European Review for Medical and Pharmacological Sciences 2019; 23: 2003-2012

TRIM66 promotes malignant progression of hepatocellular carcinoma by inhibiting E-cadherin expression through the EMT pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the regulatory role of TRIM66 in the development of hepatocellular carcinoma (HCC), and to investigate its underlying mechanism.

PATIENTS AND METHODS: A total of 88 pairs of HCC tissues and para-cancerous tissues were surgically resected. The expression of TRIM66 was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between TRIM66 expression and clinic-pathologic characteristics of HCC patients was analyzed. Follow-up data of enrolled HCC patients were collected for survival analysis. Subsequently, TRIM66 expression in HCC cells was determined by qRT-PCR as well. By constructing si-TRIM66, the biological performances of transfected HCC cells were determined using cell counting kit-8 (CCK-8), colony formation and transwell assay. Western blot was performed to measure the protein expressions of relative genes in epithelial-mesenchymal transition (EMT) pathway. Finally, HCC cells were co-transfected with si-TRIM66 and pcDNA-E-cadherin, followed by detection of invasive and migratory abilities.

RESULTS: TRIM66 was highly expressed in HCC tissues compared with that of para-cancerous tissues. High expression of TRIM66 was positively correlated with tumor stage, lymph node metastasis and distant metastasis, whereas not correlated with age and sex of HCC patients. Kaplan-Meier curves revealed that a higher expression of TRIM66 was associated with worse prognosis of HCC. Similarly, TRIM66 was also highly expressed in HCC cells. The knockdown of TRIM66 in HCC cells significantly inhibited the proliferative, invasive and migratory abilities of transfected cells. However, TRIM66 down-regulation significantly induced cell apoptosis. Western blot results showed that TRIM66 knockdown in HCC cells markedly downregulated the protein expressions of E-cadherin, N-cadherin, Vimentin and β-catenin. The inhibited migration and invasion of HCC cells resulted from TRIM66 knockdown were partially reversed by E-cadherin overexpression.

CONCLUSIONS: TRIM66 is highly expressed in HCC, which is positively correlated with tumor stage, lymph node metastasis and distant metastasis of HCC patients. In addition, TRIM66 promotes the malignant progression of HCC by inhibiting E-cadherin through the EMT pathway.

Key Words:
TRIM66, E-cadherin, HCC, Malignancy.

Introduction

Primary liver cancer is a serious disease with high incidence and poor prognosis[1]. Globally, it is estimated that the annual incidence of liver cancer accounts for 4.0% of all malignant tumors. The incidence of liver cancer is still on the rise[2]. In China, the number of liver cancer deaths every year accounts for 45% of the total number in the world. According to relevant data, the mortality rate of liver cancer in China has sharply increased in some cities and rural areas[3,4]. The incidence of liver cancer is a multi-factor, multi-stage and complex process[5]. Histological features of early-stage liver cancer have been clearly elucidated. Meanwhile, the histological origin of liver cancer is relatively simple. Hepatocellular carcinoma (HCC) is the most common histological type of liver cancer[5,6]. The differentiation degree of HCC progressively deteriorates[7,8]. Due to the rapid growth, strong invasiveness, high

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malignancy degree and easy recurrence, HCC has become a refractory malignant tumor[9,10]. Over the past 20 years, surgical and non-surgical treatments for HCC have rapidly advanced. Unfortunately, many HCC patients lose the best surgical opportunity since they are already in the advanced stage with metastasis when first diagnosed. It is reported that the 5-year survival of large HCC and small HCC is 34.6% and 62.9%, respectively[10,11]. Prevention and early diagnosis of HCC are important in improving the clinical outcomes of HCC patients. Therefore, exploring the pathogenesis of HCC and finding new molecular targets for HCC treatment are urgent problems to be solved[12].

There are more than 100 members in the tripartite-motif protein (TRIM) family, of which 70 TRIM proteins have been identified in the human genome[13]. TRIM has three domains, including one zinc finger domain (RING finger), one or two B-box domains, and one coiled-coil (CO) domain from N-terminus to C-terminus. The above three domains are collectively named as RBCC domain for short[13-15]. The C-terminus of TRIM is a variable region, which is a typical PRY/SPRY domain interacting with target proteins. The RING domain has E3 ubiquitin ligase activity. Meanwhile, it acts on target genes by interacting with the related E2 ubiquitin-binding enzyme, thus mediating the ubiquitination of itself and different substrates[15]. The B-box domain is a zinc ion-binding domain unique to TRIM protein, which mainly contains a cysteine-histidine-zinc finger motif[16]. The CO domain is located behind the B-box domain, and is mainly entangled by a plurality of a-helices. The main function of CO domain is to promote homologous or heterologous oligomerization between TRIM protein and TRIM protein or others. This may eventually stimulate the production of macromolecular polymers and formation of protein subcellular localization[16]. The TRIM protein family was originally discovered to exert antiviral functions by interfering with the viral replication process[17]. In addition, proteins in the TRIM family act as pattern recognition receptors in the process of innate immune signaling. The proteins are also capable of recognizing capsid protein of the virus and regulating pathways in the natural immune process[18]. In recent years, several studies have shown that members of the TRIM protein family are involved in tumor development and progression[14-16]. In this study, we aim to explore the clinical significance of TRIM66 in HCC.

