Knockdown of TRIM66 Suppresses the Proliferation, Migration, Invasion and Glycolysis in Cholangiocarcinoma Cells

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Research Article

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Abstract

**Background:** The tripartite motif (TRIM) family proteins feature highly conserved order of domains in the RBCC motif and most of them play an essential role in various cellular processes. Recently, increasing evidence has shown association of TRIM proteins with cancer development. In this study, we examined the expression pattern and biological functions of TRIM66 in cholangiocarcinoma (CCA).

**Methods:** Western blot was performed for the protein levels of TRIM66, E-cadherin, α-catenin, N-cadherin, vimentin, p-PI3K, PI3K, p-Akt and Akt. MTT assay, wound healing assay and transwell assay were conducted for cell proliferation, migration and invasion, respectively. Glucose uptake and lactate production were determined using specific kits.

**Results:** TRIM66 was overexpressed in CCA tissues and cell lines. In addition, knockdown of TRIM66 significantly inhibited proliferation, migration, invasion and glycolysis of CCA cells. Moreover, TRIM66 silencing obviously decreased levels of phosphorylated PI3K and Akt in CCA cells.

**Conclusion:** Our study provided a novel insight into the roles of TRIM66 in CCA and suggested TRIM66 as a promising therapeutic target for CCA treatment.

Introduction

Cholangiocarcinoma (CCA), a type of epithelial cell tumor, originates from bile ducts and is the sixth leading cause of gastrointestinal cancer in the world [1]. According to locations, CCA is divided into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC) [2]. In the last decade, the morbidity and mortality of CCA has been remarkably increasing [3]. The detection of CCA is usually unavailable at an early stage of the disease due to lack of specific symptoms, leading to an failure in a thorough tumor resection and a compromise of therapeutic effectiveness [4, 5]. Thus, it is necessary to explore novel biomarkers for early detection of CCA patients.

Aerobic glycolysis, a hallmark of human cancers, is a crucial process for unrestricted tumor growth during cancer progression [6, 7]. Even in a microenvironment rich in oxygen, cancer cells mainly depend on glycolytic pathways [8]. It has been demonstrated that cancer cells have various metabolic demands and they meet these demands via adjustment of metabolic use [9, 10]. Relying on the metabolic program of aerobic glycolysis, cancer cells sustain biomass production [11]. Increasing evidence has shown that poor prognosis of diverse tumors is related to excess glucose uptake and glycolysis [12]. But the molecular mechanisms underlying elevated glycolysis during cancer progression still remain unclear.

The tripartite motif (TRIM) protein family consists of more than 70 members and these proteins could be further divided into 12 subgroups on the basis of C-terminal domains [13]. The TRIM family proteins feature highly conserved order of domains in the RBCC motif and most of the proteins contain single RING-finger E3 ubiquitin ligases and thereby play an essential role in various cellular processes such as cell differentiation and autophagy [14, 15]. Recently, increasing evidence has shown that dysregulation of
TRIM proteins is closely associated with cancer development and some of them serve as either tumor promoters or suppressors [16, 17].

TRIM66 is a newly identified member of the TRIM family and pathological studies have demonstrated its involvement in tumorigenesis of several cancers [18, 19]. However, the specific role of TRIM66 in CCA remains unclear. Therefore, in this study, we examined the expression pattern and biological functions of TRIM66 in CCA. We found that TRIM66 was overexpressed in CCA tissues and cell lines. In addition, knockdown of TRIM66 significantly inhibited proliferation, migration, invasion and glycolysis of CCA cells. We also demonstrated that TRIM66 knockdown exerted the inhibitory effect on CCA progression partly via regulating the PI3K/Akt signaling pathway.

Materials And Methods

Patients and tissue samples

Human CCA tissues and matched normal tissues were obtained from 42 CCA patients who received surgery at the First Affiliated Hospital of Jinzhou Medical University (Jinzhou, China). No patients underwent chemotherapy or radiotherapy before surgery. Tissue samples were collected and used with written informed consent from all patients. This study was approved by the Ethics Committee of Jinzhou Medical University.

