RBM39 is a potential prognostic biomarker associated with immune infiltrates in hepatocellular carcinoma

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Abstract

RNA-binding motif protein 39 (RBM39) is a well-studied RNA-binding protein that has been reported to be associated with the process of tumorigenesis and the development of numerous cancers. However, its role in hepatocellular carcinoma (HCC) remains unclear. This study aimed to understand the prognostic value of RBM39 in HCC by investigating the relationship between RBM39 expression and clinicopathological features. The cancer genome atlas (TCGA) and gene expression omnibus (GEO) databases were used to analyze the differential expression of RBM39 between normal tissues and that of HCC. Kaplan–Meier and COX regression models were used to analyze the correlation between RBM39 expression and survival rate in the case of HCC. Moreover, gene set enrichment analysis (GSEA) was performed to identify key pathways associated with RBM39. The correlation of RBM39 with tumor immune infiltration was evaluated by single sample gene set enrichment analysis (ssGSEA) using TCGA data. The data generated by bioinformatic approach were further validated using qRT-PCR and immunohistochemistry. The CCK8 and Wound healing assays were performed to investigate the biological function of RBM39 in HCC cells. Our results indicated that there was a significant upregulation of RBM39 in HCC as compared to that of normal tissues. High RBM39 expression was significantly associated with advanced T-stage, histological grade, and pathological stage, and predicted poor overall survival (OS), disease-free survival (DSS), and progression-free interval (PFI) in HCC patients. Multivariate Cox analysis further confirmed that the upregulation of RBM39 expression was an independent prognostic factor for OS in HCC. Moreover, GSEA enrichment analysis indicated that RBM39 was functionally involved in pathways associated with cell cycle, DNA replication, P53, and primary immunodeficiency. RBM39 expression was associated with the infiltrating levels of Th2 cells and DC cells. Knockdown of RBM39 significantly inhibited the proliferation and migration of HCC cells. Altogether, these findings suggest an important role of RBM39 in the development, diagnosis, and prognosis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies, with an overall sixth-highest incidence rate and third-highest cancer-related mortality rate in the world (Sung et al., 2021). Although surgical approaches and immune-targeted therapy have greatly improved the survival rate (Moriguchi et al., 2017, Xu et al., 2022), HCC is mostly detected and diagnosed in the middle and late stages of the disease, with limited therapeutic options (Medavaram and Zhang 2018). Therefore, it is pivotal to investigate the underlying mechanisms of HCC development and progression, to identify potential prognostic biomarkers.

RBM39, also known as HCC1 or CAPERα (Xu et al., 2021), is an important serine/arginine-rich (SR) RNA binding protein. RBM39 has been identified as a pre-mRNA splicing factor that facilitates exon incorporation by regulating exon box splicing (Xu et al., 2022). Independent of its pre-mRNA splicing factor activity, RBM39 has also been shown to function as a transcriptional cofactor of steroidal nuclear receptors such as ESR1/ER-α, ESR2/ER-β, and JUN/AP-1 (Xu et al. 2021, Xu et al. 2022). Moreover,
RBM39 has been reported in numerous tumors. In particular, RBM39 was found to be upregulated in a variety of tumors such as breast cancer (Mercier et al., 2014, Campbell et al., 2018), acute myeloid leukemia (AML) (Wang et al., 2019), colorectal cancer (Sillars-Hardebol et al., 2012), and lung cancer (Chai et al., 2014). Aryl sulfonamides have demonstrated therapeutic efficiency in-vitro via recruitment of RBM39 to DCAF15, leading to ubiquitination of RBM39 and subsequent degradation, resulting in RNA splicing alterations and cell death in cancer cell lines (Ting et al., 2019). Hematopoietic or lymphoid malignancies are more sensitive to aryl sulfonamide cytotoxicity, indicating the susceptibility of these cancers to splicing changes (Han et al., 2017). In AML, RBM39 promotes the expression of mRNAs encoding HOX9 targets, which are essential for the maintenance of leukemia (Wang et al. 2019).

