RNA interference against TRIM29 inhibits migration and invasion of colorectal cancer cells

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Abstract. Colorectal cancer (CRC) is one of the most common malignancies worldwide. Tripartite motif-containing 29 (TRIM29) is a member of TRIM proteins family, which plays diverse physiological and pathological roles in humans. Recent studies found that TRIM29 expressed highly in CRC and promoted cell growth in vitro. However, its function in the metastasis of CRC has not been studied. In the present study, we confirmed the previous report that TRIM29 was upregulated in CRC tissues and high levels of TRIM29 expression were associated with poor overall survival of patients. Moreover, TRIM29 knockdown significantly reduced cancer cell proliferation via notably inducing cell cycle arrest and cell apoptosis. Silencing of TRIM29 significantly inhibited the migration and invasion ability of CRC cells. The protein levels of apoptosis-, migration- and invasion-related proteins were also changed after TRIM29 knockdown. Furthermore, phosphorylation levels of JAK2 and STAT3 were clearly reduced in TRIM29 knockdown cells, indicating a possible mechanism underlying its effects on colorectal carcinogenesis. Collectively, TRIM29 may exert oncogenic effects in CRC cells via regulating JAK2/STAT3 signaling.

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer around the world (1). Due to its insidious onset, the majority of CRC patients are diagnosed at an advanced pathological stage. Despite significant improvements in treatments for CRC over the past decades, the overall survival for those with advanced or metastatic CRC has not been sufficiently improved (2). Therefore, a better understanding of the molecular mechanism of the metastasis of CRC is important for the development of potential therapeutic approaches for this malignancy.

Tripartite motif-containing 29 (TRIM29, also known as ATDC) belongs to TRIM protein family, which is generally classified due to the presence of an N-terminal tripartite motif including a RING domain, one or two B-box motifs and a coiled-coil region (3). TRIM proteins have a wide variety of biological roles such as control of innate immune response, development and cancer (4,5). TRIM29 expression was decreased in breast cancer (6,7), and silencing of TRIM29 in breast cancer cell lines increased cell proliferation, cell motility and invasiveness (6,7). In contrast, TRIM29 expression was increased in a spectrum of cancers (8-18) and elevated TRIM29 expression was associated with reduced survival of patients with bladder (8), gastric (9) and colon cancer (13). TRIM29 can promote proliferation of gastric (10), lung (12) and pancreatic cancer cells (14,15), as well as cell invasion of lung (11) and pancreatic cancer cells (14). In CRC, upregulation of TRIM29 predicted poor survival and promoted cell growth in vitro (13). Little is known about the biological functions of TRIM29 in the metastasis of CRC.

This study confirmed the overexpression of TRIM29 in CRC tissues and the prognostic value for CRC. Downregulation of TRIM29 inhibited the migration and invasion of CRC cells. Silencing of TRIM29 suppressed cell proliferation via inducing G0/G1 phase arrest and cell apoptosis. Furthermore, gene set enrichment analysis (GSEA) with The Cancer Genome Atlas (TCGA) colon adenocarcinoma (COAD) dataset showed that TRIM29 expression was positively related with Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, which was further validated in TRIM29-silenced CRC cells. Our data provide new insights into the molecular functions of TRIM29 as well as its possible regulatory mechanisms in CRC.

Materials and methods

Patients and tissue samples. A total of 90 CRC tissue samples and non-tumorous tissue samples were obtained from patients...
by surgical resection in the Department of Clinical Laboratory, Shanghai Tongren Hospital (Shanghai, China) from July 2008 to September 2009 after giving written informed consent. The age of the patients at the time of surgery was 63.2±10.8 years for 40 women and 63.9±9.1 years for 50 men. All tissue specimens were immediately collected, snap-frozen and stored at -80°C after resection. This study was approved by the local Ethics Committee of our Hospital.

**Cell lines and culture conditions.** Human colorectal carcinoma cell lines, HCT116, SW620, SW480, SW1116, LOVO, HT29 and RKO cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). All cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U penicillin and 100 µg/ml streptomycin, and incubated in a 5% CO2 atmosphere at 37°C.

**RNA interference (RNAi).** HT-29 and SW1116 cells were transfected with TRIM29 small interference RNA (siRNA) and negative control (NC) by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), which were designed and synthesized by GenePharma (Shanghai, China). The sequences were as follows: TRIM29 siRNA, 5'-GGCCATTCTACGTCAACAA-3' and the negative control (NC), 5'-CCTAAGGTTAAGTCGCCCTCG-3'.

**Quantitative RT-PCR.** RNA was isolated from tissue samples or cultured cells using TRIzol reagent (Invitrogen) and quantified according to the manufacturer's instructions. Reverse-transcription was performed with RevertAid First Strand cDNA Synthesis kit (Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) detection of TRIM29 was performed on an ABI Prism 7300 instrument (Applied Biosystems, Foster city, CA, USA) by using the Maxima SYBR-Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Inc., Rockford, IL, USA). GAPDH was amplified as an internal control. The PCR thermal cycle conditions were as follows: an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3' and 60°C for 1 min. Specific primers of TRIM29 and GAPDH were as follows: TRIM29, 5'-GATGCTGTGGACCAAGTG-3'.
For migration assay, only 4x10^4 cells were plated onto the Transwell chamber without Matrigel.

