



# Comprehensive Assessment of Serum hsa\_circ\_0070354 as a Novel Diagnostic and Predictive Biomarker in Non-small Cell Lung Cancer

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**Background:** More and more studies have shown that circular RNAs (circRNAs) play an essential role in the occurrence and development of tumors. Hence, they can be used as biomarkers to assist in diagnosing tumors. This study focuses on exploring the role of circular RNA (hsa\_circ\_0070354) in the diagnosis and prognosis of non-small cell lung cancer (NSCLC).

**Materials and Methods:** First of all, high-throughput sequencing was used to find the difference in the expression of circular RNA between NSCLC and adjacent tissues. The circRNAs with higher differences in expression were selected to verify their expressions in tissues, cells, and serum using qRT-PCR. Secondly, the hsa\_circ\_0070354 with a significant difference was chosen as the research goal, and the molecular properties were verified by agarose gel electrophoresis and Sanger sequencing, etc. Then, actinomycin D and repeated freeze-thaw were used to explore the stability and repeatability of hsa\_circ\_0070354. Finally, the expression of hsa\_circ\_0070354 in serum of 133 patients with NSCLC and 97 normal donors was detected, and its sensitivity, specificity, and prognosis as tumor markers were statistically analyzed.

**Results:** Hsa\_circ\_0070354 was highly expressed in tissues, cells, and serum of NSCLC, and it has the characteristics of sensitivity, stability, and repeatability. The ROC curve indicates that hsa\_circ\_0070354 is superior to conventional tumor markers in detecting NSCLC, and the combined diagnosis is of more significance in the diagnosis. The high expression of hsa\_circ\_0070354 is closely related to the late-stage, poor differentiation of the tumor and the short survival time of the patients, which is an independent indicator of poor prognosis.

**Abbreviations:** ACCU, overall accuracy; AUC, area under the curve; cDNA, complementary DNA; ceRNA, competitive endogenous RNA; circRNAs, circular RNAs; COPD, chronic obstructive pulmonary disease; GO, Gene Ontology; gDNA, genomic DNA; KEGG, Kyoto Encyclopedia of Genes and Genomes; NPV, negative predictive value; NSCLC, non-small cell lung cancer; OS, overall survival; PD-1, programmed cell death protein-1; PPV, positive predictive value; ROC, receiver operating characteristic; SEN, sensitivity; SPE, specificity.

**Conclusion:** Hsa\_circ\_0070354 is not only a novel sensitive index for the diagnosis of NSCLC but also a crucial marker for bad biological behavior.

**Keywords:** circular RNA, non-small cell lung cancer, diagnosis, prognosis, biomarker

## INTRODUCTION

Previously, lung cancer has been the leading malignant tumor with the highest morbidity and mortality globally. Although the global cancer data in 2020 showed that the incidence rate of lung cancer has changed from the first place, the mortality rate was still the highest (Ma et al., 2021). In Asia, the incidence and mortality of lung cancer occupy the first place in the global data, and China is at the top of the list compared to other countries. Even though risk factors such as smoking are under control, the incidence rises in both sexes (Zukotynski et al., 2021). In all lung cancer cases, non-small cell lung cancer (NSCLC) accounts for more than 80%, with a 5-year survival rate of only about 15% (Ferlay et al., 2010; Hang et al., 2018) and a recurrence rate after radical treatment of more than 40% (Zheng et al., 2013). In the past 2 decades, an endless stream of treatments has been widely applied to NSCLC to reduce mortality and improve quality of life. Compared with conventional radiotherapy and chemotherapy, molecular targeting and immunotherapy have been preferred for patients with advanced, recurrent, and metastatic NSCLC (Ma et al., 2021). However, radical surgery for early NSCLC is still the key to curing the disease (Chaft et al., 2021). The reason for the high mortality rate of NSCLC is that patients were diagnosed at an advanced stage or local late-stage, missing the possibility of early cure. The leading causes include lack of routine physical examination, lack of biomarkers for early diagnosis, and disease surveillance methods (Qian et al., 2021). Therefore, with the progress of imaging, more sensitive and specific tumor biomarkers are needed to screen and diagnose NSCLC.

Circular RNAs (CircRNAs) are a class of non-coding RNAs derived from single or multiple exons (ENCODE Project Consortium, 2012; Zhao et al., 2019), a unique subtype of non-coding RNAs, with a covalently, closed continuous loop structure and no polarized or polyadenylated tail (Memczak et al., 2013; Rybak-Wolf et al., 2015). Unlike the traditional linear RNAs, circRNA has a high tolerance to exonuclease and is widely and stably expressed in the cytoplasm of various eukaryotic cells (Glažar et al., 2014; Zhao et al., 2019; Pei et al., 2020).

CircRNAs have always been regarded as by-products of low abundance splicing or splicing errors (Nigro et al., 1991; Cocquerelle et al., 1993). With the rapid development of high-throughput sequencing and bioinformatics technology, plenty of circRNAs have sprung up and been studied (Jeck et al., 2013; Memczak et al., 2013; Jeck and Sharpless, 2014). CircRNAs are generally considered the primary regulators of cellular processes because of their unique properties, such as richness, stability, and specific expression in cells and tissues (Di et al., 2019; Yu et al., 2020). CircRNAs can regulate gene expression at the transcriptional and post-transcriptional level by interacting with microRNAs (miRNAs) or other competing endogenous RNAs and

participate in various biological activities (Hansen et al., 2013; Peng et al., 2015; Wang et al., 2018). Growing evidence showed that abnormal expression of circRNA may lead to the occurrence and development of cancers, including NSCLC (Wan et al., 2016; Zhu et al., 2017). Besides, numerous researches have identified that circRNAs are related to the clinical characteristics of patients and play a regulatory role in NSCLC (Zhang et al., 2019).

Tumor biomarkers are essential methods for tumor screening and early diagnosis due to their multiple sampling, dynamic monitoring, economic efficiency, as well as the advantages of high sensitivity and specificity. At present, the most commonly used serum tumor biomarkers for the diagnosis of NSCLC are embryonic antigen (CEA), squamous cell carcinoma antigen (SCC), and cytokeratin 19 fragment (Cyfra21-1) (Ye et al., 2020). However, the sensitivity and specificity of the above three markers for diagnosing NSCLC are not satisfactory, especially in the early stage of the disease (Kamel et al., 2019). CircRNAs play a vital role in the occurrence and development of NSCLC and can stably exist in patients' body fluids. Combined with their high sensitivity and specificity, they are ideal choices for liquid biopsy. Therefore, circRNAs can be considered as new specific diagnostic biomarkers of NSCLC (Huang et al., 2021a). Existing studies have successively verified the feasibility of circRNAs as a biomarker for the early diagnosis of NSCLC. For example, the differential expression of circ\_0047921, circ\_0056285, and circ\_0007761 in exosomes can distinguish early NSCLC from healthy people, chronic obstructive pulmonary disease (COPD), or pulmonary tuberculosis (Xian et al., 2020). The high expression of hsa\_circ\_0102533 in peripheral blood indicated the occurrence of NSCLC (Zhou et al., 2018). Nevertheless, hsa\_circ\_0046264 was highly expressed in serum of patients with NSCLC, and it was closely related to clinical factors such as the age of onset, tumor size, disease stage, and lymph node metastasis (Liu et al., 2020). Moreover, circRNAs can also be regarded as predictors of adverse biological behaviors of lung cancer. For instance, a statistical difference between the high expression of hsa\_circ\_0060937 in serum and the occurrence of bone metastasis occurred in NSCLC (Zhang et al., 2020). The increased expression of circFARSA in plasma of patients was closely related to the migration and invasion of tumor cells as well (Hang et al., 2018). What's more, circRNAs could be used as biological indicators to guide treatment. Specifically, the high expression of circPVT1 often indicates that patients are not sensitive to chemotherapy (Lu et al., 2021). Likewise, the expressions of hsa\_circ\_0134501 and hsa\_circ\_0109320 were low in NSCLCs' plasma, and the combination of them can be used as a predictor of response to gefitinib (Liu et al., 2019a). Similarly, in the immunotherapy of NSCLC, circ-CPA4 participated in the regulation of CD8<sup>+</sup>T cell response (Hong et al., 2020), while the overexpression of circFGFR1 led to drug

resistance to programmed cell death protein-1 (PD-1) (Zhang et al., 2019).

