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Original Article

Overexpression of RBM34 Promotes Tumor Progression and Correlates with Poor Prognosis of Hepatocellular Carcinoma

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Abstract

Background and Aims: Emerging evidence suggests that RNA-binding motif (RBM) proteins are involved in hepatocarcinogenesis and act either as oncogenes or tumor suppressors. The objective of this study was to investigate the role of RBM34, an RBM protein, in hepatocellular carcinoma (HCC). ***Methods:*** We first examined the expression of RBM34 across cancers. The correlation of RBM34 with clinicopathological features and the prognostic value of RBM34 for HCC was then investigated. Functional enrichment analysis of RBM34-related differentially expressed genes (DEGs) was performed to explore its biological function. RNA sequencing (RNA-seq) was applied to identify downstream genes and pathways affected upon RBM34 knockout. The correlation of RBM34 with immune characteristics was also analyzed. The oncogenic function of RBM34 was examined in *in vitro* and *in vivo* experiments. ***Results:*** RBM34 was highly expressed in hepatocellular carcinoma and correlated with poor clinicopathological features and prognosis. RBM34 was positively associated with tumor immune cell infiltration, biomarkers of immune cells, and immune checkpoint expression. A positive correlation was also observed between RBM34, T cell exhaustion, and regulatory T cell marker genes. Knockout of RBM34 significantly inhibited cell proliferation, migration, and xenograft tumor growth, and sensitized HCC cells to sorafenib treatment. RBM34 inhibition reduced FGFR2 expression and affected PI3K-AKT pathway activation in HCC cells. ***Conclusions:*** Our study suggests that RBM34 may serve as a new prognostic marker and therapeutic target of HCC.

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Keywords: RBM34; Hepatocellular carcinoma; Oncogene; Sorafenib resistance; Immune infiltration.

Abbreviations: DEGs, differentially expressed genes; DSS, disease-specific survival; HCC, hepatocellular carcinoma; OS, overall survival; PFI, progression-free interval; qPCR, quantitative PCR; RBM, RNA-binding motif; RBPs, RNA-binding proteins.

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Introduction

Hepatocellular carcinoma (HCC) is the major type of liver cancer and the second most common cause of cancer-related deaths worldwide.[1,2] Some therapies, such as surgical resection and liver transplant, are effective for treating HCC,[3] but the prognosis is poor because of the high recurrence rate and delayed diagnosis.[4] Therefore, there is an urgent need to identify novel therapeutic vulnerabilities.

RNA-binding proteins (RBPs) include many subfamily proteins, such as RNA recognition motif (RRM), RNA-binding motif, and ribonucleoprotein (RNP) motif protein.[5,6] RBPs may contribute to cancer progression because of their ability to regulate RNA transcription, splicing, localization, stability, and translation.[7] It has been reported that dysregulation of RBPs was involved in several human cancers, including HCC.[8–10] For example, RBM43 is down-regulated in HCC and correlates with poor prognosis in patients. RBM43 is a tumor suppressor through the regulation of cyclin B1 mRNA stability in HCC.[11] The long noncoding RNA HOTAIR promotes HCC migration and invasion through the downregulation of RBM38.[12] Another study has demonstrated that RBMY contributes to the male predominance of HCC by regulating androgen receptor activity[13] and enhances HCC stemness by activating the β-catenin signaling pathway.[14] Immunotherapy has proven to be a promising strategy for treating human HCC.[15,16] However, biomarker-based patient classification for an optimum therapeutic response is an unmet need.[17] Recent studies have reported that RBPs are involved in anti-PD1 immunotherapy for HCC treatment.[18,19] However, the link between RBM family members and antitumor immunity in HCC has not been not fully studied.

A-binding motif protein (RBM34) is a member of the RBM protein family. Similar to other RBM proteins, RBM34 contains two RNA-binding domains, RNA-binding motif 1 (RNP1) and RNA-binding motif 2 (RNP2). However, whether its role in liver cancer progression or tumor suppression is similar to other RBM proteins remains unclear. In this study, we performed a comprehensive expression and survival analysis of RBM34 in multiple types of human cancer. Functional enrichment analysis was performed to explore the biological function of RBM34 in HCC. RNA-seq was performed to identify RBM34-regulated genes and pathways in HCC. Correlations between RBM34 expression and immune cell infiltration, immune cell biomarkers, or immune checkpoints in HCC were identified by bioinformatics analysis. The oncogenic function of RBM34 was validated by *in vitro* and *in vivo* experiments. The results suggest that RBM34 may be a novel therapeutic target for HCC treatment.

Methods

Cell culture and reagents

Human liver cancer cell lines Huh7 and SK-hep1 were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Clark, USA) and 1% streptomycin/penicillin (Beyotime, China) as previously described.[20] To knockout RBM34 expression, two guide RNAs (5′-CGCGGCGAACACCATTCCAGAGG-3′; 5′-GTCGCCAGTAGCTTATTTCGCGG-3′) were cloned into a lentiCRISPRv2 vector. CRISPR constructs were transiently transfected into Huh7 or Sk-hep1 cells for 24 h. The cells were then puromycin-selected for another 72 h. RBM34 mutant cells were pooled to prevent selection effects of a single clone. The primary antibody against β-actin (#AA33122) was obtained from Bioworld (Nanjing, China). Antibodies against RBM34 (#A10139), FGFR2 (#A19051), phospho-AKT1-S473 (#AP0140), AKT (#A18120) phospho-ERK1/2 (#AP0472), ERK1/ERK2 (#A11116), HRP goat anti-mouse IgG (H+L) (#AS003), and HRP goat anti- rabbit IgG (#AS029) were purchased from Abclonal.

Cell proliferation and colony formation assays

Proliferation was assayed by counting the number of cells. Briefly, identical numbers of cells were inoculated on day 0 and then counted with a hemocytometer at various times. Cells were grown for 14 days in the culture medium, the colonies were fixed in a 4% paraformaldehyde solution, stained with 0.5% crystal violet, and the colonies were counted using ImageJ software.

Cell migration and invasion assays

For wound healing assays, HCC cells were inoculated in 6-well plates (5×10^5 cells/well) and incubated at 37°C for 24 h. A sterile 10 μL pipette tip was used to make the wound after the cells were completely confluent. The scratched area was measured using ImageJ software after five random fields were captured at 0 and 48 h. For the invasiveness and immigration assays, cells were resuspended in 200 μL serum-free medium and placed in the upper compartment of the Transwell chamber with or without Matrigel. Crystal violet was used to stain migrating or invading cells. The numbers of cells in five randomly selected areas were counted by light microscopy.

Xenograft tumor growth assay

The Animal Care Committee at Xuzhou Medical University approved the animal procedures. Four-week-old specific-pathogen free BALB/c nude mice were procured from Beijing Vital River Laboratory Animal Technology. The mice were subcutaneously injected in each flank, with Huh7 (1×10^7) or SK-hep1 (5×10^6) cells in 100 μL serum-free DMEM with 0.5 v/v Matrigel (BD, San Jose, USA). Tumor size was determined as previously described.[21]

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qPCR)

qPCR was performed as previously described.[21] In brief, the total RNA from control or RBM34 knockout Huh7 cells was extracted by with TRZIOL (Invitrogen, USA) following the manufacturer's instructions and 2 μg RNA was used to synthesize cDNA by using PrimeScript RT Master Mix kit (Takara, Shiga, Japan). An Applied Biosystems 7500 Fast Real-Time PCR machine was used to perform the qPCR assays with SYBR Green probes (Takara). PrimerBank provided the primers used in the gene expression assays.[22] The following primers were used: SOAT2: 5′-ATGGAAACACTGAGACGCACA-3′ (forward), 5′-GGTAGGATTGTATAGCCTCCCG-3′ (reverse); DLK1: 5′-GCACTGTGGGTATCGTCTTCC-3′ (forward), 5′-CTCCCCGCTGTTGTACTGAA-3′ (reverse); PCSK9: 5′-CCTGGAGCGGATTACCCCT-3′(forward), 5′-CTGTATGCTGGTGTCTAGGAGA-3′(reverse); FGFR2: 5′-GGTGGCTGAAAAACGGGAAG-3′ (forward), 5′-AGATGGGACCACACTTTCCATA-3′ (reverse); LCN2: 5′-GACAACCAATTCCAGGGGAAG-3′ (forward), 5′-GCATACATCTTTTGCGGGTCT-3′ (reverse).

