



Increased *RBM12* expression predicts poor prognosis in hepatocellular carcinoma based on bioinformatics

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Background: Liver cancer is one of the major causes of cancer death worldwide, incurring high mortality and a significant financial burden on the healthcare system. Abnormal RNA-binding proteins (RBPs) have been found to be associated with carcinogenesis in liver cancer. Among these, RNA-binding motif protein 12 (*RBM12*) is located in the exon junction complex (EJC). The goal of this study was to determine what role *RBM12* plays in hepatocellular carcinoma (HCC) from a biological perspective.

Methods: The Tumor Immune Estimation Resource (TIMER) and the Human Protein Atlas database were used to examine the expression level of *RBM12*, with the UALCAN and Gene Expression Profiling Interactive Analysis (GEPIA) databases used to investigate the relationship between *RBM12* and other noteworthy clinical features. *RBM12* expression in cells and tissue samples was detected using quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis. The functional network of *RBM12* in HCC was studied using LinkedOmics and gene set enrichment analysis (GSEA), while the effects of hypomethylation on the expression of *RBM12* in HCC was investigated using methylation databases. Finally, we used TIMER and CIBERSORT to investigate the relationship between immune cell infiltration and *RBM12* in HCC.

Results: *RBM12* is highly elevated in HCC tissues and cells, and it can be used to predict the prognosis of patients with HCC. Analysis with LinkedOmics and GSEA revealed *RBM12* to be closely linked with tumor progression. Furthermore, hypomethylation was linked to an increase in *RBM12* expression in HCC, while *RBM12* was associated with immune cell infiltration.

Conclusions: This study shows that an elevated level of *RBM12* in HCC indicates a poor patient prognosis. Furthermore, according to LinkedOmics and GSEA analyses, *RBM12* was implicated in the most important hallmark pathways. Our findings suggest that *RBM12* overexpression is caused by hypomethylation and that *RBM12* plays a key role in liver cancer tumor immunity.

Keywords: RNA-binding motif protein 12 (*RBM12*); hepatocellular carcinoma (HCC); biomarker; tumor immunity; bioinformatic analysis

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Introduction

In recent decades, liver cancer has become one of the most common cancers in the world and has a high patient fatality rate. In comparison to developed countries, China has both a higher incidence and higher fatality rate of cancer. Surgery is the most common treatment for hepatocellular carcinoma (HCC), and fluorouracil, sorafenib, and other chemotherapeutic medications being commonly administered (1). Despite a decrease in the mortality rate of patients diagnosed in the early stages of the disease's progression, the overall survival (OS) for advanced-stage liver cancer patients can still be improved (2). Patients are frequently identified with advanced-stage liver cancer and are unable to obtain appropriate therapy because of a lack of highly specific and specific early diagnostic biomarkers, and thus their prognosis is poor (3). It is critical to investigate new HCC markers to enhance the prognosis of HCC patients, as well as to better understand the molecular pathways driving carcinogenesis and cancer progression.

RNA-binding proteins (RBPs) are proteins that bind RNA via 1 or more globular RNA-binding domains (RBDs) or motifs (RBMs) and can alter the function of the RNAs they bind (4). Various studies have revealed that RBPs are important regulators of posttranscriptional gene expression, and influence RNA modification and stability, including the processes of RNA splicing, localization, translation, and turnover (5). To date, more than 800 messenger RNA (mRNA) RBPs have been identified, but only a small percentage have been characterized in terms of function (6).

RBP dysregulation has been linked to the development of liver tumors and the progression of cancer (7-11). For example, RNA-binding motif protein 43 (*RBM43*) is downregulated in cases of malignancy where low expression is linked to a poor prognosis for HCC patients (7). Furthermore, *in vitro* experiments showed that *RBM38* can induce apoptosis and senescence in liver cancer cells by inhibiting proliferation and colony development and suppressing migration and invasion (10). RNA-binding motif protein 12 (*RBM12*) is an RBP belonging to the RBM family. It is a conserved protein that is widely produced in cells. Five different RBMs, two proline-rich regions, and many potential transmembrane domains are found in the *RBM12* protein (12). *RBM12* was first discovered to have an abnormal expression in meibomian cell carcinoma (13), and its disruption has been linked to psychosis and endometrial

cancer (14,15). Despite these discoveries, the specific role of *RBM12* in HCC is still unknown.

To determine the clinical significance of *RBM12* in HCC, our study used online databases to examine the expression of *RBM12* and its predictive value in patients. We also looked at the expression of *RBM12* in the cells or tissues of liver cancer, and the functional network of *RBM12* was analyzed using LinkedOmics and gene set enrichment analysis (GSEA). Next, we examined the impact of DNA methylation on *RBM12*, and finally, the link between *RBM12* and immune infiltration in HCC was elucidated. Consequently, our study is the first to explore the expression, prognostic significance, immune cell infiltration, and biological networks of *RBM12*, as well as the probable causes of the overexpression of *RBM12* in HCC. We present the following article in accordance with the REMARK reporting checklist (available at <https://dx.doi.org/10.21037/jgo-21-390>).

Methods

Tumor Immune Estimation Resource (TIMER)

The TIMER (<https://cistrome.shinyapps.io/timer>) is a tool used for analyzing the infiltration of tumors by immune cells (16). This resource can be used to assess six types of immune cell infiltrates (CD4+ T cells, B cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells). The TIMER was also used to reveal the *RBM12* expression in tumors compared with its expression in normal tissues and the distribution of gene expression in mutant and wild-type tumors in HCC.

The Human Protein Atlas

Immunohistochemistry (IHC) data from 44 distinct normal tissue types were located in the Human Protein Atlas (<https://www.proteinatlas.org>) (17). During this search, we were able to identify the expression data of *RBM12* in several human tissues.

UALCAN database analysis

The clinical data of 33 different cancer patients were located on the UALCAN database (<http://ualcan.path.uab.edu>) (18), which was further used to investigate the relationship between *RBM12* and other clinical features in HCC patients.

Table 1 Primer sequence of the target genes

Gene	Primers
<i>RBM12</i> -F	5'-TGGACATTGCGCACTTCTTC-3'
<i>RBM12</i> -R	5'-CGCATCATACCAAGCCTTGC-3'
β - <i>actin</i> -F	5'-GGCGGCACCACCATGTACCCT-3'
β - <i>actin</i> -R	5'-AGGGGCCGGACTCGTCATACT-3'

Kaplan-Meier plotter database analysis

The Kaplan-Meier plotter database (<http://kmplot.com/analysis/>) includes 5,143 samples of breast cancer, 2,437 samples of lung cancer, 1,816 samples of ovarian cancer, and 1,065 samples of stomach cancer (19). In providing various cancer samples, this database is useful for examining the relationship of genes with patient survival. Our study used the Kaplan-Meier plotter to assess the association between *RBM12* and the survival of patients with HCC.

LinkedOmics

LinkedOmics (<http://linkedomics.org/login.php>) is a free online database that contains information on the 32 cancer types that are also found in The Cancer Genome Atlas (TCGA) (20). LinkFinder, LinkInterpreter, and LinkCompare are the three analytical modules on the website. We used the LinkInterpreter module for an enrichment analysis that was based on Gene Ontology (GO), network modules, and biological pathways in order to gain new biological insights about *RBM12* in HCC.

GSEA

GSEA is a computer tool used to determine if there are statistical and consistent differences in a group of genes among two biological states. GSEA was used to analyze TCGA gene expression data from 374 liver cancer samples acquired from the UCSC Xena Functional Genomics Explorer so that the biological processes enriched by *RBM12* could be identified.

Databases related to DNA methylation

MEXPRESS (<https://mexpress.be/about.html>) is a data visualization tool that can present TCGA expressions, clinical data, and DNA methylation, as well as their

interconnections (21). TCGA, the International Cancer Genome Consortium (ICGC), and the Gene Expression Omnibus (GEO) have all contributed cancer genomic databases to the cBioPortal (<https://www.cbioportal.org/>) (22). The link between *RBM12* mRNA expression and DNA methyltransferase (DNMT) expression was found via Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (<http://gepia.cancer-pku.cn/index.html>) (23), while the Shiny Methylation Analysis Resource Tool (SMART) app (<http://www.bioinfo-zs.com/smartapp/>) is a user-friendly web application used to evaluate TCGA DNA methylation data in detail (24).

Immune infiltrates analysis

We employed CIBERSORT, a deconvolutional technique based on gene expression (<http://cibersort.stanford.edu/>), to assess the gene expression variations among sample sets (25). We assessed the immune response of 22 tumor-infiltrating immune cells (TIICs), using CIBERSORT to clarify the link between *RBM12* expression in HCC and any correlations among the TIICs.

Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analyses

The TRIzol reagent (Invitrogen Life Technologies, USA) was used for extraction of total RNA from both HCC cells (Huh7, LM3, MHCC97H) and the normal cell line of the liver, known as QSG-7701. To produce complementary DNA (cDNA), a RevertAid First Strand cDNA Synthesis Kit (TaKaRa Bio, Japan) was used for RNA reverse transcription. The primer sequences we used for qRT-PCR analysis are presented in *Table 1*. qRT-PCR analysis was completed on a LightCycler 480 II (Roche) PCR platform using a SYBR Master Mixture (TaKaRa Bio) and SYBR Master Mixture (TaKaRa Bio) according to the manufacturer's instructions. To assess the transcriptional expression, the fold change in the expression of *RBM12* compared to β -*actin* was employed (*RBM12* expression levels were determined using the $-\Delta\text{Ct}$ method), while *RBM12* protein expression in normal and HCC cell lines was determined by western blot analysis. The primary antibody was antihuman *RBM12* (1:1,000, sc-514258, Santa Cruz Biotechnology, USA), the secondary antibody was anti-mouse immunoglobulin G (IgG; 1:2,000; A0216, Beyotime, China), and the loading control was β -*actin* (1:1,000; AF0003, Beyotime, China).

Cell culture and patients

HCC tissues and corresponding nearby noncancerous tissues were obtained from 45 patients (33 men and 12 women) who had not undergone therapy before surgery at the Affiliated Hospital of Nantong University between 2018 and 2020. The included patients gave their informed consent, and the Affiliated Hospital of Nantong University's Ethics Committee approved the study (No. 2018-L006). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The samples were promptly frozen in liquid nitrogen and preserved at -80°C for subsequent use. The Shanghai Institute of Cell Biology (Shanghai, China) provided the HCC cell lines (Huh7, LM3, and MHCC97H), and Beyotime provided a normal liver cell line (QSG-7701; Shanghai, China). Cells were grown in RPMI 1640 medium or Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$) at 37°C in a 5% CO_2 environment.

Statistical analysis

All statistical analyses were conducted using SPSS 22.0 (IBM Corporation, USA) and GraphPad Prism 8.02. The mean and the standard deviation (SD) of the data were calculated, and differences between the sets of data were assessed using a one-way analysis of variance (ANOVA). All experiments were carried out in triplicate.

