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TRIM56 suppresses the malignant development of hepatocellular carcinoma *via* targeting RBM24 and inactivating the Wnt signaling

Y. YANG[1], F.-F. MAO[2], L. GUO[1], W.-X. GUO[1]

[1]Department of Hepatic Surgery VI, Third Affiliated Hospital of Second Military Medical University, Shanghai, China
[2]Department of Tongji University Cancer Center, Shanghai Tenth People's Hospital, School of Medicine, Tongji University, Shanghai, China

Yang Yang and Feifei Mao contributed equally to this work

Abstract. – **OBJECTIVE:** The aim of this study was to explore the expression pattern of TRIM56 in Hepatocellular carcinoma (HCC) patients and its influence on the prognosis, and to illustrate the molecular mechanisms of TRIM56 in regulating HCC cell behaviors.

PATIENTS AND METHODS: TRIM56 levels in HCC specimens and paracancerous specimens were detected. Then, the influences of TRIM56 on clinical data and prognosis in HCC patients were assessed. Next, the regulatory effects of TRIM56 on proliferative potential in Huh7 and Bel-7402 cells were determined, and the role of TRIM56 on the Wnt signaling was examined. Finally, biological characteristics between TRIM56 and RBM24 in HCC development were illustrated by Luciferase assay and rescue experiments.

RESULTS: TRIM56 was lowly expressed in HCC tissues and cell lines. HCC patients expressing a low level of TRIM56 suffered advanced T stage and poor survival. Besides, overexpression of TRIM56 inhibited proliferative potential of Huh7 cells, while knockdown of TRIM56 in Bel-7402 yielded the opposite result. TRIM56 was able to negatively regulate key genes in the Wnt signaling. In addition, RBM24 was proven to be the downstream target of TRIM56, which was involved in TRIM56-influenced HCC development.

CONCLUSIONS: Downregulated TRIM56 in HCC samples is closely linked to pathological staging and prognosis. TRIM56 alleviates the malignant development of HCC by inactivating the Wnt signaling and targeting RBM24.

Key Words:
TRIM56, Wnt/β-catenin signaling, Hepatocellular carcinoma (HCC), Malignant development.

Introduction

Hepatocellular carcinoma (HCC) is the fifth prevalent cancer, and it ranks third in cancer-related death globally[1-3]. Therapeutic efficacy of conventional treatment in HCC is unsatisfying, and affected patients are prone to developing radiotherapy resistance[4,5]. The pathogenesis of HCC is complicated, involving imbalanced immunity, abnormally activated signaling and genetic variations[6,7]. It is generally considered that chronic inflammation is the major reason for HCC. Over 80% HCC cases are deteriorated from chronic HBV or HCV infection[8,9]. Alcohol abuse, chemical carcinogens, aflatoxins, insulin resistance, and obesity-induced liver damage and chronic inflammation are all risk factors for HCC[10]. Molecular mechanisms of HCC require to be comprehensively clarified[11,12].

TRIM (tripartite motif) contains three conventional structural domains, including a ring domain in the N-terminal, one or two B-box domains and a coiled-coil domain in the C-terminal[13,14]. Members in the TRIM family are involved in the regulation of malignant phenotypes of cancer cells[15,16]. Previous studies[15-17] have reported the role of TRIM56 in mediating cancer development and its influence on cancer prognosis. The Wnt signaling is identified to be closely associated with the occurrence and progression of HCC. β-catenin mutations occur in human and mouse HCC[18,19]. The interaction between TRIM56 and the Wnt signaling in HCC remains unclear, which is explored in this paper in detail.

Corresponding Author: Weixing Guo, MD; e-mail: yinchzh@shanghaitech.edu.cn

Patients and Methods

HCC Samples

A total of 41 paired HCC and paracancerous tissues were surgically resected and stored at -80°C. Clinical data and follow-up data of included HCC patients were recorded. None of enrolled subjects were preoperatively treated with anti-tumor therapy. In addition, tumor staging was assessed based on the guideline proposed by the Union for International Cancer Control (UICC). This investigation was approved by the Ethics Committee of Third Affiliated Hospital of Second Military Medical University and conducted after informed consent of each subject.

Cell Lines and Reagents

HCC cell lines (Bel-7402, HepG2, MHCC88H, Bel-7402, Huh7, Hep3B) and a normal hepatocyte cell line (LO2) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 µg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted when cells were grown to 80-90% confluence.