Expression and survival analysis

The RNA sequencing data of 33 types of cancer containing clinical information were downloaded from The Cancer Genome Atlas (TCGA) database. We used the Wilcoxon rank-sum test to analyze the RBM34 mRNA expression in normal and liver cancer tissues. Immunohistochemical (IHC) staining data from the Human Protein Atlas (HPA) (https://www.proteinatlas.org/) were obtained to examine the protein level of RBM34 in liver tissues.

To evaluate the prognostic value of the *RBM34* gene in HCC patient prognosis, Kaplan-Meier curves were constructed to show the association of RBM34 with overall survival (OS), progression-free interval (PFI), and disease-specific survival (DSS) of HCC patients from TCGA. For the Kaplan-Meier curves, *p*-values and hazard ratios (HRs) with 95% confidence intervals (CIs) were generated by log-rank tests and univariate Cox proportional hazards regression.

Functional enrichment analysis

The Limma Package (version 3.40.2) of R software was used to investigate the differential expression of mRNAs. Adjusted *p*-values were analyzed to correct for false-positive results. An adjusted $p<0.05$ and log fold-change > 1 were defined as the thresholds for the screening of differentially expression genes (DEGs). To further understand the oncogenic function of RBM34, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using the ClusterProfiler package.

Correlations of RBM34 expression with immune characteristics in HCC

The Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) was used to investigate the association of RBM34 expression with the extent of immune cell infiltration, macrophage cell marker genes, and regulatory T cell (Treg) marker genes in HCC.[23] Additionally, the ESTIMATE algorithm was applied to analyze the immune and stromal scores in HCC according to corresponding transcriptional data, and the correlations of the scores with RBM34 expression were calculated.

Statistical analysis

The Kruskal-Wallis test was used to compare the difference in the expression of RBM34. Correlation analysis of RBM34 with various immune-associated genes was performed by

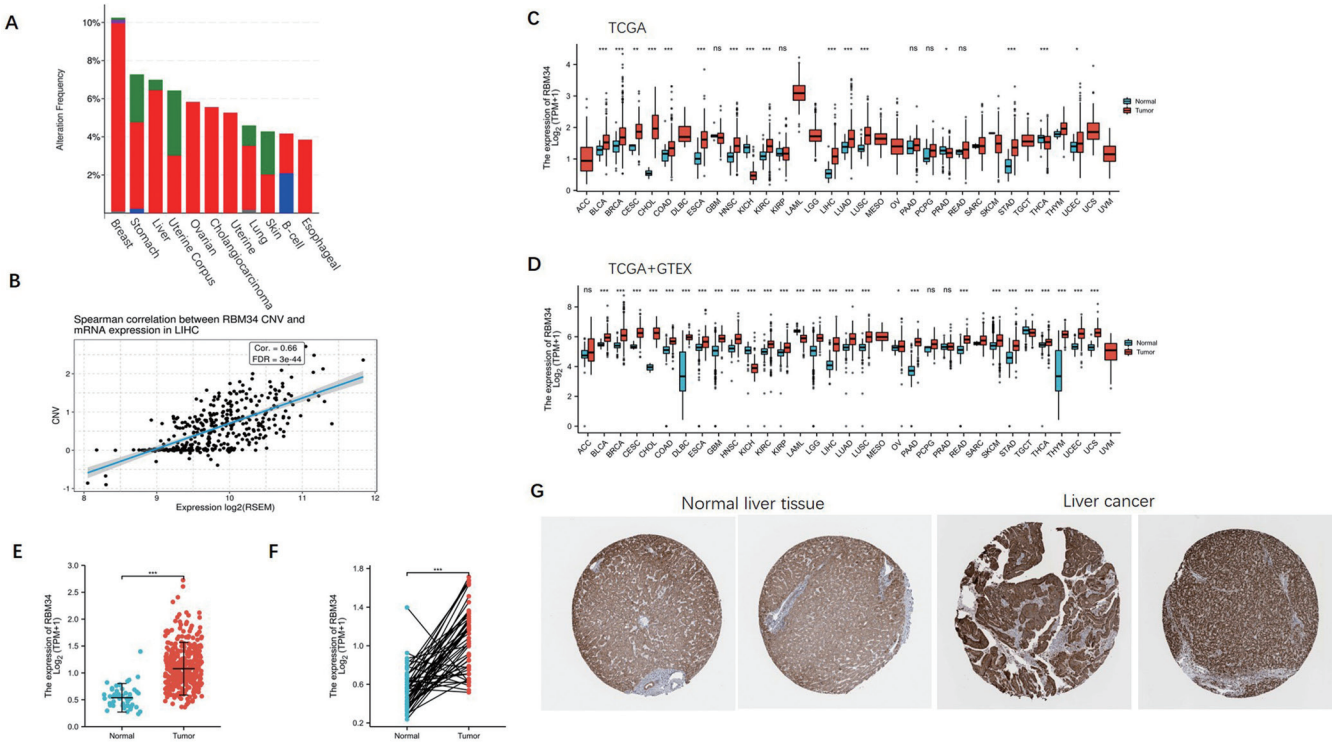


Fig. 1. RBM34 is overexpressed in HCC. (A) cBioPortal examination of TCGA datasets revealed RBM34 genomic changes across human malignancies. (B) RBM34 mRNA expression are positively correlated with its gene copy numbers. (C) The expression of RBM34 in normal and cancer tissues across different cancers. (D) RBM34 expression in normal and cancer tissues from the TCGA and GTEx databases. (E, F) RBM34 expression in HCC with nonmatched and matched normal tissues. (G) RBM34 protein expression in normal and tumor liver tissues determined by immunohistochemistry staining data from the Human Protein Atlas. *p<0.05; **p<0.01; ***p<0.001. HCC, hepatocellular carcinoma; RBM, RNA-binding motif.

Spearman's test. Survival analysis was estimated by the Kaplan-Meier method to evaluate the predictive performance of RBM34 in HCC. Log-rank tests were used to adjust the significance of the difference between different groups. p-values <0.05 and log-rank p<0.05 were considered statistically significant.

Results

RBM34 is overexpressed in HCC

Cancer genomics analysis of the TCGA HCC dataset found that gene amplification was frequent in RBM genes, especially in the RBM34 gene (Supplementary Fig. 1). We performed a pancancer analysis of RBM34 gene alteration. The analysis showed that the RBM34 gene was amplified in many human cancers, specifically in 10.2% of breast, 7% of liver cancer, and 5.6% of cholangiocarcinomas (Fig. 1A). The copy number of RBM34 was correlated with its mRNA expression (R=0.66, p=3e−44; Fig. 1B). We next examined the expression of RBM34 in 33 types of human cancer tissues and adjacent normal tissues. As shown in Fig. 1C, RBM34 was significantly upregulated in multiple cancer types, including HCC. We also analyzed its expression in the integrated TCGA and Genotype Tissue Expression (GTEx) database because of the limited adjacent normal tissues in the TCGA database. The analysis confirmed that RBM34 was overexpressed in liver cancer tissues compared with noncancerous tissue (Fig. 1D). In addition, the differential expression of RBM34 in the HCC samples compared with matched and unmatched adjacent normal tissues was also investigated. The analysis confirmed the upregulation of RBM34 in HCC (Fig. 1E, F). Moreover, the protein level of RBM34 was compared between HCC and normal tissues by IHC staining and the HPA, database. We observed that the expression RBM34 protein was higher in HCC than in normal liver tissue (Fig. 1G). The results indicate that RBM34 was elevated in HCC and may have an important role in liver cancer progression.

