjuvant chemoradiotherapy; POD, postoperative day; LOS, length of stay; pCR, pathological complete response; HAR, high anterior resection; LAR, low anterior resection; APR, abdominoperineal resection; NED, no evidence of disease.

occurrence rate was 8.6% (range from 1.2 to 20.5%) (19, 20) and 1.8 to 13.6% in robotic surgery (21, 22). Its occurrence affects the patient's quality of life, increases hospitalization costs, delays the implementation of adjuvant chemotherapy, and shortens the overall survival (22, 23). Eleven patients (10.1%) had postoperative complications, which included 3 patients

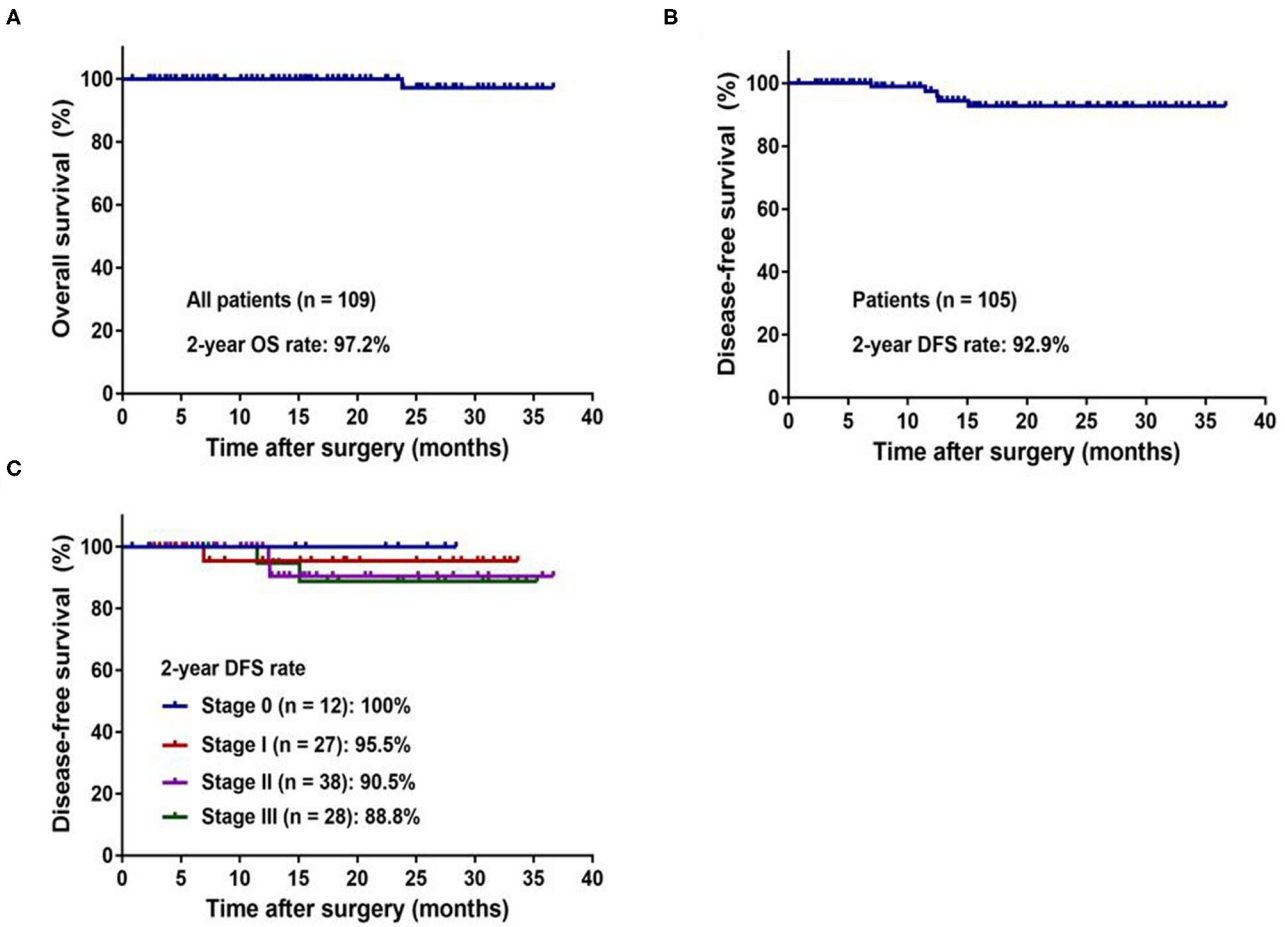

FIGURE 3 | Kaplan–Meier curves of the patients with colorectal cancer underwent robotic surgery. **(A)** 2-year overall survival for whole study population. **(B)** 2-year disease-free survival for non-metastatic patients. **(C)** 2-year disease-free survival by pathologic stage.

who suffered anastomotic leakage. Due to the advantages of robotic surgery, such as 3D magnified view, wristed instruments and stable camera platform, surgeons are able to maintain the sufficient surgical dissection plane down to the pelvic floor, which minimizes damage to marginal vessels and allows performance of the rectal division and reconstruction efficiently and safely to shorten the procedure time.

More precise surgery also helps protect the autonomic nerves and reduce the occurrence of long-term postoperative complications, including defecation, urinary and sexual dysfunction (14). Wang and coworkers (24) described a significant increase in International Prostate Symptom Score (IPSS) after surgery in the laparoscopic group, and more patients in the laparoscopic group (34.8%) perceived a severe damage in their overall level of sexual function following surgery than the patients in the robotic group (18.3%). Several studies (25, 26) also claimed that robotic TME improved the preservation of urinary and sexual functions because the arms of the robotic device are stable and highly flexible in the separation and exposure of tissues. With the high-resolution lens of the da Vinci surgical system to effectively recognize the nerve, the application of the PANP technique resulted in a significant reduction in the incidence of urinary dysfunction (4.6%) and sexual dysfunction (7.3%) in our study.

A positive circumferential margin or insufficient harvested lymph nodes leads to local recurrence (27). Although the relationship between sufficiently harvested lymph nodes and local recurrence rate is controversial, the guidelines list the harvesting of <12 lymph nodes as risk factor and noted that the performance of TME with clear surgical margins and adequate lymph node dissection were related to lower recurrence rate (28, 29). In our study, the median positive total harvested lymph nodes was 0 (range 0–22), and the total harvested lymph nodes was 12 (range 1–51). The 2-year DFS of patients in stages 0, I, II, and III were 100, 95.5, 90.5, and 88.8%, respectively, and the 2-year DFS of patients in stage III was slightly better than previous studies (65.2–82.8%) (12, 13, 17). The high quality of the procedure (no positive resection distal margin and sufficient harvested lymph nodes) and neoadjuvant treatment contributed to the remarkable oncological outcomes.

Although routine mobilization of the splenic flexure is not necessary during anterior resection for rectal cancer, it is one of the important surgical step in some of sigmoid and rectal cancer resection, which aimed to ensure a tension-free with good blood supply (30, 31). However, splenic flexure mobilization was recognized as a challenging step for robotic surgery. It was well-known that the splenic flexure anatomy is complex, which consisted of multiple vessels, surrounding vulnerable organs, such as spleen, and irregular adhesions. In addition, this step would be usually more difficult due to the lack of operating space (32). Moreover, due to limited range of motion of the robotic arms and surgical field compromising multiple quadrants, mobilization of the splenic flexure required a series of procedure, including removing robotic arms, replacing the patient cart and even reconnecting the robot system (33, 34). Therefore, when progress was difficult, we would mobilize splenic flexure by using laparoscopy approach in our center. Since there was no such case receiving splenic flexure mobilization, we were unable to provided any technical skills and surgical outcome of this step in the current study.

Several limitations should be acknowledged in the present study. First, this retrospective descriptive study included an uncontrolled, single-arm methodology and a limited number of patients from a single cohort. Although our study confirms the safety and feasibility of robotic surgery in Chinese CRC patients, the findings must be validated in a prospective, multicenter clinical trial with a large population in the future. Second, the short follow-up duration was insufficient to evaluate 5-year survival outcomes, which may have led to a misestimation of the effect of robotic surgery on OS and DFS. Considering the short follow up mean time, oncological results are derived from the pathological specimen anlaysis, that indirectely might confirm good survival rate. Additionally, selective bias undeniably exists in our cohort.

CONCLUSION

Robotic surgery is technically feasible and safe for Chinese CRC patients, especially for rectal cancer patients receiving neoadjuvant treatment because a robotic laparoscope with large magnification shows a clear surgical space for tumor resection in cases of mesorectal edema. Due to the advantages of robotic surgery, surgeons are able to perform the procedure efficiently and safely and help protect marginal vessels and the autonomic nerves, which reduces the occurrence of short-term and long-term postoperative complications and ensures clear surgical margins and adequate lymph node dissection.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (http://www.researchdata.org.cn), with the Approval Number as RDDA2021002030.

ETHICS STATEMENT

The present study was performed according to the ethical standards of the World Medical Association Declaration of Helsinki and was approved by the Institutional Review Board and Independent Ethics Committees of Sun Yat-sen University Cancer Center. The informed consent requirement was waived by the ethics committees based on the nature of this retrospective study, in which patient data were kept confidential.

AUTHOR CONTRIBUTIONS

JP and WL analyzed and interpreted the data. JL and ZP were the chief surgeons who performed the surgery, the chemotherapy, and all authors participated. JP, WL, JL, and ZP were major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Association Between Serum Carcinoembryonic Antigen Levels at Different Perioperative Time Points and Colorectal Cancer Outcomes

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Background: Whether elevated postoperative serum carcinoembryonic antigen (CEA) levels are prognostic in patients with stage II colorectal cancer (CRC) remains controversial.

Patients and Methods: Primary and sensitivity analysis populations were obtained from a retrospective, multicenter longitudinal cohort including consecutive patients without neoadjuvant treatment undergoing curative resection for stage I–III CRC. Serum CEA levels before ($CEA_{pre\text{-}m1}$) and within 1 ($CEA_{post\text{-}m1}$), 2–3 ($CEA_{post\text{-}m2–3}$), and 4–6 months ($CEA_{post\text{-}m4–6}$) after surgery were obtained, and their associations with recurrence-free survival (RFS) and overall survival (OS) were assessed using Cox regression. Sensitivity and subgroup analyses were performed.

Results: Primary and sensitivity analysis populations included 710 [415 men; age, 54.8 (11.6) years] and 1556 patients [941 men; age, 56.2 (11.8) years], respectively. Recurrence hazard ratios (HRs) in the elevated $CEA_{pre\text{-}m1}$, $CEA_{post\text{-}m1}$, $CEA_{post\text{-}m2–3}$, and $CEA_{post\text{-}m4–6}$ groups were 1.30 (95% CI: 0.91–1.85), 1.53 (95% CI: 0.89–2.62), 1.88 (95% CI: 1.08–3.28), and 1.15 (95% CI: 0.91–1.85), respectively. The HRs of the elevated $CEA_{pre\text{-}m1}$, $CEA_{post\text{-}m1}$, $CEA_{post\text{-}m2–3}$, and $CEA_{post\text{-}m4–6}$ groups for OS were 1.09 (95% CI: 0.60–1.97), 2.78 (95% CI: 1.34–5.79), 2.81 (95% CI: 1.25–6.30), and 3.30 (95% CI: 1.67–.536), respectively. Adjusted multivariate analyses showed that both in the primary and sensitivity analysis populations, elevated $CEA_{post\text{-}m2–3}$, rather than $CEA_{pre\text{-}m1}$, $CEA_{post\text{-}m1}$, and $CEA_{post\text{-}m4–6}$, was an independent risk factor for recurrence, but not for OS. The RFS in the elevated and normal $CEA_{post\text{-}m2–3}$ groups differed significantly

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among patients with stage II disease [n = 266; HR, 2.89; 95% CI, 1.02–8.24 (primary analysis); n = 612; HR, 2.69; 95% CI, 1.34–5.38 (sensitivity analysis)].

Conclusions: Elevated postoperative CEA levels are prognostic in patients with stage II CRC, with 2–3 months after surgery being the optimal timing for CEA measurement.

Keywords: colorectal cancer, carcinoembryonic antigen, adjuvant chemotherapy, recurrence risk, risk stratification

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer-related death both in men and women worldwide (1). Tumor relapse is the primary cause of poor prognosis in patients with CRC (2). Predicting the risk of relapse could allow a more targeted approach with respect to the selection of adjuvant therapies and follow-up strategies (e.g., by defining subgroups) for improving overall survival (3).

Carcinoembryonic antigen (CEA) is regarded as an essential indicator of CRC prognosis (4), and the guidelines recommend that serum CEA should be measured preoperatively and postoperatively in patients with CRC (5–9). Recent studies confirm that the preoperative and postoperative serum CEA levels are both associated with CRC outcomes, and elevated postoperative CEA levels are more prognostic than elevated preoperative CEA levels (4, 10–16). Hence, routine measurement of postoperative CEA levels is warranted.

Whether elevated postoperative CEA levels are prognostic in patients with stage II CRC remains controversial (4, 10–13). Some studies report that postoperative CEA levels have a predictive value in patients with stage II CRC (11, 12), while several others have been unable to determine the significance of postoperative CEA levels in such patients (4, 10, 13). A systematic review of published studies (4, 10–13) showed that the time points of postoperative CEA measurement varied across studies. CEA was measured within 4–12 weeks after surgery in some studies (11, 12) and within 1–12 weeks after surgery in several others (4, 10, 13). The difference in the time points of postoperative CEA measurement may be responsible for the inconsistent results, and the optimal timing for postoperative serum CEA measurement is therefore unknown.

In this study, we aimed to examine the association between serum CEA levels at different perioperative time points and CRC outcomes using a retrospective, multicenter longitudinal cohort and to determine the optimal timing for postoperative serum CEA measurement.

PATIENTS AND METHODS

Ethics Approval and Informed Consent

The ethics committee of each participating hospital approved this multicenter retrospective study. The requirement for informed consent was waived by the board, owing to the study's retrospective nature. All the patient data in the survey were anonymized. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Patients

A multicenter retrospective cohort was created. It included all consecutive patients with CRC who did not receive neoadjuvant treatment but underwent curative resection for stage I–III colorectal adenocarcinoma between January 2011 and June 2017 at two hospitals in China. A detailed description of the cohort's inclusion and exclusion criteria can be found in the **Online-Only Supplement**. Participants were included in the primary analysis population if preoperative serum CEA data and postoperative serum CEA measurements obtained within 1, 2–3, and 4–6 months after surgery were available. Participants were included in the sensitivity analysis population if postoperative serum CEA measurements obtained within 2–3 months after surgery were available. The study flowchart is shown in **Figure 1**.

Serum CEA Determination

Preoperative serum CEA level ($CEA_{pre\text{-}m1}$) was defined as the CEA level obtained closest to the time of surgery (as long as it was obtained within 4 weeks before surgery). Postoperative serum CEA level was defined as the last CEA value obtained 1 ($CEA_{post\text{-}m1}$), 2–3 ($CEA_{post\text{-}m2\text{–}3}$), and 4–6 months ($CEA_{post\text{-}m4\text{–}6}$) after surgery (a month was defined as 30 natural days). The CEA status was classified into two types as follows: normal (≤5.0 ng/mL) and elevated (>5.0 ng/mL). All CEA measurements were made with a chemiluminescence immunoassay using the Cobas 8000 e602 immunoassay analyzer (Roche Diagnostics, Tokyo, Japan) at Yunnan Cancer Hospital and an Alinity i immunoassay analyzer (Abbott Diagnostics, Chicago, IL, USA) at The Sixth Affiliated Hospital of Sun Yat-sen University, following World Health Organization standard methods (code 73/601) (17).

Surveillance Protocol and Outcome

The surveillance protocol was detailed in our previous study (18). In this study, follow-up ended on June 30, 2020. The primary outcome was recurrence-free survival (RFS). Recurrence included local recurrence and distant metastases, which were confirmed *via* a biopsy sample, positive imaging findings, or histological analyses. RFS was calculated from the date of surgery until the date of recurrence, death, or last follow-up. Data from patients who died or were lost to follow-up were treated as censored. The secondary outcome was overall survival (OS).

Covariates

Covariates included age, sex, surgical approach (open resection or laparoscopic resection), primary site, tumor differentiation,

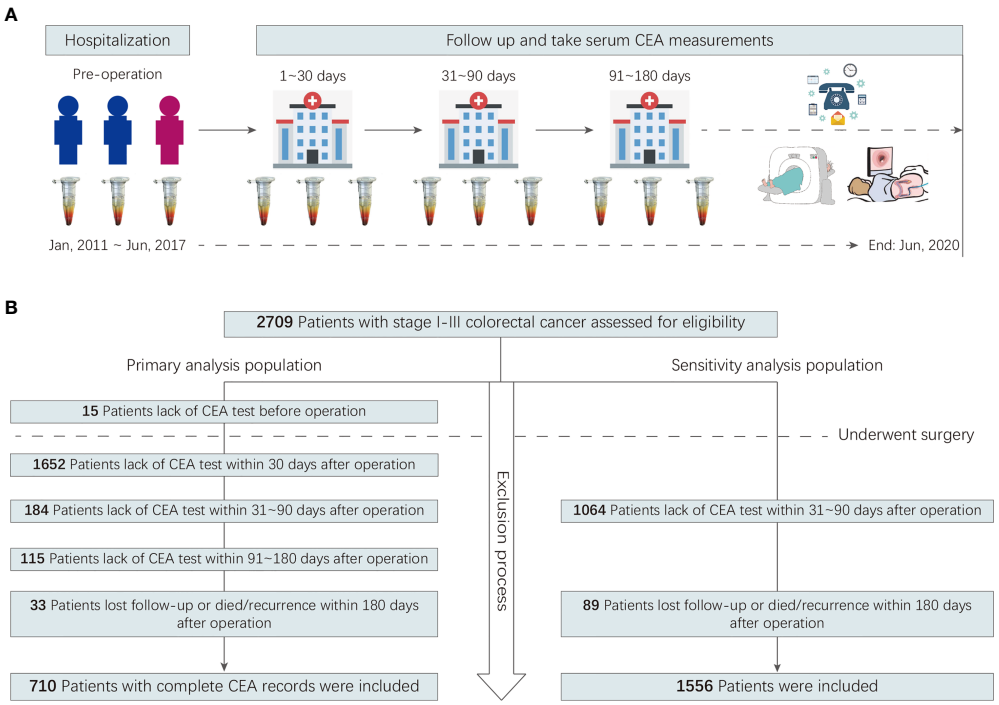


FIGURE 1 | Study flowchart.

tumor–node–metastasis (TNM) stage (I-III), lymph node yield (yes or no), mucinous (colloid) type (yes or no), the presence of lymphovascular invasion (yes or no), the presence of perineural invasion (yes or no), and the use of adjuvant chemotherapy (yes or no).

Statistical Analysis

All statistical analyses were performed using R (version 3.6.2). All tests were 2-sided, and *P* values <.05 indicated statistical significance. The mean, standard deviation (SD), and minimum and maximum values were used to describe results for continuous variables with a normal distribution (including age and body mass index [BMI]); these were further compared using the independent two-sample *t*-test. The group-specific number and percentage of patients in each category were used to describe results for categorical parameters, which were further compared using the chi-square (χ^2) test.

Differences in RFS between normal and elevated CEA groups at different time points were assessed using the Cox proportional hazards regression model. Hazard ratios (HRs) with two-sided 95% confidence intervals (CIs) were calculated for each group. Cumulative event curves were used to demonstrate the 3-year recurrence of patients with CRC, and log-rank tests were utilized to statistically analyze the differences between the two CEA groups.

To test the robustness of the risk estimates, we used two additional sensitivity analyses. (1) Multivariate Cox proportional hazards regression analysis with stepwise variable selection was performed to identify independent risk factors for recurrence and death. Three models were used: model 1 was unadjusted and constructed using $CEA_{pre\text{-}m1}$, $CEA_{post\text{-}m1}$, $CEA_{post\text{-}m2\text{–}3}$, and $CEA_{post\text{-}m4\text{–}6}$; model 2 was a version of model 1 adjusted for demographic variables; and model 3 was a version of model 2 adjusted for clinicopathological variables as well. (2) The statistical analyses used in the primary population were also performed in the expanded sensitivity analysis population.

To test for potential sources of heterogeneity, subgroup analyses were performed after stratification by age, sex, BMI, primary tumor site, tumor differentiation, mucinous (colloid) type, cancer stage, lymph node yield, the presence of lymphovascular invasion, the presence of perineural invasion, tumor deposit, $CEA_{pre\text{-}m1}$, and $CEA_{post\text{-}m1}$, with tests for interaction using the Cox regression model. Forest charts of subgroup-stratified analyses were created using the R package "forestplot."

To distinguish between high-recurrence risk and low-recurrence risk patients, associated with RFS differences, we have used maximally selected rank statistics to determine the potential threshold value of CEA (19).