Transfection

Cells were cultured to 30-40% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, cells were collected for the following use.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were pre-inoculated in a 6-well plate (200 cells/well) and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Next, visible colonies were washed in phosphate-buffered saline (PBS), fixed in methanol for 20 min and dyed in 0.1% crystal violet (Solarbio, Beijing, China) for 20 min and were captured and calculated at last.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were pre-inoculated in a 24-well plate (2×10^4 cells/well). They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100, and 30-min reaction in 400 µL of 1×ApollorR. Afterwards, cells were dyed in 4',6-diamidino-2-phenylindole (DAPI) for another 30 min. EdU-positive cells and DAPI-labeled nuclei were captured (Sigma-Aldrich, St. Louis, MO, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNAs extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were listed below: TRIM56: forward: 5'-GCTCTGGCTAGTTCTCACAGG-3' and reverse: 5'-CTTGGGCCACTTGCTCTTGA-3', RBM24: forward: 5'-CTGGATGCCGGTTGTTAAGC-3' and reverse: 5'-GCCATGGTGACCTTTGCAGT-3', and GAPDH: forward: 5'-CCTGGCACCCAGCACAAT-3' and reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western Blot

Cells were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours, and membranes were reacted with primary and secondary antibodies for indicated time. Finally, band exposure and analyses were finally conducted.

Luciferase Assay

HEK293T cells were pre-inoculated in a 24-well plate and co-transfected with NC/pcDNA3.1-RBM24 and TRIM56-WT/TRIM56-MUT, respectively. After 48 h cell culture, they were lysed for measuring Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 (V5.01) (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Chi-square test was conducted for analyzing the relationship between TRIM56 level and clinical data of HCC patients. Besides, Pearson correlation test was applied for evaluating the relationship between relative levels of TRIM56 and RBM24 in HCC tissues. Kaplan-Meier curves were depicted for survival analysis in HCC patients. $p<0.05$ was considered as statistically significant.

Results

Expression Pattern of TRIM56 In HCC

As qRT-PCR data revealed, TRIM56 was downregulated in HCC tissues and cell lines than those in controls (Figure 1A, 1B). In the 6 tested HCC cell lines, Huh7 and Bel-7402 cells expressed the pronounced differential level of TRIM56, and they were used in the following experiments.

According to the calculated median level of TRIM56 in HCC tissues collected, HCC patients were classified into high or low TRIM56 expression group, respectively. By analyzing their clinical data, it was found that TRIM56 was correlated with T stage in HCC patients (Table I). Moreover, Kaplan-Meier curves revealed that lowly expressed TRIM56 was unfavorable to progression-free survival (Figure 1C) and overall survival in HCC (Figure 1D).

TRIM56 Regulated Proliferative Potential In HCC

TRIM56 overexpression and knockdown models were established in Huh7 and Bel-7402 cells, respectively (Figure 1E). Overexpression of TRIM56 in Huh7 cells markedly decreased viability (Figure 2A), colony number (Figure 2B) and EdU-positive rate (Figure 2C). On the contrary, knockdown of TRIM56 in Bel-7402 yielded the opposite results. It was suggested that TRIM56 was able to suppress proliferative potential in HCC.

TRIM56 Regulated the Wnt Signaling In HCC

To explore the interaction between TRIM56 and the Wnt signaling, the protein levels of vital genes in the Wnt signaling were determined. Overexpression of TRIM56 downregulated β-catenin, c-Myc, RBM24, MMP-9 and cyclin D1, and upregulated GSK-3β in Huh7 cells. Transfection of sh-TRIM56 in Bel-7402 obtained the opposite trends about the expression changes of these genes (Figure 3).

Interaction Between TRIM56 and RBM24

Binding sequences in the 3'UTR of TRIM56 and RBM24 were predicted online (Figure 4A). Furthermore, overexpression of RBM24 was

Table I. Association of TRIM56 expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	No. of cases	TRIM56 expression		*p*-value
		High	Low	
Age (years)				0.567
<60	18	11	7	
≥60	23	12	11	
Gender				0.350
Male	17	11	6	
Female	24	12	12	
T stage				0.017
T1-T2	20	15	5	
T3-T4	21	8	13	
Lymph node metastasis				0.529
No	25	15	10	
Yes	16	8	8	
Distance metastasis				0.529
No	25	15	10	
Yes	16	8	8	