Furthermore, in the case of AML, aryl sulfonamides such as Indisulam have been shown to induce ubiquitination and proteasomal degradation of RBM39 by altering the substrate combination with CRL4-DCAF15 E3 ubiquitin ligase. This in turn results in direct and selective degradation of RBM39 (Hsiehchen et al., 2020). In the case of breast cancer, RBM39 has been shown to directly interact with ERα, Erβ, and AP-1/c-jun to regulate the proliferation of human breast cancer cells (Mercier et al. 2014). RBM39 has also been implicated in lung cancers, especially non-small cell lung cancer, wherein it is mainly localized in the nucleus of lung cancer cells and induces immune responses in patients diagnosed with lung cancer. Moreover, overexpression of RBM39 promotes lung cancer cell proliferation and migration (Chai et al. 2014). These findings indicate that an upregulation of RBM39 contributes to the development of numerous cancers. However, the expression and prognostic value of RBM39 in HCC and its underlying mechanisms remain largely unknown. Therefore, in this study, we utilized a bioinformatic approach using high-throughput RNA sequencing data extracted from TCGA to demonstrate the efficacy of RBM39 as a novel and effective prognostic marker for HCC.

**Materials And Methods**

**Acquisition of RBM39 expression data and its clinical relevance**

An online database TIMER2.0 (http://timer.cistrome.org/) was used to detect the mRNA levels of RBM39 in pan-cancers. The RNA-seq data (TPM format) of 374 HCC tissues and 50 normal liver tissues and the clinical information of the corresponding patients were retrieved from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). In addition, the immunohistochemistry (IHC) results of 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 expression profiles of HCC and normal livers were used to further validate the expression levels of RBM39 mRNA in HCC.

**Gene set enrichment analysis (GSEA)**

Signaling pathways associated with RBM39 expression in HCC were analyzed using R statistical software (version 3.6.3). The gene set "c2.cp.v7.2.symbols.GMT" was used. An FDR<0.25, P<0.05, and normalized enrichment score (NES) >1 were considered statistically significant.
**Immune Infiltration Analysis**

A single sample gene set enrichment analysis (ssGSEA) approach was carried out in HCC samples to analyze the correlation of RBM39 expression with 24 types of immune cells using the GSVA package in R. Spearman correlation and Wilcoxon rank sum tests were performed to assess and compare immune cell infiltration in the RBM39 high and low expression groups.

**Immunohistochemistry (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), which contained 10 HCC and 10 normal liver tissues. The tissues were dewaxed and hydrated, followed by antigen repaired and endogenous peroxidase blocked. Then the slide was incubated with primary antibody overnight at 4°C. After incubated with secondary antibody, the slide was stained by Diaminobenzidine (DAB). Finally, the staining results were evaluated independently 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 (≥50%). The intensity of staining was scored visually and stratified as follows: 1 (negative), 2 (light yellow), 3 (light brown) and 4 (dark brown). A final immunoreactivity score for each case was obtained by multiplying the percentage and intensity scores.

**Cell culture**

The human 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 DMEM medium enriched with 10% FBS, 1% penicillin and streptomycin. Cells were cultured in cell incubator containing 5% CO2 at 37°C.

**Transfection of small interfering RNA**

The small interfering RNA (siRNA) targeting RBM39 was purchased from RiboBio (Guangzhou, China). siNC, siRBM39-1 and siRBM39-2 were transfected into SMMC-7721 and Huh7 cell lines using lipo2000 (Invitrogen, USA), respectively. All manipulations were performed according to the instructions. The interference efficiency was verified by qPCR and Western Blotting. The sequences of siRNAs were as follows: siRBM39-1: 5′-GAAGCGAAGTAGAGACAGA-3′; siRBM39-2: 5′- GGAAAGGACTGGAATTGAT-3′.

**Quantitative Real-Time PCR (qRT-PCR)**

A commercial cDNA microarray was purchased from Outdo Biotechnology (Shanghai, China), including 66 HCC tissues and 21 para-cancerous tissues. The method of this microarray qPCR and the statistics of the results are as described previously (Wang et al., 2020). 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'- TCCGTTTCTTACTTTTGCTTCTC-3'.
**Western Blotting**

Cells were collected 72 hours after transfection and lysed with RIPA lysis buffer (Yase, China) and protease inhibitor (Sollerbauer, China). Protein concentrations were measured with BCA Protein Assay Kit (Yase, China) and adjusted to equal concentrations. Protein lysates were then separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, USA). The membranes were then blocked with 5% skimmed milk for 2 h at room temperature and incubated at 4 °C with anti-RBM39 (21339-1-AP, 1:1000, Proteintech, China), anti-GAPDH (10494-1-AP, 1:5000, Proteintech, China) primary antibodies overnight. The immunoblots were detected using ECL chemiluminescence kit (Yarase, China).

