

RBM45 Promoted HCC Growth and Metastasis by Stabilizing BCL2 and Twist1 mRNA

Lili Han (✉ 61439376@qq.com)

Xi'an Jiaotong University Second Affiliated Hospital Department of Oncology <https://orcid.org/0000-0003-4964-140X>

Lin Wang

Xi'an Jiaotong University Second Affiliated Hospital

Fei Wu

Xi'an Jiaotong University Second Affiliated Hospital

Research Article

Keywords: RNA-binding protein, RBM45, BCL2, Twist1, HCC

Posted Date: June 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-564605/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

The post-transcriptional of mRNA expression involved in the hepatocellular carcinoma (HCC) pathogenesis and progression need to be further explored. RBPs, the main undertaker of post-transcriptional regulatory process, has been shown to impact HCC carcinogenesis and progression. However, the role of RBP, RNA-binding motif 45 (RBM45) in hepatocarcinogenesis and its interaction with its potential target mRNA remains entirely unknown. The expression of RBM45 was significantly increased in HCC and was associated with poor clinicopathological features and clinical outcome of HCC patients. RBM45 promoted HCC cells growth, invasion, migration and EMT in vitro and in vivo. Mechanistically, RNA immunoprecipitation sequencing (RIP-seq) approach was utilized to screen the important differentially expressed RBM45 genes in HCC. Furthermore, RIP assay, pull-down assay and mRNA decay assay were carried out to uncover the effect of RBM45 on its downstream genes. And the results revealed that RBM45 mediated the stabilization of BCL2 and Twist1 mRNA via respectively binding to their 3'UTR. Further assay results suggested RBM45 promoted HCC growth and metastasis upon BCL2 and Twist1. In short, we unveiled a novel role of RBM45 in promoting hepatocarcinogenesis via the post-transcriptional regulation of BCL2 and Twist1 expression. The results proposes that RBM45 may serve as a potential therapeutic target for HCC.

Highlights

1. RBM45 is up regulated in HCC tissues and may serve as a poor prognostic marker of HCC.
2. RBM45 promotes malignant biological behavior of HCC cells in vitro and in vivo.
3. RBM45 enhanced the stabilization of BCL2 and Twist1 mRNA via respectively binding to their 3'UTR.
4. RBM45 worked as an RNA binding protein and promoted HCC growth and metastasis upon the post-transcriptional regulation of BCL2 and Twist1.

Background

Hepatocellular carcinoma (HCC), one of the most frequently diagnosed cancer in the worldwide with poor prognosis^[1,2], is a genetically complex set of diseases characterized by high proliferation and invasion. Despite intensive efforts has been made to improve our understanding about the molecular mechanism of HCC carcinogenesis, HCC patients still face unsatisfied five-year survival. Lack of comprehensive exploration of the molecular mechanisms underlying HCC may be one of the reasons. The epigenetic mechanisms has been intensively studied in the past few decades, however, the post-transcriptional of genes involved in the HCC pathogenesis and progression remain need to be further explored.

RNA binding proteins (RBPs), the main undertaker of post-transcriptional regulatory process, are critical in RNA fate determination via regulating RNA splicing, nuclear export, degradation, stabilization and translation^[3]. Recently, RBPs has been implicated as a critical regulator of an important series of cancer-related genes^[4-6]. The dysregulation of RBPs has been shown to impact HCC carcinogenesis and progression^[7-9]. RNA-binding motif 45 (RBM45), an RNA-binding protein with three RNA-recognition motifs (RRMs)^[10,11], was initially identified to contribute to the DNA damage response^[12]. Recent studies reveal that RBM45 is essential for amyotrophic lateral sclerosis, frontotemporal lobar degeneration patients with TDP-43 pathology and Alzheimer's disease^[13]. However, its biological function in human cancers, including HCC, remains largely unknown.

The study confirmed RBM45 promoted hepatic tumourigenesis in vitro and in vivo. RNA immunoprecipitation sequencing (RIP-seq) approach was utilized to screen the important differentially expressed RBM45 genes to uncover novel regulatory factors important in HCC. The results of RIP and mRNA decay assay uncovered that RBM45 mediated the stabilization of BCL2 and Twist1 via respectively binding to their 3'UTR. Further assay results suggested both of BCL2 and Twist1 were essential for RBM45 promoted HCC growth and metastasis. Our study unveils RBP RBM45 promoted HCC growth and metastasis via the post-transcriptional regulation method and proposes that RBM45/BCL2/Twist1 axis may work as a potential therapeutic target in HCC.

Results

RBM45 expression was upregulated in HCC and indicated poor prognosis

Compared to the THLE-2 immortalized human liver cells, both mRNA and protein expression levels of RBM45 were markedly up regulated in HCC cell lines (Fig. 1A). In 120 pairs of HCC samples, the RBM45 mRNA expression in HCC samples was elevated obviously, compared with that in the non-cancerous samples (Fig. 1B). Consistently, the analysis using Cancer Genome Atlas (TCGA) data also demonstrated RBM45 was increased in tumor tissues than in normal tissues (Fig. 1C). Figure 1D showed that RBM45 protein expression level was up regulated in HCC samples from 10 HCC patients. IHC assay in HCC samples also showed that RBM45 was markedly overexpressed in most of HCC tissues (79/120) (Fig. 1E). According to the cut-off value of RBM45 IHC score, HCC patients were divided into RBM45-high group and RBM45-low groups. Then, the correlation between RBM45 expression and HCC clinic pathological data was assessed. Our analysis showed that the increased RBM45 expression was closely associated with microscopic vascular invasion ($p < 0.001$), advanced TNM stage in HCC ($p < 0.001$), poor tumor differentiation ($p = 0.002$), multiple tumors ($p = 0.025$) and bigger tumor size ($p = 0.019$, Table S1). Kaplan-Meier analysis in 120 clinical samples demonstrated that higher RBM45 in HCC indicated both shorter OS ($p = 0.008$, Fig. 1F) and DFS ($p = 0.001$, Fig. 1F). In short, our results suggest that RBM45 is increased in HCC and indicates poor prognosis for HCC patient.

RBM45 promoted HCC cells growth in vitro and in vivo

We established Huh7 and Hep3B cells with stable overexpression or knockdown of RBM45 via a virus transfection approach. qRT-PCR and western blot were performed to test the transfection efficiency (Fig. 2A). The results of MTT assays demonstrated Huh7 and Hep3B cells proliferation were enhanced markedly after RBM45 expression was increased (Fig. 2B). While knockdown of RBM45 resulted in decreased proliferation of Huh7 and Hep3B cells (Fig. 2C). Flow

cytometry assay (FCM) uncovered increased RBM45 reduced Huh7 and Hep3B cells apoptosis (Fig. 2D), while decreased RBM45 promoted Huh7 and Hep3B cells apoptosis (Fig. 2E).

In addition, we established THLE-2 cells steadily overexpressing RBM45 via a virus transfection approach (Fig. S1A). MTT assays and Flow cytometry assays (FCM) also showed the increased

proliferation capacity (Fig. S1B) and decreased apoptosis of THLE-2 cells (Fig. S1C). Furthermore, an HCC xenograft model in nude mice was established to confirm our results in vivo. Figure 2F showed that xenograft tumors in ov-RBM45 group were markedly larger than those in ov-Ctrl group. The immunohistochemistry assay demonstrated that RBM45 expression was markedly up regulated in the xenograft tumor tissues in the ov-RBM45 group. These data demonstrated that RBM45 enhanced HCC growth in vitro and in vivo.

RBM45 promoted invasion and migration of HCC cells in vitro and in vivo

Transwell assays showed the migration (Fig. 3A) and invasion capacity (Fig. 3B) of Huh7 and Hep3B cells were increased markedly after RBM45 expression was up regulated. While knockdown of RBM45 resulted in decreased migration (Fig. 3C) and invasion (Fig. 3D) of Hep3B and Huh7 cells. In addition, our results also showed that the overexpression of RBM45 markedly increased the migration and invasion capacity of THLE-2 cells (Fig. S1D). We next evaluated the function of RBM45 in HCC metastasis in vivo via establishing an HCC xenograft metastasis model in nude mice. The results showed that RBM45 overexpressing increased the number and volume of metastases, suggesting RBM45 increased Hep3B cells lung metastatic capability (Fig. 3E). Immunohistochemistry assay suggested that RBM45 expression was significantly up regulated in the metastases in the ov-RBM45 group (Fig. 3F). These data demonstrated that RBM45 enhanced HCC metastasis in vitro and in vivo.

RBM45 increases the expression level of BCL2 and Twist1

We further performed transcriptomic sequencing assay in RBM45 overexpressing group (ov-RBM45) and the control group of Hep3B cells (ov-Ctrl) to screen the potential target of RBM45. Among the genes with significant changes in RBM45 overexpressing Hep3B cells (Fig. 4A), Twist1 and BCL2 caught our attention. Previous studies confirmed B-cell lymphoma-2 (Bcl2) is a key anti-apoptotic protein that promotes the survival of HCC cells^[14,15]. Twist1 is well known as a crucial epithelial mesenchymal transition (EMT) inducing transcription factor that elevated vimentin expression while suppress E-cadherin expression in HCC^[16–18]. Therefore, BCL2 and Twist1 were identified as the downstream target of RBM45 for further analysis, not only because they were the most significantly up regulated gene in response to RBM45 overexpression, but also as their crucial proliferation and metastasis-associated role in HCC.

