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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 Tim merase Chain Reaction (qRT-PCR). T relation between TRIM66 expression an n-ic-pathologic characteristics of HCC pa was analyzed. Follow-up data of enrolled patients were collected for survival analys Subsequently, TRIM66 expres HCC cell was determined by qRT-PC By constructing si-TRIM66, the bi ical p rmanc-es of transfected HCC ce ere det d using cell counting kit-8 8), c tion and transwell as t was performed to measure t otein sions of relative genes in epi al-mesench ransition (EMT) pathway. HCC cells w trans-fected with si d pcDNA-E adherin, followed by ction sive and migratory abilities.

RESU TRIM66 was h expressed in HCC ti s compared with t at of para-cancero sues. expression of TRIM66 was pos y cor ed with tumor stage, lymph nod st and di t metastasis, whereas not ed wit e and sex of HCC pa-s. Ka leie ves revealed that a high-ressio M66 was associated with wo rognos HCC. Similarly, TRIM66 was also hly expressed in HCC cells. The knock-do in HCC cells significantly in-h d the liferative, invasive and migratory ties of transfected cells. However, TRIM66 -regulation significantly induced cell apop-t estern blot results showed that TRIM66 knoc own in HCC cells markedly downreg-ul protein expre ions of E-cadherin, dherin, Vimentin and β-catenin. The inhibit-migration an vasion of HCC cells resulted n TRIM66 kn lown were partially reversed cadherin o xpression.

CLUSIO TRIM66 is highly expressed in positively correlated with tumor st ph node metastasis and distant metastasis of HCC patients. In addition, TRIM66 tes the malignant progression of HCC by 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 d in interacting with target proteins. The RI main has E3 ubiquitin ligase activity. Mea le, it acts on target genes by interacting wit related E2 ubiquitin-binding enzyme, thus m ating the ubiquitination of itself and different su strates[15]. The B-box domain is -binding domain unique to TRIM p , w mainly contains a cysteine-histid inc fin motif[16]. The CO domain is loca ind t main, and is mainly tang plurality of a-helices. The main ction o omain is to promote homolog or heterologe igomerization between otein and TR protein or others. T may tually stimulate the production f macromole olymers and formation o tein subcellula lization[16]. The TRIM ein fa ily was orig ally discovered to ex ntivira ctions by interfering with the vir licati proces In addition, proteins in the amily s pattern recognition r ptors proc f innate immune signal- e pro lso capable of recognizing cap protein e virus and regulating pathway the natural immune process[18]. In recent ye udies have shown that members e TRIM protein family are involved in tumor lopment and progression[14-16]. In this study, w to explore the clinical significance of TRI in HCC.

Epithelial-mesenchymal transition (EMT) is a process of transformation from epit to mesenchymal cells under norm hysio ical and specific pathological c ions. Exist- ing theory suggests that EMT egulate the malignant behaviors of tumors adherin and others are key transcr ion fac ulat ing the EMT processes[2] Based on t characteristics, this st ims t erify wh E-cadherin may bec a sero al marker for early diagnosis of H oin matics icted that E-cadherin y b the t genes of TRIM66. H ver, wh TR o partic- ipates in the iferation and is of HCC cells thro ed regulation E-cadherin remains nown

In this study, we ed the expression of TR 88 pairs of H issues and para-can- c tissues. The effects of TRIM66 on the bio- cal function of HCC cells were also explored. vious studie ve indicated that TRIM66 promote the sion and metastasis of tumor c us con ng tumor development. Our stud e 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. 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 suspended in the binding buffer, followed by incubation with 5 μL Annexin V-FITC and 5 μL propidium Iodide (PI) (Solarbio, Beijing, China) for 15 min in the dark. After staining, flow cytometry was performed to detect the apoptotic rate.

