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Two tripartite classification systems of CD86⁺ and CD206⁺ macrophages are significantly associated with tumor recurrence in stage II-III colorectal cancer

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Introduction: The prognostic value of tumor-associated macrophages remains unclear in colorectal cancer (CRC). Two tripartite classification systems, namely, ratio and quantity subgroups, were investigated as the prognostic stratification tools for stage II-III CRC.

Methods: We assessed the infiltration intensity of CD86⁺ and CD206⁺ macrophages in 449 cases with stage II-III disease by immunohistochemical staining. Ratio subgroups were defined by the lower- and upper-quartile points of CD206⁺/(CD86⁺+CD206⁺) macrophage ratio, including the low-, moderate-, and high-ratio subgroups. Quantity subgroups were defined by the median points of CD86⁺ and CD206⁺ macrophages and included the low-, moderate-, and high-risk subgroups. The main analysis was recurrence-free survival (RFS) and overall survival (OS).

Results: Ratio subgroups (RFS/OS: HR=2.677/2.708, all $p<0.001$) and quantity subgroups (RFS/OS: HR=3.137/3.250, all $p<0.001$) could serve as independent prognostic indicators that effectively predicted survival outcomes. More importantly, log-rank test revealed that patients in the high-ratio (RFS/OS: HR=2.950/3.151, all $p<0.001$) or high-risk (RFS/OS: HR=3.453/3.711, all $p<0.001$) subgroup exhibited decreased survival outcomes after adjuvant chemotherapy. The predictive accuracy of the quantity subgroups within 48 months was higher than that of the ratio subgroups and tumor stage (all $p<0.05$).

Conclusions: Ratio and quantity subgroups could serve as independent prognostic indicators that could potentially be incorporated into the tumor staging algorithm to improve prognostic stratification and provide better predictions of survival outcomes in stage II-III CRC after adjuvant chemotherapy.

KEYWORDS

CD86, CD206, colorectal cancer, macrophage polarization, tripartite classification system

Introduction

Colorectal cancer (CRC) is well recognized for its clinical and biological diversities (1). Approximately three-fifths of CRC cases are stage II-III disease at diagnosis (2, 3). Radical resection is the preferred option for these patients (2–5). The treatment outcome of these patients remains unsatisfactory (2–6). Approximately 30% of CRC patients with stage II-III disease will experience tumor recurrence after radical resection (2–6). The clinical and biological diversities may present great challenges in identifying high-risk CRC patients, which subsequently makes it difficult to distinguish between CRC patients who may benefit from adjuvant chemotherapy when the probability of tumor recurrence is considered.

Macrophages are a main cellular component of the immune microenvironment (7). Tumor-associated macrophages may exhibit a spectrum of polarization status, with M_1 - and M_2 -macrophages representing the ends of this spectrum. Diametrically polarized macrophages may have opposite functions in tumor progression (7, 8). M_1 -macrophages may provide a resistant role in tumorigenesis by activating tumor-killing mechanisms and amplifying Th_1 immunocyte responses (7, 8). However, M_2 -macrophages may stimulate tumor invasion and metastasis by suppressing tumor-specific immune responses (7, 8). Previous studies revealed that M_1 - and M_2 -macrophages exhibited high expression levels of CD86 and CD206 in gastrointestinal cancers, respectively (9–11). Therefore, we concluded that high infiltration of CD206⁺ macrophages, low infiltration of CD86⁺ macrophages, and a high ratio of CD206⁺/(CD86⁺+CD206⁺) macrophages would be markedly associated with advanced stage and a high rate of tumor recurrence and mortality (10–13). Actually, there are many tumor cases that may fall into a gray zone between M_1 - and M_2 -polarization. It is difficult to determine a suitable polarization phenotype for these cases. Thus, the tripartite categorization (M_1 -, mixed-, and M_2 -phenotype) may be a reasonable choice when one evaluates the polarization phenotype.

Integrating these immune markers into TNM staging might refine the prognostic significance for risk stratification and facilitate the development of better treatment strategies. Moreover, a single biomarker might not effectively characterize the complex immune microenvironment (14). In our study, we simultaneously assessed the infiltration intensity of stromal CD86⁺ and CD206⁺ macrophages by immunohistochemical staining. We developed two tripartite classification systems of CD86⁺ and CD206⁺ macrophages, namely,

ratio and quantity subgroups, as prognostic tools for tumor recurrence and mortality. The first tripartite categorization, namely, ratio subgroups, was composed of the low-, moderate-, and high-ratio subgroups based on the lower- (LQ) and upper-quartile (UQ) cutoff points of the CD206⁺/(CD86⁺+CD206⁺) macrophage ratio, correspondingly representing the M_1 -, mixed-, and M_2 -phenotype. The secondary tripartite categorization, namely, quantity subgroups, consisted of the low-, moderate-, and high-risk subgroups determined by the median cutoff points of CD86⁺ and CD206⁺ macrophages infiltration density, correspondingly embodying the M_1 -, mixed-, and M_2 -phenotype.

Materials and methods

Study participants

We retrospectively collected 449 CRC cases with stage II-III disease from two different hospitals. Of 449 patients, 310 patients underwent radical operations at Yantai Yuhuangding Hospital of China between 2012 and 2015. The remaining 139 patients underwent radical operations at Guangzhou Red Cross Hospital of China between 2013 and 2015. All participants were restaged according to the 8th edition Staging Classification of American Joint Committee on Cancer. This protocol was authorized by the Ethics Committee of Yantai Yuhuangding Hospital (Approval No.2018-118) and Guangzhou Red Cross Hospital (Approval No.2019-227-01). The inclusion criteria for this study were as follows: (a) middle-high rectal cancer or colon cancer; (b) with paraffin-embedded tumor tissue and survival information; and (c) patients with stage II-III disease. The exclusion criteria for this study were as follows: (a) low rectal cancer; (b) without paraffin-embedded tumor tissue or survival information; (c) with secondary primary tumors before and at diagnosis; (d) patients with stage I or IV disease; and (e) with neoadjuvant chemotherapy, radiotherapy, and immunotherapy. The reason is that neoadjuvant treatment may affect the infiltration number of different polarized macrophages in tumoral tissues.

