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Original Research Article

TRIM 16 gene expression regulates the growth and metastasis of human esophageal cancer

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Abstract

Purpose: *To investigate the effect of tripartite membrane protein (TRIM) 16 gene silencing on human esophageal cancer (KYSE-270) cell proliferation, invasion and metastasis.*
Methods: *Short interfering RNA (siRNA) TRIM 16 silencing fragment was transfected into KYSE-270 cells. Transfection efficiency was determined using real-time quantitative polymerase chain reaction (qRT-PCR). Cell proliferation, invasiveness and migration were measured by 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, Transwell invasion assay, and scratch test, respectively. Protein expressions of bax and bcl-2 were assayed using Western blotting.*
Results: *The gene expression of TRIM 16 was significantly upregulated in esophageal cancer cells, relative to normal human esophageal cells, but was downregulated after gene silencing. Moreover, the silencing of TRIM 16 gene led to significant reductions in KYSE-270 cell viability, migration and invasiveness, but significantly increased KYSE270 cell apoptosis ($p < 0.05$). The silencing of TRIM 16 gene also significantly upregulated bax protein expression, while downregulating the expression of bcl-2 protein ($p < 0.05$).*
Conclusion: *These results suggest that TRIM 16 gene silencing inhibits KYSE-270 cell proliferation, invasion and metastasis, and thus provide a basis for its development as a therapeutic approach for the management of esophageal cancer.*

Keywords: *Apoptosis, Cell invasion, Esophageal cancer, Gene silencing, Metastasis*

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INTRODUCTION

Esophageal cancer is the seventh leading cause of cancer deaths worldwide [1]. The incidence of esophageal cancer varies, depending on the regions of the world, from 30 cases to as high as 800 cases per 100 000 persons [2]. The high mortality associated with esophageal cancer is attributed to poor prognosis and late diagnosis [3]. While squamous cell carcinoma accounts for 95 % of the pathology of esophageal cancers worldwide, adenocarcinomas are becoming an increasingly prevalent in the western world [2]. Strategies commonly employed for the management of esophageal cancer include surgery, chemotherapy, radiotherapy and laser therapy [4]. Moreover, since the 5-year survival is very low, there is need for novel compounds that can effectively alleviate the disease [5]. Over the last one decade, studies have focused

on identification of potent biomarkers for early diagnosis of esophageal cancer [6].

Tripartite motif (TRIM) proteins, a family of about 75 proteins with E3 ligase activities, function in innate immunity, cell proliferation, differentiation, development, carcinogenesis, apoptosis, and autophagy [7]. Several TRIM proteins have been implicated in autophagy where they function as autophagy regulator–receptor. Tripartite motif (TRIM) proteins form a platform for the assembly of core autophagic machinery, including autophagy initiation (ULK1), elongation (ATG16L1), and completion (LC3B) factors, and they facilitate the interaction of the autophagic machinery with specific autophagic cargo [8]. Tripartite membrane protein 16 (TRIM 16) interacts with galectin-3 and recognizes damaged endo-membranes, leading to the assembly of autophagy machinery for selective sequestration of damaged lysosomes and phagosomes [7]. More recently, TRIM 16 has been shown to be involved in secretory autophagy where it interacts with SNARE proteins to recruit cargoes to autophagosomes [7]. Although the role of TRIM 16 has been determined in various human cancers, little or nothing is known about its role in human esophageal cancer [9,10]. The aim of this study was to investigate the effect of TRIM 16 gene silencing on KYSE-270 cell proliferation, invasion and metastasis.

EXPERIMENTAL

Materials

Bradford protein assay kit, RPMI-1640 medium, TRIZOL reagent, DNase I, SYBR Green QuantStudio (5.0) real-time system and lipofectamine 2000 were obtained from Thermo Fisher Scientific Co. (USA).

Short interfering RNA (siRNA) TRIM 16 silencing fragment was bought from RiboBio, (China). MiScript reverse transcription kit was a product of Qiagen (USA). Rabbit anti-human bax, bcl-2, β-actin and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG were purchased from Cell Signaling Technology Co. Ltd. (USA), while fetal bovine serum (FBS) and Applied Biosystems 7300 RT- PCR machine were products of Life Technologies (USA).

Cell lines and culture

Normal human esophageal cell line (HET-1A) and four different esophageal cancer cell lines (ESO26, ESO51, KYSE-270, KYSE-30) were purchased from American Type Collection Center (ATCC, USA). The cells were cultured in RPMI-1640 medium supplemented with 10 % FBS and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air until the cells attained 80 % confluency. The medium was replaced with fresh one every two days. After 1 week of incubation, the adherent confluent cells were trypsinized with 0.25 % trypsin-EDTA (2 mL), cultured again, and passaged for later use. Cells in logarithmic growth phase were selected and used in this study.