**GSEA of CRC with TRIM29 expression.** TCGA COAD dataset was obtained from https://tcga-data.nci.nih.gov/tcga/. GSEA of TCGA COAD dataset was conducted to explore the gene sets enriched in samples with higher TRIM29 expression as previously described (20). The nominal P-value and normalized enrichment score (NES) were used to sort the pathways enriched in high TRIM29 expression.

**Statistical analyses.** All experiments were carried out at least three times and data are presented as the means ± standard deviation (SD). All data were analyzed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA, USA). Differences between groups were assessed by Student’s t-test. Survival analysis was performed according to Kaplan-Meier method. Differences in overall survival between TRIM29-high and -low groups were assessed by the log-rank test. P<0.05 was considered statistically significant.

**Results**

**TRIM29 expression and prognostic value in human CRC.** We performed qRT-PCR analysis and demonstrated that TRIM29 mRNA level was significantly higher in CRC tissues (n=90) than that in paired non-tumorous tissues (Fig. 1A, P<0.0001). Similar results were obtained based on the analysis of expression data of TCGA COAD dataset from https://tcga-data.nci.nih.gov/tcga/ (Fig. 1B, P<0.0001). The 90 patients were divided into TRIM29-high and -low groups by using the median value of TRIM29 mRNA level as cut-off. Kaplan-Meier survival analysis suggested that the patients with TRIM29-high tumor had significantly poorer prognosis than patients with TRIM29-low tumor (P=0.0091, Fig. 1C). The median survival times were 38 months in the patients with TRIM29-high tumor and 56 months in TRIM29-low tumor.

**Knockdown of TRIM29 by RNAi.** We then investigated the function of TRIM29 in CRC cells using siRNA silencing technology. Firstly, we assessed TRIM29 mRNA and protein expression in seven CRC cell lines by qRT-PCR and western blotting, respectively. As shown in Fig. 2A and B, two cell lines, SW1116 and HT-29, had a higher level of mRNA and protein expression, and were chosen for further study. Furthermore, HT-29 and SW1116 cells were transfected with TRIM29 siRNA or control siRNA (NC). Gene silencing was confirmed by qRT-PCR (Fig. 2C) and western blotting (Fig. 2D), and both mRNA and protein levels of TRIM29 were reduced at least 60%.

**Silencing of TRIM29 inhibits the proliferation, but induces cell cycle arrest of human CRC cells.** To explore the effects of TRIM29 on CRC cell proliferation, CCK-8 assay was performed. Here, CCK-8 assay demonstrated that cell proliferation was significantly inhibited by TRIM29 gene silencing in HT-29 and SW1116 cells (Fig. 3A). We wondered whether TRIM29 influences the cell cycle of CRC cells. We evaluated cell cycles of both CRC cells by PI staining and flow cytometer after transfection with TRIM29 siRNA or control siRNA for 48 h (Fig. 3B). In HT-29 cells, the percentage of G0/G1 phase was significantly higher in TRIM29 siRNA transfected cells than that in control siRNA transfected
cells (72.62±0.45% vs. 47.73±0.96%, P<0.001), whereas, the percentages of S and G2/M phase were significantly lower in TRIM29 siRNA transfected cells than those in control siRNA transfected cells (10.84±1.45% vs. 30.00±0.60%, P<0.001; 14.71±0.77% vs. 18.31±0.73%, P<0.01) (Fig. 4A). Similar trend was observed in SW1116 cells. The results indicated that the silencing of TRIM29 led to cell cycle arrest and lowered the proliferation of CRC cells, which confirmed the results of CCK-8 assay.

Effect of the TRIM29 siRNA on apoptosis of CRC cells. We then determined the effect of TRIM29 knockdown on apoptosis of CRC cells by Annexin V/PI staining and flow cytometry analysis. In control siRNA transfected cells, the majority of cells were intact live cells. After TRIM29 siRNA transfection, apoptotic cells notably increased. The data demonstrated that both HT-29 and SW1116 cells exhibited a noteworthy increase in early-apoptosis rate ( Annexin V+/PI-) in TRIM29 siRNA group, compared to control siRNA group (Fig. 4A and B).
Late-apoptosis rate (Annexin V+/PI+) was also increased in TRIM29 siRNA group, while no late-apoptosis cells were observed in WT and control siRNA group. These data demonstrated that TRIM29 siRNA can markedly induce apoptosis in CRC cells.

TRIM29 is involved in p53-regulated pathways such as apoptosis (21,22). Thus, we detected the expression levels of p53 and apoptosis-related proteins (Bax, Bcl-2 and caspase-9) in CRC cells with TRIM29 siRNA treatment. As shown in Fig. 4C and D, TRIM29 siRNA significantly increased the protein levels of p53, Bax (an apoptosis promoting protein) and caspase-9 (apoptosis-related cysteine peptidase), but notably decreased the protein level of Bcl-2 (an anti-apoptosis protein). These data suggested that TRIM29 siRNA may induce apoptosis via regulating the p53 pathway.