Immunohistochemistry staining

The tumor sections (4 μm) for these 449 cases were collected for immunohistochemical staining of CD86 and CD206 in January 2020.

Tumor sections were stained for CD86 and CD206 from February to April 2020. The Benchmark-XT immunohistochemistry platform (Roche Company, Switzerland) was adopted for immunohistochemistry staining according to the standard procedure. Anti-human CD86 (Catalog Number : DF6332, 1:200, Affinity, USA) and anti-human CD206 (Catalog Number:91992S, 1:400, CST, USA) primary antibodies were utilized for immunohistochemistry staining. The enzyme-labeled anti-mouse/rabbit polymerized secondary antibody (ready to use, Roche Company, Switzerland) was further adopted.

In colorectal cancer, tumor area consists of tumor nest and stroma (13). Macrophages mainly infiltrate in the tumor stroma (13). CD86⁺ or CD206⁺ macrophages in the tumor stroma were defined as those cells that stained brown. So only macrophages that infiltrated at the invasive margins of the tumor stroma were counted by three randomly selected fields (400×) under the Leica-DM-LB2 microsystem. Two experienced researchers were blinded to the clinicopathologic information, and independently assessed the infiltrating number of these three random fields at the invasive margins for each patient. The mean number of the two counting results was utilized for the infiltrating number of per field (400×). For each patient, the mean number of three random fields was further adopted for the infiltrating intensity of CD86⁺ or CD206⁺ macrophages. Intraobserver and inter-observer agreement was well acceptable ($\kappa > 0.90$).

The definition of ratio and quantity subgroup systems

The first analysis was performed on the ratio subgroup system determined by the ratio of CD206⁺/(CD86⁺+CD206⁺) macrophages using the LQ and UQ cutoff points. The ratio subgroup system was

composed of the low- (ratio≤LQ), moderate- (LQ<ratio≤UQ), and high-ratio (ratio>UQ) subgroups.

The secondary analysis was performed on the quantity subgroup system based on the high- and low-infiltration groups of CD86⁺ (CD86^{high}, Figure 1A; CD86^{low}, Figure 1B) and CD206⁺ (CD206^{high}, Figure 1C; CD206^{low}, Figure 1D) macrophages using the median cutoff points. The quantity subgroup system was composed of the low- (CD86^{high}/CD206^{low}), moderate- (CD86^{low}/CD206^{low} & CD86^{high}/CD206^{high}), and high-risk (CD86^{low}/CD206^{high}) subgroups.

Exploration of macrophage-related gene sets based on microarray data

The microarray dataset GSE39582 was downloaded from the Gene Expression Omnibus repository, which was provided by the French national CIT program (15). The levels of gene expression were first normalized by the limma package and further log2-transformed. In this microarray dataset, fresh-frozen samples of primary tumor were collected for analyzing mRNA expression profiles by the GPL570 platform, including 460 stage II-III patients with complete clinical and survival information.

The CIBERSORT algorithm is an accurate tool for calculating the estimated proportion of M₁- and M₂-macrophages by imputing gene expression profiles of the microarray dataset GSE39582 (16). A value of $p < 0.05$ is recommended for inclusion in the further analysis (16). And 379 cases with stage II-III disease were finally fitted in the subsequent analysis. The correlation of 21 immune cell types in CRC tissues (GSE39582) was evaluated by the correlation heatmap. This patient cohort was stratified into the low- and high-infiltration groups based on the optimal cutoffs of M₁- or M₂-macrophage proportions determined by the MaxStat method, separately (17).

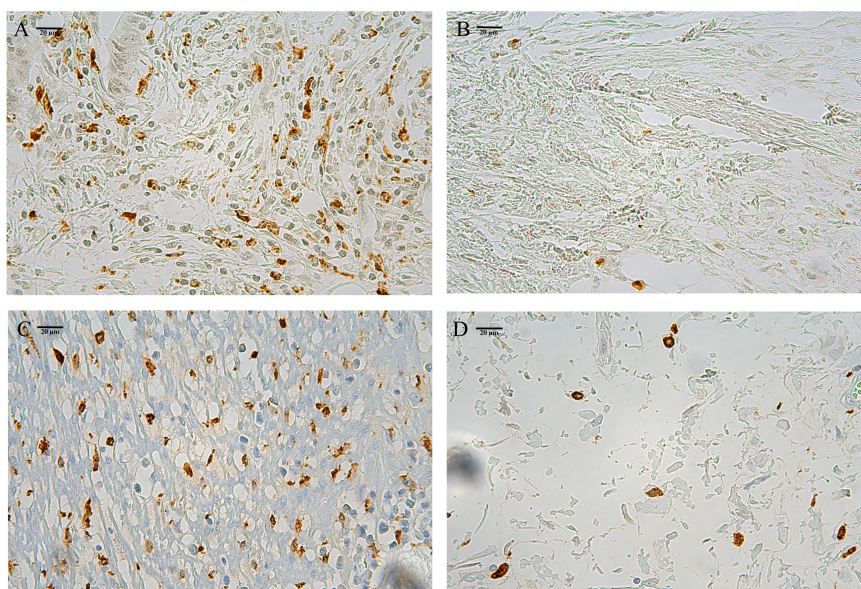


FIGURE 1
The high- and low-infiltration of stromal CD86⁺ (A, B) and CD206⁺ (C, D) macrophages.