Results

Expression levels of *RBM12* in different types of tissues and tumors

RBM12 expression levels in normal human tissues were discovered by searching The Human Protein Atlas database. The results showed a lower level of *RBM12* in the normal liver tissues (Figure 1A). The TIMER was also employed to visualize *RBM12* expression in distinct malignancies. Figure 1B describes the high *RBM12* expression in liver HCC (LIHC), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and stomach adenocarcinoma (STAD). Finally, we discovered that, with the exception of the amygdala, the

expression of *RBM12* was normally lowest in the liver but elevated in liver cancer.

RBM12 expression in HCC cells and patients

The GSE138178 and GSE112613 data sets indicated higher expressions of *RBM12* in HCC (Figure 2A,2B). In addition, we found a significantly higher expression of *RBM12* in the liver tissues of patients with cirrhosis than in patients with chronic hepatitis, but the expression showed no significant difference when compared to hepatitis B surface antigen (HBsAg) carriers (Figure 2C). During surgery, 45 paired samples were collected from liver cancer and nearby noncancerous tissues to corroborate these findings. The expression of *RBM12* mRNA in these samples was then analyzed using qRT-PCR, revealing a substantial increase of *RBM12* expression in tissues of liver cancer as compared to adjacent healthy tissues (Figure 2D). We also looked at *RBM12* mRNA levels in the Huh7, LM3, and MHCC97H HCC cell lines and in QSG-7701 (the normal cell line of the human liver; Figure 2E,2F). *RBM12* is significantly increased in HCC cells. Indirect immunofluorescence showed *RBM12* localized to the nucleoplasm (Figure 3A). IHC staining revealed high expression levels of *RBM12* in tumor tissues (Figure 3B), and *RBM12* levels were considerably greater in certain categories, such as age, gender, weight, race, tumor grade and stage, histological subtypes, and nodal metastatic status (Figure 4). Cumulatively, these findings show that *RBM12* is typically increased during HCC development.

Prognostic potential of *RBM12* in HCC

Our results suggested that enhanced *RBM12* expression is linked with poor patient prognosis (Figure 5A). Using the Kaplan-Meier plotter database, we examined the connection of *RBM12* expression with clinical features, which revealed that *RBM12* expression was linked to OS, progression-free survival (PFS), and relapse-free survival (RFS) in HCC patients (Figure 5B-5D). The clinical data of the 45 patients with liver cancer showed that *RBM12* was related to tumor size, alpha-fetoprotein (AFP) tumor marker status, pathologic stage, and vascular invasion (Table 2). The possibility of *RBM12* serving as a predictive biomarker was confirmed through univariate analysis. As shown in Table 3, *RBM12* had prognostic significance in TCGA ($P=0.02$) cohorts and was related to pathologic stage ($P=0.00$) and neoplasm cancer status ($P=0.00$). The multivariate analysis indicated that *RBM12* expression was related to age

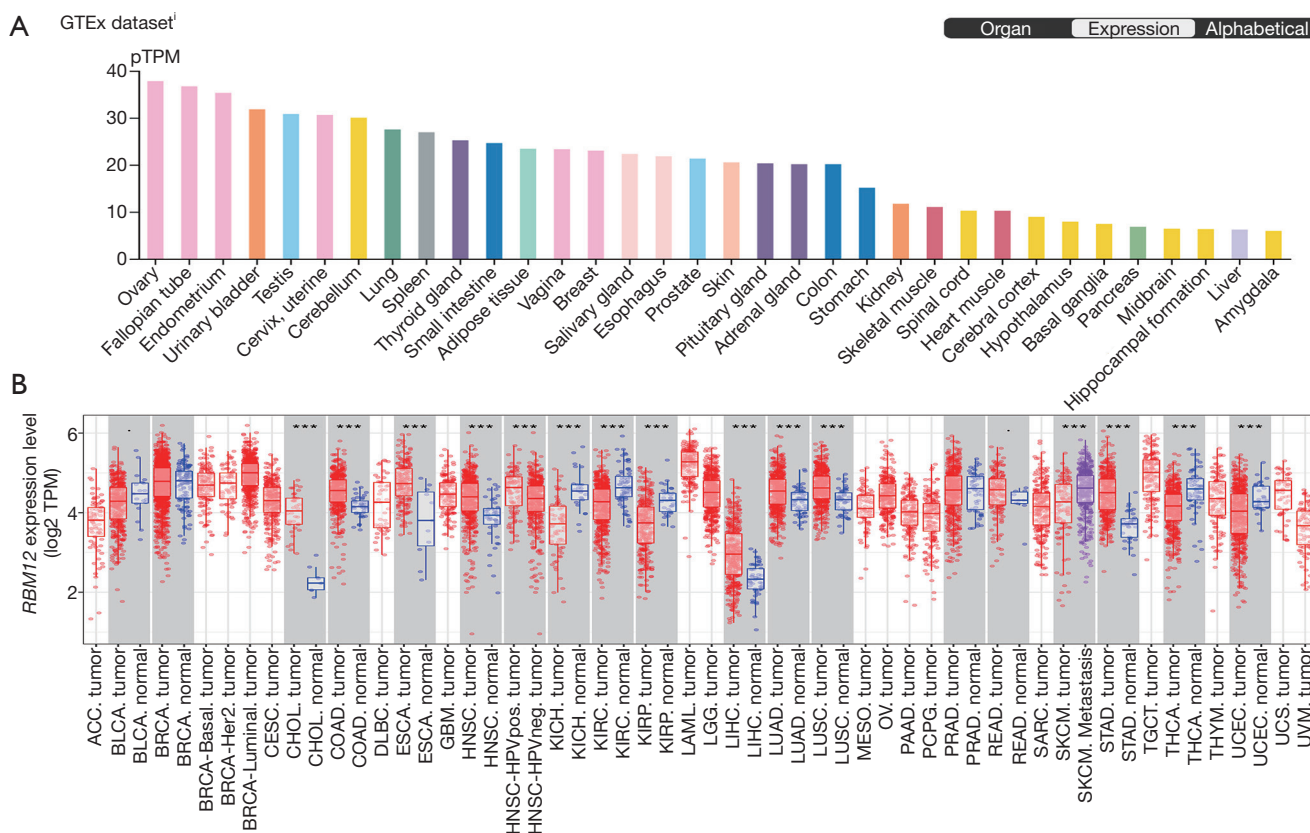


Figure 1 The expression of *RBM12* was relatively low in normal liver tissues but was increased in liver cancer tissues. (A) The expression of *RBM12* in different normal human tissues ([†], GTEx dataset RNA-seq tissue data generated by the GTEx project is reported as mean pTPM, corresponding to mean values of the different individual samples from each tissue). (B) *RBM12* expression in tumor samples and paired normal tissues (***, $P < 0.001$). *RBM12*, RNA-binding motif protein 12; GTEx, Genotype-Tissue Expression; pTPM, protein-coding TPM; TPM, transcripts per million.

($P = 0.028$), neoplasm cancer status ($P < 0.001$), and weight ($P = 0.011$; *Figure 6*). These findings suggest that *RBM12* may have impact on the prognosis of patients with liver cancer.

LinkedOmics predicts functional annotation and signaling pathways

LinkedOmics was also used to find coexpressed *RBM12* genes in the LIHC cohort. *Figure 7A* displays the significant genes, with a heat map depicting the top 50 most significant genes that have a negative or positive connection with *RBM12* expression (*Figure 7B, 7C*). *Figure 7D* depicts the *RBM12* network and 100 coexpressed genes, and the findings of functional enrichment studies of these 100 implicated genes are displayed in *Figure 7E*. Significant genes were inextricably involved in the cell cycle, peptidyl-lysine modification, *BRCA1-BARD1-BACH1*-DNA damage

complex II, and nuclear export.

Significant genes and pathways identified by GSEA

GSEA is one of the more commonly used analysis methods to study the biological function of tumors, as it can detect expression variations in gene sets rather than individual genes, and it is more reliable and flexible than are the more traditional methods like GO and Kyoto Encyclopedia of Genes and Genomes (KEGG). GSEA retrieved the expression data of *RBM12* mRNA for HCC samples from TCGA data sets. This data allowed us to better understand the biological relevance of coregulated proteins, with GSEA revealing that *RBM12* is involved in a variety of tumor progression pathways, including basal transcription factors, renal cell carcinoma, cell cycle, pancreatic cancer, and noncoding RNA (ncRNA) export from the nucleus (*Figure 8*).

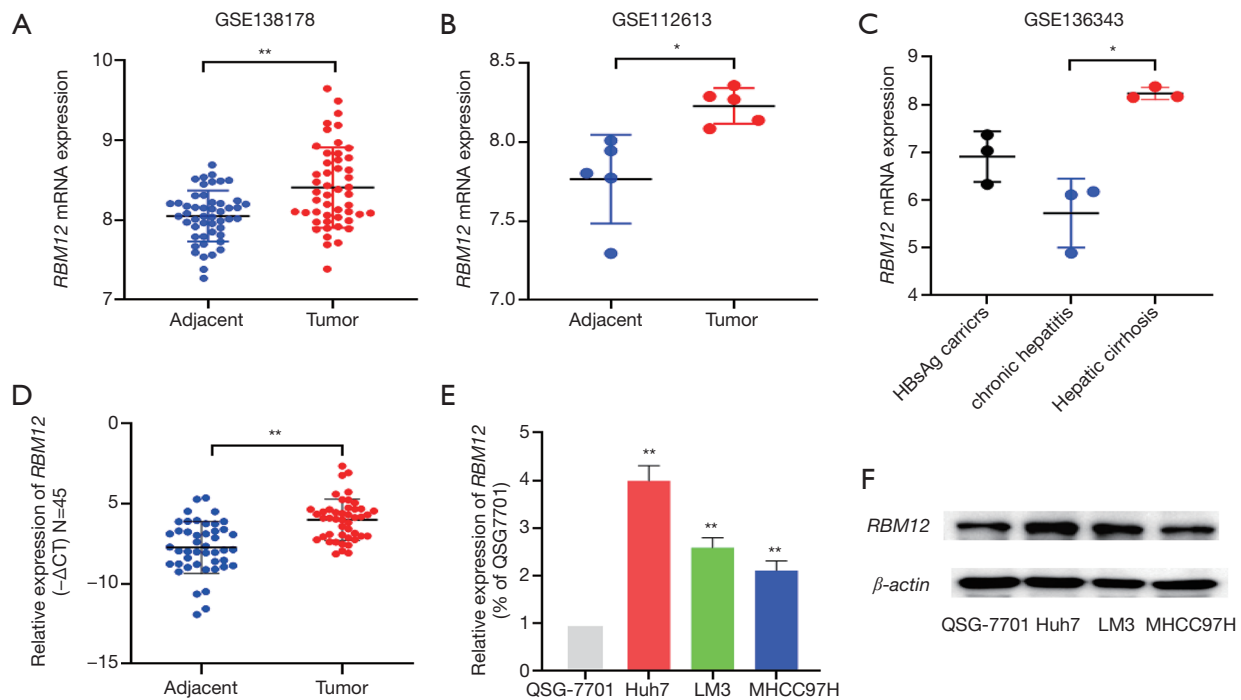


Figure 2 The increased expression of *RBM12* in HCC tissues and cells. (A,B) GSE138178 and GSE112613 showing that *RBM12* mRNA levels in liver cancer tissues were significantly increased compared to the adjacent tissues. (C) The expression of *RBM12* in patients with cirrhosis was significantly higher than that in patients with chronic hepatitis. (D) Analysis of 45 pairs of liver cancer specimens showed that *RBM12* was highly expressed in liver cancer tissues. (E,F) qRT-PCR and western blotting analysis showed that the expression of *RBM12* in HCC cell lines was higher than that in normal liver cells (*, $P < 0.05$; **, $P < 0.01$). *RBM12*, RNA-binding motif protein 12; HCC, hepatocellular carcinoma; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction.

