

79440_Auto_Edited.docx

Name of Journal: *World Journal of Gastrointestinal Oncology*

Manuscript NO: 79440

Manuscript Type: ORIGINAL ARTICLE

Basic Study

E3 Ubiquitin Ligase TRIM55 promotes metastasis of gastric cancer cells by mediating epithelial-mesenchymal transition

TRIM55 and gastric cancer

Abstract

BACKGROUND

Gastric cancer is considered a major global health problem. The role of tripartite motif containing 55 (TRIM55), a member of the TRIM protein family, in gastric cancer (GC) is unknown.

AIM

To determine the expression of TRIM55 in GC tissues and its relationship with clinicopathological characteristics, and to investigate the effects of TRIM55 on the malignant biological behavior of GC cells.

METHODS

Differential expression of TRIM55 in GC and para-cancer tissues was detected by immunohistochemistry, and the relationship between TRIM55 Level and clinicopathological characteristics and prognosis was analyzed. Gain-of-function, loss-of-function, CCK-8 assay, colony formation, transwell assay, wound healing assay, and western blot analysis were used to assess the potential role of TRIM55 in the development of GC.

RESULTS

TRIM55 expression was significantly increased in GC tissues compared with adjacent normal tissues. High expression of TRIM55 was associated with advanced pathological stage and poor prognosis. Overexpression of TRIM55 promoted invasion and metastasis of GC cells *in vitro* by regulating epithelial-mesenchymal transition (EMT), whereas knockdown of TRIM55 had the opposite effect. Our data showed that TRIM55 is highly expressed in GC tissues, and is associated with poor prognosis. TRIM55 plays the role of an oncogene in GC, and it promotes metastasis of GC through the regulation of EMT.

CONCLUSION

TRIM55 may be a possible target for the diagnosis and prognosis of GC patients.

Key Words: Tripartite motif containing 55; gastric cancer; prognosis; Epithelial-Mesenchymal Transition

Li WW, Yuan H, Kong S, Tian SB. E3 Ubiquitin Ligase TRIM55 promotes metastasis of gastric cancer cells by mediating epithelial-mesenchymal transition. *World J Gastrointest Oncol* 2022; In press

Core Tip: TRIM55 expression was elevated in GC cancer tissues. Depletion of TRIM55 in GC cells suppressed proliferation, migration and invasion of cells. Knockdown of TRIM55 affected the expression of cell EMT-related proteins. TRIM55 may serve as an oncogene in GC.

17

INTRODUCTION

Gastric cancer (GC) is one of the most common tumors and seriously affects patients' health^[1]. Comprehensive treatment based on surgery is the preferred strategy for GC ^[2]. Postoperative metastasis is the leading cause of death from GC; however, the mechanisms underlying the occurrence and the development of GC have not been fully elucidated, and there is a lack of effective markers for early diagnosis^[3]. Therefore, identifying molecular markers of GC is important for the early diagnosis, treatment, and prognostic evaluation of GC. The three-domain protein (TRIM) family is composed of many members, including the tripartite motif, which consists of a RING domain, 1 or 2 Box motifs, and a coiled-coil region (RBCC)^[4]. Some TRIM proteins are involved in the regulation of cellular transcription, cell proliferation, and tumor development; thus, they play a role in either promoting or inhibiting cancer^[5]. The structural diversity of TRIM family proteins underpins their functional diversity. TRIM55, also known as muscle-specific RING zinc finger protein 2 (Murf 2), maintains muscle development

and cardiac function. TRIM55 plays an important role in early skeletal muscle differentiation and the generation of muscle fibers^[6]. Studies have shown that mir-30-5p can inhibit muscle cell differentiation and regulate the alternative splicing of TRIM55 by targeting Muscleblind-like Protein (MBNL)^[7]. TRIM55 can regulate the TNF- α -CCL2 pathway and promote an inflammatory response in the development of mesangial proliferative glomerulonephritis^[8]; however, the role of TRIM55 in GC has not been fully elucidated.