Cell lines and cell culture

Human CCA cell lines (HUCCT1 and QBC939) and the normal bile duct epithelial cell line HiBEC were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco) and 100 μg/mL penicillin/streptomycin and incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and synthesized into cDNA using PrimeScript RT reagent kit (Takara, Japan). RT-qPCR was performed with SYBR Premix Ex Taq™ (Takara, Japan) on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The following primers were used: TRIM66, 5’-GCCCTCTGTGCTACTTACTC-3’ (forward) and 5’-GCTGGTTGTGGGTTACTCTC-3’ (reverse), GAPDH, 5’-CCATCAATGACCCCTTCATTG-3’ (forward) and 5’-CATGGGTGGGAATCATATTGGAAC-3’ (reverse). The relative expression of target genes was normalized to GAPDH. All data were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Tissues or cells were lysed in lysis buffer for protein extraction. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). An equal amount of protein was separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA).
The membranes were blocked with 5% skim milk, followed by overnight incubation at 4°C with primary antibodies against TRIM66, E-cadherin, α-catenin, N-cadherin, vimentin, p-PI3K, PI3K, p-Akt, Akt and GAPDH. After washing with TBST, the membranes were incubated with appropriate secondary antibody. All antibodies in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein bands were detected using an ECL kit (Pierce, Rockford, IL, USA) and their intensity was quantified using the ImageJ software.

**Cell transfection**

TRIM66 shRNA (shTRIM66) and the control shRNA (shNC) were designed and synthesized by GeneCopoeia (Rockville, MD, USA). Cells were cultured in a 96-well plate at a density of 1×10³ cells/well. After reaching 80% confluence, cells were transfected with shTRIM66 or shNC using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The western blot analysis was performed to confirm the transfection efficiency.

**MTT assay**

Cell proliferation was assessed using the MTT assay. In brief, cells were seeded in a 96-well plate at a density of 1×10³ cells/well. After incubation for different time, MTT (5 mg/mL, Sigma, St. Louis, MO, USA) was added to each well and cells were cultured for another 4 h. Subsequently, the supernatants were removed from each well and DMSO (Sigma) was added. The absorbance value was measured at 570 nm using a microplate reader.

**Wound healing assay**

Cells were seeded in a 6-well plate and cultured to reach 80% confluence. A sterile plastic tip was used to scratch a wound on cell monolayer and then cells were cultured for 24 h. The images of wounds were taken at the indicated time points under a microscope (200×).

**Transwell assay**

Transwell chambers were used to measure cell invasion. Cells were resuspended in serum-free medium and added to the upper chamber. The membrane was coated with Matrigel. The lower chamber was filled with 10% FBS as chemoattractant. After incubation for 24 h, cells remaining on the upper surface of the membrane were removed and invading cells on the lower surface of the membrane were fixed and stained with crystal violet. The number of invading cells in five random fields was counted under a microscope (200×).

**Measurement of glucose uptake and lactate production**

48 h after transfection, cell culture media was collected to determine glucose uptake and lactate production using a glucose assay kit (Sigma) and a lactate assay kit (Sigma), respectively. Glucose and lactate levels were calculated with normalization to corresponding protein levels.
Statistical analysis

Data were collected from three independent experiments and shown as means ± standard deviation (SD). The SPSS software version 19.0 was used for statistical analysis. Student’s *t*-test or one-way ANOVA was applied for the comparison between different groups. *P*<0.05 was considered statistically significant.

Results

TRIM66 is overexpressed in CCA tissues and cell lines

We first analyzed the expression of TRIM66 in CCA tissues using RT-qPCR and western blot analysis. The results showed that the mRNA and protein expression levels of TRIM66 in CCA tissues were significantly higher than in the matched normal tissues (Fig. 1A and 1B). Next, we investigated TRIM66 expression in CCA cell lines (HUCCT1 and QBC939). As shown in Fig. 1C and 1D, TRIM66 was up-regulated in HUCCT1 and QBC939 cell lines versus the normal bile duct epithelial cell line HiBEC.

