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ORIGINAL ARTICLE

RNA-binding protein RBM6 acts as a tumor suppressor gene to inhibit the progression of hepatocellular carcinoma

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Summary

Purpose: *To investigate whether RBM6 can serve as a suppressor gene in hepatocellular carcinoma (HCC) and affect its progression.*

Methods: *QPCR and Western blot were carried out to measure RBM6 expression in tissue samples collected from HCC patients with different tumor sizes or in different stages. The relationship between overall survival (OS) and RBM6 expression in patients with HCC was analyzed using Kaplan-Meier survival method. Meanwhile, the effects of different factors on HCC progression were evaluated through Cox regression analysis. After over-expression of RBM6 in HepG2 and HB611 cells, the cell viability, cell migration and invasion abilities and apoptosis rate were assessed by cell counting kit-8 (CCK-8), transwell assay, and flow cytometry analysis, respectively.*

Results: *RBM6 expression, markedly down-regulated in HCC tissues, showed a great relevance to tumor size, TNM stage, and histological grade, and the survival rate of patients in high RBM6 expression group was higher than those in low RBM6 expression group. Besides, Cox regression analysis revealed that RBM6 expression, tumor size, TNM stage and histological grade were four independent factors affecting the OS of HCC patients. Moreover, in vitro cell experiments demonstrated that overexpression of RBM6 significantly attenuated the cell viability as well as the invasive ability while enhanced cell apoptosis.*

Conclusions: *The low expression of RBM6 contributes to the improvement of the survival of patients with HCC. Therefore, RBM6 can serve as a tumor-suppressing gene to repress cell proliferation, migration and invasion and promote cell apoptosis, thereby affecting the progression of HCC.*

Key words: *RBM6, HCC, tumor suppressor gene*

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world [1]. The biological characteristics of high proliferation and invasion of HCC cells determine the insidious onset, rapid development and poor prognosis of HCC, leading to its mortality rate ranking third in the world [2]. In the progression of HCC, overexpression of oncogenes, mutation and inactivation of tumor suppressor genes play important roles [3].

A large number of studies have shown that changes in RBM expression play a critical regulatory role in the occurrence of various malignant tumors [4]. RBM is highly conserved and exists in almost all living cells [4]. Studies have demonstrated that RBMs can be engaged in a number of cellular functions through the modulation of RNA metabolism, including transport, splicing, translation, and precursor RNA processing [5]. At present, the regulatory role of RBM family members in tu-

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mors has been widely recognized [6]. For example, RBMS2 can inhibit the proliferation of breast cancer cells [7]. RBM10 is directly regulated by RBM5 to promote the transformation related process of small-cell lung cancer, and the measurement of RBM10 and RBM5 expressions in clinical samples may have prognostic and/or potential predictive value [8].

RBM6, located on chromosome 3p21.3 with a high homology with RBM5, plays an essential role in RNA splicing [9]. It contains two zinc finger motifs, a binuclear signal and two RNA binding motifs, indicating that the proteins encoded by RBM6 gene possess DNA/RNA binding functions [10]. Recent studies have shown that RBM6 can be used as a biomarker to predict the prognosis of pancreatic cancer [11]. However, there have been no reports on the biological function and potential mechanism of RBM6 in the pathogenesis of HCC. Therefore, this study explored RBM6 expression in HCC tissues and further analyzed the role and potential mechanism of RBM6 affecting the development of HCC *in vitro* and *in vivo*, so as to provide new ideas for clinical targeted diagnosis and treatment for this cancer.

Methods

Materials

A total of 53 HCC specimens were surgically collected, and their clinicopathological data and follow-up data were analyzed. Among the HCC patients in this study, 20 were male and 33 female; 27 were aged ≤ 60 years, and 26 > 60 years. The normal tissues adjacent to the cancer (2 cm away from the tumor) were used as controls. All specimens were confirmed by two pathologists as HCC and adjacent normal tissues. All patients had no adjuvant treatment such as intervention and chemotherapy before and after surgery, and the follow-up time was from the date of surgery to 10 years after surgery. This study was reviewed and approved by the Ethics Committee of Nanfang Hospital, Southern Medical University.