In this study, we determined the differential expression of TRIM55 in GC patients and investigated the relationship between TRIM55 and clinicopathological characteristics. We found that TRIM55 induced proliferation, migration, and invasion of GC cells. We also investigated the mechanism underlying these effects.

MATERIALS AND METHODS

Tissue samples

Tissues were obtained from 91 GC patients admitted to the Department of Gastrointestinal Surgery of Shandong Provincial Hospital Affiliated to Shandong First Medical University between July 2014 and December 2015. The tumor tissue samples and adjacent normal gastric mucosal tissue samples were validated by pathologists. Of the 91 patients, 61 were male and 30 were female, with an average age of 63.6 years. Data such as gender and age of the patient, tumor location, tumor pathological stage, and lymph node metastasis, were also collected. In addition, five fresh GC tissue samples and matched adjacent normal gastric tissues were obtained from our hospital. The research protocols were approved by the Ethics Committee of the Provincial Hospital Affiliated to Shandong First Medical University, and all the patients signed an informed consent form before surgery.

Cell culture and transfection

Human GC cell lines (AGS, MKN28, MGC803, SGC7901, HGC27, and MKN45) and the immortalized gastric mucosa cell line (GES-1) were provided by the Cell Center of the Chinese Academy of Medical Sciences. All the cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Gibco, USA). Cells were cultured in an incubator at 37°C and 5% CO₂. According to the CDS coding sequence of TRIM55, small interfering RNAs (siRNA) to knock down TRIM55 were designed and synthesized by RiboBio (Guangzhou, China), and the scramble nonsense sequence was used as the negative control (si-control). The targeting TRIM55 siRNA sequence was as follows: TRIM55 siRNA, 5'-AUCAAACUUCUCACAAAGCUC-3'. The control siRNA was not homologous to any human genome sequence. For the overexpression assay, the coding sequence of TRIM55 was amplified and cloned into a pcDNA3.1-HA vector to construct the TRIM55 overexpression system (TRIM55 plasmid). The pcDNA3.1-HA empty vector was used as the negative control in HGC27 cells.

Immunohistochemical staining

The GC tissue and adjacent tissue were embedded in paraffin and sliced into 4-μm-thick sections. Xylene was used for dewaxing and citrate buffer was used for antigen repair. After washing with PBS 4 times, the samples were sealed with BSA blocking solution at 37°C. TRIM55 primary antibody (Novus, USA) was added and incubated overnight at 4°C. According to the instructions of the immunohistochemical test kit, the secondary antibody was added and incubated for 20 min at room temperature. Then, streptavidin-peroxidase conjugate was added and the samples were incubated for 20 min. DAB was used for staining and hematoxylin was used for redyeing and dehydration. The sections were observed and photographed under a microscope and analyzed with Image analysis software. According to the staining intensity, no staining was scored as 0, light yellow was scored as 1, brownish yellow was scored as 2, and brown was scored as 3. According to the percentage of positive cells, the score for no positive cells was 0, 1%-10% was 1, 11%-50% was 2, 51-75% was 3, and 76%-100% was 4. The expression of TRIM55 was considered to be high if the multiple of the two scores was ≥ 5.

Quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed to cDNA using a reverse transcription kit (Takara, Dalian, China) following the manufacturer's instructions. The quantitative real-time polymerase chain reaction (qRT-PCR) three-step method was used, and the $2^{-\Delta\Delta C_t}$ method was used to calculate the relative expression levels of TRIM55. GAPDH was used as the endogenous control. The upstream primer sequence of TRIM55 was 5'-GGTTTGGATAGACATGGGGT-3', and the downstream primer sequence was 5'-TTCTCCTCTTGGGTTCGGGT-3'.

Cell counting kit-8 (CCK-8) assay

Cells were seeded in 96-well plates (2000 cells/well) and placed into an incubator for further culture. Ten microliters of CCK-8 reagent (Dojindo, Mashikimachi, Japan) was added to each well on days 1, 2, 3, and 4. After 4 h incubation, the absorbance value at 450 nm (OD450) was measured with a microplate reader. The growth curve of cells was drawn according to the OD value. Three independent assays were performed for each time point.

