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Protective effect of the RNA-binding protein RBM10 in hepatocellular carcinoma

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Abstract. – OBJECTIVE: To illustrate the protective effect of RBM10 on hepatocellular carcinoma (HCC) progression and the molecular mechanism.

PATIENTS AND METHODS: RBM10 levels in HCC tissues classified by tumor size and tumor node metastasis (TNM) staging were detected by quantitative real-time polymerase chain reaction (qRT-PCR) Chi-square test was conducted to reveal the relationship between RBM10 level and pathological features in HCC patients. The prognostic potential of RBM10 in HCC was assessed via the Kaplan-Meier method. Overexpression of RBM10 was achieved by transfection of LV-RBM10 in HepG2 and HCC-LM3 cells. Cell counting kit-8 (CCK-8) assay and flow cytometry were carried out to detect viability and apoptosis in HCC cells, respectively. In addition, invasive ability was assessed by transwell assay. Protein level of cleaved-caspase-3 was examined by Western blot. Regulatory effects of RBM10 on protein levels of EGFR, ERK and p-ERK were determined.

RESULTS: RBM10 was downregulated in HCC tissues. Its level was markedly lower in HCC cases with larger tumor size and stage III+IV. Low level of RBM10 predicted poor prognosis in HCC patients. Overexpression of RBM10 suppressed viability and invasiveness in HCC-LM3 and HepG2 cells, but enhanced apoptotic rate and protein level of cleaved-caspase-3. EGFR was upregulated in HCC tissues, which was negatively regulated by RBM10. Overexpression of RBM10 downregulated protein levels of EGFR and p-ERK in HCC-LM3 and HepG2 cells.

CONCLUSIONS: RBM10 is downregulated in HCC tissues, which is favorable to the prognosis in HCC patients. As a tumor suppressor, RBM10 attenuates proliferative and invasive abilities, but drives apoptosis in HCC cells, thus alleviating the progression of HCC.

Key Words:
RBM10, EGFR, Hepatocellular carcinoma (HCC).

Introduction

Hepatocellular carcinoma (HCC) is a primary malignant tumor in the liver. About 500,000 people are diagnosed as HCC, comprising 75-85% liver tumor cases[1]. Currently, HCC is the third lethal cancer in the world, posing a severe health burden[2]. The complicated development of HCC involves diverse genes, pathways and steps. Although diagnostic and therapeutic strategies have been largely improved, the prognosis in advanced HCC is very poor[3]. The invasiveness and metastasis of HCC are closely linked to the prognosis[4]. It is of significance to clarify specifically expressed genes associated with HCC, and to underly their potential functions in HCC progression. RNA-binding proteins are vital proteins responsible for regulating numbers and functions of genetic products[5]. RBM10 locates on Xp11.23, which is well known for its function in mRNA splicing[6]; RBM10 triggers cell apoptosis and inhibits proliferation[7,8]. Recently, RBM10 has been discovered as a tumor suppressor in lung cancer, osteosarcoma and endometrial cancer[9-11].

EGFR locates on chromosome 7p12, and it is a member of the family of ErbB tyrosine kinase receptors[12]. EGFR and other family members are vital regulators in tumorigenesis through mediating cell growth, cell motion and angiogenesis[13-15]. EGFR-mediated proliferation signaling is an independent factor leading to tumor progression[16]. The relevance of EGFR-related signals has been studied in many cancers, such as gliomas and breast cancer[17,18]. This study aims to explore the role of RBM10 in regulating HCC progression and the potential involvement of the EGFR signaling. Our findings provide a new idea for targeted therapy for HCC.

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Patients and Methods

Specimen Collection

This investigation was approved by the Ethics Committee of Gongli Hospital, Pudong New Area, and all patients signed written informed consent. A total of 46 cases of HCC specimens with complete pathological data and follow-up data were collected. Surgical resected HCC specimens and normal ones were frozen at -80°C for use. Inclusion criteria of HCC patients were: (a) patients were pathologically diagnosed as HCC; (b) HCC patients were treated by primary or curative hepatectomy; (c) patients did not have preoperative chemotherapy or radiotherapy, and they had no extrahepatic metastases; (d) no history of HCC treatment; (e) clinical and follow-up data were complete.