Epithelial-mesenchymal transition (EMT) is a process of transformation from epithelial cells to mesenchymal cells under normal physiological and specific pathological conditions. Existing theory suggests that EMT may regulate the malignant behaviors of tumors[19,20]. E-cadherin and others are key transcription factors regulating the EMT processes[21,22]. Based on the above characteristics, this study aims to verify whether E-cadherin may become a serological marker for early diagnosis of HCC. Bioinformatics predicted that E-cadherin may be one of the target genes of TRIM66. However, whether TRIM66 participates in the proliferation and apoptosis of HCC cells through targeted regulation of E-cadherin remains unknown.

In this study, we analyzed the expression of TRIM66 in 88 pairs of HCC tissues and para-cancerous tissues. The effects of TRIM66 on the biological function of HCC cells were also explored. Previous studies have indicated that TRIM66 can promote the division and metastasis of tumor cells, thus controlling tumor development. Our study may provide references for improving the therapeutic efficacy of HCC.

Patients and Methods

Patients and HCC Samples

A total of 88 pairs of HCC tissues and para-cancerous tissues were surgically resected. All patients were pathologically confirmed as HCC based on the 8th edition of the TNM stage of UICC/AJCC. The enrolled HCC patients did not receive preoperative tumor treatments. Informed consent was obtained from patients and their families before sample collection. This study was approved by the Ethics Committee of Chinese PLA General Hospital.

Cell Lines

Six human HCC cell lines (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7 and Hep3B) and one human normal liver cell line (LO2) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and maintained in a 5% CO_2 incubator at 37°C. The culture medium was replaced every 2-3 days. Until 80-90% of confluence, cell passage was performed using trypsin.

Cell Transfection

Si-TRIM66 and si-NC were constructed by GenePharma (Shanghai, China). HCC cells were first seeded into 6-well plates at a density of 70%. Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell Proliferation Assay

48 h after transfection, HCC cells were seeded into 96-well plates with 2000 cells per well. After culturing for 6 h, 24 h, 48 h and 72 h, respectively, cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well. After 2 hours of incubation in the dark, optical density (OD) value of each well at the wavelength of 490 nm was measured by a microplate reader.

Colony Formation Assay

48 h after transfection, HCC cells were seeded into 6-well plates with 200 cells per well. Cells were cultured in complete medium for 2 weeks. Culture medium was replaced once and twice in the first week and the second week, respectively. Until colony formation, the cells were washed with phosphate-buffered saline (PBS) twice and fixed with 2 mL of methanol for 20 min. Then the cells were washed with PBS, followed by staining with 0.1% crystal violet staining solution for 20 min. Finally, formed colonies were observed and captured using a microscope.

Flow Cytometry Analysis

The transfected HCC cells were first suspended in the binding buffer, followed by incubation with 5 μL AnnexinV-FITC and 5 μL Propidium Iodide (PI) (Solarbio, Beijing, China) for 15 min in the dark. After gentle mixture, flow cytometry was performed to detect the apoptotic rate.

Transwell Cell Migration and Invasion Assay

48 h after transfection, HCC cells were digested and re-suspended in serum-free medium. Cell density was adjusted to 5.0×10^5/mL. Transwell chamber coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) or not was placed in 24-well plates. 200 μL of cell suspension containing 1.0×10^5 cells was added to the upper chamber. Meanwhile, 700 μL of medium containing 20% FBS was added to the lower chamber. 48 hours later, the cells were fixed with 4% paraformaldehyde for 15 min until chamber removal. Subsequently, the cells were stained with 0.2% crystal violet for 20 min. The inner layer cells were carefully removed. Five fields were randomly selected for each sample. Penetrating cells were captured, and the number of cells was calculated.

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

Total RNA was extracted from transfected cells, followed by reverse transcription. QRT-PCR was performed to determine the relative mRNA levels of TRIM66 and E-cadherin. Primers used in the study were as follows: TRIM66: forward: 5'-GCCCTCTGTGCTACTTACTC-3', reverse: 5'-GCTGGTTGTGGGTTACTCTC-3'; E-cadherin: forward: 5'-TAGGTATTGTCTACTACTCTG-3', reverse: 5'-TATATCACTCTTGCTTCA-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'. The relative expression level of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method and analyzed by ABI Step One software (Applied Biosystems, Foster City, CA, USA).