In this study, through high-throughput sequencing, it was found that a dramatic difference in the expression of hsa\_circ\_0070354 between NSCLC and adjacent tissues. Our team demonstrated the expression of hsa\_circ\_0070354 in serum of patients with NSCLC for the first time, analyzed its correlation with clinical features, evaluated its diagnostic efficacy, and explored its possibility as a novel biomarker for diagnosing NSCLC.

## MATERIALS AND METHODS

### Serum and Tissue Samples

The serum samples of 133 patients with NSCLC (I-IV stage) and 97 healthy donors were collected from the affiliated Hospital of Nantong University from 2015 to 2016 for follow-up experimental analysis. The sera collected by us were those after the initial diagnosis without any treatment. The preoperative and postoperative sera were the samples within 1 week before and after the operation. A flowchart showing the inclusion/exclusion criteria and analysis pipeline of this investigation were provided in **Supplementary Figure S1**. The tissue samples of NSCLC came from the thoracic surgery of the Affiliated Hospital of Nantong University and the Affiliated Tumor Hospital of Nantong University. Special thanks to Yupeng Liu, Guan Jun Ju, Chun Cheng, and others of thoracic surgery for their strict control over the quality inspection of tissue samples, collecting tumor tissues, and paracancerous tissues (>5 cm tumor margin) with a diameter greater than 0.5 cm. More than two pathologists diagnosed all the samples, and TNM staging was performed according to the 8th edition of WHO. Every patient signed the informed consent form for collecting and preserving specimens and documenting details. All the samples were immediately kept in enzyme-free aseptic cryopreservation tubes at  $-80^{\circ}\text{C}$  for a long time.

### High Throughput Sequencing

Relying on Hipure Total RNA Minikit (Magen, Guangzhou, China), we extracted total RNA from 3 pairs of cancer and paracancerous tissues. Then, the concentration and purity of the total RNAs were evaluated by Qubit 3.0 Fluorometer (Invitrogen, California, CA, United States) and Agilent 2100 Bioanalyzer (Applied Biosystems, California, CA, United States). Ribosomal RNAs were fractionated from total RNA samples, meanwhile linear RNAs were removed (Genesee, Guangzhou, China), and RNA-seq libraries were orderly constructed by TruSeq RNA sample preparation kits (Illumina, California, CA, United States). The purified cDNA library was sequenced on the Illumina HiSeq Xten platform via the PE150 sequencing model.

### Cell Culture

Four human NSCLC cell lines, A549, H1299, HUT226, SPCA, and normal bronchial epithelial cell line HBE1 were purchased from Shanghai Institutes for Biological Sciences, China Academy of Science (Shanghai, China) and conserved in our laboratory. All

**TABLE 1** | Primer sequences for Real-Time qPCR.

Gene	Primer sequences
has_circ_0070354	Forward: 5'-TCTGGGAAATCTTTCTGGGTAA-3' Reverse: 5'-CTACCAGATGGCAGCAACAG-3'
PTPN13	Forward: 5'-TTGCTGCCATCTGGTAGTGTG-3' Reverse: 5'-TGGTGCAGTGAATGCTCGAAG-3'
GAPDH	Forward: 5'-AGAAGGCTGGGGCTCATTG-3' Reverse: 5'-GCAGGAGGCATTGCTGATGAT-3'
U6	Forward: 5'-GACTATCATATGCTTACCGT-3' Reverse: 5'-GGGCAGGAAGAGGGCCTAT-3'
18S	Forward: 5'-AGAAGGCTGGGGCTCATTG-3' Reverse: 5'-GCAGGAGGCATTGCTGATGAT-3'

cells were cultured in RPMI 1640 medium (Corning, Virginia, VA, United States) containing 10% fetal bovine serum (FBS, Gibco, South Dakota, SD, United States), 100 U/ml penicillin-streptomycin mixture (Gibco, South Dakota, SD, United States) in a humidified incubator (Thermo, Massachusetts, MA, United States) with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

### Total RNA Extraction and qRT-PCR

Serum, tissue, and cell total RNAs were extracted by TRIzol reagent (Invitrogen, Karlsruhe, Germany). According to the manufacturer's instruction, complementary DNAs (cDNAs) were reverse transcribed by Revert Aid RT Reverse Transcription Kit (Thermo Fisher Scientific, Massachusetts, MA, United States). The specific divergent primers involved in this paper were designed and synthesized by Ribobio Corporation (Suzhou, China), with the forward and reverse primer as shown in **Table 1**. RT-qPCR was performed as described in previous studies (Huang et al., 2021b). In brief, the reaction was initiated at  $95^{\circ}\text{C}$  (10 min), followed by 40 cycles at  $95^{\circ}\text{C}$  (10s) and  $62^{\circ}\text{C}$  (34s) in the 20  $\mu\text{l}$  system. PCR products were identified by Sanger sequencing.

### RNase R and Agarose Gel Electrophoresis

RNase R treatment was executed at  $37^{\circ}\text{C}$  with 4 U/ $\mu\text{g}$  of RNase R (Epicentre Biotechnologies, Wisconsin, WI, United States) for 3 min and then inactivated at  $70^{\circ}\text{C}$  for 10 min to deactivate the enzyme, after which the mixture was reversely transcribed into cDNA for qRT-PCR. 2.5% agarose gel was prepared, and then the electrophoresis bands of PCR products were observed under UV conditions after about 20 min electrophoretic at 100 V.

### Actinomycin D

The actinomycin D of 1,000 mg/ml was diluted to 2.5  $\mu\text{g}/\text{ml}$  using RPMI-1640 complete medium and replaced the ordinary medium for 24 h. After treatment, total RNA was extracted from the cells for subsequent determination at 0, 2, 4, 8, 12, 24 h, respectively.

### Nuclear and Cytoplasmic RNA Separation Assay

Up to  $5 \times 10^6$  cells were digested by trypsin and collected in a small centrifuge tube for backup use. According to the

manufacturer's protocol, RNA fractions from the nucleus and cytoplasm of cells were separated by the Ambion®PARIS™Kit (Thermo Fisher Scientific, Massachusetts, MA, United States). Nuclear and cytoplasmic RNAs were then converted to cDNAs and detected by qRT-PCR or stored in the refrigerator at  $-80^{\circ}\text{C}$ .