RBM34 is a marker of an unfavorable HCC prognosis

We next explored the clinical significance of RBM34 expression in HCC. The correlation between RBM34 and clinicopathological characteristics of HCC is shown in Table 1. A receiver operating characteristic curve (ROC) was constructed to estimate the efficacy of RBM34 to distinguish HCC and normal liver tissue. The area under the curve of RBM34 was 0.912 (95% CI: 0.871–0.952), suggesting that RBM34 was a useful HCC marker (Fig. 2A). We also examined RBM34 expression in patients with HCC of different stages, including T1 and T2 vs. T3 and T4), N0 vs. N1 and N2, and M0 vs. M1. RBM34 was not differently expressed in patients with different T and N staging (Fig. 2B, C). However, RBM34 expression was in M1 stage patients than M0 stage patients (Fig. 2D). The correlation between RBM34 expression and clinicopathologic characteristics was explored by logistic regression. RBM34 expression was significantly associated with histologic grade (G1 and G2 vs. G3 and G4). No significant associations between RBM34 and T stage (T1 and T2 vs. T3 and T4), N stage (N0 vs. N1 and N2), M stage (M0 vs. M1), and pathologic stage (stage I and stage II vs. stage III and stage IV) were observed (Fig. 2E).

Table 1. The associations between RBM34 expression and clinicopathological parameters of HCC patients from TCGA

Characteristic	Low expression of RBM34	High expression of RBM34	*p*-value
n	187	187	
T stage, *n* (%)			0.018
T1	105 (28.3)	78 (21)	
T2	37 (10)	58 (15.6)	
T3	36 (9.7)	44 (11.9)	
T4	8 (2.2)	5 (1.3)	
N stage, *n* (%)			1.000
N0	121 (46.9)	133 (51.6)	
N1	2 (0.8)	2 (0.8)	
M stage, *n* (%)			0.629
M0	122 (44.9)	146 (53.7)	
M1	1 (0.4)	3 (1.1)	
Sex, *n* (%)			0.507
Female	57 (15.2)	64 (17.1)	
Male	130 (34.8)	123 (32.9)	
Age, *n* (%)			0.502
≤60	85 (22.8)	92 (24.7)	
>60	102 (27.3)	94 (25.2)	
Pathologic stage, *n* (%)			0.094
Stage I	97 (27.7)	76 (21.7)	
Stage II	35 (10)	52 (14.9)	
Stage III	41 (11.7)	44 (12.6)	
Stage IV	2 (0.6)	3 (0.9)	
Histologic grade, *n* (%)			0.006
G1	32 (8.7)	23 (6.2)	
G2	101 (27.4)	77 (20.9)	
G3	48 (13)	76 (20.6)	
G4	4 (1.1)	8 (2.2)	
AFP (ng/mL), *n* (%)			0.005
≤400	118 (42.1)	97 (34.6)	
>400	22 (7.9)	43 (15.4)	
Age, median (IQR)	63 (53, 69)	61 (51, 68)	0.249

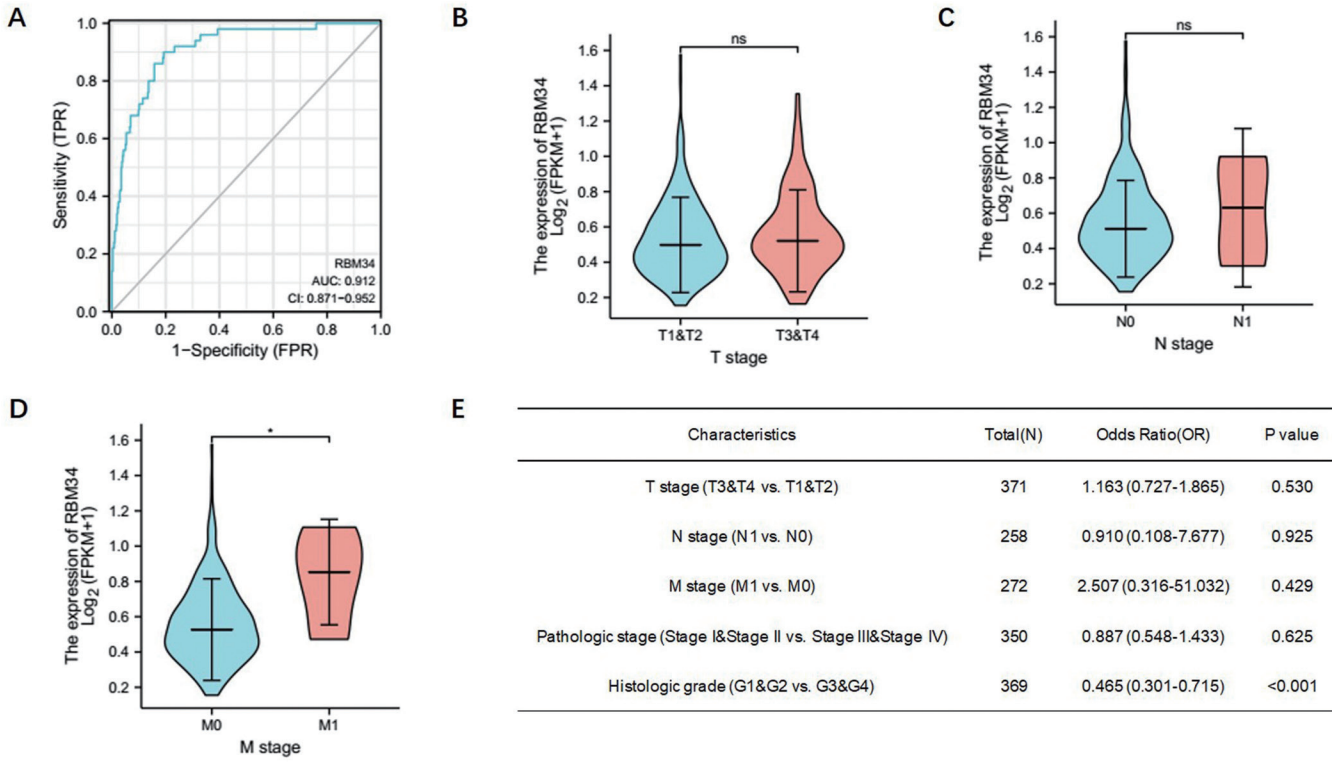
AFP, alpha fetoprotein; HCC, hepatocellular carcinoma; RBM, RNA-binding motif.

To investigate the prognostic prediction value of RBM34 in HCC patients, we assessed the relationship between RBM34 expression and patient OS, DSS, and PFI using the TCGA database. Kaplan-Meier survival analysis indicated that high RBM34 expression was significantly linked with poor OS (HR=1.97, 95% CI: 1.30–2.97, p=0.001), DSS (HR=2.00, 95% CI: 1.19–3.35, p=0.009), and PFI (HR = 1.62, 95% CI: 1.19–2.21, p=0.002; Fig. 3A–C).

We next constructed a nomogram to predict the OS of HCC patients on the basis of multivariate Cox analysis of RBM34 expression and independent clinical risk factors, including T, N, M, pathological stage, sex and histologic grade. As shown in Figure 3D, each predictive factor was assigned a score based on the actual condition. The total score predicted 1-, 3-, and 5-year survival of HCC patients. A calibration curve was constructed to validate the prediction accuracy of the nomogram, and it showed a good fit between the prediction and observation (Fig. 3E–G).

Functional inference of RBM34 in HCC

To explore the potential biological function of the RBM34 gene in HCC, we performed functional enrichment analysis based on transcriptome data from TCGA. The HCC samples from TCGA were divided into two groups, RBM34-high and RBM34-low groups by the median value of RBM34 mRNA expression. We analyzed the DEGs in the two groups with the criteria set $|\log 2FC| > 1$ and adjusted $p<0.05$, which



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Characteristics	Total(N)	Odds Ratio(OR)	P value
T stage (T3&T4 vs. T1&T2)	371	1.163 (0.727-1.865)	0.530
N stage (N1 vs. N0)	258	0.910 (0.108-7.677)	0.925
M stage (M1 vs. M0)	272	2.507 (0.316-51.032)	0.429
Pathologic stage (Stage I&Stage II vs. Stage III&Stage IV)	350	0.887 (0.548-1.433)	0.625
Histologic grade (G1&G2 vs. G3&G4)	369	0.465 (0.301-0.715)	<0.001

Fig. 2. Correlation between RBM34 expression and clinicopathological features. (A) ROC curve constructed to estimate the efficacy of RBM34 expression to distinguish HCC and normal liver tissue. (B–D) Association of RBM34 expression with T, N and M stage in HCC. (E) Correlation between RBM34 and clinicopathologic characteristics determined by logistic regression. HCC, hepatocellular carcinoma; RBM, RNA-binding motif; ROC, Receiver operating characteristic.