Transwell Cell Migration and Invasion Assay

After transfection, HCC cells were digested and suspended in serum-free medium. Cell density was adjusted to 5.0×10^5/mL. Transwell upper chamber coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) or not was placed in 24-well plates. 200 μL of cell suspension containing 1×10^5 cells was added to the upper chamber. Meanwhile, 700 μL of medium containing 20% FBS was added to the lower chamber. 48 hours later, 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 fully 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 β-actin. Primers used in the study were as follows: TRIM66: forward: 5'-GCCCTCTGTGCCTTACTC-3', reverse: 5'-GCTGGTTGTGGCCTC-3'; E-cadherin: forward: 5'-TAGCCTTGTTTACTCTG-3', reverse: 5'-TATATCACTCTTCA-3'; β-actin: forward: CTGGCACCACACAAT-3', reverse: 5'-CCGTAGGTGTCCCTTG-3'. The relative expression level of mRNA was calculated by the 2^{-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 determined 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

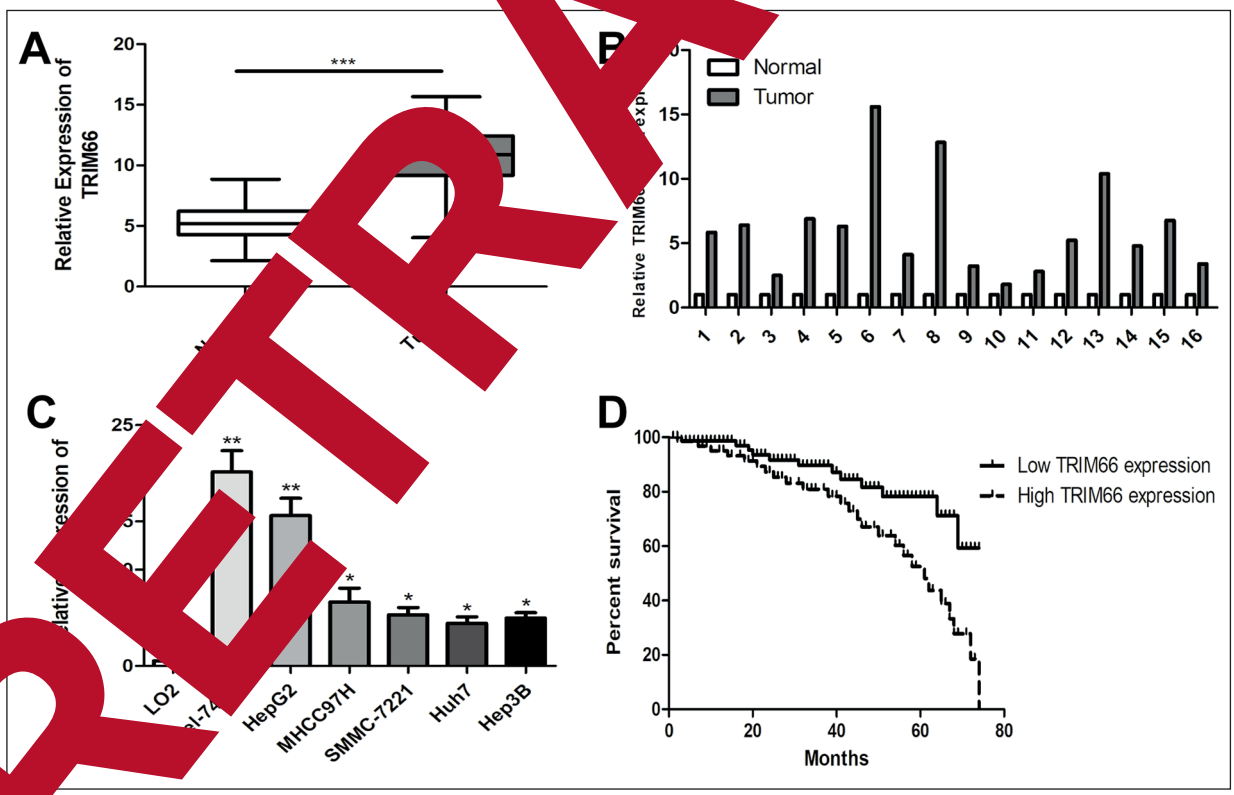
A total of 88 pairs of HCC and para-cancerous tissues were collected in this study. The expression of TRIM66 in these tissues was detected by qRT-PCR. The results showed that the expression of TRIM66 in HCC tissues was significantly higher than that of para-cancerous tissues. This indicated that TRIM66 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 expre... age, sex, tumor stage, lymph node ... distant metastasis of HCC patie... The results demonstrated that high expre... of TRIM66 was positively correlated with ... stage, lymph node metastasis a... distant ...tasis whereas not correlated w... age and sex ... patients (Table I). Fo...-up d... of en... HCC patients were ...cted f... rvival anary-sis. Kaplan-Meier ... rev...d that ...gher expression of TR...66 ...ciated ... worse prognosis of H... (Figure ...