According to qRT-PCR assays, overexpressed RBM45 did not enhance mRNA expression of BCL2 and Twist1 in HCC cells markedly (Fig. 4B). Consistently, knockdown of RBM45 could not observably reduce the mRNA expression of BCL2 and Twist1 (Fig. 4C). However, the protein expression levels of BCL2 and Twist1 were dramatically up regulated in HCC cells (Fig. 4D) or down regulated (Fig. 4E) upon RBM45 overexpression or knockdown, respectively.

RBM45 failed to regulate mRNA expression of BCL2 and Twist1 but regulates BCL2 and Twist1 protein expression in HCC cells, indicating that RBM45 modulated BCL2 and Twist1 in a post-transcriptional manner.

RBM45 directly bound to the 3'-UTR of BCL2 and Twist1 mRNA

We further investigated the stability of BCL2 and Twist1 upon RBM45 expression in HCC cells. The ov-RBM45 and ov-control Huh7 cells were treated with Actinomycin D (5mg/ml) which could bind to both double-stranded and single-stranded DNA and inhibit RNA polymerase activity, thereby inhibiting DNA-dependent RNA synthesis for differential times. The results demonstrated that overexpression of RBM45 prolonged the half-life of BCL2 mRNA from 1.98 h to 3.12 h in Huh7 cells. While, the half-life of BCL2 mRNA was reduced from 2.12 h to 1.26 h after RBM45 was decreased in Huh7 cells (Fig. 5A). In addition, similar results could be found in Hep3B cells (Fig. 5A). As shown in Fig. 5B, overexpression of RBM45 prolonged the half-life of Twist1 mRNA; while RBM45 knockdown shorted the half-life of Twist1 mRNA in Huh7 and Hep3B cells (Fig. 5B). Our results demonstrated that RBM45 promoted BCL2 and Twist1 expression via enhancing its mRNA stability.

To further explore whether RBM45 could directly bind to BCL2 and Twist1 mRNA, we performed RNA immunoprecipitation assay (RIP) and found that BCL2 and Twist1 mRNA were enriched in the RBM45 antibody precipitated complex sample whereas not in IgG (Fig. 5C and 5E). In addition, RNA pull-down results demonstrated RBM45 interacted with BCL2 and Twist1 3'-UTR (Fig. 5D and 5F). These results uncovered that RBM45 stabilized BCL2 and Twist1 via binding to their 3'-UTR.

BCL2 is an essential downstream effector of RBM45

To identify whether BCL2 is pivotal for modulating the function of RBM45 on HCC cells proliferation, we carried out a rescue assay. We established Huh and Hep3B cell lines with RBM45 knockdown stably overexpressing BCL2 (Fig. 6A), and stably silenced BCL2 in Huh7 and Hep3B cells with RBM45 overexpressing depending a virus transfection pathway (Fig. 6B). MTT assay results showed that BCL2 overexpression reversed inhibiting effect induced by RBM45 knockdown on proliferation of Huh7 and Hep3B cells (Fig. 6C), while BCL2 knockdown inhibited RBM45-induced promoting function on the proliferation of Huh7 and Hep3B cells (Fig. 6D). In addition, FCM (flow cytometry) assay results suggested that BCL2 overexpression reversed promoting effect induced by RBM45 knockdown on apoptosis of HCC cells (Fig. 6E), while decreased BCL2 reduced the inhibiting function caused by increased RBM45 on the apoptosis of HCC cells (Fig. 6F).

Although BCL2 is one of the classical pathways regulating cells apoptosis, we could not ignore that BCL2 has been well known to have a significant impact in cancer metastasis^[19,20]. Transwell assay experiments were carried out to determine whether BCL2 also acted an important downstream of RBM45, upon which RBM45 could promote HCC cells migration and invasion. The results showed that BCL2 overexpression reversed inhibiting effect of decreased RBM45

on Huh7 and Hep3B cells invasion and migration (Fig. S2A and S2B). But knockdown of BCL2 could not markedly inhibit RBM45-induced migration and invasion, while knockdown of both BCL2 and Twist1 could markedly reduce HCC cells migration and invasion capacity (Fig. S2C and S2D).

Twist1 is an essential downstream effect of RBM45

We established Huh and Hep3B cell lines with RBM45 knockdown stably overexpressing Twist1, and found overexpression of Twist1 rescued the function of RBM45 altering HCC EMT (Fig. 7A). On the other hand, we used a virus transfection pathway to silence Twist1 in Huh7 and Hep3B cells overexpressing RBM45, and found that decreased Twist1 rescued the function of RBM45 altering HCC EMT (Fig. 7B). Transwell assay indicated that Twist1 overexpression reversed inhibiting effect of decreased RBM45 on Huh7 and Hep3B cells migration and invasion (Fig. 7C). While Twist1 knockdown impaired RBM45-induced migration and metastasis of Huh7 and Hep3B cells (Fig. 7D).

MTT assay and FCM assay were performed to analysis whether Twist1 was also essential downstream effect of RBM45 on promoting HCC cells growth. The results showed that Twist1 overexpression reversed inhibiting effect induced by RBM45 knockdown on proliferation of Huh7 and Hep3B cells (Fig. 7E), and increased Twist1 reversed promoting effect induced by RBM45 knockdown on apoptosis of HCC cells (Fig. 7G). However, knockdown of Twist1 fail to inhibit overexpressed RBM45-induced growth of HCC cells, while knockdown of both BCL2 and Twist1 could markedly reduce HCC cells growth capacity (Fig. 7F and 7H).

RBM45 promoted hepatocarcinogenesis upon BCL2 and Twist1 in vivo.

We next carried out further animal experiments to verify experimental results in vitro. The results demonstrated that tumors derived from Huh7 cells with decreased RBM45 (sh-RBM45 group) were markedly smaller than those in sh-Ctrl group. Tumors derived from Huh7 cells co-transfected with sh-RBM45 vectors and ov-BCL2 vector (sh-RBM45 + ov-BCL2 group) were larger than those in sh-RBM45 group (Fig. 8A). The IHC results shows that RBM45 expression levels were decreased in the tumor tissues of the sh-RBM45 group and sh-RBM45 + ov-BCL2 group compared with the sh-Ctrl group. The expression of BCL2 and Ki67 was much lower in the sh-RBM45 group than that in the sh-Ctrl group. But compared with that in sh-RBM45 group, BCL2 and Ki67 expression was increased in the sh-RBM45 + ov-BCL2 group (Fig. 8C and 8E). HCC metastasis xenograft model in nude mice showed that Twist1 knockdown (ov-RBM45 + sh-Twist1 group) markedly decreased the number and volume of metastases, suggesting Twist1 knockdown attenuated lung metastatic induced by overexpression of RBM45 (Fig. 8B). IHC assay in the xenograft lung metastases samples showed that RBM45, Twist1, Vimentin expressions were all increased in the ov-RBM45 group tissue, but the expression of Twist1 and Vimentin was markedly recovered in the ov-RBM45 + sh-Twist1 group tumor tissue (Fig. 8D and 8F). These data highlighted that RBM45 promoted HCC growth and metastasis upon BCL2 and Twist1 in vivo.

Discussion

HCC is one of the most frequently diagnosed cancer worldwide with a poor prognosis. The complicated regulatory network involved in the mechanisms underlining HCC is not well understood, that may be responsible for the currently limited available therapies. The post-transcriptional of mRNA expression is one of the most important methods of HCC pathogenesis and progression. In this study, we firstly demonstrated that RBM45 is abnormally increased in HCC and associated with poor patient outcome. We unveiled RBM45 promoted HCC cells malignant biological behavior via the post-transcriptional regulation of BCL2 and Twist1 expression. Our results explored the function of RBM45/BCL2/Twist1 axis on HCC hepatocarcinogenesis and progression, providing a novel insight for the pathogenesis of HCC.

Recently, more and more attention has been paid to RBPs, which were discovered to function as important regulators of the tumorigenesis, growth and metastasis of human cancers [21–23]. However, the role of the novel RBP RBM45 in hepatocarcinogenesis and its interaction with its potential target mRNA remains unknown. Our detection results confirms RBM45 promotes HCC cells growth, which further supports the clinical testing results that RBM45 is increased in HCC tissue and associates with poor outcomes.