RESULTS

Patient Characteristics

In total, 710 patients were included in the primary analysis. The number of participants assessed for eligibility and the reasons for exclusion are shown in **Figure 1**. The 710 patients included 415 men (58.5%), and the mean (SD) age was 54.8 (11.6) years. The mean age and SD of female and male patients were 54.2 ± 11.2 and 55.3 ± 11.8 years, respectively. The 385 patients underwent

laparoscopic surgery, 325 underwent open surgery. A total of 699 (98.5%) patients had adjuvant chemotherapy. The median long-term follow-up duration was 49.0 [interquartile range (IQR): 38.7–66.6] months. During the follow-up period, 152 patients (21.4%) showed recurrence, with an incidence density of 24.7 per 1,000 person-years. The characteristics of the primary analysis population are shown in **Table 1**.

The median (IQR) $CEA_{pre\text{-}m1}$, $CEA_{post\text{-}m1}$, $CEA_{post\text{-}m2\text{–}3}$, and $CEA_{post\text{-}m4\text{–}6}$ levels were 3.8 (2.0–8.8), 1.8 (1.2–2.9), 2.0 (1.3–2.9), and 2.2 (1.5–3.3) ng/mL, respectively. There were 417, 648, 662, and 642 patients with normal CEA levels before and 1, 2–3, and 4–6 months after surgery and 293, 62, 48, and 68 patients with elevated CEA levels before and 1, 2–3, and 4–6 months after surgery in the primary analysis population, respectively. The proportion of patients with elevated CEA levels at different perioperative time points showed a U-shaped curve, and the proportion observed within 2–3 months after surgery was the lowest (**Figures 2A, B**).

Association of CEA Status at Different Perioperative Time Points With RFS and OS

There was an inverted U-shaped association between CEA status at different perioperative time points and RFS (**Figure 2C**). Univariate analysis showed that recurrence HRs in the elevated

TABLE 1 | Demographic and Clinicopathological Characteristics of Primary Analysis Population.

Characteristics	Total (*N* =710)	$CEA_{post\text{-}m2\text{-}3}$		*P* value
		≤5 ng/ml (*n* = 662)	>5 ng/ml (*n* = 48)	
Age, year				
Mean (SD)	54.9 (11.6)	54.7 (11.6)	57.8 (11.0)	0.06
Range	(18.0-86.0)	(18.0-86.0)	(34.0-76.0)	
Sex, no. (%) of patients				
Male	415 (58.5)	385 (58.2)	30 (62.5)	0.66
Female	295 (41.5)	277 (41.8)	18 (37.5)	
BMI[a]				
Mean (SD)	23.0 (3.1)	23.0 (3.1)	22.9 (3.5)	0.87
Range	(15.2-35.4)	(16.8-35.4)	(15.2-29.8)	
Primary site, no. (%) of patients				
Colon	463 (65.2)	428 (64.7)	35 (72.9)	0.32
Rectum	247 (34.8)	234 (35.3)	13 (27.1)	
Pathological stage, no. (%) of patients				
I	22 (3.1)	21 (3.2)	1 (2.1)	0.25
II	266 (37.5)	253 (38.2)	13 (27.1)	
III	422 (59.4)	388 (58.6)	34 (70.8)	
Tumor differentiation, no. (%) of patients				
Well	29 (4.1)	28 (4.2)	1 (2.1)	0.25
M oderate	463 (65.2)	426 (64.4)	37 (77.1)	
Poor	197 (27.7)	189 (28.5)	8 (16.7)	
Unknown	21 (3.0)	19 (2.87)	2 (4.2)	
Mucinous (colloid) type, no. (%) of patients[a]				
Yes	39 (5.5)	36 (5.4)	3 (6.3)	>0.99
No	671 (94.5)	626 (94.6)	45 (93.8)	
T stage, no. (%) of patients				
T1 & T2	59 (8.3)	55 (8.3)	4 (8.3)	0.26
T3	592 (83.4)	555 (83.8)	37 (77.1)	
T4	59 (8.3)	52 (7.9)	7 (14.6)	
N stage, no. (%) of patients				
N0	287 (40.4)	274 (41.4)	13 (27.1)	0.15
N1	289 (40.7)	265 (40.0)	24 (50.0)	
N2	134 (18.9)	123 (18.6)	11 (22.9)	
Lymph node yield, no. (%) of patients[a]				
<12	105 (14.8)	101 (15.3)	4 (8.3)	0.27
≥12	605 (85.2)	561 (84.7)	44 (91.7)	
Lymphovascular invasion, no. (%) of patients				
Yes	96 (13.5)	86 (13.0)	10 (20.8)	0.19
No	614 (86.5)	576 (87.0)	38 (79.2)	
Perineural invasion, no. (%) of patients[a]				
Yes	68 (9.6)	60 (9.1)	8 (17.0)	0.13
No	641 (90.4)	602 (90.9)	39 (83.0)	

(Continued)

TABLE 1 | Continued

Characteristics	Total (*N* =710)	CEA $_{post-m2-3}$		*P* value
		≤5 ng/ml (*n* = 662)	>5 ng/ml (*n* = 48)	
Tumor deposit, no. (%) of patients[a]				
Positive	55 (11.7)	50 (11.3)	5 (18.5)	0.41
Negative	416 (88.3)	394 (88.7)	22 (81.5)	
Adjuvant chemotherapy, no. (%) of patients				
Yes	699 (98.5)	652 (98.5)	47 (97.9)	0.54[b]
No	11 (1.5)	10 (1.5)	1 (2.1)	
Adjuvant radiotherapy, no. (%) of patients				
Yes	6 (0.8)	5 (0.8)	1 (2.1)	0.34[b]
No	704 (99.2)	657 (99.2)	47 (97.9)	

SD, standard deviation; [a]Include some missing values since some patients did not accept these examinations; [b]Result of fisher's exact test.

CEA_{pre-m1}, $CEA_{post-m1}$, $CEA_{post-m2–3}$, $CEA_{post-m4–6}$ groups were 1.30 (95% CI: 0.91–1.85), 1.53 (95% CI: 0.89–2.62), 1.88 (95% CI: 1.08–3.28), and 1.15 (95% CI: 0.91–1.85), respectively. However, this association was only significant for elevated $CEA_{post-m2–3}$ levels (P = 0.03) in the primary analysis (**Table 2**). The HRs of the elevated CEA_{pre-m1}, $CEA_{post-m1}$, $CEA_{post-m2–3}$, and $CEA_{post-m4–6}$ groups for OS were 1.09 (95% CI: 0.60–1.97), 2.78 (95% CI: 1.34–5.79), 2.81 (95% CI: 1.25–6.30), and 3.30 (95% CI: 1.67–.536), respectively (**Table S1**).

Subsequently, adjusted multivariate Cox proportional hazards regression analyses showed that elevated $CEA_{post-m2–3}$, rather than CEA_{pre-m1}, $CEA_{post-m1}$, or $CEA_{post-m4–6}$, was an independent risk factor for recurrence, but not for OS, in the primary analysis population (**Table 2** and **Table S1**). Additionally, the adjustments resulted in a slight attenuation of the risk estimates in patients with elevated $CEA_{post-m2–3}$, both in model 2 (elevated $CEA_{post-m2–3}$ *vs.* normal $CEA_{post-m2–3}$: HR, 2.38; 95% CI: 1.23–4.61) and model 3 (elevated $CEA_{post-m2–3}$ *vs.* normal $CEA_{post-m2–3}$: HR, 2.10; 95% CI: 1.02–4.32) (**Table 2**).

Figure 3 shows the cumulative incidence rates of recurrence in the normal and elevated CEA groups at different perioperative time points. There was no significant difference in the 3-year recurrence rates between those with normal and elevated CEA levels before (19.8% *vs.* 15.6%; **Figure 3A**) and 1 month (24.2% *vs.* 16.7%; **Figure 3B**) after surgery. However, patients with elevated $CEA_{post-m2–3}$ levels showed a higher cumulative incidence rate of recurrence than patients with normal $CEA_{post-m2–3}$ levels in the primary analysis (29.2% *vs.* 16.5%; **Figure 3C**). In contrast, no significant differences in the 3-year recurrence rates were observed between patients showing elevated and normal CEA levels 4–6 months after surgery (19.1% *vs.* 17.1%; **Figure 3D**).

Sensitivity Analysis

The results from the sensitivity analysis are shown in **Tables S2** and **S3**. In addition to the primary analysis population, the sensitivity analysis population also included 846 patients for whom CEA_{pre-m1}, $CEA_{post-m1}$, or $CEA_{post-m4–6}$ levels were unavailable. The results were consistent with those obtained from the primary analysis. In the sensitivity analysis population, CEA_{pre-m1} (elevated CEA_{pre-m1} *vs.* normal CEA_{pre-m1}: HR, 1.50; 95% CI: 1.17–1.92) and $CEA_{post-m4–6}$ (elevated $CEA_{post-m4–6}$ *vs.* normal $CEA_{post-m4–6}$: HR, 1.81; 95% CI: 1.25–2.62) were associated with significantly shorter RFS in the univariate analysis but not in the multivariate analysis.

Subgroup Analysis

Patients with elevated $CEA_{post-m2–3}$ tended to have a higher risk of recurrence, similar to that in the overall population (**Figure 4** and **Figure S1**), in most subgroups except for among patients with normal CEA_{pre-m1}. It should be noted that the RFS of the elevated and normal $CEA_{post-m2–3}$ groups also differed significantly among patients with stage II CRC [elevated $CEA_{post-m2–3}$ *vs.* normal $CEA_{post-m2–3}$: HR, 2.89; 95% CI: 1.02–8.24 [primary analysis population); HR, 2.69; 95% CI: 1.34–5.38 (sensitivity analysis population)]. There were no statistically significant interactions between patients' baseline characteristics and $CEA_{post-m2–3}$ (all $P > 0.05$).

Threshold Value of $CEA_{post-m2–3}$

Patients were classified into $CEA_{post-m2–3}$-low (≤ 5.14 ng/mL) or $CEA_{post-m2–3}$-high (> 5.14 ng/mL) groups based on the optimal cut-off point determined by maximally selected rank statistics (**Figure S2**). And the RFS curves were statistically different (p = 0.003) when the threshold value of CEA was 5.14 ng/mL in the sensitivity analysis population (**Figure S3**).

DISCUSSION

Our analyses of a retrospective, multicenter longitudinal cohort of patients with stage I–III CRC who underwent curative resection showed that the association between serum CEA levels and CRC outcomes varied at different perioperative time points, and $CEA_{post-m2–3}$ was more informative than CEA_{pre-m1}, $CEA_{post-m1}$, and $CEA_{post-m4–6}$. Our data also showed that elevated $CEA_{post-m2–3}$ was associated with shorter RFS. This association seemed to be independent of traditional prognostic factors and CEA levels at other perioperative time points.

We found that elevated postoperative CEA levels were more prognostic than elevated preoperative CEA levels, consistent with

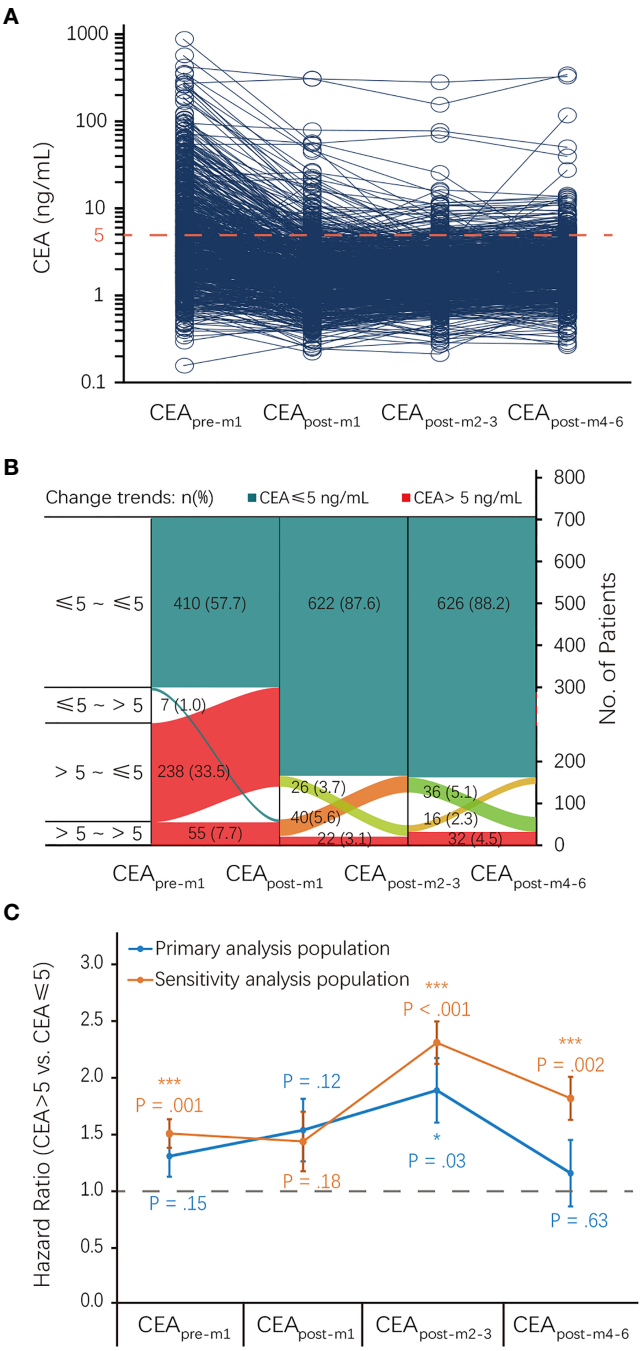


FIGURE 2 | CEA status at different perioperative time points and its association with RFS. **(A)** CEA levels of each patient at different perioperative time points. **(B)** The proportion of patients with elevated CEA levels at different perioperative time points. **(C)** Association of CEA status at different perioperative time points with RFS. CEA, carcinoembryonic antigen; CI, confidence interval; HR, hazard ratio; RFS, recurrence-free survival.

several previous studies (4, 10, 14–16). We also found, for the first time, that elevated $CEA_{post\text{-}m2\text{–}3}$ is more prognostic than elevated $CEA_{post\text{-}m1}$ and $CEA_{post\text{-}m4\text{–}6}$. It may be postulated that the prognostic value of perioperative CEA levels is more likely to depend on the proportion of CEA reflecting the biological behavior of tumors. The elevated tumor biomarker levels are due to tumor burden and differences in the biological behavior of tumors (20). Preoperative CEA levels are both related to the tumor burden and biological behavior, while postoperative CEA levels are mainly related to biological behavior. This may be why

TABLE 2 | Univariate and Multivariate Analysis of 3-year Recurrence Free Survival based on Primary Analysis Population.

Variables	Univariate analysis			Multivariate analysis (M1)[b]			Multivariate analysis (M2)[c]			Multivariate analysis (M3)[d]		
	HR	95%CI	*P* value	*HR*	95%CI	*P* value	*HR*	95%CI	*p*-value	*HR*	95%CI	*P* value
CEA (>5 *vs.*≤5), ng/ml												
CEA_{pre-m1}	1.30	0.91-1.85	0.15									
$CEA_{post-m1}$	1.53	0.89-2.62	0.12									
$CEA_{post-m2-3}$	1.88	1.08-3.28	**0.03**	1.88	1.08-3.28	**0.03**	1.91	1.210-3.34	**0.02**	1.91	1.09-3.35	**0.02**
$CEA_{post-m4-6}$	1.15	0.65-2.05	0.63									
Demographic variables												
Age, years	1.00	0.98-1.01	0.74	—	—	—						
Sex (Female *vs.* Male)	1.50	1.05-2.13	**0.03**	—	—	—	1.51	1.06-2.15	**0.02**	1.51	1.05-2.15	**0.02**
BMI[a]	0.96	0.89-1.03	0.22	—	—	—						
Clinicopathological variables												
Primary site (Rectum *vs.* Colon)	1.52	1.06-2.17	**0.02**	—	—	—	—	—	—	1.88	1.30-2.71	**<0.001**
Tumor differentiation (Well+Moderate *vs.* Poor)[a]	0.62	0.33-1.17	0.14	—	—	—	—	—	—			
Mucinous (colloid) type (Yes *vs.* No)[a]	1.19	0.58-2.44	0.64	—	—	—	—	—	—			
T stage (reference is T1+T2)												
T3	5.63	1.39-22.82	**0.02**	—	—	—	—	—	—	7.60	1.87-30.94	**0.005**
T4	7.54	1.71-33.16	**0.008**	—	—	—	—	—	—	10.59	2.37-47.37	**0.002**
N stage (reference is N0)												
N1	1.67	1.08-2.58	**0.02**	—	—	—	—	—	—	1.63	1.05-2.52	**0.03**
N2	2.72	1.70-4.35	**<0.001**	—	—	—	—	—	—	2.59	1.61-4.14	**<0.001**
Lymph node yield (≥12 *vs.*<12)[a]	1.04	0.63-1.74	0.87	—	—	—	—	—	—			
Lymphovascular invasion (Yes *vs.* No)	1.97	1.28-3.01	**0.002**	—	—	—	—	—	—			
Perineural invasion (Yes *vs.* No)[a]	1.75	1.06-2.88	**0.03**	—	—	—	—	—	—			
Tumor deposit (Positive *vs.* Negative)[a]	2.67	1.63-4.35	**<0.001**	—	—	—	—	—	—			

HR, Hazard ratio; aInclude some missing values since some patients did not accept these examinations; bM1: Unadjusted model; cM2: Model adjusted by demographic variables; dM3: Model adjusted by demographic and clinicopathological variables.
Bold indicates P value < 0.5.

elevated postoperative CEA levels are more prognostic than elevated preoperative CEA levels. In addition, the half-life of CEA varies from 3 to 7 days (21). Therefore, 3.0–18.0 weeks following surgery are required to allow for the clearance of CEA corresponding to tumor burden (16, 21). Interestingly, our data showed that the proportion of patients with elevated CEA levels within 2–3 months after surgery was the lowest. Together, these data indicate that the CEA level within 2–3 months after surgery may represent actual differences in the biological behavior of tumors. Hence, $CEA_{post-m2-3}$ is more strongly associated with CRC outcomes than $CEA_{post-m1}$ and $CEA_{post-m4-6}$.

The sensitivity and subgroup analyses supported our findings, demonstrating that the effect estimates were robust. It is important to note that the association between $CEA_{post-m2-3}$ and recurrence in patients with CRC may vary according to CEA_{pre-m1}, with an RFS advantage seen in patients with normal $CEA_{post-m2-3}$ and elevated CEA_{pre-m1} but not in patients with normal $CEA_{post-m2-3}$ and CEA_{pre-m1}. This suggests that elevated $CEA_{post-m2-3}$ may not be informative when CEA_{pre-m1} is normal. Moreover, this also implies that combined use of $CEA_{post-m2-3}$ and CEA_{pre-m1} may help clinicians in assessing the risk of recurrence better, thus allowing them to determine the optimal follow-up strategy and adjust adjuvant treatment regimens.

After subgroup analysis, our study also showed that postoperative CEA levels within 2–3 months after surgery had predictive value in patients with stage II CRC, consistent with some previous studies (11, 12). Our results confirmed that the prognostic value of serum CEA levels in patients with stage II CRC was affected by the timing of postoperative measurement. Our findings support the use of postoperative CEA measurements within 2–3 months as an indicator for the requirement of adjuvant treatment in patients with stage II CRC. And we found that the potential threshold value of $CEA_{post-m2-3}$ was 5.14 ng/mL, which was close to 5.0 ng/mL. Besides, The $CEA_{post-m2-3}$ had good prognostic value in OS analysis, though it was not significant in the multivariate model analysis. However, considering the clinical value of recurrence prediction, we believe that 2-3 months after surgery is the key time of perioperative serum CEA measurement.