identified 385 DEGS, including 179 upregulated genes and 206 down-regulated genes (Fig. 4A). The results of a hierarchical clustering analysis of the DEGs in the high and low RBM34 expression groups are shown in a heatmap (Fig. 4B). KEGG and GO enrichment analysis was used to determine the potential functions of RBM34. KEGG pathway analysis showed that the upregulated DEGs were mainly involved in oocyte meiosis and the cell cycle (Fig. 4C). GO enrichment analysis indicated that upregulated DEGs were involved in organelle fission, nuclear division, and mitotic nuclear division (Fig. 4D). To further determine the biological functions of RBM34, we performed RNA sequencing in RBM34 knockout Huh7 cells. We identified 2,149 in the control cells and the RBM34 knockout cells ($p<0.05$, |log2 FC |>1), consisting of 902 down-regulated and 1,247 upregulated genes (Fig. 4E). KEGG pathway enrichment analysis revealed that depletion of RBM34 most prominently affected pathways in cancer, including PI3K-AKT and MAPK signaling (Fig. 4F). The findings strongly suggest that RBM34 may be a novel oncogene and involved in the cancer pathways in HCC.

RBM34 correlates with immune cell infiltration in HCC

Tumor-infiltrating immune cells are often highly relevant for HCC prognosis.[24] To explore whether RBM34 has a role in the regulation of immune cell infiltration in HCC, we assessed the correlation between the genetic alteration status of RBM34 and the extent of immune cell infiltration level with the TIMER database. As shown in Figure 5A, RBM34 copy number alteration with arm-level gain resulted in decreases of CD8+ T cell and neutrophil cell infiltration in HCC. We also evaluated the correlation between RBM34 expression level and immune cell infiltration. RBM34 expression was significantly positively correlated with infiltration of B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells in HCC (Fig. 5B). The correlation between RBM34 and immune cell biomarkers in HCC was also determined to further investigate the role of RBM34 in the tumor immune microenvironment. As shown in Table 2, there was a positive correlation between RBM34 and the B cell marker CD19 ($R=0.16$, $p<0.05$), M1 macrophage marker IRF5 ($R=0.33$, $p<0.001$), neutrophil marker ITGAM ($R=0.1$, $p<0.05$), and the dendritic cell markers CD1C ($R=0.11$, $p<0.05$) NRP1 ($R=0.23$, $p<0.001$) and ITGAX ($R=0.14$, $p<0.001$) were found. These results are consistent with an association of RBM34 with immune cell infiltration in HCC. Next, we analyzed the immune, stromal, and ESTIMATE scores to explore the roles of RBM34 in the tumor immune microenvironment during tumor development. RBM34 was negatively correlated with the ESTIMATE score ($R=-0.139$, $p<0.001$) and stromal score ($R=-0.181$, $p<0.001$; Fig. 5C). There was no significant correlation between RBM34 and immune score (Fig. 5D).

RBM34 is involved in immune escape in HCC

Inhibitory immune checkpoints, such as PD1/PD-L1 and CTLA4 have key roles in suppressing the antitumor immune response in solid tumors.[25] Considering the potential oncogenic function of RBM34 in HCC, we analyzed the correlation between RBM34 and more than 40 common immune-related genes in HCC using TCGA data. As shown in Figure 6A, B, RBM34 expression was associated with 20 immune

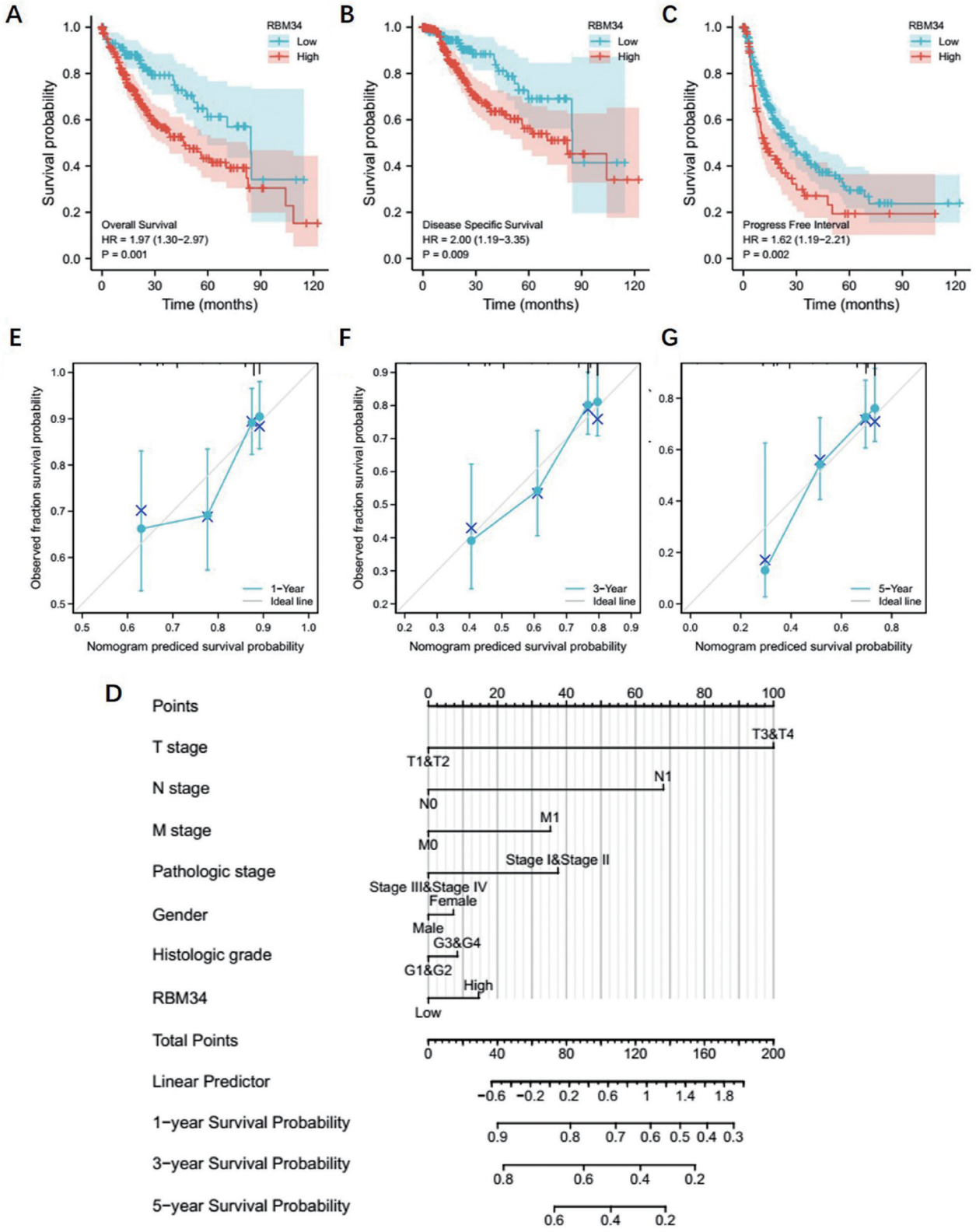

Fig. 3. Prognostic value of RBM34 for HCC patients. (A–C) Kaplan-Meier survival curves of OS, DSS, PFI between HCC patients with high and low RBM34 expression. (D) Nomogram integrating clinical characteristics and RBM34 for predicting the overall survival of HCC patients at 1, 3, and 5 years. (E–G) Nomogram calibration curves of 1-, 3-, and 5-year survival probabilities. HCC, hepatocellular carcinoma; RBM, RNA-binding motif; OS, overall survival; DSS, disease-specific survival; PFI, progress free interval.