Colony formation assay

Two thousand cells/well were plated in a six-well plate and allowed to grow in complete growth medium for 14 days. The colonies were then fixed with 4% paraformaldehyde for 15 min, followed by staining with 0.1% crystal violet solution for 10 min. The number of colonies was counted, and the average value was calculated from three independent experiments.

Transwell migration and invasion assay

GC cells at the logarithmic growth stage were trypsinized and suspended in serum-free media at a concentration of 1×10^6 cells/mL. Two hundred microliters of the cell suspension was added to the upper transwell chamber. Culture medium containing

10% FBS was added to the lower chamber. For the invasion assay, the transwell membrane was coated with Matrigel. After 48 h incubation, the cells that did not pass through the membrane in the upper chamber were removed with cotton swabs. The migrated/invaded cells were fixed with paraformaldehyde for 30 min and stained with 0.1% crystal violet solution. The cells were observed under a microscope and five random fields were selected to count the number of migrated or invaded cells.

Wound healing assay

GC cells were inoculated into a six-well plate at a density of 5×10^5 cells/well until 90% confluence was reached. Then, a sterile 100- μ L pipette tip was used to scrape the bottom surface of the plate to form a wound vertically. The cells were washed with $1 \times$ PBS, and complete growth medium was added. The wound margins were observed at 0 and 24 h in 5 randomly selected microscopic regions, and the mean cell spacing was calculated.

Western blot assays

GC cells were collected and lysed in immunoprecipitation lysis buffer. After protein quantification by the BCA method, 30 μ g of protein sample was used for electrophoresis. After transfer and blocking, the membrane was incubated overnight at 4°C with the primary antibody. Horseradish peroxidase-labeled secondary antibody was added and incubated for 1 h. The bands were visualized using chemiluminescence reagent.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 7.0 (CA, USA). All the data are represented as mean \pm standard deviation from three independent experiments. Differences among groups were compared using student's t-test and one-way analysis of variance (ANOVA). The prognostic factors were analyzed by univariate and multivariate Cox regression models. The Kaplan-Meier method was used to calculate the survival rate, and the log-rank test was used to compare different

survival curves. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

TRIM55 is highly expressed in GC samples

Immunohistochemical results showed that TRIM55 was mainly expressed in the nucleus. Figure 1 A-D shows the representative immunohistochemical staining images of TRIM55 in normal gastric mucosa tissue and cancer tissue. The expression of TRIM55 was significantly high in 91 GC tissues and low in para-cancer tissues (Table 1, $P < 0.001$). There was no significant correlation between TRIM55 expression and age at diagnosis, gender, tumor location, or Lauren type ($P > 0.05$, Table 2). However, a significant relationship was found between TRIM55 expression and tumor stage, lymph node metastasis, and T stage ($P < 0.05$, Table 2).

To further confirm the results, we performed western blotting using five GC specimens and matched normal tissues. The results showed that protein levels of TRIM55 in GC tissues were up-regulated compared to non-tumor tissues (Figure 1E).

Relationship between TRIM55 expression and prognosis of GC

The 5-year survival rate of patients with high TRIM55 expression was lower than that of those with low expression ($P = 0.026$, Figure 1F). The median overall survival of patients with high and low expression of TRIM55 was 34 mo (95%CI 13.3–62.8) and 57 mo (95%CI 25.5–84.5), respectively. Similarly, patients with high TRIM55 gene expression had a poor prognosis in the TCGA database (Figure 1G). Univariate Cox regression analysis showed that T stage, lymph node metastasis, TNM stage, and TRIM55 expression level were the influencing factors for the prognosis of GC patients (Table 3). Further multivariate Cox regression analysis showed that lymph node metastasis, TNM stage, and TRIM55 expression level were independent factors affecting the prognosis of GC (Table 3).

