



RBM39 is a potential prognostic biomarker with functional significance in hepatocellular carcinoma

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Background: RNA-binding motif protein 39 (RBM39) is a well-known RNA-binding protein involved in tumorigenesis; however, its role in hepatocellular carcinoma (HCC) remains unclear. The aim of this study was to investigate the role of RBM39 in HCC.

Methods: The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases were used to analyze the differential expression of RBM39 in HCC and normal tissues. The prognostic and diagnostic value of RBM39 in HCC was accessed by Kaplan-Meier analysis, Cox regression, and receiver operating characteristic (ROC) curve analyses. Quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry were used to validate the mRNA and protein expression of RBM39 in HCC. Moreover, gene set enrichment analysis (GSEA) was performed to identify key pathways related to RBM39. The correlation between RBM39 expression and immune cell infiltration was evaluated using a single-sample gene set enrichment analysis (ssGSEA). CCK8 and wound healing assays were performed to investigate the proliferation and migration abilities of HCC cells with RBM39 knockdown.

Results: RBM39 expression was upregulated in the HCC tissues. High RBM39 expression was significantly associated with advanced T stage, histological grade, and pathological stage and predicted poor overall survival (OS), disease-specific survival (DSS), and progress-free interval (PFI) in HCC patients. The upregulation of RBM39 expression was an independent prognostic factor for OS. Moreover, GSEA enrichment analysis indicated that RBM39 was functionally involved in pathways associated with the cell cycle, DNA replication, the p53 signaling pathway, and primary immunodeficiency. RBM39 expression was associated with infiltration of Th2 cells and dendritic cells (DC). RBM39 knockdown significantly inhibited the proliferation and migration of HCC cells.

Conclusions: These findings suggest that high RBM39 expression is associated with poor prognosis and promotes HCC cell proliferation and migration. Based on these results, RBM39 is a promising prognostic biomarker with functional significance for HCC.

Keywords: Hepatocellular carcinoma (HCC); RNA-binding motif protein 39 (RBM39); prognosis; The Cancer Genome Atlas (TCGA)

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, resulting in over 800,000 deaths per year (1). Chronic viral hepatitis caused by infections of hepatitis B virus (HBV) and hepatitis C virus (HCV), aflatoxin poisoning, chronic alcohol consumption, and nonalcoholic fatty liver disease (NAFLD) are the primary risk factors associated with HCC (2). The mechanisms underlying the development and progression of HCC remain incompletely understood. HCC arises from the accumulation of genetic mutations and epigenetic modifications in proto-oncogenes and their associated driver genes, leading to its molecular heterogeneity. Based on these research findings, targeted therapy and immunotherapy have emerged as novel approaches for the clinical management of HCC, particularly in patients with advanced disease (3,4). However, given its high degree of malignancy and insidious onset, HCC is mostly detected and diagnosed at advanced stages with limited therapeutic options (5). Therefore, it is important to investigate the underlying mechanisms of HCC development and progression and to identify predictive biomarkers for treatment selection.

RNA-binding motif protein 39 (RBM39), also known as HCC1 or CAPER α (6), is an important serine/arginine-rich (SR) RNA binding protein. RBM39 has been well defined as a splicing factor that regulates pre-mRNA alternative splicing (7). Besides, RBM39 has also been shown to function as a transcriptional cofactor of several transcription factors such as ESR1/ER- α , ESR2/ER- β , and JUN/AP-1 (6,7). Thus, RBM39 is involved in multiple biological processes, including development, cell metabolism, stress

response, and carcinogenesis. In particular, RBM39 is found to be upregulated in a variety of tumors, and the loss of RBM39 significantly inhibits the proliferation and migration of several cancers, including breast cancer (8), acute myeloid leukemia (AML) (9,10), colorectal cancer (11), ovarian carcinoma (12) and lung cancer (13). RBM39 promotes the expression of mRNAs encoding HOXA9 targets, which are essential for the maintenance of AML (9). In breast cancer, RBM39 has been shown to directly interact with ER α , Er β , and AP-1/c-Jun to regulate the proliferation of cancer cells (14). In addition, overexpression of RBM39 promotes the proliferation and migration of lung cancer cell (13). These findings indicate that the upregulation of RBM39 contributes to cancer development. Recently, RBM39 has been identified as an unexpected target of aryl sulfonamides. Mechanistically, aryl sulfonamides act as molecular glues to recruit RBM39 to DCAF15-associated E3 ubiquitin ligase, leading to ubiquitination and subsequent degradation of RBM39, thereby exhibiting potent anti-cancer activities in several preclinical models (15-17). However, in clinical trials, aryl sulfonamides have only mild to moderate clinical benefits for a few patients (18,19). The insufficient understanding of the function and mechanism of RBM39 might limit its clinical application of aryl sulfonamides. Moreover, the role of RBM39 in HCC and its underlying mechanisms remain largely unknown.

In this study, we investigated the relationship between RBM39 expression and the clinical characteristics of patients with HCC, as well as the potential prognostic value and possible biological functions of RBM39 using The Cancer Genome Atlas (TCGA) data. The effects of RBM39 on the proliferation and migration of HCC cells were also assessed. Our results highlight the oncogenic role of RBM39 in HCC, and demonstrates RBM39 as a potential prognostic marker. We present this article in accordance with the REMARK reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-2252/rc>).

Highlight box

Key findings

- RNA-binding motif protein 39 (RBM39) is a potential prognostic biomarker of hepatocellular carcinoma (HCC). Knockdown of RBM39 inhibits the proliferation and migration of HCC cells.

What is known and what is new?

- High RBM39 expression is associated with poor prognosis in HCC.
- RBM39 is correlated with immune infiltration in HCC.

What is the implication, and what should change now?

- RBM39 participates in HCC malignancy and could serve as a potential target for HCC treatment.

Methods

Acquisition of RBM39 expression data and its clinical relevance

The online database TIMER2.0 (<http://timer.cistrome.org/>) was used to analyze the mRNA levels of RBM39 in pan-cancers. The RNA-seq data in transcripts per million (TPM) reads format of 374 HCC tissues and 50 normal liver tissues and the clinical information of the corresponding patients

were retrieved from TCGA database (<https://portal.gdc.cancer.gov/>). The clinical information included age, gender, TNM stage, pathologic stage, histologic grade, serum alpha-fetoprotein (AFP) level, fibrosis Ishak score and vascular invasion status. Samples with incomplete clinical information were excluded from the analysis. In addition, immunohistochemistry (IHC) results for RBM39 protein in HCC and normal tissues were obtained from the Human Protein Atlas (HPA, <https://www.proteinatlas.org/>) online database. Datasets GSE6764 and GSE14520, retrieved from the Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>), containing the expression profiles of HCC and normal livers, were used to further validate the expression levels of RBM39 mRNA in HCC. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (2022-KY-1184-003) and informed consent was obtained from all individual participants.

Gene set enrichment analysis (GSEA)

Signaling pathways associated with RBM39 expression in HCC were analyzed by GSEA using the R statistical software (version 3.6.3). The samples were categorized into high- and low-expression groups based on the median expression level of RBM39. The expression level of RBM39 was used as the phenotype label. Gene set permutations were conducted with 1,000 random combinations for every analysis. The reference gene set used was “c2.cp.v7.2.symbols.gmt”. False discovery rate (FDR) <0.25, $P < 0.05$, and normalized enrichment score (NES) >1 were considered to be statistically significant. The signaling pathways with the highest NES were selected.

Immune infiltration analysis

A single-sample gene set enrichment analysis (ssGSEA) approach was used in HCC samples to analyze the correlation of RBM39 expression with 24 types of immune cells using the Gene Set Variation Analysis (GSVA) package in R. Spearman's correlation analysis was performed to examine the correlation between RBM39 expression and 24 types of immune cells. The infiltration of immune cells between the high- and low-expression groups of RBM39 was analyzed using the Wilcoxon rank-sum test.