Cell Culture and Transfection

Human HCC cell lines (SMMC-7721, SK-hep1, HCC-LM3 and HepG2) and immortalized normal hepatocytes (L-02) were purchased from China Center For Type Culture Collection (CCTCC, Wuhan, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 5% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) in a humidified incubator with 5% CO_2 at 37°C. Medium was replaced according to the statue of cell growth. Until cells were grown to 80%, they were digested for passage and plating. LV-RBM10 and LV-Ctrl were purchased from HANBIO (Shanghai, China). They were transfected in cells using Lipofectamine 2000 plus 5 μg/mL polybrene (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was examined at 24 h by quantitative real-time polymerase chain reaction (qRT-PCR).

QRT-PCR

Total RNAs were isolated from tissues or blood samples using RNA extraction kit (ABI, Foster City, CA, USA). The concentration and purity of RNA were determined using an ultraviolet spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After reverse transcription, complementary deoxyribose nucleic acids (cDNs) were amplified for qRT-PCR. Relative mRNA level was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences of RBM10 were: 5'-GGGGTGTCCTCTAACATTGG-3' (forward) and 5'-ATGGTCTTGCCGTCGATAGT-3' (reverse); GAPDH were: 5'-CTGGAACGGTGAAGGTGACA-3' (forward) and 5'-AAGGGACTTCCTGTAACAATGCA-3' (reverse).

Cell Counting Kit-8 (CCK-8)

1.0×10^4 cells were implanted in each well of a 6-well plate. At day 0, 1, 2 and 3, 10 μL of CCK-8 solution was added (TaKaRa, Dalian, China). After 1 h culturing in the dark, the optical density at 450 nm was measured using a microplate reader.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) on ice for 30 min. Cell lysate was centrifuged at 4°C, 1000 rpm for 10 min. Extracted protein samples were quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were electrophoresed in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses of grey values were finally conducted.

Flow Cytometry

Cells were washed in pre-cold phosphate-buffered saline (PBS) and resuspended in 1 mL of binding buffer at the density of 1×10^5 /mL. After dual-staining of Annexin V and Propidium Iodide (PI) for 15 min, cell apoptotic rate was determined using flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Transwell Assay

200 μL of serum-free suspension and 500 μL of complete medium were applied to the top and bottom of a transwell chamber, respectively, and cultured for 12 h. Cells in the bottom were subjected to methanol fixation for 15 min, and crystal violet staining for 20 min. Invasive cells were counted in 5 randomly selected fields per sample.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) and GraphPad Prism (Version X; La Jolla, CA, USA). Measurement data were expressed as $x \pm s$. Differences between groups were compared using the Student's *t*-test. The relationship between RBM10 and pathological features in HCC patients was

analyzed by χ^2-test. Kaplan-Meier method was conducted for survival analysis, followed by Log-rank test for comparing survival differences. $p<0.05$ was considered statistically significant.

Results

Downregulation of RBM10 in HCC Specimens

Relative levels of RBM10 were detected in 46 pairs of HCC and normal specimens. As qRT-PCR data revealed, RBM10 was lowly expressed in HCC specimens (Figure 1A). In addition, RBM10 level was markedly lower in HCC patients with larger than 5 cm of tumor size than those with smaller ones (Figure 1B). Stage III-IV HCC patients had lower level of RBM10 than those stage I-II patients (Figure 1C). We further analyzed the relationship between RBM10 level and pathological features in 46 HCC patients. Based on the median level of RBM10, patients were assigned into high level group and low-level group, respectively. Significant differences in tumor size and TNM staging were detected between groups (Table I). Results of univariate and multivariate analysis of clinicopathological parameters related to overall survival in HCC patients were shown in Table II. It is indicated that RBM10 could affect the tumor size and TNM staging in HCC. To determine the possible influence of RBM10 on the survival in HCC patients, Kaplan-Meier curves were plotted. It is shown that HCC patients with low level of RBM10 had poor prognosis (HR = 0.4282, p=0.0141) (Figure 1D). Collectively, RBM10 may be a tumor suppressor in HCC progression.

