Original Article

TRIM29 promotes gastric cancer cell proliferation, migration, invasion and predicts the prognosis of patients

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Abstract: Objectives Gastric cancer (GC) is the fourth most common cancer and second leading cause of cancer deaths worldwide. Tripartite motif-containing 29 (TRIM29), a member of the TRIM family of transcription factors, exhibits a contextual function in cancer. The present study aimed to study the expression, function and prognosis significance in GC. Methods Real-time PCR and Western blot were performed to analyze the expression profiles of TRIM29 in GC samples and cell lines. Kaplan-Meier analysis was done to reveal the relationship of TRIM29 expression with overall survival of the patients. Cell-based assays including MTT and flow cytometry and subcutaneous tumorigenicity were performed to investigate the function of TRIM29 in gastric cancer cell proliferation. Transwell assays were performed to investigate the function of TRIM29 in cell migration and invasion. Real-time PCR and Western blot were finally used to explore the mechanism. Results TRIM29 was upregulated in GC samples and cell lines and higher levels of TRIM29 were associated with poor overall survival of patients. RNAi guided TRIM29 knockdown significantly inhibited GC cell proliferation, induced cell cycle arrest, inhibited GC cell migration and invasion, and repressed the tumor formation in nude mice. The expression levels of p53 and β-catenin proteins were impaired by TRIM29 knockdown, indicating a possible linkage underlying the oncogenic role of TRIM29 in GC. Conclusion Our findings provide important evidence that gain of TRIM29 expression contributes to GC cell proliferation and it may serve as a potential prognostic biomarker for GC.

Keywords: Gastric cancer, TRIM29, prognosis, cell proliferation, migration and invasion, p53 and β-catenin signaling

Introduction

Gastric cancer (GC) remains as the fourth most frequent type of cancer and the second leading cause of cancer mortality worldwide, although its incidence is declining during the last decades [1, 2]. The treatment option includes surgery, radiotherapy, chemotherapy and molecular targeted therapy [3]. Its progression involves multi-factors and multi-steps, accompanied by alterations of gene expression, including activation of oncogenes and suppression of tumor suppressors. Nowadays, more and more novel coding or non-coding genes have been identified to process oncogenic or tumor suppressing potential, we do not make a breakthrough on the mechanisms of GC occurrence. The 5-year survival rate for GC patients is merely about 20% due to metastasis, recurrence and multiple drug resistance (MDR) [4, 5].

Tripartite motif-containing 29 (TRIM29) is a member of the tripartite motif (TRIM) family featured by the presence of a RING finger, B-box, and coiled-coil domains [6]. Due to the existence of other protein-protein interaction motifs like PHD, MATH and SPRY motifs [6], TRIM family proteins have significant plasticity in interaction with multiple binding partners, and are implicated in a variety of biological processes, such as development, cancer and
TRIM29 promotes gastric cancer cell proliferation

Immune response [7-9]. Examination of TRIM29 expression in many human cancers has revealed that it is up-regulated in cancers including lung cancer [10], colorectal cancer [11], pancreatic cancer [12], bladder cancer [13], thyroid carcinoma [14] and nasopharyngeal carcinoma [15]. But it is down-regulated in several other cancers including breast cancer [16] and hepatocellular carcinoma [17].

In gastric cancer, there are two reports expounding the expression, survival correlation and biological functions of TRIM29 [18, 19]. Kosaka et al addressed that TRIM29 expression was far higher in GC tissues and its increased mRNA expression was markedly associated with histological grade, lymph node metastasis and poorer survival rate [18]. Recently, Qiu et al reported that TRIM29 promoted cell proliferation, cell cycle and apoptosis resistance via RNA interference (RNAi) technologies. They further demonstrated that TRIM29 functioned by regulating Wnt/β-catenin signaling pathway and was targeted by microRNA-185 [19]. These findings all support the oncogenic roles of TRIM29 in GC. However, the in vivo data was still absent from the two reports, and the possible role or mechanism of TRIM29 in pathogenesis of GC remains to be further explored.

In the present study, we mainly analyzed the expression profile of TRIM29 in GC samples and cell lines. Then RNAi technology was applied to study the in vitro and in vivo functions of TRIM29 in GC cell proliferation, migration and invasion, aiming to provide the basis for new GC therapy methods.

Materials and methods

Patients and tissues

A total of 22 confirmed GC patients were consecutively recruited between January 2014 and December 2016 for the General Surgery at The Affiliated Cancer Hospital of Zhengzhou University. The normal gastric mucosa tissues, negative lymph node tissues, primary lesion tissues, positive lymph node tissues and metastasis lesion tissues were obtained by surgery, and examined by a pathologist. Final surgical pathology reports were obtained and recorded. All the patients had provided the written informed consent use of their samples.