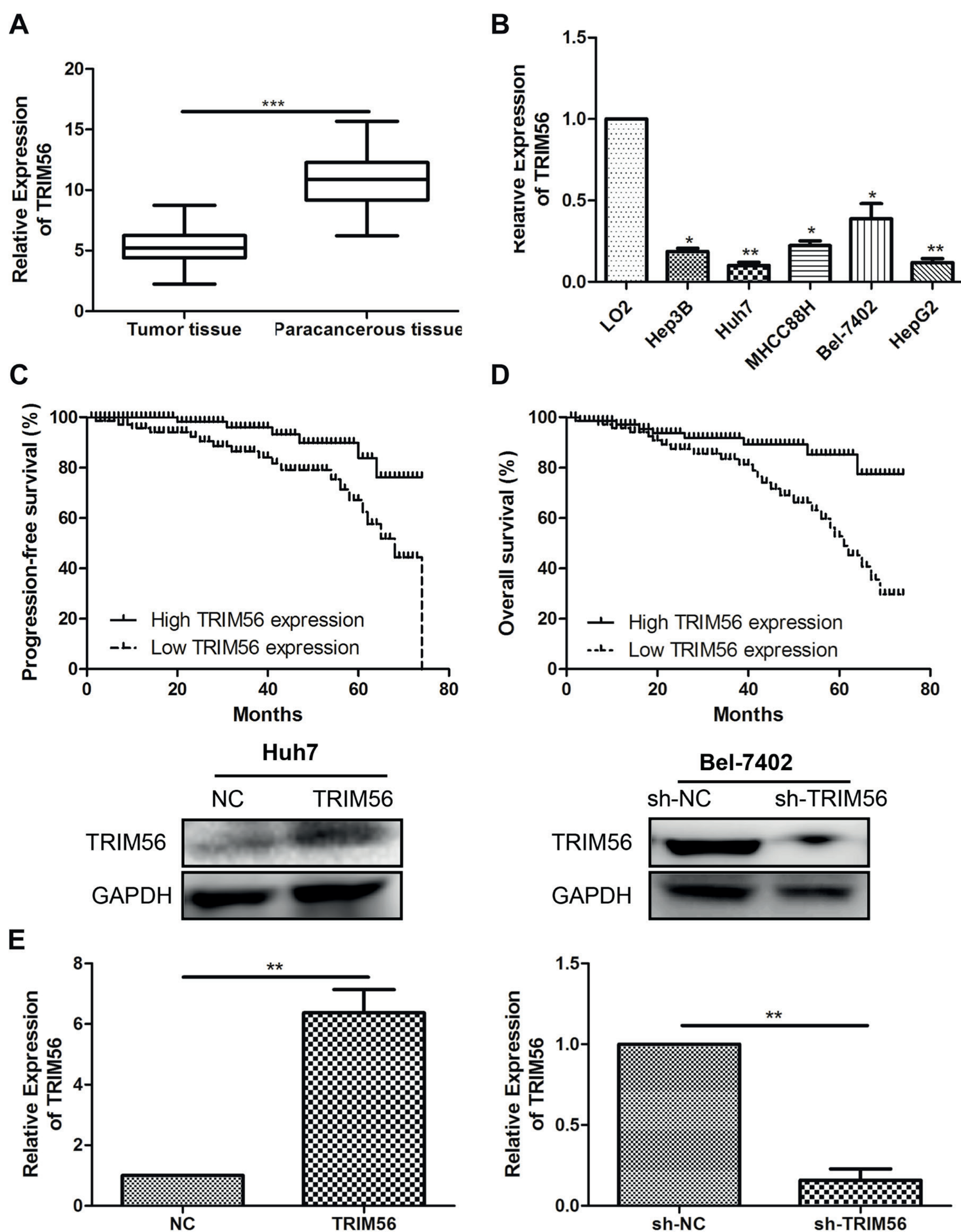

Figure 1. Expression pattern of TRIM56 in HCC. **A,** TRIM56 levels in HCC and paracancerous tissues. **B,** TRIM56 levels in HCC cell lines. **C-D,** Progression-free survival (C) and overall survival (D) in HCC patients depending on their TRIM56 levels. **E,** Transfection efficacy of pcDNA3.1-TRIM56 and sh-TRIM56 in Huh7 and Bel-7402 cells, respectively. Data were expressed as mean±SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$..