## Statistical Analysis

Statistical analyses in this study were mainly conducted by SPSS 20.0 (IBM SPSS Statistics, Chicago, USA) and GraphPad Prism v8.0 (Graphpad Software Inc., California, USA). The differences between groups were analyzed using Student's *t*-test and one-way ANOVA. The correlation of hsa\_circ\_0070354 to clinical characteristics was analyzed by chi-square test. The receiver operating characteristic (ROC) curve and area under the curve (AUC) were determined to evaluate the diagnostic performance of hsa\_circ\_0070354 in NSCLC. Before plotting the ROC curve, we performed binomial logistic regression. Univariate and multivariate Cox proportional hazards regression models were carried out to assess the clinical variables' impacts on the overall survival (OS) of NSCLC patients. Survival curves were estimated by the Kaplan-Meier method, while the log-rank test evaluated statistical significance. Data are denoted by mean value  $\pm$  standard deviation (SD) from at least three biological replicates independently. *p* values  $< 0.05$  were indicated statistical significant.

## RESULTS

### Screening and Detecting the Expression of hsa\_circ\_0070354 in NSCLC Tissues, Cells, and Sera

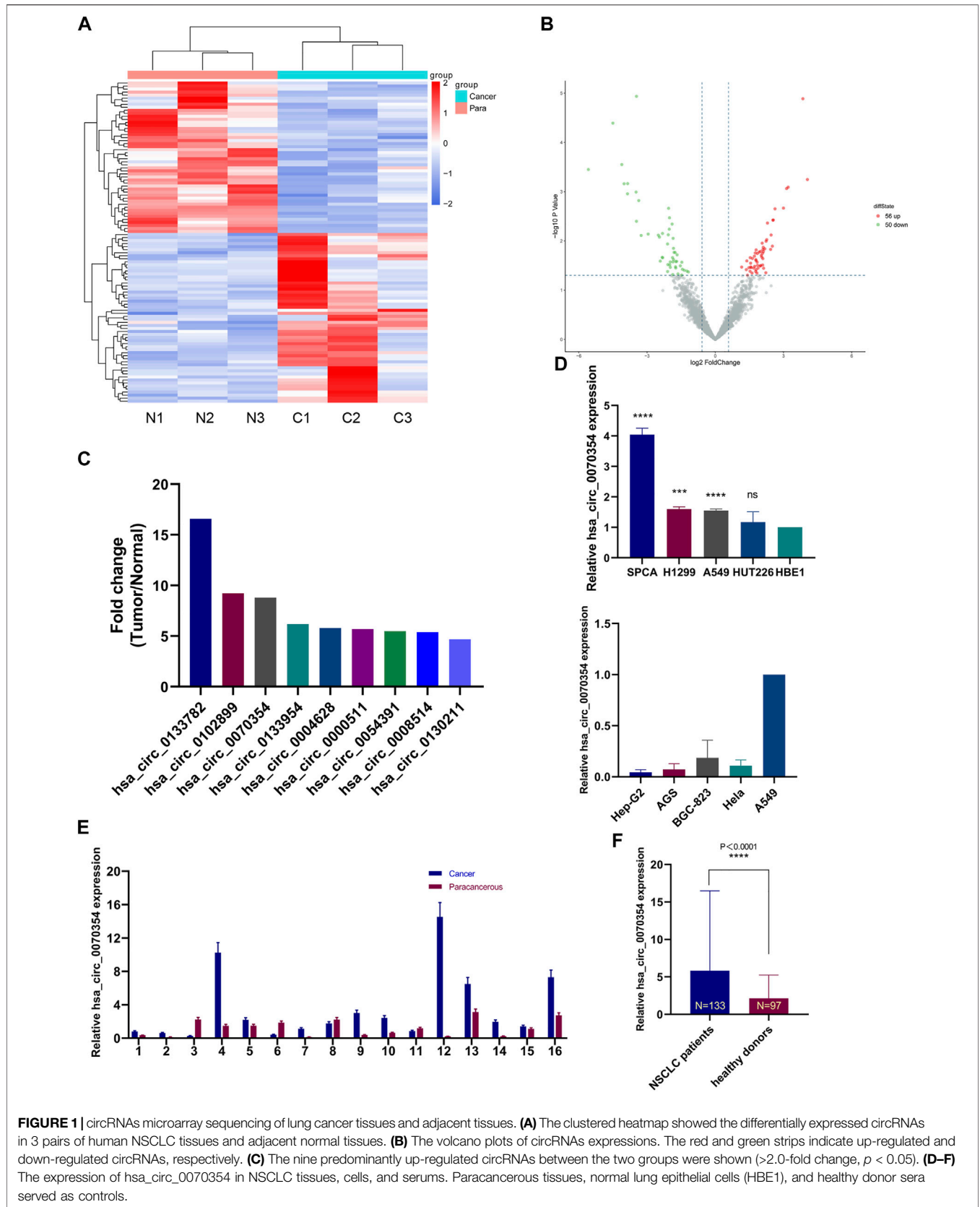
High-throughput sequencing was implemented in 3 pairs of cancerous and adjacent noncancerous tissues from NSCLC patients to determine and characterize the differential expression of circRNAs in NSCLC. A total of 2319 circRNAs were identified, of which 56 were significantly up-regulated and 50 were down-regulated when we set the filter criteria as fold-change  $\geq 2$  and *p*  $< 0.05$  (Figures 1A,B). Figure 1C showed the top 9 up-regulated circRNAs with remarkable differences, among which our study object hsa\_circ\_0070354 is included. Then the expression of hsa\_circ\_0070354 in 16 pairs of NSCLC tissue samples was verified by qRT-PCR (Figure 1E, small sample verification). Notably, 11 cases of adenocarcinoma and 5 cases of squamous cell carcinoma were concluded among the collected cases of NSCLC (Supplementary Table S1). Compared with the corresponding adjacent tissue adjacent tissues, hsa\_circ\_0070354 showed high expression in tumors. This result also corresponded to the presentation of cell lines. Similarly, compared with normal bronchial epithelial cells, the expression of hsa\_circ\_0070354 was also increased meaningfully in NSCLC cell lines (Figure 1D above), which indicated that hsa\_circ\_0070354 might be related to the presence of lung cancer. At the same time, we also detected the expression levels of hsa\_circ\_0070354 in cell lines of gastric cancer (AGS and BGC-823), hepatocellular carcinoma (Hep-G2), cervical cancer (Hela), and NSCLC

(A549), respectively (Figure 4D below). Compared with A549, their CT value was significantly higher (Figure 1D below). Under the verification of large numbers of serum samples, the expression of hsa\_circ\_0070354 was detected in the serum of 133 patients with NSCLC and 97 normal donors and verified the conclusion of its high expression (Figure 1F).