Western Blot

Total protein in cells was extracted, and the concentration of the extracted protein was detected by the bicinchoninic acid (BCA) protein determination kit (Pierce, Rockford, IL, USA). Subsequently, the extracted protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Western blot was conducted according to the standard procedures. Primary and secondary antibodies were provided by Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 software (La Jolla, CA, USA) was used for all statistical analysis. Data were expressed as mean ± standard deviation. The *t*-test was used to compare the difference between the two groups. One-way ANOVA was applied to compare the differences among different groups, followed by Post-Hoc test. The correlation between TRIM66 expression and clinical indexes of HCC patients was analyzed by chi-square test. Kaplan-Meier method was introduced to evaluate the effect of TRIM66 on the survival of HCC patients. Log-rank test was used to compare the difference between different curves. $p < 0.05$ was considered statistically significant.

Results

TRIM66 Was Highly Expressed in HCC

A total of 88 pairs of HCC and para-cancerous tissues were collected in this study. The expression of TRIM66 in these tissues was detected by qRT-PCR. The results showed that the expression of TRIM66 in HCC tissues was significantly higher than that of para-cancerous tissues. This indicated that TRIM66 might serve as an oncogene (Figure 1A, 1B). Meanwhile, TRIM66 expression was also determined in HCC cell lines. Among them, Bel-7402 and HepG2 cells expressed a relatively high level of TRIM66 (Figure 1C). Hence, these two cell lines were selected for subsequent experiments.

TRIM66 Expression Was Correlated with Clinical Stage, Lymph Node Metastasis, Distant Metastasis and Overall Survival in HCC Patients

Based on the expression level of TRIM66, the enrolled HCC patients were assigned to the high-level group and the low-level group. Chi-square analysis was conducted to evaluate the correlation between TRIM66 expression with age, sex, tumor stage, lymph node metastasis and distant metastasis of HCC patients. The results demonstrated that high expression of TRIM66 was positively correlated with tumor stage, lymph node metastasis and distant metastasis, whereas not correlated with age and sex of HCC patients (Table I). Follow-up data of enrolled HCC patients were collected for survival analysis. Kaplan-Meier curves revealed that a higher expression of TRIM66 was associated with worse prognosis of HCC (Figure 1D).

Knockdown of TRIM66 Inhibited Proliferation of HCC Cells

To further explore the regulatory effect of TRIM66 on the biological performances of HCC cells, we first transfected si-TRIM66 and corresponding si-NC into HCC cells. Transfection efficacies were verified by qRT-PCR (Figure 2A, 2B). CCK-8 and colony formation assay were carried out to detect the proliferative capacities of HCC cells. As shown in Figure 2C and 2D,