To identify the enriched gene sets between high- and low-infiltration groups of M₁- or M₂-macrophages, we performed gene set enrichment analysis (GSEA) on all the mRNAs of the GPL570 platform using hallmark gene sets (18). We performed 1000 random sample permutations using the GSEA desktop application (version 4.3.0) to determine whether the members of a given gene set were associated with M₁- or M₂-macrophage infiltration. A threshold value of *p*<0.01 was considered significant.

Statistical analysis

The chi-square test was utilized to evaluate the correlation between the tripartite categorizations and these clinicopathologic factors. A threshold value of *p*<0.05 was considered significant. The main endpoints included recurrence-free/overall survival (RFS/OS). The R software (version 4.2.1) was utilized for data analysis.

Kaplan-Meier analysis and log-rank test were utilized to assess survival differences among the three risk subgroups. Multivariate Cox analysis was used to determine whether ratio and quantity subgroups were independent of those clinicopathologic variables. Receiver operating characteristic (ROC) curves were performed to assess the prediction abilities of tumor stage, ratio and quantity

subgroups (19). To construct ROC curves by the pROC package (19), patients with a duration of ≤48 months were excluded if they still did not experience tumor recurrence at the final follow-up. The recurrence-free time of the remaining cases was divided into either ≤48 months or >48 months.

Results

Prognostic values of the ratio subgroup system

Survival differences among the three ratio subgroups

The ratio of CD206⁺/(CD206⁺+CD86⁺) macrophages ranged from 0.019 to 0.993. Based on the lower- (0.285) and upper-quartile (0.709) points, 449 patients were stratified into the low- (n=112), moderate- (n=225), and high-ratio (n=112) subgroups. Clinicopathologic factors among the three ratio subgroups are shown in Table 1. As shown in Figures 2A, B, the ratio subgroup system (high- vs. moderate- vs. low-ratio) was significantly associated with worse RFS (hazard ratio [HR] =2.620, 95% confidence interval [CI]=1.991-3.447; *p*<0.001) and OS (HR=2.625, 95% CI=1.945-3.541; *p*<0.001).

TABLE 1 Clinical and pathological characteristics of 449 CRC patients among different risk subgroups.

Variable	Ratio Subgroups				Quantity Subgroups			
	low ratio (n=112)	moderate ratio (n=225)	high ratio (n=112)	<i>p</i> .value	low risk (n=112)	moderate risk (n=228)	high risk (n=109)	<i>p</i> .value
Age								
<66 y	53	115	70	0.054	63	115	60	0.533
≥66 y	59	110	42		49	113	49	
Gender								
male	69	124	69	0.377	70	130	62	0.590
female	43	101	43		42	98	47	
Mucinous Component								
no	106	196	100	0.104	108	200	94	0.021
yes	6	29	12		4	28	15	
Primary Locations								
colon	53	110	41	0.092	45	115	44	0.096
rectum	59	115	71		67	113	65	
Tumor Stage								
II	55	111	40	0.045	63	108	35	0.001
III	57	114	72		49	120	74	
Adjuvant Chemotherapy								
yes	74	146	74	<0.001	77	144	73	<0.001
no	38	79	38		35	84	36	

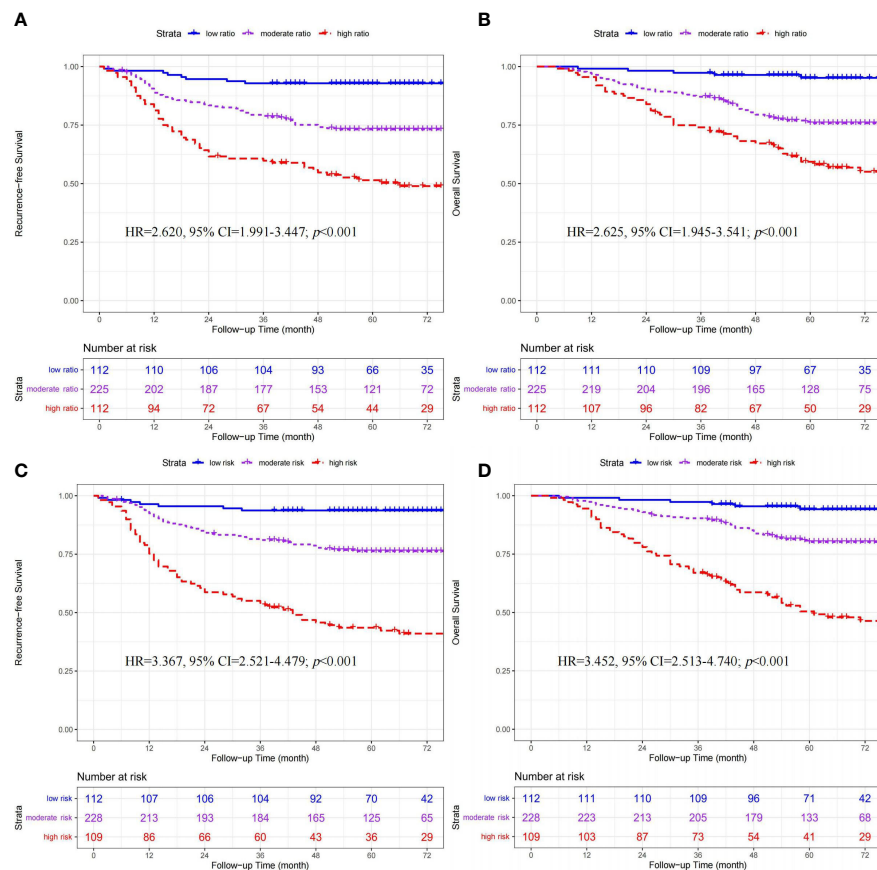


FIGURE 2 Kaplan-Meier curves of recurrence-free survival and overall survival stratified by ratio (A, B) and quantity (C, D) subgroups in 449 CRC patients.