### Molecular Properties and Methodological Evaluation of hsa\_circ\_0070354 in NSCLC

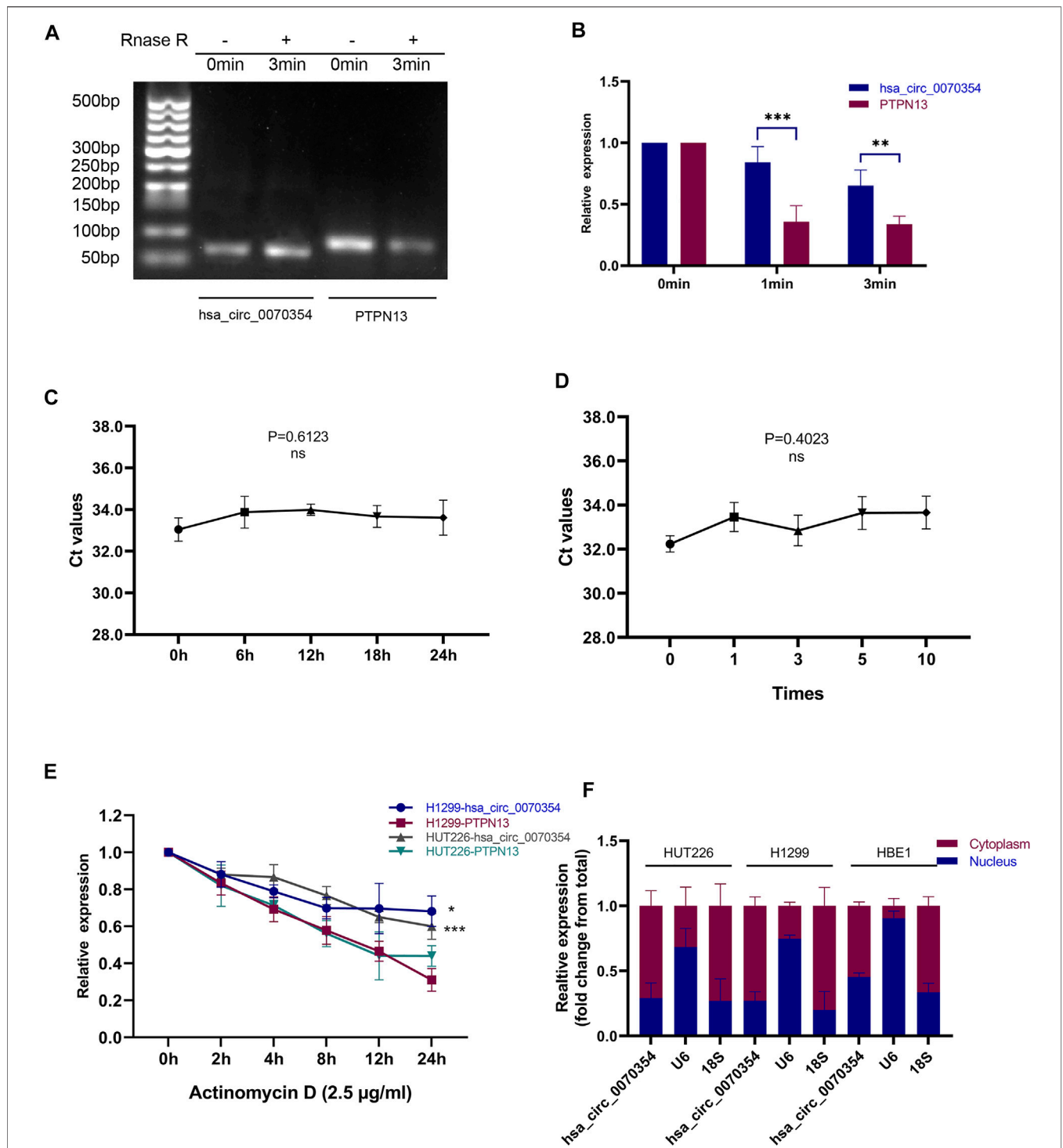
The high expression of hsa\_circ\_0070354 has a clear relationship with cancer, providing the possibility of hsa\_circ\_0070354 as a tumor biomarker. Due to the lack of previous studies, to further clarify the properties of hsa\_circ\_0070354, we have completed the following explorations. Initially, the Ensembl genome database (GRCh37/hg19) was applied to verify hsa\_circ\_0070354 located on chr4\_87593517\_87610343\_+, with a predicted length of 431 bp (Figure 2A). In order to ensure that hsa\_circ\_0070354 was not *trans*-splicing or genomic rearrangement, we designed divergent and convergent primers to amplify the circRNA 0070354 and its source gene PTPN13 linear mRNA, respectively. The results of PCR amplification pointed out hsa\_circ\_0070354 was amplified from cDNA, excluding the existence of genomic DNA (gDNA), while convergent primers amplified PTPN13 from both cDNA and gDNA (Figure 2B). Consistently, agarose gel electrophoresis presented a single electrophoretic band, which was about the same size as the primer amplification product about 70 bp (Figure 2B). In addition, the presence of hsa\_circ\_0070354 was proved by RT-PCR (Figure 2C), and the back splicing junction of hsa\_circ\_0070354 in PCR products was confirmed by Sanger sequencing (Figure 2D).

Since circRNA is more stable than linear RNA and is not easily degraded by exonuclease, the expression of hsa\_circ\_0070354 and linear PTPN13 were detected by qRT-PCR after being treated with RNase R. The results presented that compared with linear PTPN13, the degradation of hsa\_circ\_0070354 was not significant (Figures 3A,B). The serum samples from 20 healthy controls were randomly mixed with equal volume, placed at room temperature for 24 h (Figure 3C), frozen, and thawed 0, 1, 3, 5, 10 times repeatedly (Figure 3D). Interestingly, the results showed no significant difference in cycle threshold (CT) value of hsa\_circ\_0070354 after these operations. As we are known, actinomycin D could inhibit the synthesis of new RNAs. To further evaluate the stability, the expression of hsa\_circ\_0070354 and linear PTPN13 was detected at the indicated time points after treatment with actinomycin D. The results signified that hsa\_circ\_0070354 had a longer half-life after treatment with actinomycin D (Figure 3E). All these results illustrated that hsa\_circ\_0070354 is a circRNA and the stability met the requirement of tumor biomarker. It is known that the circular RNA in the nucleus mainly regulates the transcription of parental genes, while the circular RNA in the cytoplasm primarily acts as a competitive endogenous RNA (ceRNA) (Rong et al., 2017; Xu et al., 2020). Through the nuclear-cytoplasmic separation experiment, we found that hsa\_circ\_0070354 is mainly located in the cytoplasm