Overexpression of RBM10 Suppressed HCC Proliferation

We detected RBM10 levels in HCC cell lines and normal hepatocytes by qRT-PCR. It is shown that RBM10 was markedly downregulated in HCC cell lines (Figure 2A). HCC-LM3 and HepG2 cells expressed the lowest level of RBM10 compared to other tested HCC cell lines, and they were used for generating RBM10 overexpression models by transfection of LV-RBM10 (Figure 2B). CCK-8 assay showed decreased viability in HCC-LM3 and HepG2 cells overexpressing RBM10, suggesting the inhibited proliferative ability (Figure 2C).

Overexpression of RBM10 Induced Apoptosis and Suppressed Invasiveness in HCC

Flow cytometry demonstrated that overexpression of RBM10 enhanced apoptotic rate in HCC-LM3 and HepG2 cells (Figure 3A). Consistently, protein level of cleaved-caspase-3

Table I. Correlation analysis between RBM10 expression and clinicopathological parameters of HCC patients.

Clinicopathologic features	No. of cases	RBM10 expression		*p*-value
		Low (n=23)	High (n=23)	
Age (years)				0.767
≤60	21	11	10	
>60	25	12	13	
Gender				0.369
Male	19	8	11	
Female	27	15	12	
Tumor size				0.036*
≤5 cm	19	6	13	
>5 cm	27	17	10	
TNM stage				0.035*
I-II	22	7	15	
III-IV	24	16	8	
Vascular invasion				0.116
Negative	15	5	10	
Positive	31	18	13	
Histological classification				
Low grade	19	10	9	0.765
Medium and High grade	27	13	14	

HBV: hepatitis B virus.

