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ORIGINAL RESEARCH

Integrated Bioinformatics Analysis and Validation of the Prognostic Value of *RBM10* Expression in Hepatocellular Carcinoma

Shu-Jie Pang[1,*], Zhe Sun[1,*], Wen-Feng Lu[1,*], Hui Si-Ma[1], Zhi-Peng Lin[2], Yang Shi[1], Ying-Cheng Yang[1], Xi-Jun Zhao[1], Guang-Shun Yang[1], Guang-Zhi Jin[3], Ning Yang[1]

[1]Department V of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, 200438, People's Republic of China; [2]Department of Hepatobiliary Surgery, The 940th Hospital of CPLA Joint Logistics Support Force, Lanzhou, 730050, People's Republic of China; [3]Hongqiao International Institute of Medicine, Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200336, People's Republic of China

*These authors contributed equally to this work

Correspondence: Ning Yang, Department V of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, 200438, People's Republic of China, Tel +86 21 81877591, Fax +86 21 6556 6851, Email lancet00@163.com; Guang-Zhi Jin, Hongqiao International Institute of Medicine, Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, 200336, People's Republic of China, Email jgzhi@hotmail.com

Background: *RBM10*'s function in hepatocellular carcinoma (HCC) has rarely been addressed. We intend to explore the prognostic significance and therapeutic meaning of *RBM10* in HCC in this study.

Methods: Multiple common databases were integrated to analyze the expression status and prognostic meaning of *RBM10* in HCC. The relationship between *RBM10* mRNA level and clinical features was also assessed. Multiple enrichment analyses of the differentially expressed genes between *RBM10* high- and low- transcription groups were constructed by using R software (version 4.0.2). A Search Tool for Retrieval of Interacting Genes database was used to construct the protein–protein interaction network between *RBM10* and other proteins. A tumor immune estimation resource database was employed to identify the relationship between *RBM10* expression and immune cell infiltrates. The prognostic value of *RBM10* expression was validated in our HCC cohort by immunohistochemistry test.

Results: The transcription of *RBM10* mRNA was positively correlated with tumor histologic grade ($p < 0.001$), T classification ($p < 0.001$), and tumor stage ($p < 0.001$). High transcription of *RBM10* in HCC predicted a dismal overall survival ($p = 0.0037$) and recurrence-free survival ($p < 0.001$). Kyoto Encyclopedia of Genes and Genomes, Gene Ontology, and Gene Set Enrichment Analysis all revealed that *RBM10* was involved in the regulation of cell cycle, DNA replication, and immune-related pathways. Tumor immune estimation analysis revealed that *RBM10* transcription was positively related to multiple immune cell infiltrates and the expressions of *PD-1* and *PD-L1*.

Conclusion: *RBM10* was demonstrated to be a dismal prognostic factor and a potential biomarker for immune therapy in HCC in that it may be involved in the immune-related signaling pathways.

Keywords: *RBM10*, hepatocellular carcinoma, prognosis, differentially expressed gene, integrated bioinformatics analysis

Background

Liver cancer ranks the seventh most prevalent malignancy and the second leading cause of cancer-related mortality worldwide.[1] Hepatocellular carcinoma (HCC) is the predominant type of primary liver cancer, accounting for 85–90% of all primary liver cases.[2] Currently, surgical resection remains the mainstay of treatment for early-stage HCC. However, the resection rate of HCC is relatively low because most HCC cases were already in advanced stages at diagnosis mainly due to the lack of specific symptoms.[3,4] Despite tremendous therapeutic advancements in recent years, the prognosis of

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HCC remains dismal due to its aggressive biological characters.[5] Thus, it is of great significance to identify new diagnostic and prognostic markers for early detection, treatment, and prognosis prediction of the disease.

RNA-binding protein 10 (RBM10) is an important member of the RNA-binding protein family, containing two RNA recognition motifs for alternative RNA splicing, which could lead to the regulation of target gene expression after transcription.[6] Depending on its function in RNA splicing, *RBM10* participates in the regulation of multiple biological processes, including cell apoptosis, proliferation, cell cycle arrest, and inflammatory response.[7–10] Most previous studies on *RBM10* have mainly focused on the relationship between its mutation status and disease occurrence. For instance, it was found that *RBM10* mutation was the root cause of Talipes equinovarus, atrial septal defect (ASD), Robin sequence (TARP) syndrome.[11] Some recent studies demonstrated that the expression level of *RBM10* was associated with the regulation of cancer proliferation and metastasis.[12] Some studies in various cancer types suggest that *RBM10* may act as a tumor suppressor.[13–15] However, the role of *RBM10* in HCC remains uncertain. Therefore, clarification of the specific role of *RBM10* in HCC may throw light on the early detection and prognosis monitoring of HCC. The present study aimed to clarify the prognostic significance and potential role of *RBM10* in HCC based on existing public databases, and validate its prognostic value in our HCC cohort by using multiple bioinformatics analysis methods.

Materials and Methods

Raw Data of HCC Patients in Common Databases and Our Cohort

mRNA transcriptional data of HCC and normal liver tissues were downloaded from The Cancer Genome Atlas (TCGA) or Gene Expression Omnibus (GEO) databases (GSE112971, GSE60502). As both two datasets contain RNA-seq data of unpaired or paired HCC-normal tissues respectively which could testify the result of RBM10 expression difference between LIHC and normal liver tissue derived from the TCGA database, the above two datasets were analyzed in the current study. Survival data and clinical features were extracted from the clinical records of the patients in the TCGA. Clinicopathological characteristics and survival data of our HCC patient cohort were retrospectively collected and reviewed. They included 262 patients who received curative surgery in our hospital between January 2007 and January 2009. They were followed up postoperatively till July 2014. Of them, 21 patients were lost to follow-up, and finally, 241 patients were included for analysis.