As post-transcriptional regulation provides an additional level of control that dictates HCC progression, understanding how RBM45 control HCC progression as a RBPs may provide a new perspective in HCC. In terms of mechanism research, transcriptomic sequencing assay focused our attention on BCL2, a main player in the intrinsic apoptotic pathway, may be a crucial target of RBM45. The B cell lymphoma/leukaemia – 2 (*Bcl-2*) is an oncogene that inhibits apoptosis. Apoptosis-related protein BCL2, belong to BCL family, is upregulated in a variety of human cancers and promotes tumor growth via favoring cancer cells survival through inhibiting cell death [24–26]. The results of RIP assay and RNA pull-down assay explored RBM45 directly bind to BCL2 3'-UTR. Stability assay further uncovered RBM45 promoted BCL2 expression via enhancing its mRNA stability, providing a novel understanding of the mechanism by which RBM45 promotes HCC tumorigenesis. Furthermore, BCL2 not only constitute a fundamental part of suppression system against a variety of cell death, but also increased the resistance of tumorigenic cells to most cytotoxins [27–29]. Thus, our findings may provide a new strategy for inhibiting BCL2 to suppress HCC.

Our data demonstrated the positive correlation between RBM45 and HCC invasion in vitro and in vivo, and identified the posttranscriptional regulatory function of RBM45 on Twist1 by leading to Twist1 stability via directly binding with its 3'-UTR in HCC. Twist1, belong to the basic-helix-loop-helix transcription factor family, is one of the EMT-induced transcription factors that commonly promotes cells migration and invasion in human cancers [30]. Twist1 has been confirmed regulates EMT-associated genes via directly binding to their promoters. Numerous studies reveal that Twist1 activated mesenchymal cell phenotype-associated genes, such as snail and vimentin [31, 32], while Twist1 suppresses the epithelial phenotype-related genes, such as E-cadherin [33]. In addition, Twist1 involved in causing up regulation of AKT which is important to facilitate cancer metastasis via binding to special pieces of the E box in its promoter [34]. Our research work unveils that Twist1 was largely up regulated by RBM45 in HCC, and the inhibited migration and invasion induced by knockdown of RBM45 could be markedly rescued by overexpression of Twist1. In short, this study elucidate the essential role of Twist1 in modulating the invasion promoting function of RBM45 and provides new insights into the upstream of Twist1 in HCC.

Since BCL2 and Twist1 interact closely according to previous reports ^[20], the role of BCL2 in modulating the function of RBM45 on HCC cells migration and invasion should not be ignored. Our data showed that inhibited migration and invasion induced by decreased RBM45 could be markedly rescued by overexpression of BCL2 in Huh7 and Hep3B cell lines. That is entirely possible, Sun et al have revealed that Bcl-2 facilitates the nuclear transport of Twist1 as an important cofactor of Twist1 in HCC ^[19]. It is suggested that BCL2 is also essential for RBM45 in modulating HCC invasion. This process may be achieved through co-activation the transcription and up-regulation the expression of Twist1 as the previously reported. However, in HCC cells with overexpressed RBM45, single knockdown of BCL2 failed to inhibit RBM45-induced migration and invasion, while co-knockdown of BCL2 and Twist1 results in the reduction of HCC cells migration and invasion. The reason maybe though BCL2 was decreased, Twist1 was still positively regulated by overexpressed RBM45, resulting in Twist1 was still upregulated and continuously promoted the migration and invasion of HCC cells. The previous study reported that the research results of BCL2 inhibitor showed good activity against cancer cells in vitro and in vivo, but subsequent clinical trials did not show significant efficacy ^[35, 36]. Combined with our results, it deserves attention that we should not only focus on the overexpression of BCL2 alone in the treatment of HCC, but also should take precautions against its upstream RBM45 at the same time.

As Tan et al reported that BCL2 was increased followed Twist1 overexpressed and decreased in the Twist1 knockdown cells, suggesting that BCL2 worked as an important downstream effector of the FZD7-Twist1 axis^[37]. Moreover, Kwon et al uncovered that Twist1 induced resistance to chemical treatment on gastric cancer cells via the modulation of Akt and Bcl-2 ^[38]. These results remind us that Twist1 may involve in mediating the function of RBM45 on HCC cells growth. This study bears out our hypothesis that Twist1 contributes to the oncogenic function of RBM45 in HCC growth as an essential downstream effect.

Our study identified for the first time that RBP RBM45 is abnormally increased in HCC and associated with poor patient outcome. We unveiled RBM45 promoted HCC cells malignant biological behavior by post-transcriptionally increasing the stability of BCL2 and Twist1 via respectively binding to their 3'UTR mRNA. We also uncovered the significant role of BCL2 and Twist1 in the cancer promoting effect of RBM45 in HCC. The newly identified RBM45 /BCL2/Twist1 axis provides novel therapeutic targets for HCC.

Methods Or Materials And Methods

Clinical tissues

Clinical tissues were collected from 120 HCC patients who accepted operative treatment between February 2013 and February 2014 at The Second Affiliated Hospital of Xi'an Jiaotong University. The samples were kept in liquid nitrogen for the following detection. The clinicopathological features of the HCC patients was shown in Table. S1. The cutoff value of RBM45 expression was 2.01 according to Receiver operating characteristic analysis.

Immunohistochemistry

The Immunohistochemistry assay were carried out follow the steps as our previously reported ^[7]. The anti-RBM45, anti-BCL2, anti-Twist1, anti-Ki67, anti-E-cadherin and anti-Vimentin primary antibodies, as well as the secondary anti-rabbit antibody were all purchased from Beijing Biosynthesis Biotechnology co., LTD.

RNA isolation, reverse transcription and quantitative realtime PCR (qRT-PCR)

Total RNA was isolated from tissue samples or cells using RNeasy reagent (Qiagen, Shanghai, China). A reverse transcription kit (Takara, Otsu, Shiga, Japan) was then used to reverse transcribe RNA samples into cDNA for qRT-PCR. qRT-PCR was performed as previous described ^[7].

Cell culture and transfection

The cell lines used in this study, including HepG2, Hep3B, Huh7, MHCC-97H, SMMC-7721 and THLE-2 were all bought from FuHeng Cell Center (Shanghai, China). They were routinely cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Wisent, China) with 10% fetalbovine serum (FBS, Gibco). The lentivirus vectors used to overexpress RBM45, BCL2, Twist1 and these control vector, as well as RBM45, BCL2 and Twist1 specific sh-RNA vector were bought from Genepharma (Shanghai, China). After the cells seeded in 6-well plates reached 30%-40% confluence, the transfection processes were carried out followed the manufacturer's instructions. Then, the next assays were performed after 48 h.

Western blotting

The protein samples were extracted from HCC cells or tissues using lysis Buffer (CST, MA).

Western blotting assay was carried out as described previously ^[7]. The primary antibodies used in this study including anti-Twist1 (#46702; 1:1000), anti-E-cadherin (#3195; 1:200), anti-Vimentin (#574S; 1:200), were bought from Cell Signaling Technology, Inc. Anti-BCL2 (ab196495; 1:1000), GAPDH antibody (ab6922; 1:200) and anti-RBM45 (AV41154; 1:500), were purchased from Abcam and Sigma-Aldrich, respectively.

MTT assay

HCC cells (100 cells/well) in 100 µl medium were added into 96-well plates. At the indicated times (24, 48 and 72 h), the HCC cells in each well was incubated with 10 µl MTT solution at 37°C. After 4 hours, the medium was replaced by new medium with 200 µl dimethyl sulfoxide. At last, the results was detected under a plate reader at a 492 nm wavelength.

Flow cytometry

The HCC cells were counted and added into 6-well plates according to 3×10^5 cells per well, and the cells were treated in groups. Annexin-v /PI double-stained by BD LSRFortessa (BD Biosciences).

Cell migration assays

A total of 2×10^4 HCC cells in 200 μ l FBS-free DMEM were seeded onto the top chamber of the Transwell Chambers (Millipore, Bedford, Massachusetts, USA). DMEM with 10% FBS was treated into the bottom chamber. After 48 h, HCC cells failed to invaded through the membrane were removed. HCC cells successfully invaded through the membrane and adhered to the underside of the membrane were successively fixed by methanol and stained with crystal violet solution. At last, the cells invaded through the membrane were counted in 10 random fields under a microscope.

Cell invasion assays

The steps are the same as above, except 50 μ g Matrigel bought from Becton Dickinson Bioscience (Bedford, USA) was covered onto the top surface of the membrane before the HCC cells were seeded onto the membrane.

mRNA decay assay

HCC cells and actinomycin D (Sigma, St. MO, USA) were added into 6-well plates. BCL2 and Twist1 RNA expression were detected by qRT-PCR at 1, 2, 3, 4, 5, 6 h or 2, 4, 6, 8, 10, 12 h (according to the length of half-life) after treated with indicated vectors.

RNA immunoprecipitation (RIP assay)

RIP assay was carried out follow the protocol of the Magna Immunoprecipitation Kit (Millipore, Billerica). A total of 2×10^7 HCC cells were lysed by Lysis Buffer. Then magnetic beads embedded with antibodies against RBM45 (Sigma-Aldrich) or mouse IgG (Millipore) was added into the cell lysates. After incubated at 4°C for 12h with continuous rotation, the mixture were added with proteinase K to remove proteins. After reversed transcribed using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher), the transcripts were detected by qRT-PCR.