Knockd... TRIM66 Inhi...ed Prolife...on o... Cells

To further explo... regulatory effect of TR... the biologic... formances of HCC c... we first transfected si-TRIM66 and cor-...onding si-NC into HCC cells. Transfection ...cacies were v...ed by qRT-PCR (Figure 2A, ... CCK-8 an... olony formation assay were c... out to ...t the proliferative capacities of H... shown in Figure 2C and 2D,



...e 1. TRIM66 was highly expressed in HCC. ***A, B,*** Expression of TRIM66 in HCC and para-cancerous tissues detected by ...CR. ***C,*** Expression of TRIM66 in HCC cell lines detected by qRT-PCR. ***D,*** Kaplan-Meier curves were introduced based ... 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.
Male	43	28		
Female	45	22		
T stage				5
T1-T2	49	33		
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		
Yes	22	8		

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 (Figu E).

Knockdown of TRIM66 Induced Apoptosis in HCC Cells

Flow cytometry was utilized to determine apoptotic rate of HCC cells. Double-staining Annexin V-FITC/PI pointed o apoptotic rate in HCC cells transfe with RIM66 was significantly higher those sfected with controls (Figure 2F).

Knockdown of T 66 In d Migration and asion of H lls

After transfe -TRIM66 in C cells, migratory an vasi ilities were accessed using trans ell assay. T ount of penetrating HCC s was significa ecreased after TRIM6 nockd wn, suggesti inhibited migrato bility ure 3A, 3B). Invasive ability of cells also rkedly suppressed by TRIM down (re 3C, 3D).

kdow M66 in HCC Cells Ch ed Exp ions of Relative Genes in Pathway

d that the EMT pathway might volved in TRIM66-mediated HCC development. The protein expressions of relative genes i EMT pathway were detected by Western blot. was found that the TRIM66 knockdown

HCC cells ma ly downregulated the protein essions of E herin, N-cadherin, Vimentin a tenin (re 4A).

TRIM66 Regulated E-Cadherin in HCC Cells

matics predicted that TRIM66 might ter 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).