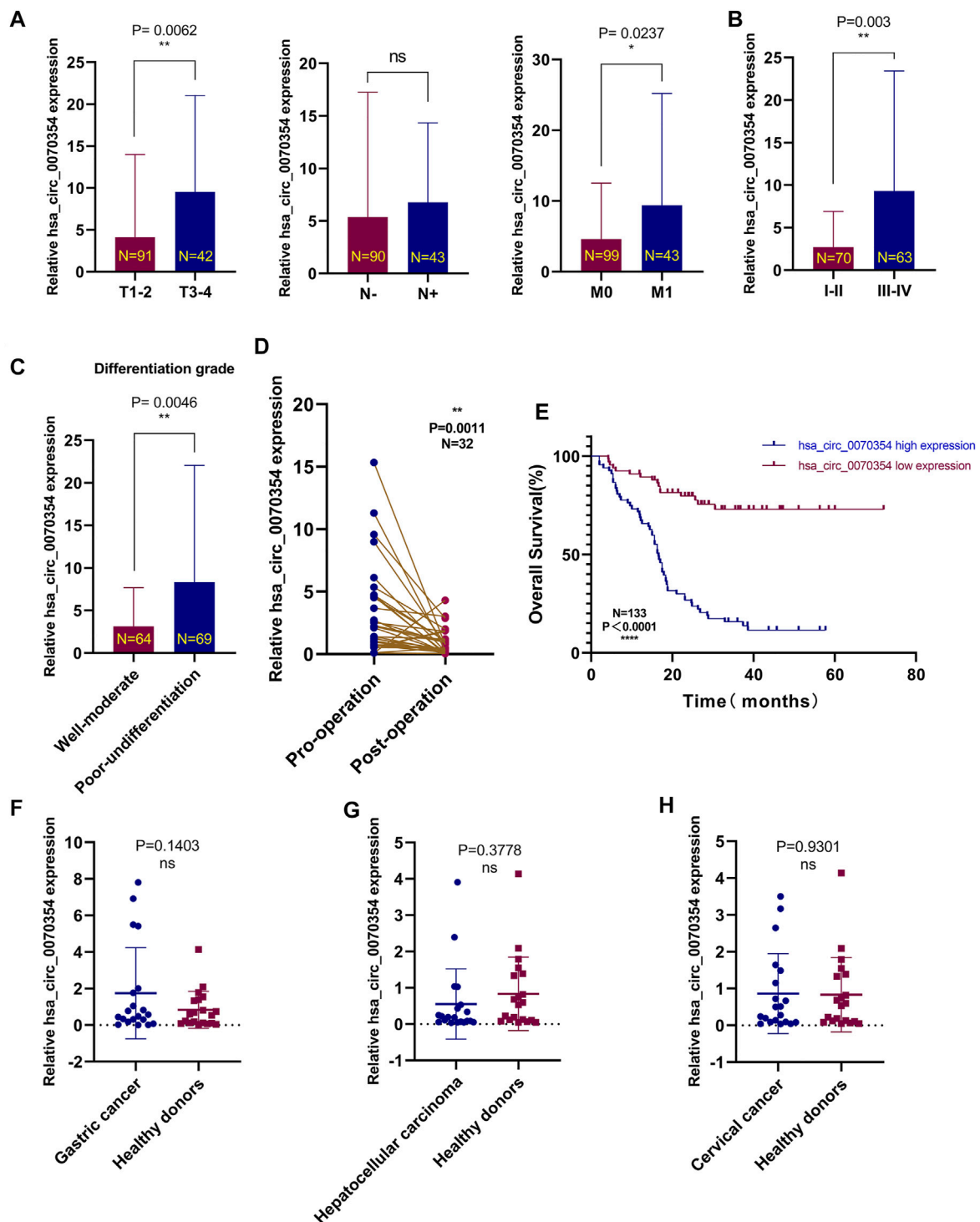
**FIGURE 3 |** Characteristics of hsa\_circ\_0070354 as a serum tumor biomarker. **(A,B)** qRT-PCR analyses were conducted to determine the abundances of hsa\_circ\_0070354 and linear PTPN13 mRNA in A549 cells after treatment with RNase R at the indicated time points. **(C,D)** The expressions of hsa\_circ\_0070354 in mix serum which placed at room temperature at the indicated time points **(C)** or frozen and thawed 0, 1, 3, 5, 10 times **(D)**. **(E)** The RNA expressions of hsa\_circ\_0070354 and linear PTPN13 mRNA in A549 cells were analyzed by qRT-PCR after treatment with 2.5 µg/ml actinomycin D for 2, 4, 8, 12, 24 h. **(F)** Nuclear-cytoplasmic RNA fractionation assay showed that hsa\_circ\_0070354 was mainly localized in the cytoplasm of A549 cells. 18S was used as a cytoplasmic protein control while U6 was considered as a nuclear control. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

**TABLE 2 |** The association between hsa\_circ\_0070354 expression and clinicopathologic parameters in 133 NSCLC specimens.

Parameters	Total	hsa_circ_0070354 expression		p Value
		Low (<median)	High (≥median)	
Age				0.850
< 55	39	20	19	
≥ 55	94	46	48	
Gender				1.000
Male	63	31	32	
Female	70	35	35	
Smoking status				0.581
Non-smoker	89	46	43	
Smoker	44	20	24	
Pathological Type				0.179
Squamous	38	15	23	
Adenocarcinoma	95	51	44	
Differentiation grade				0.002**
Well-moderate	64	41	23	
Poor-undifferentiation	69	25	44	
Tumor size				0.000***
<5 cm	91	57	34	
≥5 cm	42	9	33	
Lymph node metastasis				0.001**
Negative	90	54	36	
Positive	43	12	31	
Distant metastasis				0.028*
Negative	99	55	44	
Positive	34	11	23	
TNM stage				0.000***
I-II	70	54	16	
III-IV	63	12	51	
Ki-67 expression				0.005**
< 30%	78	47	31	
≥ 30%	55	19	36	
Pleural invasion				0.186
Negative	93	50	43	
Positive	40	16	24	
Nerve invasion				0.244
Negative	131	64	67	
Positive	2	2	0	
Vascular invasion				0.682
Negative	102	52	50	
Positive	31	14	17	
Spread through Air Spaces				0.858
Negative	83	42	41	
Positive	50	24	26	
CEA				0.038*
Negative(<5 ng/ml)	66	39	27	
Positive(≥5 ng/ml)	67	27	40	
SCC				0.469
Negative(<1.5 μg/L)	86	45	41	
Positive(≥1.5 μg/L)	47	21	26	
Cyfra21-1				0.007**
Negative(<2.08 ng/ml)	94	54	40	
Positive(≥2.08 ng/ml)	39	12	27	
CA199				0.115
Negative(<37 U/ml)	126	65	61	
Positive(≥37 U/ml)	7	1	6	
Serum ferritin				0.483
Negative(<204 μg/L)	78	41	37	
Positive(≥204 μg/L)	55	25	30	

Statistical analyses were performed by the Pearson  $\chi^2$  test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 was considered significant.





**FIGURE 4 |** High hsa\_circ\_0070354 expression in the NSCLC serums and prognostic significance. **(A)** The expression levels of hsa\_circ\_0070354 in serum specimens of NSCLC at different stages of tumor size, lymph node metastasis, and distant metastasis. **(B,C)** The expression levels of hsa\_circ\_0070354 in serum specimens of NSCLC at different stages of TNM stage **(B)** and differentiation degree **(C)**. **(D)** The expression of hsa\_circ\_0070354 in patients with NSCLC before and after operation. **(E)** Prognostic analysis of hsa\_circ\_0070354 expression in 133 NSCLC patients. **(F-H)** The expression level of hsa\_circ\_0070354 in 20 cases of patients with gastric cancer, hepatocellular carcinoma, cervical cancer, and healthy donors, respectively. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , NS  $p > 0.05$ .

**TABLE 3 |** Univariate and multivariate Cox regression analysis of hsa\_circ\_0070354 and clinical variables predicting survival from 133 NSCLC specimens.