RNA pull-down

The fragments, including 3'-UTR, CDS and 5'-UTR were amplified by PCR. After prepared by PCR, the biotinylated RNA probes were extracted using the mixture of chloroform and phenol. A total of 150g HCC cells lysate were incubated with 500ng biotinylated transcripts at 37°C for 2 h with continuous rotation. Streptavidin-conjugated Dynabeads (Invitrogen) were used to isolate the mixture. At last, the protein was collected and measured by Western blotting.

Animal model assays

Animal model assays in this study were carried out under the guidelines of Experimental Animal Ethical Committee of Xi'an Jiaotong University. Nude mice (male, aged 5 weeks, weighing 13–15 g) were bought from the Animal Center of Xi'an Jiaotong University and feed in a standard specific-pathogen-free environment. For the xenograft tumor model, 5 nude of mice per conditions was used in each experiment, 1×10^7 indicated HCC cells were inoculated into the right flank of the mice. The tumor volume was calculated with the formula $\text{length} \times \text{width}^2 \times 0.5$ every 3 days. After about one month, the mice were sacrificed and the xenografts were surgically removed. For the tumor metastasis model, 1×10^7 indicated HCC cells were inoculated into the lateral tail veins of mice. After 4 weeks, the mice were sacrificed, the number of nodules in the lung was observed using monitoring of the luciferase signals.

Statistical analysis

The statistical analyses in this study was performed using version 19.0 of SPSS statistical software, and the results are expressed as a mean \pm standard deviation. $*p < 0.05$ with a two-sided test was defined as statistically significant. The data between 2 two groups was evaluated using t-test, the difference among multiple groups was tested using one-way analysis (ANOVA with Dunnett's test). The correlation between RBM45 expression and clinicopathological features of HCC patient was analyzed using Chi-square tests (χ^2). The overall survival and disease-free survival of HCC patients were assessed using Kaplan-Meier method (the log-rank test).

Abbreviations

RBPS
RNA Binding Protein
RBM45
RNA-binding motif 45
HCC
Hepatocellular Carcinoma
OS
Overall Survival
DFS
Disease free survival
BCL2
B-cell lymphoma-2

Declarations

Ethical Approval and Consent to participate

The Ethics Committee of the Medical School of Xi'an Jiaotong University reviewed and approved the study, and written informed consent was obtained from each participant at each examination phase. The study complied with the principles of the Helsinki Declaration. The animal experiments carried out in this study were approved by the Experimental Animal Ethical Committee of Xi'an Jiaotong University.

Consent for publication

All authors agreed on the manuscript.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its Additional files.

Competing interests

No potential conflicts of interest were disclosed.

Funding

This study was funded by National Natural Science Foundation of China (No. 82003122).

Acknowledgements

The authors would like to thank Prof. Lumin Wang of Xi'an Jiaotong University (Xi'an, China) for providing the expert opinions.

Authors' information

Lili Han, Address: No.157 Xiwu Road, Department of Oncology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province 710004, China; e-mail: hanlili621@mail.xjtu.edu.cn; Tel number: 15332396006; Fax number: 029-87679515.

Lin Wang, Address: No.157 Xiwu Road, Department of Oncology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province 710004, China; e-mail: 2236506322@qq.com; Tel number: 15877234707; Fax number: 029-87679515.

Fei Wu, Address: No.157 Xiwu Road, Department of Oncology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province 710004, China; e-mail: 1936005658@qq.com; Tel number: 15771965751; Fax number: 029-87679515.

References

1. Torre L (2015) A., et al. Global cancer statistics, 2012. *CA Cancer J Clin* 65:87–108
2. Zhou M et al (2019) Mortality, morbidity, and risk factors in China and its provinces, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* 394:1145–1158
3. Gerstberger S, Hafner M, Tuschl T (2014) A census of human RNA-binding proteins. *Nat Rev Genet* 15:829–845
4. Zhang J et al (2020) RBP EIF2S2 Promotes Tumorigenesis and Progression by Regulating MYC-Mediated Inhibition via FHIT-Related Enhancers. *Mol Ther* 20:30090–30093
5. Azam SH et al (2019) Quaking orchestrates a post-transcriptional regulatory network of endothelial cell cycle progression critical to angiogenesis and metastasis. *Oncogene* 38:5191–52103
6. Dahlem C et al (2019) The Insulin-Like Growth Factor 2 mRNA Binding Protein IMP2/IGF2BP2 is overexpressed and Correlates with Poor Survival in Pancreatic Cancer. *Int J Mol Sci* 20:3204
7. Han LL, Huang C, Zhang SQ (2019) The RNA-binding protein SORBS2 suppresses hepatocellular carcinoma tumourigenesis and metastasis by stabilizing RORA mRNA. *Liver Int* 39:2190–2203
8. Zhao L et al (2019) RNA-binding protein RPS3 contributes to hepatocarcinogenesis by post-transcriptionally up-regulating SIRT1. *Nucleic Acids Res* 28:2011–2028
9. Zhang Z et al (2019) RNA-binding protein ZFP36/TTP protects against ferroptosis by regulating autophagy signaling pathway in hepatic stellate cells. *Autophagy* 11:1–24
10. Tamada H et al (2002) cDNA cloning and characterization of Drb1, a new member of RRM-type neural RNA-binding protein. *Biochem Biophys Res Commun* 13:96–104
11. Mashiko T et al (2016) Developmentally regulated RNA-binding protein 1 (Drb1)/RNA-binding motif protein 45 (RBM45), a nuclear-cytoplasmic trafficking protein, forms TAR DNA-binding protein 43 (TDP-43)-mediated cytoplasmic aggregates. *J Biol Chem* 291:14996–15007
12. Gong J et al (2017) RBM45 competes with HDAC1 for binding to FUS in response to DNA damage. *Nucleic Acids Res* 15:12862–12876
13. Collins M et al (2012) The RNA-binding motif 45 (RBM45) protein accumulates in inclusion bodies in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with TDP-43 inclusions (FTLD-TDP) patients. *Acta Neuropathol* 124:717–732
14. Strasser A, Vaux DL (2018) Viewing BCL2 and cell death control from an evolutionary perspective. *Cell Death Differ* 25:13–20
15. Agani H et al (2017) Antiapoptotic Bcl-2 protein as a potential target for cancer therapy: A mini review. *Artif Cells Nanomed Biotechnol* 44:1212–1221

16. Meng J et al (2018) Twist1 Regulates Vimentin through Cul2 Circular RNA to Promote EMT in Hepatocellular Carcinoma. *Cancer Res* 78:4150–4162
17. Xiao T et al (2018) Protease-activated receptor-1 (PAR1) promotes epithelial-endothelial transition through Twist1 in hepatocellular carcinoma. *J Exp Clin Cancer Res* 37:185
18. Sun T et al (2010) Expression and functional significance of Twist1 in hepatocellular carcinoma: its role in vasculogenic mimicry. *Hepatology* 51:545–556
19. Sun T et al (2011) Promotion of tumor cell metastasis and vasculogenic mimicry by way of transcription coactivation by Bcl-2 and Twist1: a study of hepatocellular carcinoma. *Hepatology* 54:1690–1706
20. Liu K et al (2015) Hypoxia induced epithelial-mesenchymal transition and vasculogenic mimicry formation by promoting Bcl-2/Twist1 cooperation. *Exp Mol Pathol* 99:383–391
21. Castello A et al (2012) Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* 149:1393–1406
22. Shibata T, Arai Y, Totoki Y (2018) Molecular genomic landscapes of hepatobiliary cancer. *Cancer Sci* 109:1282–1291
23. Gerstberger S, Hafner M, Tuschl T (2014) A census of human RNA-binding proteins. *Nat Rev Genet* 15:829–845
24. Wei C et al (2015) MicroRNA-497 induces cell apoptosis by negatively regulating Bcl-2 protein expression at the posttranscriptional level in human breast cancer. *Int J Clin Exp Pathol* 8:7729–7739
25. Reszec J et al (2004) Expression of cell proliferation and apoptosis markers in papillomas and cancers of conjunctiva and eyelid. *Ann N Y Acad Sci* 1030:419–426
26. Delbridge AR et al (2016) Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. *Nat Rev Cancer* 16:99–109
27. Xiao L et al (2017) Cytoplasmic RAP1 mediates cisplatin resistance of non-small cell lung cancer. *Cell Death Dis* 8:e2803
28. Yang F et al (2018) MicroRNA-7-5p Promotes Cisplatin Resistance of Cervical Cancer Cells and Modulation of Cellular Energy Homeostasis by Regulating the Expression of the PARP-1 and BCL2 Genes. *Med Sci Monit* 24:6506–6516
29. Watanabe A et al (2018) BCL2 and BCLxL are key determinants of resistance to antitubulin chemotherapeutics in melanoma cells. *Exp Dermatol* 22:518–523
30. Yang J et al (2014) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 117:927–939
31. Cho YA et al (2019) Twist and Snail/Slug Expression in Oropharyngeal Squamous Cell Carcinoma in Correlation With Lymph Node Metastasis. *Anticancer Res* 39:6307–6316
32. Meng J et al (2018) Twist1 Regulates Vimentin through Cul2 Circular RNA to Promote EMT in Hepatocellular Carcinoma. *Cancer Res* 78:4150–4162
33. Onder TT et al (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* 68:3645–3654
34. Pereira L et al (2015) Implications of Akt2/Twist crosstalk on breast cancer metastatic outcome. *Drug Discov Today* 20:1152–1158
35. Wilson WH et al (2010) Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. *Lancet Oncol* 11:1149–1159
36. Zhu Y et al (2016) Identification of a novel senolytic agent, navitoclax, targeting the Bcl-2 family of anti-apoptotic factors. *Aging Cell* 15:428–435
37. Tan M et al (2019) The FZD7-TWIST1 axis is responsible for anoikis resistance and tumorigenesis in ovarian carcinoma. *Mol Oncol* 13:757–780
38. Kwon CH et al (2017) TWIST mediates resistance to paclitaxel by regulating Akt and Bcl-2 expression in gastric cancer cells. *Tumour Biol* 39:10104