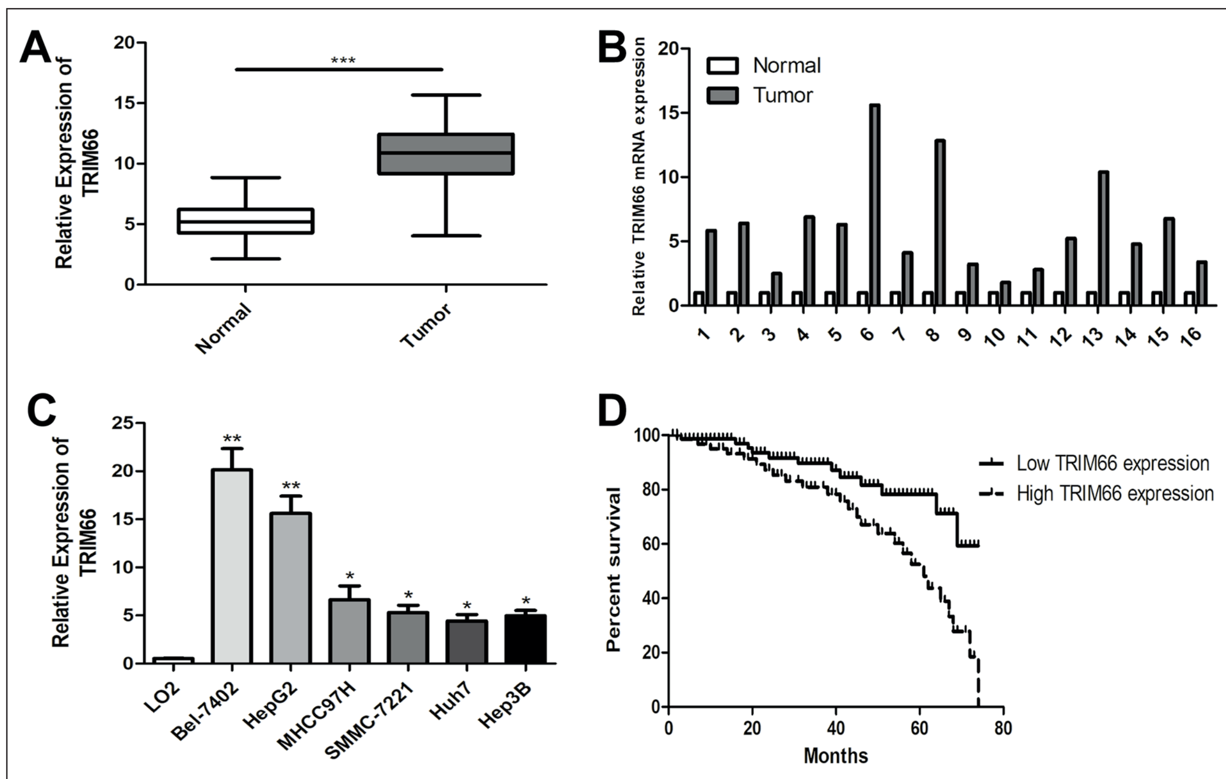

Figure 1. TRIM66 was highly expressed in HCC. ***A, B,*** Expression of TRIM66 in HCC and para-cancerous tissues detected by qRT-PCR. ***C,*** Expression of TRIM66 in HCC cell lines detected by qRT-PCR. ***D,*** Kaplan-Meier curves were introduced based on TRIM66 expression in HCC patients. Data were expressed as mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table I. Association of TRIM66 expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	Number of cases	TRIM66 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.540
< 60	38	23	15	
≥ 60	50	27	23	
Gender				0.124
Male	43	28	15	
Female	45	22	23	
T stage				0.025
T1-T2	49	33	16	
T3-T4	39	17	22	
Lymph node metastasis				0.029
No	51	34	17	
Yes	37	16	21	
Distance metastasis				0.018
No	66	43	23	
Yes	22	8	14	

HCC cells transfected with si-TRIM66 showed significantly lower proliferative rate than those transfected with si-NC. Consistently, the colony formation ability of HCC cells was remarkably decreased after TRIM66 knockdown (Figure 2E).

Knockdown of TRIM66 Induced Apoptosis in HCC Cells

Flow cytometry was utilized to determine the apoptotic rate of HCC cells. Double-staining of Annexin V-FITC/PI pointed out that the apoptotic rate in HCC cells transfected with si-TRIM66 was significantly higher than those transfected with controls (Figure 2F).

Knockdown of TRIM66 Inhibited Migration and Invasion of HCC Cells

After transfection of si-TRIM66 in HCC cells, migratory and invasive abilities were accessed using transwell assay. The amount of penetrating HCC cells was significantly decreased after TRIM66 knockdown, suggesting inhibited migratory ability (Figure 3A, 3B). Invasive ability of HCC cells was also markedly suppressed by TRIM66 knockdown (Figure 3C, 3D).

Knockdown of TRIM66 in HCC Cells Changed Expressions of Relative Genes in EMT Pathway

We speculated that the EMT pathway might be involved in TRIM66-mediated HCC development. The protein expressions of relative genes in the EMT pathway were detected by Western blot. It was found that the TRIM66 knockdown in HCC cells markedly downregulated the protein expressions of E-cadherin, N-cadherin, Vimentin and β-catenin (Figure 4A).

TRIM66 Regulated E-Cadherin in HCC Cells

Bioinformatics predicted that TRIM66 might interact with E-cadherin. Hence, E-cadherin expression in HCC tissues and cell lines was assessed by qRT-PCR and Western blot. The results found that the expression of E-cadherin in HCC tissues was significantly lower than that of para-cancerous tissues (Figure 4B). Similarly, E-cadherin was lowly expressed in HCC cells than that of LO2 cells (Figure 4C). Furthermore, the correlation between TRIM66 expression and E-cadherin expression was analyzed in 16 collected HCC tissues. The data showed that the TRIM66 expression was negatively correlated with E-cadherin expression in HCC tissues at both mRNA and protein levels (Figure 4D).

To elucidate the regulatory roles of TRIM66 and E-cadherin in HCC cells, they were co-transfected with si-TRIM66 and pcDNA-E-cadherin. The mRNA and protein levels of E-cadherin in co-transfected HCC cells were determined by qRT-PCR and Western blot, respectively (Figure 5A, 5B). Subsequently, invasive and migratory abilities were accessed after co-transfection in cells. The results indicated that inhibited migration and invasion of HCC cells resulted from TRIM66 knockdown were partially reversed by E-cadherin overexpression (Figure 5C, 5D).