The major bile acid biosynthesis and the flavonoid metabolic pathway were found to be among the gene sets enriched in the low expression phenotypic group (Table 4).

DNA methylation affects *RBM12* mRNA levels

RBM12 gene amplification is rare in HCC (Figure 9A,9B), which implies that other processes are responsible for the enhanced *RBM12* expression in HCC. Promoter hypomethylation contributes to tumorigenesis via the transcriptional activation of oncogenes.

MEXPRESS was used to investigate the methylation status of the *RBM12* gene in HCC. Many methylation sites were discovered in the promoter region of the *RBM12* gene (Figure 10A). Using the cBioPortal online tool, we discovered a negative relationship between *RBM12* mRNA and promoter methylation in HCC (Figure 10B). According to an HCC cohort analysis from TCGA, *RBM12* promoter methylation in HCC tissues was considerably lower when

compared to normal pancreas tissues (Figure 10C). We further discovered a link between *RBM12* mRNA expression and DNMT mRNA expression levels in HCC patients (Figure 10D). Figure 11A visualizes the chromosomal distribution of every 5'-C-phosphate-G-3' (CpG) associated with the *RBM12* gene in HCC, and Figure 11B,C show the differences in CpG expression between normal tissue and tumor tissue samples and its effect on prognosis. Our findings suggested that *RBM12* is upregulated in HCC due to abnormal promoter methylation.

The link between *RBM12* and immune cell infiltration in HCC

The TIMER database analysis indicated the expression of *RBM12* to be substantially correlated with different types of immune cell infiltration (Figure 12A). We aimed to determine whether the tumor immune microenvironment in liver cancer with a high level of *RBM12* was different

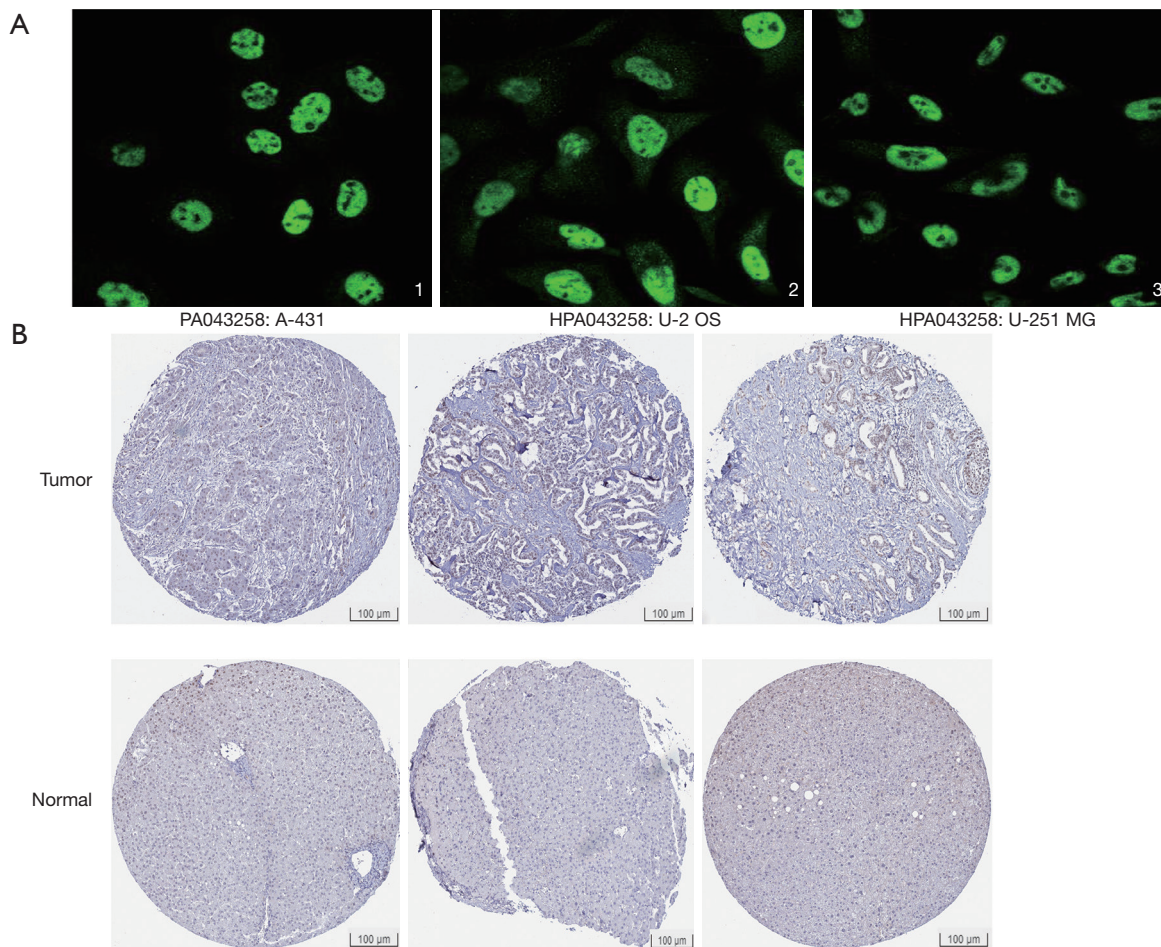


Figure 3 Representative images of staining of HCC patient cells from the Human Protein Atlas database. (A) Indirect immunofluorescence showed RBM12 localized to the nucleoplasm, A-431, U-2 OS, and U-251 MG cell lines. (B) RBM12 expression in three liver cancer tissues and three normal liver tissues. Representative images of IHC staining. HCC, hepatocellular carcinoma; RBM12, RNA-binding motif protein 12; IHC, immunohistochemistry.

from that with a low level. Based on *RBM12*, the 374 tumor samples were divided into two groups, with 187 samples in the group of high expression and 187 samples in the group of low expression. In order to determine the levels of the 22 different types of immune cells, we used CIBERSORT to analyze the gene expression of the downloaded samples. The CIBERSORT algorithm, which was applied to the 22 immune cell subtypes, assisted in determining differences in the expression levels of the cell subtypes between the high and low *RBM12* expression groups. It was found that the ratios of resting mast cells, monocytes, and CD8⁺ T cells were significantly higher in the low expression group. Conversely, the proportions of M0 macrophages and resting memory CD4⁺ T cells were significantly higher in the

high expression group (Figure 12B,12C). Furthermore, we demonstrated the correlation between several immune cells and *RBM12* in HCC (Figure 13). In 22 types of immune cells, the levels of highly activated natural killer (NK) cells, neutrophils, M0 macrophages, follicular helper T cells, and regulatory T cells level were related to poor OS, while increased resting memory CD4⁺ T cells, monocytes, and CD8⁺ T cells were related to better OS (Figure 14).

Discussion

Mortality rates from liver cancer are high all around the world (26). Despite the accessibility of several successful clinical treatments, patient OS rates remain low (2). The

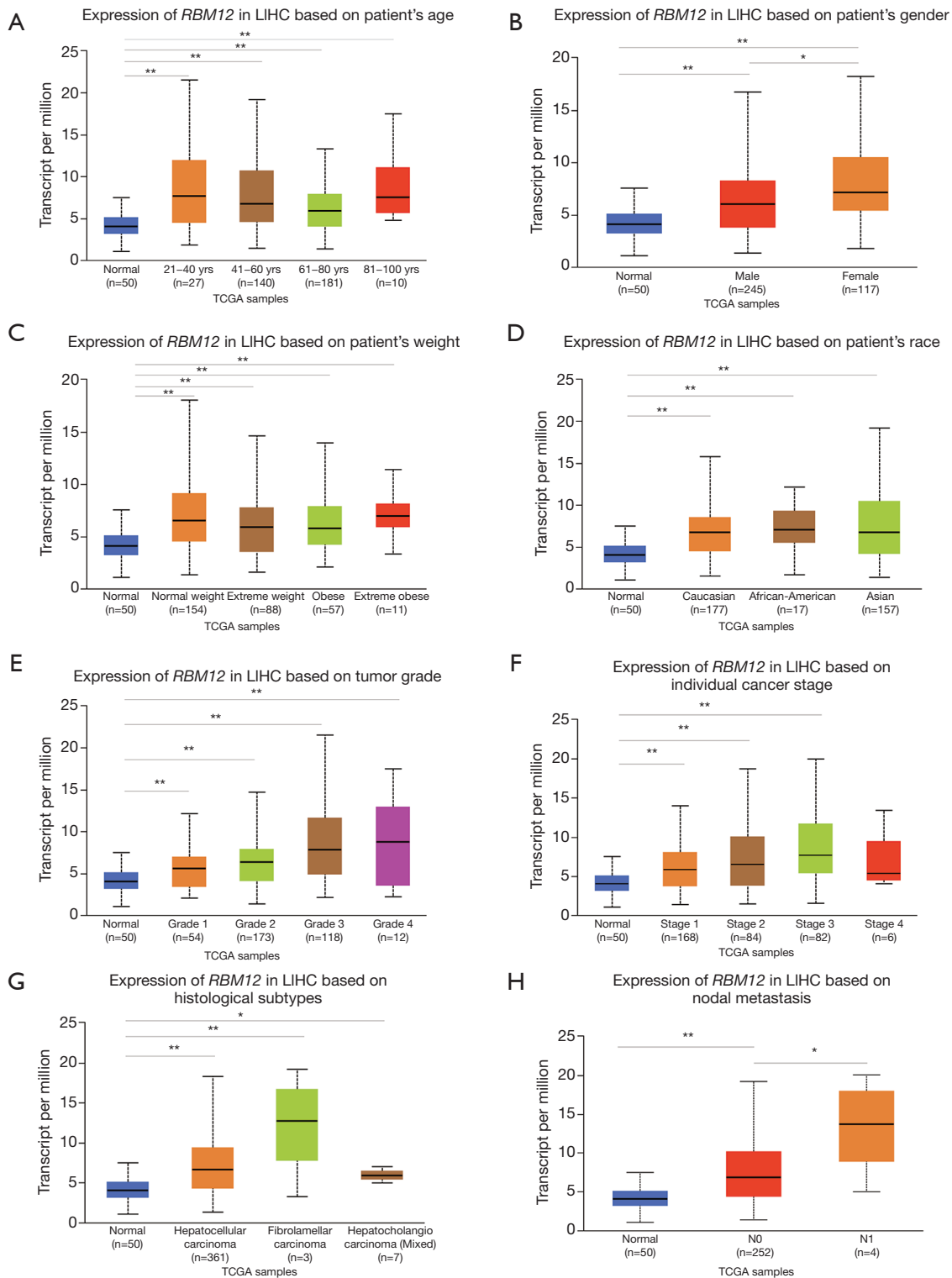


Figure 4 The expression of *RBM12* is related to a patient's clinical characteristics. Box plot showing the relationship of age (A), gender (B), weight (C), race (D), tumor grade (E), stage (F), histological subtypes (G), and nodal metastasis status (H) with *RBM12* expressions in liver cancer, respectively (*, $P < 0.05$; **, $P < 0.01$). *RBM12*, RNA-binding motif protein 12; LIHC, liver hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.