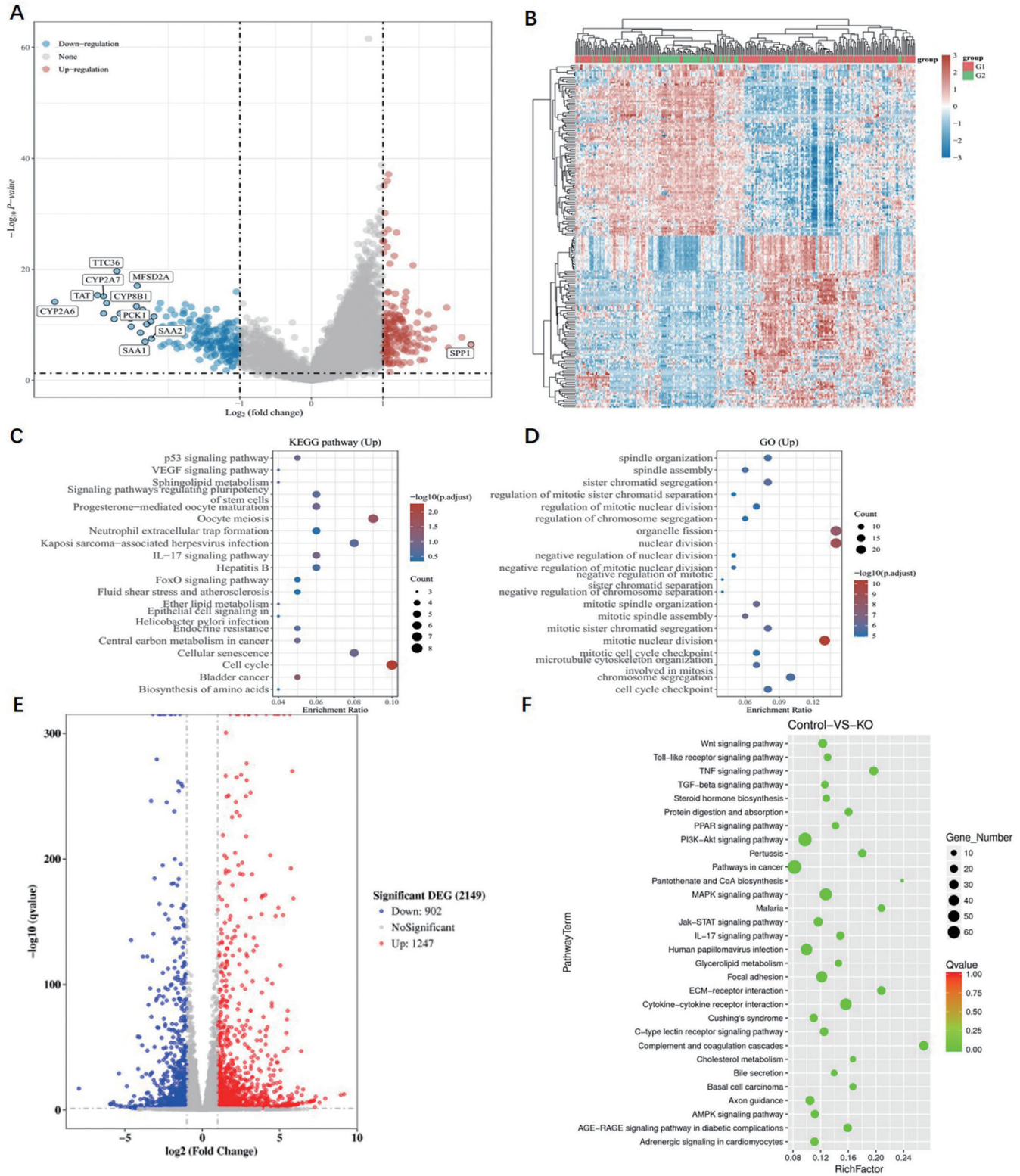

Fig. 4. Enrichment analysis of RBM34 expression-correlated DEGs in HCC. (A) Volcano plot of DEGs in high- and low-RBM34 groups. (B) The heatmap shows the top 50 up- or down-regulated DEGs between high- and low-RBM34 groups. (C, D) KEGG and GO enrichment analysis of RBM34-associated upregulated DEGs. (E) The most substantially differentially expressed genes by genome-wide transcriptome analysis between Ctr and rbm34 knockout Huh7 cells (n=3) are shown in a volcano plot of statistical significance (p<0.05) against fold-change (ratio of Knockout/Control group). (F) KEGG pathway enrichment analysis of differently expressed genes (n=3). HCC, hepatocellular carcinoma; RBM, RNA-binding motif; DEGs, differentially expressed genes.

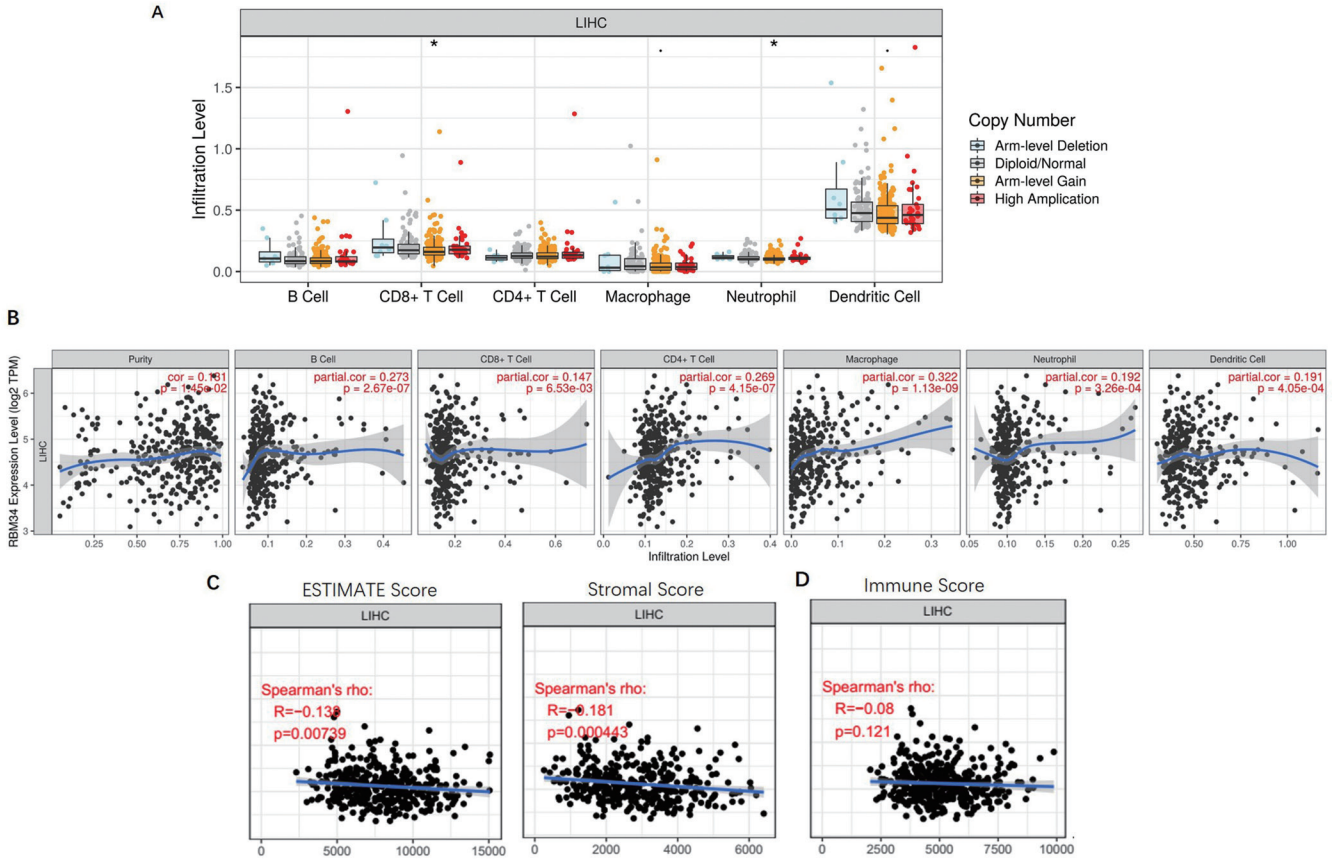

Fig. 5. Correlation between RBM34 and immune cell infiltration in HCC. (A) Immune cell infiltration with different copy numbers of RBM34 in HCC by the TIMER. (B) Correlation of RBM34 expression with various immune cell infiltration levels was investigated by TIMER. (C) The ESTIMATE algorithm showed a positive correlation of RBM34 expression with the stromal and ESTIMATE scores. (D) Correlation of RBM34 with the immune score. HCC, hepatocellular carcinoma; RBM, RNA-binding motif.

checkpoint markers. T cell exhaustion marker genes such as CTLA4 and PDCD1 were positively correlated with RBM34 expression, suggesting that RBM34 may be involved in T cell function in HCC.