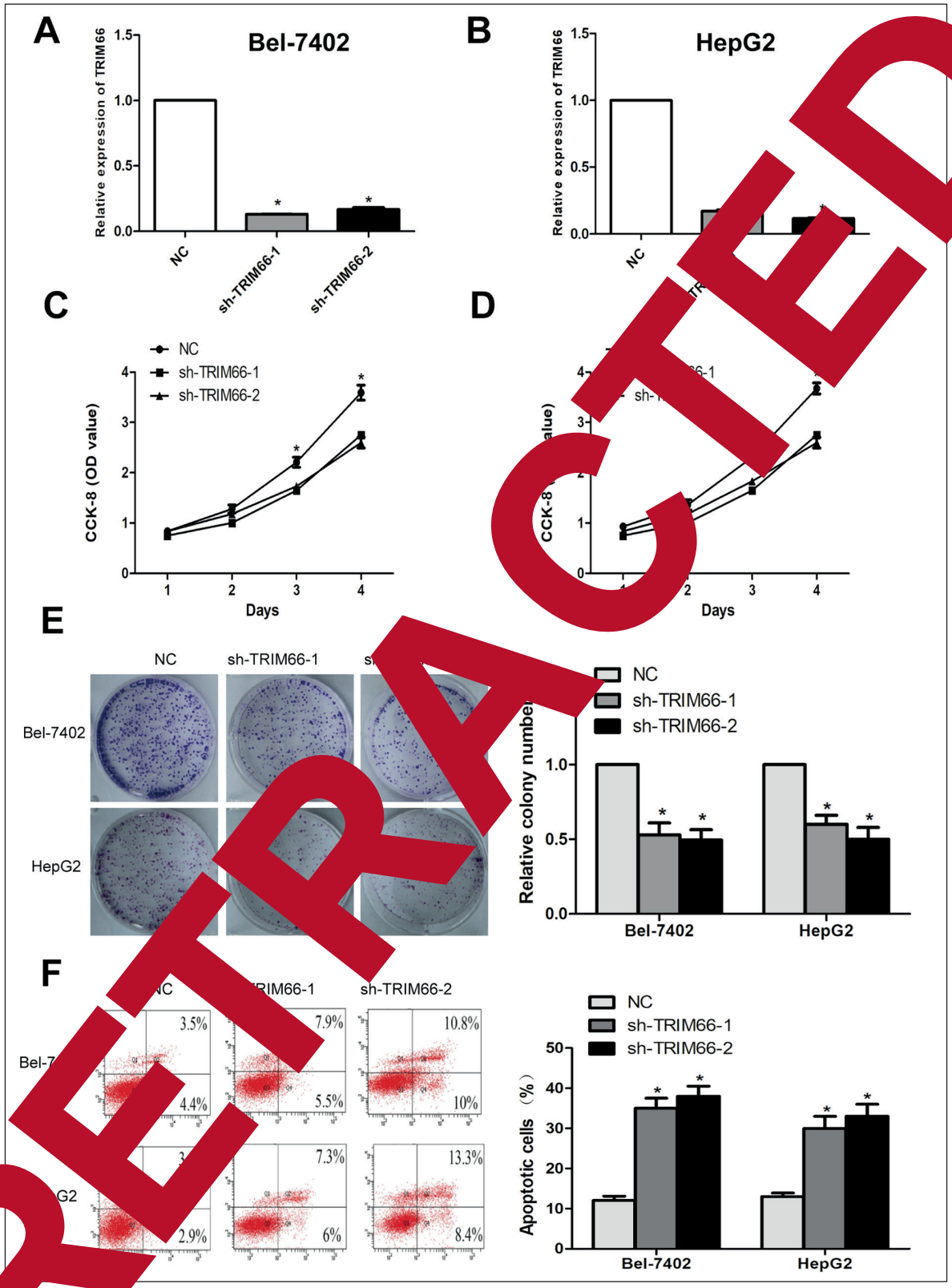


Figure 2. Knockdown of TRIM66 inhibited proliferation of HCC cells. ***A, B,*** Transfection efficacies of si-TRIM66 and corresponding si-NC in HCC cells. ***C, D,*** Proliferative rate of HCC cells transfected with si-TRIM66 or si-NC detected by CCK-8 assay. ***E,*** Colony formation ability of HCC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