Cell lines and cell culture

Five GC cells (SGC-7901, AGS, HGC-27, MGC80-3, NCI-N87) were grown in 1640 or DMEM supplemented with 10% fetal bovine serum (HyClone) according to ATCC protocols. Cells were maintained in a humidified incubator at 37°C and 5% CO2.

Immunohistochemical staining

The 5 μm tissue sections were dewaxed, rehydrated, and retrieved in 10 mM citrate buffer pH6.0 (10 mM citrate buffer and 0.05% Tween-20). The sections were immersed in 3% hydrogen peroxide for 20 min and stained with anti-TRIM29 antibody (1:100) with the non-immunized goat serum at 4°C overnight. After incubating with secondary antibodies (1:3000, Beyotime) for 1 h, the sections were stained with DAB (Beyotime). The sections were photographed under a microscope.

Reverse transcription (RT) and quantitative real-time PCR

Total RNA was isolated from tissues using TRizol reagent (Life Technologies). First-strand cDNA was synthesized from 2 mg of total RNA using oligo (dT)n (Takara) and reverse transcriptase (Takara). Quantitative real-time PCR analysis of TRIM29 expression was carried out using 2 ml of cDNA and the SYBR Green Master Mix (TOYOBO), as recommended by the manufacturer. GAPDH was used as internal controls.

SDS-PAGE and western blot

Total cell lysates were extracted from cells by RIPA buffer (Beyotime). After boiled for 10 min at 95°C, protein samples were loaded onto a 9% SDS-PAGE gel, followed by transferred to NC membrane (Amersham Bioscience). Then the membrane was blocked in PBST with 5% non-fat milk and 0.05% Tween-20. After washing for three times with PBST the membrane was incubated with certain primary antibodies: anti-TRIM29 (Cell Signaling Technology), anti-p53 (Santa Cruz), anti-p21 (Cell Signaling Technology), anti-β-catenin (Santa Cruz) and anti-β-actin (Santa Cruz) at 4°C over night. After washing for three times with PBST, the blots were incubated with certain secondary antibodies (Beyotime) at RT for one hour. Finally, the protein-antibody complexes were visualiz-
TRIM29 promotes gastric cancer cell proliferation

Figure 1. The expression of TRIM29 is increased in GC samples. A. The mRNA expression of TRIM29 was increased in primary lesions, positive lymph nodes and metastasis lesions groups of GC samples by quantitative real-time PCR assay. GAPDH was used as internal controls. B. The protein expression of TRIM29 was increased in primary lesions, positive lymph nodes and metastasis lesions groups of GC samples by western blot assay. β-actin was used as internal controls. C. The protein expression of TRIM29 was increased in primary lesions of GC samples, compared to normal gastric mucosa by immunohistochemical staining. D. The overall survival time was analysis by Kaplan-Meier analysis between TRIM29 negative and positive groups. 1: normal gastric mucosa, 2: negative lymph node, 3: primary lesion, 4: positive lymph node, 5: metastasis lesion. N=22, **, P<0.05, ***, P<0.01.
TRIM29 promotes gastric cancer cell proliferation

ed by a chemiluminescence detection system (Millipore).

Short interfering RNAs and transfections

TRIM29 siRNA and control siRNA were purchased from Santa Cruz. Transfections were performed by Lipofectamin-2000 reagent (Life Technology) followed by the manufacturer's instruction.

MTT assay

SGC-7901 or MGC80-3 cells were transfect- ed with control siRNA or TRIM29 siRNA at varying time: 12 h, 24 h, 48 h and 72 h. Then 20 μl of sterile MTT (5 mg/ml, sigma) was added and incubation for another 4 h at 37℃. After that, 150 μl of DMSO was added to each well and the plates were thoroughly mixed for 20 min. Spectrometric absorbance at a wavelength of 490 nm was measured on a microplate reader.

Cell cycle analysis

SGC-7901 or MGC80-3 cells were fixed by 70% ethanol and stained with propidium iodide for 30 min. After that, the cells were immediately analyzed by cell flow cytometry. Histograms represent the percentage of cells in each phase of the cell cycle (G0/G1 phase, S phase, and G2/M phase).