Knockdown of TRIM66 inhibits the proliferation, migration and invasion of CCA cells

We explored the effect of TRIM66 on CCA cells by its knockdown. The efficiency of transfection by specific shRNA was determined by the western blot analysis. As shown in Fig. 2A, the protein expression of TRIM66 was sharply reduced in HUCCT1 cells after transfection. We next examined the effect of TRIM66 knockdown on CCA cell proliferation, migration and invasion. The MTT assay showed that TRIM66 knockdown significantly decreased the proliferation rate of HUCCT1 cells in comparison with the control cells (Fig. 2B). The wound healing and transwell assays showed that the migratory and invasive abilities of HUCCT1 cells were markedly inhibited after TRIM66 knockdown in comparison with the control group (Fig. 2C and 2D).

Knockdown of TRIM66 reversed the EMT process in CCA cells

We also examined the effect of TRIM66 knockdown on the EMT process. The protein expression of EMT-related markers in CCA cells was measured using the western blot analysis. The results indicated that TRIM66 knockdown significantly increased the expression levels of E-cadherin and α-catenin but decreased the expression levels of N-cadherin and vimentin in HUCCT1 cells (Fig. 3A and 3B).

Knockdown of TRIM66 inhibits the glycolysis of CCA cells

To explore the effect of TRIM66 knockdown on glycolysis in CCA, we compared the differences in metabolic parameters after HUCCT1 cells were transfected with shTRIM66 or shNC. As shown in Fig. 4A and 4B, knockdown of TRIM66 remarkably reduced glucose uptake and lactate production of HUCCT1 cells.

Knockdown of TRIM66 inhibits the activity of PI3K/Akt signaling pathway
The PI3K/Akt signaling pathway has been demonstrated to play a significant role in development of various cancers including CCA [20, 21]. Therefore, we investigated the effect of TRIM66 knockdown on the PI3K/Akt pathway. The results showed that knockdown of TRIM66 obviously decreased the expression levels of phosphorylated PI3K and Akt in HUCCT1 cells (Fig. 5A and 5B).

**Knockdown of TRIM66 inhibits CCA cell proliferation, invasion and glycolysis via the PI3K/Akt signaling pathway**

To further explore the mechanism underlying TRIM66-mediated cell proliferation, invasion and glycolysis, HUCCT1 cells were treated with LY294002 (PI3K/Akt inhibitor). As shown in Fig. 6A-6D, LY294002 treatment potentiated the inhibitory effect of TRIM66 knockdown on HUCCT1 cell proliferation, invasion and glycolysis.

**Discussion**

CCA, one of the most common hepatic tumors, accounts for almost 3% of all gastrointestinal cancers in the world [1]. Patients with CCA suffer from a poor prognosis and high mortality due to a late diagnosis of the disease [3]. Thus, exploration of early and accurate diagnostic approaches is urgently needed for a favorable outcome of CCA patients.

Recently, many studies have demonstrated the potential value of TRIM proteins in cancer progression. For example, Yin et al. reported association of TRIM32 with lung cancer and contribution of TRIM32 overexpression to high proliferative and invasive abilities [22]. Similarly, Tong et al. found that TRIM59 functioned as a powerful driver of tumorigenesis in ovarian cancer [23]. In this study, we showed that TRIM66 was overexpressed in CCA tissues and cell lines and TRIM66 knockdown inhibited CCA cell proliferation, migration and invasion. In consistent with our results, the study by Zhang et al. demonstrated up-regulation of TRIM66 in hepatocellular cancer and the inhibitory effect of TRIM66 silencing on hepatocellular cancer cell proliferation and invasion [18]. Like Zhang, Cao et al. reported that TRIM66 served as a promoter in the malignant progression of prostate cancer [19]. These observations suggested that TRIM66 may play an oncogenic role in cancer progression.