Figures

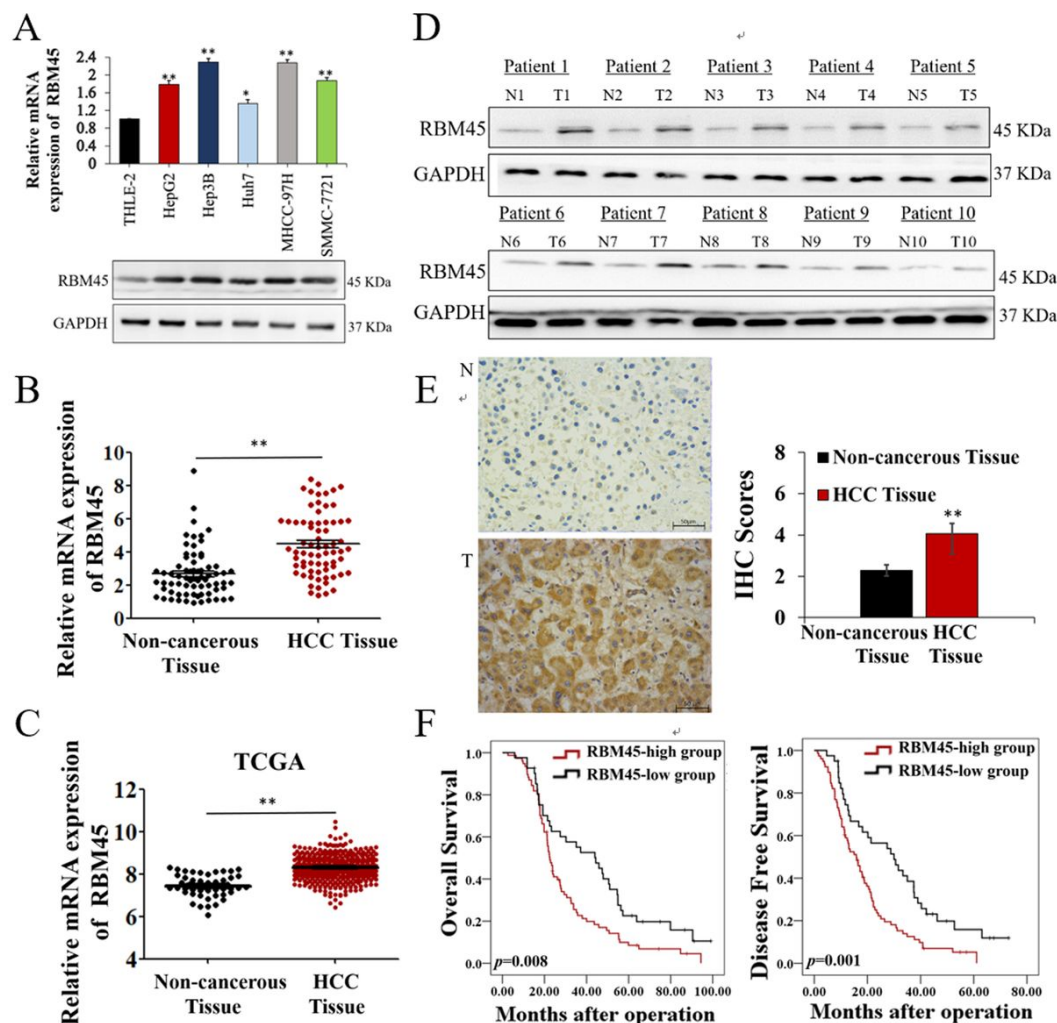


Figure 1

Increased RBM45 expression in HCC and its association with poor prognosis. A. RBM45 expression levels in various human HCC cell lines and the THLE-2 immortalized human liver cells. B. The expression of RBM45 in 120 samples was analyzed by qRT-PCR. C. Analysis of RBM45 mRNA expression in normal tissue and HCC tissues using data from the TCGA database. D. RBM45 expression in HCC tissues was increased compared with that in the paired distant noncancerous tissues in ten HCC patients by western blot analysis. E. The expression of RBM45 in samples was analyzed by an immunohistochemistry assay (magnification 200 \times). F. Comparison of OS and DFS between HCC patients with high and low RBM45 expression levels using data of 120 clinical samples. * $p<0.05$, ** $p<0.01$.

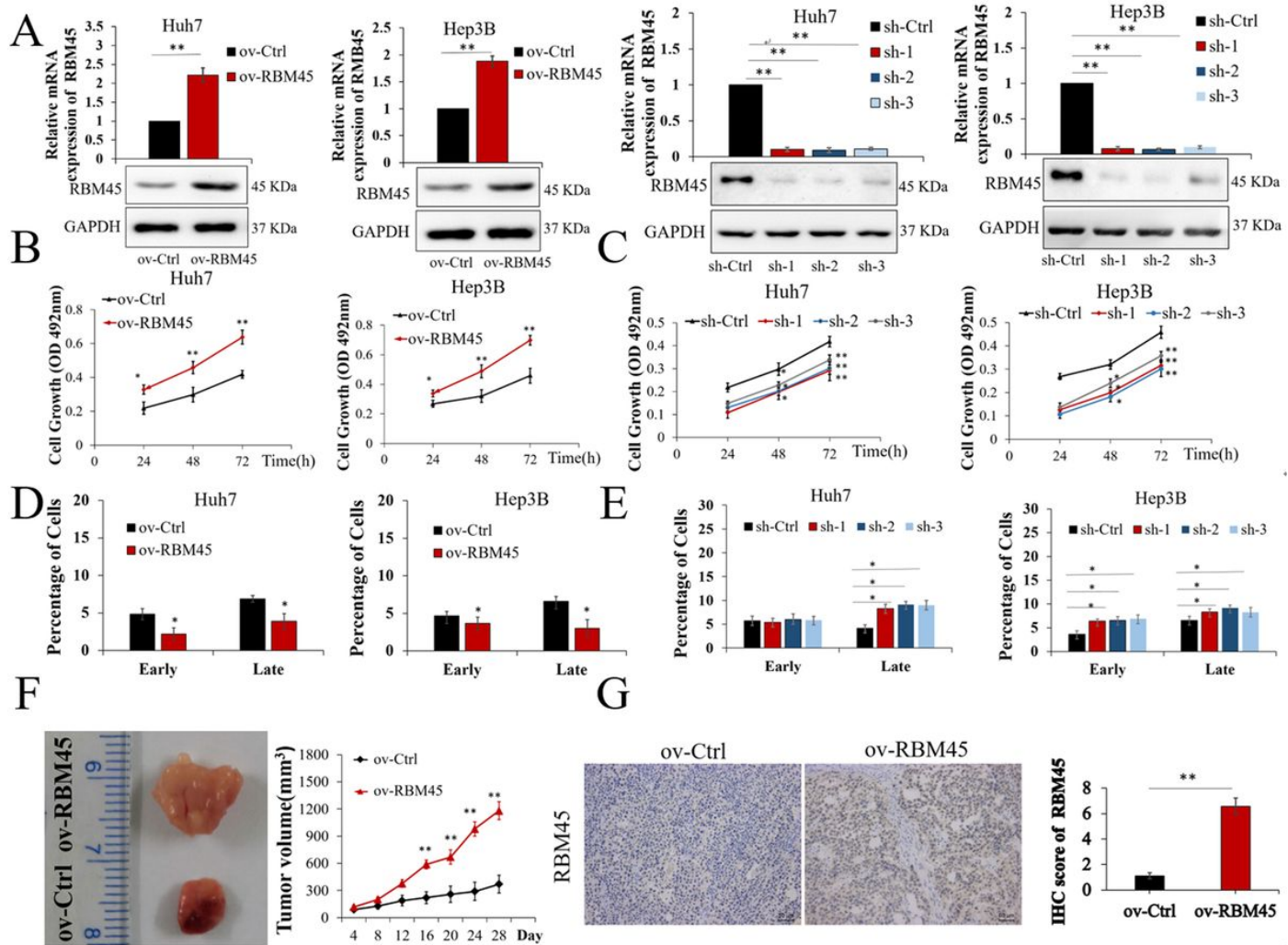


Figure 2

RBM45 promoted HCC growth. A. RBM45 expression was significantly increased following the transfection of the ov-RBM45 vectors into Huh7 and Hep3B cells; RBM45 expression was decreased after the transfection of RBM45 -shRNAs into Huh7 and Hep3B cells. B. MTT assay showed that RBM45 overexpression promoted Huh7 and Hep3B cells proliferation. C. MTT assay showed that RBM45 knockdown inhibited Huh7 and Hep3B cells proliferation. D. Overexpression of RBM45 reduced Huh7 and Hep3B cells apoptosis. E. Inhibition of RBM45 promoted Huh7 and Hep3B cells apoptosis. F. Increased RBM45 promoted HCC xenograft tumor growth. G. IHC assay showed RBM45 expression in the xenograft tumor tissue in different groups (magnification, 200 \times). * $p < 0.05$, ** $p < 0.01$.