IHC

The protein level of RBM39 in HCC was detected by IHC using a commercial tissue microarray (Zhongke Guang Hua Intelligent Biotechnology Co., Xi'an, China) with 10 HCC and 10 normal liver tissues. The tissues were dewaxed and hydrated, followed by antigen repair and endogenous peroxidase blocking. The slides were then incubated with the primary antibody overnight at 4 °C. After incubation with secondary antibody, the slides were stained with diaminobenzidine (DAB). Finally, the staining results were independently evaluated by two pathologists. RBM39 expression was scored according to the percentage of positively stained tumor cells and the intensity of RBM39 staining. The percentage of immunoreactive tumor cells was scored as follows: 1 (<10%), 2 (10–25%), 3 (26–49%) and 4 ($\geq 50\%$). The staining intensity was scored visually and stratified as follows: 1 (negative); 2 (light yellow); 3 (light brown) and 4 (dark brown). The final immunoreactivity score for each case was obtained by multiplying the percentage with the intensity score.

Cell culture

The HCC cell lines, Huh7 and SMMC-7721, were purchased from the Shanghai Institute of Life Science Cell Bank Center (Shanghai, China). The cells were cultured in dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and streptomycin in an incubator with 5% CO₂ at 37 °C. Cells in the logarithmic growth phase were utilized for subsequent experiments.

Transfection of small interfering RNA (siRNA)

The siRNA targeting RBM39 were purchased from RiboBio (Guangzhou, China). siRNAs were transfected into SMMC-7721 and Huh7 cell lines using lipofectamine 2000 (Invitrogen, Carlsbad, USA). All manipulations were performed in accordance with the manufacturer's instructions. Briefly, cells were seeded onto plates and cultured until they reached 50% confluence. Subsequently, siRNAs were mixed with lipofectamine 2000 reagent and incubated at room temperature for 20 min. Cells were then transfected with the mixture for 48 h at a final siRNA concentration of 100 nM. The sequences of the siRNAs were as follows: siRBM39-1,

5'-GAAGCGAAGTAGAGACAGA-3'; siRBM39-2, 5'-GGAAAGGACTGGAATTGAT-3'.

Quantitative real-time polymerase chain reaction (qRT-PCR)

A commercial cDNA microarray was purchased from Outdo Biotechnology (Shanghai, China), which came with the cDNA of 66 HCC and 21 paracancerous tissues. qRT-PCR was performed as described previously (20). Briefly, the cDNA was amplified using SYBR-Green PCR Master Mix (TAKARA, Tokyo, Japan) on a QuantStudio 5 system (ABI, Carlsbad, USA) under the conditions as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The level of RBM39 mRNA was calculated by $2^{-\Delta\Delta C_t}$ method using β -actin as internal control. The sequences of the specific primers used for amplification were as follows: β -actin forward, 5'-GAAGAGCTACGAGCTGCCTGA-3'; β -actin reverse, 5'-CAGACAGCACTGTGTTGGCG-3'; RBM39 forward, 5'-CAATGCTTGAGGCTCCTTACA-3'; RBM39 reverse, 5'-TCCGTTCCCTTACTTTTGCTTCTC-3'.

Western blotting

Cells were collected 72 h after transfection and lysed using RIPA lysis buffer (Epizyme, Shanghai, China) and a protease inhibitor (Sollerbauer, Beijing, China). Protein concentrations were measured using the BCA Protein Assay Kit (Epizyme) and adjusted to equal concentrations. Protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, USA). The membranes were blocked with 5% skimmed milk for 2h at room temperature and incubated overnight at 4 °C with anti-RBM39 (21339-1-AP, 1:1,000, Proteintech, Wuhan, China) and anti-GAPDH (10494-1-AP, 1:5,000, Proteintech) primary antibodies. The immunoblots were detected using ECL chemiluminescence kit (Epizyme).

CCK8 assay

HCC cells were plated in 96-well plates at a density of 2×10^4 cells per well and incubated for 24, 48, 72 and 96 h. Subsequently, 10 μ L of the CCK-8 reagent (GK10001, Gpibo, Montclair, USA) was added to each well. The cells were incubated for 1.5 h at 37 °C and the absorbance was measured at 450 nm. Five replicates were used for each

group and the experiment was repeated three times.

Wound healing assay

The migratory ability of HCC cells was evaluated using a wound-healing assay. When the cells occupied 95% of the area of the 12-well plate bottom, the monolayer was scraped with a 10 μ L pipette tip. After washing the cells twice with phosphate-buffered saline (PBS), the cell culture medium was replaced with DMEM. The cells were cultured for another 24 h before imaging. Cell migration was determined by the migration index using Image J software [migration index = (initial scratch area – final scratch area)/initial scratch area].

Statistical analysis

Statistical data obtained from TCGA were merged and processed using the R3.6.3. The Wilcoxon rank-sum test and Wilcoxon signed-rank test were employed to compare the expression levels of RBM39 between the HCC group and the control group. Welch one-way ANOVA was used to access the correlation between RBM39 expression and clinicopathological factors, followed by the Bonferroni correction or *t*-test. Patients were categorized into high- and low-expression groups according to median RBM39 mRNA levels. Chi-squared test and logistic regression analysis were used to analyze the impact of clinicopathological factors on RBM39 expression. Univariate and multivariate Cox regression analyses and Kaplan-Meier curves were used to assess the prognostic value of RBM39 expression. Variables that were found to be significant ($P < 0.05$) in the univariate analysis were included in the multivariate analysis. The hazard risk (HR) of individual factors was estimated along with a 95% confidence interval (CI). The diagnostic value of RBM39 was evaluated through receiver operating characteristic (ROC) analysis using the pROC package (21). The area under the curve (AUC) values were calculated, ranging from 0.5 to 1.0, indicating a discrimination ability of 50–100%. For the *in vitro* experiments, data were analyzed using GraphPad Prism V9.3.1. The Student's *t*-test was used to analyze the differences between the two groups. Statistical significance was set at P value < 0.05 .

Results

RBM39 is upregulated in HCC

To explore the possible role of RBM39 in tumorigenesis,

we first evaluated RBM39 expression in pan-cancer using TIMER2.0. The results indicated that RBM39 is upregulated in numerous cancers, including cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), prostate adenocarcinoma (PRAD), rectal adenocarcinoma (READ), stomach adenocarcinoma (STAD), and HCC (*Figure 1A*). Subsequently, gene expression data were extracted from TCGA HCC dataset, including 50 normal tissue samples and 374 HCC tissue samples. RBM39 mRNA expression in normal and tumor tissues in both unpaired and paired tissues was analyzed. The results indicated a significant upregulation of RBM39 mRNA in HCC tissues compared to that in normal tissues (*Figure 1B,C*). These results were further validated using the GSE6764 and GSE14520 datasets from the GEO database (*Figure 1D,E*). In addition, the HPA database identified increased protein levels of RBM39 in HCC (*Figure 1F*).

Relationship between the expression of RBM39 mRNA and the clinical features of HCC patients

As shown in *Figure 2*, elevated RBM39 expression was significantly correlated with gender ($P=0.004$), N stage ($P=0.02$), histological grade ($P<0.001$), pathological stage ($P=0.01$), and AFP level ($P<0.001$). Patients were categorized into high- and low-expression groups according to median RBM39 mRNA levels, and their corresponding clinical parameters are summarized in *Table 1*. The correlation between RBM39 levels and clinical parameters was also analyzed. Our results indicated a significant association between RBM39 expression and age ($P=0.03$), gender ($P=0.02$), histological grade ($P=0.002$), and AFP levels ($P<0.001$). Moreover, logistic analysis revealed an association between increased RBM39 expression and advanced histological grading [odds ratio (OR): 2.047, 95% CI: 1.344–3.162, $P=0.001$] and high AFP levels (OR: 2.952, 95% CI: 1.653–5.426, $P<0.001$) (*Table 2*).