The large size of the multicenter cohort ensured that our findings were robust when applied to different conditions, which is a major strength of our study. One limitation, however, is that different immunoassay analyzers were used for CEA measurements at the two centers. Even though harmonization of the CEA results obtained using the two immunoassay analyzers has not yet been achieved (22), the normal CEA ranges for both immunoassay analyzers are 0.0–5.0 ng/mL (17). We analyzed CEA levels as a dichotomized variable. Hence, the primary results of this study should not be affected by CEA testing methods. Another limitation is that the proportion of patients who were not treated with adjuvant chemotherapy was too low (1.5% and 8.4% in the primary and sensitivity analysis populations, respectively). Therefore, the results may not be generalizable to patients not receiving adjuvant chemotherapy. Finally, we did not control for other factors that can lead to false-positive CEA elevation (23), such

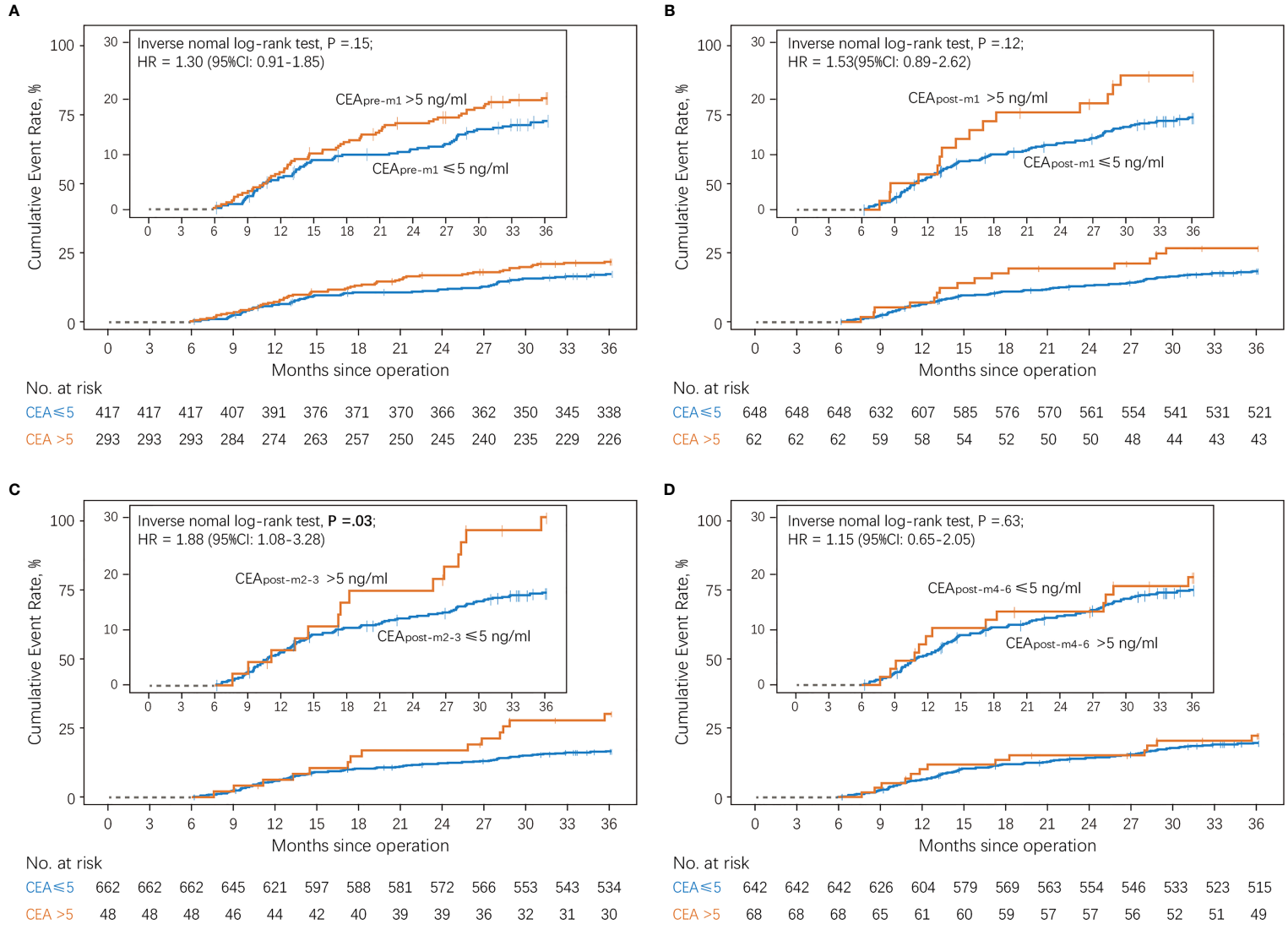
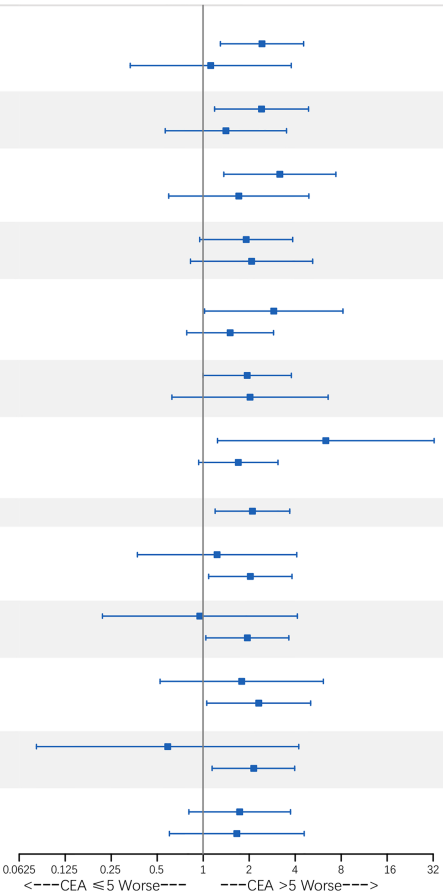


FIGURE 3 | Cumulative incidence of recurrence according to serum CEA levels compared using a log-rank test **(A)** Patients with normal *vs.* elevated preoperative CEA levels (CEA_{pre-m1}). **(B)** Patients with normal *vs.* elevated CEA levels 1 month after surgery ($CEA_{post-m1}$). **(C)** Patients with normal *vs.* elevated CEA levels 2–3 months after surgery ($CEA_{post-m2–3}$). **(D)** Patients with normal *vs.* elevated CEA levels 4–6 months after surgery ($CEA_{post-m4–6}$). CEA, carcinoembryonic antigen.

Subgroup	No. of Patients (%)	Hazard Ratio[b]	95%CI	P value	P value for Interaction
Age					
< 65 year	556 (78.3)	2.43	1.30–4.54	**.006**	.39
⩾ 65 year	154 (21.7)	1.12	0.33–3.77	.85	
Sex					
Male	415 (58.5)	2.41	1.19–4.89	**.02**	.37
Female	295 (41.5)	1.41	0.56–3.51	.74	
BMI[a]					
< 24	304 (64.5)	3.17	1.36–7.39	**.007**	.07
⩾ 24	167 (35.5)	1.71	0.60–4.92	.32	
Primary site					
Colon	463 (65.2)	1.91	0.95–3.85	.07	.90
Rectum	247 (34.8)	2.07	0.83–5.21	.12	
Pathological stage					
II	266(37.5)	2.89	1.02–8.23	**.04**	.33[c]
III	422(59.4)	1.5	0.78–2.89	.23	
Tumor differentiation[a]					
Well+ Moderate	492 (71.4)	1.94	1.00–3.78	.05	.95
Poor	197 (28.6)	2.03	0.62–6.57	.24	
Mucinous (colloid) type[a]					
Yes	39 (5.5)	6.34	1.24–32.47	**.03**	.20
No	671 (94.5)	1.70	0.93–3.09	.08	
Lymph node yield[a]					
⩾ 12	605 (85.2)	2.10	1.20–3.69	**.01**	>.99
Lymphovascular invasion					
Yes	96 (13.5)	1.23	0.37–4.10	.73	.47
No	614 (86.5)	2.04	1.09–3.81	**.03**	
Perineural invasion[a]					
Yes	68 (9.6)	0.95	0.22–4.13	.95	.37
No	641 (90.4)	1.95	1.04–3.64	**.04**	
Tumor deposit[a]					
Positive	55 (11.7)	1.79	0.52–6.12	.36	.64
Negative	416 (88.3)	2.31	1.06–5.05	**.04**	
CEA $_{pre-m1}$					
⩽ 5 ng/ml	417 (58.7)	0.59	0.08–4.22	.60	.22
> 5 ng/ml	293 (41.3)	2.14	1.14–3.97	**.02**	
CEA $_{post-m1}$					
⩽ 5 ng/ml	648 (91.3)	1.73	0.81–3.73	.16	.95
> 5 ng/ml	62 (8.7)	1.66	0.60–4.58	.33	



Note: [a]Include some missing values since some patients did not accept these examinations; [b]Hazard Ratio: (CEA$_{post-m2-3}$>5 vs. CEA$_{post-m2-3}$⩽5) ; [c]Test for linear trend used.

FIGURE 4 | Forest plot of CEA$_{post-m2–3}$ stratified by clinicopathological variables in the primary analysis population. Note: [a] Includes some missing values since some patients did not accept these examinations; [b] HR: (CEA >5.0 *vs.* ≤5.0 ng/mL); [c] Test for linear trend used. *P* values for interaction were calculated using the Cox regression model. HR and 95% CIs are provided and are visually represented by the squares and error bars. CEA, carcinoembryonic antigen; CEA$_{post-m2–3}$, serum CEA levels 2–3 months after surgery; CI, confidence interval; HR, hazard ratio; RFS, recurrence-free survival.

as tobacco use (24), as this was challenging to accurately ascertain from the patients.

In conclusion, our study provides evidence that elevated CEA$_{post-m2–3}$, rather than CEA$_{pre-m1}$, CEA$_{post-m1}$, and CEA$_{post-m4–6}$, is associated with CRC outcomes. The optimal timing for perioperative serum CEA measurement is 2–3 months after surgery for patients with CRC, and CEA$_{post-m2–3}$ can be used as a predictor of RFS. Our findings suggest that prolonged adjuvant chemotherapy and more frequent follow-ups should be considered to reduce the risk of relapse in CRC patients with elevated CEA$_{post-m2–3}$.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Yunnan Cancer Hospital (KY201824). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DY had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design, DY. Acquisition, analysis, or interpretation of data, ZL, ZW, XP, SY, DZ, ML, XC, QS, LC, and DY. Drafting of the manuscript, ZL, ZW, XP, and SY. Critical revision of the manuscript for important intellectual content, ZL,

ZW, XP, SY, DZ, ML, XC, QS, LC, and DY. Statistical analysis, ZL, ZW, and DY. Administrative, technical, or material support, DZ, ML, XC, QS, and LC. Study supervision, DY. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.722883/full#supplementary-material

Supplementary Figure 1 | Forest plot of $CEA_{post\text{-}m2\text{–}3}$ stratified by clinicopathological variables in the sensitivity analysis population. Note: [a] Includes some missing values since some patients did not accept these examinations; [b] HR: (CEA >5.0 *vs.* ≤5.0 ng/mL); [c] Test for linear trend used. *P* values for interaction were calculated using the Cox regression model. HR and 95% CIs are provided and are visually represented by the squares and error bars. CEA, carcinoembryonic antigen; $CEA_{post\text{-}m2\text{–}3}$, serum CEA levels 2–3 months after surgery; CI, confidence interval; HR, hazard ratio; RFS, recurrence-free survival.

Supplementary Figure 2 | The optimal cut-off to CEA as high-recurrence risk and low-recurrence risk patient was determined by maximally selected rank statistics method.

Supplementary Figure 3 | Kaplan-Meier survival curves of RFS of CEA-low *vs* CEA-high categories in the sensitivity analysis population. CEA-low: $CEA_{post\text{-}m2\text{–}3} \leq$ 5.14 ng/mL; CEA-highd: $CEA_{post\text{-}m2\text{–}3} >$ 5.14 ng/mL

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Proteomics-Based Identification of Candidate Exosomal Glycoprotein Biomarkers and Their Value for Diagnosing Colorectal Cancer

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Early diagnosis and treatment of colorectal cancer (CRC) significantly improves the survival rate and quality of life. Here we screened for differences in glycoproteins associated with tumor-derived exosomes and validated their clinical value to serve as liquid biopsy biomarkers to diagnosed early CRC. Exosomes were extracted from paracancerous tissues, cancer tissues, and plasma. LC-MS/MS proteomic and glycoproteomics analyses were performed using an LTQ-Orbitrap Elite mass spectrometer. The differences in glycoproteins associated with exosomes of paracancerous tissues and cancer tissue were determined, and their levels in plasma exosomes were determined. Statistical analysis was performed to evaluate the diagnostic efficacy of exosome-associated glycoproteins for CRC. We found that the levels of fibrinogen beta chain (FGB) and beta-2-glycoprotein 1 (β2-GP1) in the exosome of CRC tissue were significantly higher compared with those of paracancerous tissues exosome. The areas under the receiver operating characteristic (ROC) curves of plasma exosomal FGB and β2-GP1 as biomarkers for CRC were 0.871 (95% CI = 0.786–0.914) and 0.834 (95% CI = 0.734–0.901), respectively, compared with those of the concentrations of carcinoembryonic antigen concentration [0.723 (95% CI = 0.679–0.853)] and carbohydrate antigen19-9 concentration [0.614 (95% CI = 0.543–0.715)]. Comprehensive proteomics analyses of plasma exosomal biomarkers in CRC identified biomarkers with significant diagnostic efficacy for early CRC, which can be measured using relatively non-invasive techniques.

Keywords: colorectal cancer, exosome, receiver operating characteristic, fibrinogen beta chain, beta-2-glycoprotein

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INTRODUCTION

Colorectal cancer (CRC) is the third most common malignant tumor and is a serious global threat to human health (1). Early diagnosis of CRC is clinically significant, because it significantly improves patients' survival rate and their quality of life. For example, the 5-year survival rates are 90% for patients with early-stage CRC and 13.1% for those diagnosed with late-stage CRC. Unfortunately, the lack of symptoms and biomarkers for early-stage CRC mainly explains the inability to diagnose early CRC, which excludes the possibility to provide such patients with potentially life-saving treatment.

Available approaches for screening for CRC are mainly based on endoscopic analysis of the mucosae followed by biopsy and fecal occult blood test (FOBT). These techniques are inherently limited, particularly for early diagnostic. Furthermore, endoscopic exams are invasive, costly, and associated with discomfort and procedural risk. Although the FOBT is non-invasive and affordable, its insufficient sensitivity and specificity prevent its use as a stand-alone diagnostic test (2, 3). Serum biomarkers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are considered among the best available prognostic markers for CRC (3, 4). However, their low sensitivity and specificity limit their use as biomarkers for early diagnosis, and their expression levels are only applied for post-resection monitoring of patients already diagnosed with cancer. Certain new serological biomarkers such as non-coding RNAs are being evaluated for their clinical diagnostic value. Therefore, non-invasive, rapid, simple, and effective biomarkers for early diagnosis and for monitoring of the prognosis of patients with CRC are urgently required.

Recently developed as biomarker, exosomes are attracting much attention. Exosomes, which are derived from blood cells, dendritic cells, tumor cells, and other sources under physiological and pathological conditions, comprise complex membrane packets containing molecules such as miRNAs, mRNAs, lncRNAs, proteins, and bioactive lipids (5, 6). Exosomes released from donor cells into the cancer microenvironment affect the functions of target cells (6, 7). Their variations in abundance and half-lives in all biological fluids contribute to the potential of exosomes to serve as a source of biomarkers for early diagnosis, monitoring, and prognosis of patients with cancer. Evidence suggests that cancer-derived exosomes may contribute to tumorigenesis and metastasis as well as serving as biomarker (8, 9). Moreover, their presence in most body fluids makes exosomes potential candidates as clinical biomarkers for the early detection of different cancers, particularly because relatively non-invasive techniques can be used for this purpose (10–14). Most studies on exosomes as biomarker focused on associated nucleic acids, which can be amplified *in vitro* to enable high sensitive detection. Furthermore, the glycoprotein-associated exosomes may serve as biomarkers that can be readily measured using available detection technologies.

Protein glycosylation affects protein conformation, stability, spatial conformation, biological activity, transport, and localization required for diverse biological processes such as molecular recognition, cellular communication, and signal transduction (15–18). Glycoproteins, which are covalently linked through glycosidic bonds to oligosaccharides, are associated with the pathogenesis and progression of infectious diseases, tumors, cardiovascular disease, liver disease, kidney disease, diabetes, and certain genetic diseases (19–23). Moreover, glycoproteins on the cell surface are shed into the extracellular environment or enter the circulation and therefore can be used as biomarkers for abnormalities, which may be helpful for clinical diagnosis. The levels of certain glycoproteins in body fluids undergo disease-specific changes, which can be helpful for early diagnosis, guiding treatment, and prognosis. However, N-glycosylation proteins are present at low levels, which along with their structural complexity, makes them extremely difficult to detect. Therefore, it is a challenging but important task to detect and analyze the glycoproteins in plasma exosomes of patients with CRC.

Here we conducted proteomic and glycoproteomics analyses of three pairs of paracancerous tissues and their corresponding cancer-tissues exosomes. ELISA was used to detect selected glycoproteins associated with plasma exosome for the purpose of evaluating their potential to serve as biomarkers, with the ultimate goal to improve the early diagnosis of CRC.

MATERIALS AND METHODS

Extraction of Tissue Exosomes

Banked frozen human tissue samples, including three pairs of paracancerous tissues and CRC tissues, were obtained from the Department of Gastrointestinal Surgery, Shanghai Tongji Hospital, Tongji University School of Medicine. Tissue samples were transported in ice and then added to 1640 medium precooled at 4°C. The tissues were cut into 1 mm^3 pieces and kept on ice. Collagenase IV (350 μl, 1 mg/ml) and 2 μl of 0.2% (w/v) DNase I were added to the tissues, which were gently mixed, incubated in a constant temperature shaker (100 rpm/min) at 37°C for 60-90 min, and then stored 4°C. Processing time was ≤90 min. The tissue homogenates were then centrifuged at 3,000 × g at 4°C for 30 s, and the supernatant was centrifuged at 13,000 × g at 4°C for 10 min. The supernatant was passed through a 0.22 μm filter, mixed the PEG6000 (16% w/v) (1:1, v/v), gently mixed, incubated overnight at 4°C, and then centrifuged at 13,000 × g at 4°C for 30 min. The precipitate containing exosomes was centrifuged at 13,000 × g at 4°C for 5 min and suspended in.

Extraction of Plasma Exosomes

Subjects granted their written informed consent for donating plasma (EDTA-K2) samples and pathological information. Plasma exosomes were prepared from blood samples of 30 patients with CRC enrolled between January 2016 and July 2016, as well as from 20 healthy individuals matched for sex and age (test and validation sets, respectively). Patients' detailed clinical data are summarized in **Table 1**. Preoperative blood samples were collected into tubes containing an anticoagulant and centrifuged at 3,000 × g for 15 min at 4°C. The supernatant

TABLE 1 | Characteristics of subjects (n.s., not significant).

Characteristics	Controls (n = 20)	CRC (n = 30)	p
Gender, n (%)	12 (60)	17 (56.7)	0.453
Smoking, n (%)	6 (30)	12 (40)	0.027
Drinking, n (%)	7 (35)	10 (33.3)	0.068
FGB (ng/L)	14.61 ± 3.12	24.34 ± 3.65	<0.01
β2-GP1 (ng/L)	23.46 ± 4.21	35.93 ± 5.61	<0.01
CEA (ng/ml)	3.40 ± 1.88	15.10 ± 5.80	0.021
CA19-9 (U/ml)	9.71 ± 3.52	18.96 ± 4.51	0.027

(250 µl) was added to a new tube, to which Exo-Quick^{TM} solution (EXOQ5A-1; SBI System Biosciences, USA) (63 µl) was added. The mixture was mixed, kept at room temperature for 30 min, and then centrifuged at 1,500 × g for 30 min. The supernatant was discarded, and the pellets were resuspended at 1,500 × g for 5 min. The pellets containing total exosomes were resuspended in 100 µl of phosphate-buffered saline (PBS).

Transmission Electron Microscopy

Isolated exosomes were resuspended in PBS, and 20 µl of the suspension was placed on a carbon-coated copper grid, which was incubated for10 min at room temperature. Next, the grid was washed using sterile distilled water, and 2% uranyl-oxalate solution was placed on the grids for 1 min and dried in air. The samples were observed using an electron microscope (JEOL-JEM1400, Tokyo, Japan).

Nanoparticle Tracking Analysis

To measure the size and quantities of isolated particles, the suspension (1×10^7/ml and 1×10^9/ml) were examined using ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) equipped with a 405 nm laser. Videos were recorded (60 s, frame rate of 30 s), and particle movement was analyzed using NTA software (ZetaView 8.02.28).

Western Blot Analysis

Exosome suspension was diluted with 5× sodium dodecyl sulfonate (SDS) buffer and was boiled for 10 min. Western blot analysis employed 10% SDS-polyacrylamide gel electrophoresis, 50 µg protein/lane. CD63 and TSG101 served as positive controls, and Calnexin served as a negative control. The rabbit polyclonal antibody CD63 (ab68418, 1:1000), TSG101 (ab30871, 1:1000), and Calnexin (ab22595, 1:1000) were purchased from Abcam (Cambridge, UK). After, samples were incubated with primary antibodies (overnight at 4°C), followed by the addition of an IgG goat anti-rabbit secondary antibody (1:2,000, A21020, Abbkine, Scientific Co., Ltd., Wuhan, China) for 1 h at 37°C. Immunocomplexes were detected using an enhanced chemiluminescence reagent (1856190; Thermo Scientific, USA).

Proteomics and Glycoproteomics Analysis of Exosomes

Protein extraction: Exosomes were suspended in water, and proteins were precipitated using a solution containing chloroform: methanol: water (1:3:4, v/v). The middle layer containing a white precipitate of protein was washed twice with methanol.