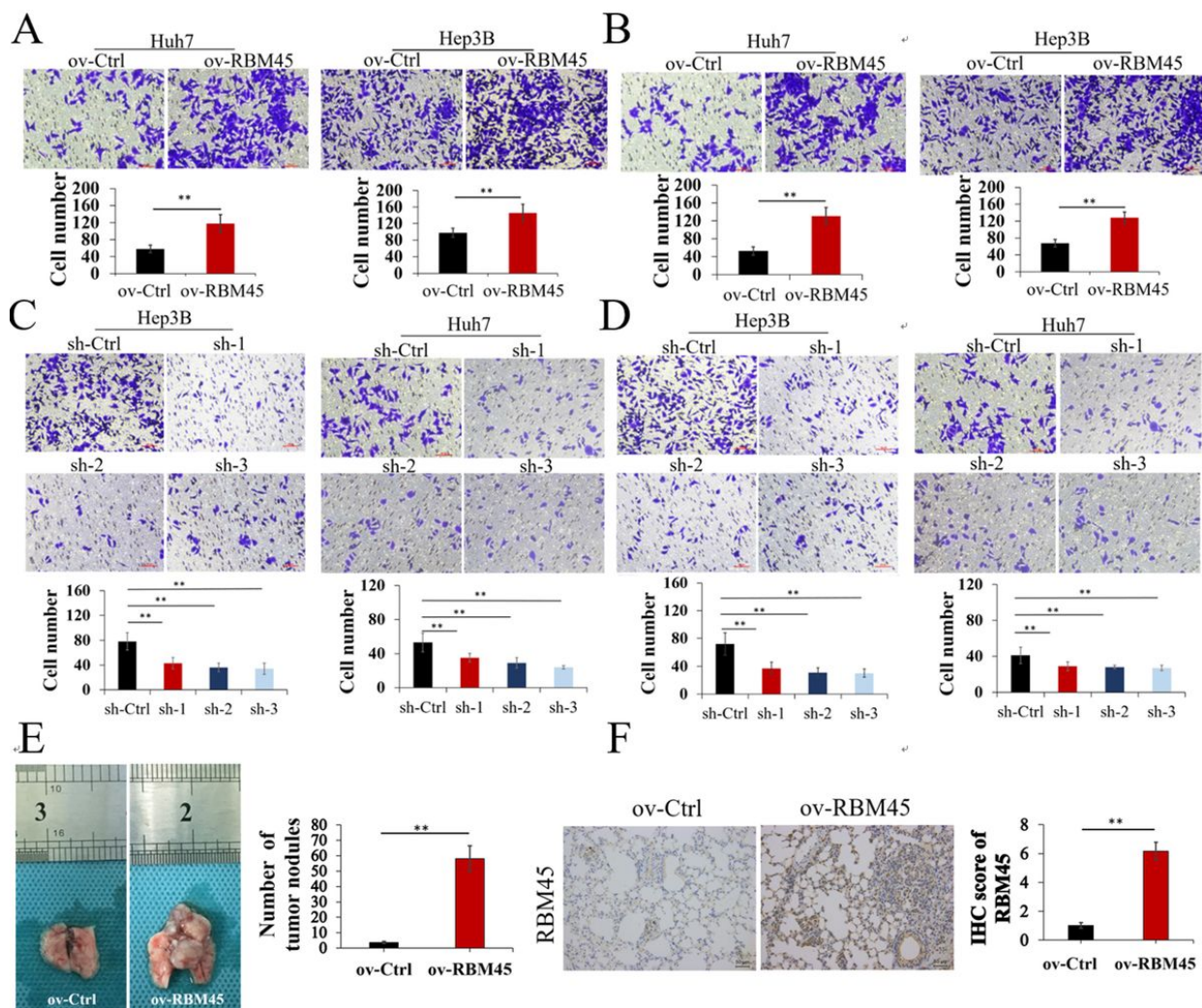


Figure 3

RBM45 promoted HCC metastasis. A,B. Transwell assay showed that RBM45 overexpression promoted HCC cells migration and invasion. C,D. Transwell assay showed that RBM45 knockdown inhibited HCC cells migration and invasion. The invaded cells were quantified by counting the cells in 10 random fields (magnification 200×). Data are presented as the mean ± SE of three independent experiments. E. RBM45 overexpression promoted the lung metastatic capability of HCC cells. F. IHC assay showed RBM45 expression in metastatic xenograft model tissue in different groups (magnification, 200×). * $p < 0.05$, ** $p < 0.01$.

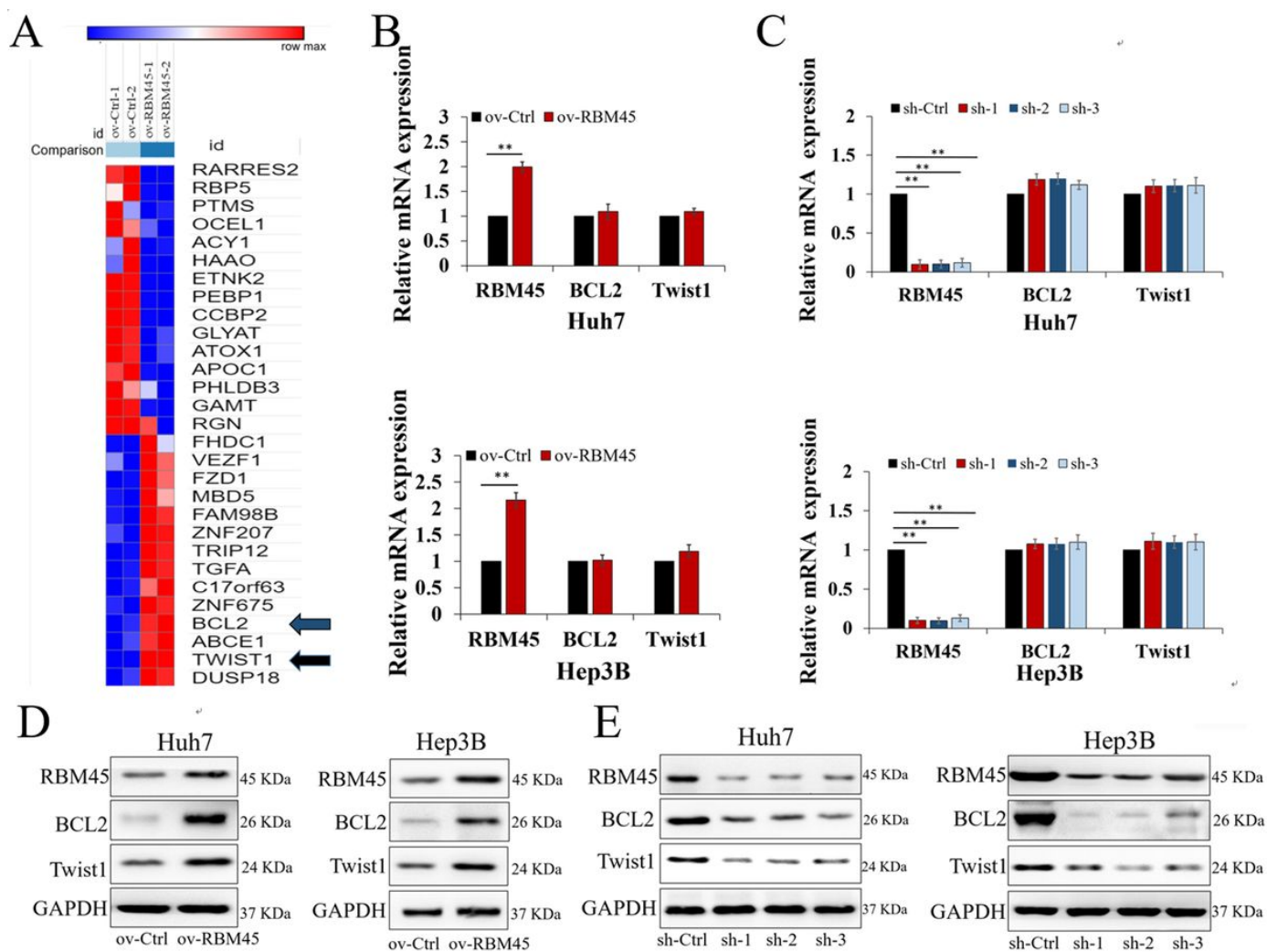


Figure 4

RBM45 increases the expression level of BCL2 and Twist1. A. Heat map of log2-transformed fold changes in the genes with the most dramatic changes upon RBM45 overexpression. B. C. The expression of RBM45, BCL2 and Twist1 in Huh7 and Hep3B cells were analyzed by qRT-PCR. D.E. The expression of RBM45, BCL2 and Twist1 in Huh7 and Hep3B cells were analyzed by western blot. * $p < 0.05$, ** $p < 0.01$.