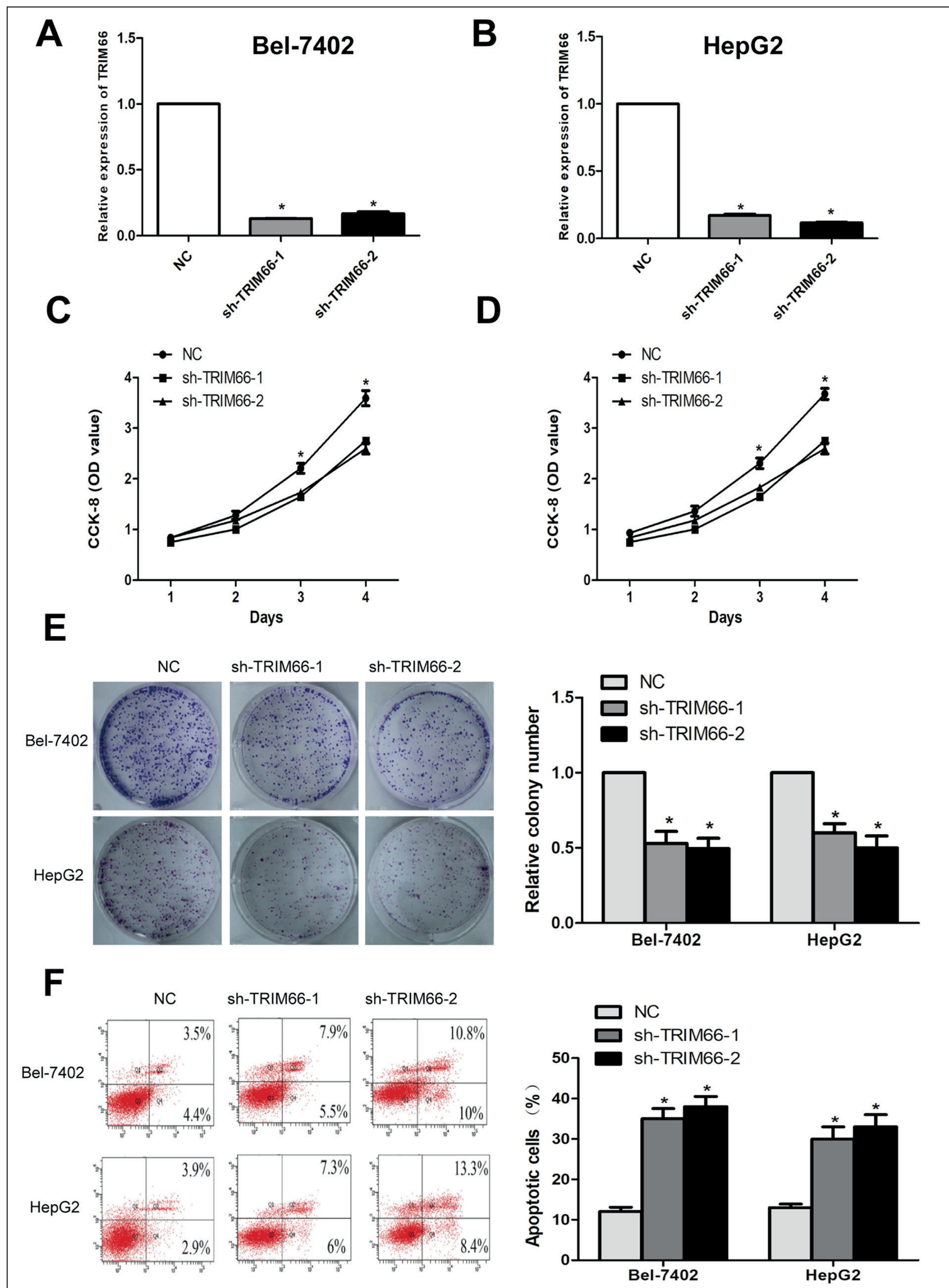

Figure 2. Knockdown of TRIM66 inhibited proliferation of HCC cells. ***A, B,*** Transfection efficacies of si-TRIM66 and corresponding si-NC in HCC cells. ***C, D,*** Proliferative rate of HCC cells transfected with si-TRIM66 or si-NC detected by CCK-8 assay. ***E,*** Colony formation ability of HCC cells transfected with si-TRIM66 or si-NC. ***F,*** Apoptotic rate of HCC cells transfected with si-TRIM66 or si-NC. Data were expressed as mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