Multivariate COX analysis of ratio subgroups and other clinicopathologic factors

Multivariate analysis demonstrated that the ratio subgroup system remained an independent factor for RFS (HR=2.677, 95% CI=2.028-3.533; $p < 0.001$) and OS (HR=2.708, 95% CI=1.998-3.670; $p < 0.001$) (Table 2).

The prognostic value of ratio subgroups for CRC patients receiving adjuvant chemotherapy

In 294 patients receiving chemotherapy (Shown in Figures 3A, B), significant differences in RFS (HR=2.950, 95% CI=2.502-4.127; $p < 0.001$) and OS (HR=3.151, 95% CI=2.620-4.591; $p < 0.001$) were found among the three ratio subgroups (high- vs. moderate- vs. low-ratio).

Prognostic values of the quantity subgroup system

Survival differences among the three quantity subgroups

According to the median cutoff points, 449 patients were classified into three quantity subgroups, including the low- (CD86^{high}/CD206^{low}, n=112), moderate- (CD86^{low}/CD206^{low} &

CD86^{high}/CD206^{high}, n=228), and high-risk (CD86^{low}/CD206^{high}, n=109) subgroups. The clinicopathologic characteristics among the three ratio subgroups are presented in Table 1. As shown in Figures 2C, D, the quantity subgroup system (high- vs. moderate- vs. low-risk) was significantly correlated with worse RFS (HR=3.367, 95% CI=2.521-4.479; $p < 0.001$) and OS (HR=3.452, 95% CI=2.513-4.740; $p < 0.001$).

Multivariate COX analysis of quantity subgroups and other clinicopathologic factors

Multivariate analysis demonstrated that the quantity subgroup system remained an independent indicator for RFS (HR=3.137, 95% CI=2.342-4.200; $p < 0.001$) and OS (HR=3.250, 95% CI=2.357-4.483; $p < 0.001$) (Table 2).

The prognostic value of quantity subgroups for CRC patients receiving adjuvant chemotherapy

In 294 patients receiving chemotherapy (Shown in Figures 3C, D), significant differences in RFS (HR=3.453, 95% CI=2.447-4.873; $p < 0.001$) and OS (HR=3.711, 95% CI=2.521-5.461; $p < 0.001$) were found among the three quantity subgroups (high- vs. moderate- vs. low-risk).

TABLE 2 Multivariate Cox analysis of macrophage-based risk subgroups, clinicopathologic factors and survival.

Variable	Recurrence-free Survival		Overall Survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Ratio Subgroups				
ratio subgroups (high vs. moderate vs. low ratio)	2.677 (2.028-3.533)	<0.001	2.708 (1.998-3.670)	<0.001
age (≥66 y vs. <66 y)	1.337 (0.910-1.964)	0.139	1.743 (1.145-2.654)	0.010
gender (female vs. male)	1.212 (0.843-1.742)	0.299	1.067 (0.716-1.588)	0.753
mucinous component (yes vs. no)	0.861 (0.448-1.656)	0.655	0.851 (0.410-1.766)	0.665
primary locations (rectum vs. colon)	0.738 (0.508-1.072)	0.111	0.748 (0.500-1.119)	0.158
tumor stage (III vs. II)	3.345 (2.174-5.146)	<0.001	3.580 (2.218-5.779)	<0.001
adjuvant chemotherapy (no vs. yes)	0.756 (0.506-1.132)	0.174	0.779 (0.508-1.197)	0.255
Quantity Subgroups				
quantity subgroups (high vs. moderate vs. low risk)	3.137 (2.342-4.200)	<0.001	3.250 (2.357-4.483)	<0.001
age (≥66 y vs. <66 y)	1.086 (0.743-1.589)	0.670	1.455 (0.962-2.201)	0.076
gender (female vs. male)	1.095 (0.764-1.569)	0.622	0.949 (0.639-1.409)	0.796
mucinous component (yes vs. no)	0.693 (0.359-1.338)	0.275	0.678 (0.326-1.414)	0.300
primary locations (rectum vs. colon)	0.792 (0.547-1.147)	0.217	0.792 (0.530-1.181)	0.253
tumor stage (III vs. II)	2.804 (1.821-4.317)	<0.001	3.088 (1.914-4.984)	<0.001
adjuvant chemotherapy (no vs. yes)	0.788 (0.527-1.179)	0.247	0.792 (0.515-1.217)	0.287

HR, hazard ratio, CI, confidence interval.

ROC curve analysis for ratio and quantity subgroups

As shown in Figure 4, the predictive accuracy of the quantity subgroups within 48 months was higher than that of the ratio subgroups (area under the curve [AUC]: 0.731 vs. 0.687, $p=0.037$) and tumor stage (AUC: 0.731 vs. 0.651, $p=0.016$). Despite the lack of significant difference, the predictive accuracy of the ratio subgroups tended to be higher than that of tumor stage (AUC: 0.687 vs. 0.651, $p=0.278$).