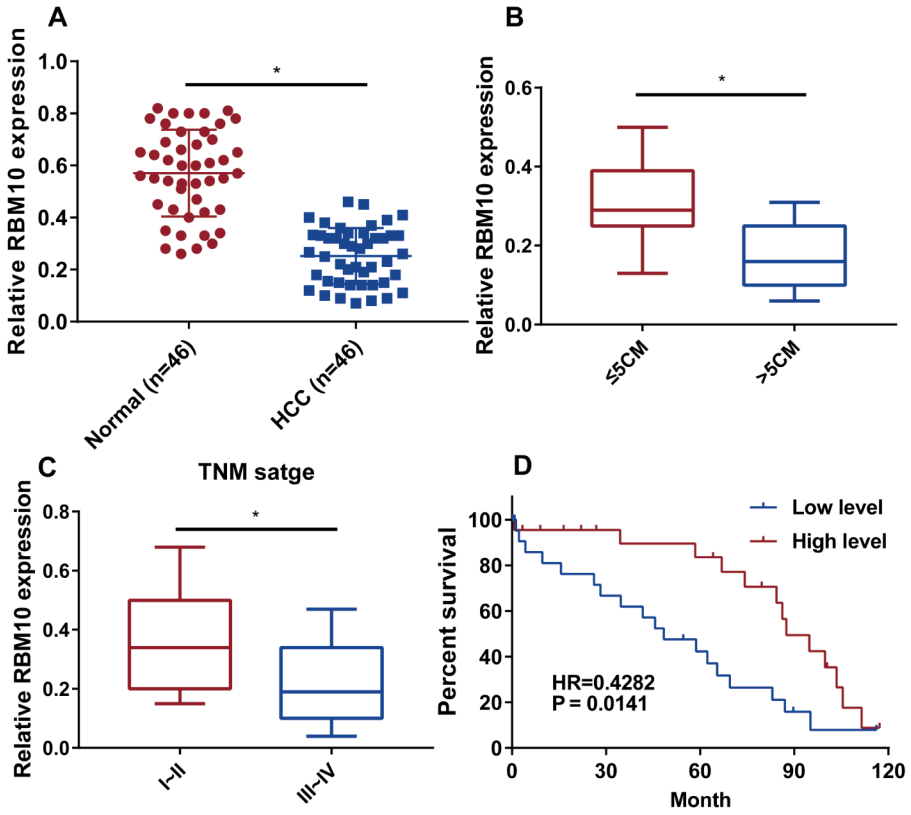

Figure 1. Downregulation of RBM10 in HCC specimens. **A,** RBM10 expressions in HCC specimens (n=46) and normal ones (n=46); **B,** RBM10 expressions in HCC specimens with large tumor size (≤5 cm) or small ones (>5 cm); **C,** RBM10 expressions in stage I~II and stage III~IV HCC cases; **D,** Overall survival in HCC patients based on RBM10 levels.

Table II. Univariate and multivariate analysis of clinicopathological parameters related to overall survival in HCC patients..

	Univariate		Multivariate	
Variables	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Age (≤60/>60)	1.258 (0.678, 2.112)	0.367	1.112 (0.701, 2.012)	0.342
Gender (Female/Male)	0.769 (0.345, 2.001)	0.678	0.698 (0.256, 2.342)	0.603
AFP (≤400/>400)	3.889 (2.497, 6.691)	<0.001	3.234 (1.423, 5.921)	<0.001
HBV (Absent/Present)	0.897 (0.445, 1.567)	0.701	0.821 (0.521, 1.672)	0.687
Tumor Size (>5 cm/≤5 cm)	1.886 (1.112, 3.321)	0.034*	1.623 (0.891, 2.121)	0.125
Vascular invasion (Positive/Negative)	2.678 (1.345, 3.980)	<0.001	2.123 (0.789, 4.012)	0.231
Tumor differentiation (Moderate-Well/Poor)	0.385 (0.198, 0.702)	0.003*	0.462 (0.232, 0.887)	0.012*
CDCA2 expression (High/Low)	3.342 (1.784,7.892)	<0.001	3.541 (2.034, 8.765)	<0.001