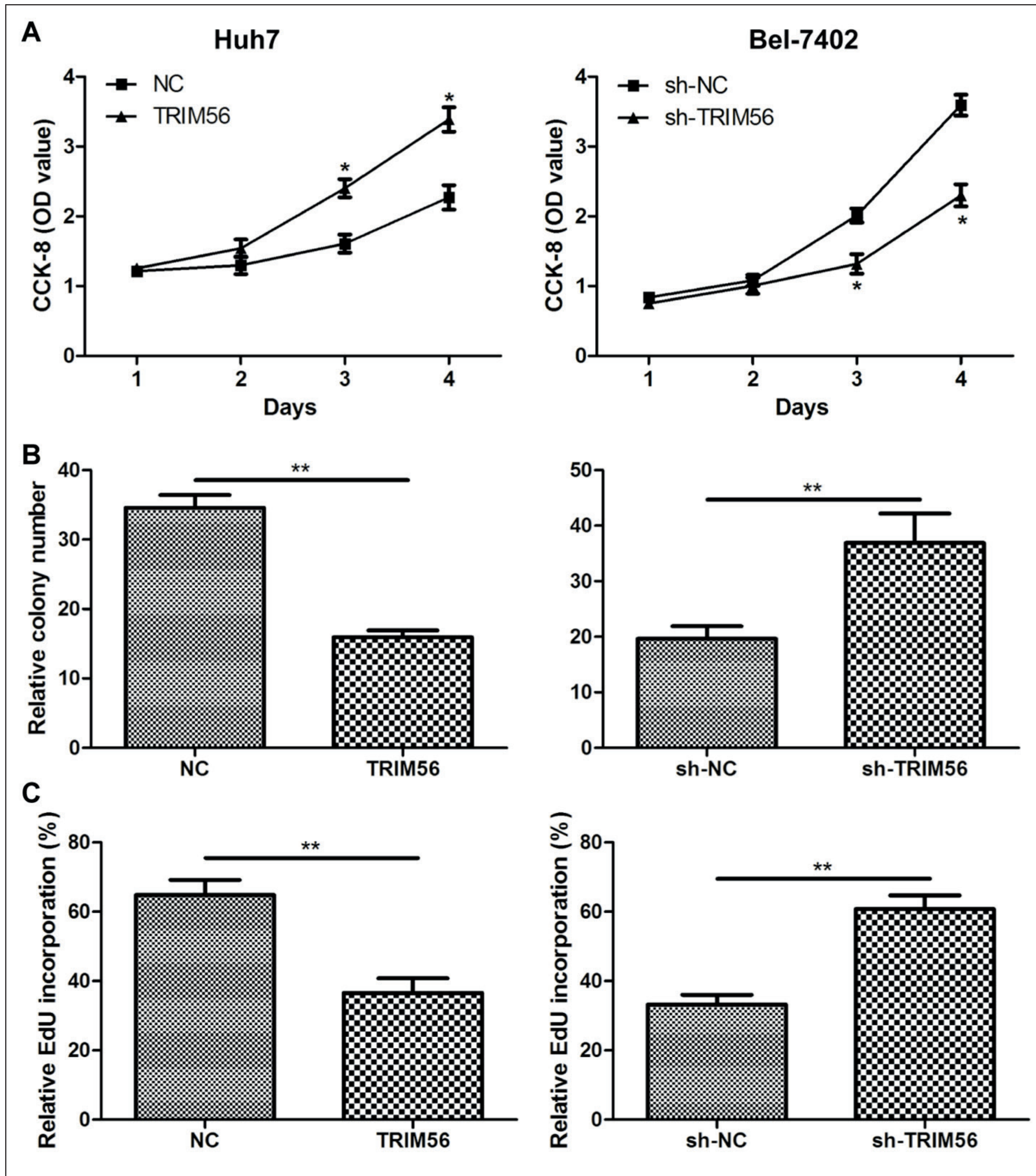

Figure 2. TRIM56 regulated proliferative potential in HCC. **A,** Viability in Huh7 and Bel-7402 cells influenced by TRIM56. **B,** Colony number in Huh7 and Bel-7402 cells influenced by TRIM56. **C,** EdU-positive rate in Huh7 and Bel-7402 cells influenced by TRIM56. Data were expressed as mean±SD. $^{*}p<0.05$, $^{**}p<0.01$.

able to decrease luciferase activity in wild-type TRIM56 vector, confirming that RBM24 was the downstream target of TRIM56 (Figure 4B). Contrary to TRIM56, RBM24 was highly expressed in HCC cell lines and tissues (Figure 4C, 4D). A negative correlation was identified between relative levels of TRIM56 and RBM24 in HCC samples (Figure 4E).