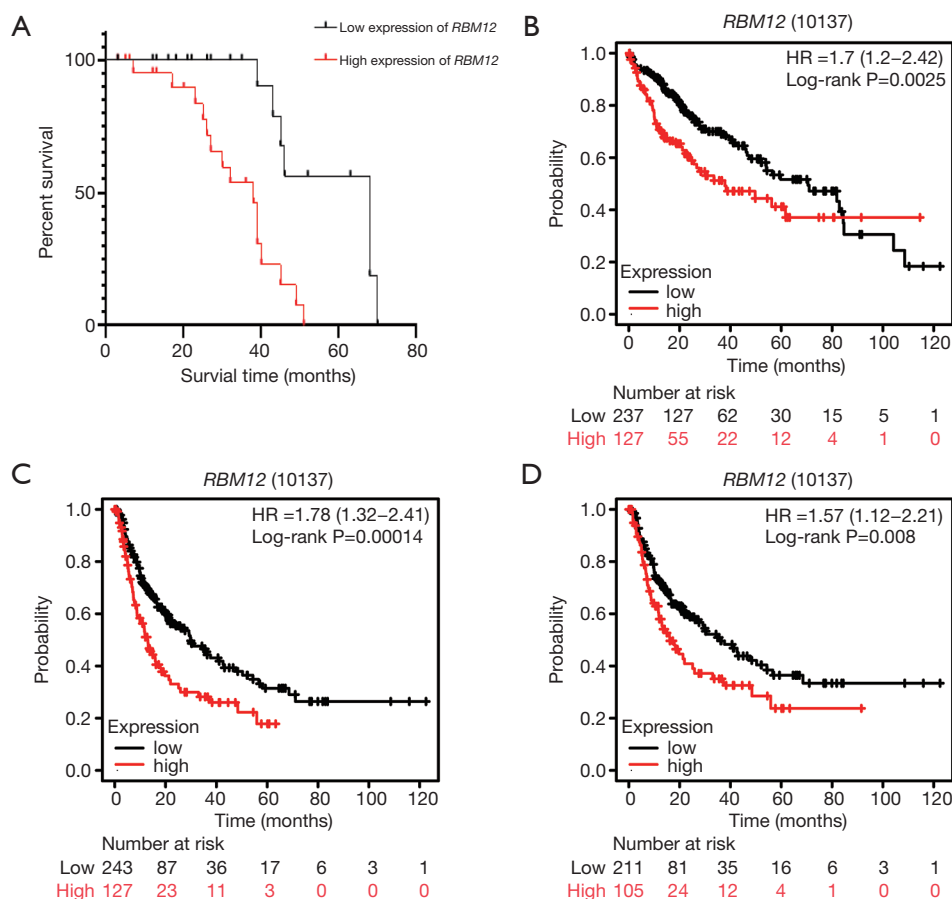


Figure 5 A high expression of *RBM12* indicates poor prognosis in HCC patients. (A) The prognostic analysis of 45 HCC patients showed that patients with a high expression of *RBM12* had poor prognosis. Analysis of the relationship between *RBM12* mRNA expression and OS (B), PFS (C), and RFS (D) in HCC patients, according to data from the Kaplan-Meier plotter database. *RBM12*, RNA-binding motif protein 12; HCC, hepatocellular carcinoma; OS, overall survival; PFS, progression-free survival; RFS, relapse-free survival.

majority of people with early-stage liver cancer exhibit no typical symptoms. AFP positivity is found in fewer than 70% of liver cancer patients, and is frequently employed as a diagnostic tool for the early detection of HCC (27). Nonalcoholic fatty liver disease (NAFLD) has become more prevalent due to modern lifestyles and dietary habits. Furthermore, effective hepatitis virus vaccination treatment procedures have become more accepted, and the etiology of liver illnesses has changed, the metabolic risk factors of HCC, including metabolic syndrome, obesity, type II diabetes and NAFLD, are increasing and may become the main causes of HCC (26). In light of these characteristics, novel HCC markers are required to improve the early detection rates of the disease.

Despite the ubiquity of HCC, the pathogenic mechanisms that cause it are still unknown (28). RBPs, which regulate

gene expression at the posttranscriptional level (4), are important in the development of HCC (7–11), while the core protein, *RBM12* has been linked to the onset of a variety of diseases (13–15). Even though these contributing factors have been recognized by the scientific community, no in-depth research into the role of *RBM12* in HCC has been conducted. Thus, a bioinformatics *RBM12* analysis of liver cancer was performed in this study.

In database study, *RBM12* was found to be overexpressed at the mRNA level in cirrhosis and HCC tissues. In addition, qRT-PCR, western blotting, and sample immunohistochemistry analysis suggested the upregulation of *RBM12* in HCC. Furthermore, *RBM12* was shown to be positively linked to age, gender, weight, race, grade, stage, status for nodal metastasis, and histological subtypes. *RBM12* was also shown to have a high diagnostic value

Table 2 Association of *RBM12* mRNA expression and clinicopathological features in HCC

Parameters	RBM12 expression			P value
	Cases (n=45)	Low level (n=22)	High level (n=23)	
Age (years)				0.4631
≤60	16	9	7	
>60	29	13	16	
Gender				0.5237
Male	35	18	17	
Female	10	4	6	
Tumor size (cm)				0.0014**
≤5	26	18	8	
>5	19	4	15	
Tumor number				0.6522
≤1	23	12	11	
>1	22	10	12	
AFP (μg/L)				0.0005**
≤20	17	14	3	
>20	28	8	20	
Pathologic stage				0.0273*
I-II	32	19	13	
III-IV	13	3	10	
Vascular invasion				0.0005**
No	25	18	7	
Yes	20	4	16	
Cirrhosis				0.5589
No	12	5	7	
Yes	33	17	16	

*, P<0.05; **, P<0.01. RBM12, RNA-binding motif protein 12; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein.

in the case of HCC, with high levels of *RBM12* being connected to poorer OS, PFS, and RFS. Furthermore, univariate and multivariate analyses of clinical data obtained from TCGA revealed significant differences in *RBM12* expression during the pathologic stage (P<0.00), in neoplasm cancer status (P<0.00), age (P=0.028) and weight (P=0.011). As a result, we believe *RBM12* is a useful diagnostic and prognostic marker for liver cancer.

A functional module from LinkedOmics was used to investigate the *RBM12* coexpression mode in the LIHC cohort. According to GO terms, *RBM12* coexpressed genes primarily participate in the cell cycle, peptidyl-lysine modification, *BRCA1-BARD1-BACH1*-DNA damage complex II, and nuclear export. We also analyzed TCGA data on liver cancer patients to discover the expression changes in *RBM12* gene sets. The results demonstrated that *RBM12* is involved in various pathways, including basal transcription factors, the cell cycle, and ncRNA export from the nucleus. Our findings suggest that elevated levels of *RBM12* in HCC patients may affect both cell cycle control and nuclear export. In multiple networks of genes coexpressed with *RBM12*, cell cycle regulation was a primary enrichment pathway. Cell cycle disruptions induce unlimited proliferation, which is an underlying mechanism of HCC (29,30). Whether *RBM12* affects a patient's prognosis by regulating the cell cycle remains to be further studied. The export of mRNA from the nucleus to the cytoplasm is an important regulatory phase in protein expression. Abnormal RNA export has been observed in primary human cancer specimens, and these cargo RNAs code for proteins that are involved in almost every aspect of cancer (31). *RBM12* is most likely involved in these processes, but more research is needed to determine the exact mechanisms of its involvement.

According to TCGA data, *RBM12* gene amplification is uncommon in HCC, implying that alternative processes are responsible for the elevated *RBM12* expression in HCC (32). Promoter hypomethylation contributes to tumorigenesis through the transcriptional activation of oncogenes (33), and in HCC, DNA methylation changes play a crucial role in regulating pathological and physiological processes (34). We investigated the DNA methylation status of *RBM12* in HCC and discovered that *RBM12* expression was closely correlated with DNMT expression. Moreover, several methylated CpG sites were significantly correlated with the outcomes of HCC patients. Epigenetic markers are established dynamically and reversibly, and epigenome-targeted medicines are also expanding the therapeutic portfolio for solid tumors (35). Patients with various forms of cancer, including HCC patients, have been treated solely with DNMT inhibitors or a combination of DNMT inhibitors and immunotherapy (36). Our findings suggest that DNA changes that affect the transcriptional levels of the *RBM12* gene may influence HCC carcinogenesis, implying that DNMT inhibitors could be an effective therapy for HCC patients.

Tumor immunotherapy is a relatively recent treatment

Table 3 Univariate analysis of OS in TCGA cohort

Parameters	Hazard ratio	95% CI	P value
<i>RBM12</i>	5.24	1.54–17.8	0.02
Age at initial diagnosis	1.01	1.00–1.03	0.16
Gender	0.83	0.58–1.18	0.47
Height	1.00	0.98–1.02	0.84
Neoplasm histologic grade	0.86	0.60–1.23	0.59
Pathologic m	0.59	0.41–0.85	0.01
Pathologic n	0.66	0.46–0.95	0.05
Pathologic stage	0.46	0.32–0.66	0.00
Pathologic treatment	0.47	0.32–0.67	0.00
Person neoplasm cancer status	2.40	1.64–3.49	0.00
Postoperative ablation embolization treatment	0.83	0.44–1.54	0.73
Postoperative medication and treatment	1.19	0.62–2.28	0.74
Weight	0.99	0.98–1.00	0.33

OS, overall survival; TCGA, The Cancer Genome Atlas; *RBM12*, RNA-binding motif protein 12.