Role of RBM39 in survival of HCC

Subsequently, the prognostic value of RBM39 in HCC was evaluated by Kaplan-Meier analysis using TCGA data. As shown in *Figure 3A-3C*, high RBM39 mRNA expression was associated with poor overall survival (OS), disease-specific survival (DSS), and progress-free interval (PFI) in HCC. In addition, the prognostic value of RBM39 for

OS was evaluated in different HCC subtypes. As shown in *Figure 3D-3K*, elevated levels of RBM39 were significantly associated with poor OS in patients with T3/T4 ($P=0.004$), N0 ($P<0.001$), M0 ($P<0.001$), pathological stage III/IV ($P=0.01$), histological grade G1/G2 ($P=0.01$), and G3/G4 ($P=0.02$). Furthermore, the findings of the univariate Cox regression analysis indicated that T stage ($P<0.001$), M stage ($P=0.01$), and pathological stage ($P<0.001$) were important parameters that determined the survival time in HCC patients. Multivariate Cox regression analysis further indicated that RBM39 was an independent predictor (HR, 1.668; 95% CI: 1.053–2.644, $P=0.02$) (*Table 3*). Taken together, these results suggest that RBM39 expression is a potential prognostic biomarker of HCC.

The diagnostic value of RBM39 for HCC

ROC curve analysis was performed to assess the diagnostic value of RBM39 in HCC. The AUC was estimated to be 0.916, indicating that RBM39 has a high diagnostic value (*Figure 4A*). In addition, subgroup analysis showed a high diagnostic value of RBM39 expression in patients in the T phase (T1/T2), T phase (T3/T4), N phase (N0), and M phase (M0), with corresponding AUC values of 0.915, 0.919, 0.919 and 0.919, respectively (*Figure 4B-4E*). These results further illustrate the potential early diagnostic value of RBM39.

Signaling pathways associated with RBM39 in HCC

Given the relevance of RBM39 to clinical features and its prognostic and diagnostic value, GSEA was performed using data from TCGA database to further explore the potential role of RBM39 in HCC. Our results indicate a strong association between the upregulation of RBM39 and several signaling pathways, including the cell cycle, DNA replication, P53 signaling pathway, and primary immunodeficiency (*Figure 5* and *Table 4*). The genes associated with RBM39 expression in each pathway are listed in *Table S1*.

Correlation between RBM39 expression and immune infiltration

Over the past decade, a growing body of evidence has suggested the important role of immune-infiltrating cells in cancer (22). To investigate the association of RBM39 expression with immune infiltration in HCC, we analyzed

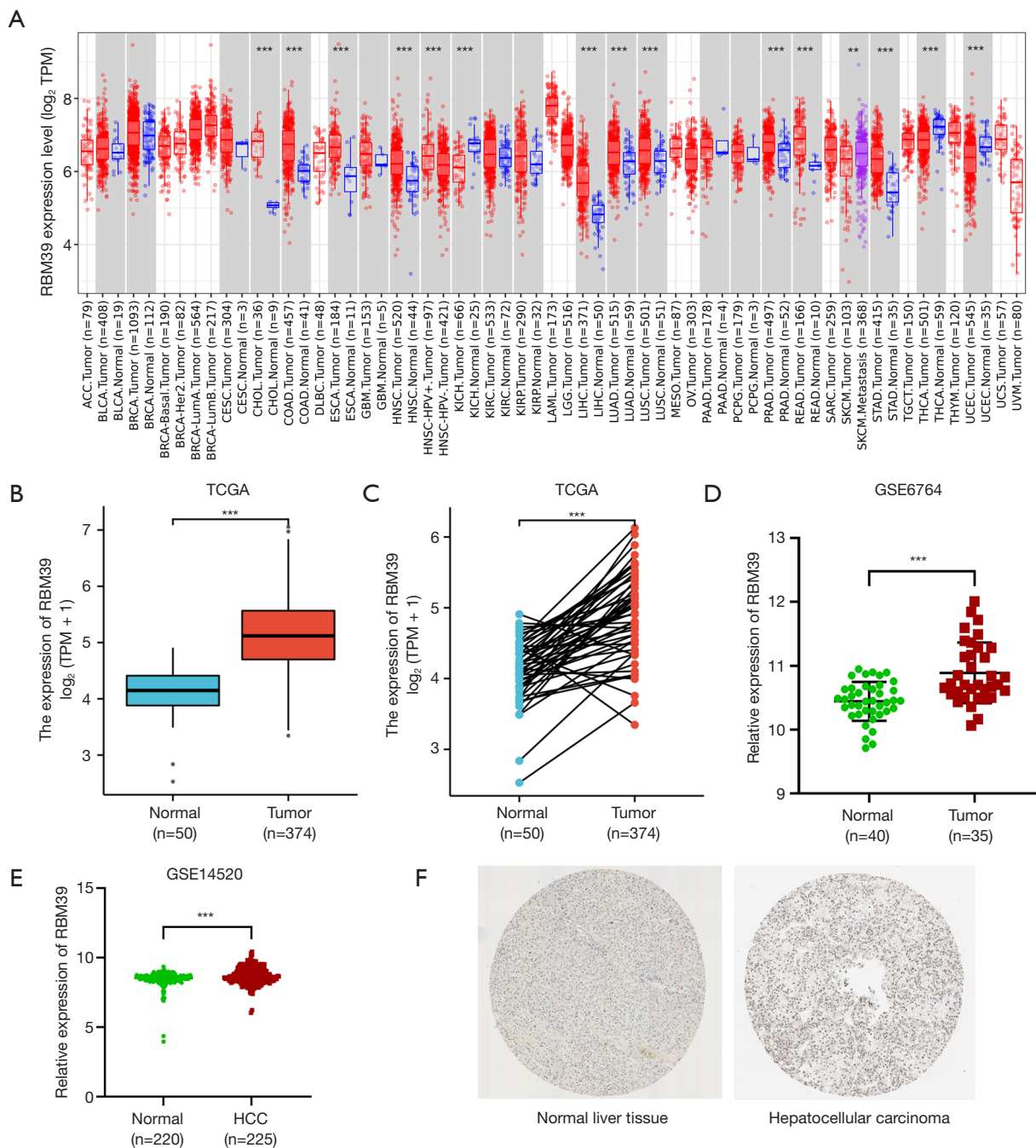


Figure 1 The expression level of RBM39 in HCC. (A) The expression level of RBM39 in different cancers in the TIMER2.0 database. (B) RBM39 mRNA level in normal livers and liver cancer tissues in TCGA database. (C) The RBM39 mRNA level in 50 pairs of liver cancer tissues and matched normal livers in TCGA database. (D) The mRNA expression of RBM39 was significantly increased in HCC in the GSE6764 dataset. (E) The mRNA expression of RBM39 was significantly increased in HCC in the GSE14520 dataset. (F) Representative immunohistochemistry images of RBM39 in normal liver and HCC tissues from HPA database (<https://www.proteinatlas.org/>). The links of the images were as follows: <https://www.proteinatlas.org/ENSG00000131051-RBM39/tissue/liver#img>, <https://www.proteinatlas.org/ENSG00000131051-RBM39/pathology/liver+cancer#img>. **, $P < 0.01$; ***, $P < 0.001$. RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; HPA, Human Protein Atlas; TPM, transcripts per million.