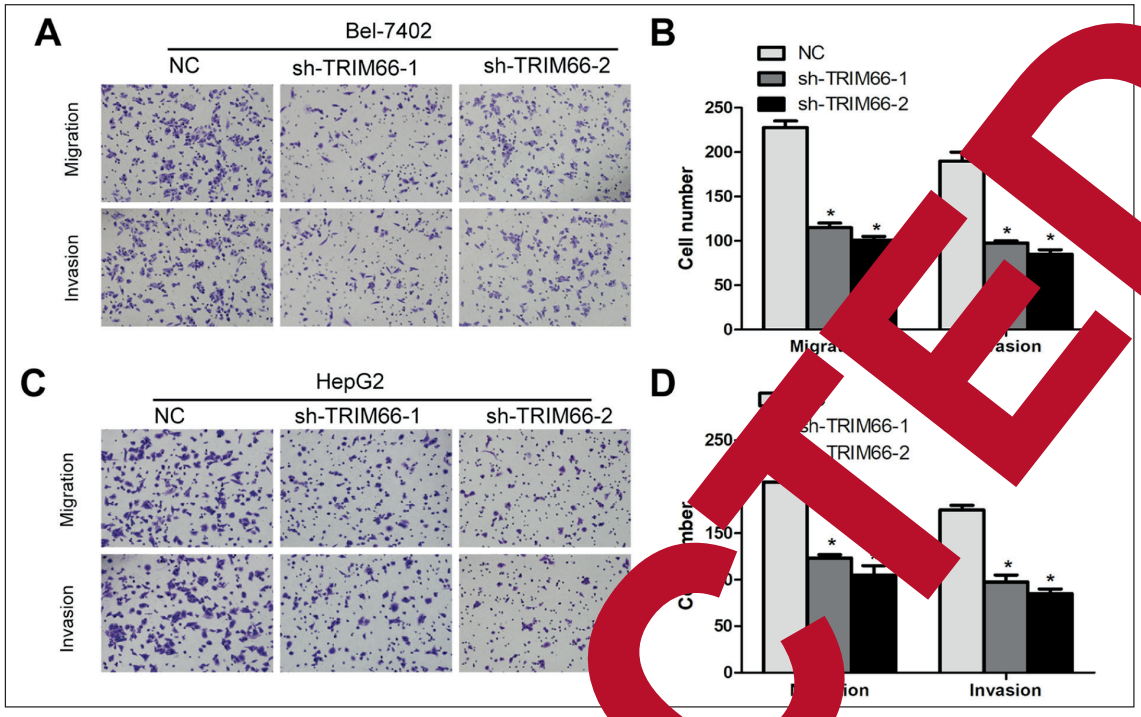


Figure 3. Knockdown of TRIM66 inhibited migration and invasion [illegible], Migration and invasion of Bel-7402 cells transfected with si-TRIM66 or si-NC. ***C, D,*** Migration and invasion [illegible]s transfected with si-TRIM66 or si-NC. Data were expressed as mean ± SD, *$p < 0.05$, **p [illegible]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