Cell culture and transfection

A normal liver cell line (L-02) and HCC cell lines (SMMC-7721, HepG2, HB611, and HHCC) were provided by the Cell Resource Center of the Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China), and were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) and 1% penicillin / streptomycin in a 37°C cell incubator with 5% CO_2. Lentivirus transfection was performed with recombinant lentiviral vectors carrying the RBM6 gene (Lv-RBM6) or empty plasmid (Lv-Ctrl) (Hanheng Biotechnology, Shanghai, Co., Ltd., Shanghai, China).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from tissues or cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a BeyoRTTMII cDNA synthesis kit (TaKaRa, Tokyo, Japan), and then amplified using a SYBR Green kit on a PCR instrument, with glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The primers were synthesized by Shanghai Shengong Company (Shanghai, China) with the sequence: RBM6: F: 5'-AGGCTGAG-GAGAAGGAGGAG-3' and R: 5'-TCTTGGCTC-CCACGAAAAGG-3'. GAPDH: F: 5'-AATGGGCAGCCGTTAGGAAA-3', R: 5'-GCGCC-CAATACGACCAAATC-3' downstream.

Western blot

Pre-chilled radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) was used to extract total protein from cells or tissues, and quinolinic acid (BCA) kit (Beyotime, Shanghai, China) was used to quantify total protein. Thereafter, Western blot analysis was carried out based on standard procedures. Finally, Image J software (NIH, Bethesda, MD, USA) was used to analyze the target band.

Cell counting kit-8 (CCK-8) assay

Cells were plated in 96-well plates (2×10^3 cells/well) in 200 μL culture medium. CCK-8 assay (Dojindo, Kumamoto, Japan) was performed based on the manufacturer's instructions.

Transwell assay

Cell invasion experiment: Cells after 24 h of transfection were digested with trypsin and resuspended in serum-free medium. 100 μL of Matrigel (Corning, Corning, NY, USA) diluted with serum-free medium was pre-plated in the bottom of the upper chamber of the Transwell chamber, and incubated at 37°C for 30 min. Then, the migrating cells were counted and observed after stained by crystal violet under a microscope, and 5 fields of view were randomly selected.

Cell migration experiment: The steps were the same as the cell invasion experiment except pre-plated matrigel.

Apoptosis rate detected by flow cytometry

The cells were collected 24 h after heat treatment, washed with 2 times pre-chilled PBS at 4°C, and resuspended with 1 ml of binding buffer. The cell density were adjusted to 1×10^5 / mL, and cell apoptosis was detected by flow cytometry after 15 min of staining by Annexin V and propidium iodide (PI).

Statistics

Data were analyzed using SPSS 20.0 statistical software (IBM, Armonk, NY, USA). Measurement data were presented as mean±standard deviation, and t-test was used to compare data between the two groups. Count data were compared using the x^2 test. P less than 0.05 was considered statistically significant.

Results

Low expression of RBM6 in HCC tissues

QPCR detection indicated that RBM6 was remarkably downregulated in HCC tissue specimens in comparison to adjacent normal ones (Figure 1A). According to the size of tumor tissues and TNM staging, we divided 53 cases of HCC tissue into ≤5 cm group and >5 cm group and TNM stage I-II group and III-IV group. It was found that RBM6 showed a significant reduction in tumor samples>5 cm (Figure 1B). In addition, RBM6 expression in stage III+IV group was also remarkably lower than that in stage I+II group (Figure 1C). To determine whether RBM6 can affect the OS of patients with HCC, we followed up patients for up to 10 years. Kaplan-Meier analysis revealed that the OS of patients with highly-expressed RBM6 was higher than of those with lowly-expressed RBM6 (HR=0.5538, p=0.0239) (Figure 1D). Western blot analysis further confirmed a consistent result in the protein expression of RBM6 (Figure 1E). The above results suggested that RBM6 can not only suppress the progression of HCC, but also benefit the OS of HCC patients.

Correlation between RBM6 expression and clinicopathological characteristics of HCC patients

We analyzed the general data of 53 patients with HCC but detected no significant association between RBM6 expression and patient age or gender (p>0.05). However, RBM6 showed a significantly increased expression in HCC tumor tissues>5cm, in TNM stage III to IV, or with high-grade of differentiation comparing to those ≤5 cm, in TNM stage I to II, or with poorly differentiated grades (p<0.05). In the two groups with and without lymph node metastasis, a slight statistical difference in RBM6 expression was detectable (p=0.04937) (Table 1). These observations revealed that the expression of RBM6 might have effects on tumor size, TNM stage and histological grade of HCC.