Identification of Differentially Expressed Genes (DEGs)

DEGs between 374 liver cancer tissue samples and 50 normal liver tissue samples were identified with software R (version 4.0.3) by using the Limma package.[16] $|\log FC| > 0.6$ and $p < 0.001$ were regarded as the thresholds to recognize the DEGs. The volcano plot was constructed by employing the R package "ggpubr".

Functional Enrichment Analysis

To determine the DEGs pattern between HCC patients with high and low *RBM10* transcription, HCC patients in the TCGA database were divided into two groups according to the median *RBM10* transcription value. DEGs were determined by using the Limma package with the absolute value of logFC (log fold change) ≥ 1 and p value ≤ 0.001. The functions of these identified DEGs were explored by Gene Ontology (GO)[17] and Kyoto Encyclopedia of Genes and Genomes (KEGG)[18] pathway enrichment analysis. GO analysis mainly focused on three components: biological process (BP), cellular component (CC), and molecular function (MF), and KEGG analysis mainly evaluated the pathways that DEGs may be involved in. The results were visualized using cluster Profiler and ggplot2 R packages (threshold: $p < 0.05$).

Construction of the Protein–Protein Interaction (PPI) Network

The PPI network was constructed by utilizing the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; string-db.org/).[19] In the current study, only experimentally validated interactions with a combined score > 0.7 were selected as significant.

Gene Set Enrichment Analysis (GSEA)

The GSEA 4.1.0 software was utilized to carry out GSEA with the gene set "c2.cp.kegg.v6.2.symbols.gmt".[20] The permutation number was established as 1000, and a p-value < 0.05 was considered significant. The enrichment plot of GSEA was constructed by using the R package of ggplot.

Relationship Between *RBM10* Transcription and Immune Cell Infiltration

The relationships between *RBM10* transcription and immune cell infiltration and immunotherapy markers were determined by using the Tumor Immune Estimation Resource (TIMER) database.[21]

Immunohistochemistry (IHC)

Tumor sections were incubated with commercial rabbit polyclonal antibodies against *RBM10* (Abcam, ab224149) at 1:50 dilution overnight at 4°C. Then, the sections were incubated with the secondary antibody (1:1000 dilution; Thermo Fisher Scientific, A-10042, Massachusetts, USA) at 37°C for 1 h, and then covered by 3, 3-diaminobenzidine (DAB) (ZLI-9032, Zhongshan Biotech, Beijing, China). Subsequently, all fields were observed under the light microscope (Olympus 600 auto-biochemical analyzer, Tokyo, Japan). The control experiment without the primary antibody demonstrated that the signals observed were specific. The IHC staining intensity was quantified by software Image-Pro Plus, and the best cut-off value to identify *RBM10* high expression and low expression was defined as the median of the intensity values.

Statistical Analysis

Survival curves were generated from the Gene Expression Profiling Interactive Analysis (GEPIA) website[22] based on data in the TCGA database. The relationships between the clinicopathological characteristics and *RBM10* expression were evaluated by Student's *t*-test. For our HCC cohort, Kaplan–Meier (K-M) survival curves were constructed with GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, United States). $p < 0.05$ was considered as statistically significant in the current study.

Results

RBM10 is Up-Regulated in Various Tumor Types

RBM10 transcriptions in multiple tumor types were determined by using the online service of TIMER 2.0. As shown in Figure 1, *RBM10* transcription was up-regulated in a variety of tumor types, especially HCC (black framed) as compared with that in their normal counterparts.

Transcriptional Level of *RBM10* in HCC

Raw data of LIHC RNA-seq (Fragments Per Kilobase Million, FPKM) were downloaded from the TCGA database and analyzed with R software. Transcriptional levels of *RBM10* in 374 HCC tissue samples and 50 normal liver tissue samples were extracted and compared. It was found that *RBM10* transcription was up-regulated in the HCC tissue as compared with that in the normal liver parenchyma with a p-value < 0.0001 (Figure 2A). Similarly, in the paired HCC-normal liver tissue, *RBM10* was also highly expressed in the HCC tissue ($p < 0.0001$) (Figure 2B). These results were validated in the GEO database (Figure 2C and D). A volcano plot was constructed to show DEGs, and *RBM10* was lined out (Figure 2E).

Relationship Between Transcriptional Levels of *RBM10* and Clinicopathological Characteristics and Prognosis of HCC Patients

Survival data and RNA-seq data in the TCGA-LIHC patients were combined to investigate the potential prognostic meaning of *RBM10* transcription. Compared with HCC patients with low transcriptional levels of *RBM10*, HCC patients with high transcriptional levels of *RBM10* were associated with a dismal overall survival (OS) (Figure 3A) and recurrence free-survival (RFS) (Figure 3B). On account of the poor prognostic biomarker characteristic of *RBM10*, we speculated that high transcriptional levels of *RBM10* might be related to some aggressive biological features of HCC.