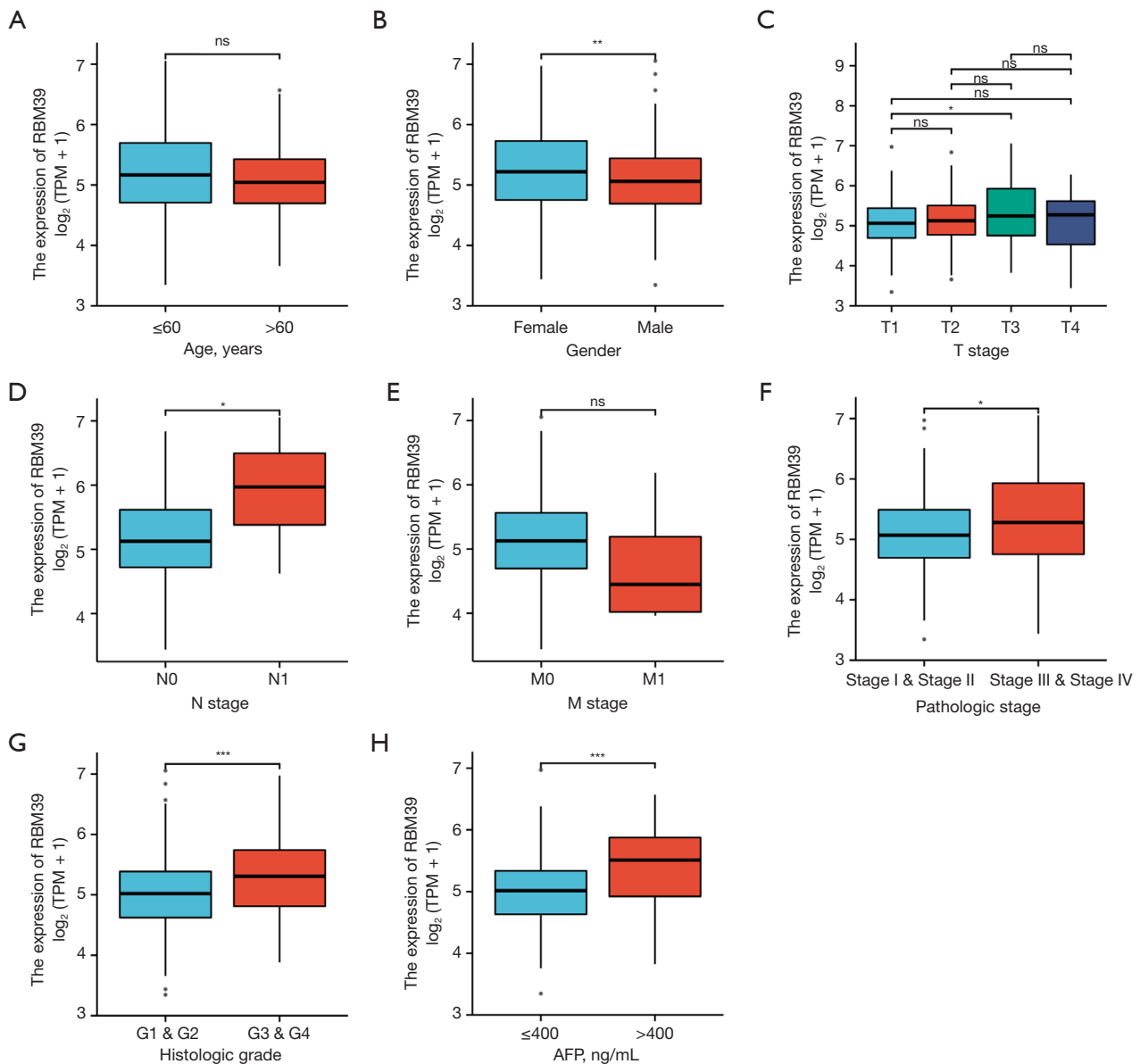


Figure 2 RBM39 expression in HCC according to different clinical characteristics: (A) age, (B) gender, (C) T stage, (D) N stage, (E) M stage, (F) pathological stage, (G) histological grade, and (H) AFP level. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; TPM, transcripts per million.

the correlation between RBM39 and the infiltration levels of 24 types of immune cells using the ssGSEA algorithm (Figure 6A). RBM39 expression positively correlated with Th2 cells (Spearman's $R = 0.345$, $P < 0.001$) (Figure 6B), while negatively correlated with DC (Spearman's $R = -0.362$, $P < 0.001$) (Figure 6C). In addition, differences in immune infiltration of Th2 and DC in the RBM39 high- and low-

expression groups were analyzed. The results showed that the infiltration levels of Th2 cells were significantly higher ($P < 0.001$) (Figure 6D), whereas the infiltration levels of DC ($P < 0.001$) were significantly lower (Figure 6E) in the RBM39 high-expression group than in the RBM39 low-expression group. These findings suggest a vital role for RBM39 in immune infiltration of HCC.

Table 1 Relationship between RBM39 expression and clinical features of HCC patients

Characteristic	Low expression of RBM39	High expression of RBM39	P
Age (years), n (%)			0.03
≤60	78 (21.1)	99 (26.8)	
>60	107 (28.9)	86 (23.2)	
Gender, n (%)			0.02
Female	50 (13.5)	71 (19.1)	
Male	135 (36.5)	115 (31)	
T stage, n (%)			0.67
T1	94 (25.4)	87 (23.6)	
T2	47 (12.7)	47 (12.8)	
T3	35 (9.5)	45 (12.2)	
T4	6 (1.6)	7 (1.9)	
N stage, n (%)			0.62
N0	125 (38.8)	127 (49.6)	
N1	1 (0.4)	3 (1.2)	
M stage, n (%)			0.62
M0	132 (48.9)	134 (49.6)	
M1	3 (1.1)	1 (0.4)	
Pathologic stage, n (%)			0.18
Stage I	90 (25.9)	81 (23.3)	
Stage II	45 (13)	41 (11.8)	
Stage III	35 (10.1)	50 (14.4)	
Stage IV	4 (1.2)	1 (0.3)	
Histologic grade, n (%)			0.002
G1	38 (10.4)	17 (4.6)	
G2	93 (25.4)	84 (23)	
G3	47 (12.8)	75 (20.5)	
G4	5 (1.4)	7 (1.9)	
AFP (ng/mL), n (%)			<0.001
≤400	120 (43.2)	93 (33.5)	
>400	20 (7.2)	45 (16.2)	
Fibrosis Ishak score, n (%)			0.47
0	46 (21.7)	28 (13.2)	
1/2	15 (7.1)	16 (7.5)	
3/4	14 (6.6)	14 (6.6)	
5/6	47 (22.2)	32 (15.1)	

Table 1 (continued)

Table 1 (continued)

Characteristic	Low expression of RBM39	High expression of RBM39	P
Tumor status, n (%)			0.14
Tumor free	106 (30.1)	95 (27)	
With tumor	67 (19)	84 (23.9)	
Vascular invasion, n (%)			0.31
No	112 (35.6)	94 (29.8)	
Yes	52 (16.5)	57 (18.1)	

RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein.

Table 2 Logistic regression analysis of the correlation between RBM39 expression and clinical features

Characteristics	Total (N)	OR (95% CI)	P value
T stage (T3/T4 vs. T1/T2)	371	1.343 (0.839–2.162)	0.22
N stage (N1 vs. N0)	258	2.953 (0.373–60.136)	0.35
M stage (M1 vs. M0)	272	0.328 (0.016–2.601)	0.33
Pathologic stage (III/IV vs. I/II)	350	1.457 (0.901–2.370)	0.12
Histologic grade (G3/G4 vs. G1/G2)	369	2.047 (1.344–3.162)	0.001
AFP (>400 vs. ≤400 ng/mL)	280	2.952 (1.653–5.426)	<0.001
Fibrosis Ishak score (1/2&3/4&5/6 vs. 0)	215	1.298 (0.735–2.313)	0.37
Vascular invasion (yes vs. no)	318	1.353 (0.852–2.154)	0.20
Tumor status (with tumor vs. tumor free)	355	1.408 (0.924–2.151)	0.11

RBM39, RNA-binding motif protein 39; AFP, alpha-fetoprotein; OR, odds ratio; CI, confidence interval.

Validation of the mRNA and protein levels of RBM39 in HCC by qRT-PCR and IHC

To validate the above bioinformatics findings, we detected the mRNA level of RBM39 in a cDNA microarray containing 66 HCC tissues and 21 para-cancerous tissues by qRT-PCR, and the protein level of RBM39 in a tissue microarray containing 10 normal liver tissues and 10 HCC tissues by IHC. The results showed that the expression level of RBM39 was significantly higher in HCC tissues than in normal tissues at both mRNA (Figure 7A) and protein levels (Figure 7B). However, we were unable to demonstrate any significant association between RBM39 mRNA levels and clinicopathological features of HCC (Table S2). This may be because of the small sample size. We further analyzed the relationship between RBM39 mRNA expression and OS in HCC using Kaplan-Meier analysis. Although not statistically significant, we observed lower OS in HCC patients with high RBM39 expression levels (Figure 7C). In

conclusion, the results of qRT-PCR and IHC were similar to the findings of the bioinformatics analysis.