Cell transfection

Esophageal cancer (KYSE-270) cells were seeded in 6-well plates at a density of 2 x 10^4 cells/well until they attained 80 % fusion. The cells were subsequently cultured in serum-free medium with equal volume of siTRIM 16 (TRIM 16 silenced group) or siNC (negative control group), each at 10 µmol/L. Incubation was carried out at room temperature for 6 h. Lipofectamine 2000 was dissolved in serum-free medium and incubated at room temperature for 10 min to form a mixture. The mixture was then added to cells in each group, and cultured at 37 ^oC in a humidified atmosphere of 5 % CO_2 and 95 % air for 48 h. Normal cell culture without siTRIM 16 or siNC served as normal control group. The transfection efficiency was determined using qRT-PCR.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

After 48 h of cell transfection, mRNA expression of TRIM 16 was determined using qRT-PCR. Trizol RNA extraction reagent was used to extract total RNA from cells of each group, while cDNA synthesis kit was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for the estimation of the expression level of TRIM 16. Variation in the cDNA content was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The qRT-PCR reaction conditions were: pre-denaturation at 95 ^oC for 5 min, PCR reaction at 95 ^oC for 5 sec and 60 ^oC for 30 sec, and a total of 40 cycles. The PCR reaction mixture (20 µL) consisted of 6.4 µL of dH_2O, 1.6 µL of gene-specific primer (10 µM), 2 µL of synthesized cDNA, and 10 µL of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter, and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression level of the gene. The primers used for qRT-PCR are shown in Table 1.

Table 1: Sequence of primers used for qRT-PCR

Gene	Direction	Primer sequence
TRIM 16	Forward	5'-TGGCCGAGCTTCCTCTGGGA-3'
	Reverse	5'-ATGAATGGTCCCCAAGCACTC-3'
GAPDH	Forward	5' -GTCTCCTCTGACTTCAA-3'
	Reverse	5' ACCACCCTGTTGCTGT-3'

Cell viability assay

The viability of KYSE-270 cells transfected with siNC or siTRIM 16 was determined using MTT assay. After 24 h of transfection, KYSE-270 cells were seeded in 96-well plates at a density of 2 x 10^5 cells/well and cultured in Dulbecco's modified Eagle medium (DMEM) for 24 h. Then, 20 µL of 5 g/L MTT solution was added to the wells, followed by incubation for another 4 h. The medium was finally replaced with 150 µL of 0.1 % dimethyl sulfoxide (DMSO) solution, agitated at 50 oscillations/min for 10 min, and absorbance of the samples was read in a microplate reader at 570 nm. Cell viability was calculated as shown in Equation 1:

Cell viability (%) = (Ae/Ac)100 …….. (1)

where Ae and Ac are the absorbance of the samples derived from the experimental and control groups, respectively.

Apoptosis assay

The KYSE-720 cells transfected with siNC or siTRIM 16 were seeded at a density of 0.6 x 10^5 cells/well in 6-well plates and cultured for 24 h. The cells were thereafter washed with phosphate-buffered saline (PBS), and thoroughly mixed with 300 µL binding buffer. The cells were then stained with 5 µL each of Annexin V-fluorescein isothiocyanate and propidium iodide within 25 min at room temperature in the dark. The DAPI-stained cells were examined under a fluorescent microscope. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 485 nm.

***In vitro* cell invasion assay**

The degree of invasiveness of KYSE-270 cells transfected with siNC or siTRIM 16 was determined using Transwell invasion assay. The cells were placed in Transwell chamber coated with substrate at a concentration of 5 × 10^5 cells/mL, and were cultured in serum-free medium. The lower chamber contained medium with 10 % FBS. After 48 h, cells that passed through the matrix gel membrane were fixed and stained with crystal violet, followed by photographing and counting using an inverted microscope.

***In vitro* cell migration assay (scratch test)**

Esophageal cancer (KYSE-270) cells in logarithmic growth phase were seeded in 6-well plates until they attained 80 % confluency, and scratches were made on the cell monolayers. After washing thrice with serum-free medium, the cells were further cultured for 24 h, and then observed and photographed.