Protein Digestion: Proteins were digested using FASP method and dissolved in 4% SDS, 50 mM DTT in 50 mM Tris-HCl (pH8.0). The solution was subsequently heated at 95°C water bath for 10 min, diluted with 8 M urea in 100 mM Tris-HCl, pH8.5 (UA solution) (final SDS concentration <0.5%), transferred to an Amicon 30-kD aultracentrifugal filter unit (MRCF0R030, Merck), and centrifuged at 14,000 × g for 30 min. Alkylation was performed by adding 50 µl of UA solution with 50 mM iodoacetamide to the filter unit, followed by incubation in the dark for 30 min at room temperature. After centrifugation at 14,000 × g for 10 min, 100 µl of UA solution was added to the filter unit, which was centrifuged four times. The filter unit was then washed three times with 100 µl of 50 mM NH_4HCO_3. Next, proteins were digested by adding 100 µl of 50 mM NH_4HCO_3 containing sequencing-grade trypsin (enzyme to protein ratio = 1:50) to the filter unit and incubating at 37°C for 14 h. Peptides were eluted using 100 µl of 50 mM NH_4HCO_3 and were collected by centrifugation at 14,000 × g for 10 min. This step was repeated five times. The peptides were further purified using a prepacked C18 ZipTip micro-column.

Glycopeptide enrichment: The enrichment of glycopeptides was performed using an iSPE HILIC cartridge. Briefly, the HILIC cartridge was prewashed with 300 µl of 0.1% TFA and equilibrated with 600 µl of 80% ACN containing 0.1% TFA. Peptide samples dissolved in 400 µl of 80% ACN containing 0.1% TFA were loaded onto the cartridge. The flow-through was reloaded onto the column twice, and the column was then washed with 1.2 ml of 80% ACN containing 1% TFA. The glycopeptides were sequentially eluted with 750 µl of 0.1% TFA, 60 µl of H_2O, 60 µl of 25 mM NH_4HCO_3, and 60 µl of 50% ACN. The fractions were combined followed by lyophilization and stored at –20°C. Glycopeptide was then dissolved by 50 mM NH_4HCO_3 in ^{18}O water and digested using PGNase F at 37°C for 16 h.

Proteomic and glycoproteomics analyses were performed using an LTQ-Orbitrap Elite mass spectrometer (ThermoFisher) equipped with an EASY-Spray source and a nano-LC UltiMate 3000 high-performance liquid chromatography system (Thermo Fisher). Each sample was separated using reversed-phase (RP)-HPLC fractionation on an EASY-Spray PepMap C18 column (length, 50 cm; particle size, 2 µm; pore size, 100 Å; Thermo Fisher), using a 120 min gradient as follows: 2 to 50% solvent B, flow rate of 300 nl/min (mobile phase A, 1.95% acetonitrile, 97.95% H_2O, 0.1% formic acid). A full-scan survey MS experiment (*m/z* range from 375 to 1,600; automatic gain control target, 1,000,000 ions; resolution at 400 *m/z*, 60,000; maximum ion accumulation time, 50 ms) was

performed using an Orbitrap mass analyzer. The 10 most intense ions were selected and fragmented in the LTQ mass spectrometer (automatic gain control target value, 10,000) *via* collision-induced dissociation (CID) with 100 ms maximum ion accumulation. Raw data were analyzed using Proteome Discoverer 1.4 (Thermo Fisher) to query the human Uniprot/TrEMBL database (2016_02 Release, 20,198 reviewed entries). Modifications were as follows: static modification of *via* carbamidomethyl (Cys, + 57.0214 Da); dynamic modification of glycosylation (Asn, + 2.9882 Da), oxidation (Met, + 15.9949 Da), and acetylation (Lys, + 42.0106 Da). Trypsin was selected as the proteolytic, and up to two missed cleavages were allowed. The mass tolerance was set 20 ppm for the precursor ions and 0.5 Da for the fragment ions. The false discovery rate = 1% for peptide and protein identification.

ELISA Quantification of Glycoproteins

Exosomes were precipitated in 100 μl of RIPA lysis solution on ice for 30 min, oscillated, and fully mixed. The samples were diluted three times with 1×PBS. The levels of plasma exosomal FGB and β2-GP1 were determined using sandwich immunoassay after generating a standard curve using serial dilution of FGB and β2-GP1 (JL47995/JL19205, Jianglai Biotechnology Co., LTD, Shanghai, China). Briefly, samples (100 μl) were added to the ELISA plate, incubated for 60 min at 37°C, washed three times, after which solution B was added for 30 min at 37°C, plates were washed five times, followed by the addition of 90 μl of substrate, incubated for 15 min at 37°C, and addition 50 μl of termination solution. Absorbance at 450 nm was immediately measured.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0. Exosomal glycoproteins were evaluated to identify patients of CRC and healthy individuals. Clinicopathological diagnoses served as the gold standard to assess the diagnostic significance of exosomal glycoprotein levels according to the results of receiver operating characteristic (ROC) curves analysis. $P < 0.05$ indicates a significant difference.

RESULTS

Identification of Exosomes Extracted From Tissue and Plasma

Subjects' detailed information (30 patients with CRC and 20 healthy controls) is listed in **Table 1**. TEM analysis of plasma and tissue samples revealed the presence of round, cup-shaped, double-membrane-bound, vesicle-like structure (**Figure 1A**). NTA revealed that the diameters of spherical nanoparticles moving under Brownian motion ranged between 30 and 150 nm (**Figure 1B**). Western blot analysis of these samples detected the exosome markers CD63 and TSG101, but not the negative control, Calnexin (**Figure 1C**).

Identification of Glycoproteins Specific for Colorectal Cancer

The workflow of the study (**Figure 2**) shows the screening (I) and verification (II) phase. In phase I, tissue exosomes were collected from patients with CRC and digested with trypsin for LC-MS/MS analysis. Database searches identified the corresponding glycoproteins. In phase II, ELISA was used to detect the levels of selected glycoproteins.

Identification of Tissue Exosomal Total Proteins and Glycoproteins

Analysis of tissue exosomes pooled from three patients with CRC unambiguously identified 985 proteins in cancer tissues and 1,022 proteins in paracancerous tissue, among which 420 were identified in tissue exosomes of each source (**Figure 3A**). Furthermore, 565 and 602 proteins were unique to paracancerous tissue exosomes or cancer tissue exosomes, respectively. We unambiguously identified 181 glycoproteins in cancer tissue and 161 glycoproteins in paracancerous tissue, among which 113 and 93 glycoproteins, respectively, were unique (**Figure 3B**).

Functional Classification of Glycoproteins of Tissue Exosomes

Gene Ontology analysis revealed that the frequencies of glycoprotein functions in paracancerous tissue exosomes were as follows: binding (53.20%), catalytic activity (32.30%), receptor activity (5.10%), transporter activity (3.80%), signal transducer activity (3.20%), structural molecule activity (1.90%), and antioxidant activity (0.60%) (**Figure 4A**). The functions of glycoproteins in cancer tissue exosomes were as follows: binding (52.10%), catalytic activity (32.60%), receptor activity (4.90%), transporter activity (4.20%), signal transducer activity (2.80%), structural molecule activity (2.10%), and antioxidant activity (1.40%) (**Figure 4B**). The functions of the majority of shared glycoproteins in tissue exosomes were as follows: binding (54.10%), catalytic activity (33.30%), receptor activity (4.50%), transporter activity (2.70%), signal transducer activity (2.70%), structural molecule activity (1.80%), and antioxidant activity (0.90%) (**Figure 4C**).

Mass Spectrometry of Glycosylation Site in Glycoproteins in CRC Tissue Exosomes

N-glycosylation sites of tissue exosome samples were labeled using ^{18}O during PNGase F digestion process. The asparagine (Asn) linked to glycan is converted to an aspartic acid residue paracancerous, and the oxygen atom in the hydroxyl moiety of the functional group of Asp is replaced with ^{18}O. Thus, LC-MS/MS analysis detects a 2.9883 Da difference between glycosylated and unglycosylated Asp residues. Furthermore, through the CID fragmentation of the peptide, b and y ions are generated that confirm the peptide structure. **Figure 5A** shows the glycosylation of Asn394 of fibrinogen beta, consistent with six published data. **Figure 5B** shows the glycosylation of Asn183 and Asn193 of beta-2-glycoprotein 1, consistent with four published data.

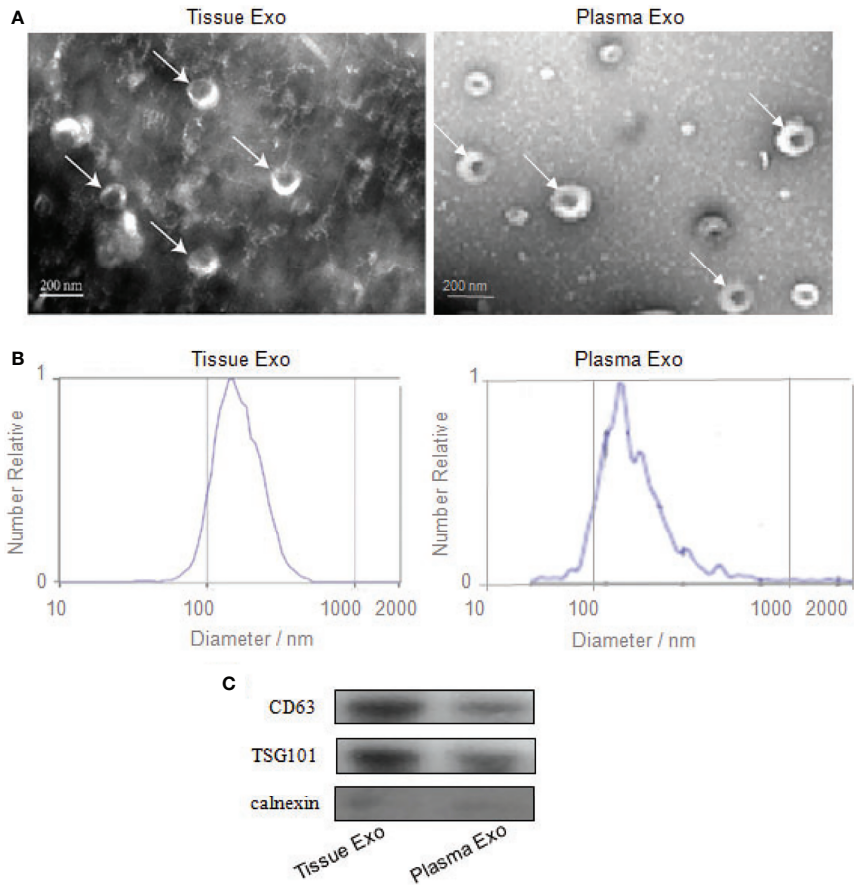

FIGURE 1 | Identification of exosomes from tissue and plasma. **(A)** Transmission electron microscopy confirmed the presence of exosomes. Scale bar=200 nm. **(B)** Nanoparticle-tracking analysis determined the sizes of exosomes. **(C)** Western blotting analyzed the exosomes-enriched positive protein markers of CD63 and TSG101, and negative protein marker of Calnexin.

Elisa Analysis of the Glycoproteins as Biomarkers for Early Diagnosis of CRC

We next used an ELISA to determine the glycoprotein levels of plasma exosomes of selected patients and controls. The levels of FGB and β2-GP1 were significantly higher in patients with CRC compared with those of healthy controls ($p<0.01$) (**Figure 6A**).

To understand if analyzing the levels of plasma exosomal glycoproteins served as diagnostic biomarkers, we evaluated the diagnostic efficacies of FGB and β2-GP1 in plasma exosomes and compared them with the values of CEA and CA19-9 (**Table 2**). The discriminatory power of each putative biomarker was further evaluated using (ROC) area-under-the-curve (AUC) analysis. The data show that the AUC value (0.871) of FGB directly isolated from plasma exosomes was higher compared with the values of serum CEA and CA19-9 (0.625 *vs.* 0.614). Furthermore, the AUC value of β2-GP1 (0.834) was higher compared with the values of CA19-9 and CEA. Moreover, combining the levels of the two plasma exosomal glycoproteins achieved a higher AUC compared with the values of CA19-9 and CEA (**Figure 6B**).

DISCUSSION

Upon diagnosis, the majority of patients with CRC present with advanced- to middle- and late-stage disease, mainly because of undetectable previous symptoms and the absence of specific biomarkers that detect early disease. Despite advances in clinical diagnosis and therapy, most patients experience very low survival rate. To address this serious problem, here we applied a novel targeted mass spectrometry proteomic approach to screen exosomal glycoproteins as potential biomarkers for early CRC. For example, no study, to our knowledge, reports the quantitation of differences in the abundances of exosomal glycoproteins between those of patients with CRC compared with controls, particularly using the specific combination of instruments.

In this study, we detected large amounts of lipids compared with those of proteins during extraction, and the CRC group had more glycoproteins than the control group (**Figure 3**). The associated targets of differentially expressed glycoproteins in cancer and paracancerous tissue exosomes were predicted, and

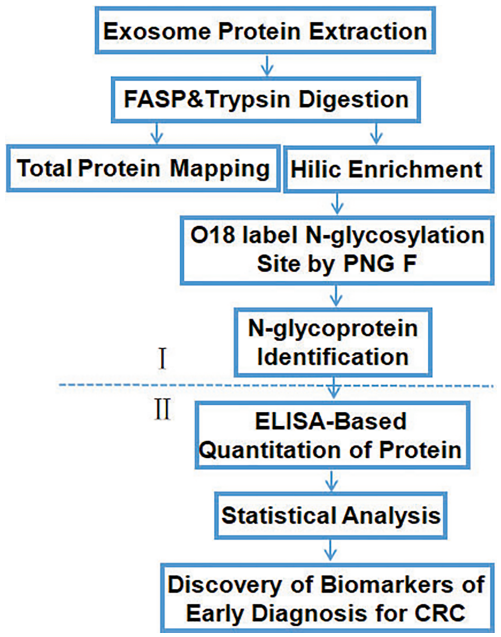

FIGURE 2 | Workflow of the identification and quantification of exosomal glycoproteins in healthy controls and patients with CRC.

their functional annotation was carried out using GO enrichment analysis. The results showed that they were involved in several potential biological pathways, including binding, catalytic activity, receptor activity, transporter activity, signal transducer activity, structural molecule activity, and antioxidant activity (**Figure 4**). The antioxidant activity was significantly different in paracancerous tissues and their corresponding cancer-tissue exosomes, indicating that the antioxidant activity of glycoproteins may be increased in patients with CRC. We identified glycosylated Asn394 of FGB and glycosylated Asn183 and Asn193 of β2-GP1 (**Figure 5**). Our protein identification and quantitation techniques are not high-throughput, and a relatively small sample set was used. Further verification of candidate glycoprotein markers was therefore required. For this purpose, we performed ELISA analysis, which confirmed that glycosylated forms of FGB and β2-GP1 were present at higher levels in the plasma exosome of patients with CRC compared with those of controls (**Figure 6A**).

The glycoprotein fibrinogen, which is synthesized and secreted mainly by hepatocytes, comprise three pairs of distinct polypeptide chains linked by disulfide bonds, termed α, β, and γ-chains (24). High fibrinogen levels serve as an important risk factor and clinical marker for thrombotic diseases. Furthermore, increased levels of plasma fibrinogen correlate with cancer metastasis, recurrence, and shorter survival (25). Plasma fibrinogen serves as an important tumor biomarker for cancers of the digestive tract, which is non-invasively measured, making it suitable for initial screening or combined with other biomarkers for cancer diagnosis or prevention (26).

The assessment of fibrinogen content and fibrinolysis product in plasma contributes to the diagnosis of cancer and the evaluation of therapy, tumor progression, tumor stage, and survival (27). FGB, which is cleaved to fibrin during the formation of blood clots, is present at higher levels in poor responders with rectal cancer, and a clinical validation study confirmed the predictive value of FGB (28). FGB levels significantly differ in the urinary tracts of patients with bladder cancer compared with those of controls and are elevated in bladder cancer tissue compared with those of morphologically normal tissue, indicating that FGB is a potential biomarker for bladder cancer (29).

Here we show that FGB-Asn394 is glycosylated, and its levels in patients with CRC significantly differed from those of healthy controls (**Figure 5**). Furthermore, plasma exosomal FGB achieved a higher value for diagnosis of early CRC compared with those of CEA and CA19-9 (**Figure 6B**).

The plasma glycoprotein β2-GP1 circulates in blood, primarily in free form, which contributes to triglyceride metabolism, blood coagulation, and homeostasis (30–32). Moreover, β2-GP1 inhibits apoptosis, LDL oxidation, and cholesterol accumulation in vascular cells, suggesting that β2-GP1 may regulate vascular functions (33, 34). β2-GP1 contributes to angiogenesis and is required to downregulate

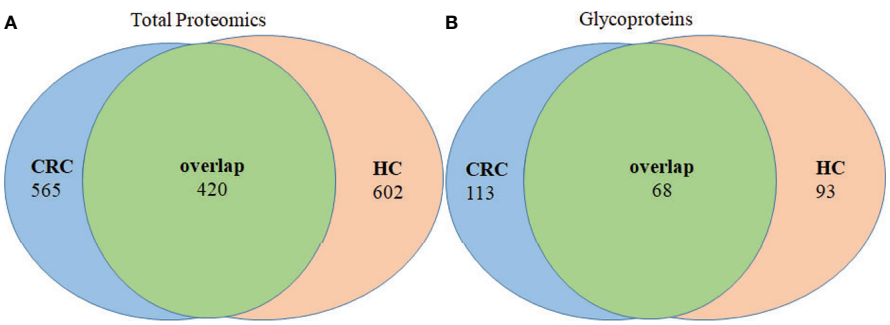

FIGURE 3 | Venn diagram of proteins of paracancerous tissues and cancer-tissue exosome samples isolated from patients with CRC. **(A)** The distribution of total proteins and **(B)** unique glycoproteins.

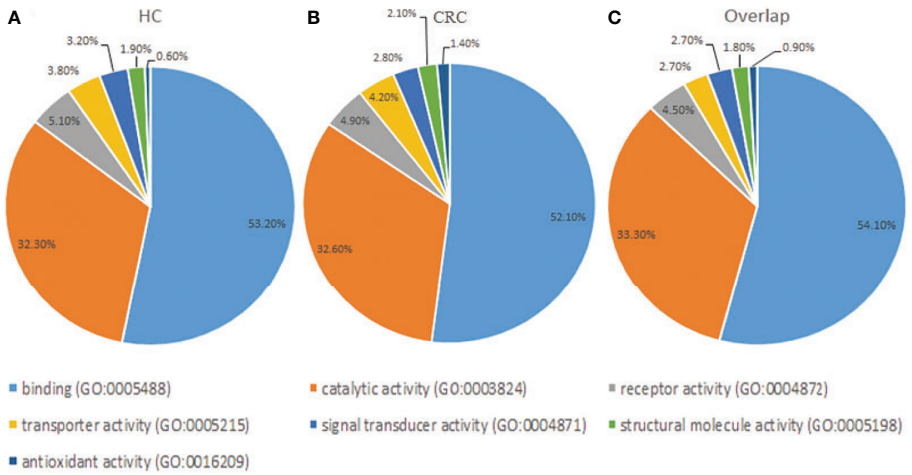

FIGURE 4 | Gene Ontology analysis of the distribution of glycoproteins according to molecular function (http://exocarta.org/exosome_markers_new). **(A)** Paracancerous tissue exosomes (HC). **(B)** Cancer tissue exosome (CC). **(C)** Common cancer tissue and paracancerous tissue exosomes (Overlap). The frequencies of the glycoprotein functional categories are presented as percentages.

VEGF-induced cell growth and migration *in vitro* and *in vivo*, and inhibits the phosphorylation of VEGFR2, ERK1/2, and Akt (35). The circulating levels of β2-GP1 INHIBIT tumor growth and exert antiangiogenic effect on melanomas, bladder cancer, and prostate cancer, suggesting that β2-GP1 is a potential marker of the efficacies of angiogenesis-targeted therapy and diagnosis (36–39).

Here we detected the glycosylation of β2-GP1 residues Asn183 and Asn193 and found that β2-GP1 was present in higher levels in plasma exosomes of patients with CRC compared

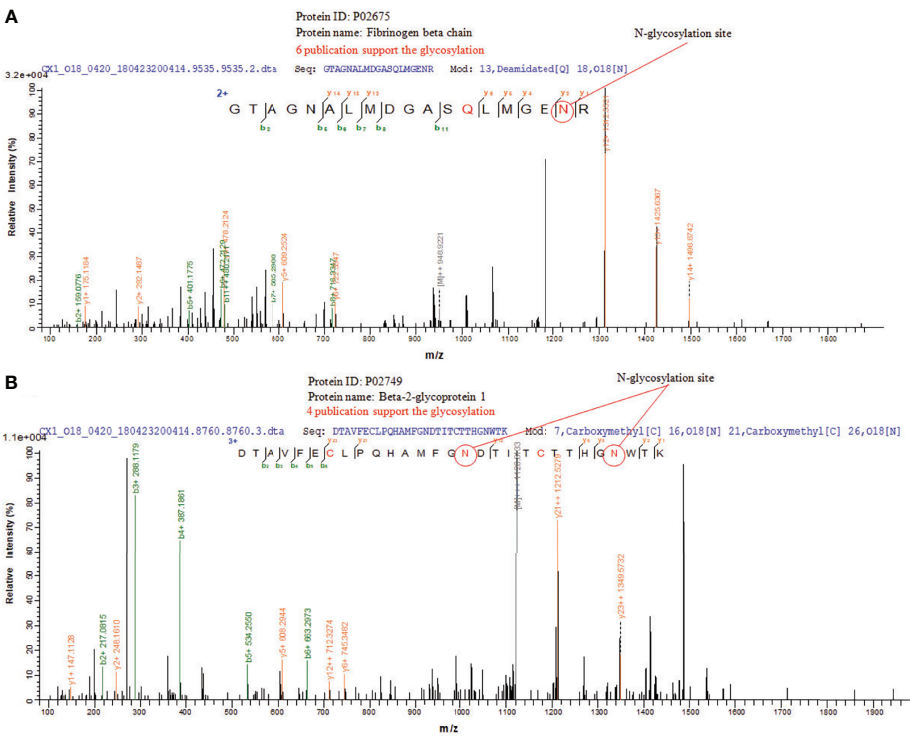

FIGURE 5 | LC-MS/MS of N-glycosylations site of glycoproteins in CRC tissue exosomes. **(A)** Fibrinogen beta chain and **(B)** beta-2-glycoprotein 1.