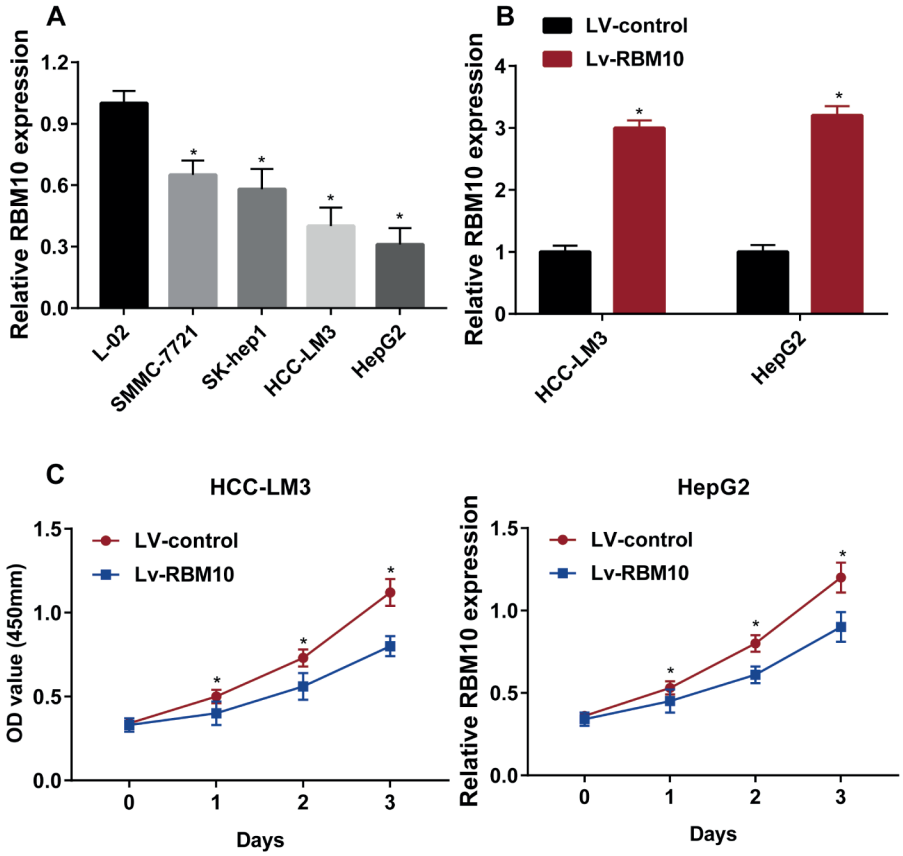

Figure 2. Overexpression of RBM10 suppressed HCC proliferation. **A,** RBM10 expressions in HCC cell lines; **B,** Transfection efficacy of LV-RBM10 in HCC-LM3 and HepG2 cells; **C,** Viability in HCC-LM3 and HepG2 cells overexpressing RBM10.

was upregulated in HCC cells overexpressing RBM10 (Figure 3B). It is indicated that overexpressed RBM10 triggered apoptosis in HCC. In addition, transwell assay revealed a decline in invasive ability after overexpression of RBM10 in HCC cells (Figure 3C).

RBM10 Suppressed Proliferative Ability in HCC by Activating the EGFR Signaling

To further explore the possible mechanism of RBM10 on affecting HCC cell functions, we examined the *in vitro* activation of the EGFR signaling. It is detected that EGFR was upregulated in HCC tissues than normal ones (Figure 4A). EGFR was downregulated in HCC cells overexpressing RBM10 (Figure 4B). As Western blot analysis uncovered, protein levels of EGFR and p-ERK were downregulated by overexpression of RBM10, while ERK level was not influenced by RBM10 regulation (Figure 4C). The above data have confirmed that RBM10 inactivated the EGFR signaling in HCC cells.

Discussion

HCC is considered as the most common primary liver tumor in the world, which is usually deteriorated from chronic inflammation and regenerative necrosis[19]. Overactivation of oncogenes and mutations/deficiency of tumor suppressors are important during the carcinogenesis of HCC[20]. Searching biomarkers for tumor progression is conductive to understand the pathogenesis of HCC and to develop therapeutic targets.

RBPs have been proven to be vital during tumor progression[21]. Yang et al[22] discovered that ectopically expressed RBM5 contributes to inhibiting growth and invasiveness of prostate cancer cells LNCap. Wang et al[23] pointed out that RBM6 is a tumor suppressor responsible for alleviating malignant growth of laryngeal cancer. RBM10 is a family member of RBPs, which was initially confirmed in 1995[24]. It is mutant in certain types of tumors, including pancreatic cancer, breast cancer, colorectal carcinoma and melanoma[25-28].

Our results uncovered that RBM10 was lowly expressed in HCC tissues and cell lines. Its level was especially lower in HCC cases with a larger tumor size and stage III~IV cases. In addition, lowly expressed RBM10 was unfavorable to the prognosis in HCC patients.

The role of RBM10 in blocking cell cycle progression and inducing apoptosis has been previously reported[29-34]. Han et al[35] indicated that overexpression of RBM10 stimulates apoptosis in the osteosarcoma cell line U2OS. Decreased proliferative ability and increased apoptosis in hypertrophic primary chon-