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	p Value	HR	95% CI	p Value
Age (<55 vs ≥55)	1.252	0.742–2.113	0.401	—	—	—
Gender (Male vs. Female)	0.800	0.505–1.268	0.343	—	—	—
Smoking status (Non-smoker vs. Smoker)	0.524	0.304–0.903	0.020*	1.571	0.720–3.426	0.256
Pathological Type (Squamous vs. Adenocarcinoma)	1.423	0.835–2.423	0.194	—	—	—
Differentiation grade (Well-moderate vs. Poor-undifferentiation)	2.445	1.498–3.990	<0.001***	1.054	0.477–2.328	0.896
Tumor size (<5 cm vs. ≥5 cm)	2.526	1.592–4.007	<0.001***	1.336	0.717–2.487	0.361
Lymph node metastasis (Negative vs. Positive)	2.935	1.846–4.666	<0.001***	0.664	0.339–1.303	0.234
Distant metastasis (Negative vs. Positive)	2.611	1.622–4.201	<0.001***	1.575	0.578–4.287	0.347
TNM stage (I–II vs. III–IV)	5.666	3.309–9.700	<0.001***	0.317	0.112–0.892	0.029*
Ki-67 expression (<30% vs. ≥30%)	2.459	1.541–3.924	<0.001***	0.977	0.453–2.108	0.953
Pleural invasion (Negative vs. Positive)	1.335	0.827–2.156	0.237	—	—	—
Nerve invasion (Negative vs. Positive)	0.854	0.119–6.156	0.876	—	—	—
Vascular invasion (Negative vs. Positive)	2.526	1.549–4.118	<0.001***	0.462	0.140–1.519	0.203
Spread through Air Spaces (Negative vs. Positive)	1.752	1.103–2.781	0.017*	0.793	0.324–1.940	0.611
CEA (<5 ng/ml vs. ≥5 ng/ml)	2.147	1.328–3.473	0.002**	1.260	0.641–2.478	0.502
SCC (<1.5 µg/L vs. ≥1.5 µg/L)	1.070	0.665–1.721	0.781	—	—	—
Cyfra21-1 (<2.08 ng/ml vs. ≥2.08 ng/ml)	1.668	1.031–2.697	0.037*	1.103	0.592–2.057	0.757
CA199 (<37 U/ml vs. ≥37 U/ml)	2.435	1.050–5.646	0.038*	0.715	0.235–2.169	0.553
Serum ferritin (<204 µg/L vs. ≥204 µg/L)	1.381	0.872–2.186	0.169	—	—	—
hsa_circ_0070354 (Negative vs. Positive)	5.641	3.222–9.876	<0.001***	5.087	2.442–10.600	<0.001***

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 was considered significant.

screening of other small tumor samples. In conclusion, hsa\_circ\_0070354 likely participates in the progression of NSCLC, and the high expression of hsa\_circ\_0070354 can be used as a poor prognostic factor.

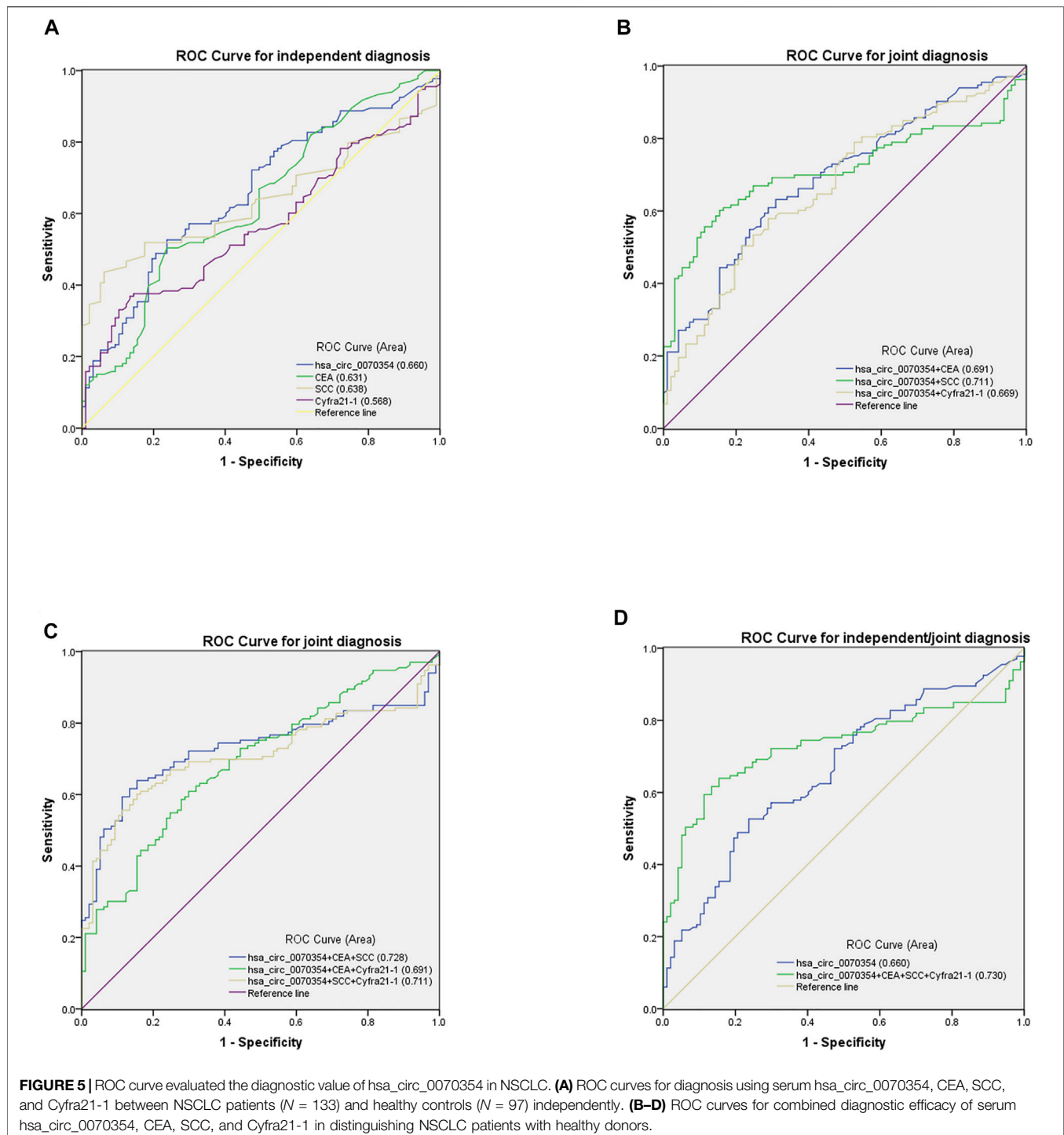
### Evaluation of the Diagnostic Accuracy of Serum hsa\_circ\_0070354 Independent and Combined Diagnostic Model in NSCLC

In previous essays, hsa\_circ\_0070354 has been confirmed to have the molecular properties of tumor biomarkers and was related to poor tumor prognosis. And yet, its efficacy in the diagnosis of NSCLC needs to be further explored. Compared with the mature lung cancer markers such as CEA and SCC, the diagnostic efficiency of hsa\_circ\_0070354 is the key factor in evaluating it as a novel diagnostic and predictive biomarker. The ROC curve showed that the AUC of hsa\_circ\_0070354 was 0.660 (95% CI: 0.589–0.730), which was higher than 0.631 of CEA (95% CI: 0.559–0.703), 0.638 of SCC (95% CI: 0.557–0.709), and 0.568 of Cyfra21-1 (95% CI: 0.495–0.642) (Figure 5A). Figures 5B,C demonstrated the AUC of hsa\_circ\_0070354 combined with the other three tumor markers, respectively. Compared with the single diagnosis, the AUC of the combined diagnosis of hsa\_circ\_0070354 and CEA increased to 0.691 (95% CI: 0.623–0.758), as well as the AUC of combined diagnosis with SCC and Cyfra21-1 increased to 0.711 (95% CI: 0.644–0.779) and 0.669 (95% CI: 0.599–0.733) respectively, which were both higher than the single diagnosis (Figure 5B). Similarly, the three-combination diagnosis of AUC was remarkably higher than that of a single diagnosis (Figure 5C). What is particularly striking is that in Figure 5D, the AUC of the combination of hsa\_circ\_0070354 and the other three mature tumor markers reached 0.730 (95% CI: 0.663–0.796), which was significantly

higher than that of the single diagnosis of hsa\_circ\_0070354, and also higher than the combined diagnosis of the two or three. Subsequently, we also analyzed the sensitivity (SEN), specificity (SPE), overall accuracy (ACCU), positive predictive value (PPV), as well as negative predictive value (NPV) of single and combined diagnostic models (Table 4). According to our statistics, the SEN, SPE, ACCU, PPV and NPV of hsa\_circ\_0070354 were 52.63, 76.29, 62.61, 75.27 and 54.01%, respectively, while the combined diagnosis of the four tumor markers were 63.91, 84.54, 72.61, 85.00 and 63.08%. Therefore, hsa\_circ\_0070354 is superior to traditional tumor markers in diagnosis, and the combined diagnosis has higher sensitivity and specificity for the diagnosis of NSCLC.