Univariate and multivariate Cox regression analyses of OS in patients with HCC

To determine whether the OS of HCC patients is affected by other factors, we performed univariate and multivariate Cox regression analyses on the clinical characteristics of the patients. It was found that the HR values of tumor size, TNM stage, histological grade, and RBM6 expression on the OS

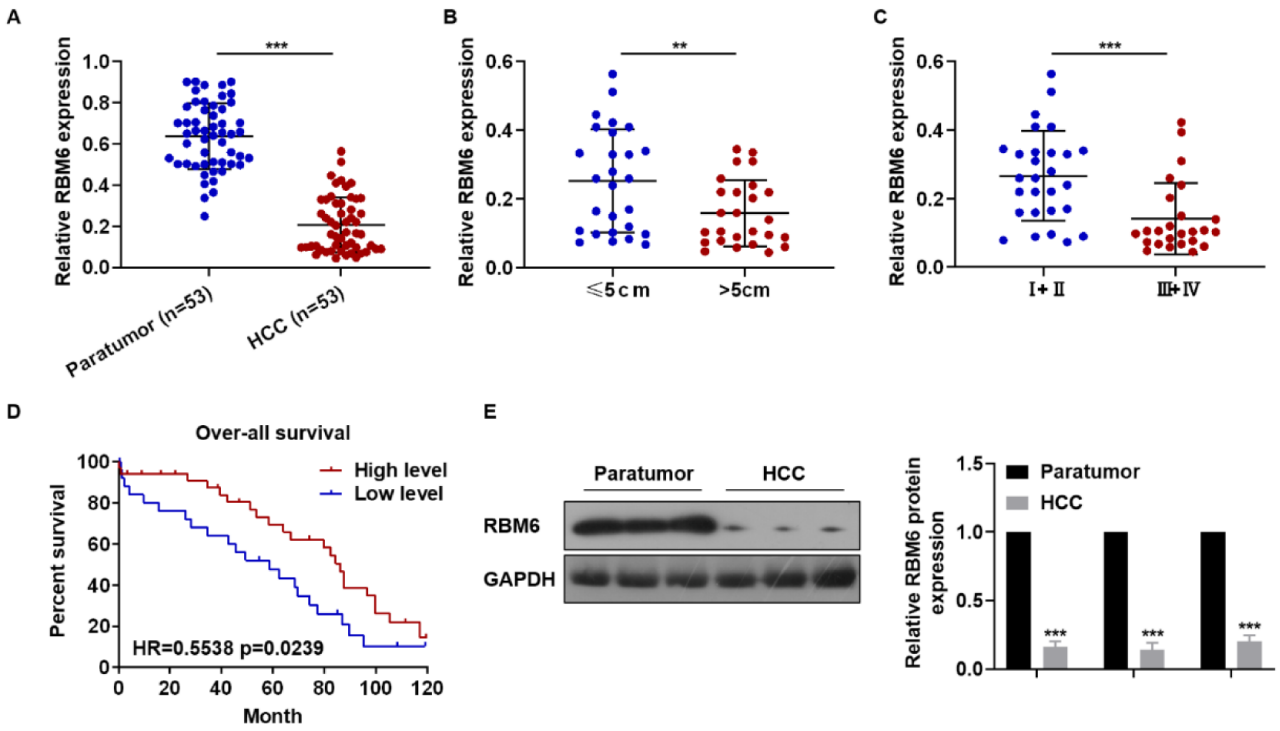

Figure 1. RBM6 was underexpressed in HCC tissues. **A:** In 53 pairs of HCC tissues and adjacent tissues, qRT-PCR detected RBM6 was significantly downregulated in HCC tissues. **B:** qRT-PCR detected that RBM6 was significantly downregulated in tumor tissues larger than 5 cm. **C:** The mRNA expression of RBM6 in tumor stage III+IV was significantly lower than that in stage I + II. **D:** The survival rate of patients in RBM6 high expression group was higher than that in RBM6 low expression group. **E:** Western blot showed that the protein expression of RBM6 in HCC tissues was significantly lower than that in adjacent tissues. (**p<0.01, ***p<0.001).

of the patients were 1.631, 1.384, 1.208, and 0.608, respectively ($p<0.05$) in univariate analysis, while they were 1.492, 1.335, 1.302, and 0.760, respectively in multivariate analysis ($p<0.05$) (Table 2), further indicating that these four indexes are all independent factors influencing patient OS.