Tregs facilitate tumor development by upregulating checkpoint inhibitors and contributing to systemic immune dysfunction and antitumoral activity in HCC.[26,27] We therefore examined the associations between RBM34 and Tregs marker genes based on TIMER2.0. there was a positive correlation between RBM34 and the Tregs markers interleukin (IL)2RA and CD25 (R=0.102, p=0.0497; Fig. 6C). RBM34 was positively correlated with checkpoint molecule markers of Tregs, such as PDCD1 (PD-1, R=0.184, p=0.0354), CTLA-4 (R=0.202, p<0.001), TNFRSF18 (GITR, R=0.225, p<0.001) and HAVCR2 (TIM3, R=0.109, p=0.0358), suggesting that RBM34 suppressed the T cell effector function. In addition, the Treg secreted T cell suppressive cytokine TGFβ1 was positively correlated with RBM34 (R=0.188, p<0.001), which further highlights the potential immune cell inhibitory effects of RBM34. The results further suggest that RBM34 plays an essential role in immune escape in the tumor microenvironment of HCC.

Depletion of RBM34 suppresses HCC cell proliferation and migration

To determine the functional role of RBM34 in HCC, we knocked out RBM34 expression in Huh7 and SK-hep1 cells with the CRISPR/cas9 gene-editing tool. To avoid selection effects, polyclonal cells were used. Down-regulated RBM34 expression was validated in western blots (Fig. 7A). The results clearly showed that knockout of RBM34 significantly inhibited the proliferation of both Huh7 and SK-hep1 cells (Fig. 7B). Colony formation of Huh7 and SK-hep1 was also decreased in RBM34 knockout compared with control cells (Fig. 7C). We next assessed its role in regulating HCC cell migration. Wound healing assays showed that knockout of RBM34 significantly inhibited cell migration (Fig. 7D). Transwell assays also showed significant suppression of cell invasion in cells with depletion of RBM34 (Fig. 7E).

RBM34 knockout sensitizes HCC cells to sorafenib

To determine the downstream targets of RBM34, we analyzed RNA-seq data, and selected several top upregulated and down-regulated genes for validation by qPCR. *SOAT2*, *DLK1*, *PCSK9*, and *FGFR2* expression was significantly decreased, and *LCN2* was upregulated in RBM34 knockout cells (Fig. 8A). In addition, we observed a positive correlation between FGFR2 expression and RBM34 expression in HCC by analyzing the TCGA database (R=0.192, p<0.001; Fig. 8B). We next examined FGFR2 protein expression in HCC cells without RBM34. FGFR2 was decreased in RBM34-knockout Huh7 cells compared with the control cells (Fig. 8C). It has been reported that FGFR2 was involved in HCC progression and sorafenib resistance.[28,29] To

Table 2. Correlation between RBM34 and immune cell biomarkers in HCC by the TIMER database

Immune cell	Biomarker	*R*-value	*p*-value
B cell	*CD19*	*0.162947845*	*0.001637862*
	CD79A	0.049380089	0.34287658
CD8+ T cell	CD8A	0.037986123	0.465721115
	CD8B	0.026794975	0.606931508
CD4+ T cell	CD4	−0.064619062	0.214329153
M1 Macrophage	NOS2	−0.063819327	0.22006721
	IRF5	*0.3308735*	*6.29E−11*
	PTGS2	0.052770144	0.310725319
M2 Macrophage	CD163	−0.056188426	0.28037987
	VSIG4	−0.013171946	0.800372984
	MS4A4A	−0.008733436	0.866855955
Neutrophil	CEACAM8	0.004106146	0.937173086
	ITGAM	*0.105643346*	*0.041985682*
	CCR7	0.014597322	0.779300726
Dendritic cell	HLA-DPB1	0.054553426	0.294634945
	HLA-DQB1	0.062025515	0.23333493
	HLA-DRA	0.062567066	0.229271221
	HLA-DPA1	0.042422753	0.415227225
	CD1C	*0.112104278*	*0.03086541*
	NRP1	*0.229628812*	*7.90E−06*
	ITGAX	*0.135262982*	*0.009092517*

Italic values indicate statistical significance. HCC, hepatocellular carcinoma; RBM, RNA-binding motif.

investigate whether RBM34 expression was also associated with sorafenib resistance in HCC, we first analyzed the correlation between RBM34 expression and survival of HCC patients with sorafenib treatment. High RBM34 expression was correlated with worse survival in HCC patients with sorafenib treatment, suggesting that RBM34 may confer sorafenib resistance in HCC (Fig. 8D). To confirm that, we compared sorafenib-induced apoptosis in RBM34 knockout and control cells. Knockout of RBM34 enhanced sorafenib-induced apoptosis (Fig. 8E). FGFR2 has been shown to be involved in AKT and ERK signaling pathways.[30] We examined whether knockout of RBM34 affect those two pathways in HCC cells treated with sorafenib. Sorafenib had a strong inhibitory effect on AKT activation in RBM34 knockout cells. However, only marginal inhibition of ERK activation was observed in the sorafenib-treated RBM34 knockout cells (Fig. 8E). The results suggest that depletion of RBM34 enhanced sorafenib anticancer effectiveness in HCC cells by inhibiting AKT activity.

Suppression of RBM34 inhibits xenograft HCC tumor growth

To investigate whether RBM34 regulates HCC tumor growth *in vivo*, RBM34 knockout and control HCC cells were subcutaneously implanted into nude mice. Depletion of RBM34 significantly decreased tumor growth (Supplementary Fig. 2A). Tumor size and tumor weight were suppressed when RBM34 was knocked out (Supplementary Fig. 2B, C), indicating that RBM34 had an essential role in promoting HCC progression.

Discussion

Previous studies have demonstrated that RBPs are globally dysregulated in HCC.[7] Emerging studies have reported that several RBM proteins are involved in HCC progression or suppression. However, RBM34 is still a poorly characterized RNA-binding motif protein, and its role in HCC is unknown. RBM34 has been previously identified as a putative upstream gene correlating with CDKN3 in HCC.[31] In another study, RBM34 was found to be a candidate gene to govern CHK1 splicing.[32] During the transformation of cirrhosis to HCC, RBM34 may be activated by the oncogene CDH13.[33] Those studies suggest that RBM34 may have an important role in hepatocarcinogenesis. Our bioinformatics analysis of RBM34 expression using the TCGA database showed that RBM34 was highly expressed in multiple cancer types, including HCC. Furthermore, we found that the high expression of RBM34 was negatively correlated with OSS, PFS, and DFI. The high expression of RBM34 was also associated with worse clinicopathological features in HCC. The findings support the hypothesis that RBM34 may be a novel oncogene and a prognostic marker of HCC. The oncogenic function of RBM34 was further confirmed by *in vitro* cell proliferation and migration assays. Knockout of RBM34 significantly impaired cell growth and migration. Depletion of RBM34 also inhibited xenograft tumor growth *in vivo*, further highlighting its role in promoting HCC progression.