### Exploration of the Downstream and Function Prediction of hsa\_circ\_0070354

Previous studies have implied that circRNAs have four main functions: acting as a miRNA sponge, interacting with RNA binding proteins, regulating gene transcription, and encoding peptides. circRNAs contain a massive number of miRNA binding sites, which act as miRNA sponges and adjust gene expression indirectly (Huang et al., 2021a). Hsa\_circ\_0070354 was mainly located in the cytoplasm and may participate in the competitive inhibition of endogenous RNA as a microRNA sponge. Yet, its participation in the occurrence and development of NSCLC is a very complex process, which is worth our further in-depth study. Here, we analyze its potential function and regulation mechanism through the prediction of the relevant database. The Gene Ontology (GO) functional enrichment analysis of the target gene shows that 354 may play a potential role in RNA binding, splicing, and processing (Figure 6A). The enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes



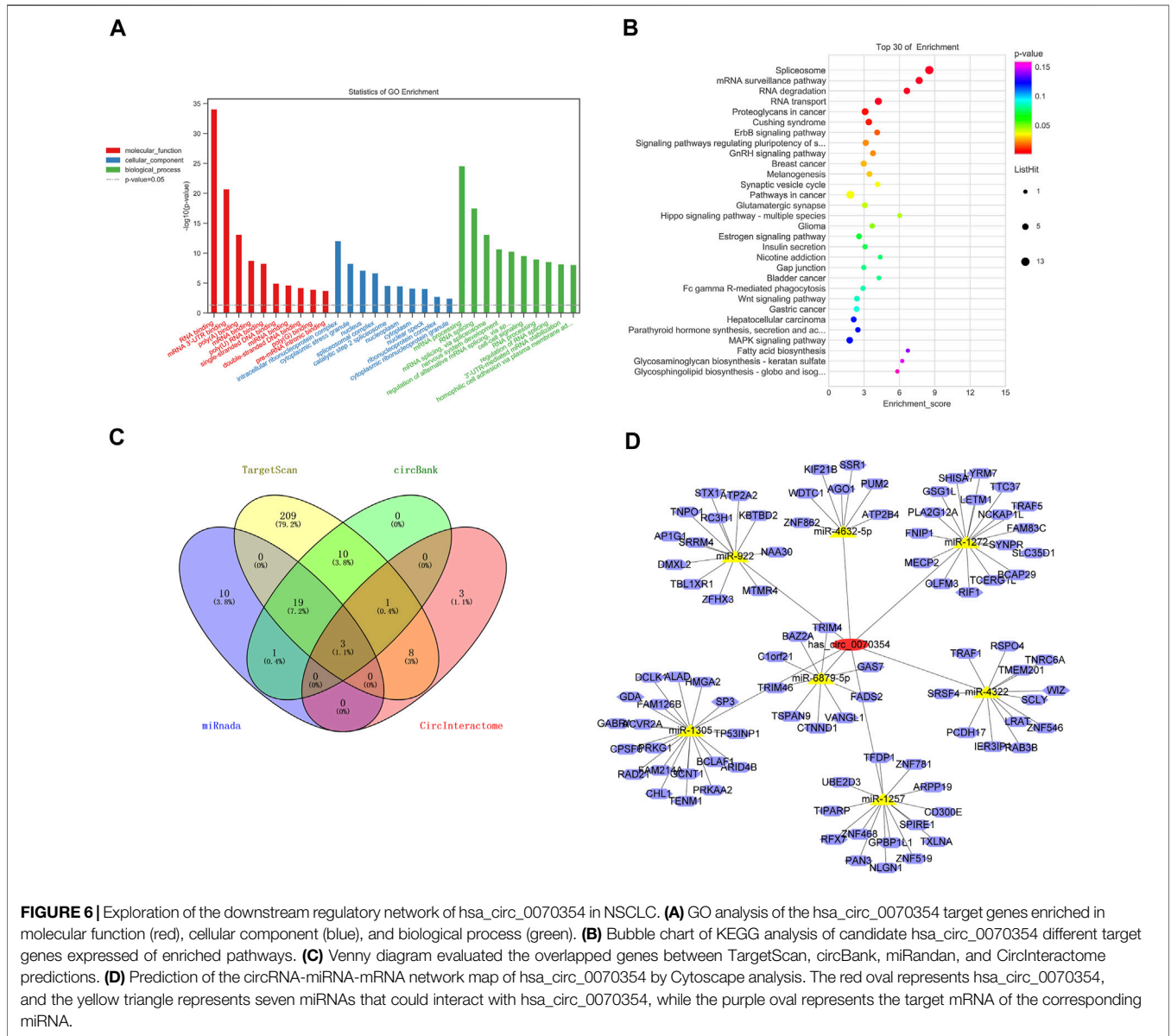
(KEGG) signal pathway showed that hsa\_circ\_0070354 was significantly enriched in a variety of tumors, and the ErbB and MAPK signal pathway was also the focus of our subsequent attention (**Figure 6B**). The Venny diagram shows our prediction of downstream miRNAs in four common databases. The three miRNAs obtained by repeated intersection are miR-1257, miR-1272, and miR-1305 (**Figure 6C**). According to the results of databases and bioinformatics analysis, we predict the possible

circRNA-miRNA-mRNA axis. As shown in **Figures 5A,D** total of 7 miRNAs (3 above, also including miR-922, miR-4322, miR-4632-5p, and miR-6879-5p) and their corresponding mRNAs are presented, which may provide a research direction for the progress of NSCLC regulation by hsa\_circ\_0070354. In any case, based on the above results, more experimental work is needed to confirm the regulation mechanism of hsa\_circ\_0070354.

**TABLE 4 |** Use the expression levels of has\_circ\_0070354, CEA, SCC and Cyfra21-1 to distinguish NSCLC patients from healthy donors.