Transwell assay

Gastric cells were harvested after transfect- ed with control siRNAs or TRIM29 siRNAs for 24 h. Then cells were suspended with serum-free medium, then seeded in the upper chamber of 8 μm pore size Transwell (Corning Inc., Coring, NY, USA) at the density of 1 × 10^5 cells per chamber for migration and invasion assay, respectively. For invasion assay, the upper chambers were precoated with BD Matrigel. The lower chambers were filled with medium containing 10% FBS and incubated for 24 h. Cells remaining on the upper chamber were removed carefully by swabs, the migrating cells in the lower chamber were fixed with 4% formaldehyde, and stained with 0.5% crystal violet solution. The stained cells were imaged with an inverted microscope, and counted at five randomly selected visual fields for each experimental condition. Each experiment was performed in triplicate.

In vivo xenograft tumor growth

4-week-old female nude mice [BALB/cA-nu (nu/ nu)] were obtained from Shanghai Experimental Animal Center and maintained in pathogen-free conditions. The mice were separated into two groups and were subcutaneously injected with control or TRIM29 knockdown MGC80-3 cells (2 × 10^6) at each flank. Tumor volumes were measured with a caliper every 6 days. The mice were killed and the tumor weight was measured with a scale at the sixtieth day.

Statistical analysis

All data were analyzed by SAS v9.2 software and presented as mean ± SD. Kaplan-Meier analysis was performed to analysis the survival time between TRIM29 negative and positive
TRIM29 promotes gastric cancer cell proliferation

Western blot revealed that TRIM29 mRNA and protein were both significantly increased in primary lesions, positive lymph nodes and metastasis lesions of GC samples, when compared with that of normal gastric mucosa and negative lymph nodes (P<0.01~0.001, Figure 1A and 1B). The results of immunohistochemistry staining revealed that, TRIM29 was mainly localized in the nuclei of GC cells, and its expression was much more dominant in the primary lesion than that in the neighboring normal gastric mucosa (Figure 1C). By Kaplan-Meier analysis, we found that GC patients with higher TRIM29 expression had a significantly shorter overall survival time than those with lower TRIM29 levels (P<0.01, Figure 1D). These results indicated a tumor promoting function of TRIM29 in GC, finely in line with the former studies [18, 19].

TRIM29 protein is highly expressed in GC cell lines

We further compared the relative TRIM29 mRNA and protein expression levels among 5 different GC cell lines (SGC-7901, AGS, HGC-27, MGC80-3, NCI-N87) and the human normal gastric mucosa cells. As shown in Figure 2A and 2B, in comparison with normal gastric mucosa cells, TRIM29 mRNA and protein levels were significantly increased in the 5 different GC cell lines (P<0.01~0.001). Together with the above results of TRIM29 expression in GC samples, these data all strongly suggested that highly expressed TRIM29 might be important for GC progression.

TRIM29 knockdown attenuated cell malignant progression in vitro

To investigate the function of TRIM29 in GC cells, we applied RNAi to knockdown intra-
TRIM29 promotes gastric cancer cell proliferation

MTT assay was performed to evaluate the cell proliferation potential of these cells. The plotted growth curves indicated that knockdown of TRIM29 (TRIM29 siRNA-transfected) resulted in decreased proliferation in both SGC-7901 and MGC80-3 cells when compared with Control siRNA groups (P<0.05~0.001, Figure 3A). We further analyzed the effect of TRIM29 knockdown on cell cycle using flow cytometry. TRIM29 silencing cells displayed significant G0/G1 arrest and G2/M phase reduction (P<0.05~0.01, Figure 3B), which indicated that TRIM29 knockdown inhibited GC proliferation as the result of G0/G1 arrest. Furthermore, transwell assay without or with BD Matrigel was performed to validate the effect of TRIM29 knockdown on cell migration and invasion. The results showed significant reductions in cell migration ability in SGC-7901 cells and cell invasion ability in MGC80-3 cells (P<0.01, Figure 3C and 3D).

TRIM29 knockdown inhibited xenograft growth of GC cells in vivo

To further confirm the role of TRIM29 in the tumorigenesis of GC, a subcutaneous tumorigenicity assay was performed in nude mice. TRIM29 silenced MGC80-3 cells were subcutaneously inoculated into the back region of nude mice at two sides. Tumor sizes were measured every 4 days. As shown in Figure 4A, the growth rate of TRIM29 knockdown MGC80-3 xenografts was slower than that in control group. Six weeks later, the transplanted tumors were excised, and the tumor weight were evaluated. The results revealed a significant decrease in the average tumor weight of xenografts resulting from TRIM29 knockdown (Figure 4B and 4C). Real-time PCR and Western blot both validated the decreased level of TRIM29 mRNA and protein in TRIM29 silenced MGC80-3 cells (Figure 4D and 4E). These results indicated that TRIM29 knockdown repressed the tumor formation of GC cells in vivo.