Integrated analysis of ratio and quantity subgroups

As shown in Figures 5A, B, 304 (66.7%) of 449 patients had an evaluation of the same risk level according to two tripartite categorization systems. Among these 304 patients, there were 73 patients with a low risk of tumor recurrence in both the low-ratio and low-risk subgroups, 154 patients with a moderate risk of tumor recurrence in both the moderate-ratio and moderate-risk subgroups, and 77 patients with a high risk of tumor recurrence in both the high-ratio and high-risk subgroups.

These 449 patients were stratified into five risk subgroups based on an integrated analysis of ratio and quantity subgroups, including very low (low-risk & low-ratio, $n=73$), low (moderate-risk & low-ratio, $n=39$; low-risk & moderate-ratio, $n=39$), moderate (moderate-risk & moderate-ratio, $n=154$), high (high-risk & moderate-ratio, $n=32$; moderate-risk & high-ratio, $n=35$), and very high (high-risk & high-ratio, $n=35$) risk subgroups (Figures 5A, B).

As shown in Figures 5C, D, significant differences in RFS (HR=1.927, 95% CI=1.649-2.251; $p<0.001$) and OS (HR=1.930, 95% CI=1.629-2.286; $p<0.001$) were found among these five risk subgroups (very-high vs. high vs. moderate vs. low vs. very-low).

Macrophage-related gene sets based on microarray data analysis

The M₁-related gene sets

The infiltration of M₁-macrophages was enriched in nine gene sets for the high-infiltration group (Shown in Figure 6A, Supplementary Table S1). No gene set was significantly enriched in the low M₁-infiltration group. The GSEA results implied that the interferon- α response, mitotic spindle, IL6/Jak/Stat3 signaling, E2F

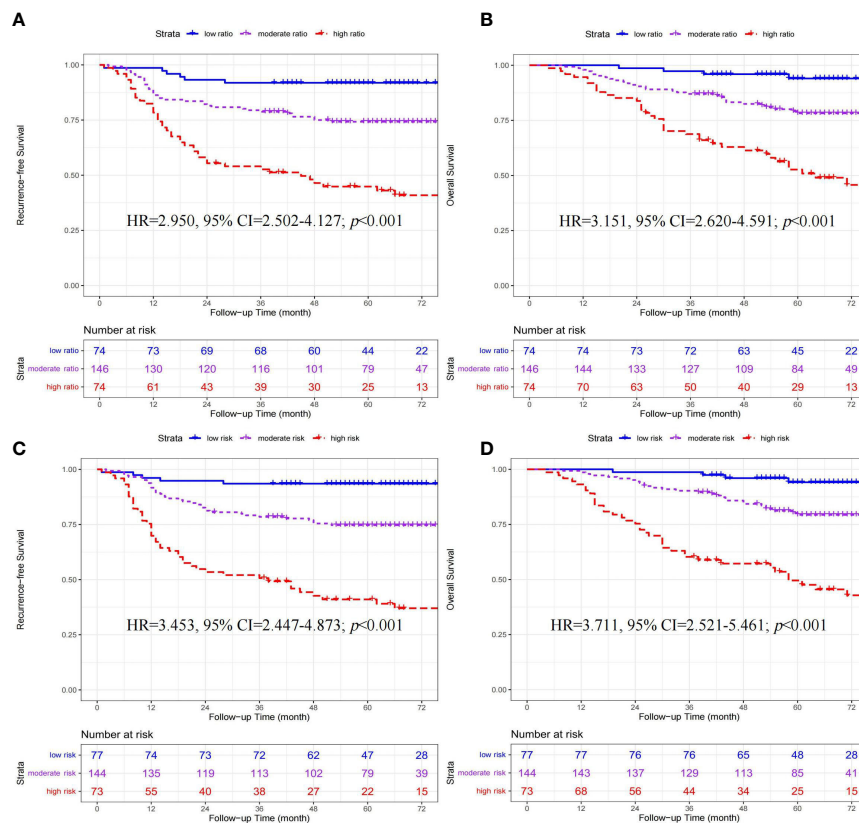


FIGURE 3 Kaplan-Meier curves of recurrence-free survival and overall survival stratified by ratio (A, B) and quantity (C, D) subgroups in 294 CRC patients receiving adjuvant chemotherapy.

targets, allograft rejection, DNA repair, myc targets V1, complement, and interferon- γ response pathways were significantly correlated with the high M₁-infiltration. The relationship between M₁-macrophages and other immune cell subtypes was shown in Figure 7.

The M₂-related gene sets

The infiltration of M₂ macrophages was involved in eight gene sets for the high-infiltration group and ten gene sets for the low-infiltration group (Shown in Figure 6B, Supplementary Table S1). We observed that epithelial mesenchymal transition, TGF- β signaling, apical junction, kras signaling up, protein secretion, hypoxia, angiogenesis, and hedgehog signaling pathways were significantly correlated with the high M₂-infiltration group. The GSEA results implied that the mitotic spindle, E2F targets, IL6/Jak/Stat3 signaling, DNA repair, G2-M checkpoint, myc targets V2, MTORC1 signaling, myc targets V1, inflammatory response, and TNF- α /NF-KB signaling pathways were significantly associated with the low M₂-infiltration group. The relationship between M₂-macrophages and other immune cell subtypes was shown in Figure 7.

Discussion

Although the TNM staging system can provide essential prognostic information for determining therapeutic regimens, it

does not incorporate any immune microenvironment information into the staging algorithm. It's noted that even CRC patients with the same stage might exhibit conflicting results (2–6). In our study, we constructed the tripartite classifications of ratio and quantity subgroups by the integrated analysis of CD86⁺ and CD206⁺ macrophages. And the ratio and quantity subgroups could effectively stratify these CRC patients with stage II-III disease into three risk groups with a low-, moderate-, and high-risk of tumor recurrence. The ratio and quantity subgroups could effectively predict treatment recurrence and mortality independent of tumor stage and other clinicopathologic factors. Compared with ratio subgroups and tumor stage, quantity subgroups may have the optimal prediction ability of tumor recurrence within 48 months. Based on the further combined analysis of ratio and quantity subgroups, stage II-III CRC could be stratified into five risk subgroups (very-high, high, moderate, low, and very-low) with significant differences in RFS and OS.