Effects of TRIM29 knockdown on cell migration and invasion. We determined the effect of TRIM29 knockdown on the migration and invasion of CRC cells. Fig. 5A and B show that in both HT-29 and SW1116 cells, the migration and invasion abilities were not significantly different in WT and control siRNA cells, while the migration and invasion potential of the...
TRIM29 siRNA transfected cells was significantly decreased comparing with the control group. These data suggested that TRIM29 might be involved in the migration and invasion of CRC cells.

The expression of migration-related proteins was also analyzed by western blotting (Fig. 5C and D). The protein levels of MMP-9, Snail and Twist were significantly down-regulated by TRIM29 knockdown in HT-29 and SW1116 cells.

Silencing of TRIM29 suppressed JAK2/STAT3 pathway. To explore the gene sets enriched in samples with higher TRIM29 expression, we performed GSEA to identify the associated signaling pathways using high throughput RNA-sequencing data of the COAD dataset of TCGA (Fig. 6A). We found that the JAK/STAT signaling pathways were associated with TRIM29 expression. To explore the effect of TRIM29 on JAK2/STAT3 signaling in CRC cells, we evaluated the activation of JAK2 and STAT3 by western blotting. In CRC cells transfected with control siRNA, we observed high level of JAK2 and STAT3 phosphorylation. On the contrary, in cells transfected with TRIM29 siRNA, JAK2 and STAT3 phosphorylation significantly reduced (Fig. 6B and C). These data suggested that TRIM29 may exert effects on apoptosis, migration and invasion of CRC cells by activating the JAK2/STAT3 pathway.
Discussion

It has been demonstrated that the expression of TRIM29 proteins was closely related with the development of various malignant tumors (6-18). Jiang et al showed that TRIM29 expression was increased in primary CRC tumors, poor survival (both overall and disease-free) is associated with increased TRIM29 expression, and TRIM29 knockdown leads to reduced cell proliferation and colony forming ability of RKO cells (13). In the current study, we partially confirmed these results (13), on the effects of TRIM29 on cell cycle, cell apoptosis, migration and invasion of CRC cells in vitro and explored the possible regulatory mechanisms.

p53, as one of the most common tumor suppressor genes in human cancer, is involved in cell growth arrest, DNA repair and cell apoptosis (23). Previous reports have shown that TRIM29 could inhibit p53-mediated functions (21,22). Increased cell apoptosis has been observed in TRIM29-silenced gastric cancer cells (10). Here, TRIM29 siRNA treatment led to a significant increase of cell apoptosis, as well as expression of p53, Bax and caspase-9, and a notable reduction of Bcl-2 expression (Fig. 4). Our data implied that TRIM29 siRNA may induce cell apoptosis through upregulating p53 expression although further exploration is needed for the precise mechanism.

Cell invasion and migration are key steps that lead to the metastasis and poor prognosis of tumors (24). TRIM29 inhibits cell motility and invasiveness of breast cancer cells (7), while promotes that of pancreatic (14) and lung cancer cells (11). The discrepancy may show that TRIM29 has different functions dependent on different cell types. Silencing of TRIM29 significantly inhibited the migration and invasion of CRC cells (Fig. 5), which may partially explain the poorer overall survival of patients with higher TRIM29 expression. MMP-9, an important member of the MMPs, plays a critical role in cancer invasion and metastasis (25). TRIM29 was reported to promote lung cancer cell invasion via upregulating MMP-9 (11). Snail and Twist, inducers of epithelial-mesenchymal transition (EMT), were previously shown to be involved in the pathogenesis of tumors (26). TRIM29 is a regulator of EMT in pancreatic intraepithelial neoplasia lesions (16), whereas, TRIM29 inhibits Twist1 expression and EMT of breast cancer cells (7). In this study, we showed that TRIM29 silencing triggered a decrease in the expression of
MMP-9, Snail and Twist (Fig. 5). Therefore, we hypothesize that TRIM29 promotes the migration and invasion of CRC cells through regulation of MMP-9, Snail and Twist.

JAK2/STAT3 signaling pathway, which is frequently constitutively activated in cancer (27-30), has been shown to involve in cell apoptosis, cell cycle arrest and cell invasion of CRC cells (31). A recent study reported that TRIM8, a member of TRIM proteins, could enhance the STAT3-dependent signal pathway by inhibiting the function of PIAS3 (32). In the present study, we found a notable decrease of JAK2/STAT3 activation (Fig. 6), which suggested that TRIM29 may exert its anti-apoptotic, anti-cell cycle arrest and cell invasion-promoting functions via JAK2/STAT3 signaling pathways.

In summary, we found that TRIM29 expression in CRC tissues was significantly higher than in non-tumorous tissues, and it correlated with overall survival of patients. More importantly, our results demonstrated a possible pro-tumorigenic role of TRIM29 via JAK2/STAT3 signaling in CRC. The study may contribute to a better understanding of CRC and provide a new therapeutic target of this disease.

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References