Western blotting

The protein expressions of bax and bcl-2 in KYSE-270 cells were assayed using Western blotting. The cells were washed with PBS and lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitor. The resultant lysate was centrifuged at 15, 000 rpm for 15 min at 4 °C, and the protein concentration of the supernatant was determined using Bradford protein assay kit. A portion of total cell protein (30 µg) from each sample was separated on 8 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C, and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies of bax, bcl-2 and β-actin, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1.5 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Bio-Rad gel imaging system. The respective protein expression levels were normalized to that of standard β-actin.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad prism (7.0). Groups were compared using Student's *t*-

test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Expression levels of TRIM 16 gene in normal esophageal and cancer cells

The mRNA expression of TRIM 16 was significantly higher in esophageal cancer cells than in normal human esophageal cells, and was also higher in KYSE-270 cells than in the other three esophageal cancer cell lines ($p < 0.05$). However, siRNA silencing of TRIM 16 gene led to the downregulation of TRIM 16 mRNA expression and reduction in the viability of KYSE-270 cells ($p < 0.05$). These results are shown in Figure 1.

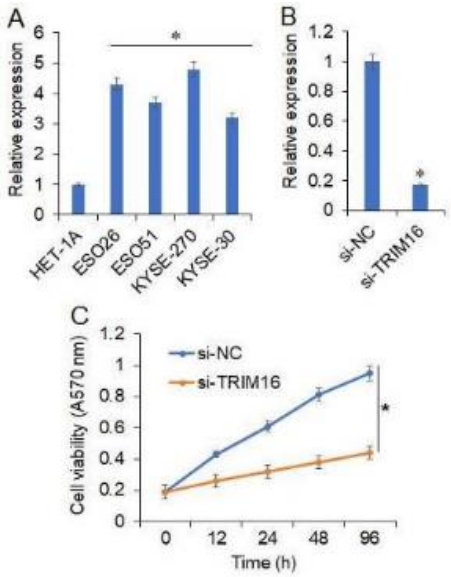

Figure 1: Levels of expression of TRIM 16 gene in normal and esophageal cancer cells. (A): Levels of expression of TRIM 16 mRNA in normal and esophageal cancer cells; (B): mRNA expression of TRIM 16 in KYSE-270 cells transfected with siNC or siTRIM 16; and (C): Viability of KYSE270 cells transfected with siNC or siTRIM 16, as measured using MTT assay. *P < 0.05, compared with control group

Effect of TRIM 16 gene silencing on KYSE270 cell apoptosis

As shown in Figure 2, TRIM 16 gene silencing (transcriptional repression) significantly increased KYSE270 cell apoptosis ($p < 0.05$). There were more apoptotic cells in TRIM 16 silenced group than in control group ($p < 0.05$).

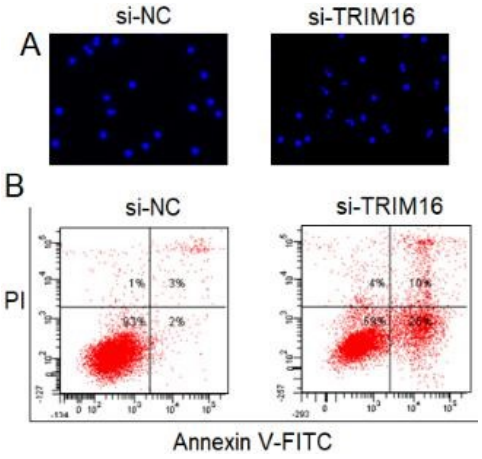

Figure 2: Effect of TRIM 16 gene silencing on KYSE270 cell apoptosis. **(A):** KYSE-270 cell apoptosis, as measured via DAPI staining; **(B):** Flow cytometric analysis of apoptosis in KYSE-270 cells

Effect of TRIM 16 gene silencing on the levels of expression of apoptosis-related proteins in KYSE-270 cells

The silencing of TRIM 16 gene significantly upregulated bax protein expression, but it downregulated the expression of bcl-2 protein ($p < 0.05$). Moreover, it significantly increased bax/bcl-2 protein ratio ($p < 0.05$; Figure 3 and Figure 4).

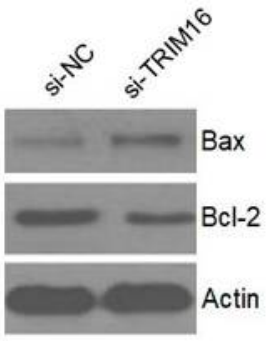

Figure 3: Effect of TRIM 16 gene silencing on the levels of expression of apoptosis-related proteins in KYSE-270 cells

Effect of TRIM 16 gene silencing on metastasis of esophageal cancer cells

As shown in Figure 4 and Figure 5, TRIM 16 gene silencing significantly reduced the migratory and invasive capabilities of KYSE-270 cells ($p < 0.05$). The percentage of migrated and/or invaded cells was reduced 5 folds in TRIM 16 silenced group.