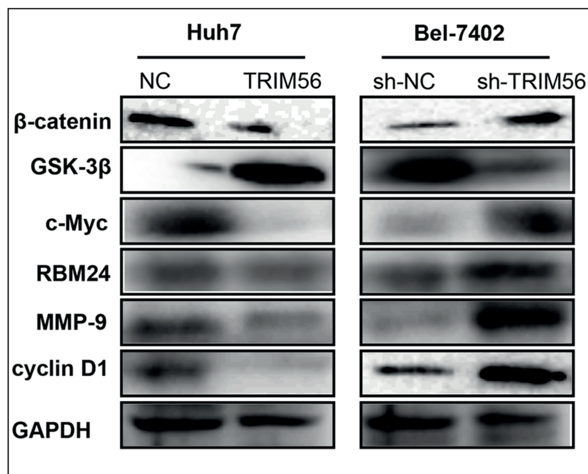

Figure 3. TRIM56 regulated the Wnt signaling in HCC. Protein levels of β-catenin, GSK-3β, c-Myc, RBM24, MMP-9 and cyclin D1 in Huh7 and Bel-7402 cells influenced by TRIM56.

RBM24 Reversed the Influence of TRIM56 on Proliferative Potential In HCC

The involvement of RBM24 in the malignant development of HCC was explored. Transfection efficacy of pcDNA3.1-RBM24 and si-RBM24 was tested in Huh7 and Bel-7402 cells, respectively (Figure 5A). Compared with those overexpressing TRIM56, Huh7 cells co-overexpressing TRIM56 and RBM24 displayed higher viability (Figure 5B) and a large colony number (Figure 5C). In addition, lower viability and a small colony number were seen in HCC cells with co-silence of TRIM56 and RBM24 than those with solely TRIM56 knockdown (Figure 5B, 5C).

Discussion

Various factors should be considered in HCC treatment, especially tumor size and pathological staging[1-3]. A great number of HCC patients are diagnosed until advanced stage, leading to a poor prognosis[4-6]. Surgical resection or liver transplantation is the best therapeutic approach for HCC. Nevertheless, only 10-20% HCC cells can be cleared by surgery. Once HCC cells cannot be completely cleared, it is difficult for