Knockdown of RBM39 inhibits the proliferation and migration of HCC cells

To further explore the biological function of RBM39 in HCC development, we knocked down RBM39 in SMMC-7721 and Huh7 cells using siRNAs and verified the knockdown efficiency by qRT-PCR (Figure 8A, 8B) and western blotting (Figure 8C, 8D). Subsequently, we examined the effect of RBM39 knockdown on HCC cells using CCK8 assay. As shown in Figure 8E, 8F, the knockdown of RBM39 significantly inhibited the proliferation of SMMC-7721 and Huh7 cells. In addition, the results of the wound healing assay showed that the migration ability of HCC cells was inhibited after RBM39 knockdown (Figure 8G, 8H). These findings indicate that RBM39 may be involved in the

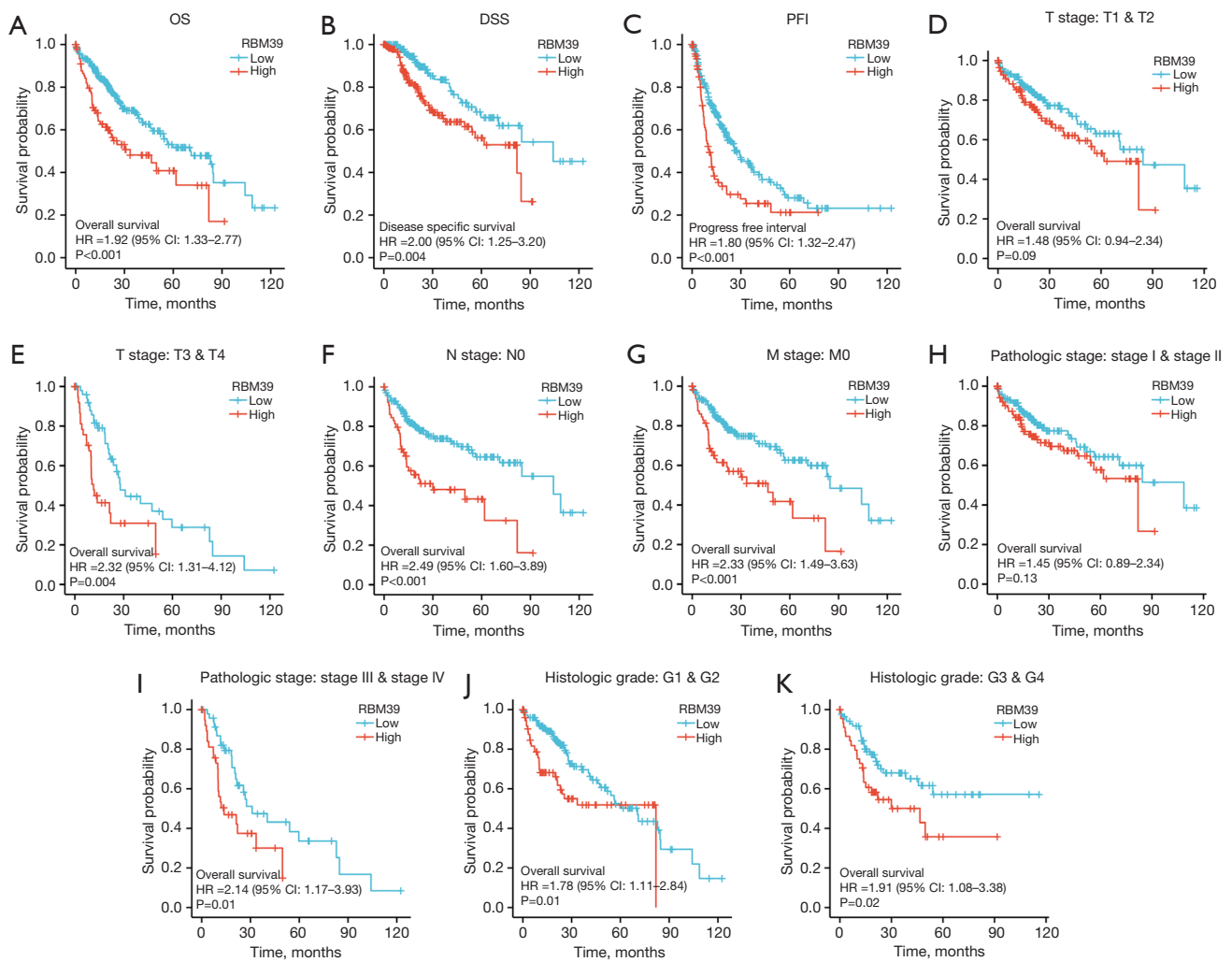


Figure 3 Kaplan-Meier curves of OS, DSS and PFI in patients with HCC according to the high or low expression level of RBM39. (A) OS; (B) DSS; (C) PFI. The association between RBM39 expression and OS in HCC patients with (D) T stage (T1/T2), (E) T stage (T3/T4), (F) N stage (N0), (G) M stage (M0), (H) pathological stage (I/II), (I) pathological stage (III/IV), (J) histological grade (G1/G2), and (K) histological grade (G3/G4). OS, overall survival; DSS, disease-specific survival; PFI, progress-free interval; HCC, hepatocellular carcinoma; HR, hazard ratio; RBM39, RNA-binding motif protein 39.

Table 3 Univariate and multivariate Cox regression analysis of RBM39 expression and clinical characteristics

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
T stage (T3/T4 vs. T1/T2)	370	2.598 (1.826–3.697)	<0.001	1.809 (0.244–13.414)	0.56
N stage (N1 vs. N0)	258	2.029 (0.497–8.281)	0.32		
M stage (M1 vs. M0)	272	4.077 (1.281–12.973)	0.01	1.684 (0.391–7.250)	0.48
Pathologic stage (III/IV vs. I/II)	349	2.504 (1.727–3.631)	<0.001	1.378 (0.187–10.150)	0.75
Histologic grade (G3/G4 vs. G1/G2)	368	1.091 (0.761–1.564)	0.63		
Fibrosis Ishak score (1/2&3/4&5/6 vs.0)	214	0.772 (0.465–1.281)	0.31		
Vascular invasion (yes vs. no)	317	1.344 (0.887–2.035)	0.16		
RBM39 (high vs. low)	373	1.478 (1.041–2.099)	0.02	1.668 (1.053–2.644)	0.02

RBM39, RNA-binding motif protein 39; HR, hazard ratio; CI, confidence interval.

