Article

TRIM28 is Overexpressed in Osteosarcoma and Associated with Tumor Progression

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Abstract: Tripartite motif containing 28 (TRIM28) is a transcriptional co-factor with pleiotropic biological activities, especially in tumor cells. In this study, expression levels of TRIM28 in osteosarcoma (OS) tissues and the correlations with clinicopathological variables were investigated in 87 OS cases, meanwhile, cell and animal experiments were performed to detect the biological functions of TRIM28. The results showed that TRIM28 was overexpressed in OS tissues compared with normal bones, and TRIM28 expression correlated significantly with tumor diameter ($\chi^2 = 4.06, p=0.04$), metastasis ($\chi^2 = 11.10, p<0.01$) and clinical stage ($\chi^2 = 15.50, p<0.01$). Furthermore, patients with high TRIM28 level always had poor survival ($p<0.01$). Functionally, TRIM28 knockdown significantly inhibited cell ability of proliferation and migration in OS cell lines of in vitro and in vivo experiments. Additionally, TRIM28 could significantly inhibited E-cadherin expression in reducing the stability of the TRIM28 mRNA. Therefore, TRIM28 overexpression was correlated with malignant progression of OS and poor survival of OS patients, and TRIM28 pathway may be a therapeutic target for OS.

Keywords: TRIM28; osteosarcoma; progression; E-cadherin

1. Introduction

Osteosarcoma (OS) is a primary high-grade malignant solid bone neoplasm of children and adolescent, and is the leading cause of cancer-relevant death in children worldwide, with the rapid proliferation and strong tendency for invasion and metastasis[7, 12]. Although the advanced diagnostic techniques and treatment for OS have been applied over the past few decades, 5-year postoperative survival rate is still very low, and targeted therapy for improving survival has not been well established[15, 16]. Therefore, exploiting the in-depth molecular mechanism and finding novel treatment strategies are crucial for OS therapy.

Tripartite motif containing 28 (TRIM28), also known as KRAB domain-associated protein 1 (KAP1) or transcriptional intermediary factor 1 beta (TIF1β), serves as a transcriptional co-factor by interacting with KRAB-containing zinc finger proteins (KRAB-ZFP)[2, 4]. Meanwhile, TRIM28 has pleiotropic biological activities, and regulates its target genes at both transcriptional and posttranscriptional levels[9]. TRIM28 could affect the transcriptional activity of KRAB-ZFP-specific loci, trans-repression or epigenetic modulation of chromatin structure, and then regulate many genes expression[2]. Meanwhile, TRIM28 could serve as a SUMO/ubiquitin E3 ligas and regulate apoptosis[14]. To date, many studies have indicated that TRIM28 plays an important role in the proliferation and migration of tumor cells, and higher levels of TRIM28 expression have been observed in liver, gastric, lung, breast, pancreatic, ovarian and prostate cancer[1, 5, 6, 8, 10, 11, 13, 14, 17, 18, 20-22]. Despite many efforts to elucidate the cellular functions and associated molecular mechanisms of TRIM28, the relation between TRIM28 and OS has not been reported.

In this study, we investigated the expression level of TRIM28 in OS tissues, and analyzed the correlation of TRIM28 expression with clinicopathological variables and survival of OS patients.
Meanwhile, in vivo and in vitro experiments were performed to detect the effect of TRIM28 on cell biological behavior of OS cell lines.

2. Results

2.1. TRIM28 is overexpressed in OS and is associated with tumor progression

To compare the expression level of TRIM28 in OS specimens and adjacent non-tumor bone tissues, 87 cases of human OS were collected and immunohistochemistry was used. The results showed that TRIM28 mainly localized to the cell nucleus (Figure 1A), and the expression level of TRIM28 was significantly higher in OS tissues than adjacent non-tumor bone tissues (Figure 1B, p<0.01).

We also analyzed the association of TRIM28 expression levels with clinicopathological variables of cancer, including age, sex, tumor diameter, metastasis, clinical stage, and survival status. For statistical analysis, the sections were scored as lower expression (positive stain cells≤50%) and higher expression (positive stain cells>50%), and the results were summarized in Table 1. The results demonstrated that higher TRIM28 expression was significantly associated with tumor diameter ($\chi^2 = 4.06$, $p = 0.04$), metastasis ($\chi^2 = 11.10$, $p < 0.01$) and clinical stage ($\chi^2 = 15.50$, $p < 0.01$) (Table 1, Figure 1C-E). The results suggested that TRIM28 expression correlated with aggressive clinical features in OS.