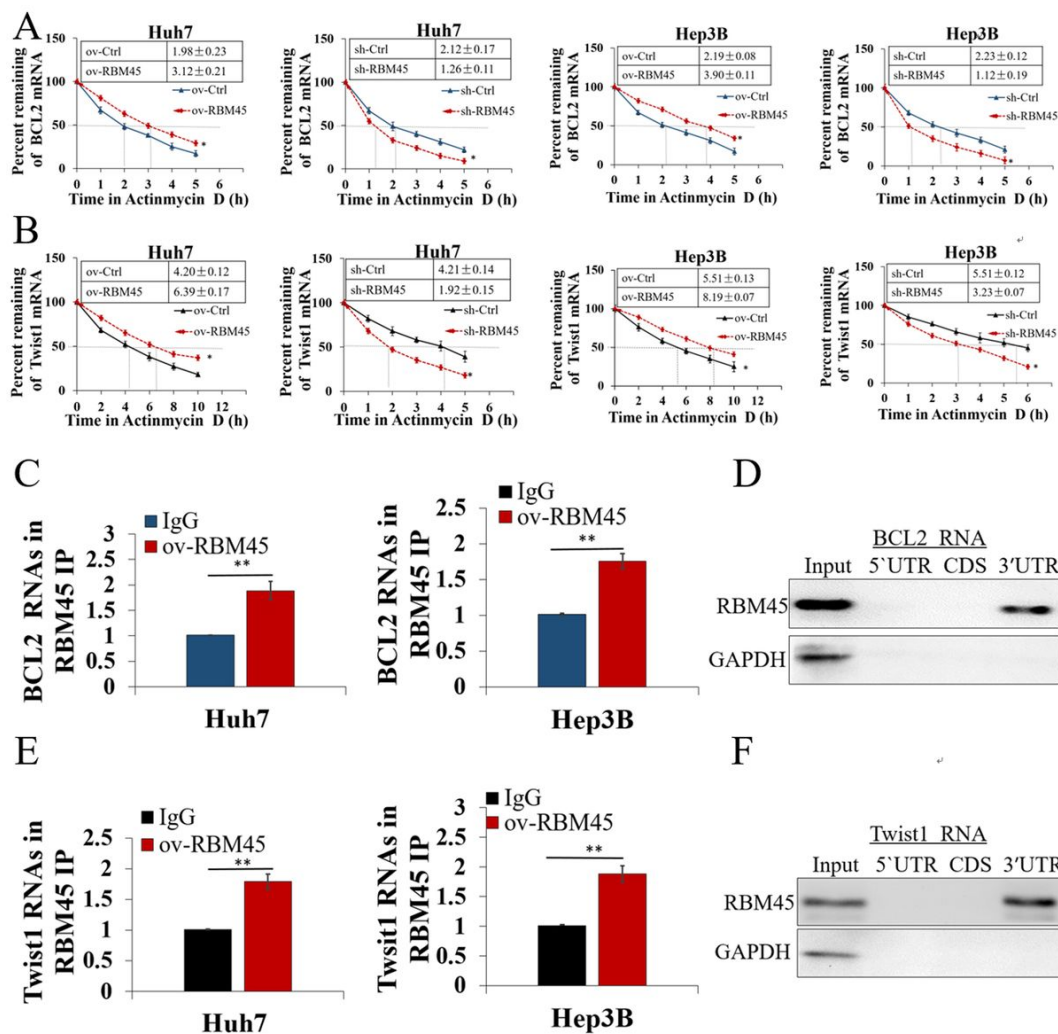


Figure 5

RBM45 directly bind to the 3'-UTR of BCL2 and Twist1 mRNA. A. B. The half-life of BCL2 and Twist1 mRNA was increased after RBM45 overexpression and reduced after RBM45 knockdown in Huh7 and Hep3B cells. C. RIP assays showed that RBM45 directly bound to BCL2 mRNA. D. RNA pull-down results showed that RBM45 was directly associated with the 3' UTR of BCL2 mRNA. E. RIP assay showed that RBM45 directly bound to Twist1 mRNA. F. RNA pull-down results showed that RBM45 was directly associated with the 3' UTR of Twist1 mRNA. * $p < 0.05$, ** $p < 0.01$.

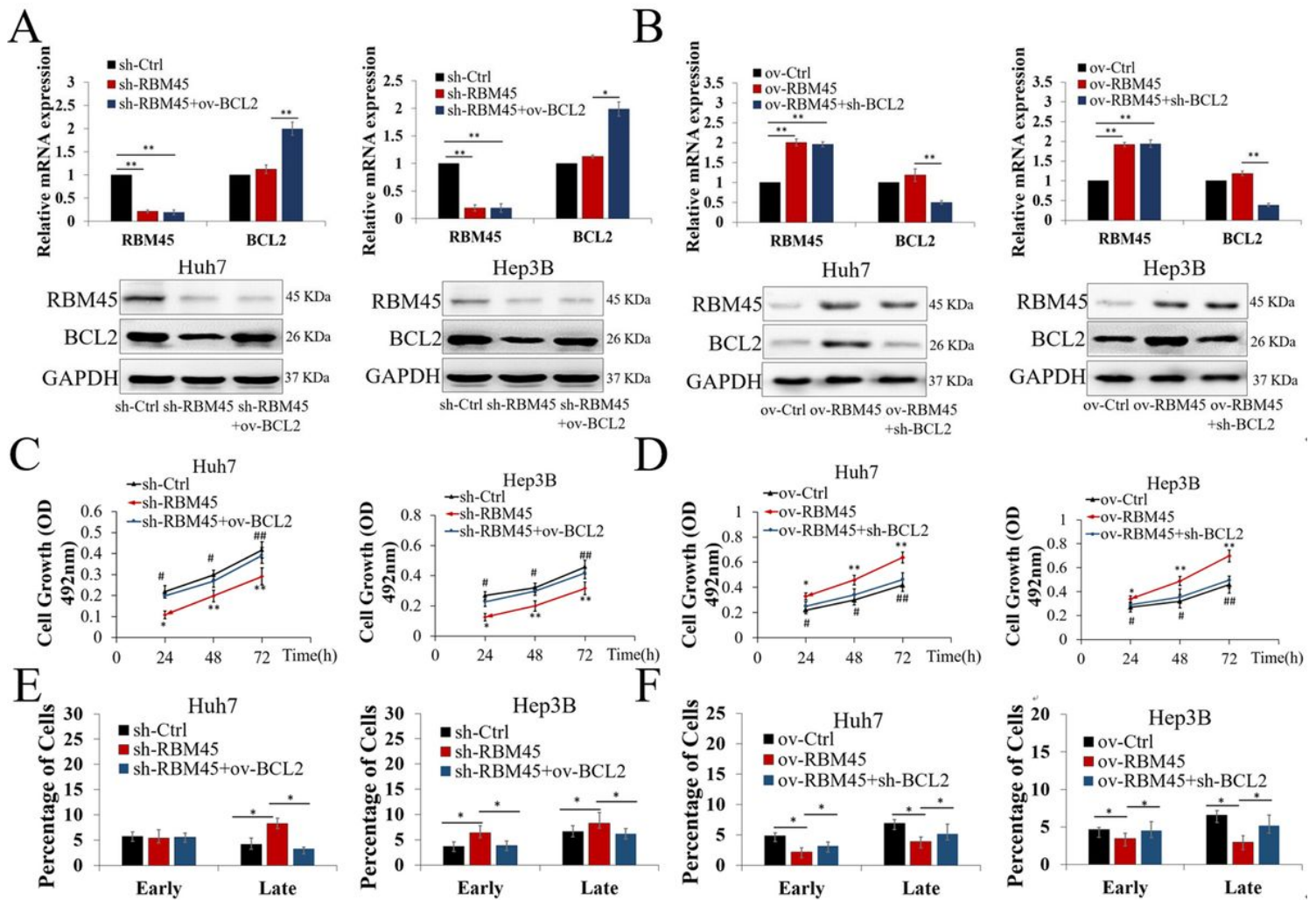


Figure 6

RBM45 promoted HCC cells proliferation upon BCL2. A.B. The expression of RBM45 and BCL2 in Huh7 and Hep3B cells were analyzed by qRT-PCR and western blot assays (** $p < 0.01$). C. Overexpression of BCL2 rescued the effect of decreased RBM45 on Huh7 and Hep3B cells proliferation (* $p < 0.05$, ** $p < 0.01$, sh-RBM45 vs. sh-Ctrl; # $p < 0.05$, ## $p < 0.01$, sh-RBM45 +ov-BCL2 vs. sh-RBM45). D. Knockdown of BCL2 rescued the effect of increased RBM45 on Huh7 and Hep3B cells proliferation (* $p < 0.05$, ** $p < 0.01$, ov-RBM45 vs. ov-Ctrl; # $p < 0.05$, ## $p < 0.01$, sh-RBM45 +ov-BCL2 vs. sh-RBM45). E. Overexpression of BCL2 rescued the effect of decreased RBM45 on Huh7 and Hep3B cells apoptosis (* $p < 0.05$). F. Knockdown of BCL2 rescued the effect of increased RBM45 on Huh7 and Hep3B cells apoptosis (* $p < 0.05$).