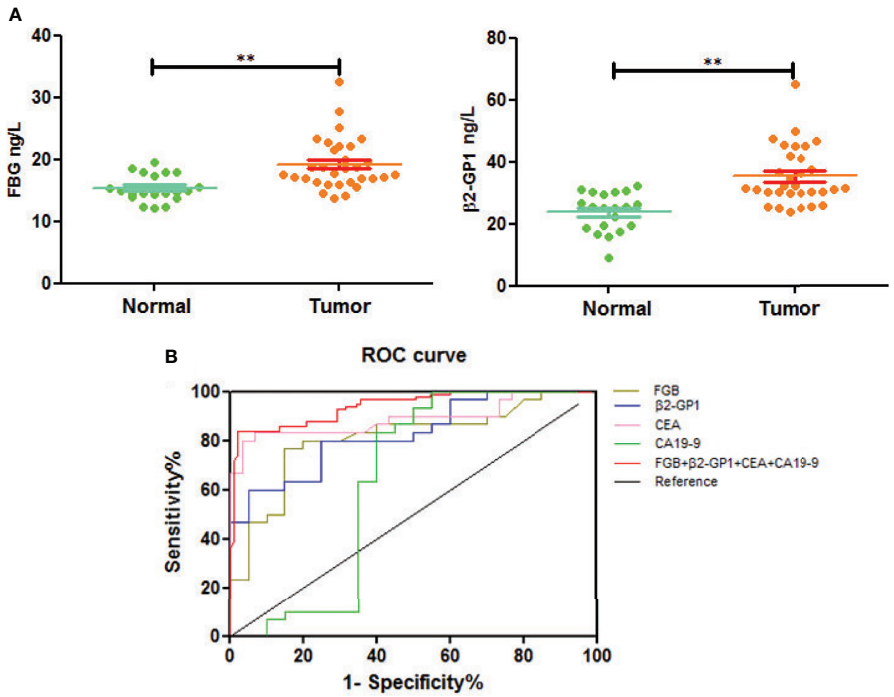

FIGURE 6 | Verification of plasma exosomal glycoproteins as biomarkers for early diagnosis of CRC. **(A)** The levels of fibrinogen beta chain and beta-2-glycoprotein 1 in HC and CRC. **p < 0.01. **(B)** ROC curve of plasma exosomal fibrinogen beta chain and beta-2-glycoprotein 1.

with those of controls. The levels of plasma exosomal β2-GP1 achieved higher efficacy for diagnosis of early CRC compared with those of CEA and CA19-9. Moreover, glycosylated FGB and β2-GP1 were identified to be in tissue exosomes and were present at higher levels in plasma exosomes of CRC compared with controls. Furthermore, FGB and β2-GP1 achieved higher sensitivity and specificity for the diagnosis of CRC compared with CEA and CA19-9. The cutoff values of the ROC curves (**Table 2**) reflect a trade-off between sensitivities and specificities. Additionally, we also analyzed the possibility of FGB and β2-GP1 as a panel to diagnose CRC. The results showed that the AUC value was markedly higher than FGB or β2-GP1 alone when discriminating CRC patients from controls (**Table 2**), suggesting the panel to be a better biomarker for CRC diagnosis.

There are limitations to the present study. First, a large amount of lipid during extraction may have affected the quality of the specimen and thus diminished the accuracy of the results, requiring further improvements in the method used to extract tissue exosomes. Second, the subject population comprising patients at multiple centers is required to support the application of standard liquid biopsy biomarkers for the diagnosis of early CRC. Third, the majority of the patients had advanced (T2–T4) disease. Future studies will therefore consider early-stage patients.

The combination of proteomic techniques and databases for screening and validation of plasma exosomal glycoproteins related to CRC shows that glycoproteins were enriched in tissue exosomes. The overexpression of FGB and β2-GP1 in patients with CRC compared with the control group achieved higher sensitivity and specificity for the diagnosis of CRC compared with the levels of CEA and CA19-9. FGB and β2-GP1 may therefore serve as biomarkers for diagnosing patients with early-stage CRC.

TABLE 2 | ROC of plasma exosomal glycoproteins as biomarkers for early diagnosis of CRC.

marker	FBG	β2-GP1	FBG+β2-GP1	CEA	CA19-9
AUC (95% CI)	0.871 (0.786–0.914)	0.834 (0.734–0.901)	0.915 0.845–0.987	0.723 (0.679–0.853)	0.614 (0.543–0.715)
Cutoff	18.6 ng/L	30.6 ng/L		4.7 ng/ml	27.0 U/ml
Sensitivity (%)	68.35	71.55	63.84	48.43	53.67
Specificity (%)	86.27	85.51	93.54	81.23	83.14
Positive likelihood ratio (%)	93.22	91.15	96.2	79.25	77.31
Negative likelihood ratio (%)	73.26	70.11	75.6	54.32	51.28

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The study was approved by the Ethics and Research Committee of Tongji Hospital and conducted according to the ethical guidelines of the 1975 Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

ZS and SJ helped in the design of the research study and helped in acquiring data, analyzing data, and writing the manuscript. JT and JW helped in acquiring data, analyzing data, and writing the manuscript. WQ and AS helped in analyzing data and writing the manuscript. PJ and DiL helped in data acquisition and analysis. DoL, XW, and WX helped in the design and conception of the research study, data acquisition, and data analysis. All authors contributed to the article and approved the submitted version.

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Risk Factors for Anorectal Dysfunction After Interspincteric Resection in Patients With Low Rectal Cancer

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Purpose: The objective of this study was to explore the risk factors for anorectal dysfunction after intersphincteric resection in patients with low rectal cancer.

Methods: A total of 251 patients who underwent intersphincteric resection from July 2014 to June 2020 were included in this study, for which the Kirwan's grade, Wexner score, and anorectal manometric index were used to evaluate the anorectal function and other parameters including demographics, surgical features, and clinical and pathological characteristics. These parameters were analysed to explore the potential risk factors for anorectal function after intersphincteric resection.

Results: In the 251 included patients, 98 patients underwent partial intersphincteric resection, 87 patients underwent subtotal intersphincteric resection, and 66 patients underwent total intersphincteric resection. There were 53 (21.1%) patients who had postoperative complications, while no significant difference was observed between the three groups. Furthermore, 30 patients (45.5%) in the total intersphincteric resection group were classified as having anorectal dysfunction (Kirwan's grade 3–5), which was significantly higher than that in the partial intersphincteric resection group (27.6%) and subtotal intersphincteric resection group (29.9%). The mean Wexner score of patients that underwent total intersphincteric resection was 7.9, which was higher than that of patients that had partial intersphincteric resection (5.9, $p = 0.002$) and subtotal intersphincteric resection (6.4, $p = 0.027$). The initial perceived volume was lower in the total intersphincteric resection group than in the partial and subtotal intersphincteric resection groups at 1, 3, and 6 months after intersphincteric resection. In addition, the resting pressure, maximum squeeze pressure, and maximum tolerated volume in the total intersphincteric resection group were worse than those in the partial and subtotal groups at 3 and 6 months after intersphincteric resection. Univariate and multivariate analyses suggested that an age ≥65, total intersphincteric resection, and preoperative chemoradiotherapy were independent risk factors for anorectal dysfunction ($P = 0.023$, $P = 0.003$, and $P = 0.008$, respectively). Among the 66 patients who underwent total intersphincteric resection, 17 patients received preoperative chemoradiotherapy, of which 12 patients (70.6%) were classified as having anorectal dysfunction.

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Conclusion: The current study concluded that age ≥65, total intersphincteric resection, and preoperative chemoradiotherapy were risk factors for anorectal dysfunction after intersphincteric resection. The morbidity of anorectal dysfunction after total intersphincteric resection for patients who received preoperative chemoradiotherapy was relatively high, and the indication should be carefully evaluated.

Keywords: anorectal dysfunction, intersphincteric resection, risk factors, preoperative chemoradiation, low rectal cancer

INTRODUCTION

Abdominoperineal resection is regarded as a standard procedure for curative surgical treatment in patients with low rectal cancer. In recent years, anus-preserving surgeries, including intersphincteric resection (ISR) and transanal total mesorectal excision (Ta_TME), have been widely performed for low rectal cancer and can significantly avoid a permanent stoma (1–3). With the development and application of laparoscopic and robotic systems for the resection of low rectal cancer, the ISR has become one of the most popular anus-preserving procedures. Previous evidence has indicated that its clinical and oncological outcomes are similar to abdominoperineal resection (APR), and the anal functional outcome is suggested to be acceptable (1, 4, 5). However, many patients suffer from anorectal dysfunction after ISR, especially total ISR, resulting in a conversion to a permanent colostomy and a reduction in the quality of daily life (6). Previous studies have shown that ∼42% of patients experience major bowel dysfunction after ISR, indicating that the functional outcomes may be the main risk of undergoing ISR rather than oncological outcomes (7). Furthermore, ISR can be classified as partial ISR, subtotal ISR, and total ISR according to the resected grade of the internal sphincter. Partial ISR is defined as the distal resection line of the internal sphincter at the dentate line, subtotal ISR is located between the intersphincteric groove and dentate line, and total ISR is located at the intersphincteric groove (8). The internal anal sphincter should be partially or totally removed in different ISRs, wherein this sphincter was reported to contribute ∼55% of anal pressure, and its removal resulted in varying degrees of anorectal dysfunction (9). To explore the potential factors that might influence anorectal function after ISR, we retrospectively analysed clinicopathological characteristics, surgical features, postoperative complications, and functional indicators in this study.

METHODS

Patients

The present study included consecutive patients with low rectal cancer who underwent laparoscopic or robotic-assisted ISR from July 2014 to June 2020 at the Southwest Hospital affiliated with Army Medical University (<city>Chongqing</city>, China). Inclusion criteria were (1) an age of 18–70 years, (2) a distance between the lower edge of the tumour and Hilton line of 1–5 cm, (3) preoperatively evaluated well-differentiated adenocarcinoma, (4) estimated TNM stage (8th edition) p/yp$T_{1-3}N_{0-2}M_0$, and (5) an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2. The exclusion criteria were (1) synchronous cancer or metachronous cancer during follow-up, (2) rectal cancer associated with inflammatory bowel disease or hereditary rectal cancer, and (3) local tumour recurrence in 2 years. Patients with preoperatively estimated T4 or stage III disease received a long course of preoperative chemotherapy or chemoradiotherapy (CRT). After this, the estimated T stage was below T3, and non-external sphincter infiltration was determined according to preoperative enhanced rectal MRI and endoscopic ultrasonography evaluation.

Demographics and perioperative clinicopathological characteristics were investigated and compared in order to explore the risk factors for anorectal dysfunction after ISR.

Surgical Procedure

The ISR was performed by laparoscopic or robotic surgical systems according to previously reported methods (10). First, dissection was performed by the abdominal route, then the levator ani muscle hiatus was entered, and a division was created between the loose internal and external sphincter spaces to the level of the dentate line *via* the anal or abdominal route. Patients enrolled in this study who went through partial and subtotal ISR underwent transabdominal procedures, and total ISR was performed through transanal transabdominal procedures. The transanal dissection contains a circumferential incision of the mucosa at the Hilton line. Through a careful circumferential dissection and the protection of the external anal sphincter and levator ani muscle, confluence at the level of the abdominal dissection and total ISR were completed. After the removal of the specimen, bowel reconstruction was performed using an end-to-end procedure *via* a stapled anastomosis in the partial and subtotal ISR groups, a handsewn coloanal anastomosis with absorbable interrupted sutures in the total ISR group, and a diverting ileostomy.

Postoperative Follow-Up and Evaluation

Postoperative complications were recorded and classified as Clavien–Dindo grades. Anastomotic complications, including anastomotic leakage, anastomotic bleeding, and anastomotic stricture, were analysed to evaluate the risk factors for anorectal dysfunction. The manometric measurements were evaluated before a surgery and every 3 months after surgery. The clinical, pathological, and functional outcomes were evaluated every 3 months in 2 years after surgery *via* an

outpatient service. Anorectal manometry was performed by High-resolution manometry (XDJ-S8G) (KAILIGUANGDIAN LLC, Hefei, Anhui, China), of which the resting pressure (RP), maximum squeeze pressure (MSP), initial perceived volume (IPV), and maximum tolerated volume (MTV) values were assessed to evaluate sphincteric and faecal function (11). Wexner scores (12) and Kirwan classification (13) were recorded before ISR and every 3 months after stoma closure.

Statistical Analysis

Categorical data are presented as the number of cases evaluated, and quantitative data are reported as the mean ± SD. A chi-square test was used to evaluate categorical variables, and a Fisher's exact test or Student's *t*-test was used for continuous variables. The factors related to potential risk factors were analysed by binary logistic regression analysis, and odds ratios (ORs) with 95% CIs were calculated. The Cox proportional hazards model was used to define prognostic factors related to anorectal dysfunction. Covariates with $p < 0.05$ were selected for the multivariate model. All statistical analyses were performed using SPSS 22 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ were regarded as statistically significant.

Ethics

The institutional review board of the Southwest Hospital Affiliated to the Army Medical University approved the study protocol (KY2019138). All methods in this study were performed in accordance with relevant guidelines and regulations. Written informed consent was obtained from all included patients.

RESULTS

Patient Enrollment

As the flow diagram of patient selection shows (**Figure 1**), a total of 266 patients were included according to the inclusion criteria, while 11 patients were excluded according to the exclusion criteria, and four patients were lost to follow-up. Thus, a total of 251 patients were enrolled in this study, of which 98 patients underwent partial ISR, 87 patients underwent subtotal ISR, and 66 patients underwent total ISR. The median follow-up was 26 (6–72) months.

Operative and Clinicopathological Characteristics of Patients

For the enrolled patients in this study, the demographics and clinical characteristics, including sex, age, body mass index (BMI), American Society of Anesthesiologists (ASA) classification, haemoglobin level, albumin level, and preoperative CRT, were compared according to different surgical procedures. The results showed that there was no significant difference in any parameter between these three groups (**Table 1**). Preoperative CRT was recommended for patients with T3–4 or stage III rectal cancer or suspected anal sphincter invasion according to preoperative MRI. Tumour regression after preoperative CRT was assessed by the tumour regression grade provided by the American Joint Committee on Cancer (AJCC) and the College of American Pathologists (14).

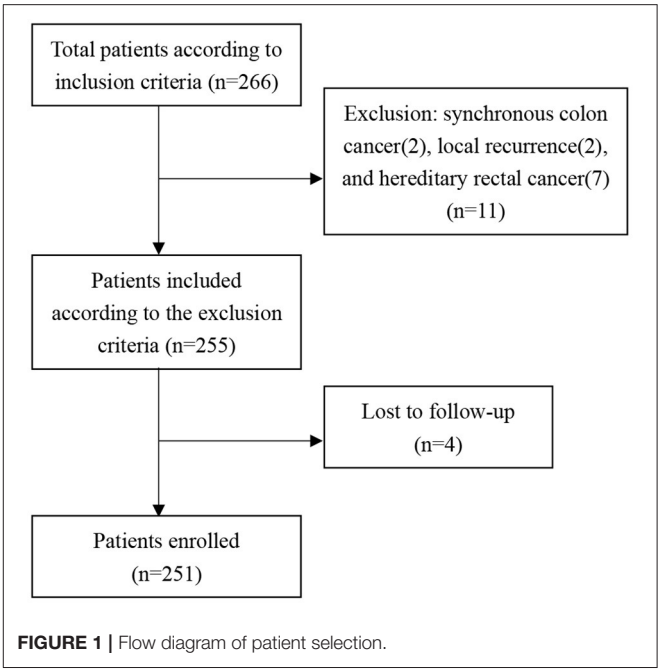

FIGURE 1 | Flow diagram of patient selection.

Operative and Pathological Characteristics of Patients Who Underwent ISR

The operative and pathological outcomes are presented in **Table 2**. We pathologically evaluated the resection of the external anal sphincter (EAS) for every patient after ISR and found that the EAS was reserved for all patients who underwent partial ISR. The EAS was partially resected for 9 out of 87 patients who underwent subtotal ISR and 19 out of 66 patients who underwent total ISR. In the partial ISR group, 45 patients received robotic ISR and 53 patients received laparoscopic ISR. In the subtotal ISR group, 49 patients received robotic ISR and 38 patients received laparoscopic ISR. In the total ISR group, 58 patients received robotic ISR and 8 patients received laparoscopic ISR. The proportion of robotic surgeries was significantly higher ($p < 0.001$) in the total ISR group. No significant difference was found in the anastomosis level from the anal verge, operation time, estimated blood loss, tumour differentiation, T or N stage according to the 8th edition of AJCC cancer staging criteria (15), the number of lymph nodes (LNs) harvested, and distal resection margin according to our results. The circumference margin of all the patients was pathologically proven as oncologically negative. A total of 92 patients were pathologically diagnosed with low differentiation after surgery, and 57 patients were stage III-IV, which were also included in the analysis for the evaluation of the risk factors for anorectal dysfunction.

Postoperative Complications

Data on postoperative complications are shown in **Table 3**. No grade IV or V complications were observed in these 251 patients. There were 21 (21.4%) patients in the partial ISR group, 18 (20.7%) patients in the subtotal ISR group, and 14 (21.2%) patients in the total ISR group who had

TABLE 1 | Demographics and clinical characteristics of patients.

Variables	Partial ISR $n = 98$	Subtotal ISR $n = 87$	Total ISR $n = 66$	*p*-value P-ISR vs. S-ISR/P-ISR vs. T-ISR/S-ISR vs. T-ISR*
Sex				0.891/0.662/0.760
Female	37	32	22	
Male	61	55	42	
Age (years)				0.554/0.178/0.288
Mean (SD)	61.8 (9.1)	60.3 (10.3)	58.6 (8.8)	
BMI (kg/m²)				0.875/0.472/0.447
Mean (SD)	22.4 (3.9)	22.2 (4.1)	23.1 (3.5)	
Preoperative CRT				0.612/0.519/0.882
Yes	21	19	17	
No	77	58	49	
ASA				0.946/0.068/0.084
I/II	66	59	53	
III/IV	32	28	13	
Haemoglobin (g/dL)				0.779/0.924/0.682
Mean (SD)	113.7 (18.5)	109.5 (16.3)	112.9 (20.4)	
Albumin (g/dL)				0.694/0.799/0.921
Mean (SD)	36.7 (5.4)	37.8 (5.1)	37.5 (4.7)	

ISR, interspincteric resection; SD, standard deviation; ASA, American Society of Anesthesiologists; BMI, body mass index; CRT, chemoradiotherapy.
**All parameters were appropriately compared using Pearson's $\chi 2$ test or Fisher's exact test with two-sided verification and an unpaired Student's t-test: P-ISR vs. S-ISR, partial ISR vs. subtotal ISR; P-ISR vs. T-ISR, partial vs. total ISR; S-ISR vs. T-ISR, subtotal ISR vs. total ISR.*

postoperative complications. Anastomotic complications, including anastomotic leakage, anastomotic bleeding, and anastomotic stricture, were compared, and no significant difference was observed between these groups.

Anorectal Function Evaluation After Stoma Closure

All patients enrolled in this study simultaneously underwent temporary ileostomy and ISR, and all of these patients underwent stoma closure 3–6 months after the first operation. To evaluate defecatory function after ISR, we assessed Kirwan's grade and Wexner score for every patient 3 months after stoma closure. As shown in **Table 4**, the daily bowel frequency in the partial ISR group was 4.2 ± 2.3, that in the subtotal ISR group was 4.3 ± 2.7, and that in the total ISR group was 5.5 ± 3. The bowel frequency in the total ISR group was slightly higher than that in the partial and subtotal groups, but no statistical significance was found. The faecal continent was classified as Kirwan's grade 1–2, while the faecal incontinent was classified as Kirwan's grade 3–5. As shown in **Table 4**, 27 patients in the partial ISR group (27.6%), 26 patients in the subtotal ISR group (29.9%), and 30 patients in the total ISR group (45.5%) suffered from anorectal dysfunction (Kirwan's grade 3–5). Compared with the partial and subtotal ISR groups, the total ISR group had a significantly higher faecal incontinence rate ($p = 0.018$ and 0.048, respectively). A similar result was observed for the Wexner score, and the mean score in the total ISR group (7.9 ± 5.2) was significantly higher ($p = 0.002$ and 0.027, respectively) than that of the partial ISR group (4.2 ± 2.3) and subtotal ISR group (4.3 ± 2.7).