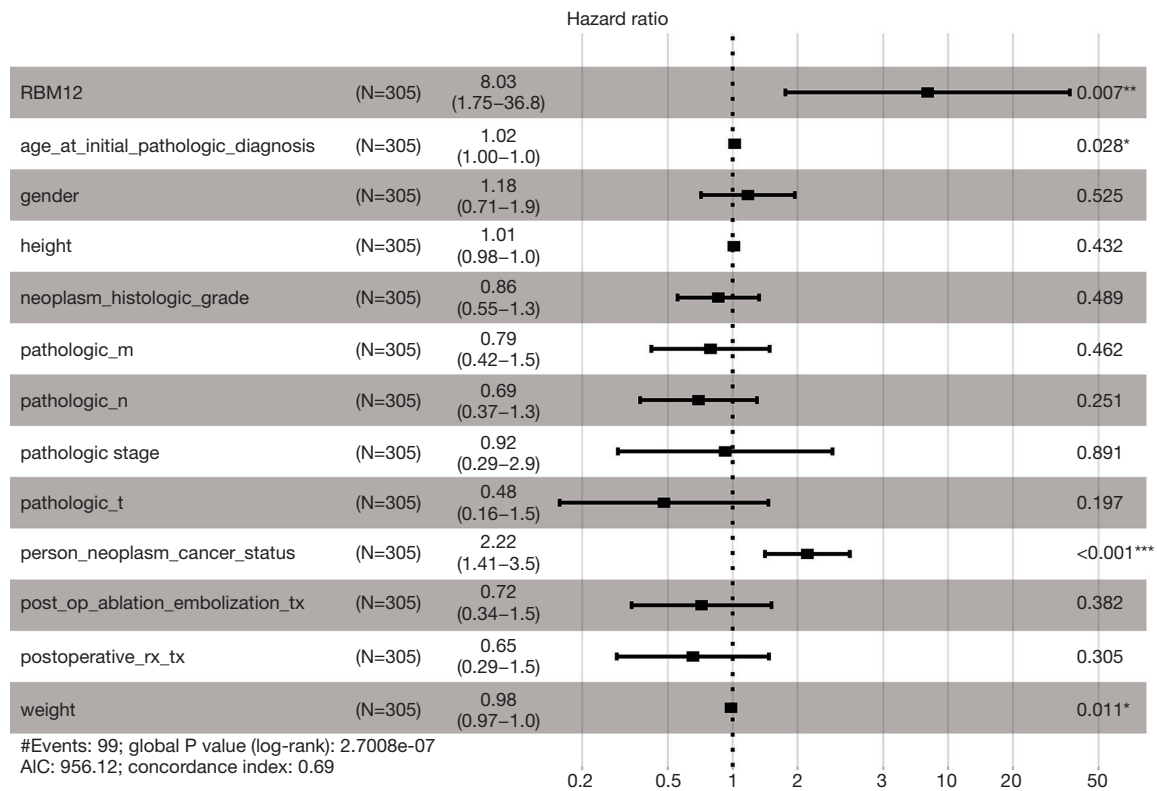


Figure 6 Multivariate analysis of HCC patients in TCGA. The multivariate analysis of *RBM12* was related to age (P=0.028), neoplasm cancer status (P<0.001), and weight (P=0.011). (*, P<0.05; **, P<0.01; ***, P<0.001). HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; *RBM12*, RNA-binding motif protein 12; AIC, Akaike information criterion.

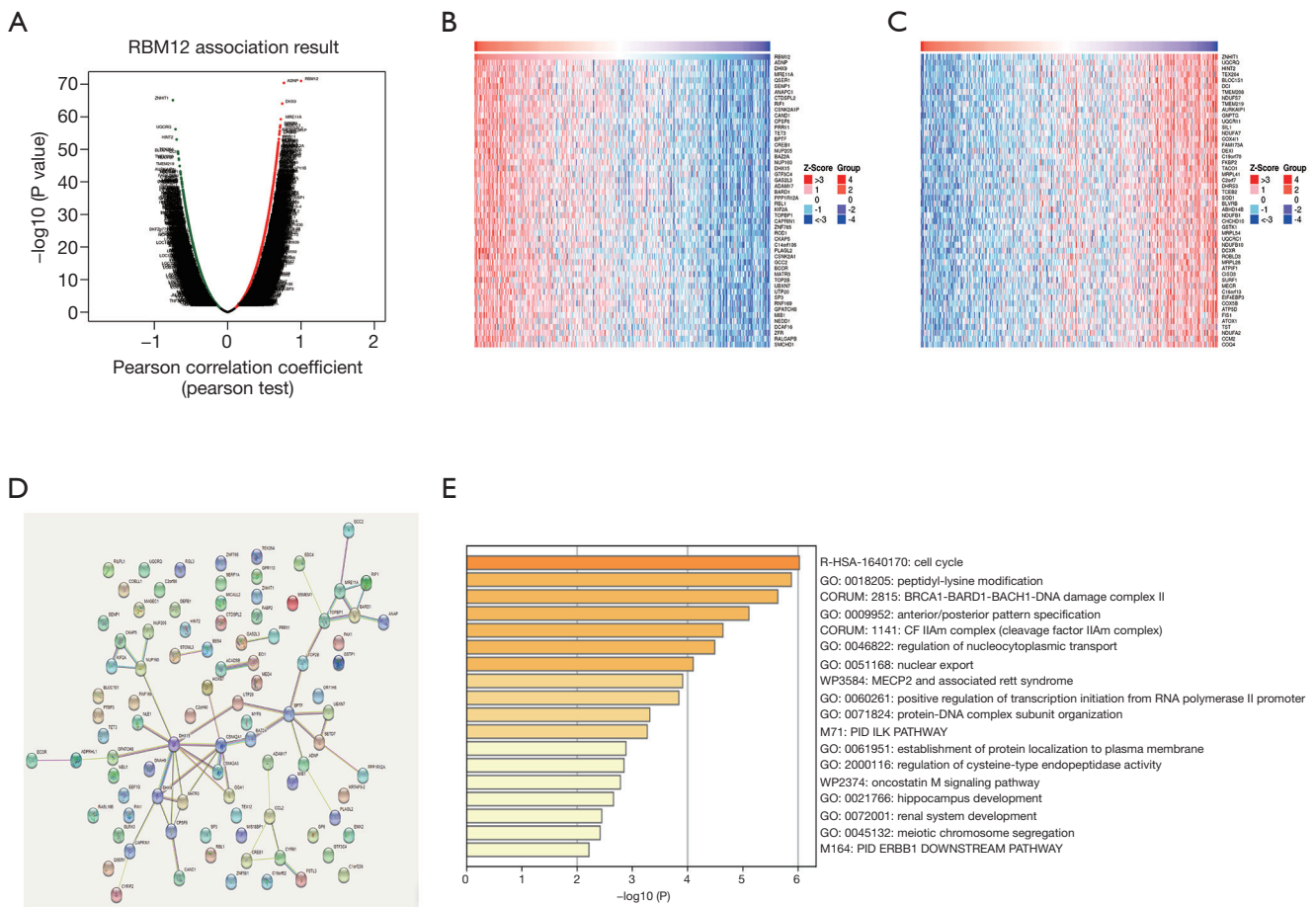


Figure 7 Significant related genes and pathways of *RBM12* in HCC obtained by LinkedOmics. (A) The correlated genes identified by Pearson’s correlation coefficient in the LIHC cohort. (B,C) Heat maps showing the top 50 genes positively and negatively correlated with *RBM12* in LIHC. Red dots indicate positively correlated genes and green dots indicate negatively correlated genes. (D) Protein-protein interaction network of *RBM12*. (E) Heatmap of GO analysis including BP, CC, and MF. *RBM12*, RNA-binding motif protein 12; HCC, hepatocellular carcinoma; LIHC, liver hepatocellular carcinoma; GO, Gene Ontology; BP, biological process; CC, cellular components; MF, molecular function.

option that is gaining credibility in the context of HCC treatment (37,38). Immunotherapy is a promising area of research into the tumor microenvironment for clinicians in search of therapeutic targets and biomarkers for prognosing and diagnosing HCC (39). The TIMER database was used in our research to find links between immune infiltration levels and *RBM12* expression in liver cancer, and according to our findings, the associations of *RBM12* with dendritic cells, macrophages, B cells, and T cells are the strongest. Furthermore, our CIBERSORT analysis demonstrated a link between *RBM12* expression and immune cell infiltration. Our results show that the levels of M0 macrophages and resting memory CD4+ T cells were

significantly higher in the high expression group, whereas the levels of resting mast cells, monocytes, and CD8+ T cells were significantly higher in the low expression group. In HCC patients, immune cell infiltration has a significant impact on survival (40), and we showed that increased abundance of resting memory CD4+ T cell, monocytes, and CD8+ T cells may be related to better OS. Taken together, these findings show that *RBM12* is crucial to HCC immune infiltration, cell regulation, and recruitment.