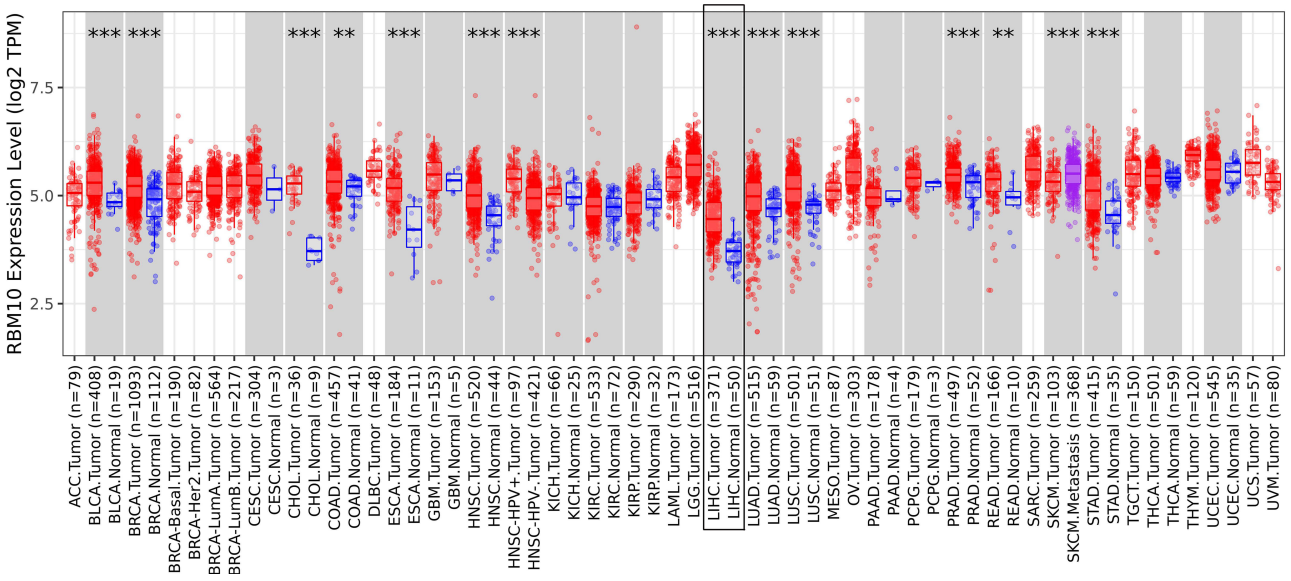

Figure 1 *RBM10* is up-regulated in various tumor types. **represents $p < 0.01$; *** represents $p < 0.001$.

Thus, the relationships between *RBM10* transcription and clinicopathological characteristics of HCC patients were analyzed, and the results showed that transcriptional levels of *RBM10* were only related to tumor T-stage and TNM stage rather than gender, age, or G-stage (Figure 3C–G).

GO and KEGG Enrichment Analysis

Compared with the low *RBM10* transcription group, 751 up-regulated genes and 83 down-regulated genes were discovered. As shown in Figure 4A and B, GO enrichment analysis revealed that the DEGs almost mapped to the DNA replication GO terms, such as DNA replication and DNA-dependent ATPase activity. KEGG enrichment analysis also displayed the enrichment of DNA replication (Figure 4C and D). GSEA analysis was also performed to validate the signaling pathway that *RBM10* may be involved in. The results showed that the DEGs in the high *RBM10* transcription group were mainly enriched in the cell cycle, DNA replication-related pathways and immune-related pathways (Figure 4E).

Interaction Analysis of the PPI Network

The STRING database was used to construct a PPI network between *RBM10* and other proteins. Only experimentally validated interaction with a combined score ≥ 0.7 was considered significant. As shown in Figure 5, *RBM10* was implicated in the combination with Cornichon (CNIH), DLG (Discs Large MAGUK Scaffold Protein), and Splicing Factor (SF) family.

RBM10 Transcription is Related to Tumor Immune Cell Infiltration and Immune Marker Transcription

Knowing that *RBM10* was engaged in immune-related pathways, as illustrated in Figure 4E, we hypothesized that *RBM10* was linked to HCC tumor immunity. Thus, the TIMER database was used to explore the relationship between *RBM10* transcription and immune cell infiltration. It was found that *RBM10* transcription was positively related to multiple immune cell (eg $CD8^+$ cell and macrophage) infiltration to the HCC tissue (Figure 6A). In addition, we found that *RBM10* transcription was positively correlated with the transcription of *PD1 (PDCD1)* and *PD-L1 (CD274)* (Figure 6B), suggesting that *RBM10* may play a therapeutic role in immunotherapy for HCC.