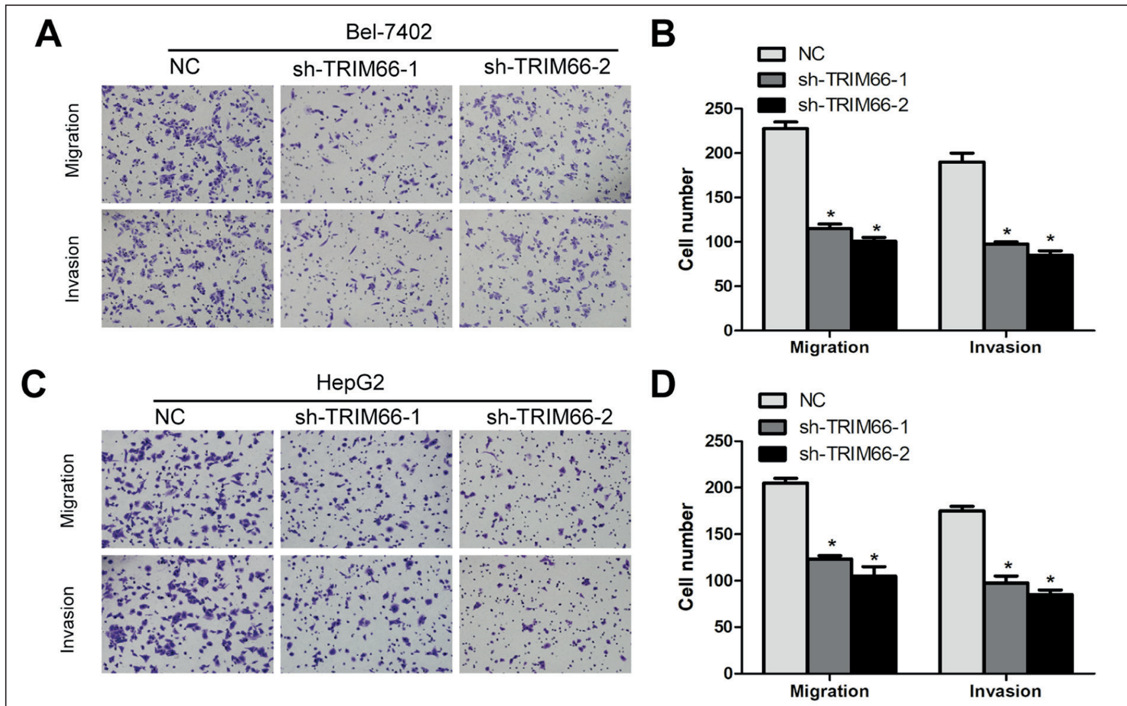

Figure 3. Knockdown of TRIM66 inhibited migration and invasion of HCC cells. ***A, B,*** Migration and invasion of Bel-7402 cells transfected with si-TRIM66 or si-NC. ***C, D,*** Migration and invasion of HepG2 cells transfected with si-TRIM66 or si-NC. Data were expressed as mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

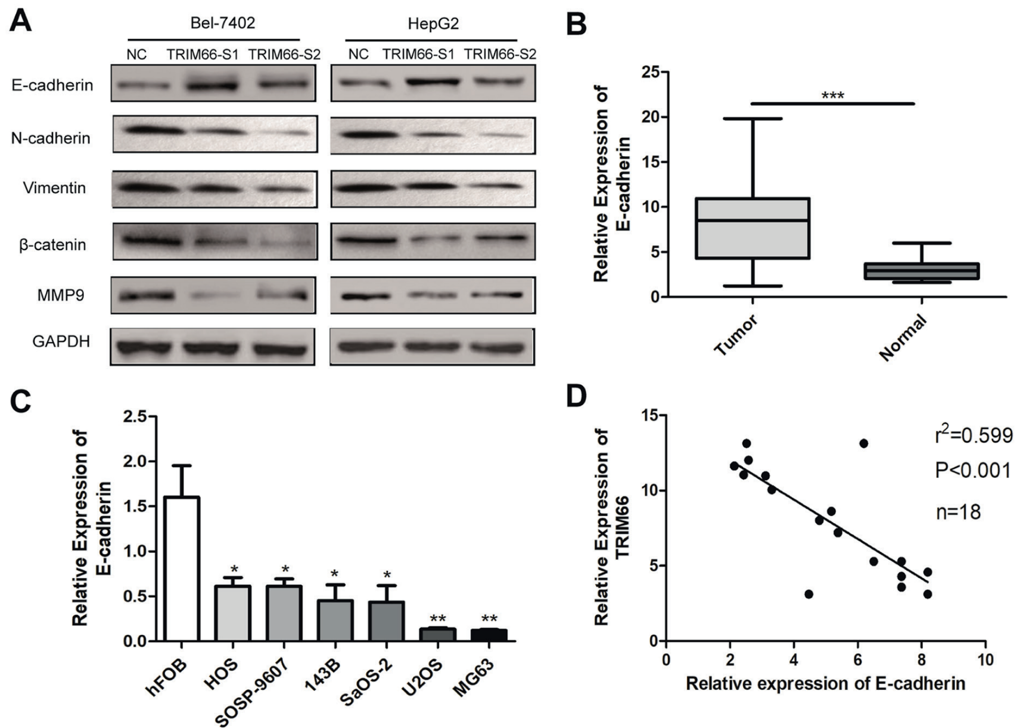

Figure 4. Correlation between E-cadherin and TRIM66 in HCC cells. ***A,*** Protein expressions of E-cadherin, N-cadherin, Vimentin and β-catenin in HCC cells after TRIM66 knockdown. ***B,*** E-cadherin expression in HCC and para-cancerous tissues detected by qRT-PCR. ***C,*** Expression of E-cadherin in HCC cell lines detected by qRT-PCR. ***D,*** Negative correlation between expressions of TRIM66 and E-cadherin in HCC tissues both at mRNA and protein levels. Data were expressed as mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