Conclusions

Overall, this is the first study to establish *RBM12* as a novel

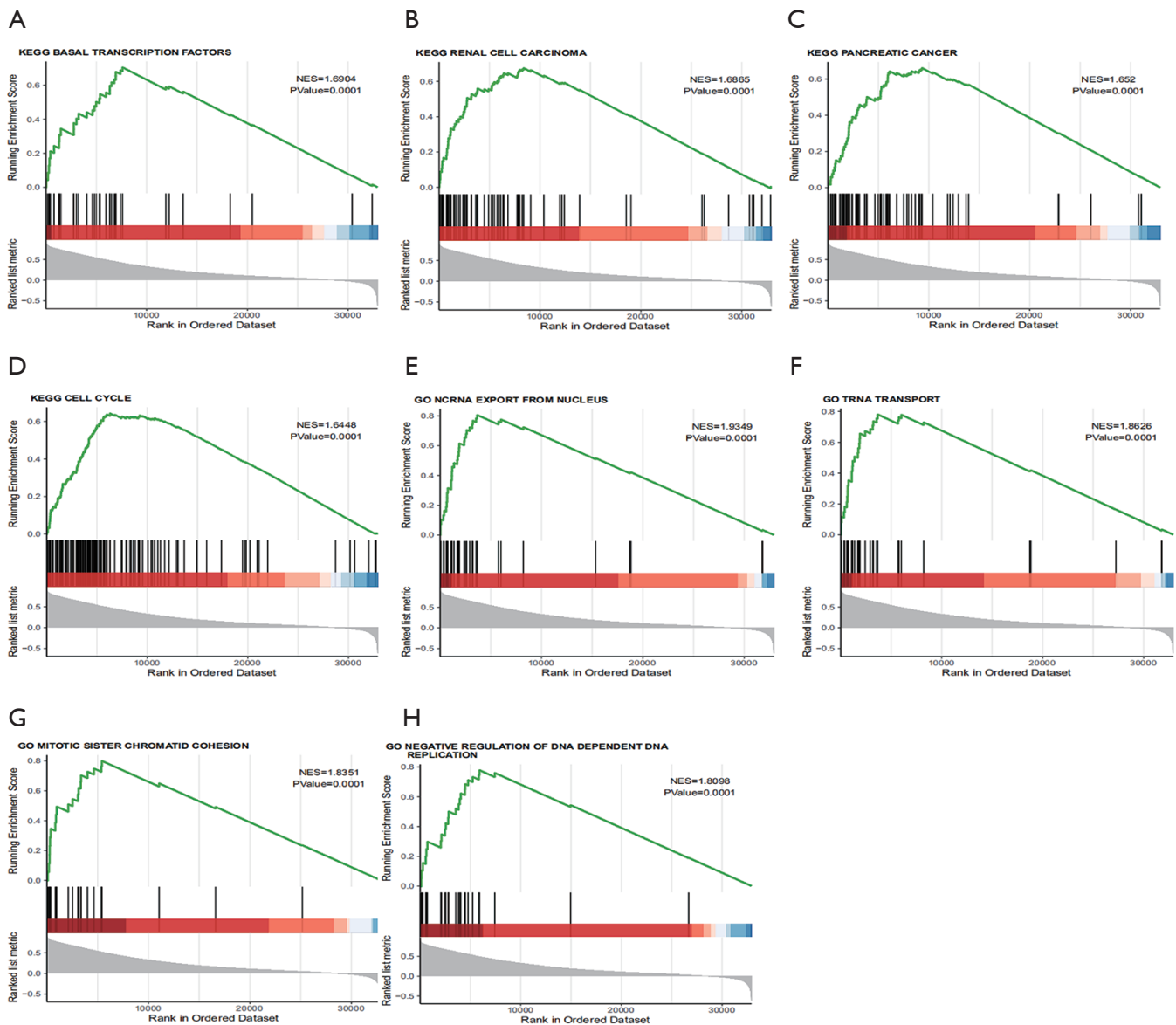


Figure 8 GO and KEGG pathway enrichment analysis of *RBM12*. KEGG pathways including (A) Enrichment of genes in the KEGG basal transcription factors by GSEA. (B) Enrichment of genes in the KEGG renal cell carcinoma by GSEA. (C) Enrichment of genes in KEGG pancreatic cancer by GSEA. (D) Enrichment of genes in the KEGG cell cycle by GSEA, and go terms contain (E) Enrichment of genes in the GO ncRNA export from nucleus by GSEA. (F) Enrichment of genes in the GO tRNA transport by GSEA. (G) Enrichment of genes in the GO mitotic sister chromatid cohesion by GSEA. (H) Enrichment of genes in the GO negative regulation of DNA-dependent DNA replication by GSEA. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; tRNA, transcription RNA.

HCC biomarker. Our findings revealed that *RBM12* levels are considerably higher in HCC, with high *RBM12* levels being predictive of poor prognosis. LinkedOmics and GSEA analysis found *RBM12* to be significantly associated with tumor progression, particularly in regard to basal

transcription factors, the cell cycle, and nuclear export. In addition, we found that abnormal DNA methylation may increase oncogenic *RBM12* expression. In conclusion, our findings show that *RBM12* is a suitable target for liver cancer immunotherapy.

Table 4 Signaling pathways most significantly negative correlated with *RBM12* expression based on their NES, P value and FDR

Parameters	NES	P value	FDR q value
KEGG_PRIMARY_BILE_ACID_BIOSYNTHESIS	-2.1298	0.006289	0.007927
KEGG_ASCORBATE_AND_ALDARATE_METABOLISM	-1.6891	0.020833	0.022422
GO_FLAVONOID_METABOLIC_PROCESS	-3.0862	0.005814	0.017394
GO_TRIGLYCERIDE_RICH_LIPOPROTEIN_PARTICLE_REMODELING	-2.7910	0.004329	0.013940
GO_ARACHIDONIC_ACID_MONOOXYGENASE_ACTIVITY	-2.7863	0.006667	0.019478
GO_SPHERICAL_HIGH_DENSITY_LIPOPROTEIN_PARTICLE	-2.7442	0.001440	0.005740
GO_VERY_LOW_DENSITY_LIPOPROTEIN_PARTICLE_REMODELING	-2.7271	0.002631	0.009422
GO_HAPTOGLOBIN_HEMOGLOBIN_COMPLEX	-2.6354	0.002631	0.009422
GO_FLAVONOID_GLUCURONIDATION	-2.6259	0.001730	0.006655
GO_HIGH_DENSITY_LIPOPROTEIN_PARTICLE_REMODELING	-2.5859	0.008771	0.024175

NES, normalized enrichment score; FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

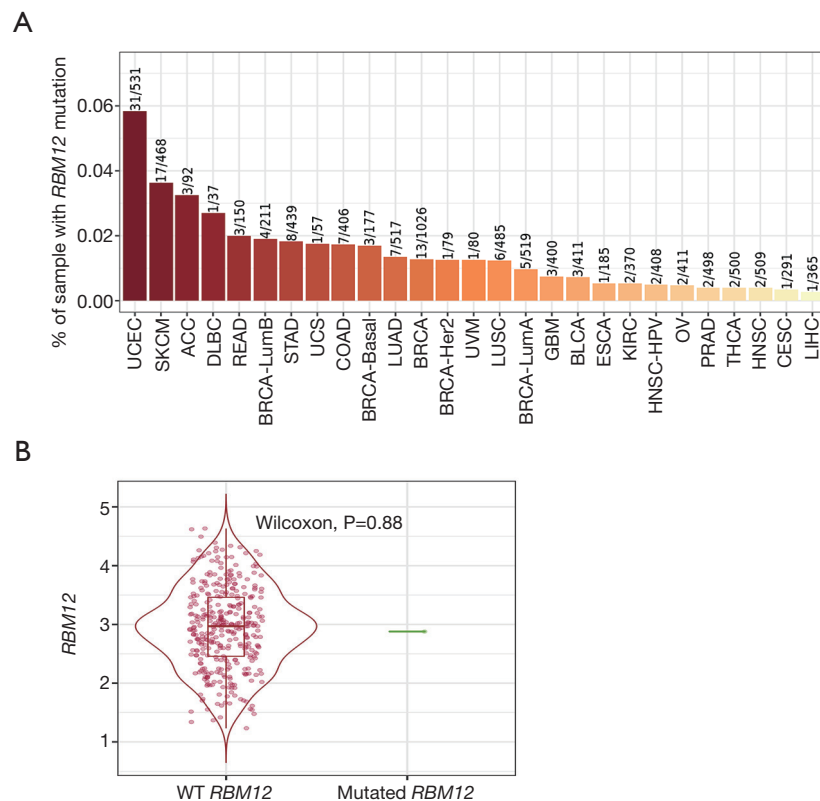
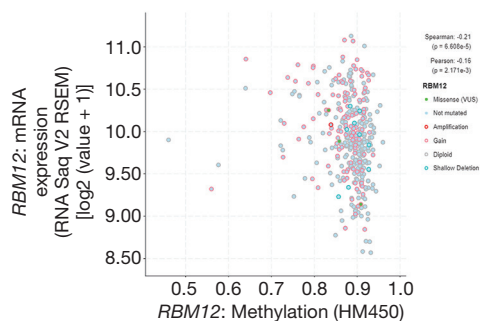


Figure 9 Mutation of *RBM12* in HCC and other tumors. (A) Compared with other tumors, *RBM12* has the lowest mutation rate in HCC. (B) *RBM12* mutation is uncommon in HCC. *RBM12*, RNA-binding motif protein 12; HCC, hepatocellular carcinoma.

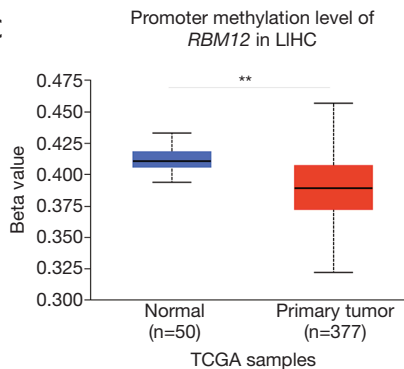
A



B



C



D

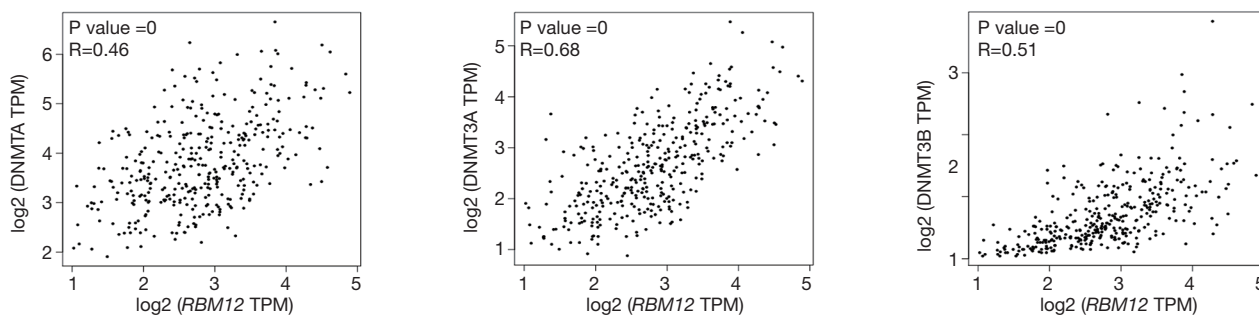
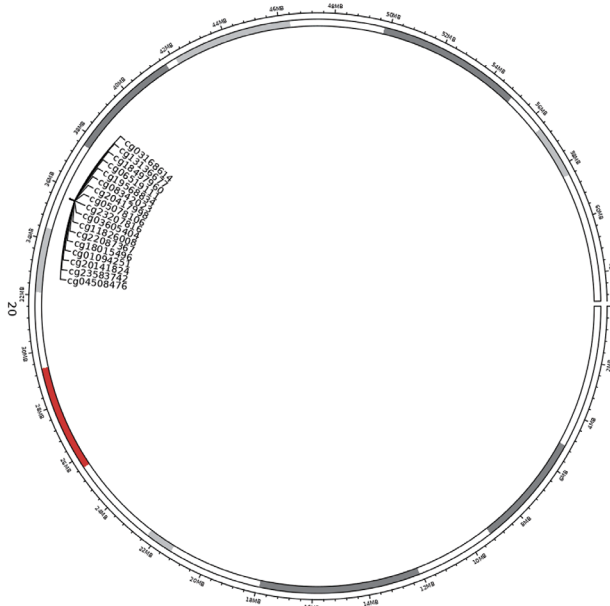