**CCK8 Assay**

The HCC cells were plated into 96-well plated at a density of $2 \times 10^3$ cells per well, and incubated for 24h, 48h, 72h and 96h. Subsequently, 10ul of CCK-8 reagent (GK10001 glpbio USA) was added to each well. Then, the cells were incubated for 1.5 h at 37°C and absorbance were measured at 450 nm. Five replicates were used for each group, and the experiment were repeated for three times.

**Wound healing assay**

Migration ability of HCC cells was evaluated by wound healing assay. When the cells took up 95% area of the 12-well plate bottom, the monolayer was scraped with a 10ul pipette tip. After cells had been washed twice with phosphate-buffered saline (PBS), the cell culture medium was replaced with basic DMEM medium.

**Statistical analysis**

The statistical data obtained from TCGA were merged and processed using R3.6.3. Welch's one-way ANOVA test, Welch's t-test, and independent samples t-test were used to analyze the expression levels of RBM39 among different groups. The chi-square test and logistic regression analysis were performed to analyze the correlation of RBM39 mRNA expression with clinical characteristics of HCC patients, including age, gender, TNM stage, histological grade, pathological stage, AFP, and tumor status. Univariate and multivariate Cox regression analyses were performed to assess the prognostic value of RBM39 expression and other clinicopathological factors on the overall survival (OS) of HCC. The hazard ratio (HR) and 95% confidence interval (CI) were measured to assess the risk of each factor. Kaplan-Meier curves were plotted to assess the prognostic value of RBM39. The diagnostic value of RBM39 was further analyzed using ROC curve analysis. The statistical analysis in vitro experiments was operated by GraphPad Prism V9.3.1. Student's t-test was used to analyze the differences of the data. A P-value <0.05 was considered statistically significant.

**Result**
**Expression levels of RBM39 mRNA in HCC**

To begin with, the difference in RBM39 expression in different tumors and normal tissues was analyzed using the TIMER2.0 online data platform. The results indicated that RBM39 was upregulated in numerous cancers including stomach adenocarcinoma (STAD), lung adenocarcinoma (LUAD), colon adenocarcinoma (COAD), prostate adenocarcinoma (PRAD), and HCC (Figure 1A). In this study, we aimed to analyze the expression levels of RBM39 in HCC using the TCGA database. Subsequently, gene expression data were extracted from the TCGA HCC dataset, including a total of 50 cases of normal tissues and a total of 374 cases of HCC tissues. Wilcoxon test was further used to compare the differences in RBM39 mRNA expression between normal and tumor tissues in both unpaired and paired tissues. The results indicated significant upregulation of RBM39 mRNA in HCC as compared to that in normal tissues (Figure 1B and C). Moreover, these results were further validated using GSE6764 and GSE14520 datasets from the GEO database (Figures 1D and E). In addition, the HPA database helped us identify an increased protein level of RBM39 in HCC as compared to that in normal liver tissue (Figure 1F).

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

The clinical information of patients diagnosed with HCC from the official website of TCGA mainly includes age, gender, TNM stage, pathological stage, histological grading, AFP, tumor status, and so on. As shown in Figure 2, elevated levels of RBM39 expression show a significant correlation with gender (P = 0.01), N stage (P = 0.021), histological grade (P < 0.001), pathological stage (P = 0.004), AFP (P <0.001) and tumor status (P = 0.011). Patients diagnosed with HCC were categorized into high and low-expression groups according to the median value of RBM39 mRNA expression, and their corresponding clinical parameters are summarized in Table 1. The correlation of RBM39 expression with clinical parameters such as age, gender, TNM stage, pathological stage, histological grade, AFP, fibrosis Ishak score, vascular invasion, and tumor status was analyzed. Our results indicated a significant association of RBM39 expression with age (P=0.037), gender (P=0.029), histological grade (P=0.002), and AFP level (P <0.001). Moreover, the logistic analysis revealed the association of an increased level of RBM39 expression with advanced histological grading (OR: 2.047 (1.344-3.162), P=0.001) and high AFP level (OR:2.952 (1.653-5.426), P<0.001), as summarized in Table 2.