	SEN (%)	SPE (%)	ACCU (%)	PPV (%)	NPV (%)
has_circ_0070354	52.63 (70/133)	76.29 (74/97)	62.61 (144/230)	75.27 (70/93)	54.01 (74/137)
CEA	50.38 (67/133)	77.32 (75/97)	61.74 (142/230)	75.28 (67/89)	53.19 (75/141)
SCC	35.34 (47/133)	94.85 (92/97)	60.43 (139/230)	90.38 (47/52)	51.69 (92/178)
Cyfra21-1	29.32 (39/133)	90.72 (88/97)	55.22 (127/230)	81.25 (39/48)	48.35 (88/182)
has_circ_0070354 + CEA	63.16 (84/133)	69.07 (67/97)	65.65 (151/230)	73.68 (84/114)	57.76 (67/116)
has_circ_0070354 + SCC	60.15 (80/133)	74.23 (72/97)	66.09 (152/230)	84.21 (80/95)	57.60 (72/125)
has_circ_0070354 + Cyfra21-1	57.89 (77/133)	71.13 (69/97)	63.48 (146/230)	73.33 (77/105)	55.20 (69/125)
has_circ_0070354 + CEA + SCC	63.91 (85/133)	84.54 (82/97)	72.61 (167/230)	85.00 (85/100)	63.08 (82/130)
has_circ_0070354 + CEA + Cyfra21-1	60.90 (81/133)	70.10 (68/97)	64.78 (149/230)	73.64 (81/110)	56.67 (68/120)
has_circ_0070354 + SCC + Cyfra21-1	60.15 (80/133)	84.54 (82/97)	70.43 (162/230)	84.21 (80/95)	60.74 (82/135)
has_circ_0070354 + CEA + SCC + Cyfra21-1	63.91 (85/133)	84.54 (82/97)	72.61 (167/230)	85.00 (85/100)	63.08 (82/130)

SEN, sensitivity; SPE, specificity; ACCU, overall accuracy; PPV, positive predictive value; NPV, negative predictive value.



## DISCUSSION

Generally, the incidence and malignancy of NSCLC are high, and the 5-year survival rate is lower than other malignant tumors, which is mainly due to the enormous heterogeneity of NSCLC and the insufficient understanding of the biological of NSCLC, leading to the lack of effective biomarkers for diagnosis and targeted therapies (Fu et al., 2020). Besides, the survival of NSCLC is closely related to staging. Studies have shown that as patients develop from stage I to IV, the 5-year survival rate of NSCLC decreases from 82 to 6% (Goldstraw et al., 2016). Most of the patients have been in the advanced or locally advanced stage at the time of diagnosis and have missed the best opportunity for surgical treatment, mainly conservative palliative treatment. Despite that, the research on targeted therapy and immunotherapy for advanced patients is advancing by leaps and bounds. The lack of targets and low mutation rate is still the treatment dilemma. The high cost of immunotherapy and unpredictable side effects also deter many patients. Even so, the increased drug resistance of targeted therapy and immunotherapy is also the limitation of treatment, and tumors develop irreversible outcomes (Hong et al., 2020). For these reasons, early detection and improving the accuracy of diagnosis are conducive to improve the prognosis and survival rate of patients, which not only depends on the popularization of physical examination and computerized tomography (CT) but also requires the exploration and research of humoral tumor biomarkers which are non-radiation, dynamic monitoring, simple and easy to popularize. It is an urgent issue to identify new specific biomarkers for NSCLC to aid in diagnosis and clinical decision.

CircRNA was first observed in the 1970s (Hsu and Coca-Prados, 1979), but its biological function was considered unclear for a long time (Cocquerelle et al., 1993; Zaphiropoulos, 1997). Until 2012, with the advancement of high-throughput sequencing and bioinformatics analysis, the distribution and function of circRNAs in different cells and tissues were progressively discovered, not just an idle by-product of mRNA splicing (Djebali et al., 2012; Salzman et al., 2012). Since then, the researchers focused on circRNAs have shifted attention to clarifying their associations with human diseases, including cancers. Some kinds of literature have reported that the abnormal expressions of circRNAs were associated with the clinical characteristics of tumors and might become biomarkers of NSCLC (Kristensen et al., 2018; Qu et al., 2018; Di et al., 2019). In this study, we determined for the first time that hsa\_circ\_0070354 was higher in tumor tissues, cells, and serum than in adjacent normal tissues, cell lines, or healthy human sera. At the same time, we found that the high expression of hsa\_circ\_0070354 was significantly positively correlated with the late TNM stage and poor differentiation of NSCLC, indicating a poor prognosis and survival time. Combined with the biological characteristics of sensitivity, stability, and ease to be detected in body fluids, hsa\_circ\_0070354 would be a promising biomarker of NSCLC and has a bright prospect for early diagnosis and treatment of lung cancer in the

future. The relevant information at the beginning of our research came from some circRNA databases, such as circBank, circBase and circular RNA Interactome. Through circBase, we found that hsa\_circ\_0070354 studies included Rybak2015 and Salzman2013. Except for A549 cell line, which was consistent with our experimental results, it was only expressed in the brain parenchyma, such as the cerebellum, occipital\_lobe and frontal\_cortex, but no evidence was found in other cancers. In the exosomes database, such as ExoRbase, we have not queried the relevant included information. The existing information supports the specific expression of hsa\_circ\_0070354 in NSCLCs. In addition, the transition from a single biomarker to the joint diagnosis of multiple biomarkers is the development direction of tumor markers. Our results revealed that the combined diagnosis of multiple tumor markers has significantly higher sensitivity and specificity than a single biomarker. The combination of hsa\_circ\_0070354 with other tumor markers is expected to improve further the accuracy of hsa\_circ\_0070354 in the diagnosis, prognosis, and prediction of NSCLC. Generally, circRNAs are formed by reverse splicing of pre-mRNAs through several acknowledged mechanisms, namely lariat-driven circularization (Eger et al., 2018), intron pairing-driven circularization (Liu et al., 2019b), RBP-mediated circularization (Conn et al., 2015), and intronic circularization (Zhang et al., 2013). The relationship between host gene and circRNA is complex and diverse. However, the expression levels of circRNAs and the abundance of mRNA produced by the parental gene were not significantly related (Barrett and Salzman, 2016; Chen et al., 2019). By querying TCGA resource, it can be easily noted that the host gene of hsa\_circ\_0070354 (PTPN13) show negative association with cancer prognosis (HR<1), which is contrary to hsa\_circ\_0070354 (HR>1). Due to the limitations of database speculation and the incidence characteristics of NSCLC in Asia, the expression of PTPN13 in Chinese population needs further large sample study. This different expression trend inspired us to explore the further regulatory axis between hsa\_circ\_0070354 and its host gene PTPN13 in depth.

In the study of the mechanisms of circRNAs, the ceRNA hypothesis proposes that RNA transcripts share the same miRNA response elements, leading to the competition with miRNA combinations and then regulating the expressions (Salmena et al., 2011). In our study, we combined the results of bioinformatics analysis and sequencing to screen the miRNA and mRNA binding to hsa\_circ\_0070354. Unfortunately, the high expression of hsa\_circ\_0070354 in the biological, behavioral regulation of NSCLC remains unclear. The analysis between hsa\_circ\_0070354 and clinicopathological features suggested it was related to tumor size and metastasis, corresponding to biological behaviors such as cell proliferation and invasion, which provides a direction for further exploration. Whether the hsa\_circ\_0070354-miRNA-mRNA axis is involved in regulating NSCLC cell proliferation, migration and invasion will be the direction of our next exploration and study. Although no study has confirmed that circRNA would be a new therapeutic target for tumors, the effects of circRNAs on the proliferation,



metastasis, and drug resistance of lung cancer indicate that they may become a target of therapeutic, which is worthy of our profound understanding. CircRNAs are expected to make significant contributions to cancer prevention, diagnosis, and treatment in the future.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI SRA repository, accession number PRJNA 781220.

## ETHICS STATEMENT

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

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## AUTHOR CONTRIBUTIONS

YL, CC and KH control over the quality inspection of tissue samples and collect tissues. All authors have read and approved the final 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/fgene.2021.796776/full#supplementary-material>

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