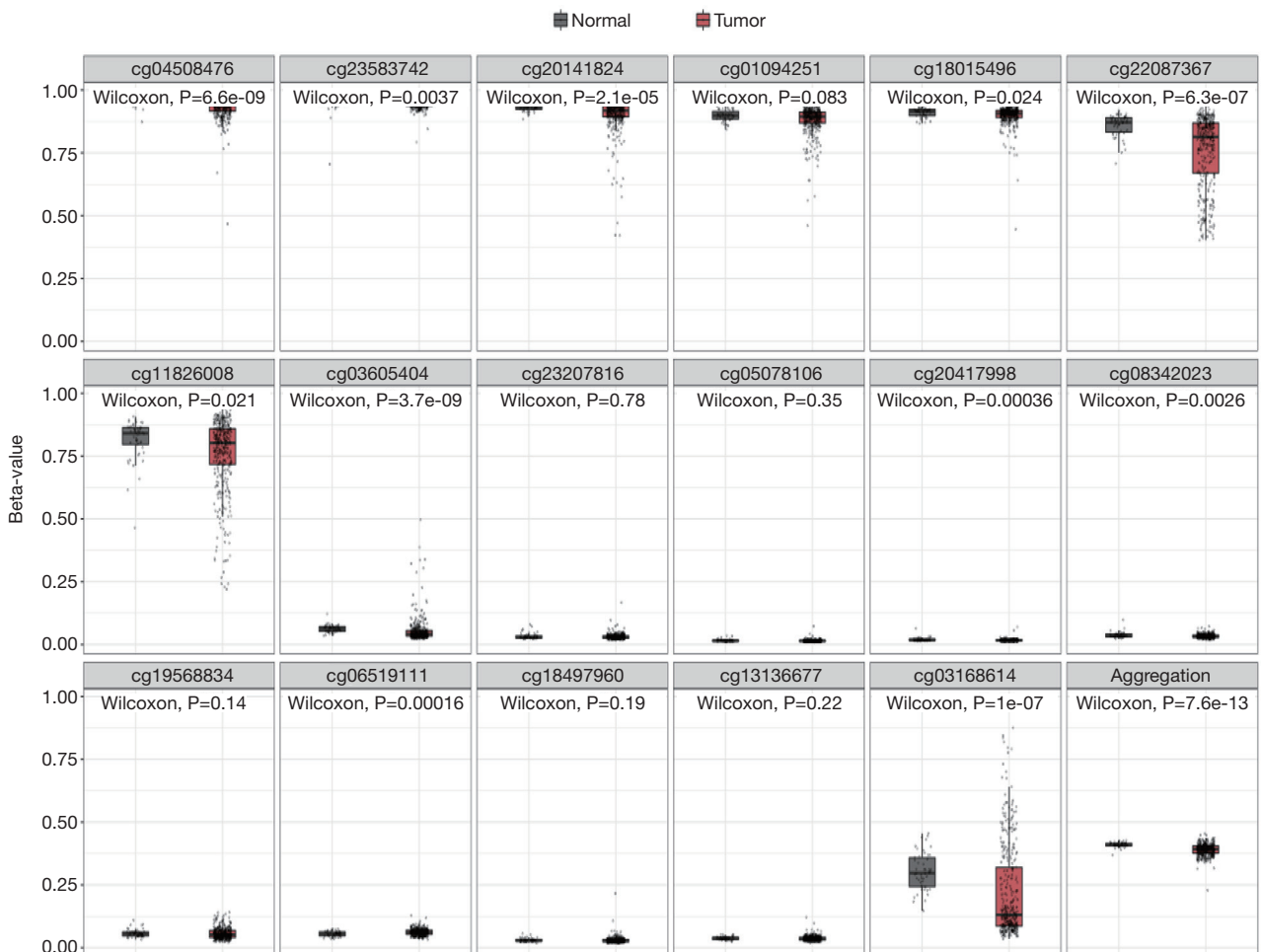
Figure 10 Promoter hypomethylation results in *RBM12* overexpression. (A) *RBM12* DNA methylation modification in HCC. (B) The correlation between *RBM12* mRNA expression and *RBM12* promoter methylation in TCGA's LIHC cohort ($R = -0.16$; $P < 0.001$). (C) *RBM12* promoter methylation status of normal liver tissues ($n = 50$) and liver cancer tissues ($n = 377$) in TCGA's LIHC cohort (**, $P < 0.01$). (D) Correlation between *RBM12* mRNA expression and DNMT expression. *RBM12*, RNA-binding motif protein 12; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; LIHC, liver hepatocellular carcinoma; DNMT, DNA methyltransferase; TPM, transcripts per million.

A



B

Comparison of methylation value between normal and tumor samples



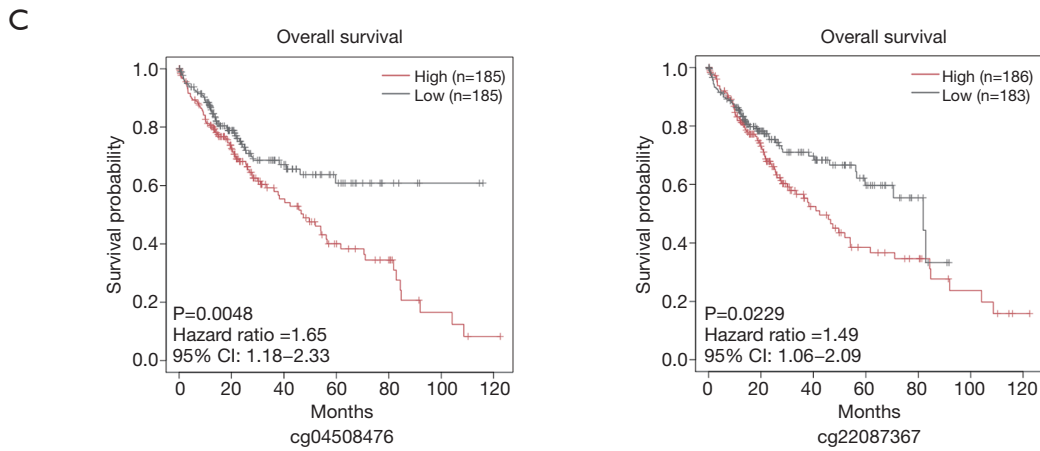
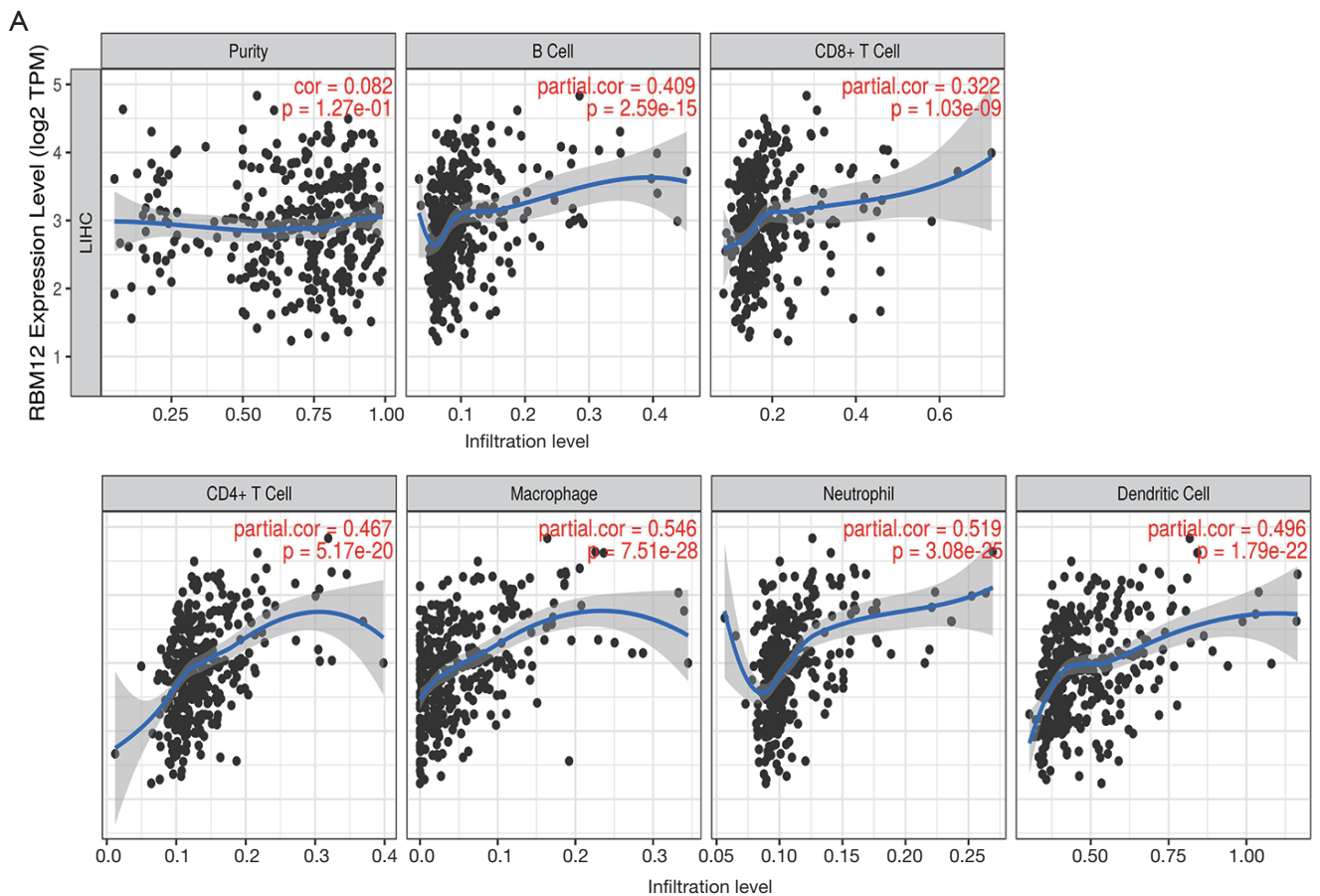


Figure 11 Identification of methylation sites associated with *RBM12*. (A) Chromosomal distribution of the methylation probes associated with *RBM12*. (B) Methylation level of methylation sites in liver cancer tissues and adjacent normal tissues. (C) The DNA methylation levels of cg04508476 and cg22087367 were correlated with patient prognosis. *RBM12*, RNA-binding motif protein 12.