**Role of RBM39 in Survival of HCC**

Subsequently, the prognostic value of RBM39 in HCC was evaluated using Kaplan-Meier survival analysis using TCGA data. As shown in Figures 3 A to C, high expression of RBM39 mRNA is associated with poor OS, disease-free survival (DFS), and disease-free progression (DFI) in HCC. In addition, the prognostic value of RBM39 was evaluated in OS of different subtypes of HCC, according to clinical features such as TNM stage, pathological stage, and histological grade. As shown in Figure 3D to K, the elevated levels of RBM39 were found to be significantly associated with poor OS in patients with T3/T4 (P=0.004), N0 (P<0.001), M0 (P<0.001), pathological stage S3/S4(P=0.014), histological grade G1/G2 (P=0.016), and G3/G4 (P=0.025). Furthermore, the findings of Univariate Cox regression analysis indicated that the T
stage ($P<0.001$), M stage ($P=0.017$), and pathological stage ($P<0.001$) were important parameters that determine 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, $P=0.029$) (Table 3). Taken together, these results suggest that RBM39 expression could be a potential biomarker for predicting the overall survival in patients diagnosed with HCC.

**The diagnostic value of RBM39 for HCC**

ROC curve analysis was performed to assess the diagnostic value of RBM39 in HCC. The area under the curve (AUC) was estimated to be 0.916, indicating that RBM39 has a high diagnostic value in HCC (Figure 4A). In addition, subgroup analysis showed a strong correlation of the diagnostic value of RBM39 expression with 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 to E). These results further illustrate the potential early diagnostic value of RBM39.

**Signaling pathways associated with RBM39 in HCC**

Given the relevance of RBM39 to the clinical features, prognosis, and diagnostic value of HCC, GSEA was performed using data from the TCGA database to further understand the relevant role of RBM39 in HCC. Our results indicated a strong association of an upregulation of RBM39 with several signaling pathways including 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 Supplementary Table 1.

**Relationship between RBM39 and tumor-infiltrating immune cells in HCC**

Over the past decade, a growing body of evidence suggests the important role of immune-infiltrating cells in cancer (Gentles et al., 2015). Tian et al. estimated the score distribution of tumor-infiltrating immune cells in HCC using a CIBERSORT algorithm. Their findings an upregulation of most immune checkpoints and their corresponding ligands in HCC as compared to that of normal tissue (Tian et al., 2022). Therefore, to investigate the association of RBM39 expression with immune infiltration in HCC, we analyzed the correlation between RBM39 and the infiltration levels of 24 types of immune cells using the ssGSEA algorithm (Figure 6A). RBM39 expression was found to be positively correlated with Th2 cells (Spearman $R = 0.345$, $p < 0.001$) (Figure 6B). However, DC cells were found to be negatively correlated with RBM39 expression (Spearman $R = -0.362$, $p < 0.001$) (Figure 6C). In addition, the differences in immune infiltration of Th2 cells and DC cells in RBM39 high and low expression groups were also analyzed (Figure 6D to E). The results showed that the infiltration levels of Th2 cells were significantly higher ($P < 0.001$), while the infiltration levels of DC cells ($P < 0.001$) were significantly lower in the RBM39 high expression group as compared to the RBM39 low expression group. These findings suggest a vital role of RBM39 in immune infiltration associated with HCC.
Validation of bioinformatics results 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 elevated in HCC tissues compared to normal tissues at both the mRNA and protein levels (Figure 7A and B). However, we were unable to demonstrate any significant association between RBM39 mRNA levels and the clinicopathological features of HCC (Supplementary Table 2). This may be due to the small sample size. We further analyzed the relationship between RBM39 mRNA expression and overall survival in HCC using Kaplan-Meier analysis. Although not significant, we observed lower overall survival in HCC patients with high RBM39 expression level (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 the development of HCC, we knocked down RBM39 in SMMC-7721 and Huh7 cells using siRNAs and verified the knockdown efficiency using qRT-PCR and western blot (Figure 8A and B). Subsequently, we examined the effect of RBM39 knockdown on the proliferation of HCC cells by CCK8 assay. As shown in the Figure 8C, knockdown of RBM39 significantly inhibited the proliferation ability of SMMC-7721 and Huh7 cells. In addition, the results of the wound healing assay showed that the migration ability of the HCC cells were inhibited after knocking down RBM39 (Figure 8D). These findings indicated that knockdown of RBM39 inhibited the proliferation and migration of HCC cells, suggesting that RBM39 may be involved in the 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 (Xu et al. 2022). Mutations and dysregulated expression of these proteins are associated with numerous human diseases, including cancer. RBM39 is an evolutionarily conserved RBP in animals that are functionally involved in transcriptional co-regulation and alternative RNA splicing. It is upregulated in numerous tumors such as AML (Wang et al. 2019), breast cancer (Mercier et al. 2014, Puvvula et al., 2021), and lung cancer (Chai et al. 2014, Li et al., 2017) and is often associated with a poor prognosis. However, the correlation of RBM39 expression with the clinical characteristics and survival of HCC patients remains unclear.