Anorectal Manometric Measurements in Patients Who Underwent ISR

To objectively assess the anorectal sensitivity and contractility, we measured the RP, MSP, IPV, and MTV for every patient before ISR and 1, 3, and 6 months after ISR. The results in **Table 5** show no difference in every parameter before surgery. At 1 month after ISR, the IPV for the total ISR group was significantly lower than that of the partial and subtotal groups. At 3 and 6 months after ISR, almost all parameters for total ISR were lower than those of the other two groups, indicating that the total resection of the internal anal sphincter could strongly affect anorectal function. We also found that the manometric measurements could recover slowly, not only in the partial and subtotal ISR groups but also in the total group.

Factors Influencing Defecatory Function After ISR

To define the risk factors for anorectal dysfunction after ISR, we analysed the potential factors mentioned in **Tables 1**, **2** by univariate and multivariate analyses. The univariate analysis indicated that an age >65, T (3–4) stage, total ISR procedure, preoperative CRT, and distal resection margin were potential risk factors. All these statistically significant parameters were included in a multivariate analysis, and the results showed that age ($p = 0.023$), total ISR ($p = 0.003$), and preoperative CRT ($p = 0.008$) were independent risk factors for anorectal dysfunction after ISR (**Table 6**).

TABLE 2 | Operative features and pathological characteristics in patients.

Variables	Partial ISR *n* = 98	Subtotal ISR *n* = 87	Total ISR *n* = 66	*p*-value P-ISR vs. S-ISR/P-ISR vs. T-ISR/S-ISR vs. T-ISR*
Partial resection of EAS				**0.001/<0.001/0.003**
Yes	0	9	19	
No	98	78	47	
Anastomosis level from AV (cm)				**<0.001 in all**
Mean (SD)	4.7 (1.2)	3.0 (0.6)	1.8 (0.4)	
Operation time (min)				0.543/0.087/0.223
Mean (SD)	179.7 (22.7)	188.9 (31.3)	204.4 (27.8)	
Estimated blood loss (ml)				0.352/0.848/0.292
Mean (SD)	86.1 (19.1)	92.8 (21.8)	85.2 (23.2)	
Tumour differentiation				0.730/0.326/0.205
Low	37	35	20	
Moderate and high	61	52	46	
Surgical procedure				0.158/<0.001/<0.001
Robotic	45	49	58	
Laparoscopic	53	38	8	
T stage				0.709/0.084/0.171
1–2	72	66	56	
3–4	26	21	10	
N stage				0.101/0.097/0.863
0	78	77	59	
1–2	20	10	7	
No. of LN harvest				0.401/0.572/0.336
Mean (SD)	21.1 (4.3)	23.2 (5.5)	19.5 (3.8)	
Distal resection margin (mm)				0.271/0.063/0.104
Mean (SD)	19.1(5.4)	18.5(3.4)	16.8 (3.1)	

ISR, interspincteric resection; EAS, external anal sphincter; AV anal verge; SD, standard deviation; LN, lymph node.

**All parameters were appropriately compared using Pearson's $\chi 2$ test or Fisher's exact test with two-sided verification and an unpaired Student's t-test: P-ISR vs. S-ISR, partial ISR vs. subtotal ISR; P-ISR vs. T-ISR, partial vs. total ISR; S-ISR vs. T-ISR, subtotal ISR vs. total ISR.*

Bold values mean statistically significant.

The Safety of Total ISR for Patients Who Received Neoadjuvant CRT

The above results suggested that total ISR and preoperative CRT were both independent risk factors for anorectal dysfunction after ISR, which reminded us to explore the safety of total ISR for patients who received preoperative CRT. In this study, 66 patients underwent total ISR, of which 17 patients received preoperative CRT. Among these 17 patients, 12 patients (70.6%) were classified as having anorectal dysfunction (Kirwan's grade 3–5), indicating that preoperative CRT may be a crucial risk factor for anorectal dysfunction after total ISR (**Table 7**).

DISCUSSION

The ISR or Ta_TME have suggested procedures for the surgical treatment of patients with low or extremely low rectal cancer. In particular, ISR is very hard to perform in conventional open surgeries, but the application of laparoscopic, or especially robotic systems, makes this procedure become easier and more familiar to surgeons (16, 17). In this study, the robotic system was more commonly used for the treatment of total ISR, indicating that the robotic system could operate better in small spaces. The clinical outcomes were evaluated and reported to be safe in many studies, while anorectal complications, including oedematous haemorrhoids, anal stenosis, and neorectal mucosal prolapse, were more common after ISR (18). Regarding the oncological outcome, ISR showed comparable overall survival with APR for patients with low rectal cancer, especially for patients at stage I–II (4). It was reported that patients who underwent ISR also showed a relatively higher local recurrence rate, while a deeper analysis found that these local recurrences were mostly observed in T3 or T4 patients. Additionally, the local recurrence rate was comparable in T1 or T2 patients, indicating that the ISR should be carefully evaluated and chosen for these patients (1, 19). For cT3 or cT4 patients, radiotherapy and CRT followed by ISR is an option that has been proven to be oncologically safe (20). In this study, a total of 57 patients were postoperatively diagnosed as being in the T3–4 stage, of which 20 patients received folinic acid, fluorouracil, and oxaliplatin (FOLFOX) chemotherapy, and the

TABLE 3 | Postoperative complications in patients.

Variables	Partial ISR *n* = 98	Subtotal ISR *n* = 87	Total ISR *n* = 66	*p*-value P-ISR vs. S-ISR/P-ISR vs. T-ISR/S-ISR vs. T-ISR*
Clavien–Dindo grade				0.807/0.714/0.568
I–II	17 (17.3%)	14 (16.1%)	12 (18.2%)	
III	4 (4.1%)	4(4.6%)	2 (3.0%)	
IV–V	0	0	0	
Anastomotic leakage				0.817/0.917/0.915
Yes	7 (7.1%)	7 (8.0%)	5 (7.6%)	
No	91 (92.9%)	80 (92.0%)	61 (92.4%)	
Anastomotic bleeding				0.585/0.991/0.621
Yes	3 (3.1%)	4 (4.6%)	2 (3.0%)	
No	95 (96.9%)	83 (95.4%)	64 (97.0%)	
Anastomotic stricture				0.882/0.620/0.729
Yes	3 (3.1%)	3 (3.4%)	3 (4.5%)	
No	95 (96.9%)	84 (96.6%)	63 (95.5%)	
Others				0.730/0.326/0.205
Yes	8	5	5	
No	90	82	61	

ISR, interspincteric resection.
**P-ISR vs. S-ISR, partial ISR vs. subtotal ISR; P-ISR vs. T-ISR, partial vs. total ISR; S-ISR vs. T-ISR, subtotal ISR vs. total ISR.*

TABLE 4 | The anorectal function was evaluated 3 months after stoma closure.

Variables	Partial ISR *n* = 98	Subtotal ISR *n* = 87	Total ISR *n* = 66	*p*-value P-ISR vs. S-ISR/P-ISR vs. T-ISR/S-ISR vs. T-ISR*
Bowel frequency				0.388
Mean (SD)	4.2 (2.3)	4.3 (2.7)	5.5 (3.0)	
Kirwan's grade#				0.726/**0.018/0.048**#
1	41	37	24	
2	30	24	12	
3	15	12	14	
4	10	10	10	
5	2	4	6	
Wexner score mean (SD)	5.9 (3.9)	6.4 (4.4)	7.9 (5.2)	0.374/**0.002/0.027**
Continent (Kirwan's 1–2)	4.3 (1.3)	4.7 (2.0)	4.7 (1.8)	
Incontinent (Kirwan's 3–5)	10.8 (3.7)	11.1 (4.0)	11.3 (4.5)	

ISR, interspincteric resection; SD, standard deviation.
**All parameters were appropriately compared using Pearson's $\chi 2$ test or Fisher's exact test with two-sided verification and an unpaired Student's t-test: P-ISR vs. S-ISR, partial ISR vs. subtotal ISR; P-ISR vs. T-ISR, partial vs. total ISR; S-ISR vs. T-ISR, subtotal ISR vs. total ISR.*
 # Kirwan's grade was compared between grades 1–2 and grades 3–5.
Bold values mean statistically significant.

other 37 patients received Xeloda (capecitabine) and oxaliplatin (XELOX) chemotherapy. None of the patients enrolled in this study received postoperative first-line radiotherapy, because the functional safety of postoperative radiotherapy is not well-proven for patients who received ISR.

In addition to the clinical and oncological outcomes, the anorectal functional outcome was another essential indicator for the safety evaluation of ISR. The present studies showed that the excision of the internal anal sphincter had negative effects on short- and long-term anorectal function, and some patients even suffered from complete incontinence resulting in a conversion to a permanent colostomy (6, 21). Kirwan's grade and Wexner score were applied to evaluate the defecatory function after ISR. Patients with liquid or solid incontinence (Kirwan's grade 3–5) were classified as having anorectal dysfunction. Given that the patients included in this study simultaneously underwent

TABLE 5 | Anorectal manometric measurements after ISR.

Variables mean (SD)	Partial ISR $n = 98$	Subtotal ISR $n = 87$	Total ISR $n = 66$	*p*-value P-ISR vs. S-ISR/P-ISR vs. T-ISR/S-ISR vs. T-ISR*
Pre-				
RP (mmHg)	55.8 (8.1)	56.3 (7.8)	55.8 (8.2)	0.788/0.916/0.697
MSP (mmHg)	175.8 (19.5)	176.6 (14.6)	178.6 (18.9)	0.952/0.584/0.628
IPV (ml)	45.7 (8.6)	47.2 (10.1)	44.6 (9.9)	0.577/0.369/0.272
MTV (ml)	158.5(18.4)	159.5 (22.1)	162.3 (20.1)	0.912/0.754/0.804
Post-1-month				
RP (mmHg)	29.3 (7.8)	29.4 (6.5)	22.4 (5.4)	0.933/0.128/0.086
MSP (mmHg)	85.2 (14.9)	84.6 (22.1)	69.0 (11.3)	0.754/0.079/0.102
IPV (ml)	26.5 (5.4)	26.6 (5.8)	15.5(4.7)	0.152/**0.001/<0.001**
MTV (ml)	67.5 (7.6)	64.8 (9.9)	65.4 (10.8)	0.425/0.511/0.878
Post-3-month				
RP (mmHg)	36.4 (8.1)	33.9 (9.2)	22.7 (6.0)	0.255/**0.006/0.014**
MSP (mmHg)	110.2 (13.2)	113.4 (15.2)	70.8 (9.1)	0.864/**<0.001/<0.001**
IPV (ml)	31.2 (6.4)	30.8 (6.6)	18.5 (5.4)	0.751/**0.016/0.034**
MTV (ml)	83.6 (8.8)	81.8 (9.6)	76.3 (9.2)	0.776/**0.037**/0.087
Post-6-month				
RP (mmHg)	43.5 (7.4)	40.8 (8.2)	33.5 (5.7)	0.259/**0.022/0.041**
MSP (mmHg)	141.3 (17.8)	136.8 (18.0)	83.9 (12.3)	0.385/**<0.001/<0.001**
IPV (ml)	36.5 (7.7)	33.4 (8.5)	25.6 (6.8)	0.263/**0.004/0.010**
MTV (ml)	96.6 (16.1)	91.5 (12.3)	81.1 (9.0)	0.122/**0.015/0.033**

ISR, interspincteric resection; SD, standard deviation; RP, resting pressure; MSP, maximum squeeze pressure; IPV, initial perceived volume; MTV, maximum tolerated volume. Pre-, preoperative; Post-x-months, x months after ISR.
Bold values mean statistically significant.
P-ISR vs. S-ISR, partial ISR vs. subtotal ISR; P-ISR vs. T-ISR, partial vs. total ISR; S-ISR vs. T-ISR, subtotal ISR vs. total ISR.

TABLE 6 | Univariate and multivariate analyses for the risk of anorectal dysfunction.

Anorectal dysfunction	Univariate			Multivariate		
Variables	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***P***
Male (vs. female)	1.17	0.76–1.88	0.573			
Age ≥65 years (vs. <65 years)	1.66	1.22–2.11	**0.021**	1.53	1.20–1.89	**0.023**
BMI <18.5 kg/m^2 (vs. ≥18.5 kg/m^2)	1.08	0.63–1.77	0.833			
ASA III/IV (vs. I/II)	1.52	0.89–2.21	0.121			
Hb<120 (vs. ≥120)	0.88	0.49–1.95	0.577			
Alb <35 (vs. ≥35)	1.44	0.78–2.0.5	0.126			
T stage (1–2 vs. 3–4)	0.54	0.22–0.87	**0.012**	0.87	0.45–1.21	0.161
N stage (0 vs. 1–2)	0.91	0.53–1.27	0.377			
Robotic ISR (vs. laparoscopic ISR)	0.87	0.57–1.31	0.422			
Anastomotic complication (yes vs. no)	1.35	0.88–2.34	0.087			
Total ISR (vs. partial, subtotal)	5.16	2.38–8.78	**<0.001**	4.78	2.21–8.66	**0.003**
Preoperative CRT (yes vs. no)	3.55	1.89–6.46	**<0.001**	3.11	1.88–7.11	**0.008**
Histology (low vs. moderate, high)	1.12	0.66–1.54	0.342			
Distal resection margin	0.54	0.22–0.85	**0.025**	0.61	0.23–1.12	0.081

ISR, interspincteric resection; OR, odds ratio; ASA, American Society of Anesthesiologists; BMI, body mass index; CRT, chemoradiotherapy; CI, confidence interval; Hb, haemoglobin; Alb, albumin.
Bold values mean statistically significant.

ISR and ileostomy, the defecatory function was assessed at 3 months after stoma closure. The results in **Table 4** show that the anorectal dysfunction morbidity in patients who underwent total ISR was relatively higher than that in the partial ISR group (30/66 vs. 27/98, $p = 0.048$) and subtotal group (30/66 vs. 26/87, $p = 0.018$). Similar results were observed in postoperative bowel

TABLE 7 | Anorectal function for patients who underwent total ISR.

Variables	CRT + $n = 17$	CRT - $n = 49$	p-value
Kirwan's grade#			**0.016**
1	2	22	
2	3	9	
3	5	9	
4	4	6	
5	3	3	

ISR, interspincteric resection; CRT +, patients who received preoperative chemoradiotherapy; CRT -, patients who did not receive preoperative chemoradiotherapy.
 # Kirwan's grade was compared between grades 1–2 and grades 3–5.
Bold values mean statistically significant.

frequency and Wexner scores. Patients who underwent total ISR had a higher bowel frequency and higher mean Wexner score than the other two groups, indicating that the excision extension of the internal anal sphincter may correlate to anorectal function after ISR. In addition, the pathological results showed that nine patients (10.3%) who underwent subtotal ISR and 19 patients (28.8%) who underwent total ISR had all underwent partial external anal sphincter excision during the surgery, which may be another risk factor for anorectal dysfunction. From the functional results, we found that the defecatory function was comparable between the partial and subtotal groups, suggesting that, for postoperative anorectal function, the excision extension may be the main risk factor. Postoperative complications, especially anastomotic complications, might influence anorectal function (22). To explore the relationship between anastomotic complications and anorectal function, we analysed anastomotic leakage, anastomotic bleeding, and anastomotic stricture. The result showed that there was no significant difference in postoperative complications between different ISRs. Univariate analysis showed that the anastomotic complications were not risk factors for anorectal dysfunction.

In addition to the subjective evaluation parameters, anal manometry was applied to objectively evaluate the anorectal contractility and sensitivity in this study. Manometric parameters including RP, MSP, IPV, and MTV are widely used to assess anorectal function after ISR, which can objectively reflect defecatory function (4, 8, 23). In this study, the preoperative manometry was measured as the baseline and showed no difference between the different ISR groups. The measurements after ISR, especially 3 and 6 months after ISR, showed that almost every parameter was weaker in the total ISR group than in the other groups, which suggested similar results as the Kirwan's grade and Wexner score indicated. The present studies showed that the manometric values were reduced after ISR, while they could mostly recover to a continental level in 12–24 months (4, 5). In addition, from the results of postoperative manometric measurements, we found that postoperative IPV was lower than preoperative IPV. The IPV value is related to rectal sensitivity and defecation-control ability, and ISR would decrease such rectal sensitivity and defecation-control ability. The IPV results indicated that the defecation-control ability of a patient was severely damaged after ISR, which may play a more important than the anal sensitivity of the impact on IPV. The IPV increased with the time after surgery, indicating that the defecation-control ability of the patient recovered with time. The manometric results in this study also showed that anorectal function recovered after surgery, and the values at 6 months were better than those at 1 and 3 months after ISR. Compared to the baseline, the reduction was still apparent, especially in patients who underwent total ISR. Both the Wexner scores and the manometric measurements showed that the anorectal function recovery was time-dependent, and anorectal function would be recovered to a similar and acceptable level approximately 12–24 months after ISR (4, 5, 24). The manometric results also suggested that the values after ISR in the partial ISR and subtotal ISR groups were comparable and significantly better than those in the total ISR group, indicating that even the partial reservation of the internal anal sphincter could contribute to anorectal function after ISR.

Apart from the excision of the internal anal sphincter, other potential risk factors were explored. Denost et al. reported that the distance of the tumour from the anal ring being >1 cm and the anastomoses being higher than 2 cm above the anal verge were independent predictors of good faecal continence for patients who received ISR (25), according to a cohort of 101 patients. Other studies reported that age, tumour stage, preoperative CRT, operative approach, level of ISR, and the reconstruction of the rectum might influence faecal incontinence after ISR (1, 26). In this study, we analysed the clinicopathological characteristics, surgical features, postoperative complications, and functional indicators to systemically evaluate the risk factors for anorectal dysfunction. The univariate and multivariate analyses suggested that an age $\geq$65 ($p = 0.023$), total ISR ($p = 0.003$), and preoperative CRT ($p = 0.008$) were risk factors for anorectal dysfunction. We evaluated the safety of total ISR for patients who received preoperative CRT and found that 70.6% of patients in this subgroup suffered from anorectal dysfunction, which was relatively high morbidity.

In conclusion, the anal functional outcome after partial ISR and subtotal ISR is acceptable for patients with low rectal cancer, which could increase the anus-preserving rate. The indication of total ISR, especially for patients who receive preoperative CRT, should be strictly and carefully evaluated and defined. The safety of total ISR for patients who receive preoperative CRT should be further explored. There are some limitations to this study. First, this study is not a randomised clinical trial, and bias may exist. Second, this study is a retrospective study, and a prospective controlled trial should be carried out for further exploration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board of the Southwest

Hospital Affiliated to Army Medical University approved the study protocol (KY2019138). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LM and ZF: data collection, management, analysis, and manuscript writing. WZ and LP: data management and analysis. XL, DM, and WY: data collection. WX: project development, data analysis, manuscript writing, and editing. TB: project development, data analysis, and manuscript editing. All authors contributed to the article and approved the submitted version.

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A Prognostic Nomogram for T3N0 Rectal Cancer After Total Mesorectal Excision to Help Select Patients for Adjuvant Therapy

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Background: The recurrence rate of T3N0 rectal cancer after total mesorectal excision (TME) is relatively low, meaning that not all patients need adjuvant therapy (AT) (radiotherapy, chemotherapy, or chemoradiotherapy).

Methods: Patients diagnosed with pT3N0M0 rectal cancer after TME were analyzed using the SEER database, of which 4367 did not receive AT and 2794 received AT. Propensity score matching was used to balance the two groups in terms of confounding factors. Cox proportional hazards regression analysis was used to screen independent prognostic factors, which were then used to establish a nomogram. The patients were then divided into three groups with X-tile software according to their risk scores. We enrolled 334 patients as external validation.

Results: The C-index of the model was 0.725 (95% confidence interval: 0.694–0.756). We divided the patients into three different risk layers based on the nomogram prediction scores, and found that AT did not improve the prognosis of low- and moderate-risk patients, while high-risk patients benefited from AT. External validation data also support the above conclusions.

Conclusion: This study developed a nomogram that effectively and comprehensively evaluates the prognosis of T3N0 rectal cancer patients after TME. After using the nomogram, we recommend AT for high-risk patients, but not for low- and moderate-risk patients.

Keywords: T3N0 rectal cancer, nomogram, prognosis, adjuvant therapy, TME

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BACKGROUND

Colorectal cancer is the third most common cancer and the second leading cause of cancer-related deaths worldwide, among which 2/3 of cases are colon cancer and 1/3 are rectal cancer (1, 2). Current guidelines recommend neoadjuvant chemoradiotherapy (NCRT) combined with total mesorectal excision (TME) and adjuvant therapy (AT) for locally advanced rectal cancer (RC); however, the treatment for patients with early-stage RC (T3NO) is controversial.