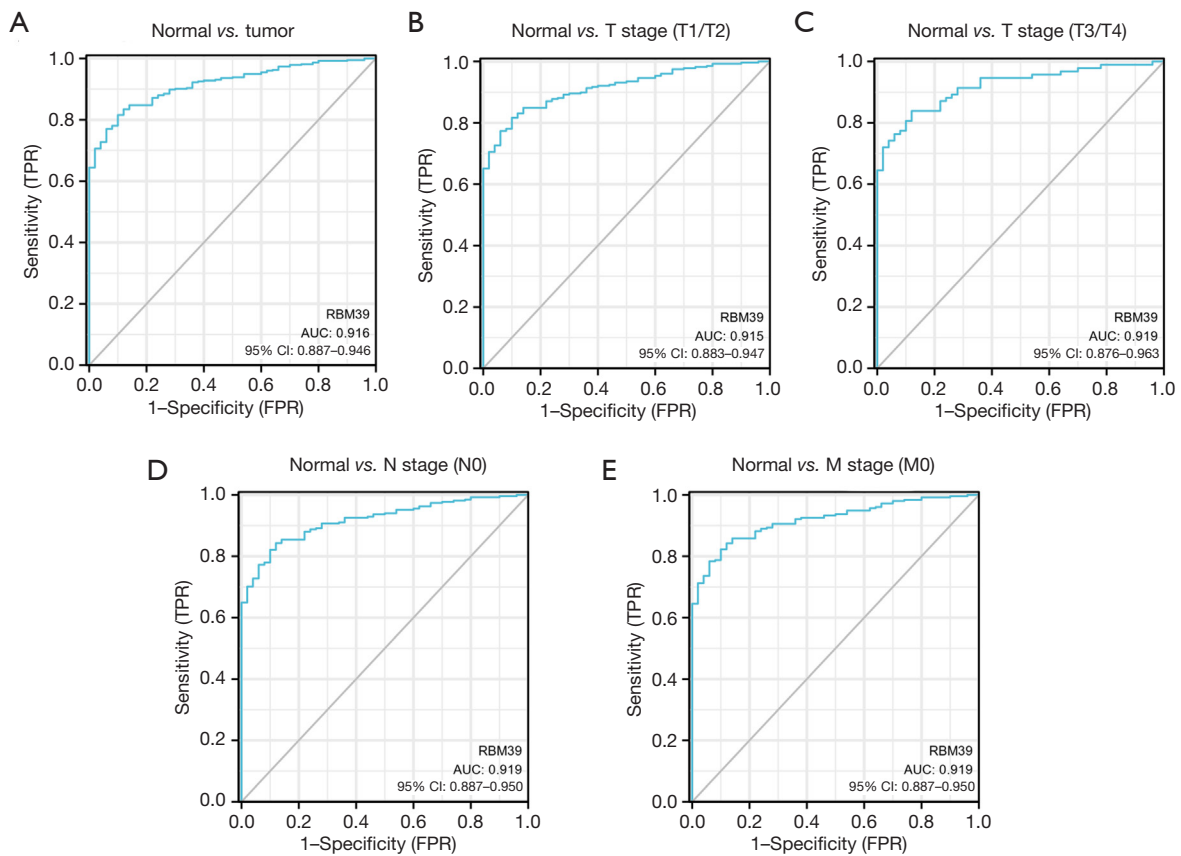


Figure 4 ROC curves indicate that RBM39 is a potential diagnostic biomarker for differentiating (A) HCC, (B) T stage (T1/T2), (C) T stage (T3/T3), (D) N stage (N0), and (E) M stage (M0) from normal livers. ROC, receiver operating characteristic; RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma; AUC, area under the curve; CI, confidence interval; TPR, true positive rate; FPR, false positive rate.

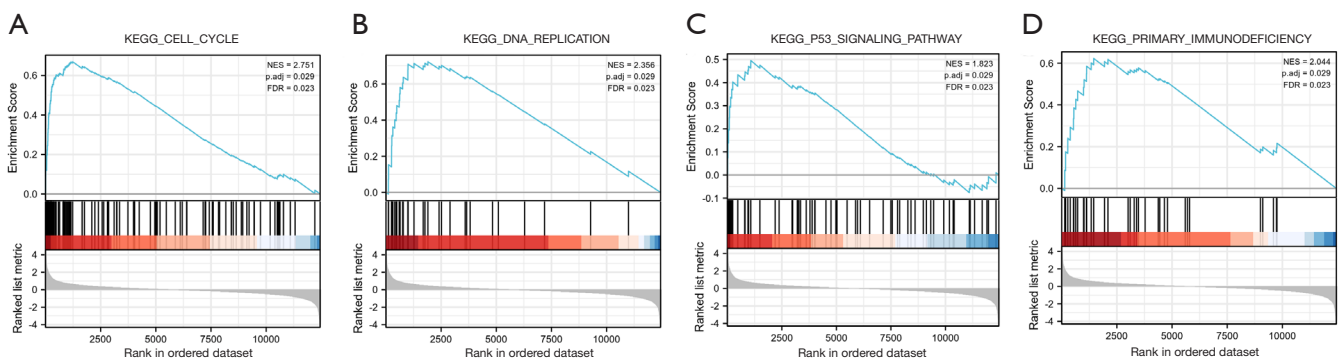


Figure 5 GSEA analysis indicated that high expression of RBM39 was significantly correlated with (A) cell cycle, (B) DNA replication, (C) P53 signaling pathway, and (D) primary immunodeficiency pathways in TCGA HCC dataset. GSEA, gene set enrichment analysis; RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.

Table 4 Gene sets enrichment of high RBM39 mRNA expression level in the TCGA HCC dataset

KEGG pathways	ES	NES	Nominal P value	FDR q value
KEGG_CELL_CYCLE	0.669	2.751	0.0029	0.0228
KEGG_DNA_REPLICATION	0.722	2.356	0.0025	0.0228
KEGG_P53_SIGNALING_PATHWAY	0.496	1.823	0.0026	0.0228
KEGG_PRIMARY_IMMUNODEFICIENCY	0.623	2.044	0.0026	0.0228

The NES is the enrichment score for the gene set after it has been normalized by gene numbers. The NES is calculated by dividing the actual ES score by the mean ES of all random combinations of gene sets. RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; FDR, false discovery rate; ES, Enrichment Score; NES, Normalized Enrichment Score.

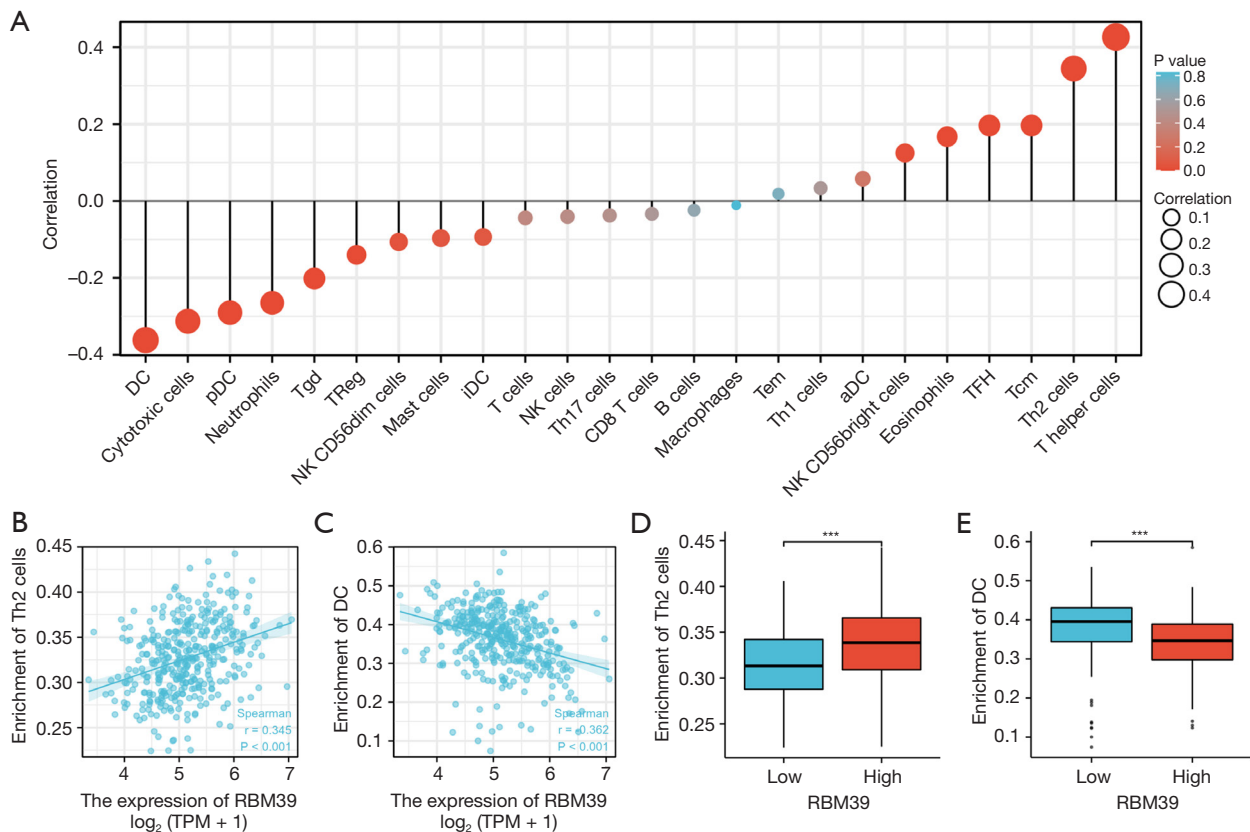


Figure 6 Relationship between RBM39 expression and immune cell infiltration. (A) Correlation between RBM39 expression level and the relative abundances of 24 types of immune cells. (B,C) The correlation between RBM39 expression and Th2 cells and DC cells. (D,E) The relationship between high and low RBM39 expression and the infiltration levels of Th2 cells and DC cells. ***, $P < 0.001$. RBM39, RNA-binding motif protein 39; DC, dendritic cell; TPM, transcripts per million.

malignant progression of HCC.