In this study, we used a bioinformatic approach using the TGCA database to reveal that there is an upregulation of RBM39 expression in HCC tissues as compared to that in normal tissues. Upon further investigation of the relationship between RBM39 and clinical characteristics of patients diagnosed with HCC, we observed a significant correlation between RBM39 expression with gender, age, AFP, and histological grade of the patients. An increased expression of RBM39 is associated with a poor survival rate in acute myeloid leukemia and breast cancer (Xu et al. 2021, Xu et al. 2022). In this study, we
demonstrated the high expression of RBM39 to be significantly associated with advanced pathological stages of HCC using logistic analysis. Prognostic analysis using Kaplan-Meier curves further demonstrated that high expression of RBM39 was significantly associated with poor OS, DSS, and PFI in HCC. Cox regression analysis further confirmed that RBM39 expression could be considered as an independent prognostic factor for OS in HCC. A subsequent ROC analysis indicated that RBM39 could be considered a potential biomarker for the diagnosis of HCC in patients. In addition, our experimental results further validated the findings of our bioinformatics analyses. Altogether, these results indicate that RBM39 could be used as a potential biomarker to predict overall survival in HCC patients. However, whether RBM39 can be used in combination with clinical features and other biomarkers to build a predictive risk model remains to be further investigated.

RBM39 is overexpressed in a wide range of cancers (Eleouet et al., 2023), and it has been reported that knockdown of RBM39 reduced the proliferation of cancer cells in AML (Wang et al., 2019), breast (Mercier et al., 2014), colorectal (Han et al., 2017, Uehara et al., 2017), gastric (Lu et al., 2021), neuroblastoma (Singh et al., 2021, Nijhuis et al., 2022), prostate (Melnyk et al., 2020) and multiple myeloma (Tong et al., 2020). 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. However, the exact underlying mechanism needs to be further investigated.

Recent studies have reported the major role of the immune microenvironment in tumorigenesis and progression. In the case of liver cancer, in addition to tumor cells, the tumor microenvironment includes numerous immune cells including T cells, B cells, tumor-related macrophages, tumor-related neutrophils, tumor-related fibroblasts, dendritic cells, extracellular matrix, and other related molecules (Kurebayashi et al., 2018). 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. During tumorigenesis and progression, there is a shift from the Th1/Th2 balance to Th2 (Maazi and Akbari 2017), which facilitates a tumor-supportive environment that can promote cancer development, progression, metastasis, and immune escape. In addition, Th2 can promote the conversion of M1 macrophages into immunosuppressive M2 macrophages (DeNardo et al., 2009), which further leads to the suppression of the host immune system, promoting tumorigenesis. Furthermore, Th2 cells suppress the host immune cells by secreting IL-4 and IL-10 molecules, inducing tumorigenesis (Zhao et al., 2015). Our results suggest that overexpression of RBM39 in HCC promotes Th2 cell infiltration, which further promotes tumorigenesis, development, and metastasis.