Although NCRT can bring better survival prognosis to RC patients, it also increases the incidence of late adverse events and postoperative complications (3, 4). Willem et al. (4) found that while NCRT reduced the local recurrence rate for resectable RC, it had no effect on overall survival (OS). Frasson et al. (5) found that T3NO RC patients did not benefit from NCRT. The local recurrence rate of T3NO RC patients is only approximately 10%. Therefore, it is now considered potentially more suitable to provide direct surgery combined with AT for patients with such low recurrence risk, thereby avoiding the side effects of overtreatment (6–10). The 5-year OS of T3N0 patients after surgery is 74–84%, and such a high survival rate means that not all patients (especially those who underwent complete radical resection) will benefit from AT. T3N0 patients' local recurrence rate is only 3.6% after TME (9, 11). Due to the lack of randomized controlled trials (RCTs) of AT in T3N0 patients, this study focused on the clinical effect of AT in T3N0 patients at high-risk of recurrence after TME without NCRT.

This study analyzed clinicopathological factors from the SEER database and evaluated the prognosis of patients with T3N0 RC. Furthermore, the patients were divided into low-, moderate-, and high-risk groups according to a novel nomogram score to select the population that could most benefit from AT.

METHOD

Patient Cohort

SEER*Stat (version 8.3.6) software was used to search 7161 patients with pT3N0M0 RC diagnosed from 2004 to 2016. The inclusion criteria were (1): pathologically diagnosed RC (ICD-O-3: C19.9, C20.9) (2); complete follow-up and survival data (3); no NCRT (4); underwent TME; and (5) primary RC. Finally, the patients were divided into two groups according to whether they received AT: the non-AT group (n=4367) and the AT group (n=2794). The included clinicopathological variables were: age, sex, race, marital status, tumor grade, size, primary site, histology, lymph nodes retrieved, carcinoembryonic antigen (CEA) level, tumor deposits, perineural invasion, radiotherapy and chemotherapy information, and survival information. Patients were further excluded if information for the above variables was unknown.

The external validation group include 334 pT3N0M0 RC patients at our center between 2008 and 2013. The inclusion criteria and clinicopathological variables were the same as for the SEER group.

Statistical Analysis

Associations of clinicopathological factors with the two groups were analyzed by the chi-square test. To balance potential confounding biases of the included cases, only significant clinicopathological factors according to the chi-square test were included in the propensity score matching (PSM). The non-AT group and the AT group were subjected to nearest neighbor matching according to 1:1 (12). Survival analysis was performed by the Kaplan–Meier method and the log-rank test.

Establishing the Nomogram

First, univariate and multivariate COX analyses were performed to find correlations between the clinicopathological variables and OS in the non-AT group. Next, significant variables according to Cox multivariate analysis (P<0.05) were included to establish a nomogram. The effectiveness of the nomogram was tested by determining it discriminatory ability by the concordance index (C-index) (13); we also compared the C-index of the nomogram, lymph nodes retrieved, and CEA to evaluate the clinical effectiveness of the model. The calibration curve intuitively displays the consistency between the predicted survival rate and the actual survival data. Decision curve analysis (DCA) was used to evaluate the net clinical benefit as compared with lymph nodes retrieved and CEA. According to the risk score of the nomogram, all cases from the two groups were divided into three groups (high-, moderate-, and low-risk) by X-tile software (14). SPSS 24.0 (IBM, Armonk, NY, USA) and R software (version 3.5.1) were used for the statistical analyses conducted in this study, with P<0.05 used to denote that the difference was statistically significant.

RESULTS

Patient Demographics

Before PSM, a total of 7161 pT3N0M0 patients who completed TME were included, including 4,367 patients without AT and 2,794 patients with AT (**Figure 1**). The median survival was 59 months (range: 0–155) and the number of deaths was 2,632 (36.8%). Chi-square analysis showed that patients with AT were significantly correlated with age, sex, marital status, grade, tumor size, primary site, histology, lymphatic invasion, CEA, tumor deposits, and perineural invasion (all P<0.05). After including variables related to AT for the PSM, the final patient number was 5588, including 2794 patients in the non-AT group and 2794 patients in the AT group (**Table 1**). The median survival was 64 months (range: 0–155) and there were 1,859 deaths (33.3%).

The prognosis of patients who received AT was better than that of the non-AT group (5-year survival rate: 79.9% *vs*. 66.8%, P<0.05, **Figure 2A**). After PSM, the prognosis of patients who received AT was still higher than that of the non-AT group (5-year survival rate: 79.9% *vs*. 71.0%, P<0.05, **Figure 2B**).

Nomogram Construction

A COX hazards ratio model for patients without AT was constructed (**Table 2**), and univariate analysis showed that age, sex, race, marital status, tumor grade, size, primary site, histology, lymph nodes retrieved, CEA, tumor deposits, and perineural invasion were correlated with OS (all P<0.05). Next, these variables were included in the multivariate analysis, which showed that age, sex, race, marital status, tumor grade, size, primary site, lymph nodes retrieved, and CEA were independent prognostic factors (P<0.05). Based on these results, a nomogram was constructed to predict the 3- and 5-year survival rates of T3N0 RC after TME (**Figure 3**).

Testing the Effectiveness of the Nomogram

A nomogram incorporating the above nine risk factors was constructed to judge the prognosis T3N0 RC and had a

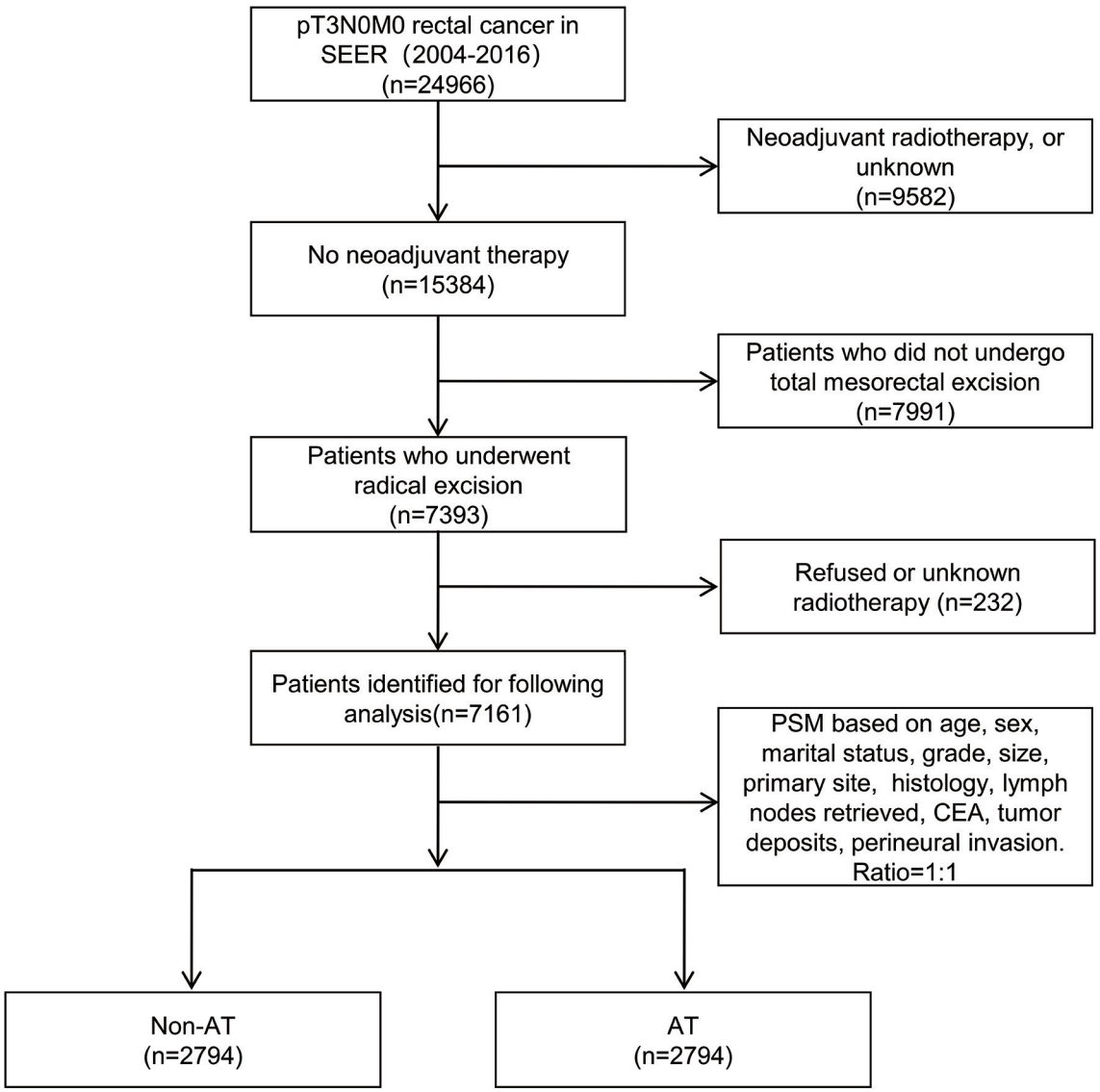

FIGURE 1 | Flowchart of the selection process of included patients.

C-index of 0.725 [95% confidence interval (CI): 0.694–0.756], which is significantly higher than the C-index of prognosis judged by lymph nodes retrieved and CEA [0.581 (95% CI: 0.550–0.612) and 0.547 (95% CI: 0.514–0.580), respectively]. The calibration curve of the 3- and 5-year OS nomogram showed that the predicted survival probability was consistent with the actual survival probability. The net benefits of the nomogram for different decision thresholds were higher than those of the lymph nodes retrieved and CEA system (**Figure 4**).

Overall Patient Risk Stratification System

Next, we calculated risk scores for each patient in the two groups with the nomogram (**Table 3**) and used X-tile software to take two cut-off values that divided the patients into three risk groups (**Figure 5**), a low-risk group (score ≤146, *n*=1331), a moderate-risk group (score 147–177, *n*=1331), and a high risk-group (score ≥178, *n*=2926). Five-year survival rates for the low-, moderate-, and high-risk groups were 91.2%, 86.6%, and 63.8%, respectively, which were statistically significant differences ($P<0.001$, **Figure 2C**).

We also divided the non-AT group into three groups using the current scoring system, a low-risk group (*n*=652), moderate-risk group (*n*=596), and high-risk group (*n*=1546). Five-year survival rates of these groups were 91.8%, 87.5%, and 56.4%, respectively, which were statistically significant differences ($P<0.01$, **Figure 2D**). In the AT group, the 5-year survival rates of the low- (*n*=679), moderate- (*n*=735), and high-risk (*n*=1380) groups were 90.7%, 85.9%, and 71.8%, respectively, which were statistically significant differences ($P<0.01$, **Figure 2E**).

TABLE 1 | Characteristics of patients.

Variable	Unmatched Cohort				Matched Cohort			P value
	Total [n(%)]	Non-AT [n(%)]	AT [n(%)]	P value	Total [n(%)]	Non-AT [n(%)]	AT [n(%)]	
Age	7161	4367	2794	<0.001	5588	2794	2794	<0.001
<65	3292	1497 (34.3)	1795 (64.2)		3291	1496 (53.5)	1795 (64.2)	
≥65	3869	2870 (65.7)	999 (35.8)		2297	1298 (46.5)	999 (35.8)	
Sex				<0.001				0.250
Male	4119	2402 (55.0)	1717 (61.5)		3392	1675 (59.9)	1717 (61.5)	
Female	3042	1965 (45.0)	1077 (38.5)		2196	1119 (40.1)	1077 (38.5)	
Race				0.875				0.279
White	5817	3552 (81.3)	2265 (81.1)		4504	2239 (80.1)	2265 (81.1)	
Black	625	384 (8.8)	241 (8.6)		523	282 (10.1)	241 (8.6)	
API	654	390 (8.9)	264 (9.4)		516	252 (9.0)	264 (9.4)	
Other	65	41 (1.0)	24 (0.9)		45	21 (0.8)	24 (0.9)	
Marital status				<0.001				0.037
Married	3947	2216 (50.7)	1731 (62.0)		3371	1640 (58.7)	1731 (62.0)	
Unmarried	1010	589 (13.5)	421 (15.1)		863	442 (15.8)	421 (15.1)	
Unknown	2202	1562 (35.8)	642 (22.9)		1354	712 (25.5)	642 (22.9)	
Grade				0.001				0.275
Well/moderately	6262	3867 (88.6)	2395 (85.7)		4817	2422 (86.7)	2395 (85.7)	
Poorly/undifferentiated	755	428 (9.8)	327 (11.7)		644	317 (11.3)	327 (11.7)	
Unknown	144	72 (1.6)	72 (2.6)		127	55 (2.0)	72 (2.6)	
Size (cm)				<0.001				0.004
<3	871	505 (11.6)	366 (13.1)		691	325 (11.6)	366 (13.1)	
≥3	5965	3702 (84.8)	2263 (81.0)		4612	2349 (84.1)	2263 (81.0)	
Unknown	325	160 (3.6)	165 (5.9)		285	120 (4.3)	165 (5.9)	
Primary site				<0.001				<0.001
Rectosigmoid junction	3884	2686 (61.5)	1198 (42.9)		2585	1387 (49.6)	1198 (42.9)	
Rectum	3277	1681 (38.5)	1596 (57.1)		3003	1407 (50.4)	1596 (57.1)	
Histology				0.034				0.303
Adenocarcinoma	6729	4117 (94.3)	2612 (93.5)		5223	2611 (93.5)	2612 (93.5)	
Mucinous adenocarcinoma	381	229 (5.2)	152 (5.4)		317	165 (5.9)	152 (5.4)	
Signet ring cell carcinoma	13	5 (0.1)	8 (0.3)		12	4 (0.1)	8 (0.3)	
Other	38	16 (0.4)	22 (0.8)		36	14 (0.5)	22 (0.8)	
Lymph nodes retrieved				0.001				0.019
< 12	2049	1183 (27.1)	866 (31.0)		1648	782 (28.0)	866 (31.0)	
≥ 12	5069	3161 (72.4)	1908 (68.3)		3907	1999 (71.5)	1908 (63.8)	
Unknown	43	23 (0.5)	20 (0.7)		33	13 (0.5)	20 (0.7)	
CEA (ng/ml)				0.001				0.170
≤5	2512	1466 (33.6)	1046 (37.4)		2045	999 (35.8)	1046 (37.4)	
>5	1625	984 (22.5)	641 (22.9)		1260	619 (22.2)	641 (22.9)	
Unknown	3024	1917 (43.9)	1107 (39.7)		2283	1176 (42.0)	1107 (39.7)	
Tumor deposits				<0.001				0.016
Negative	2836	1798 (41.2)	1038 (37.2)		2141	1103 (39.5)	1038 (37.2)	
Positive	54	23 (0.5	31 (1.1)		46	15 (0.5)	31 (1.1)	
Unknown	4271	2546 (58.3)	1725 (61.7)		3401	1676 (60.0)	1725 (61.7)	
Perineural invasion				0.027				0.300
Negative	2608	1643 (37.6)	965 (34.5)		1981	1016 (36.4)	965 (34.5)	
Positive	208	121 (2.8)	87 (3.1)		164	77 (2.8)	87 (3.1)	
Unknown	4345	2603 (59.6)	1742 (62.4)		3443	1701 (60.8)	1742 (62.4)	

AT, adjuvant therapy; API, Asian/Pacific Islander; CEA, carcinoembryonic antigen.

Evaluating the Efficiency of AT for Patients in Different Groups

We further investigated the benefit of AT in patients with different risk stratification (**Table 4**). The results showed that patients in the low-risk group did not benefit from AT (hazard ratio [HR]: 0.89, 95% CI: 0.65–1.21, $P>0.05$, **Figure 2F**). Patients in the moderate-risk group also did not benefit from AT (HR: 1.04, 95% CI: 0.81–1.32, $P>0.05$, **Figure 2G**). In contrast, patients in the high-risk group benefited from AT (HR: 0.61, 95% CI: 0.54–0.67, $P<0.001$, **Figure 2H**).

Evaluating the Efficiency of AT for Patients in the External Validation Group

The external validation group included 216 patients without AT and 118 patients with AT. The median survival was 83 months (range: 0–396) and the number of deaths was 192 (57.5%). The prognosis of patients who received AT was better than that of the non-AT group (5-year survival: 61.6% *vs.* 75.3%; $P<0.001$) (**Figure S1A**). According to the above scoring system, the external validation group were also divided into low, moderate, and high-risk groups. Five-year survival rates of all patients for

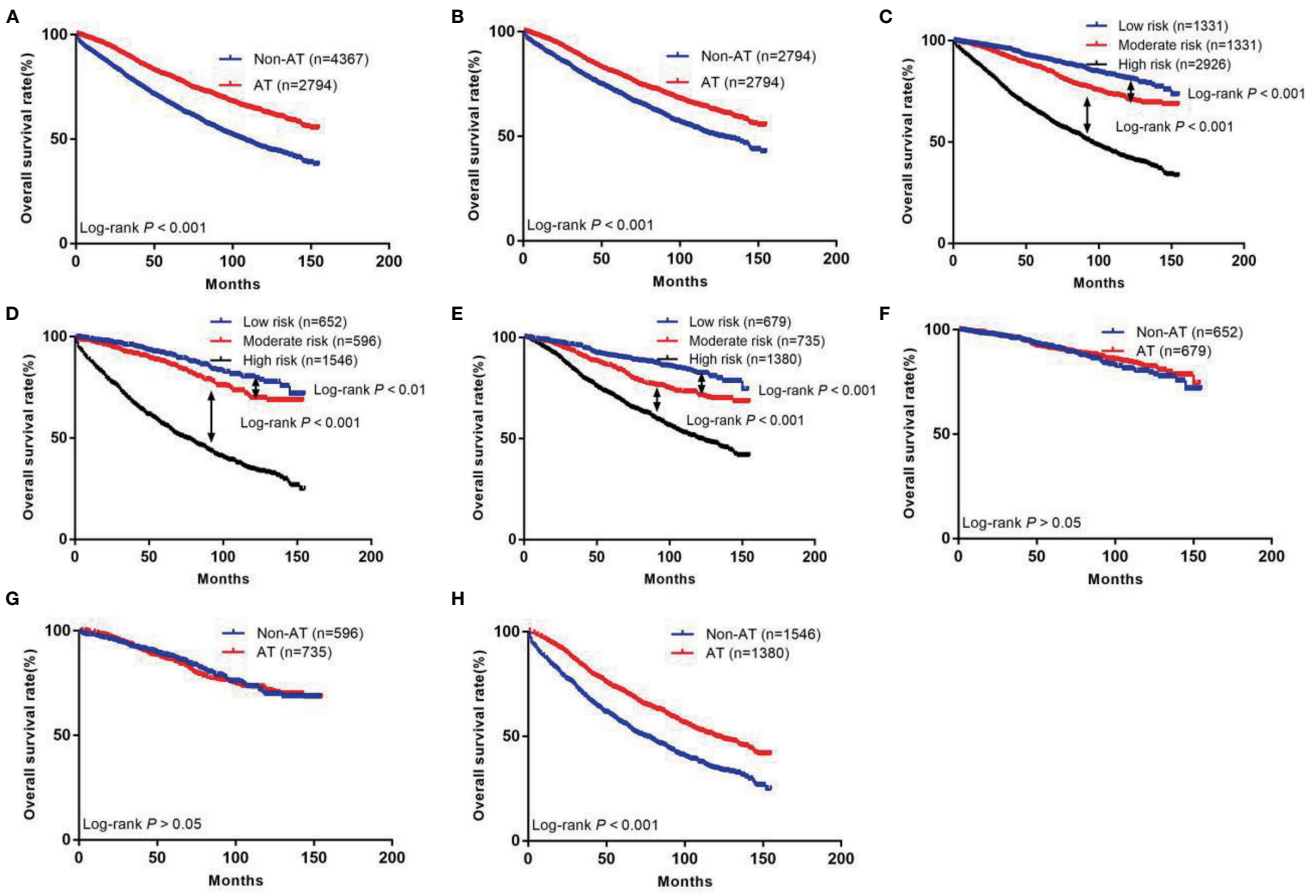

FIGURE 2 | The Kaplan-Meier curves of OS for patients in our study. **(A)** All patients; **(B)** Patients after PSM; **(C)** OS in different subgroups of all patients; **(D)** OS in different subgroups of non-AT group; **(E)** OS in different subgroups of AT group; **(F)** OS for patients with or without AT in low risk group; **(G)** OS for patients with or without AT in moderate risk group; **(H)** OS for patients with or without AT in high risk group.

the low-, moderate-, and high-risk groups were 92.1%, 87.4%, and 55.9%, respectively (**Figure S1B**). Five-year survival rates of non-AT group for the low-, moderate-, and high-risk groups were 83.3%, 86.5%, and 52.6%, respectively (**Figure S1C**). Five-year survival rates of AT group for the low-, moderate-, and high-risk groups were 100.0%, 88.8%, and 63.2%, respectively (**Figure S1D**).