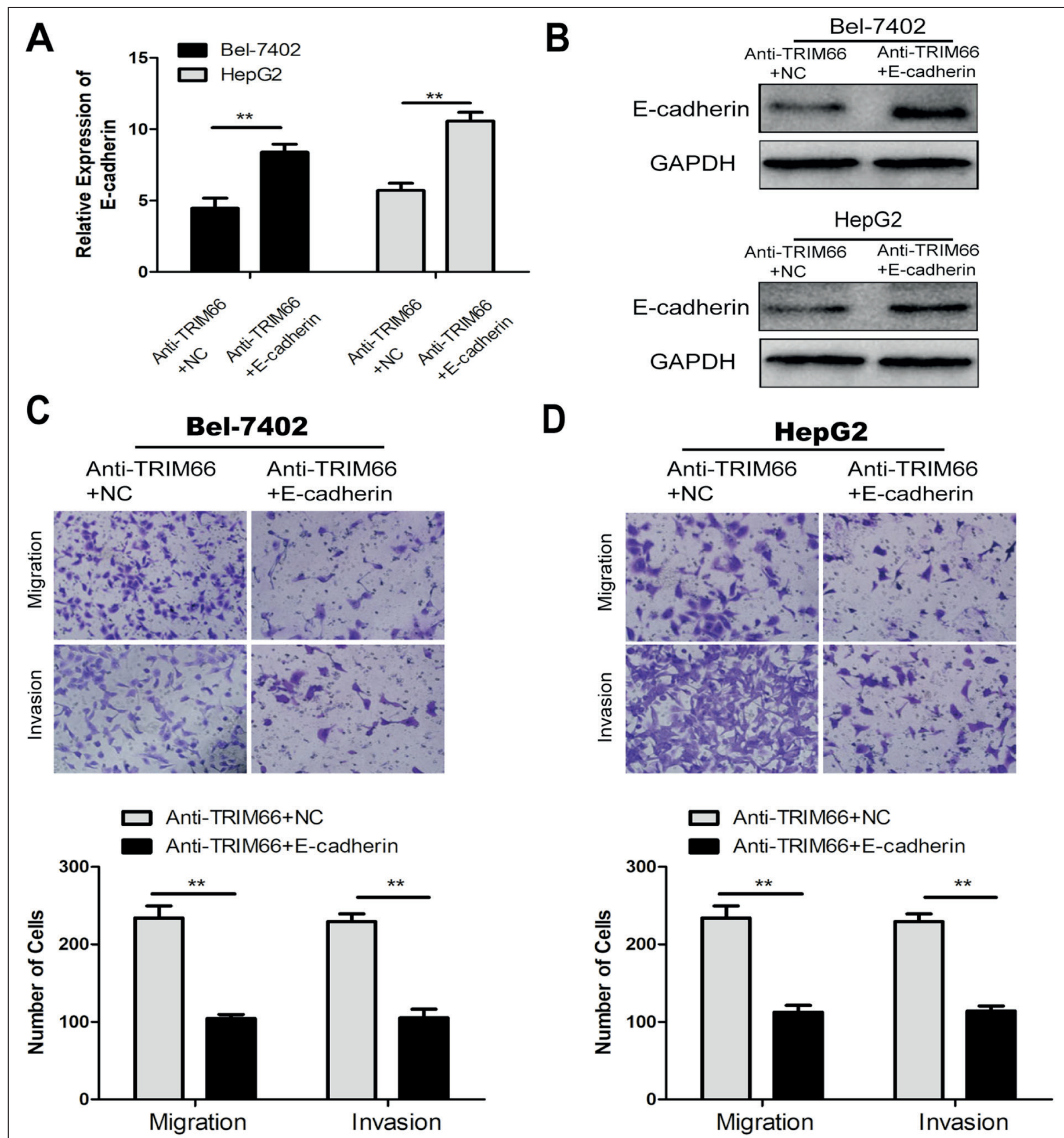

Figure 5. TRIM66 regulated E-cadherin in HCC cells. ***A, B,*** Transfection efficacy of pcDNA-E-cadherin in HCC cells verified by qRT-PCR and Western blot. ***C, D,*** Migration and invasion of HCC cells co-transfected with si-TRIM66 and pcDNA-E-cadherin. Data were expressed as mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