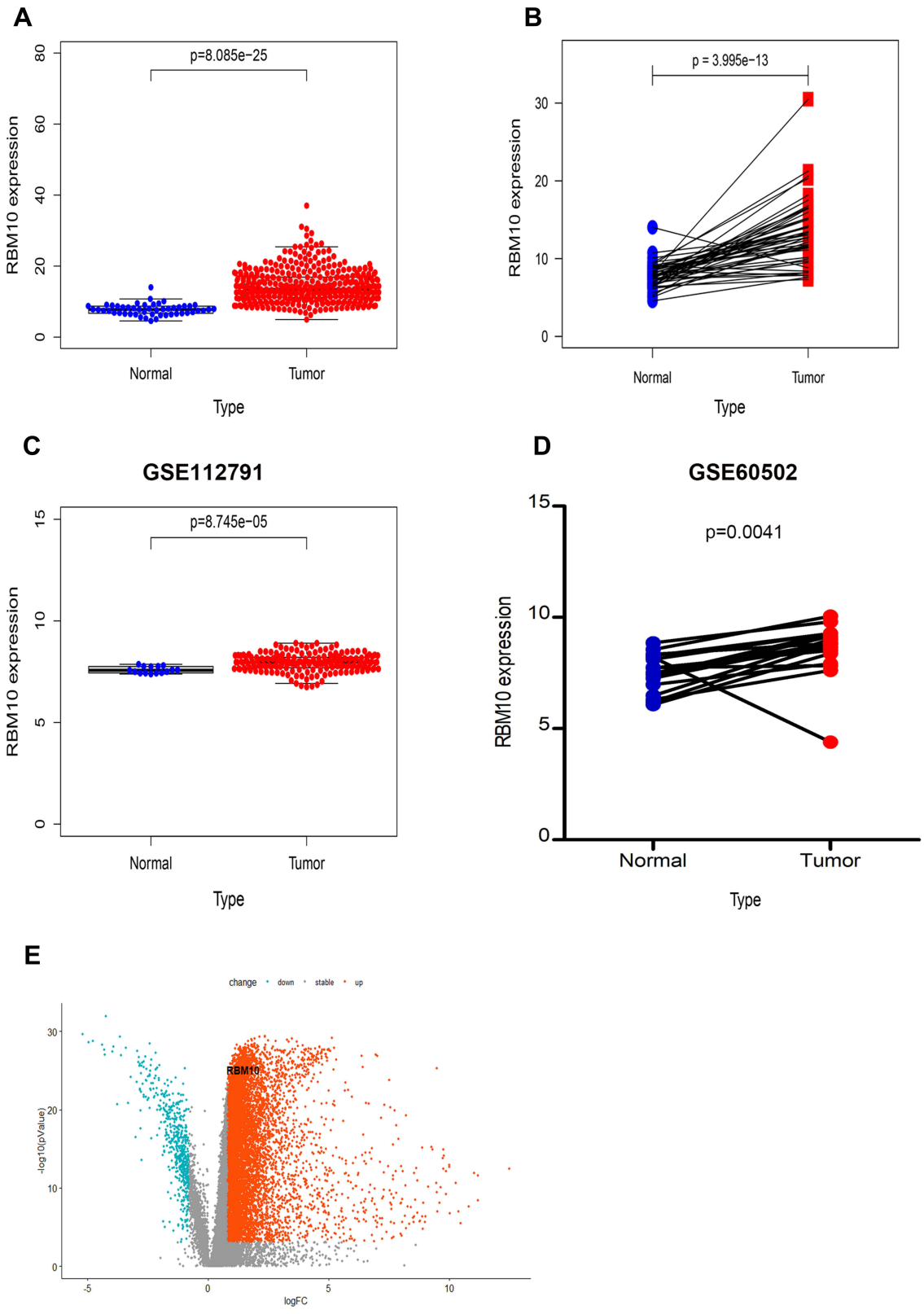

Figure 2 Transcriptional characteristics of *RBM10* in HCC samples. (**A**) Differences in *RBM10* transcription between HCC and normal liver tissues (data originated from the TCGA, n_{tumor}=374, $\overline{X}$±S 14.38±4.70; n_{normal}=50, $\overline{X}$±S 7.77±1.58; p < 0.001); (**B**) Differences in *RBM10* transcription between 50 paired HCC-normal tissues (data obtained from the TCGA, n_{pair}=50, Tumor $\overline{X}$±S 13.40±4.22; Normal $\overline{X}$±S 7.77±1.58; p < 0.001); (**C**) *RBM10* transcription in HCC patients from the GEO datasets (GSE112791, n_{tumor}=183 $\overline{X}$±S 7.94±0.42; n_{normal}=15, $\overline{X}$±S 7.58±0.16; p < 0.001); (**D**) *RBM10* transcription in 18 paired HCC and normal liver tissues from the GEO datasets (GSE60502, n_{pair}=18, Tumor $\overline{X}$±S 8.59±1.22; Normal $\overline{X}$±S 7.49±0.83; p = 0.0041); (**E**) Volcano plot based on DEGs between HCC and normal liver tissues (data originated from the TCGA).
Abbreviation: $\overline{X}$±S= mean±standard deviation.