Functional enrichment analysis of RBM34-associated genes indicated that upregulated DEGs were mainly involved in the cell cycle process, suggesting that RBM34 might promote HCC progression by regulating the cell cy-

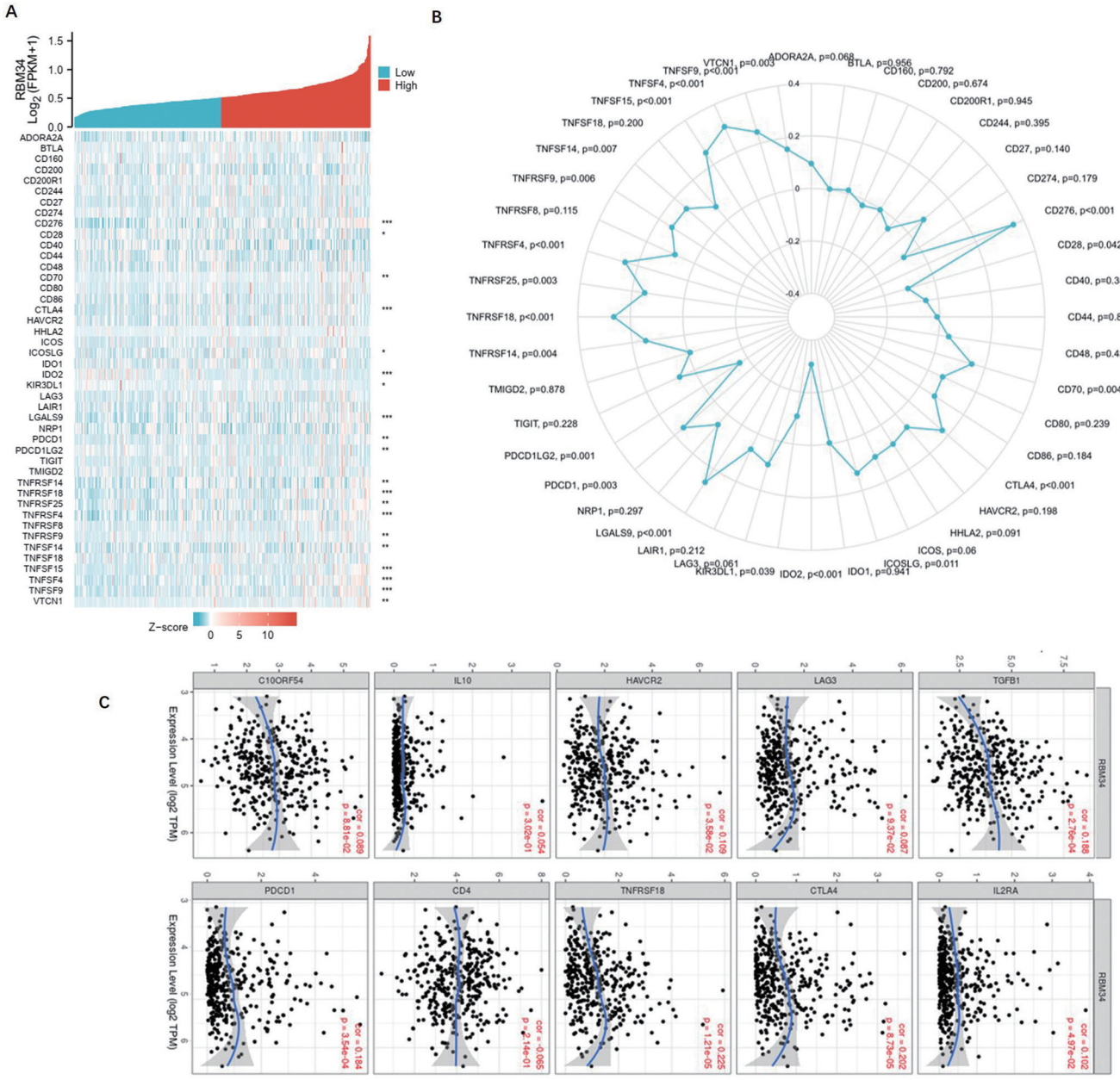

Fig. 6. Correlation between RBM34 and immune checkpoints gene in HCC. (A) Heatmap showing the correlation between RBM34 and over 40 immune checkpoint genes in HCC. (B) Radar chart of the correlations between RBM34 expression with over 40 common immune checkpoint genes in HCC. (C) Correlations of RBM34 expression and immune marker genes of Tregs in HCC based on the TIMER database. HCC, hepatocellular carcinoma; RBM, RNA-binding motif.

cle. We performed RNA-seq to identify downstream pathways regulated by RBM34. Notably, we found that knockout of RBM34 mainly affected cancer-associated pathways, including the PI3K-AKT, and MAPK signaling pathways. In addition, Wnt and TGF-beta signaling pathways, which have important roles in HCC,[34–36] was also affected by RBM34 knockout.

Sorafenib is a multikinase inhibitor that promotes apoptosis, inhibits angiogenesis, and inhibits tumor cell growth. It is currently an effective first-line treatment for late-stage HCC. Unfortunately, the emergence of sorafenib drug resistance is increasingly widespread.[37] Analysis of the TCGA database found that RBM34 overexpression was correlated with poor prognosis in sorafenib-treated HCC patients. We further confirmed this finding by performing functional experiments *in vitro*. Knockout of RBM34 significantly increased sorafenib-induced apoptosis. The results suggest that RBM34 was involved in sorafenib resistance and may be a therapeutic target in HCC patients.

Our RNA-seq analysis identified FGFR2 as a putative target gene of RBM34, which was further verified by qPCR and western blotting. Activated FGFRs activates downstream signaling, such as the PI3K-AKTM, MAPK, and STAT pathways, which are the main oncogenic pathways that participate in HCC initiation and progression.[38,39] Indeed, functional enrichment analysis found that the PI3K-AKT and