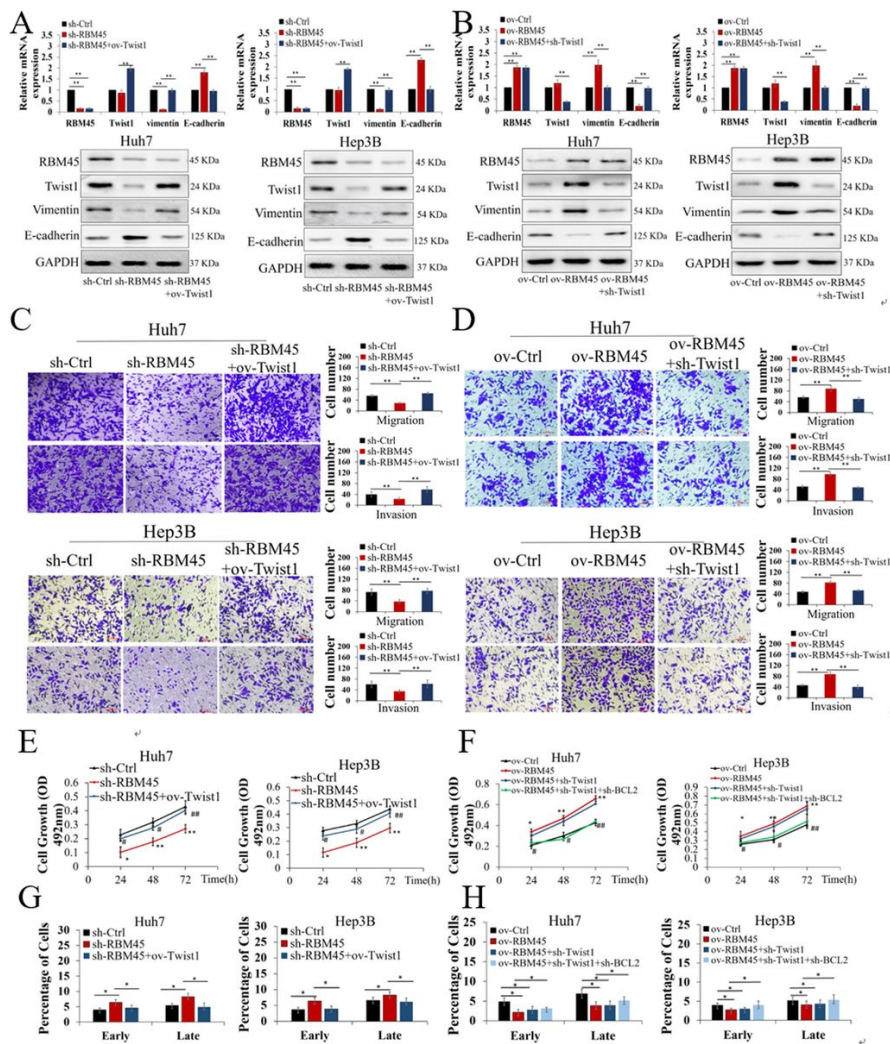


Figure 7

Twist1 is an essential downstream effector of RBM45. A. Overexpression of Twist1 rescued the function of RBM45 altering HCC EMT (** $p < 0.01$). B. knockdown of Twist1 rescued the function of RBM45 altering HCC EMT (** $p < 0.01$). C. Overexpression of Twist1 rescued the effect of decreased RBM45 on HCC cell migration and invasion ability (** $p < 0.01$). D. Knockdown of Twist1 rescued the effect of increased RBM45 on HCC cell migration and invasion ability (** $p < 0.01$). E.G. Overexpression of Twist1 rescued the function of RBM45 altering HCC cells proliferation and apoptosis (* $p < 0.05$, ** $p < 0.01$, ov-RBM45 vs. ov-Ctrl; # $p < 0.05$, ## $p < 0.01$, ov-RBM45 + sh-BCL2 + sh-Twist1 vs. ov-RBM45). F.H. Co-knockdown of BCL2 and Twist1 instead of single knockdown of Twist1 rescued the effect of increased RBM45 on HCC cells proliferation and apoptosis (** $p < 0.05$).

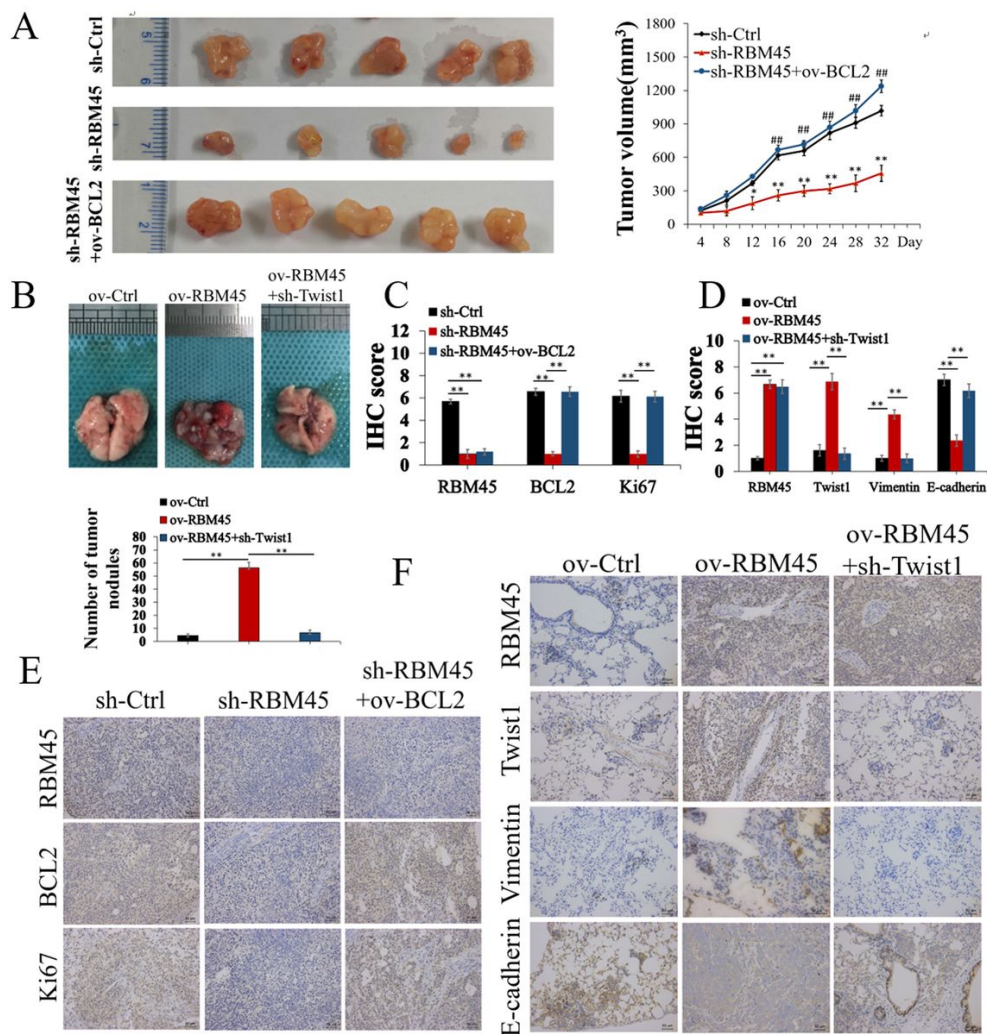


Figure 8

RBM45 promoted hepatocarcinogenesis upon BCL2 and Twist1 in vivo. A. Decreased RBM45 inhibited HCC xenograft tumor growth (* $p < 0.05$, ** $p < 0.01$), while BCL2 overexpression counteracted the inhibiting effect of sh-RBM45 (# $p < 0.05$, ## $p < 0.01$). B. Increased RBM45 promoted lung metastatic capability of Huh7 cells in vivo (** $p < 0.01$), while Twist1 knockdown counteracted the effect of RBM45 (** $p < 0.01$). C.E. IHC assay showed RBM45, BCL2 and Ki67 expression in xenograft tumor tissue in different groups (magnification, 200 \times). D.F. IHC assay showed RBM45, Twist1, Vimentin and E-cadherin expression in metastatic xenograft model tissue in different groups (magnification, 200 \times) (* $p < 0.05$).

Supplementary Files

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

- [RBM45TableS1..docx](#)
- [S1.jpg](#)
- [S2.jpg](#)