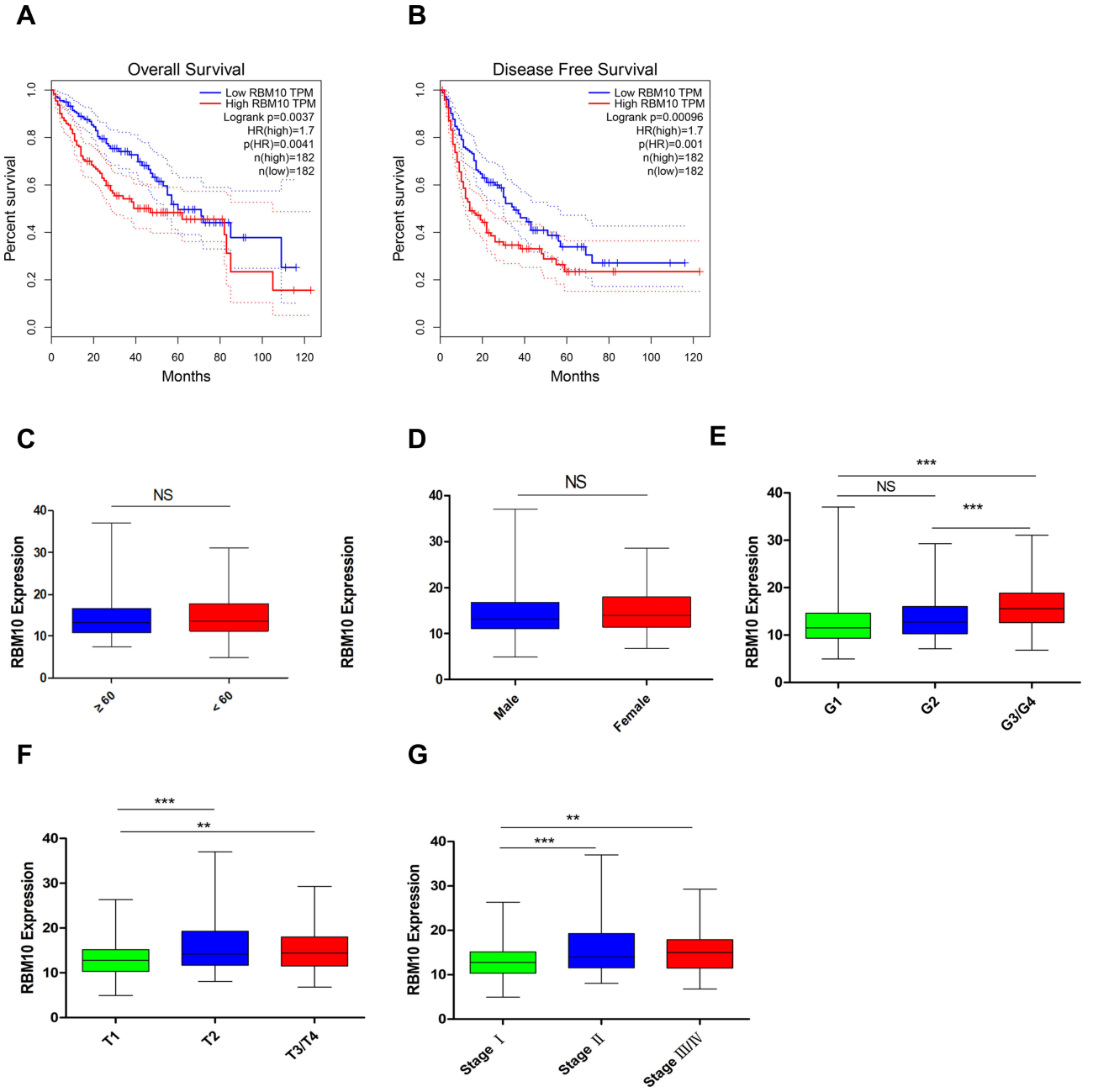

Figure 3 Elevated transcriptional levels of *RBM10* predict dismal prognosis and correlate to advanced clinicopathological features of tumor. (**A**) Up-regulation of *RBM10* is correlated with a worse OS (data originated from TCGA, p = 0.0037); (**B**) Up-regulation of *RBM10* is correlated with a worse DFS (data originated from TCGA, p < 0.001); The relationship between *RBM10* transcription and different clinicopathological features (data originated from the TCGA); (**C**) Age ($n_{\geq 60}$=201, $\overline{X}$±S 14.23±0.33; $n_{<60}$=169, $\overline{X}$±S 14.543±0.36; p = 0.53); (D) Gender (n_{male}=249, $\overline{X}$±S 14.11±0.30; n_{female}=121, $\overline{X}$±S 14.87±0.42; p = 0.14); (**E**) Histologic grade (n_{G1}=55, $\overline{X}$±S 12.89±5.27; n_{G2}=177, $\overline{X}$±S 13.55±4.11; $n_{G3/4}$=134, $\overline{X}$±S 16.06±4.80; ***p < 0.001, p^{NS}=0.357); (**F**) Tumor classification (n_{T1}=181, $\overline{X}$±S 13.27±3.77; n_{T2}=94, $\overline{X}$±S 15.87±5.73; $n_{T3/4}$=91, $\overline{X}$±S 15.15±5.00; ***p < 0.001,**p = 0.002); (**G**) TNM stage (n_1=171, $\overline{X}$±S 13.30±3.79; n_2=86, $\overline{X}$±S 15.65±5.74; $n_{3/4}$=90, $\overline{X}$±S 15.20±4.90; ***p < 0.001,**p = 0.002); **represents p < 0.01; *** represents p < 0.001.

Abbreviations: $\overline{X}$±S, mean±standard deviation; NS, none significance.

High Expression of *RBM10* Correlates with Aggressive Tumor Characteristics and Predicts a Poor Prognosis

To testify the results originated from the public database, IHC was employed to assess the expression characteristic of *RBM10* in our HCC cohort. Meanwhile, the Human Protein Atlas (HPA) website was checked to compare the characteristics of *RBM10* expression in normal liver tissue samples and HCC tissue samples. The result showed that