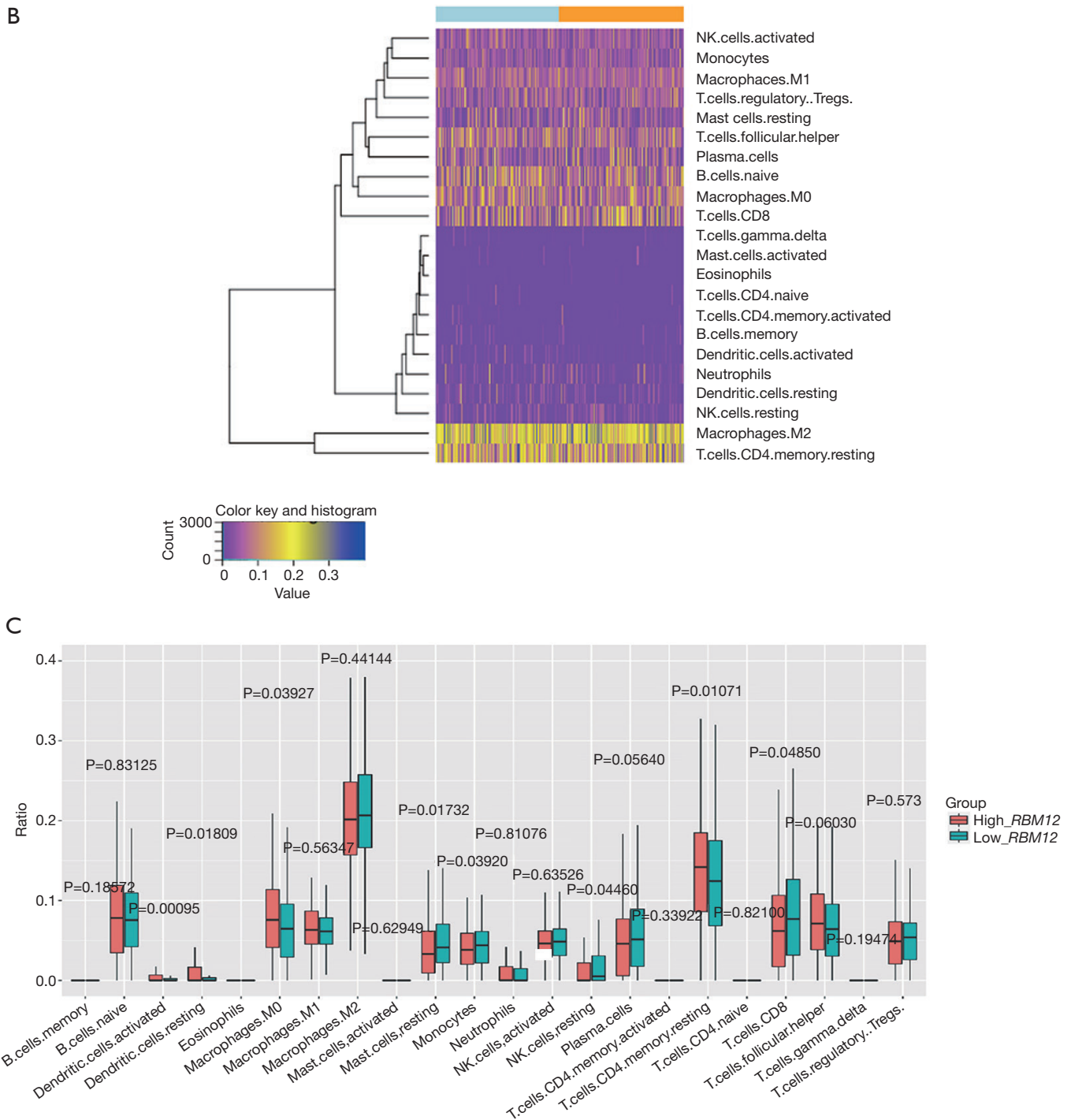


Figure 12 Correlation of *RBM12* expression with immune infiltration levels in HCC. (A) *RBM12* expression showed significant positive correlations with infiltrating levels of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells in HCC. (B) Heat map of immune infiltration. (C) The association between different kinds of TIICs and the high and low *RBM12* expression groups. *RBM12*, RNA-binding motif protein 12; HCC, hepatocellular carcinoma; TIIC, tumor-infiltrating immune cell.

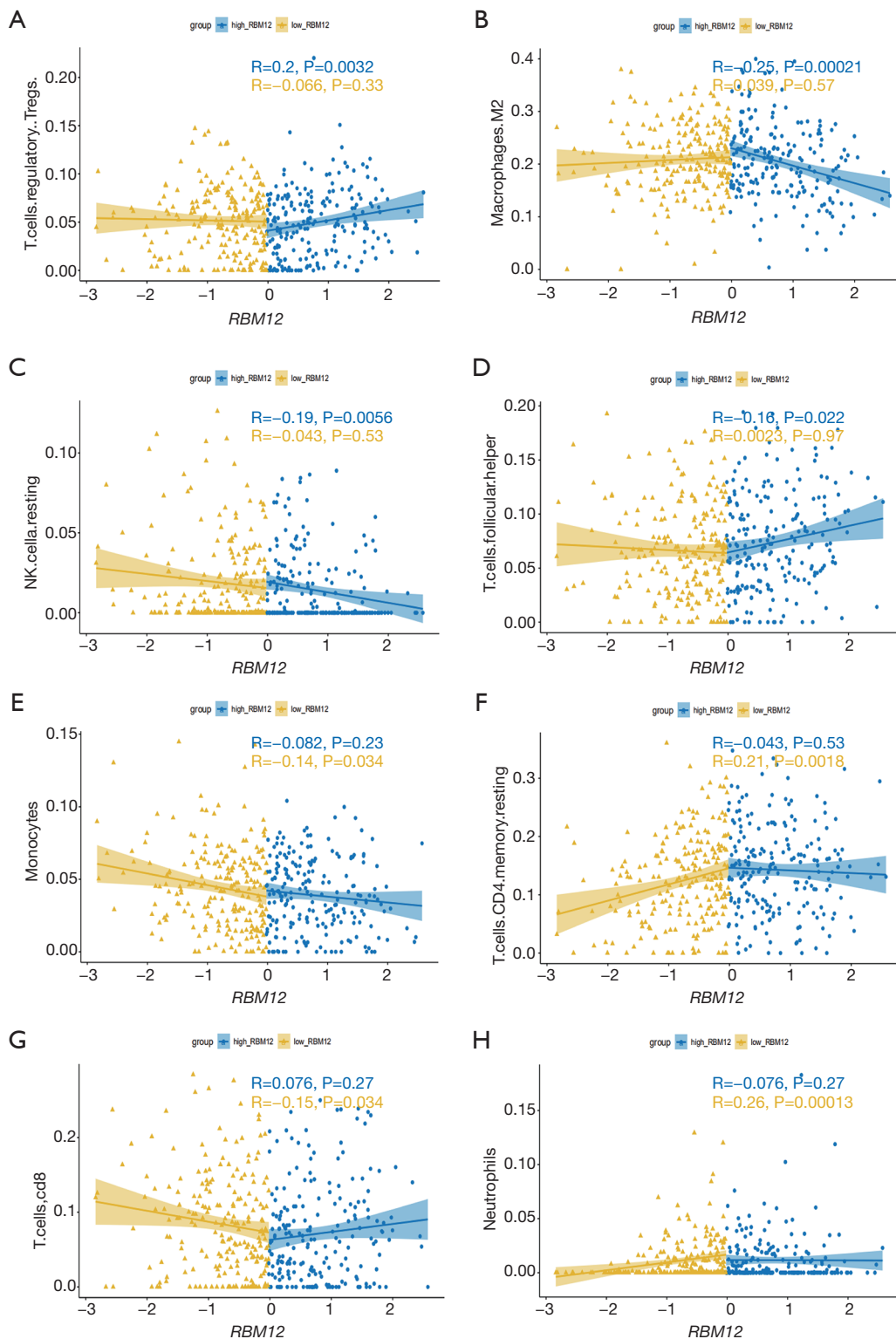


Figure 13 The expression of TIICs in liver cancer is related to *RBM12*. In the high expression group of *RBM12*, regulatory T cells (A), M2 macrophages (B), resting NK cells (C), and follicular helper T cells (D) were related to the expression of *RBM12*; meanwhile, in the low *RBM12* expression group, monocytes (E), resting memory CD4+ T cells (F), CD8+ T cells (G), and neutrophils (H) were related to the expression of *RBM12*. TIIC, tumor-infiltrating immune cell; *RBM12*, RNA-binding motif protein 12; NK, natural killer.

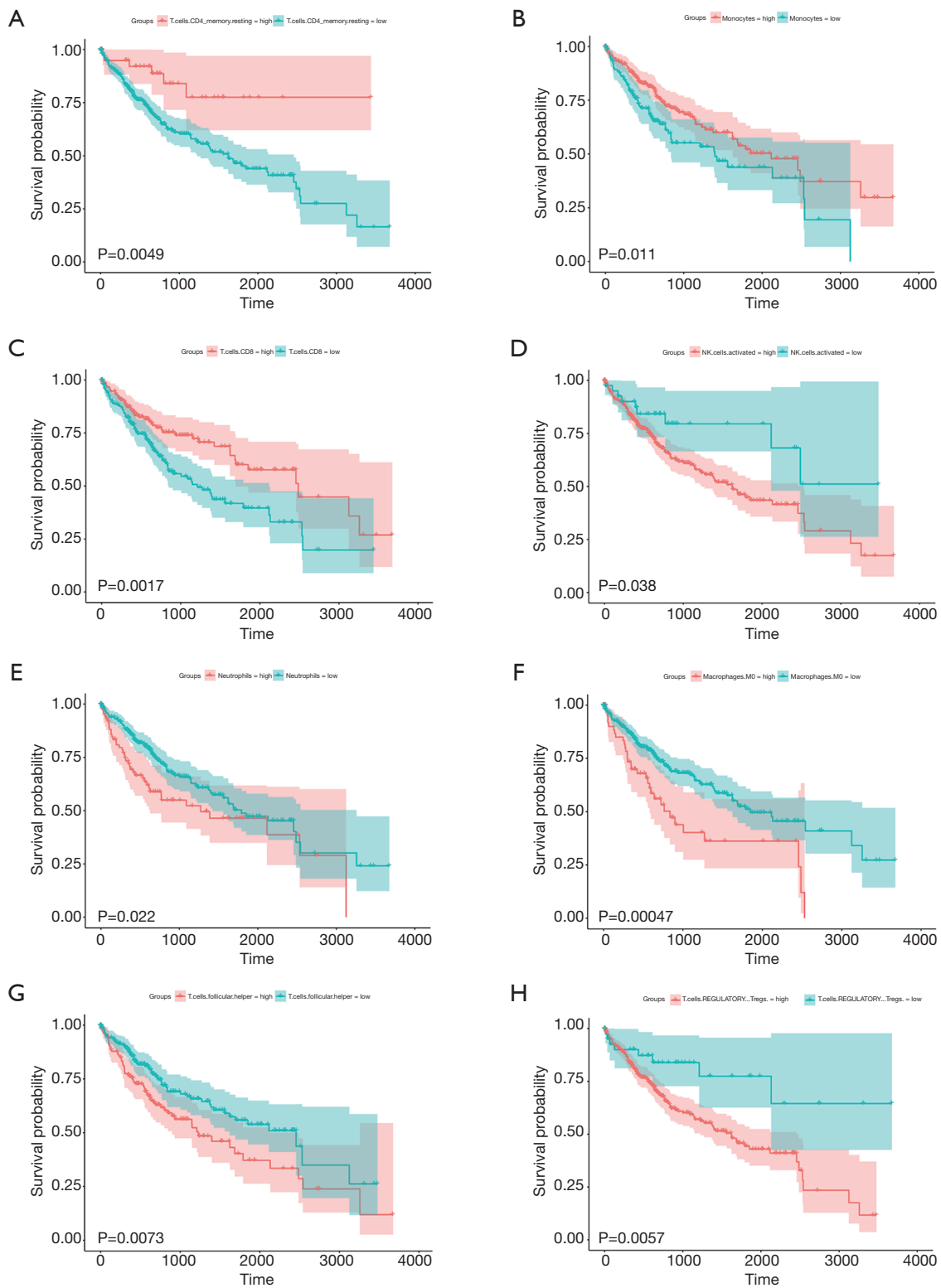


Figure 14 The effect of TIICs on prognosis. (A) Resting memory CD4+ T cells, (B) monocytes, (C) CD8+ T cells, (D) activated NK cells, (E) neutrophils, (F) M0 macrophages, (G) follicular helper T cells, and (H) regulatory T cells. TIIC, tumor-infiltrating immune cell; NK, natural killer.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to this study are appropriately investigated and resolved. The included patients gave their informed consent, and the Ethics Committee of the Affiliated Hospital of Nantong University approved the study (No. 2018-L006). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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