TRIM55 protein is upregulated in GC cell lines

The protein level of TRIM55 was analyzed in the immortalized gastric mucosal epithelial cell line GES-1 and six GC cell lines (AGS, MKN28, MGC803, SGC7901, HGC27, and MKN45). The results showed that the expression level of TRIM55 in GC cell lines was significantly higher than that in GES-1 cells (Figure 2A). Among them, the expression level of TRIM55 was highest in SGC7901 and lowest in the HGC27 cell line.

Transfection efficiency of TRIM55 siRNA and TRIM55 overexpressed plasmid

To analyze the effects of TRIM55 knockdown, SGC7901 (cells with high TRIM55 expression) GC cells were transfected with TRIM55 siRNA. The results showed that both mRNA and protein expressions of TRIM55 were significantly lower in cells transfected with TRIM55 siRNA compared to those in the control group (Figure 2B-C). HGC27 cells with low endogenous TRIM55 expression were transfected with TRIM55 overexpressed plasmid to determine the effects. We found that cells transfected with the overexpressed plasmid had higher mRNA and protein levels of TRIM55 compared to those transfected with the empty vector (Figure 2D-E).

TRIM55 promotes proliferation of GC cells

The CCK-8 assay was used to determine the proliferation of GC cells. The results showed that SGC7901 cell proliferation was significantly inhibited on days 2, 3, and 4 after TRIM55 knockdown ($P < 0.001$, Figure 3A). However, the proliferation of HGC27 cells was significantly increased on days 2, 3, and 4 ($P < 0.001$, Figure 3B) when TRIM55 was overexpressed in these cells. These results suggest that TRIM55 can promote the proliferation of GC cells.

The colony formation assay showed that the colony number was significantly reduced after siRNA transfection in SGC7901 cells (Figure 3C). Similarly, overexpression of TRIM55 in HGC27 cells enhanced the ability of cells to form colonies (Figure 3D).

TRIM55 promotes invasion and migration of GC cells

¹ The transwell migration and invasion assay results showed that TRIM55 knockdown significantly reduced the migration and invasion of SGC7901 cells (Figure 4A). Furthermore, the number of migrated and invaded HGC27 cells was significantly increased after TRIM55 overexpression (Figure 4B). The wound-healing assay revealed that knockdown of TRIM55 significantly impaired the migration ability of SGC7901 cells (Figure 5A), while HGC27 migration ability was enhanced after overexpression of TRIM55 (Figure 5B). These results suggest that TRIM55 could promote the invasion and migration of GC cells.

TRIM55 promotes the migration and invasive growth of GC cells by inducing EMT.

¹ To further explore the potential mechanism of how TRIM55 promotes the progression of GC, we analyzed the expression of EMT-related proteins by western blot analysis. The results showed that in the SGC7901 cell line, E-cadherin expression was significantly up-regulated after TRIM55 knockdown, while N-cadherin, Vimentin, ZEB1, and Snail expression were significantly down-regulated compared with the control group (Figure 6A). Furthermore, the opposite results were observed when TRIM55 was overexpressed in the HGC27 cell line (Figure 6B). These results confirmed that TRIM55 promotes the invasion and metastasis of GC cells by inducing EMT.

DISCUSSION

¹⁵ TRIM55 belongs to the TRIM protein family, which plays an important role in the development and progression of tumors. TRIM proteins have three characteristic domains and function as an E3 ligase; they also mediate ubiquitination and regulate intracellular pathophysiological and tumor-related processes by degrading target molecules. TRIM family members exhibit oncogenic and tumor-suppressive capacities in different human cancer types by regulating signal transduction pathways^[9, 10]. ⁷ Epithelial-mesenchymal transition (EMT) is a transitional process in which epithelial

cells lose intercellular adhesion and polarity, and they acquire mesenchymal cell characteristics, enhanced cell motility, and migration ability^[11, 12].

The progression of EMT is regulated by translational factors and epigenetic modification^[13]. Also, microRNAs and long non-coding RNAs are also involved in EMT regulation as post-translational regulators^[14]. The tumor cells or other stromal cells can secrete exosomes. Exosomes are extracellular vesicles with a lipid bilayer containing proteins, lipids and functional RNAs, which can transfer information between tumor cells or between tumor cells and the tumor microenvironment, thereby regulating the EMT process^[15, 16]. As EMT plays essential physiological roles, EMT-targeted therapy combined with conventional chemotherapy can improve the sensitivity of tumor cells to drugs.