The results showed that external validation group in the low-risk group did not benefit from AT (hazard ratio [HR]: 0.45, 95% CI: 0.05–4.33, P>0.05, **Figure S1E**). Patients in the moderate-risk group also did not benefit from AT (HR: 0.47, 95% CI: 0.19–1.16, P>0.05, **Figure S1F**). In contrast, patients in the high-risk group benefited from AT (HR: 0.63, 95% CI: 0.46–0.88, P=0.01, **Figure S1G**).

DISCUSSION

The postoperative recurrence rate of RC is as high as 40%, and the 5-year survival rate is not greater than 50% (15). TME reduces the local recurrence rate to less than 10% and increases the cancer-free survival rate to more than 70% (16, 17). Although NCRT significantly reduces the local recurrence rate to less than 7%, patients' 5-year distant metastasis rate still exceeds 20%. The adverse reactions of NCRT may lead to a decline in quality of life and a financial burden, and delay follow-up treatment, which may lead to shorter life expectancy (18–21). For T3N0 patients with relatively low recurrence rates, the application of NCRT is controversial. A German study showed that there was no significant difference in 10-year OS (59.6% *vs.* 59.9%, P=0.85) between the NCRT group and the adjuvant radiotherapy group, and there was no difference in the distant metastasis rate between the two groups (29.8% *vs.* 29.6%, P=0.9) (22). Thus, NCRT may not be the best treatment; direct TME surgery can obtain a good prognosis and a lower postoperative recurrence rate, and TME plus AT may be an ideal treatment for patients with a higher risk of recurrence. Our study used a large sample database to screen factors that were associated with the prognosis of T3N0 patients, and then developed a nomogram to evaluate the risk score of patients and guide their AT accurately and individually.

TABLE 2 | The univariate and multivariate analyses of factors associated with overall survival.

Variable	univariate Cox regression		multivariate Cox regression	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age				
<65	1		1	
≥65	3.886 (3.398-4.444)	<0.001	3.421 (2.967-3.944)	<0.001
Sex				
Male	1		1	
Female	0.861 (0.760-0.974)	0.018	0.752 (0.661-0.856)	<0.001
Race				
White	1		1	
Black	1.091 (0.901-1.321)	0.371	1.205 (0.991-1.466)	0.062
API	0.653 (0.510-0.837)	0.024	0.780 (0.607-1.001)	0.051
Other	0.135 (0.019-0.956)	0.045	0.259 (0.036-1.841)	0.177
Marital status				
Married	1		1	
Unmarried	1.073 (0.897-1.284)	0.441	1.284 (1.067-1.544)	0.008
Unknown	1.680 (1.471-1.919)	<0.001	1.571 (1.366-1.806)	<0.001
Grade				
Well/moderately	1		1	
Poorly/undifferentiated	1.402 (1.183-1.662)	<0.001	1.270 (1.071-1.506)	0.006
Unknown	1.317 (0.885-1.961)	0.174	1.045 (0.700-1.561)	0.829
Size (cm)				
<3	1		1	
≥3	1.394 (1.136-1.711)	0.001	1.477 (1.198-1.819)	<0.001
Unknown	1.835 (1.334-2.524)	<0.001	1.633 (1.182-2.257)	0.003
Primary site				
Rectosigmoid junction	1		1	
Rectum	1.790 (1.582-2.025)	<0.001	1.193 (1.049-1.358)	0.007
Histology				
Adenocarcinoma	1			
Mucinous adenocarcinoma	1.400 (1.116-1.756)	0.004		
Signet ring cell carcinoma	0.712 (0.100-5.058)	0.734		
Other	2.005 (0.953-4.217)	0.067		
Lymph nodes retrieved				
< 12	1		1	
≥12	0.524 (0.464-0.591)	<0.001	0.639 (0.564-0.724)	<0.001
Unknown	0.826 (0.392-1.743)	0.616	0.800 (0.376-1.705)	0.564
CEA (ng/ml)				
≤5	1		1	
>5	1.486 (1.266-1.745)	<0.001	1.397 (1.189-1.642)	<0.001
Unknown	1.264 (1.098-1.456)	0.001	1.237 (1.073-1.426)	0.003
Tumor deposits				
Negative	1			
Positive	3.241 (1.601-6.561)	0.001		
Unknown	1.415 (1.213-1.651)	<0.001		
Perineural invasion				
Negative	1			
Positive	1.503 (0.969-2.333)	0.069		
Unknown	1.391 (1.187-1.630)	<0.001		

API, Asian/Pacific Islander; CEA, carcinoembryonic antigen.

There is still no unified view of the prognosis of young RC patients. Some studies have shown that young patients have histopathological features such as late onset, aggressive disease, and worse prognosis than older patients (23–28). But there is also a view that older patients have poor prognosis (29–31), which suggests that age is a controversial prognostic factor in RC. Our study found that patients ≥65-years old had a poor prognosis (HR: 3.42, 95% CI: 2.97–3.94, P<0.001). One possible reason is that older patients are less sensitive to sensation, which makes the clinical manifestations of RC in the elderly more atypical and easier to ignore, resulting in later staged disease in many older patients. In addition, the proportion of the elderly who have received radical surgery is low, due to the poor tolerance to surgery and a large number of comorbidities, leading to a higher incidence and mortality of perioperative diseases in the elderly and a poor prognosis.

Studies have shown that colorectal cancer incidence is higher in men than in women, which may be associated with estrogen levels. Young and middle-aged women with higher estrogen levels have a decreased risk of colorectal cancer risk, and the

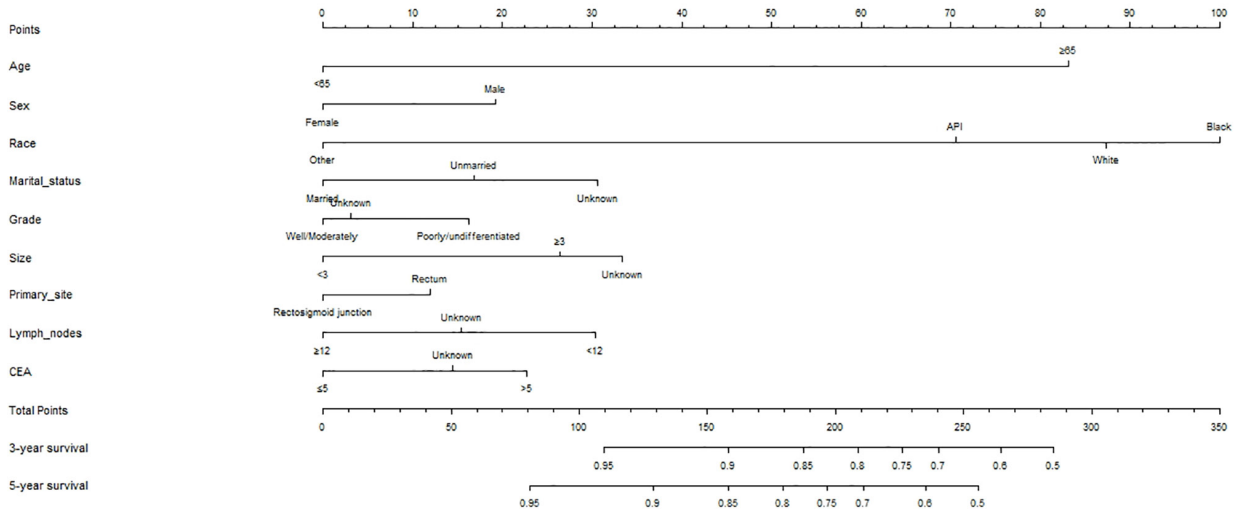

FIGURE 3 | Oncologic nomogram for T3N0 rectal cancer patients after TME.

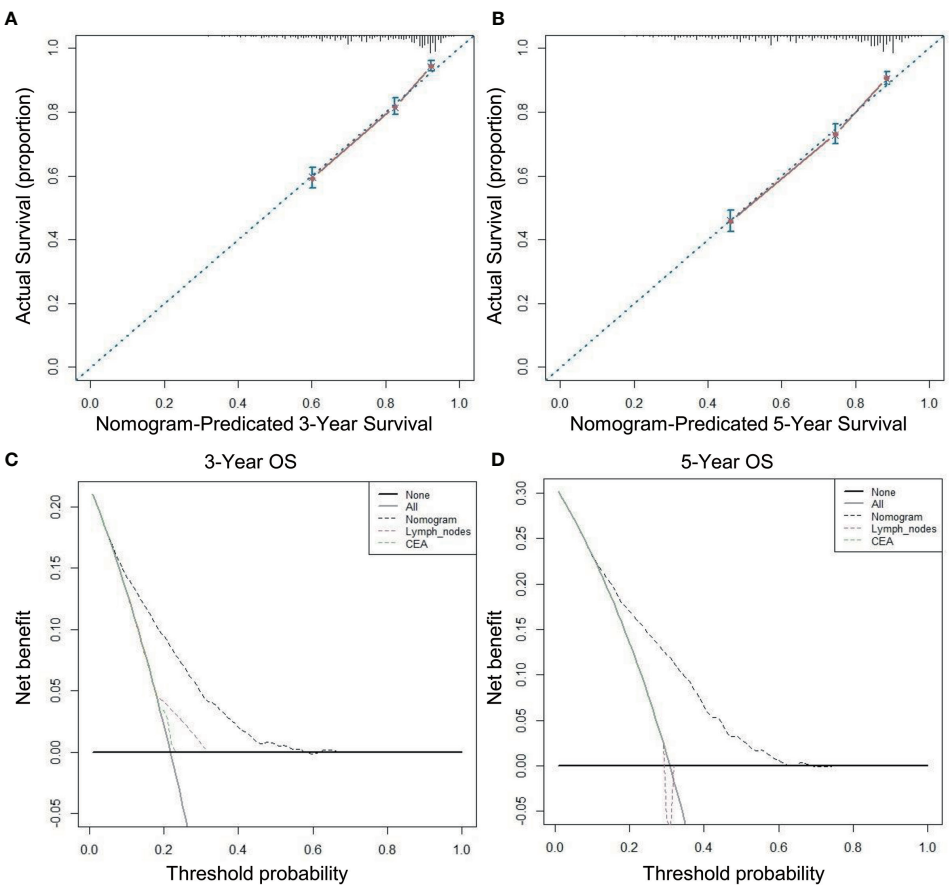

FIGURE 4 | Calibration curves and decision curve for OS prediction: **(A)** 3-year OS calibration curve in our cohort; **(B)** 5-year OS calibration curve in our cohort; **(C)** Nomogram were compared to the lymph nodes retrieved and CEA in terms of 3-year OS in our decision curve analysis; **(D)** Nomogram were compared to the lymph nodes retrieved and CEA in terms of 5-year OS in our decision curve analysis.

TABLE 3 | Point assignment of each component and prognostic score for T3N0 rectal cancer.

Group	Score	Estimated 3-y OS (%)	Estimated 5-y OS (%)
Age			
<65	0		
≥65	83		
Sex			
Male	19		
Female	0		
Race			
White	87		
Black	100		
API	71		
Other	0		
Marital status			
Married	0		
Unmarried	17		
Unknown	31		
Grade			
Well/moderately	0		
Poorly/undifferentiated	16		
Unknown	3		
Size (cm)			
<3	0		
≥3	26		
Unknown	33		
Primary site			
Rectosigmoid junction	0		
Rectum	12		
Lymph nodes retrieved			
< 12	30		
≥12	0		
Unknown	15		
CEA (ng/ml)			
≤5	0		
>5	23		
Unknown	14		
Total score			
	110	95	
	158	90	
	187	85	
	209	80	
	226	75	
	240	70	
	265	60	
	285	50	
	81		95
	129		90
	158		85
	180		80
	197		75
	211		70
	235		60
	256		50

API, Asian/Pacific Islander; CEA, carcinoembryonic antigen.

cumulative protection of higher estrogen levels can be extended up to 20 to 25 years after menopause (32–34). Our study was consistent with the literature in that female patients had a better prognosis (HR: 0.75, 95% CI: 0.66–0.86, *P*<0.001) (35).

Pulte et al. (36) found that blacks and Indians had worse outcomes than whites, which is consistent with our results. Our study found that the prognosis of unmarried patients was worse than that of married patients, consistent with previous studies (37–39), which may be related to the lower proportion of unmarried patients participating in RC screening, lower enthusiasm for treatment, and lower proportion of patients receiving surgery and AT.

Currently, serum CEA is the most important tumor marker applied in clinical colorectal cancer management. Serum CEA levels can predict the prognosis and recurrence of colorectal cancer, and the later the disease stage is, the higher serum CEA levels are, and increased CEA is correlated with poor tumor differentiation (40–42). Our study also found that CEA (HR: 1.40, 95% CI: 1.19–1.64, *P*<0.001) and poor tumor differentiation (HR: 1.27, 95% CI: 1.07–1.51, *P*=0.006) are poor prognostic factors for T3N0 RC.

Tumor size is related to the time of tumor existence, invasion, and distant metastasis, and therefore, also to poor prognosis (43). Our study found that the prognosis of tumors ≥3 cm was worse (HR: 1.48, 95% CI: 1.20–1.82, *P*<0.001). As the boundary between the colon and rectum, rectosigmoid junction cancer may be different from RC and colon cancer in terms of pathogenesis, treatment, and prognosis. It is generally believed that the prognosis of diploid DNA tumors is better than that of aneuploid tumors. Diploid status was more common in proximal colorectal cancer than in distal colorectal cancer. The benefit of 5-FU treatment is greater for proximal colorectal cancer, but less for distal colorectal cancer. Therefore, from proximal colorectal cancer to distal colorectal cancer to RC, the prognosis of patients is gradually worse (44). Our study also confirmed that the prognosis of RC was worse than that of rectosigmoid junction cancer (HR: 1.19, 95% CI: 1.05–1.36, *P*=0.007).

The number of lymph nodes retrieved after surgery is closely related to the postoperative pathological stage of RC patients. Retrieving few lymph nodes may be related to an insufficient degree of lymph node dissection during surgery, and even lead to lymph nodes that are positive in the surgical area are not dissected, which affects prognosis. It is suggested in the guidelines that at least 12 lymph nodes should be detected to ensure that there is no bias in staging (45, 46). There is already evidence that in patients with lymph node-negative colorectal cancer, a higher number of lymph nodes retrieved is associated with prognosis (47). Many studies have found that in stage II/III colorectal cancer, the prognosis of patients with <12 lymph nodes retrieved is worse than that of patients with >12 lymph nodes retrieved (48–51). Our study also found that the prognosis of patients with >12 lymph nodes retrieved was relatively better (HR: 0.64, 95% CI: 0.56–0.72, *P*<0.001). New lymph node staging indicators such as metastatic lymph nodes ratio, log odds of positive lymph nodes, negative lymph node count, and lymph node micrometastasis may predict prognosis more accurately (52–56).

Our nomogram of multiple prognostic factors was established through large sample data and more comprehensively incorporates factors that affect the prognosis of T3N0 than the number of lymph nodes retrieved [C-index: 0.581 (95% CI: 0.550–0.612)], and CEA [C-index 0.547 (95% CI: 0.514–0.580)], and our nomogram [C-index: 0.725 (95% CI: 0.694–

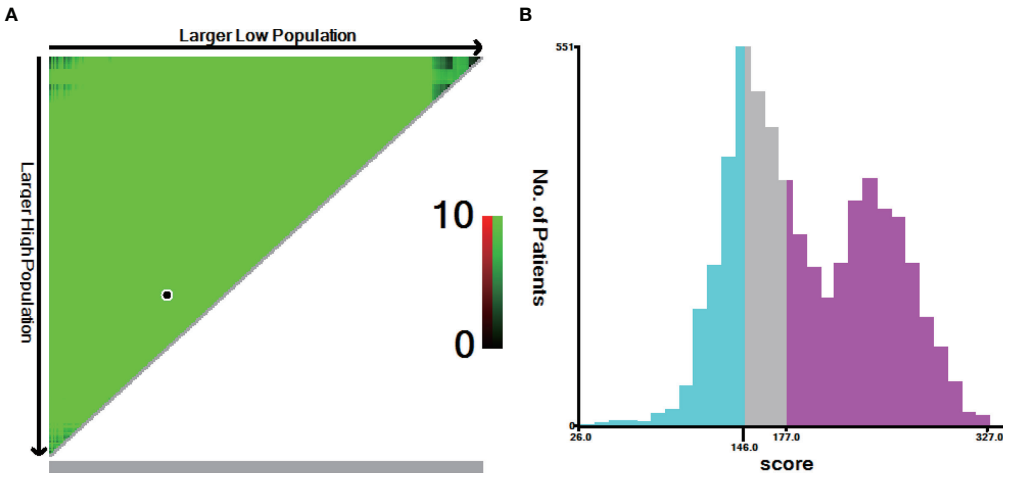

FIGURE 5 | X-tile analysis for risk stratification: **(A)** The optimal cut-off value; **(B)** Numbers of patients in low, moderate and high risk subgroups.

TABLE 4 | Risk stratification in non-AT and AT group.

Survival status	Non-AT Group			P value	AT Group			P value
	Low risk[n (%)]	Moderate risk [n (%)]	High risk [n (%)]		Low risk [n (%)]	Moderate risk [n (%)]	High risk [n (%)]	
Live	569 (87.3)	481 (80.7)	673 (43.5)	<0.001	595 (87.6)	580 (78.9)	831 (60.2)	<0.001
Death	83 (12.7)	115 (19.3)	873 (56.5)		84 (12.4)	155 (21.1)	549 (39.8)	

AT, adjuvant therapy.

0.756)] better predicts the 3- and 5-year survival rates of T3N0 RC. We applied DCA to further confirm that the nomogram was superior to the number of lymph nodes retrieved and CEA in predicting the OS of T3N0 RC patients after TME.

Whether T3N0 RC can benefit from AT is controversial, and so far, no RCT has studied whether T3N0 RC can benefit from AT. Paula et al. (10) found that postoperative adjuvant chemotherapy had no survival benefit compared with patients without chemotherapy (HR: 0.88, 95% CI: 0.52–1.56, *P*=0.66). Kim et al. (57) found that the 5-year OS of patients receiving postoperative adjuvant chemotherapy was lower than that of patients without chemotherapy (79.3 *vs.* 83.0, *P*=0.92). However, Quinn et al. (9) found that postoperative chemotherapy (HR: 0.74, 95% CI: 0.62–0.89, *P*=0.001) and chemoradiotherapy (HR: 0.57, 95% CI: 0.50–0.65, *P*<0.001) improved the prognosis of patients compared with surgery alone. Our findings suggest that AT improved patient survival both before and after PSM. Because AT is often used in patients with poor prognostic factors, they benefit more from AT, resulting in the overall results showing that AT improves prognosis. However, this does not mean that all patients need AT, which requires us to select those who will really benefit from AT for precise and individualized treatment. Our nomogram comprehensively analyzed factors that influence the prognosis and recurrence of T3N0 RC, and scored the impact of each risk subgroup: low, moderate, and high. Moreover, in our non-AT and AT groups, there were obvious survival differences in the low-, moderate-, and high-risk subgroups, indicating that our risk stratification was reasonable and effective. To determine which subgroups of patients benefit from AT, we found that the 5-year survival rate of low-risk patients receiving AT was lower than that of patients without AT (90.7% *vs.* 91.7%, *P*>0.05), so we do not recommend AT for low-risk patients. The 5-year survival rate of patients with moderate risk who received AT was lower than that of patients without AT (85.9% *vs.* 87.5%, *P*>0.05). We also do not recommend AT for such patients because the harm of AT for low and moderate risk patients exceeds the benefit. The 5-year survival rate of high-risk patients who received AT was higher than that of patients without AT (71.8% *vs.* 56.4%, *P*<0.001), we suggest that high-risk patients receive AT. Our external validation data also showed that low and moderate -risk patients did not benefit from AT (*P*>0.05), while high-risk patients did (*P*<0.05).

This study has several limitations. This was a retrospective study, and some patients fail to be included in this study due to missing data, which may cause bias. Currently, there is no large-scale RCT study on whether T3N0 RC benefits from AT, and there is no prognostic survival nomogram that incorporates the above clinical pathological factors. The most important thing is that we use this nomogram to stratify the risk of patients, which is of great significance for individualized guidance of clinical AT, as was the goal of this work.

CONCLUSION

Age, sex, race, marital status, tumor grade, size, primary site, lymph nodes retrieved, and CEA are independent prognostic factors for T3N0 RC patients after TME. Through our innovative risk score stratification, we recommend high-risk patients receive AT, while AT is not recommended for low- and moderate-risk patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

XW designed the research. SZ took part in designing the research. SZ collected the data, analyzed the date. CZ analyzed the date and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.698866/full#supplementary-material

Supplementary Figure 1 | The Kaplan-Meier curves of OS for patients in external validation group. **(A)** All patients; **(B)** OS in different risk subgroups of all patients; **(C)** OS in different risk subgroups of non-AT group; **(D)** OS in different risk subgroups of AT group; **(E)** OS for patients with or without AT in low risk group; **(F)** OS for patients with or without AT in moderate risk group; **(G)** OS for patients with or without AT in high risk group.

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