At present, tumor stage and the clinicopathologic factors remain the most important factors in the decision-making process of adjuvant chemotherapy. The 5-year survival benefit of postoperative chemotherapy is only 2%-5% for stage II CRC (20–22). And the assessment system of recurrent risk should be improved to optimize the treatment strategy in stage II CRC. In clinical practice, all CRC patients with stage III disease might be unreasonably given the long duration

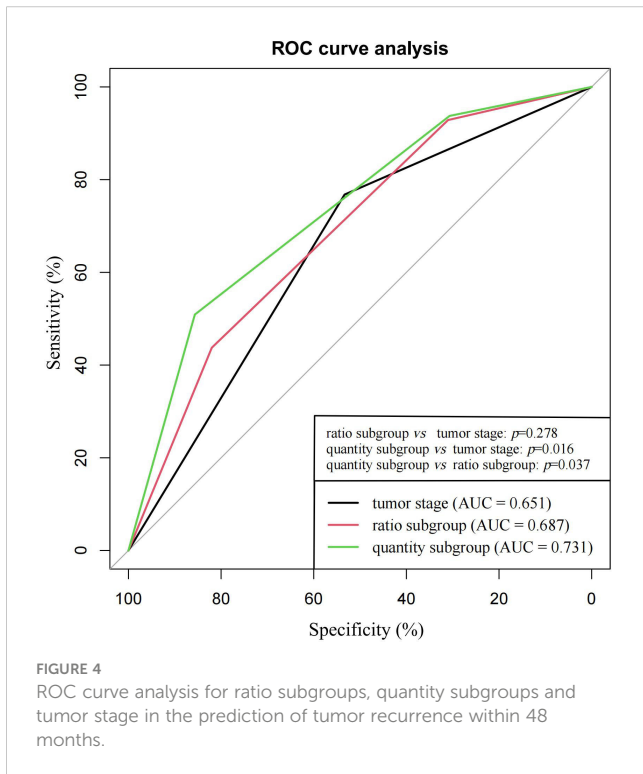
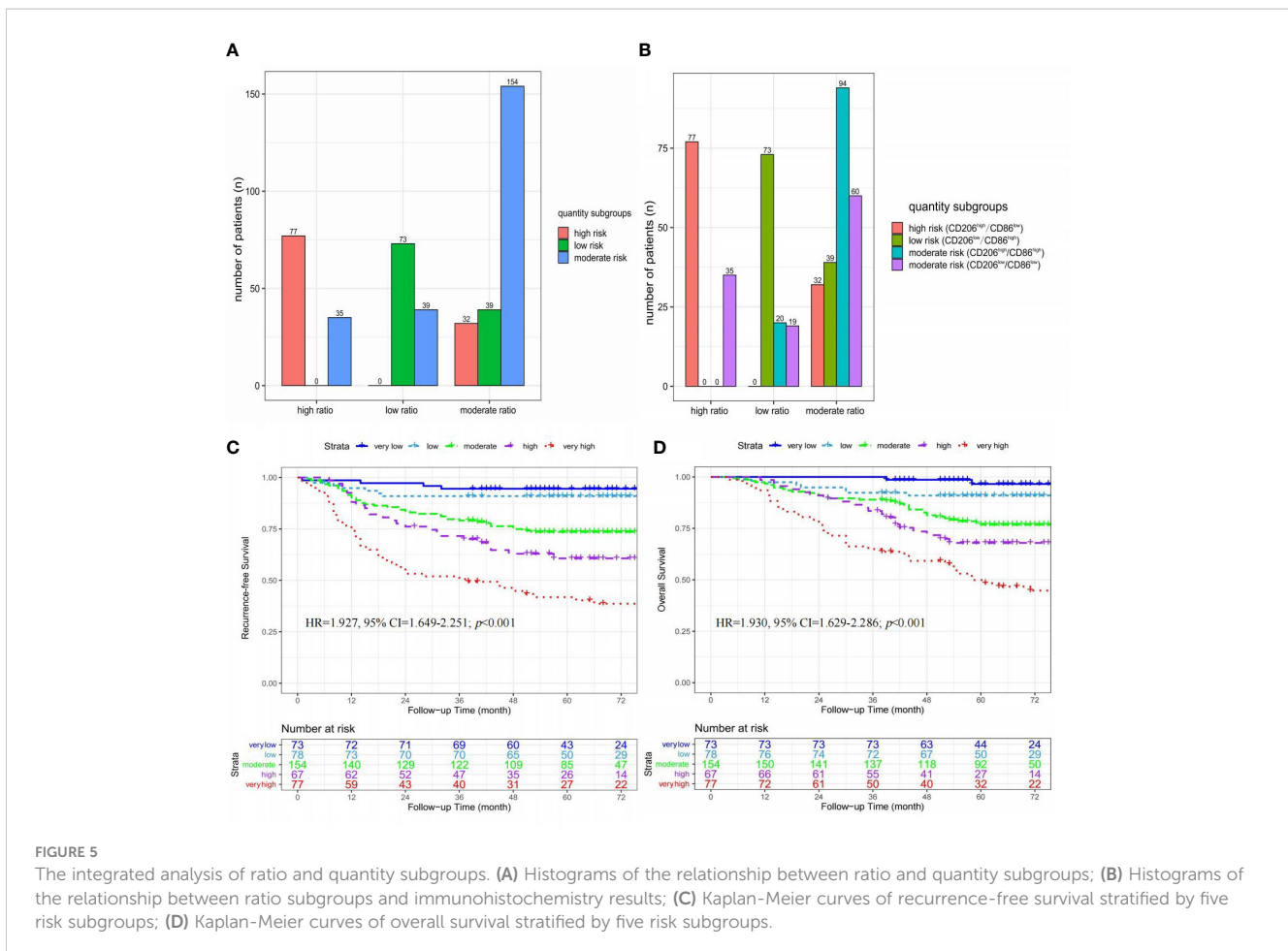


FIGURE 4
ROC curve analysis for ratio subgroups, quantity subgroups and tumor stage in the prediction of tumor recurrence within 48 months.