HCC is the most common type of liver cancer, ranking fifth in tumor incidence worldwide[1]. Asia is a high-incidence area of HCC. As the largest country in Asia, the number of liver cancer patients in China has increased in recent years. The mortality rate of liver cancer in China is second only to gastric cancer[2,4]. Currently, surgical and interventional treatments are effective methods for HCC patients. However, the recurrence rate of HCC is still relatively high, and its five-year survival is unsatisfactory[10,11]. Many factors may influence the prognostic out-

come of HCC, such as poor cell differentiation, portal vein invasion and intrahepatic tumor metastasis[8,9]. A number of studies have shown that genetic factors, including gene heterozygous deletion and gene allelic mutations, exert an essential role in the HCC development[6]. Hence, effective prevention and early diagnosis of HCC contribute to improving the postoperative survival of these patients[7,8]. Further researches on the etiology of HCC, especially the role of genetic genes, will help to provide new ideas for the therapeutic treatment of HCC[7,8,12].

TRIM66 has a typical RING domain and exerts the role of ubiquitin E3 ligase. It may regulate the expressions of other proteins through ubiquitination, thereby affecting cell growth. TRIM66 is also a transcriptional mediator and a nuclear protein receptor, which can interact with various nuclear receptors, including retinoid X receptor (RXR), retinoic acid, progesterone receptor (PR), vitamin D3 (VDR), estrogen (ER), etc.[13-16]. At present, abnormal expression of TRIM66 has been found in various tumors. This suggests its potential role in the malignant progression of tumors[23-25]. In our study, we examined TRIM66 expression in HCC tissues and cell lines at transcriptional and protein levels. The results showed that TRIM66 was highly expressed in HCC tissues and cells. These experimental results indicated that high expression of TRIM66 exerted an extremely important role in the HCC development. To verify the effect of TRIM66 on the biological behaviors of HCC cells, flow cytometry, CCK-8, colony formation and transwell assay were conducted. TRIM66 knockdown significantly decreased proliferative, invasive and migratory abilities, but induced apoptosis of HCC cells. The above experimental findings demonstrated that TRIM66 could promote the HCC development. However, its specific molecular mechanism is still unclear.

Since the concept of EMT has been proposed, more and more studies have shown that EMT is closely related to the occurrence and development of epithelial malignant tumors. Meanwhile, it has received wide attention in tumor researches[19,20]. At present, experimental evidence has indicated the pivotal role of EMT in primary and secondary metastasis of HCC, colon cancer, lung cancer, prostate cancer, liver cancer, pancreatic cancer, etc.[19,20]. Therefore, it is of significance to elucidate the regulatory effect of EMT, to search for novel therapeutic targets for malignant tumors[26,27]. EMT gene is expected to play a benign role in tumor therapy[27]. In this experiment, Western blot results showed that the expression levels of key proteins in the EMT pathway were markedly downregulated after TRIM66 knockdown. This indicated that TRIM66 could promote the invasion and migration of HCC cells through the EMT pathway.

E-cadherin is a type I transmembrane glycoprotein that is mainly expressed in epithelial cells. Its intracellular domain contains 150 amino acids. Meanwhile, the extracellular domain contains five repeat amino acid sequences (EC) with 110 amino acids in each EC[21,22]. The loss of the E-cadherin protein in the cell surface of most epithelial tumors is significantly associated with increased invasiveness. Moreover, the loss of E-cadherin stimulates the motility, invasion and EMT of the cells[28]. Upregulated E-cadherin is capable of reversing the metastatic statue of tumor cells[28,29]. In this study, we found that tissues with low expression of E-cadherin had the characteristics of poor differentiation, lymph node metastasis and high clinical stage, which were consistent with previous studies. Rescue experiments found a mutual regulation between TRIM66 and E-cadherin in HCC cells. In-depth researches on biological roles of E-cadherin in the incidence and progression of tumors are beneficial to improve diagnostic, therapeutic and prognostic approaches[28]. This may undoubtedly bring good news to tumor patients and their families.

To demonstrate whether TRIM66 promoted the development of HCC by regulating the EMT pathway, we examined the protein expressions of E-cadherin, N-cadherin, Vimentin, β-catenin and MMP9 in HCC cells after knockdown of TRIM66 by Western blot. The results indicated that TRIM66 significantly promoted the invasion and migration of HCC cells through the EMT pathway. Additionally, we also found that the expression level of E-cadherin was markedly downregulated by TRIM66 knockdown in HCC. These results suggested the potential role of E-cadherin in TRIM66-mediated malignant progression of HCC.

Conclusions

We demonstrated that TRIM66 is highly expressed in HCC, which is positively correlated with tumor stage, lymph node metastasis and distant metastasis of HCC patients. Furthermore, TRIM66 promotes the malignant progression of HCC by inhibiting E-cadherin through the EMT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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