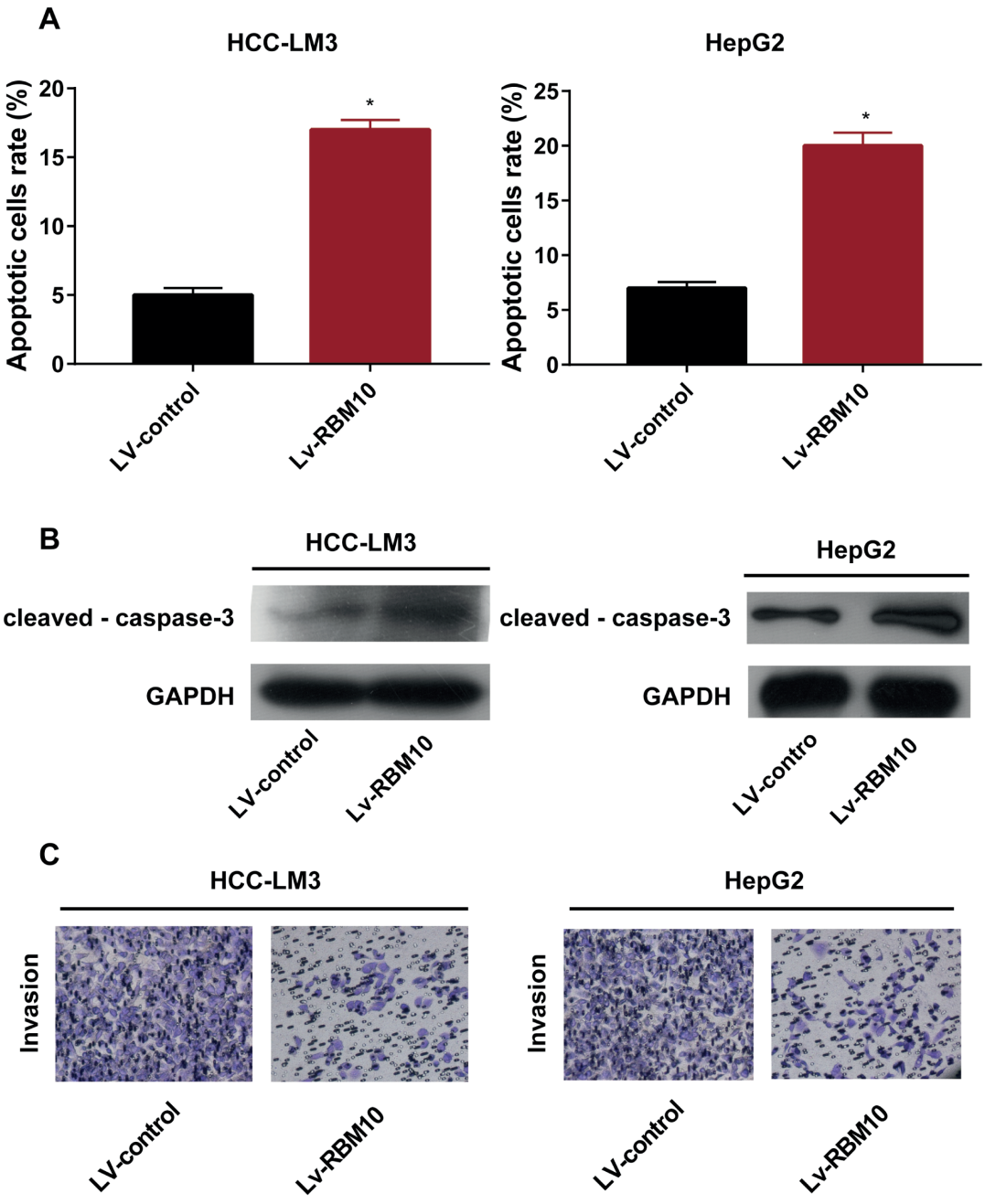

Figure 3. Overexpression of RBM10 induced apoptosis and suppressed invasiveness in HCC. **A,** Apoptotic rate in HCC-LM3 and HepG2 cells overexpressing RBM10; **B,** Protein level of cleaved-caspase-3 in HCC-LM3 and HepG2 cells overexpressing RBM10; **C,** Invasion in HCC-LM3 and HepG2 cells overexpressing RBM10 (magnification: 40×).