With regard to solid tumors including CCA, cancer cells are characterized by rapid proliferation and abundant metabolic needs and these demands are met upon enhancement of glucose uptake and aerobic glycolysis [6, 7]. In this study, our study found that knockdown of TRIM66 inhibited CCA cell glycolysis by reducing glucose uptake and lactate production. These results showed that glycolysis may play a crucial role in cancer development.

In the subsequent experiments, we explored the mechanism underlying TRIM66-mediated CCA progression. The PI3K/Akt signaling is an essential intracellular signal transduction pathway and functions as an important regulator of various biological functions such as protein synthesis and cell metabolism [24-26]. In addition, activation of the pathway is involved in tumorigenesis of diverse cancers [20, 21]. In this study, we found that knockdown of TRIM66 significantly reduced the
phosphorylation levels of PI3K and Akt in CCA cells. We also found that the suppressive effect of TRIM66 knockdown on CCA cell proliferation, migration, invasion and glycolysis was enhanced after treatment with LY294002 (PI3K/Akt inhibitor). Thus, we reasonably inferred that the PI3K/Akt pathway was part of the mechanisms underlying TRIM66-regulated CCA progression.

In conclusion, the present study was the first to investigate the biological functions of TRIM66 in CCA. We demonstrated that TRIM66 was overexpressed in CCA tissues and cell lines. Moreover, knockdown of TRIM66 inhibited CCA cell proliferation, migration, invasion and glycolysis partly via regulating the PI3K/Akt signaling pathway. Taken together, our study provided a novel insight into the roles of TRIM66 in CCA and suggested the potential value of TRIM66 as a therapeutic target for CCA treatment.

Declarations

Authors’ contributions

LJ and WWY designed the study. YQL and YL performed the experiments and analyzed the data. TY and DSL prepared the manuscript. NL edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Written informed consents were obtained from all participants, and this study was permitted by the Ethics Committee of Jinzhou Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1

TRIM66 is overexpressed in CCA tissues and cell lines. (A, B) TRIM66 mRNA and protein expression were detected in CCA tissues and matched normal tissues. (n=42). (C, D) TRIM66 mRNA and protein expression were detected in CCA cell lines (HUCCT1 and QBC939) and the normal bile duct epithelial cell line HiBEC. *p < 0.05

Figure 2

Knockdown of TRIM66 inhibits the proliferation, migration and invasion of CCA cells. (A) The protein expression of TRIM66 in HUCCT1 cells was measured by western blot analysis after shRNA transfection. (B) HUCCT1 cell proliferation was detected using the MTT assay. (C) HUCCT1 cell migration was
evaluated using the wound healing assay. (D) HUCCT1 cell invasion was examined using the transwell assay. *p < 0.05

**Figure 3**

Knockdown of TRIM66 reversed the EMT process in CCA cells. (A, B) The protein expression of E-cadherin, α-catenin, N-cadherin and vimentin in HUCCT1 cells was measured by the western blot analysis. *p < 0.05

**Figure 4**
Knockdown of TRIM66 inhibits the glycolysis of CCA cells. (A) Glucose uptake was decreased in HUCCT1 cells after TRIM66 knockdown. (B) Lactate production was reduced in HUCCT1 cells after TRIM66 knockdown. *p < 0.05

Figure 5

Knockdown of TRIM66 inhibits the activity of PI3K/Akt signaling pathway. (A) The protein expression of p-PI3K, PI3K, p-Akt and Akt in HUCCT1 cells was assessed using the western blot analysis. (B) Quantification of p-PI3K/PI3K and p-Akt/Akt. *p < 0.05
Figure 6

Knockdown of TRIM66 inhibits CCA cell proliferation, invasion and glycolysis via the PI3K/Akt signaling pathway. (A) Cell proliferation was detected in the presence or absence of LY294002 (20 μM). (B) Cell invasion was measured in the presence or absence of LY294002 (20 μM). (C, D) Glucose uptake and lactate production were determined in the presence or absence of LY294002 (20 μM). *p < 0.05 vs. shNC, #p < 0.05 vs. shTRIM66