RBM6 suppressed the proliferation of HCC cells

To specify the relevant mechanism through which RBM6 affects the development of HCC, we performed *in vitro* cell experiments. qPCR detection of RBM6 expression in normal liver cells and HCC cell lines found that HepG2 cells had the lowest expression of RBM6, followed by HB611 (Figure 2A) Therefore, in this experiment, we selected this two HCC cell lines as the research objects. After overexpression of RBM6, RBM6 gene level detected by qPCR was remarkably increased (Figure 2B), while the protein expression of Ki67 measured by western blot was remarkably downregulated (Figure 2C), indicating that RBM6 may inhibit Ki67 protein expression. In addition, we also performed CCK-8 experiments and found that cell viability was remarkably reduced after overexpression of RBM6 (Figure 2D), suggesting that RBM6 is able to suppress the proliferation ability of HCC cells.

Table 1. Correlation between the expression of RBM6 and clinicopathological features in patients with liver cancer (n=53)

Clinicopathologic features	*n*	*RBM6 expression*		*p*
		Low (n=26)	*High (n=27)*	
Age (years old)				0.3348
≤60	27	15	12	
>60	26	11	15	
Gender				0.9148
Male	20	10	10	
Female	33	16	17	
Tumor size, cm				0.0002
≤5	25	19	6	
>5	28	7	21	
TNM stage				0.0089
I-II	23	16	7	
III-IV	30	10	20	
Histological classification				0.0196
Low grade	24	16	8	
Medium and High grade	29	10	19	
Lymph node metastasis				0.04937
No	26	14	12	
Yes	27	12	15	

Table 2. Univariate and multivariate Cox regression analyses affecting overall survival (OS) in patients with liver cancer

Variables	*Univariate analysis*			*Multivariate analysis*		
	p	*HR*	*95% CI*	*p value*	*HR*	*95% CI*
Age	0.324	0.956	0.674,1.154	0.365	0.845	0.851,1.428
Gender	0.512	0.667	0.415,1.216	0.657	0.727	0.385,1.487
Tumor size	0.021	1.631	1.418,1.985	0.029	1.492	1.162,2.243
Stage	0.016	1.384	1.069,1.795	0.024	1.335	1.181,1.927
Histological classifucation	0.027	1.208	1.075,1.849	0.038	1.302	1.033,1.791
Lymph node metastasis	0.824	1.008	0.815,1.387	0.931	1.016	0.526,1.215
RBM6	0.007	0.608	0.221,0.804	0.016	0.760	0.514,0.975