Discussion

RNA-binding proteins (RBPs) are a large class of proteins that play a significant role in gene transcription and

alternative splicing (7). Mutations and dysregulation of these proteins are associated with numerous human diseases, including cancer. RBM39 is an evolutionarily conserved RBP in animals that is functionally involved in transcriptional coregulation and alternative RNA splicing. It is upregulated in numerous tumors and is often associated

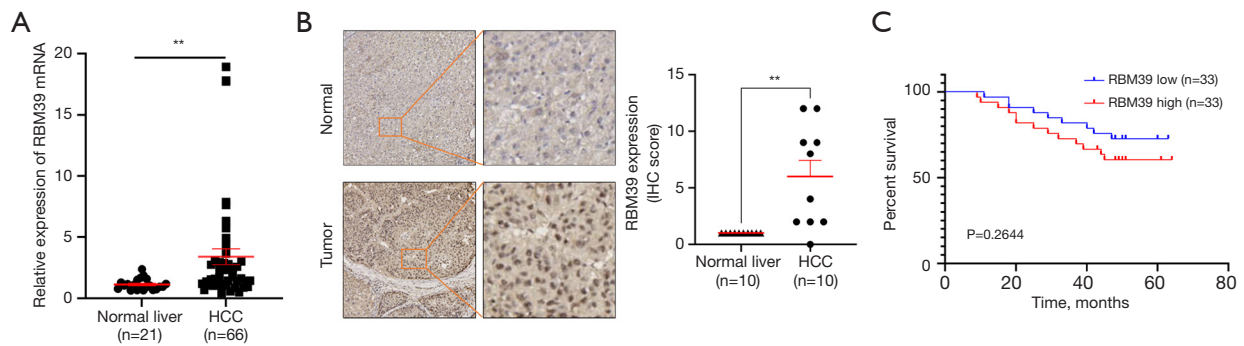


Figure 7 RBM39 was upregulated in HCC and was associated with poor OS. (A) qRT-PCR analysis of RBM39 mRNA in 66 HCC tissues and 21 para-cancerous normal livers. (B) Expression and scoring of RBM39 protein, as detected by IHC in 10 HCC and 10 normal liver tissues ($\times 100$). (C) Kaplan-Meier curve for OS of HCC patients according to the high or low level of RBM39 mRNA. **, $P < 0.01$. RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma; OS, overall survival; IHC, immunohistochemistry; qRT-PCR, quantitative real-time polymerase chain reaction.

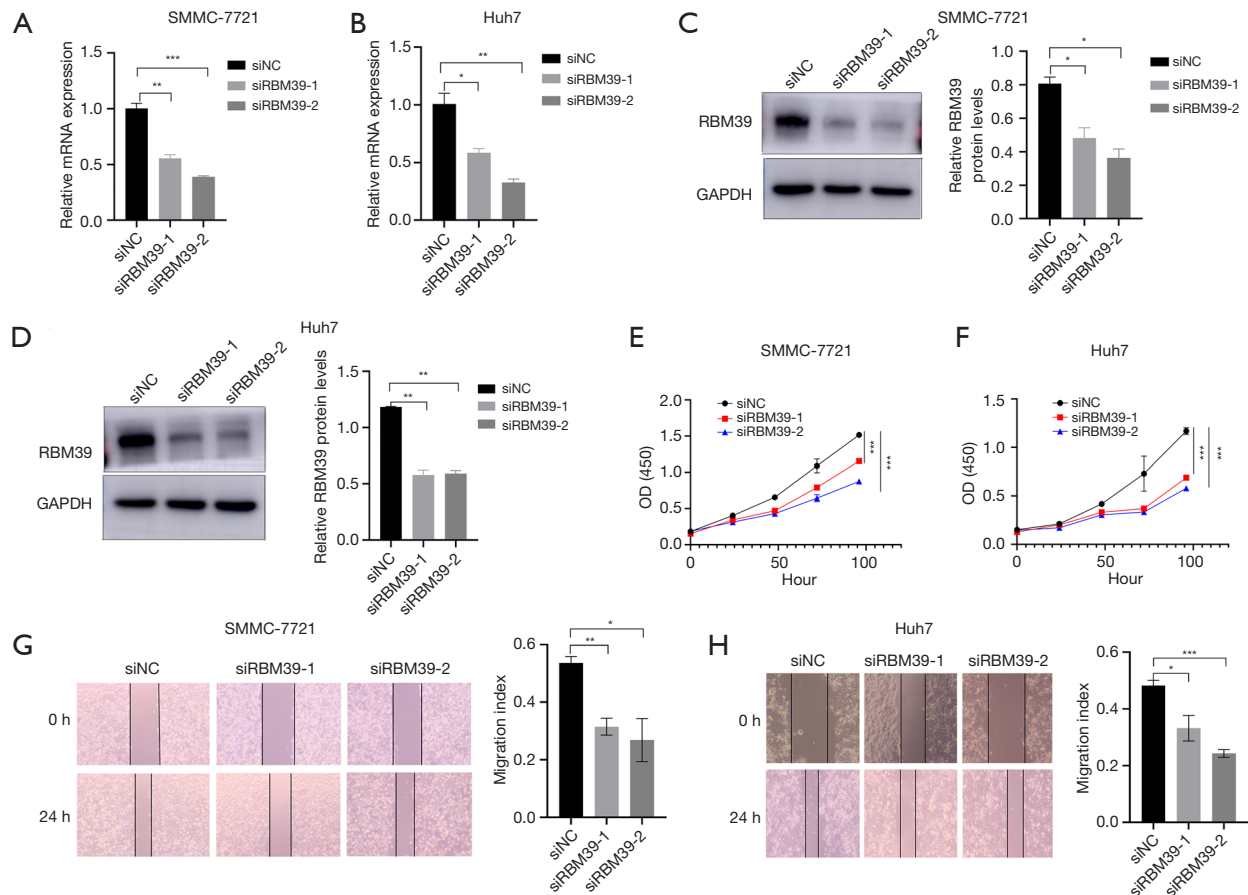


Figure 8 Knockdown of RBM39 inhibits the proliferation and migration of HCC cells. Interference efficiency of siRBM39 was assessed by qRT-PCR (A,B) and western blot (C,D) after transfection in SMMC-7721 and Huh7 cells. (E,F) Cell proliferation of SMMC-7721 and Huh7 cells after RBM39 knocking down, as determined by CCK8 assays. (G,H) Wound healing assays were performed in SMMC-7721 and Huh7 cells with RBM39 knocking down. The images were captured under an optical microscope ($\times 40$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction.

with a poor prognosis. However, the correlation between RBM39 expression and clinical characteristics and survival of patients with HCC remains unclear.

In this study, we performed a bioinformatics investigation using the TCGA database and found that RBM39 was upregulated in HCC. Upon further investigation of the relationship between RBM39 and clinical characteristics, we observed a significant correlation between RBM39 expression, AFP levels, and the histological grade of patients with HCC. Prognostic analysis using Kaplan-Meier curves further demonstrated that high expression of RBM39 predicted poor OS, DSS, and PFI in HCC. Cox regression analysis further confirmed that RBM39 expression can be considered an independent prognostic factor for OS. Subsequent ROC analysis indicated that RBM39 could be considered as a potential biomarker for HCC diagnosis. In addition, our experimental results validated the findings of the bioinformatics analyses. Taken together, these results indicate that RBM39 can be used as a potential biomarker to predict OS in patients with HCC.

RBM39 is overexpressed in a wide range of cancers (23), and it has been reported that knockdown of RBM39 reduces the proliferation of cancer cells in AML (10), breast cancer (14), colorectal cancer (16), gastric cancer (24), neuroblastoma (25,26), prostate cancer (27) and multiple myeloma (28). In this study, we found a similar effect of RBM39 in HCC. The proliferation and migration abilities of HCC cells were significantly reduced when RBM39 was knocked down, suggesting a cancer-promoting effect of RBM39.