Molecular mechanisms of TRIM29 in the cell growth of GC

We finally investigated the molecular mechanisms of TRIM29 in controlling GC cell proliferation. According to the previous literature, TRIM29 might negatively regulate p53 [20] and positively regulate β-catenin expression [19], two important molecules in cancer development. The expression of p53 and β-catenin was determined in the above mentioned GC cell lines and normal gastric mucosa cells. As shown in Figure 5A and 5B, in comparison with normal gastric mucosa cells, p53 mRNA was significantly decreased, while β-catenin mRNA was obviously increased in the 5 different GC cell lines (P<0.001). In SGC-7901 and MGC80-3 cells, TRIM29 siRNA transfection led to elevated p53, p21 and reduced β-catenin mRNAs/proteins (Figure 5C and 5D). Consistent with the former reports, our data suggested that inhibition of p53 and activation of β-catenin signaling might be required for TRIM29’s oncogenic effects.
TRIM29 promotes gastric cancer cell proliferation

Discussion

TRIM29 has been found to be associated with many forms of aggressive disease including human cancers. Abnormal expression of TRIM29 was observed in a several cancers [10-17], among which, an association was found between TRIM29 expression and reduced patient survival [18, 21]. Finely in accordance with these, we also found that TRIM29 was upregulated in GC samples and cell lines and higher levels of TRIM29 were associated with poor overall survival of patients. Actually, in GC, Kosaka’s finding was almost the same with ours, except that they found increased TRIM29 expression markedly associated with lymph node metastasis [18]. In the present study, we compared the expression of TRIM29 in 22 sets of GC samples, including normal gastric mucosa, negative lymph node, primary lesion, positive lymph node and metastasis lesion. However, we did not find significant expression difference of TRIM29 mRNA or protein between paired primary lesions and metastasis lesions. Therefore, we did not concentrate on the role of TRIM29 in GC cell migration and invasion. This might attribute to the sample size we used here, or it might be specific to human species.

In support of the oncogenic role of TRIM29, we found that TRIM29 knockdown inhibited GC cell proliferation, induced cell cycle arrest, inhibited GC cell migration and invasion and repressed the tumor formation in nude mice. Although it was clear in the previous report that TRIM29 knockdown resulted in significantly reduced cell proliferation, colony formation, as well as G1-S cell cycle arrest [18]. Our present study added the first evidence of the in vivo function of TRIM29 in GC.

Regarding to the molecular mechanisms of TRIM29’s role in GC, we learned from the literatures that overexpression of TRIM29 in pancreatic cancer lines promoted cell growth in vitro and metastatic activity in vivo stemming from stimulation of Wnt/β-catenin signaling [22]. On the other hand, TRIM29 could bind to p53, the famous tumor suppressor by inhibiting cancer cell proliferation and cell cycle, and inhibit its transcriptional activity [23]. We validated the expression of p53, p21 and β-catenin in a panel of GC cell lines, and TRIM29-silenced cells. The results revealed that p53 and p21 expression was negatively while β-catenin expression was positively correlated with TRIM29 expression, indicating a possible linkage underlying the oncogenic role of TRIM29 in GC.

In summary, we documented that TRIM29 expression is increased in GC samples and cell lines, and that TRIM29 knockdown elevated p53, p21 expression while reduced β-catenin expression, thus to drive enhanced cell proliferation and cell cycle. Our study strengthened the oncogenic role of TRIM29 in GC and pro-

Figure 5. Molecular mechanisms regulated by TRIM29 in GC cells. A. The mRNA expression of p53 and β-catenin was increased in five GC cell lines compared with normal gastric mucosa by quantitative real-time PCR assay. GAPDH was used as internal controls. B. The mRNA expression of p53 and β-catenin was analyzed by quantitative real-time PCR when knockdown of TRIM29 in SGC-7901 and MGC80-3 cells. C. The protein expression of p53, p21 and β-catenin was analyzed by western blot when knockdown of TRIM29 in SGC-7901 and MGC80-3 cells. ***: P<0.001.
TRIM29 promotes gastric cancer cell proliferation

provided the molecular basis by inhibiting tumor suppressive p53 signaling meanwhile activating β-catenin signaling.

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Disclosure of conflict of interest

None.

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TRIM29 promotes gastric cancer cell proliferation