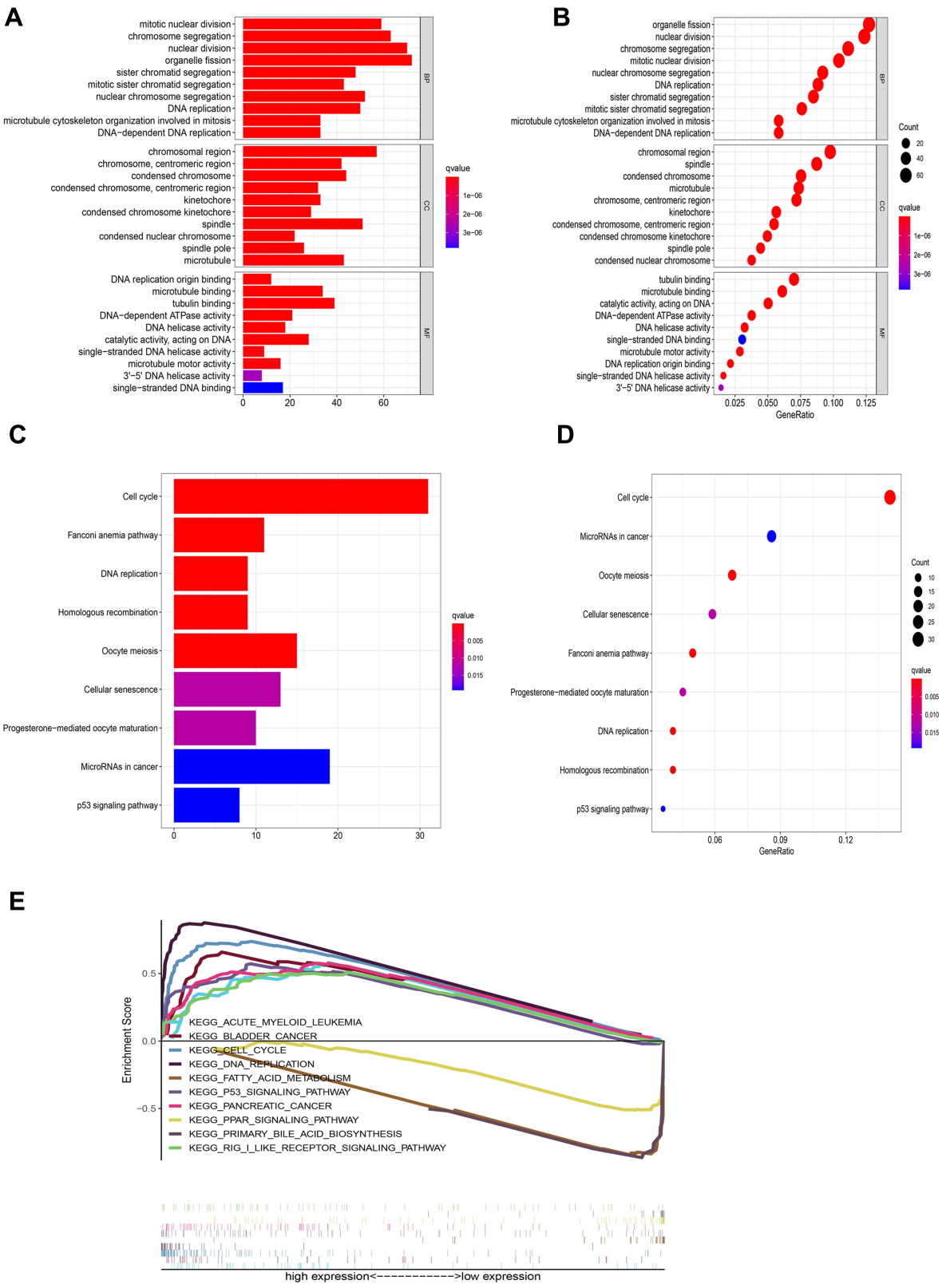

Figure 4 Enrichment plots of GO, KEGG, and GSEA. (**A**) GO pathways (bar plot); (**B**) GO pathways (bubble plot); (**C**) KEGG pathways (bar plot); (**D**) KEGG pathways (bubble plot); (**E**) GSEA pathways.

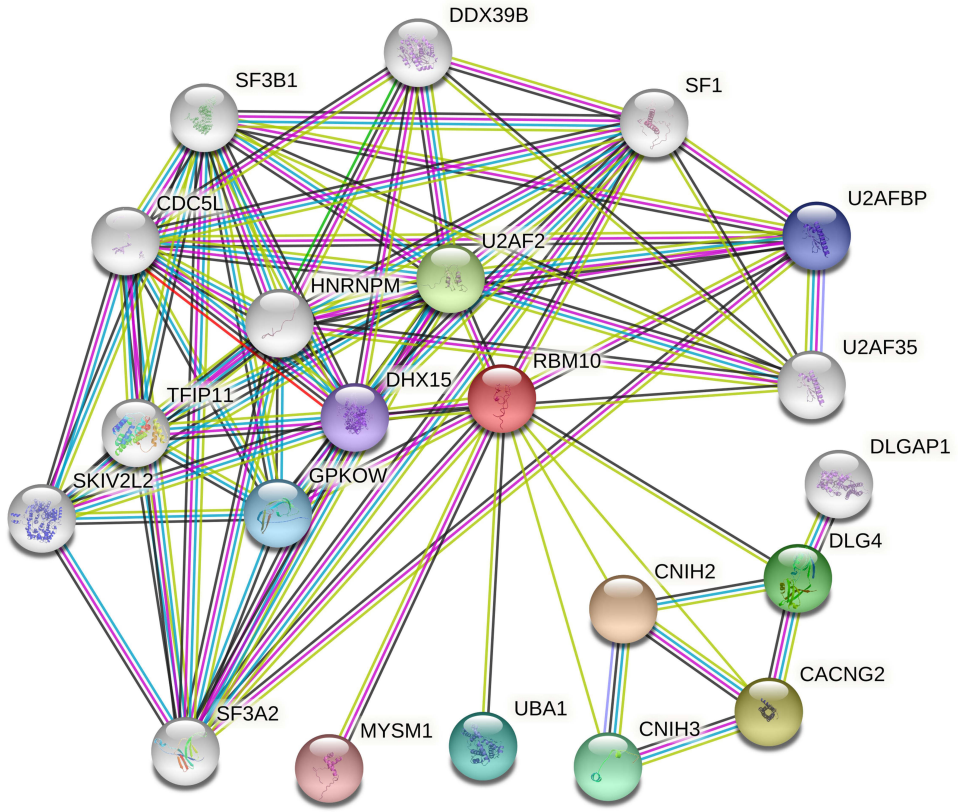

Figure 5 Protein–protein interaction network between *RBM10* and other proteins.

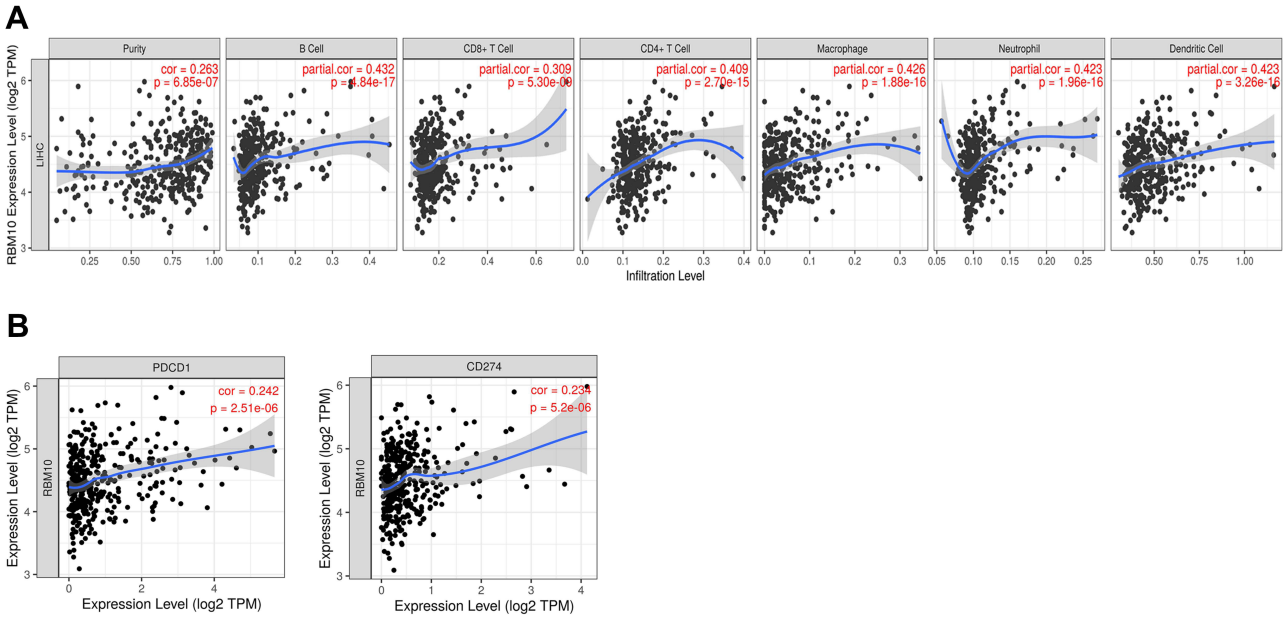

Figure 6 *RBM10* relates to tumor immune cell infiltration and immune marker expression. (**A**) *RBM10* transcription and tumor immune cell infiltration; (**B**) *RBM10* transcription is positively correlated with *PD-1* and *PD-L1* expression.