As antigen-presenting cells (APCs) of the immune system, DC cells are central regulators of the adaptive immune response (Gardner and Ruffell 2016). They play a central role in initiating antigen-specific immunity and tolerance (Steinman 2012). In cancer, DC cells 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 (Huang et al., 2022). Our results 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 cells in presenting tumor antigens to promote tumor immunity. In a recent study, Lu et al. demonstrated the promotion of antitumor immunity by pharmacological modulation of splicing using RBM39 degraders by enhancing the response to immune checkpoint blockade (Lu et al., 2021). Therefore, RBM39 may serve as a potential target of immunotherapy in HCC.

GSEA results demonstrated the association of RBM39 expression with primary immunodeficiency pathways. This further confirmed the enrichment of immune-related genes in HCC, which, combined with the relationship between RBM39 and tumor immune infiltrating cells, suggests that high RBM39 expression promotes 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 such as cell cycle, DNA replication, and p53. P53 is an important regulatory factor of cell cycle and DNA replication (Yang et al., 2019, Zhang et al., 2019). The cell cycle is associated with numerous cell cycle proteins such as cell cycle-dependent protein kinases that are activated at specific times to drive cells to complete the cell cycle. However, dysregulation in the cell cycle can lead to aberrant cellular proliferation, which can further lead to tumorigenesis (O'Leary et al., 2016). A typical example of disruption of the surveillance mechanism during tumor development is the mutation of the p53 gene. A large body of evidence suggests that deletion or mutational inactivation of the p53 gene plays an important role in tumor development (Ranjan and Iwakuma 2016, Engeland 2018). In this study, upregulation of RBM39 was found to be significantly associated with cell cycle, DNA replication, and p53 signaling pathways, suggesting that RBM39 plays an important role in the development of HCC. However, the underlying mechanism of RBM39 in each pathway needs to be further explored.

**Conclusion**

In this study, we investigated the expression levels of RBM39 and evaluated its role in HCC progression as well as its prognostic value. We found an increase in the expression of RBM39 predicted poor survival and was identified as an independent risk factor for OS in HCC patients. Knockdown of RBM39 inhibits the proliferation and migration of HCC cells. In addition, our results indicated that RBM39 may promote tumor progression through its association with numerous pathways such as cell cycle, DNA replication, and p53 signaling pathways, and regulation of immune cell infiltration in HCC. These findings provide a novel insight into the role of RBM39 in HCC development and progression. However, further studies involving functional experiments are required to validate the findings of this study.

**Declarations**

**Ethics approval and consent to participate**

The experiment was approved by the Ethics committee of the First Affiliated Hospital of Zhengzhou University. (Ethics Batch Number 2022-KY-1184-003)
Consent for publication

Not applicable.

Competing interests

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.

Author Contributions

FC, PW and CZ conceived and designed the study and wrote the initial draft of the manuscript. FC, WW, and CZ collected and analyzed the data. All authors contributed to the article and approved the submitted version.

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Availability of data and materials


Acknowledgements

Not applicable.

References


**Tables**

Table 1 Relationship between RBM39 expression and clinical features in HCC patients.
<table>
<thead>
<tr>
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<th>Low expression of RBM39</th>
<th>High expression of RBM39</th>
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<td>Age, n (%)</td>
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<tr>
<td>G1</td>
<td>38 (10.4%)</td>
<td>17 (4.6%)</td>
<td>0.002</td>
</tr>
<tr>
<td>G2</td>
<td>93 (25.4%)</td>
<td>84 (23%)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>47 (12.8%)</td>
<td>75 (20.5%)</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>5 (1.4%)</td>
<td>7 (1.9%)</td>
<td></td>
</tr>
<tr>
<td>AFP (ng/ml), n (%)</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 2 Logistic regression analysis of the correlation between RBM39 expression and clinical features.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (N)</th>
<th>Odds Ratio (OR)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T stage (T3/T4 vs. T1/T2)</td>
<td>371</td>
<td>1.343 (0.839-2.162)</td>
<td>0.220</td>
</tr>
<tr>
<td>N stage (N1 vs. N0)</td>
<td>258</td>
<td>2.953 (0.373-60.136)</td>
<td>0.351</td>
</tr>
<tr>
<td>M stage (M1 vs. M0)</td>
<td>272</td>
<td>0.328 (0.016-2.601)</td>
<td>0.338</td>
</tr>
<tr>
<td>Pathologic stage (III/IV vs. I/II)</td>
<td>350</td>
<td>1.457 (0.901-2.370)</td>
<td>0.127</td>
</tr>
<tr>
<td>Histologic grade (G3/G4 vs. G1/G2)</td>
<td>369</td>
<td>2.047 (1.344-3.162)</td>
<td>0.001</td>
</tr>
<tr>
<td>AFP(ng/ml) (&gt;400 vs. ≤ 400)</td>
<td>280</td>
<td>2.952 (1.653-5.426)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrosis ishak score (1/2&amp;3/4&amp;5/6 vs. 0)</td>
<td>215</td>
<td>1.298 (0.735-2.313)</td>
<td>0.371</td>
</tr>
<tr>
<td>Vascular invasion (Yes vs. No)</td>
<td>318</td>
<td>1.353 (0.852-2.154)</td>
<td>0.201</td>
</tr>
<tr>
<td>Tumor status (With Tumor vs. tumor free)</td>
<td>355</td>
<td>1.408 (0.924-2.151)</td>
<td>0.112</td>
</tr>
</tbody>
</table>