(six months) of postoperative chemotherapy indistinguishably (23–25). In our study, the log-rank test revealed that CRC patients in the high-ratio or high-risk subgroup exhibited the worst RFS and OS, CRC patients in the low-ratio or low-risk subgroup exhibited the optimal RFS and OS after adjuvant chemotherapy. These two tripartite classifications might enable medical oncologists to precisely stratify stage II-III CRC for avoiding overtreatment or undertreatment in some specific patients.

Tumor-associated macrophages exhibit a spectrum of polarization status, with M₁- and M₂-macrophages representing the ends of this spectrum. A meta-analysis of 29 studies demonstrated that high infiltration of CD68⁺ pan-macrophages at invasive margins was significantly associated with better survival, while high infiltration of M₂-macrophages in tumor center was significantly associated with poor prognosis in CRC patients (26). Nevertheless, the clinical significance of M₁-macrophages in CRC was still controversial (26). Actually, most macrophages belong to a mixed M₁/M₂ phenotype (27, 28). The combined analysis of M₁- and M₂-macrophages provides more comprehensive information on tumor prognosis. Yang et al. demonstrated that high ratio of CD163⁺/CD68⁺ macrophages was significantly associated with poor prognosis in patients with CRC (29). Feng et al. found that high ratio of CD206⁺/CD68⁺ macrophages was significantly associated with poor survival and could be used for a better



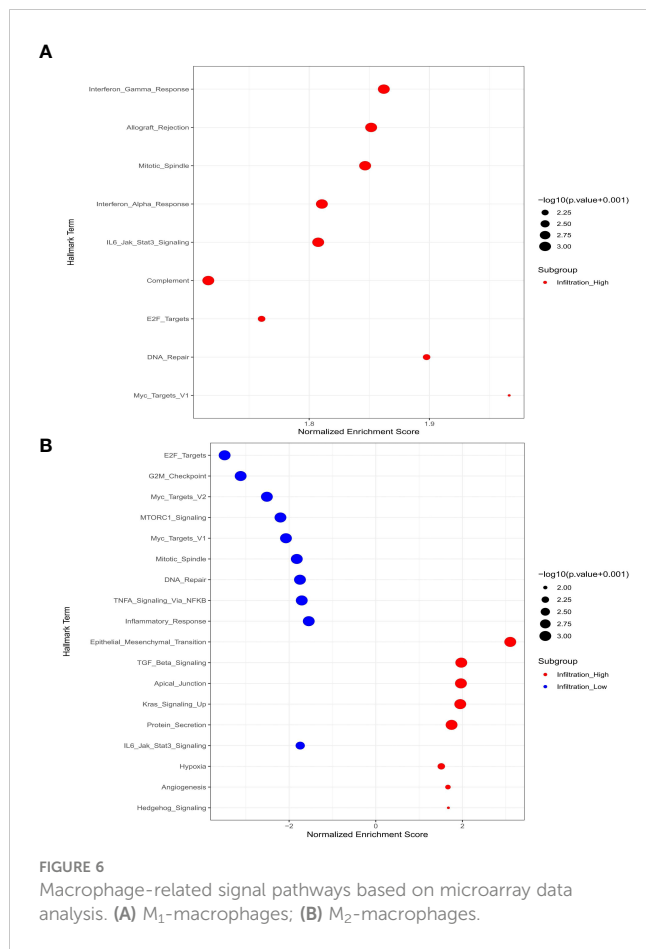


FIGURE 6
Macrophage-related signal pathways based on microarray data analysis. (A) M₁-macrophages; (B) M₂-macrophages.

predictive biomarker for adjuvant chemotherapy in stage II CRC (13). In this study, CRC patients in the moderate-risk/moderate-ratio subgroup had intermediate RFS and OS, and these patients exhibited a relatively functional counterbalance regulated by M₁- and M₂-macrophages. The low-risk/low-ratio subgroup represented a polarization profile of M₁-macrophages and were associated with a favorable prognosis. The GSEA results implied that the tumor-killing mechanism of M₁-macrophages was potentially derived from immune activation of the IFN- α (30) and IFN- γ (31) response pathways. However, the high-risk/high-ratio subgroups might demonstrate a polarization profile of M₂-macrophages and serve as a poor prognosis. The GSEA results also implied that the protumor mechanism of M₂-macrophages might be achieved by activating the epithelial mesenchymal transition (32), TGF- β signaling (33), hedgehog signaling (34), angiogenesis (35), and hypoxia (36) pathways. These findings might further confirm the opposite functions of diametrically polarized macrophages.