TRIM proteins were found to be associated with EMT in various types of cancer. TRIM11 protein was upregulated in GC tissue and cell lines, and it could promote cell proliferation, migration, invasion, and EMT of GC by activating β -catenin signaling^[17]. TRIM47 mainly influenced the EMT signaling pathway, was highly expressed in GC, and was associated with poor prognosis of patients^[18]. In GC, TRIM44 expression was also increased in GC tissues and cell lines, and it regulated GC cell metastasis by altering the expression of EMT-associated factors^[19].

Previous studies have demonstrated that the role of TRIM55 in tumors is tissue specific. For example, a study revealed that TRIM55 expression was significantly suppressed in lung adenocarcinoma tissues and tumor cells. TRIM55 exerted its tumor-suppressive effect by increasing the degradation of Snail protein *via* ubiquitination^[20]. TRIM55 was downregulated in hepatocellular carcinoma (HCC) tissue and associated with tumor stage and poor prognosis. Overexpression of TRIM55 can suppress the migration and invasion of HCC cells through EMT and the MMP2 pathways^[21].

To the best of our knowledge, our study is the first to determine the expression levels and biological functions of TRIM55 in GC. We demonstrated that TRIM55 could be a potential new biomarker for diagnosing and evaluating GC patients. First, we performed immunohistochemical staining, and the results showed that TRIM55 was

highly expressed in GC tissues and that TRIM55 expression was related to the T stage, lymph node metastasis, and TNM stage of GC. Survival analysis showed that the 5-year survival rate of GC patients with high expression of TRIM55 was significantly reduced. Cox regression analysis also confirmed that TRIM55 expression was an independent prognostic factor in GC. These results suggest that TRIM55 may be involved in the occurrence and development of GC. Furthermore, to confirm the biological functions of TRIM55 in GC, we performed a gain and loss of function experiment *in vitro*. Results from the CCK-8 assay, colony formation assay, transwell, and wound healing assays indicated that inhibition of TRIM55 decreases the proliferation and invasion of GC cells, whereas the overexpression of TRIM55 promotes these processes in GC cells. Finally, we used western blot analysis to confirm that knockdown or overexpression of TRIM55 could alter the EMT-related protein, suggesting that TRIM55 could regulate the EMT process. TRIM proteins could serve the ubiquitination function to stabilize or dislocate target proteins in various cellular compartments^[4]. Ubiquitination is a post-transcriptional modification that labels the target proteins to be degraded at the proteasome level. Thus, TRIM family members determine both tumor suppressor and oncogenic roles by affecting the signal pathways in cancer development and progression. For example, TRIM29 and TRIM8 exhibited contextual function in different cancers^[22-24]. They negatively or positively regulate tumorigenesis and tumor progression by affecting pathways. In our study, we showed that TRIM55 is highly expressed in gastric tumors and cultured tumor cells. TRIM55 has E3 ubiquitin ligase activity and whether it can regulate the EMT-related proteins through ubiquitination requires further investigation.

However, some limitations exist in our study. First, the detection of TRIM55 was based on a single-center clinical cohort, and the functional experiments were only performed *in vitro*. Future studies should enroll more patients and utilize animal models to confirm our conclusions. Second, TRIM55 has E3 ubiquitin ligase activity and whether it can regulate the EMT-related proteins through ubiquitination requires further study.

CONCLUSION

In summary, our study analyzed the expression of TRIM55 in GC and demonstrated that TRIM55 could promote GC cell proliferation, migration, and invasion *via* the EMT process. Overexpression of TRIM55 could be an independent factor predicting poor survival, and TRIM55 may serve as a potential therapeutic target for GC. In addition, whether TRIM55 can affect EMT through other molecular mechanisms remains to be examined in future studies.