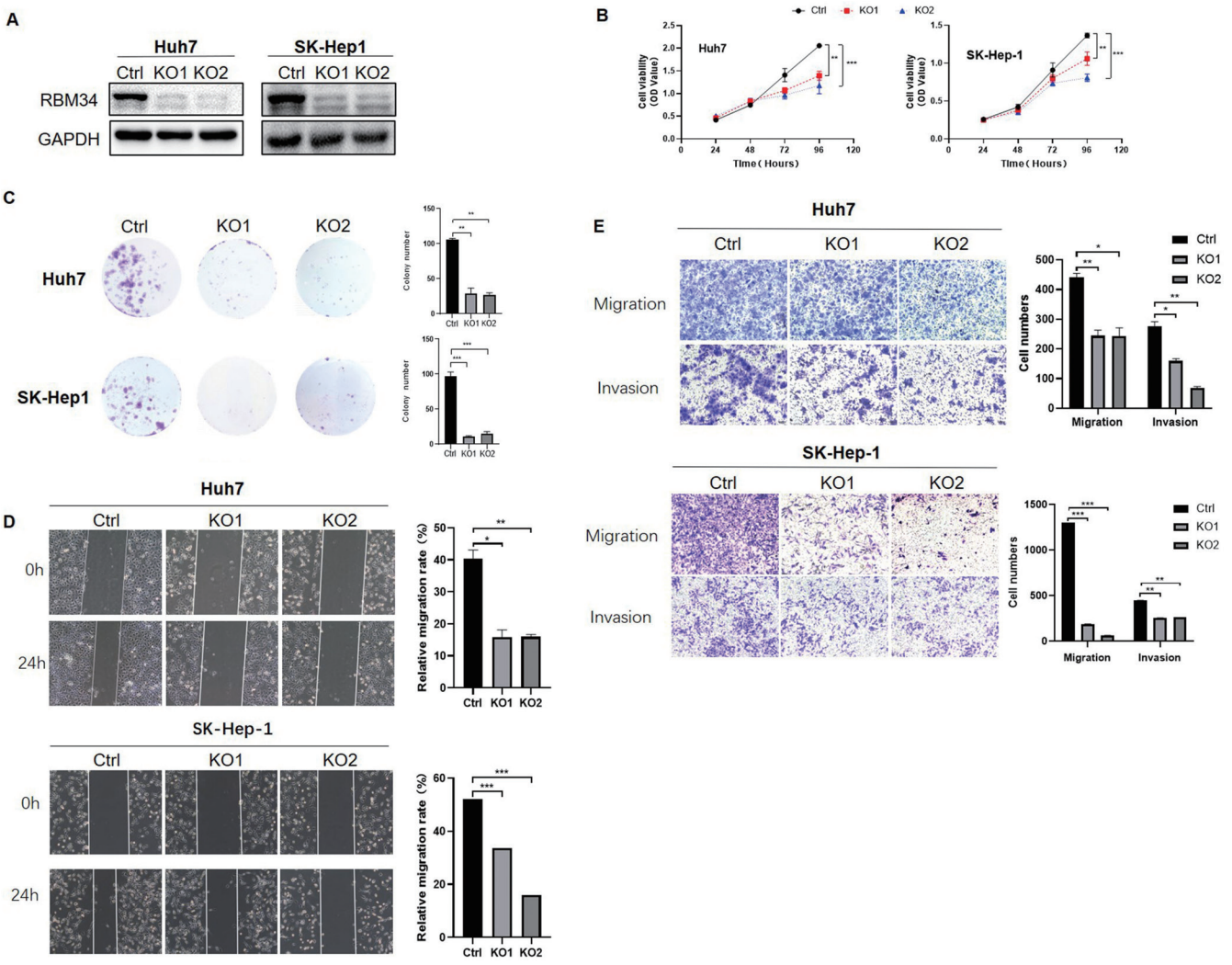

Fig. 7. Validation of the oncogenic function of RBM34 by *in vitro* experiments. (A) RBM34 knockout was confirmed by western blot assays in Huh7 and SK-hep1 cells. (B) CCK-8 assays showed reduced cell proliferation in RBM34 knockout cells. (C) RBM34 knockout attenuated colony formation of HCC cells. (D) Cell migration was assayed by wound healing. (E) Cell migration and invasion of HCC cells were determined by Transwell assays. HCC, hepatocellular carcinoma; RBM, RNA-binding motif.

MAPK pathways were affected in RBM34 knockout HCC cells. Depletion of RBM34 enhanced the inhibitory effects of sorafenib in the PI3K-AKT pathway. The findings suggest that knockout of RBM34 sensitized HCC cells to sorafenib treatment by downregulation of FGFR2-mediated PI3K-AKT pathway activity.

There is accumulating evidence for immune cell infiltration as predictive of prognosis and therapeutic efficacy in cancer patients.[40] Our results show that the amplified gene copy number of RBM34 resulted in decreased CD8+ T cell and neutrophil cell infiltration. In addition, we found that RBM34 was significantly positively correlated with B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil, and dendritic cell infiltration in HCC. Moreover, RBM34 was also positively correlated with B cell and macrophage biomarkers. The results suggest that the oncogenic function of RBM34 in HCC may be partially mediated by immune cell infiltration.

Blockade of the immune checkpoints PD1, PD-L1, or CTLA4 has recently emerged as a promising immunotherapy strategy for HCC treatment.[41–43] We thus assessed the correlation of RBM34 with immune checkpoints. Elevated RBM34 was positively associated with PD1 and CTLA4 in HCC, suggesting that targeting RBM34 may have a synergistic effect with immunotherapy for the treatment of HCC. In addition, our study also revealed that RBM34 promoted T cell exhaustion and Tregs activation in HCC. The findings suggest that elevated RBM34 contributed to an immunosuppressive microenvironment in HCC.

In summary, we found that RBM34 upregulated HCC. Highly expressed RBM34 predicted poor prognosis in HCC patients. The oncogenic activity of RBM34 was mediated by regulating cell proliferation, migration, and invasion. In addition, depletion of RBM34 sensitized HCC cells to sorafenib treatment. Therefore, RBM34 may serve as a novel prognostic biomarker and offers a new insight into the exploration of new immunotherapeutic strategies.

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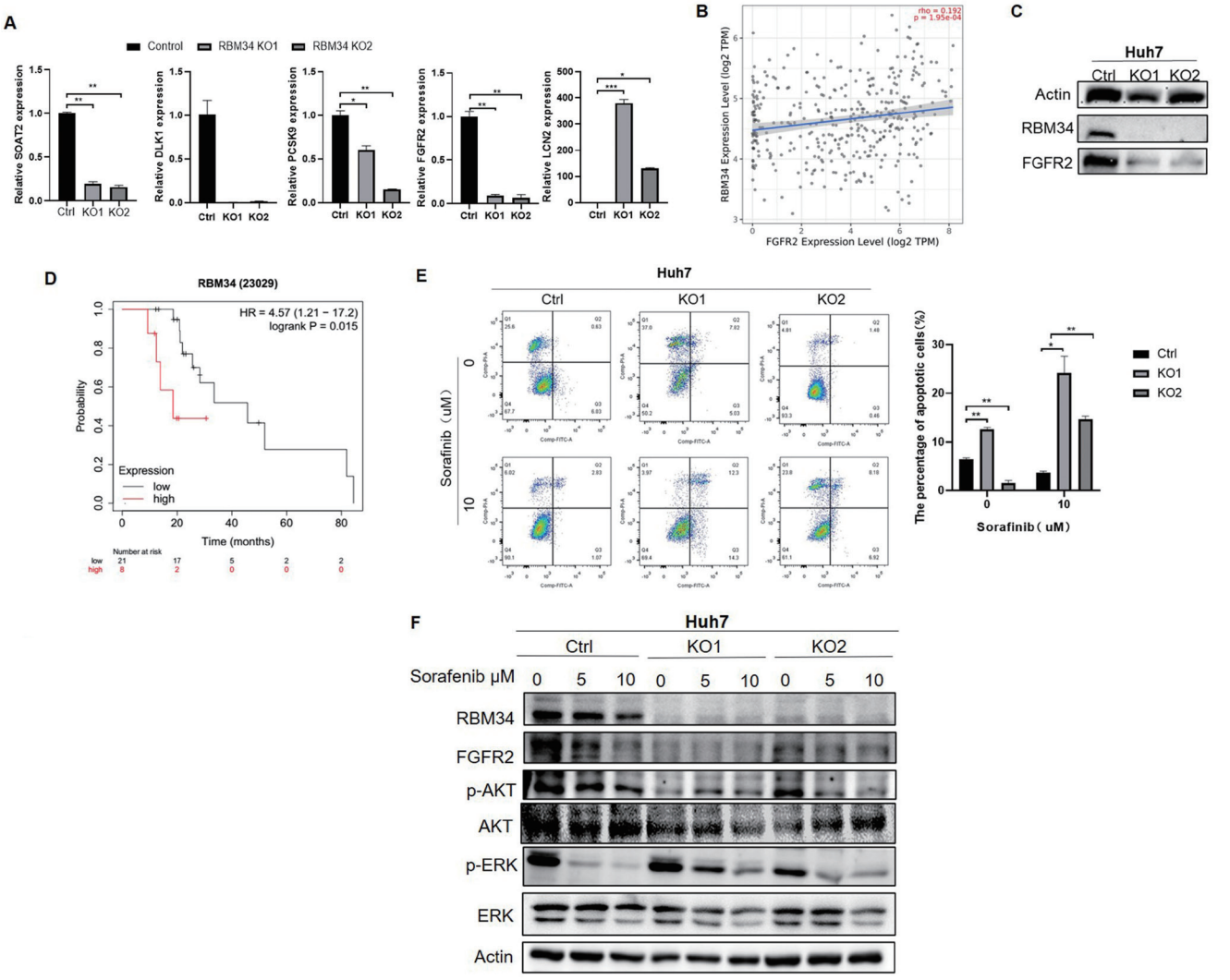

Fig. 8. Suppression of RBM34 enhances sorafenib sensitivity in part by downregulating FGFR2. (A) qPCR assays validate the target genes of RBM34 in Huh7 cells. (B) Correlation between RBM34 and FGFR2 expression by bioinformatics analysis using the TCGA database. (C) FGFR2 protein expression in HCC cells was assayed in western blots. (D) Kaplan-Meier plots of overall survival in HCC patients with different RBM34 expression levels and sorafenib treatment. (E) Flow cytometry analysis of sorafenib-induced apoptosis in Huh7 cells with or without RBM34 expression. (F) Sorafenib treatment significantly inhibits AKT activity and not ERK activity in RBM34 knockout Huh7 cells compared with control cells. HCC, hepatocellular carcinoma; RBM, RNA-binding motif.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (NL), acquisition of data (WW, RZ, NF), analysis and interpretation of data (NL, WW, NF), drafting of the manuscript (NL), and study supervision (NL, LZ). All authors have made a significant contribution to this study and have approved the final manuscript.

Ethical statement

Animal experiments were approved by the Animal Care Committee of Xuzhou Medical University.

Data sharing statement

The generated or analyzed during this study have been included in this manuscript, further inquiries can be directed to the corresponding authors.

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