The ratio and quantity subgroups might complement each other in stage II-III CRC. Firstly, 35 CRC cases were classified into both the high-ratio (ratio>p75) and moderate-risk (CD86^{low}/CD206^{low}) subgroups. The quantity subgroups might be more scientific and credible for these patients with a moderate risk of postoperative recurrence. Secondly, 19 CRC cases in both the low-ratio (ratio \leq p25) and moderate-risk (CD86^{low}/CD206^{low}) subgroups might have a moderate risk of postoperative recurrence. The quantity subgroups could contribute to defining the actual risk of these 19 cases. Thirdly,

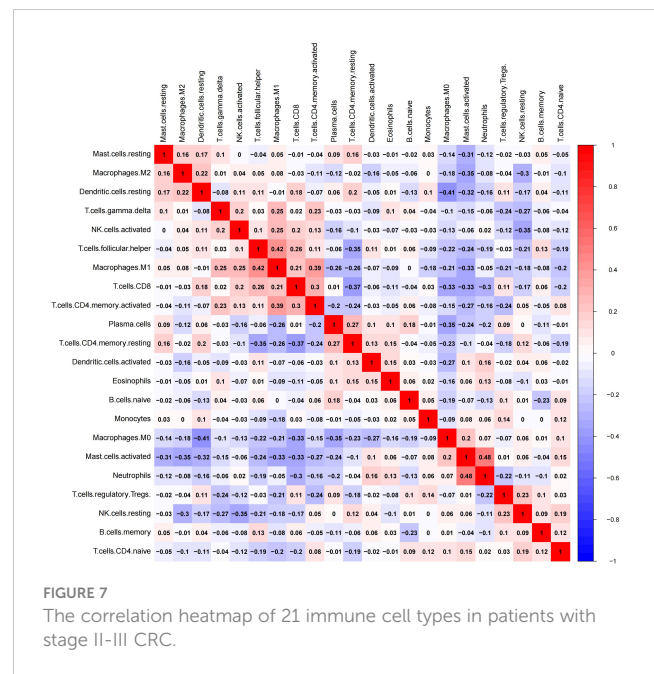


FIGURE 7
The correlation heatmap of 21 immune cell types in patients with stage II-III CRC.

20 CRC cases in both the low-ratio (ratio \leq p25) and moderate-risk (CD86^{high}/CD206^{high}) subgroups might have a low risk of postoperative recurrence. The ratio subgroups could contribute to defining the actual risk of these 20 cases. Fourthly, 32 CRC cases in the low-risk (CD86^{high}/CD206^{low}) subgroup and 39 CRC cases in the high-risk (CD86^{low}/CD206^{high}) subgroup were classified into the moderate-ratio (p25<ratio \leq p75) subgroup. Most of these CRC cases might exhibit a mixed M₁/M₂ phenotype with an intermediate risk of postoperative recurrence.

In addition to their prognostic roles, growing evidences have revealed that macrophages represent a new anticancer target (37, 38). As a selective inhibitor of CSF1R kinase, GW2580 reduces M₂-macrophage infiltration and normalizes the disorganized peritoneal vasculature in GW2580-treated ascites of ovarian cancer (37). GW2580 also enhances the anticancer and antiangiogenic effects of an anti-VEGFR-2 antibody in mouse tumor models (38). As a synthetic vitamin A derivative, fenretinide suppresses M₂-macrophages by inhibiting STAT6 phosphorylation and further preventing the tumorigenesis of colon carcinoma (39). IFN- γ recovers the M₁-phenotype through the increased expression of CD86, enhancement of the infiltration of cytotoxic T cells, and the transformation of an immunosuppressive phenotype into an immunostimulatory phenotype in IFN- γ -treated ascites of ovarian cancer (40). These findings imply that macrophage-targeted therapy may represent a promising strategy.

This study has several potential limitations. Firstly, our study design was retrospective. The prognostic significance of chemotherapy regimens and cycle was not included in the analysis. Secondly, as an unresolved issue (41), the semi-quantitative method for immunohistochemical staining might not completely reflect the actual intensity of macrophage infiltration. Thirdly, this is also an unresolved issue for the immune cells with a very accurate marker of immunohistochemical staining. Although most of M₁-macrophages expressed this biomarker of CD86, it

doesn't mean that all CD86⁺ cells are M1-macrophages (42, 43). And a small proportion of CD86⁺ cells might belong to M2b macrophages and other immune cells (42–44). So far, there is no study of colorectal cancer to explore the expression difference of CD206 in different macrophage subtypes (M2a, M2b and M2c). According to the previous study results of macrophage polarization (13, 45, 46), we adopted CD86 and CD206 to work as the biomarkers for M1- and M2-macrophages, respectively.

In conclusion, both ratio and quantity subgroups effectively stratify CRC patients with stage II–III disease into three subgroups with a low, moderate, and high risk of treatment relapse and mortality. Compared with ratio subgroups and tumor stage, quantity subgroups could more effectively predict treatment recurrence with 48 months. In addition, immunohistochemical staining of CD86⁺ and CD206⁺ macrophages is easy, inexpensive and rapid and can be performed in most hospitals. Upon further assessment in multiple-center prospective studies, these prognostic biomarkers of ratio and quantity subgroups will contribute to the implementation of precision treatment strategies for stage II–III CRC.

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

This study was authorized by the Ethics Committee of Yantai Yuhuangding Hospital (Shandong, China) and Guangzhou Red Cross Hospital (Guangdong, China). The patients/participants provided their written informed consent to participate in this study.

Author contributions

FZ, JC, and GX conceived and designed this study. GX, JL, and YM performed sample detection and the experiments. GX and YM collected the data and performed the statistical analysis. GX and QW carried out this manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1136875/full#supplementary-material>

SUPPLEMENTARY TABLE 1

The GSEA results of macrophage-related pathways based on microarray data analysis. NES, Normalized Enrichment Score, FDR, False Discovery Rate.

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