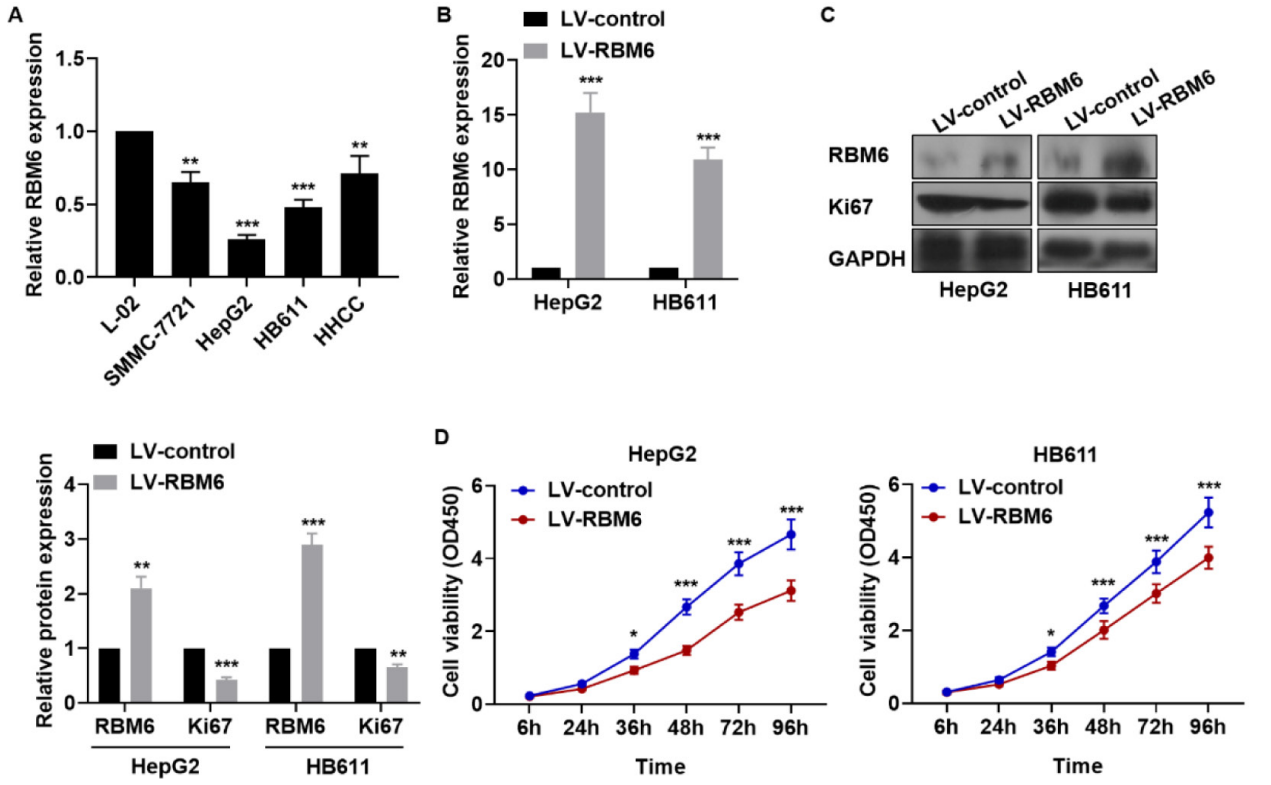

Figure 2. RBM6 inhibited the proliferation of HCC cells. **A:** qRT-PCR was used to detect the expression of RBM6 in normal liver cells (L-02) and liver cancer cell lines (SMMC-7721, HepG2, HB611 and HHCC). **B:** mRNA expression levels in HepG2 and HB611 were significantly increased after overexpression of RBM6. **C:** Western blot was used to detect the protein expressions of RBM6 and Ki67 after overexpressing RBM6 in HepG2 and HB611 cells. **D:** CCK8 showed a marked decrease in cell viability after overexpressing RBM6 in HepG2 and HB611 cells. (**p<0.01, ***p<0.001).