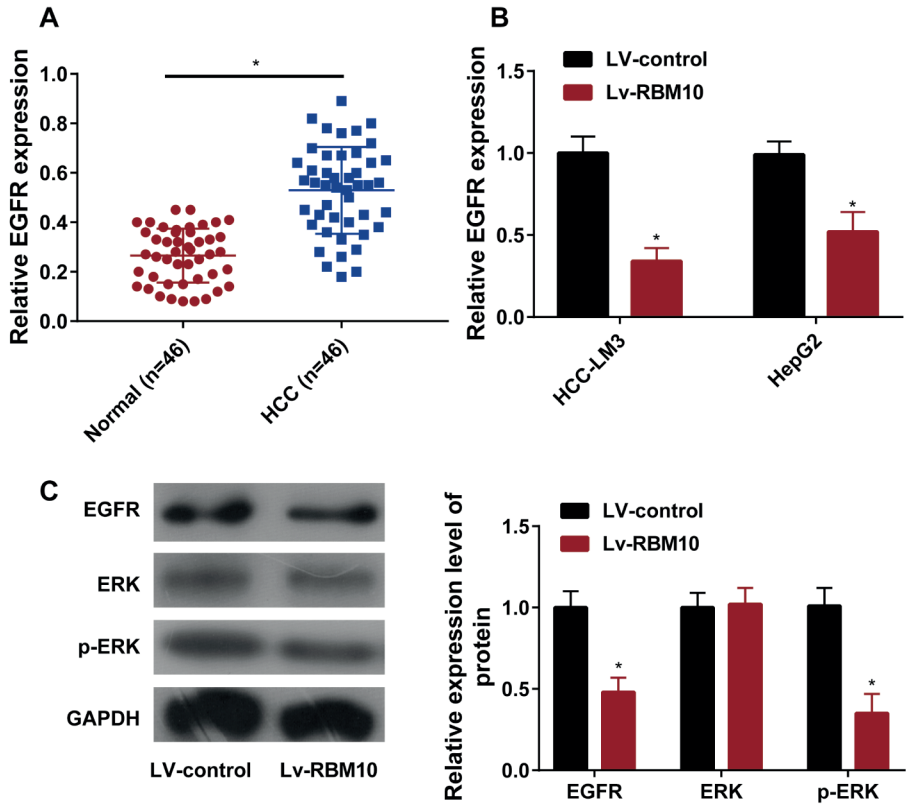

Figure 4. RBM10 suppressed proliferative ability in HCC by activating the EGFR signaling. **A,** EGFR expressions in HCC specimens (n=46) and normal ones (n=46); **B,** EGFR expressions in HCC-LM3 and HepG2 cells overexpressing RBM10; **C,** Protein levels of EGFR, ERK and p-ERK in HCC-LM3 and HepG2 cells overexpressing RBM10.

drocytes are closely linked to the overexpression of RBM10[36]. Caspase-3 is an executive factor for initiating apoptosis[37]. Under the normal circumstance, Caspase-3 normally exists in the form of zymogen, which is activated to the cleaved-caspase-3 during the process of apoptosis[38]. In this paper, overexpression of RBM10 enhanced apoptotic rate and upregulated cleaved-caspase-3 in HCC-LM3 and HepG2 cells, and inhibited invasiveness. EGFR has tyrosine kinase activity and is a transmembrane glycoprotein. EGFR and its family members are involved in carcinogenesis by driving malignant phenotypes of cells[39,40]. The EGFR signaling is thought to be related to tumor formation and development[41]. In triple negative breast cancer, upregulated EGFR triggers cancer cell growth *via* inducing phosphorylation of PKM2[42]. Identically, EGFR was upregulated in HCC tissues we collected, which was negatively regulated by RBM10. Interestingly, overexpression of RBM10 remarkably inactivated the EGFR signaling in HCC cells. We suggested that RBM10 served as a tumor suppressor in HCC progression through regulating the EGFR signaling. This study for the first time explored the vital function of RBM10 in HCC progression. Serving as a tumor suppressor gene, RBM10 is able to alleviate the malignant progression of HCC, which can be utilized as a novel prognostic biomarker and therapeutic target.

Conclusions

Summarily, RBM10 is downregulated in HCC tissues, which is favorable to the prognosis in HCC patients. As a tumor suppressor, RBM10 attenuates proliferative and invasive abilities, but drives apoptosis in HCC cells, thus alleviating the progression of HCC. It can be utilized as a potential target for clinical treatment of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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