Recent studies have reported that the immune microenvironment plays a major role in tumorigenesis and cancer progression. In addition to tumor cells, the tumor microenvironment (TME) includes numerous immune cells including T cells, B cells, tumor-related macrophages, tumor-related neutrophils, tumor-related fibroblasts, dendritic cells (DCs), extracellular matrix, and other related molecules (29). A comprehensive understanding of the intricate interactions between TME and tumor cells is imperative in order to devise novel and effective therapeutic strategies for HCC. Patients with HCC commonly experience immune system impairment, resulting in the accumulation of immune suppressive cells, such as regulatory T cells and Th2 cells, within the TME (30). Conversely, the essential immune cell subsets necessary for an effective anti-tumor immune response are typically reduced (30). During tumorigenesis and progression, there

is a shift from the Th1/Th2 balance to the Th2-dominant immunity (31), which facilitates a tumor-supportive environment that can promote cancer development, progression, metastasis, and immune escape. In addition, Th2 cells secrete IL-4 and IL-10 molecules, and promote the conversion of M1 macrophages into immunosuppressive M2 macrophages (32,33), which further leads to suppression of the host immune system and promotes tumorigenesis. To identify the potential role of RBM39 in the immune microenvironment in HCC, we performed an immune microenvironment analysis and found that there was a significant positive correlation between RBM39 expression with the level of Th2 cell infiltration, indicating that RBM39 might participate in the tumor immune escape by mediating Th2 cell infiltration.

As antigen-presenting cells (APCs) of the immune system, DC are central regulators of the adaptive immune response (34). They play a central role in initiating antigen-specific immunity and tolerance (35). In cancer, DC can present tumor-associated antigens on MHC molecules. Simultaneously, proximal tumor DC cells take up new antigens during tumorigenesis and present them to cognate T cells to initiate anti-tumor T cell responses (36). Recently, Lu *et al.* demonstrated that the splicing modulation induced by RBM39 degrader indisulam generated neoantigens that triggered anti-tumor T cell response, thereby inhibited tumor growth and enhanced checkpoint blockade in a manner dependent on host T cells and peptides presented on tumor MHC class I (37). In this study, we found a significant negative correlation between RBM39 and DC cells. This indicates that the upregulation of RBM39 in HCC affects and interferes with the functional role of DC in presenting tumor antigens to promote tumor immunity. Alterations in RNA splicing within cancer cells generate neoepitopes that serve as neoantigens. These neoantigens have demonstrated the ability to elicit robust anti-tumor immune responses (38). In a recent study, a vaccine designed to target personalized neoantigens has exhibited promising safety and efficacy in suppressing the recurrence of HCC (39). RBM39, a well-characterized splicing factor, has been demonstrated to exhibit a correlation with the abundance of neoantigens across different types of cancer (40). Thus, it is valuable to investigate whether targeting RBM39 could potentially augment the anti-tumor immunity of HCC through its involvement in neoantigen production.

In recent years, combination therapy involving targeted

therapy, specifically tyrosine kinase inhibitors (TKIs), along with immunotherapy utilizing immune checkpoint inhibitors (ICIs), has shown remarkable effectiveness in advanced HCC by reprogramming the immunosuppressive TME (41). However, the application of these therapies is hindered by the inter-individual and intra-tumoral heterogeneity of HCC, as well as the lack of reliable biomarkers for accurately predicting therapeutic responses. In this study, we discovered a correlation between RBM39 and immune infiltration in HCC, suggesting its potential as both a promising therapeutic target and a predictive biomarker for HCC treatment.

GSEA results demonstrated the association of RBM39 expression with primary immunodeficiency pathways, which, combined with the relationship between RBM39 and tumor immune infiltrating cells, suggests that high RBM39 expression might promote tumor immune escape. However, it is important to note that the effect of RBM39 on tumorigenesis may not be confined to the regulation of immune responses, as there is a significant association between tumor RBM39 expression and other pathways. P53 is an important regulatory factor of cell cycle and DNA replication (42). Dysregulation in cell cycle can lead to aberrant cellular proliferation and tumorigenesis (43). In this study, upregulation of RBM39 was found to be significantly associated with cell cycle, DNA replication, and p53 signaling pathways. Notably, these pathways are targeted by casitabine. Previous studies have demonstrated the efficacy and safety of metronomic capecitabine as a second-line systemic therapy for HCC patients who experienced discontinuation of sorafenib due to either toxicity or tumor progression (44,45). Future studies should investigate the association between RBM39 and capecitabine, which might enhance the potential of systemic treatment for HCC patients who are currently ineligible for approved systemic therapies.

Conclusions

In this study, we found that that upregulation of RBM39 predicted poor survival and was correlated with immune infiltration. In addition, elevated RBM39 expression was associated with numerous pathways, such as the cell cycle, DNA replication, the p53 signaling pathway, and primary immunodeficiency. Moreover, RBM39 knockdown inhibited proliferation and migration of HCC cells. These findings highlight the oncogenic role of RBM39 and demonstrates RBM39 as a potential prognostic marker of HCC.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-2252/rc>

Data Sharing Statement: Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-2252/dss>

Peer Review File: Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-2252/prf>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-23-2252/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics committee of The First Affiliated Hospital of Zhengzhou University (2022-KY-1184-003). Informed consent was obtained from all individual participants.

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Supplementary

Table S1 GSEA analysis of genes associated with the expression of RBM39

Description	NES	P. adjust	Q value	Core enrichment
KEGG_CELL_CYCLE	2.751	0.0286	0.0228	CDC45/CDC20/CCNB2/CCNA2/CDK1/MAD2L1/TTK/CHEK1/CCNB1/MCM5/PTTG1/MCM2/CDC25A/CDC6/PLK1/BUB1B/ESPL1/CCNE1/ORC6/ORC1/CCNE2/MCM6/MCM4/DBF4/SKP2/CDC25B/BUB1/MYC/PCNA/E2F1/CDKN2A/CDC7/MCM7/SFN/HDAC2/E2F3/CDKN2C/PKMYT1/CDC25C/CDK4/MCM3/RAD21/CHEK2/TFDP1/E2F5/YWHAZ
KEGG_DNA_REPLICATION	2.356	0.0286	0.0228	MCM5/MCM2/MCM6/MCM4/FEN1/RFC4/PCNA/RNASEH2A/DNA2/POLE2/POLA2/MCM7/RFC2/PRIM1/PRIM2/MCM3/POLD1/RFC3/POLD2/POLE3/RPA3
KEGG_P53_SIGNALING_PATHWAY	1.823	0.0286	0.0228	CCNB2/RRM2/CDK1/CHEK1/CCNB1/GTSE1/CCNE1/CCNE2/SERPINB5/CDKN2A/SFN/CYCS/CDK4/BID/ADGRB1/CHEK2
KEGG_PRIMARY_IMMUNODEFICIENCY	2.044	0.0286	0.0228	ICOS/TAP1/LCK/IL2RG/IL7R/CD3D/CD40LG/CD8A/PTPRC/CD3E/ADANG/UNG/TAP2/CD19

GSEA, gene set enrichment analysis; RBM39, RNA-binding motif protein 39; NES, Normalized Enrichment Score.

Table S2 Correlation between RBM39 expression and clinical characteristics of 66 HCC patients

Characteristic	Low expression of RBM39	High expression of RBM39	P
Gender, n (%)			>0.99
Female	5 (7.58)	4 (6.06)	
Male	28 (42.42)	29 (43.94)	
Age, n (%)			0.196
≤60	24 (36.36)	19 (28.79)	
>60	9 (13.64)	14 (21.21)	
Pathological stage, n (%)			0.284
I-II	21 (31.82)	25 (37.88)	
III	12 (18.18)	8 (12.12)	
T stage, n (%)			0.428
T1	21 (31.82)	24 (36.36)	
T2-T3	12 (18.18)	9 (13.64)	
N stage, n (%)			>0.99
N0	32 (48.48)	33 (50.00)	
N1	1 (1.52)	0 (0.00)	
M stage, n (%)			>0.99
M0	33 (50.00)	32 (48.48)	
M1	0 (0.00)	1 (1.52)	
Clinical stage, n (%)			>0.99
I-II	28 (42.42)	29 (43.94)	
III-IV	5 (7.58)	4 (6.06)	
Recurrence, n (%)			0.460
No recurrence	18 (27.27)	15 (22.73)	
Relapse	15 (22.73)	18 (27.27)	

RBM39, RNA-binding motif protein 39; HCC, hepatocellular carcinoma.