ARTICLE HIGHLIGHTS

Research background

TRIM55 plays important role in hepatocellular carcinoma and lung adenocarcinoma. However, little is known about the role of TRIM55 in gastric cancer.

Research motivation

To discover the targets for the diagnosis, treatment and prognosis prediction of GC.

Research objectives

To explore the biological function of TRIM55 and its underlying molecular mechanism in GC.

Research methods

The expression of TRIM55 was determined by qRT-PCR and Western blot. CCK-8 assay, colony formation, wound healing assay and transwell assay were used to investigate the TRIM55 function.

Research results

TRIM55 expression levels were significantly increased in GC cell lines and tissues. High expression of TRIM55 was correlated with poor prognosis of GC patients. Knockdown

of TRIM55 in GC cell lines inhibited proliferation, colony formation, migration and invasion in vitro. TRIM55 can regulate the expression of EMT-related proteins in GC cells.

Research conclusions

TRIM55 functions as an oncogene through promoting cell proliferation, migration and invasion in GC.

Research perspectives

TRIM55 may be a new potential target in GC treatment.

13%

SIMILARITY INDEX

PRIMARY SOURCES

- | | | |
|---|--|-----------------|
| 1 | www.ncbi.nlm.nih.gov
Internet | 168 words — 5% |
| 2 | pesquisa.bvsalud.org
Internet | 33 words — 1% |
| 3 | f6publishing.blob.core.windows.net
Internet | 31 words — 1% |
| 4 | Linlin Chen, Congwen Jin, Hao Liu, Rongmei Feng, Zhengdong Li, Jiasheng Zhang. "Analysis of the role of Ly-1 antibody reactive in different cancer types", Bioengineered, 2021
Crossref | 30 words — 1% |
| 5 | www-x-bio-bigdata-x-net.img.abc188.com
Internet | 26 words — 1% |
| 6 | Yue Chen, Liang Li, Xiaoxing Qian, Yongsheng Ge, Geliang Xu. "High expression of TRIM11 correlates with poor prognosis in patients with hepatocellular carcinoma", Clinics and Research in Hepatology and Gastroenterology, 2017
Crossref | 18 words — 1% |
| 7 | ojs.ptbioch.edu.pl
Internet | 17 words — < 1% |
| 8 | www.mdpi.com
Internet | |

17 words — < 1%

9 jhoonline.biomedcentral.com
Internet

16 words — < 1%

10 Chengyong Wang, Qinglian Wang, Zuquan Weng. "LINC00664/miR - 411 - 5p/KLF9 feedback loop contributes to the human oral squamous cell carcinoma progression", Oral Diseases, 2021
Crossref

15 words — < 1%

11 Juntao Sun, Tianyu Zhang, Mengmeng Cheng, Liwen Hong et al. "TRIM29 facilitates the epithelial-to-mesenchymal transition and the progression of colorectal cancer via the activation of the Wnt/ β -catenin signaling pathway", Journal of Experimental & Clinical Cancer Research, 2019
Crossref

15 words — < 1%

12 s3.amazonaws.com
Internet

14 words — < 1%

13 Amir Hosein Jafarian, Melika Kooshki Forooshani, Leila Takallou, Nema Mohamadian Roshan. "CD10 expression in gastric carcinoma is correlated with tumor grade and survival", Universa Medicina, 2019
Crossref

13 words — < 1%

14 Guo Zhao, Chuan Liu, Xin Wen, Gan Luan, Longxiang Xie, Xiangqian Guo. "The translational values of TRIM family in pan-cancers: from functions and mechanisms to clinics", Pharmacology & Therapeutics, 2021
Crossref

13 words — < 1%

15 www.pubfacts.com
Internet

13 words — < 1%

16

"Posters", Journal of Digestive Diseases, 2017

Crossref

12 words — < 1%

17

www.spandidos-publications.com

Internet

12 words — < 1%

EXCLUDE QUOTES

ON

EXCLUDE SOURCES

< 12 WORDS

EXCLUDE BIBLIOGRAPHY

ON

EXCLUDE MATCHES

< 12 WORDS