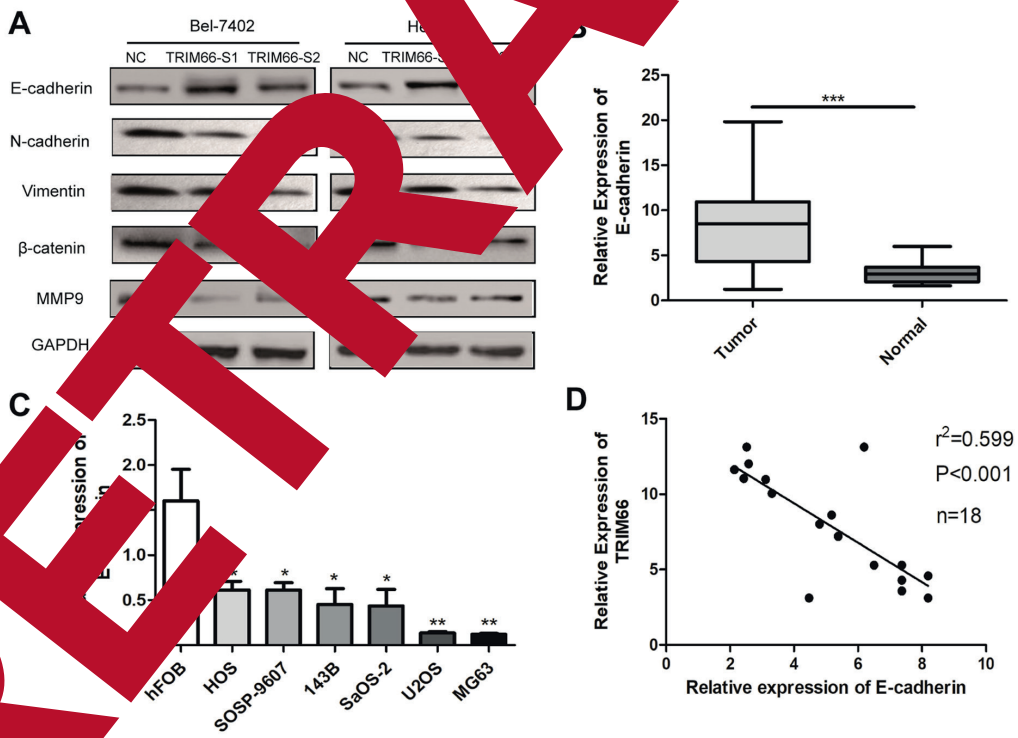
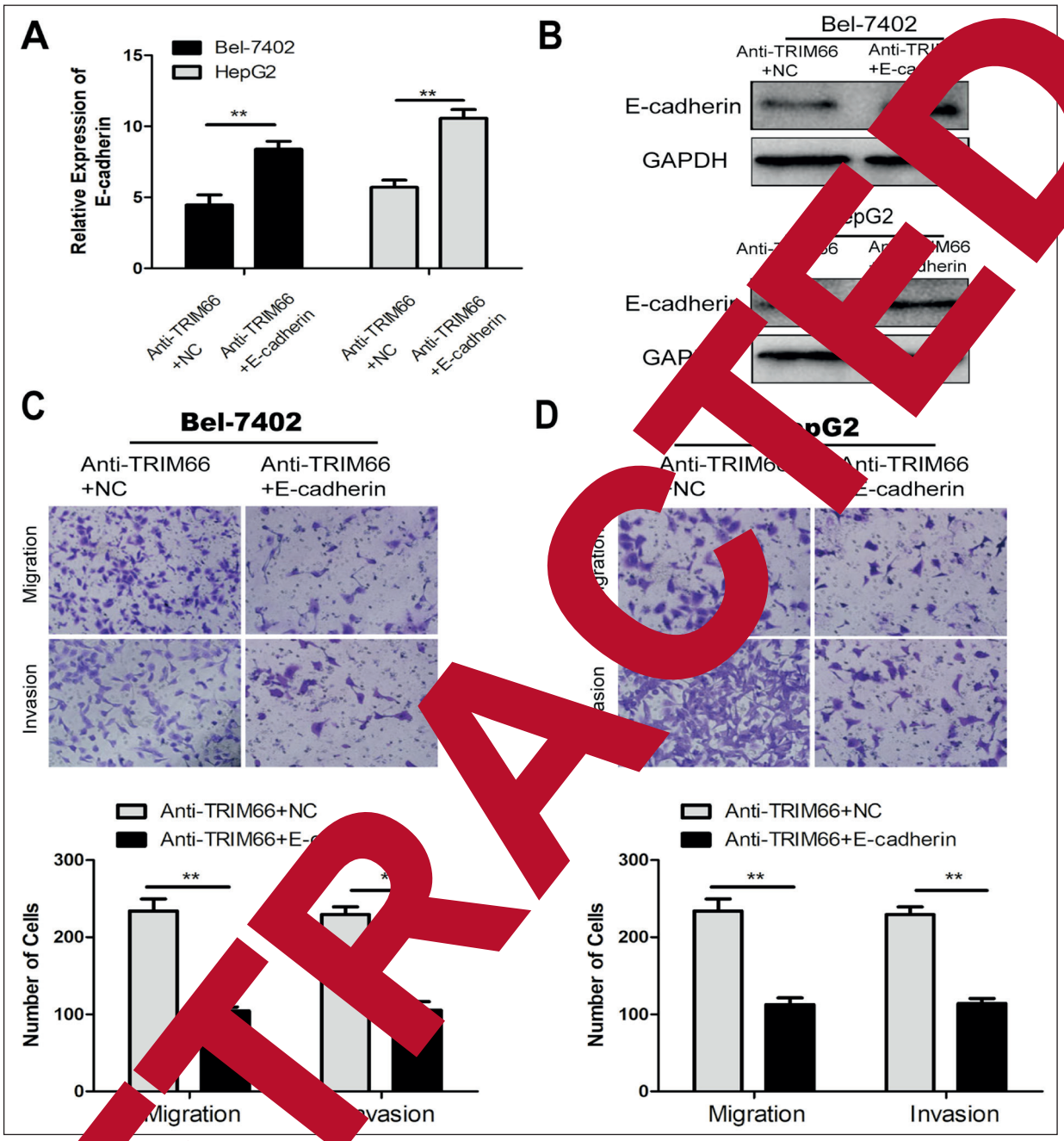