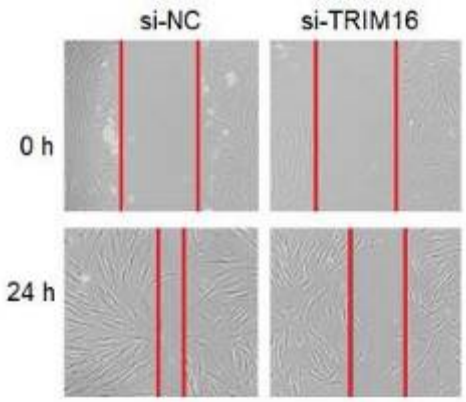

Figure 4: Effect of TRIM 16 gene silencing on KYSE-270 cell migration. Wound healing assay showing the migration of KYSE-270 cells

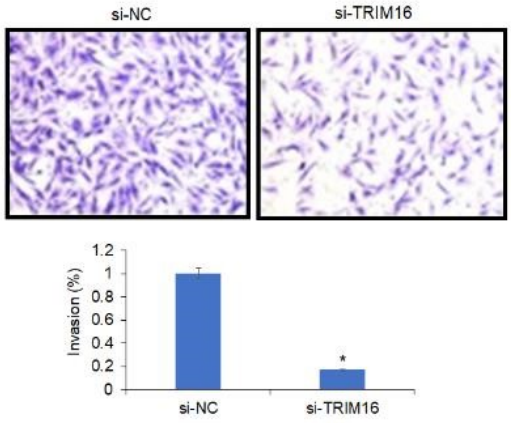

Figure 5: Effect of TRIM 16 gene silencing on KYSE-270 cell invasion. Transwell invasion assay showing the invasion of KYSE-270 cells; $^{*}p < 0.05$, compared with control group

DISCUSSION

The incidence of esophageal cancer is on the rise with over 12,000 Americans developing this disease each year. The incidence of esophageal cancer varies with age, sex, and race [11]. Advances in medical and surgical therapy have led to improvements in the survival of patients. However, improvement in survival is dependent on a better understanding of the relationship between environmental factors and the disease itself [12]. The incidence of esophageal cancer fluctuates throughout various regions of the world, and it has the largest variability of any known malignancy. High rates are found in people living in northeast China, north central Asia, Afghanistan and northern Iran. Esophageal cancer is ranked the sixth most deadly cancer in terms of overall mortality caused by cancers at the global level [2]. Esophageal cancer may be classified according to the site of origin. The malignancy may originate in squamous cells, or in columnar cells that line the esophageal lumen. Squamous cell carcinoma may occur throughout the length of the esophagus, whereas adenocarcinoma generally occurs just above the esophagogastric junction [13].

Dysregulation of different genes and proteins have been implicated in the pathogenesis of cancers [14]. One of such genes is TRIM 16 [15,16]. Tripartite motif (TRIM) proteins function in innate immunity, cell proliferation, differentiation, development, carcinogenesis, apoptosis, and autophagy [7,8]. Moreover, TRIM 16 gene has been shown to function as a dimeric unit [17]. The aim of this study was to investigate the effect of TRIM 16 gene silencing on KYSE-270 cell proliferation, invasion and metastasis. The results showed that the gene expression of TRIM 16 was significantly upregulated in esophageal cancer cells, when compared to normal human esophageal cells, but it was downregulated after gene silencing. These results indicate that TRIM 16 may play a role in the pathogenesis of esophageal cancer, and they are in agreement with those of previous reports [15-17]. The results obtained in this study suggest that TRIM 16 gene silencing-induced reduction in the viability of human esophageal cancer cells may be partly due to induction of apoptosis. The regulation of apoptosis by TRIM proteins is well established [18]. Increased bax/bcl-2 protein ratio is a positive signal for eukaryotic cell apoptosis [19]. In this study, TRIM 16 gene silencing significantly upregulated bax protein expression, while it downregulated the expression of bcl-2 protein.

Metastasis is a major biochemical behavior of cancer cells. Moreover, most strategies employed in the treatment of cancer exert their effects via inhibition of metastasis [20]. The results of scratch test and Transwell invasion assay showed that TRIM 16 gene silencing significantly reduced the migratory and invasive capabilities of KYSE-270 cells. It significantly reduced KYSE-270 cell clones, an indication that the silencing of TRIM 16 gene may inhibit esophageal cancer cell metastasis and invasiveness.

CONCLUSION

The results of this study suggest that TRIM 16 gene silencing inhibits KYSE-270 cell proliferation, invasion and metastasis. Thus, the identification of drug molecules that can target TRIM 16 gene could pave way for the

development of effective treatments for esophageal cancer.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The whole study was designed and supervised by Meihua Wang. The manuscript was drafted by Yufang Chen. The experiment was performed by Yufang Chen, Ziyan Li, Jingyao Zeng, Zhiyi Xu. The data was mainly collected by Jingyao Zeng, Zhiyi Xu.

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