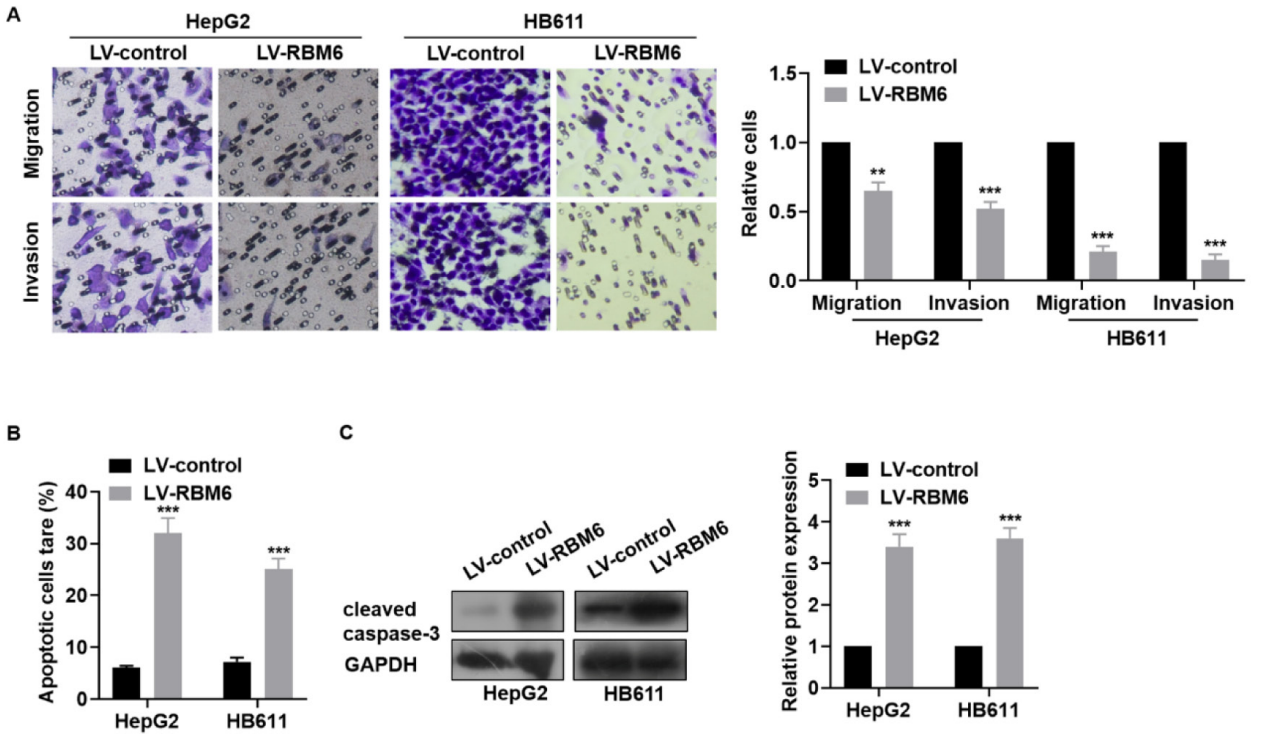

Figure 3. RBM6 inhibited the invasion and migration of HCC cells and promoted apoptosis. **A:** After RBM6 was overexpressed in HepG2 and HB611 cells, transwell detected that cell migration and invasion abilities were significantly reduced; **B:** Flow cytometry showed that cell apoptosis rate was significantly increased. **C:** The protein expression level of cleaved caspase-3 was significantly increased (**p<0.01, ***p<0.001).

RBM6 inhibits metastasis but promotes apoptosis of HCC cells

Figure 3A and 3B showed that overexpression of RBM6 significantly attenuated the invasiveness of HCC cell lines HepG2 and HB611 while elevated their apoptosis rate, revealed by transwell assay and flow cytometry analysis (Figure 3B). Furthermore, the expression of cleaved caspase-3 protein was markedly enhanced in cells overexpressing RBM6 (Figure 3C).

Discussion

The progression of HCC is the result of the interaction of activation of various oncogenes, inactivation of tumor suppressor genes and exogenous stimulating factors, among which inactivation of tumor suppressor genes is an important mechanism leading to the progression of HCC [12,13]. For example, studies have shown that the inactivation of p16, a tumor suppressor gene, can induce excessive cell proliferation and cell cycle acceleration, so that DNA enters S phase prematurely before repair, leading to tumor genesis [14]. Previous studies have confirmed that p16 expression may be absent or abnormal in HCC tissues, which may be an important link in the pathogenesis of HCC [15].

Studies have indicated that members of the RBM protein family are key regulators of tumor development and progression [16]. For example, Yang et al [17] revealed that RBM5 inhibited the proliferative and invasive abilities of prostate cancer cells. Bechara et al [18] showed that knockdown of RBM5 attenuated the colony-forming capacity of HeLa cells in cervical cancer. Recently, research has reported that RBM6 was downregulated in laryngeal cancer tissue and cell lines; however, its overexpression inhibited cell proliferation and invasion, while promoted its apoptosis [19]. The above conclusions are consistent to the results we observed in our study that RBM6, low expressed in HCC tissues, inhibited the progression of HCC, while upregulation of RBM6 markedly improved the postoperative OS of HCC patients.

Ki67 is a nuclear protein encoded by the MKI-67 gene, which is related to ribosomal RNA transcription and can serve as one of the markers of cell proliferation in relevant studies [20]. Ki67 was expressed to varying degrees in G1, S, G2 and M phases of cell proliferation, and decreased remarkably after mitosis, but was not expressed in G0 phase [21]. Relevant clinical studies have pointed out that it is closely related to tumor progression and metastasis [22-24]. Caspases are a protease family with a large number of members. Currently, there are at least 11 caspase protein family members in human body, among which caspase-3 plays the role of apoptotic executive factor in the process of cell apoptosis [25]. Caspase-3 normally exists in the form of zymogen but can be activated to cleaved caspase-3 when cell apoptosis occurs, thus playing a role in promoting cell apoptosis [26]. Our *in vitro* cell experiments revealed that Ki67 protein was remarkably downregulated, while cleaved caspase-3 protein was conversely upregulated after overexpression of RBM6 in HCC cells, indicating that RBM6 may inhibit the proliferation of HCC cells and promote cell apoptosis through affecting the protein expression of Ki67 and cleaved caspase-3.

Conclusions

To sum up, this experiment indicated for the first time that RBM6 expression was remarkably downregulated in HCC tissues, and was correlated with tumor size, TNM stage, and histological grade. Further *in vitro* experiments demonstrated that RBM6 promoted cell apoptosis and participated in the progression of HCC via inhibiting the proliferation capacity and metastasis of HCC cells. At the same time, RBM6 is an independent factor affecting the OS of HCC patients, which is conducive to the improvement of the OS of patients. Our research provides a new clinical approach for the development of candidate sites for targeted treatment of HCC.

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Conflict of interests

The authors declare no conflict of interests.

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