RBM10 was highly expressed in a certain number of HCC tissue samples (Figure 7A). And, our cohort also revealed a similar result (Figure 7B). After integrating the expression levels of *RBM10* in HCC tissues with clinicopathologic characteristics and prognosis of HCC patients in our cohort, we found that high expression of *RBM10* were also correlated with a more advanced tumor stage (Figure 7C) and microvascular invasion (MVI) (Figure 7D). In addition, elevated expression of *RBM10* was correlated to a worse OS and DFS (Figure 7E and F).

Discussion

The prognosis of HCC remains dismal due to its aggressive biological nature. Even after radical treatment, the 5-year RFS is only about 30%.[23] It is therefore urgent to discover new recurrence-related and therapeutic biomarkers to improve the outcome of recurrence surveillance and postoperative targeted therapy of HCC. In the present study, we tried to explore whether *RBM10* could serve as a prognostic and therapeutic biomarker for HCC.

Recent studies[24,25] have demonstrated that multiple members of the RBM family can suppress tumor progression. However, the role of *RBM10* in different tumors remains disputable. Previous studies reported that *RBM10* acted as a tumor suppressor in breast cancer, osteosarcoma, and pancreatic cancer.[14,26,27] Loiselle et al[28] reported that *RBM10* could promote lung cancer cell proliferation and other transformation-associated processes. However, the prognostic significance of *RBM10*in HCC has rarely been discussed. The present study aimed to explore the prognostic meaning and biological function of *RBM10* in HCC by using the common data in a variety of databases.

In the present study, we used public data in GEO and TCGA databases in combination with our HCC patient cohort to uncover the expression pattern and prognostic significance of *RBM10*. The result showed that *RBM10* was up-regulated in the HCC tissue as compared with that in the normal liver tissue. Furthermore, high transcription of *RBM10* in HCC predicted a dismal DFS and OS. Our data revealed that high expression of *RBM10* was correlated with an advanced tumor stage and microvascular invasion, suggesting that *RBM10* may play a role in promoting HCC progression and serve as an aggressive biological marker of HCC. *RBM10* is a member of the RBM family that can perform a function in targeted RNA metabolism and pre-mRNA splicing concerning the cell cycle.[6] In this way, the *RBM10* family can participate in the regulation of tumor cell proliferation and metastasis. However, further molecular biology research is needed to determine how *RBM10* promotes HCC progression.

To gain more insights into the signaling pathway in which *RBM10* may be involved, we performed KEGG and GO enrichment analyses and found that *RBM10* truly participated in regulating gene expressions in terms of cell cycle and DNA replication in HCC. Various classical splicing regulators have been shown to display oncogenic activities.[29,30] A previous study[31] revealed that misregulation of *RBM5, RBM6*, and *RBM10* affected tumor progression by targeting NUMB in lung cancer. Interestingly, Zhao et al[32] reported that *RBM10* could suppress tumor cell proliferation and played a protective role in HCC. Their results are not consistent with the results obtained from the common databases. In consideration of the absence of a comprehensive mechanism-related research, a high-quality biomolecular study is required to testify the regulatory function of *RBM10* in HCC.

In the present study, GSEA showed that *RBM10* had an impact on multiple immune-related signaling pathways. In addition to its interaction with the *DLG* family, *RBM10* was also reported to be high-expressed in immune cells, especially memory CD8 T-cells.[33] All these results suggest that *RBM10* may play a part in immune regulation in HCC. Consistently, *RBM10* was observed to be positively correlated with $CD8^+$ T cell infiltration and expression of *PD-1* and *PD-L1*, suggesting that *RBM10* may be a biomarker for immunotherapy of HCC.

Conclusion

In the current study, we analyzed multiple common databases in combination with our HCC cohort and found that up-regulated *RBM10* was a dismal prognostic factor and a potential biomarker for immunotherapy, which may be engaged in immune-related signaling pathways. However, further clinical and fundamental biological studies are warranted to verify the function of *RBM10* in HCC.