Table 3 Univariate and multivariate Cox regression analysis of RBM39 expression and clinical characteristics.
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (N)</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T stage (T3/T4 vs. T1/T2)</td>
<td>370</td>
<td>2.598 (1.826-3.697)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.809 (0.244-13.414)</td>
<td>0.562</td>
</tr>
<tr>
<td>N stage (N1 vs. N0)</td>
<td>258</td>
<td>2.029 (0.497-8.281)</td>
<td>0.324</td>
</tr>
<tr>
<td>M stage (M1 vs. M0)</td>
<td>272</td>
<td>4.077 (1.281-12.973)</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.684 (0.391-7.250)</td>
<td>0.484</td>
</tr>
<tr>
<td>Pathologic stage (III/IV vs. I/II)</td>
<td>349</td>
<td>2.504 (1.727-3.631)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.378 (0.187-10.150)</td>
<td>0.753</td>
</tr>
<tr>
<td>Histologic grade (G3/G4 vs. G1/G2)</td>
<td>368</td>
<td>1.091 (0.761-1.564)</td>
<td>0.636</td>
</tr>
<tr>
<td>Fibrosis ishak score (1/2&amp;3/4&amp;5/6 vs.0)</td>
<td>214</td>
<td>0.772 (0.465-1.281)</td>
<td>0.316</td>
</tr>
<tr>
<td>Vascular invasion (Yes vs. No)</td>
<td>317</td>
<td>1.344 (0.887-2.035)</td>
<td>0.163</td>
</tr>
<tr>
<td>RBM39 (High vs. Low)</td>
<td>373</td>
<td>1.478 (1.041-2.099)</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.668 (1.053-2.644)</td>
<td>0.029</td>
</tr>
</tbody>
</table>

**Figures**
The expression level of RBM39 in HCC. (A) The expression level of RBM39 in different human tumors 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.
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) tumor status. Ns, not significant, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 
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) DFS, (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).
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/T4), (D) N stage (N0), and (E) M stage (M0) from normal livers. 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.

Figure 6
Relationship between RBM39 expression and immune infiltration. (A) Correlation between RBM39 expression level and the relative abundances of 24 types of immune cells. (B)-(C) The correlation of Th2 cells and DC cells with RBM39 expression. (D)-(E) Infiltration levels of Th2 cells and DC cells in different RBM39 expression.

Figure 7

RBM39 was upregulated in HCC and was associated with poor OS. (A) RT-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 pairs of HCC and normal liver tissues. (C) Kaplan-Meier curve for OS of HCC patients according to the high or low level of RBM39.
Figure 8

Knockdown of RBM39 inhibits the proliferation and migration of HCC cells. (A, B) Interference efficiency was assessed by qRT-PCR and western blot after transfection of siRBM39 or siNC in SMMC-7721 and Huh7 cells. (C) Cell proliferation of SMMC-7721 and Huh7 cells after RBM39 knocking down, as determined by CCK8 assays. (D) Wound healing assays were performed in SMMC-7721 and Huh7 cells with RBM39 knocked down.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.pdf
- originalfigureof8B.pdf