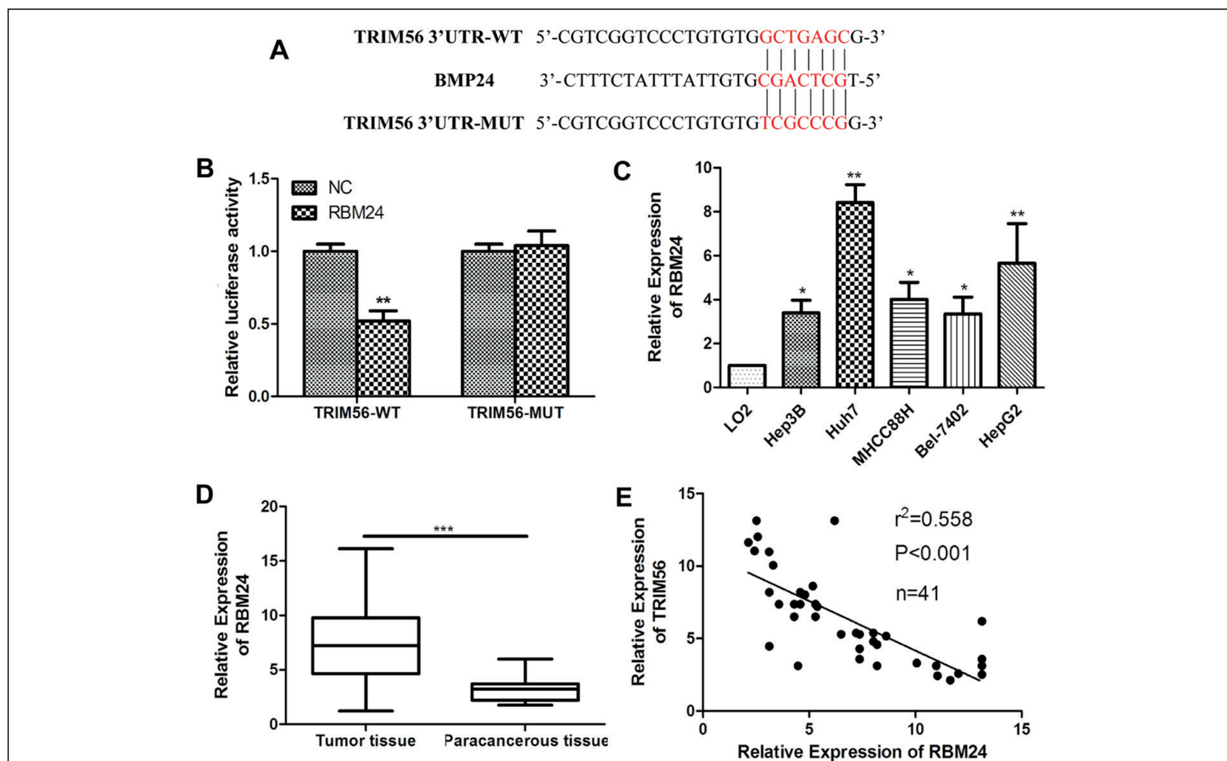

Figure 4. Interaction between TRIM56 and RBM24. **A,** Binding sequences in the 3'UTR of TRIM56 and RBM24. **B,** Luciferase activity in cells co-transfected with NC/pcDNA3.1-RBM24 and TRIM56-WT/TRIM56-MUT, respectively. **C,** RBM24 levels in HCC cell lines. **D,** RBM24 levels in HCC and paracancerous tissues. **E,** A negative correlation between relative levels of TRIM56 and RBM24 in HCC tissues. Data were expressed as mean±SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$..