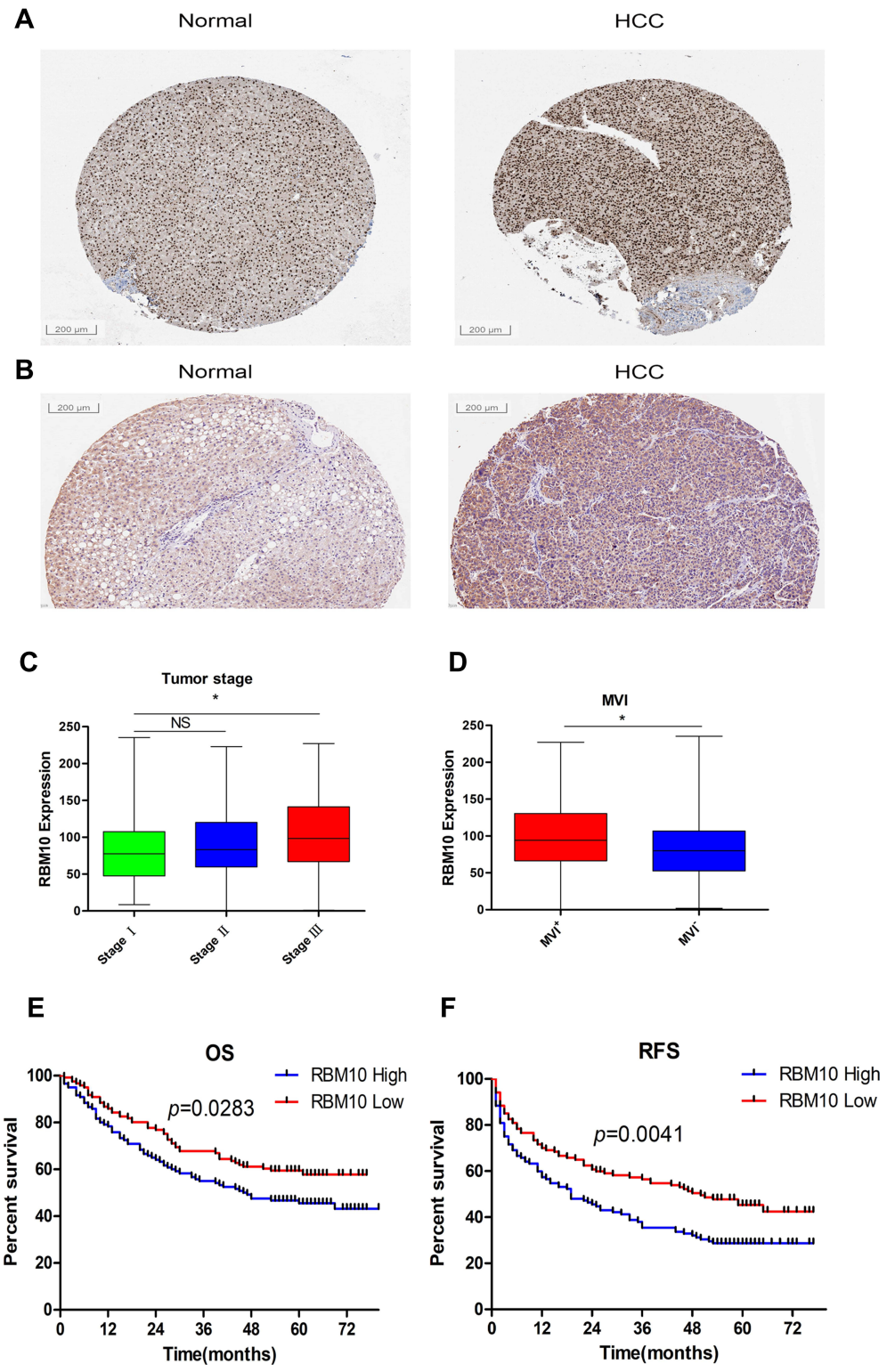

Figure 7 Expression characteristics of *RBM10* and its prognostic value in our HCC cohort revealed by IHC test. (**A**) *RBM10* protein is over-expressed in HCC by IHC in the HPA website; (**B**) *RBM10* protein is over-expressed in HCC by IHC in our HCC cohort; (**C**) Elevated expression of *RBM10* correlated to advanced TNM stage (n_1=82, $\overline{X}$±S 83.62±48.36; n_2 =125, $\overline{X}$±S 89.37±42.53; n_3=34, $\overline{X}$±S 105.08±51.77;*p = 0.023); (**D**) Elevated expression of *RBM10* correlated to positive status of MVI (n_{MVI+}=88, $\overline{X}$±S 98.12±5.20; n_{MVI-}=153, $\overline{X}$±S 84.75±3.57; *p = 0.03); (**E**) Up-regulation of *RBM10* is correlated with a dismal OS in our HCC cohort (No. of high expression patients=120, median OS 47 months; No. of low expression patients=121, median OS 57 months, p = 0.0283); (**F**) Up-regulation of *RBM10* predicts a dismal DFS in our HCC cohort (No. of high expression patients=120, median DFS 19 months; No. of low expression patients=121, median DFS 50 months; p = 0.0041). *represents $p < 0.05$.

Abbreviations: NS, none significance; $\overline{X}$±S, mean±standard deviation; n_{MVI+}, represents the number of patients with positive status of microvascular invasion; n_{MVI-}, represents the number of patients with negative status of microvascular invasion.

Abbreviations

HCC, hepatocellular carcinoma; RBM10, RNA-binding protein 10; TEV, talipes equinovarus, ASD, atrial septal defect, TARP, Robin sequence syndrome; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; DEGs, Differentially expressed genes; FC, Foldchang; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function; PPI, protein–protein interaction; Search Tool for the Retrieval of Interacting Genes/Proteins; GSEA, Gene Set Enrichment Analysis; TIMER, Tumor Immune Estimation Resource; IHC, immunohistochemistry; HPA, Human Protein Atlas; GEPIA, Gene Expression Profiling Interactive Analysis; K-M, Kaplan–Meier; OS, overall survival; RFS, recurrence free survival; MVI, microvascular invasion; CNIH, Cornichon; DLG, Discs Large MAGUK Scaffold Protein; SF, splicing factor.

Data Sharing Statement

Our cohort data are available from the corresponding author (Ning Yang, email: lancet00@163.com) upon reasonable request. Public datasets were analyzed in this work. These data can be found in their official websites: http://cancergenome.nih.gov; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112791; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi.

Ethics Approval and Consent to Participate

This retrospective study was approved by the research ethics committee of the Eastern Hepatobiliary Surgery Hospital and complied with the Declaration of Helsinki Principles. Informed consent was obtained from all patients for their data to be used in the study.

Author Contributions

NY, GJ, and GY contributed to the conception and design of the study. SP, ZS, and WL organized the database and performed the statistical analysis. HS, ZL, YS, YY, and XZ took part in the statistical analysis. NY and GJ wrote the first draft of the manuscript. All authors contributed to data analysis, drafting, or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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