Figure 4. Correlation between E-cadherin and TRIM66 in HCC cells. ***A,*** Protein expressions of E-cadherin, N-cadherin, Vimentin [illegible]catenin in HCC cells after TRIM66 knockdown. ***B,*** E-cadherin expression in HCC and para-cancerous tissues detected by qRT-P[illegible]. [illegible]xpression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



Figur TRIM6 ulated E-cadherin in HCC cells. ***A, B,*** Transfection efficacy of pcDNA-E-cadherin in HCC cells veri qRT-P d West n blot. ***C, D,*** Migration and invasion of HCC cells co-transfected with si-TRIM66 and pcDNA-E-ca Da re expre s mean ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

scussion

ost common type of liver cancer, ing fifth in tumor incidence worldwide[1]. is a high-incidence area of HCC. As the l country in Asia, the number of liver canc 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 expres in HCC tissues and cell lines at transcripti protein levels. The results showed that T 66 was highly expressed in HCC tissues and These experimental results indicated that h expression of TRIM66 exerted an xtremely in portant role in the HCC deve To verify the effect of TRIM66 on the logica haviors of HCC cells, flow cyto y, CCK colony formation and transwel we TRIM66 knockdown mini eased proliferative, invasive migra bilities, but induced apoptosi HCC cells. ove experimental find onstrated tha RIM66 could promot e H velopment. However, its specific olecular me m is still unclear.

Since t oncept of EM een proposed, more a ore studies have shown that EMT is close ated t occurrence and development of ial n nant t rs. Meanwhile, it has receiv ttentio tumor researches[19,20]. At presen erim evidence has indicated otal ro in primary and secondary met sis of H colon cancer, lung cancer, pros cancer, liver cancer, pancreatic cancer, et e, it is of significance to elucidate egulatory effect of EMT, to search for novherapeutic targets for malignant tumors[26,27]. E ene is expected to play a benign role in tum erapy[27]. In this experiment, Western blot results showed that the expression levels of key proteins in the EMT pathway were downregulated after TRIM66 kno wn. indicated that TRIM66 could p ote the invasion and migration of HCC through the EMT pathway.

E-cadherin is a type I ansmem gly coprotein that is mainly pressed in e cells. Its intracellular ain co ins 150 no acids. Meanwhil e ext ular domain contains five repea a sequen (EC) with 110 amino ds i C[21,22]. loss of the E-cadheri tein in t su e of most epithelial tu is significan iated with increased ess. Moreov the loss of E-cadhe stimu he motility, invasion and EMT of the cells[28]. U ated E-cadherin is capabl versing the m tic statue of tumor c . In this study, we found that tissues with expression of E-cadherin had the characteris- of poor diff iation, lymph node metastand high clin stage, which were consistent w vious s es. Rescue experiments found a m n between TRIM66 and E-cadherin in HCC cells. In-depth researches on biol ical roles of E-cadherin in the incidence and n of tumors are beneficial to improve agnc c, 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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