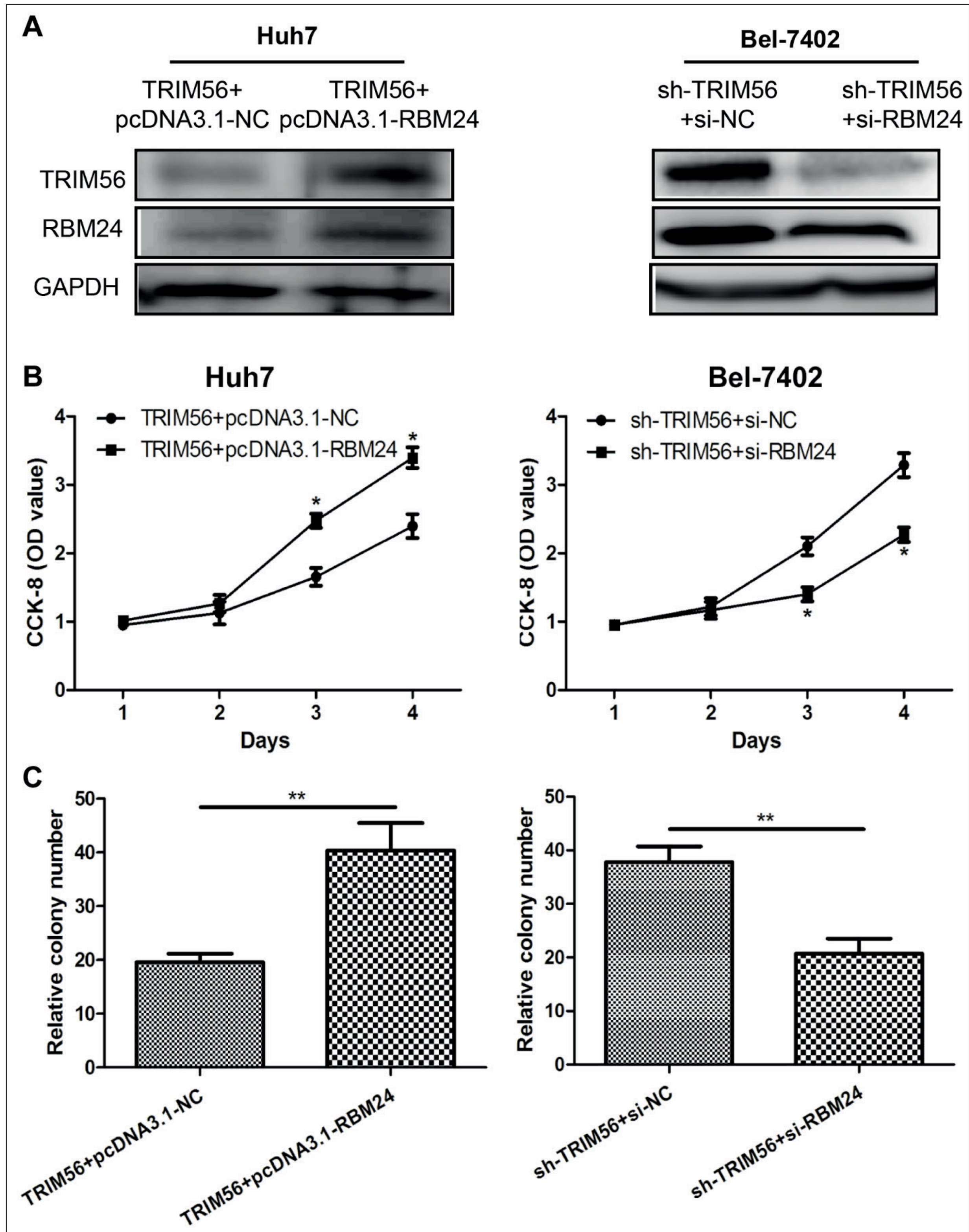

Figure 5. RBM24 reversed the influence of TRIM56 on proliferative potential in HCC. **A,** Protein levels of TRIM56 and RBM24 in Huh7 and Bel-7402 cells influenced by TRIM56 and RBM24. **B,** Viability in Huh7 and Bel-7402 cells influenced by TRIM56 and RBM24. **C,** Colony number in Huh7 and Bel-7402 cells influenced by TRIM56 and RBM24. Data were expressed as mean±SD. $^{*}p<0.05$, $^{**}p<0.01$.

patients to survive longer than 6 months[7-9]. Recently, multi-kinase inhibitory drugs have been developed to effectively prolong the survival in HCC patients, proving the feasibility of molecular treatment for HCC[10-12].

Abnormally expressed TRIM56 has been discovered in different types of tumors[20,21]. Here, it was found that TRIM56 was downregulated in HCC tissues and cell lines, suggesting that TRIM56 may contribute to alleviate the malignant development of HCC. By analyzing clinical data and follow-up data of included HCC patients, it was found that lowly expressed TRIM56 indicated advanced T stage and poor prognosis. *In vitro* evidence has shown that TRIM56 suppressed proliferative potential in HCC cells.

As a classical cancer-associated pathway, dysfunctional Wnt signaling results in uncontrolled cell proliferation and thus leads to carcinogenesis[18,19]. β-catenin is the key component of the Wnt signaling[19]. Deficiency of the Wnt signaling causes phosphorylation at different sites on the β-catenin domain, which is regulated by multiple proteins (i.e., APC, Axin, GSK-3β and CK1). As a result, cytoplasmic level of β-catenin remains relatively low[22,23]. The appearance of nuclear β-catenin is a sign of activation of cancer-associated pathways, and it exerts a vital role in cancer cell behaviors[24]. The findings of this study depicted that TRIM56 was able to inactivate the Wnt signaling. Furthermore, the downstream target of TRIM56 was searched. RBM24 was verified as the target of TRIM56, and the two exerted a negative correlation between each other.

The findings of this study revealed that TRIM56 was down regulated in HCC tissues and cell lines, and a lower level of TRIM56 predicted more advanced tumor grading of HCC patients. *In vitro* experiments verified that knockdown of TRIM56 promoted the proliferative ability of HCC cells, while overexpression of TRIM56 obtained the opposite result. Therefore, the objective of this study was firstly to elucidate the anti-oncogenic role of TRIM56 in the progression of HCC, as well as the specific mechanism of TRIM56 regulating RBM24. Notably, *in vitro* experiments showed that RBM24 was able to reverse the regulatory effects of TRIM56 on proliferative potential in HCC. As a result, the carcinostasis of TRIM56 on proliferative ability by RBM24 was verified in HCC cells.

Conclusions

In brief, downregulated TRIM56 in HCC samples is closely linked to pathological staging and prognosis. TRIM56 alleviates the malignant development of HCC by inactivating the Wnt signaling and targeting RBM24.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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