We have showed that TRIM28 was correlated with malignant progression of OS, and cell proliferation is considered to play an important role in aggressive process. Ki67 always has been used as proliferation marker in many cancer cells, also including OS. So in this study, we investigated the Ki67 expression level in OS, and analyzed the correlation of TRIM28 and Ki67. We found that there was a significantly positive correlation between TRIM28 and Ki67 expression, and the OS tissues with TRIM28 higher expression always had Ki67 overexpression (Figure 1F, G).

Table 1 Correlations between TFEB expression and clinicopathological variables

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<td></td>
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Figure 1 TRIM28 expression in OS and the associations with tumor progression. A, the representative picture of TRIM28 expression in OS tissues detected by immunohistochemistry. B, the expression levels of TRIM28 in OS tissues and counterpart bone tissues. C, the expression levels of TRIM28 in different groups of tumor diameter (<5cm and ≥5cm). D, the expression levels of TFEB in different groups of metastasis. E, the expression levels of TRIM28 in different groups of clinical stage (I/IIA and IIB/III). F, the representative immunohistochemistry picture of TRIM28 and Ki67 expression in the same cases. G, the correlation of TRIM28 and Ki67 expression all OS tissues. (IHC, 40X)

2.2. TRIM28 expression is associated with poor survival of OS patients

Firstly, Kaplan-Meier survival analysis and COX analysis were carried out in these 87 cases to investigate the relationship between TRIM28 expression and patient’s survival. The results of Kaplan-Meier survival analysis showed that OS patients with TRIM28 higher expression in tumor tissues not only always had poor overall survival (p<0.01, Figure 2A), but also had poor survival in progression free survival (p<0.01, Figure 2B). In addition, TRIM28 maybe as a predictor of survival in multivariate analysis (hazard ratio=0.36, 95% confidence interval=0.12-1.14, p=0.08), when entered into a model containing all clinicopathologic variables for COX analysis, although the P-value was p=0.08 (Table 2). Furthermore, Kaplan-Meier analysis was also performed using the R2 genomics database of Kuijjer-127 (available online:http://hgserver1.amc.nl/cgi-bin/r2/main.cgi) to detect the effect of TRIM28 mRNA on OS patient’s survival. As expected, the higher expression level of TRIM28 mRNA was also associated with the survival of OS patients, P-value of overall survival was p=0.15 and P-value of metastasis free survival was p=0.09 (Figure 2C, D). All these results suggested that higher TRIM28 levels predicted a worse prognosis and TRIM28 could serve as a promising biomarker for OS.
Table 2 Multivariate analysis of survival in all population

<table>
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<th>95.0% CI for Exp(B)</th>
<th>P</th>
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<td>0.71 - 3.60</td>
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<td>Sex (male vs. female)</td>
<td>1.08</td>
<td>0.54 - 2.17</td>
<td>0.83</td>
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<td>Tumor diameter (&lt;5 vs. ≥5)</td>
<td>0.87</td>
<td>0.34 - 2.22</td>
<td>0.77</td>
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<td>Metastases (No vs. Yes)</td>
<td>0.81</td>
<td>0.34 - 1.92</td>
<td>0.63</td>
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<tr>
<td>Stage(I/IIA vs. IIB/III)</td>
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<td>0.10 - 1.30</td>
<td>0.12</td>
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<tr>
<td>TRIM28 (Low vs. High)</td>
<td>0.36</td>
<td>0.12 - 1.14</td>
<td>0.08</td>
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</table>

Figure 2 TRIM28 expression is associated with poor survival of OS patients. A, the significance of TRIM28 expression level in overall survival. B, the significance of TRIM28 expression level in progression free survival. C, the significance of TRIM28 expression level in overall survival using the R2 genomics database. D, the significance of TRIM28 expression level in metastasis free survival using the R2 genomics database.

2.3. The effects of TFEB knockdown in tumor cell ability of proliferation and migration

Now that, TRIM28 was overexpressed in OS tissues, and TRIM28 was associated with tumor malignant progression and patient’s poor survival, but the underlying mechanism was still not very clear. In this study, gene silencing of TRIM28 through lentiviral transduction system and shRNA was established in OS cell lines U2OS and MG63. Western blot analysis was first performed to detect
the silencing efficiency of two distinct shRNAs targeting TRIM28 in OS cell lines, the results found that TRIM28 was down regulated in both U2OS (Figure 3A) and MG63 (Figure 3B) cell line. Meanwhile, PCR was also performed to investigate the silencing efficiency of shTRIM28 in TRIM28mRNA level. And we found that the levels of TRIM28mRNA were also decreased after lentiviral transduction system was transducted to OS cells in both U2OS (Figure 3C) and MG63 (Figure 3D). To investigate the role of TRIM28 in cell biology function, cell proliferation analysis, colony formation and wound healing analysis were performed in shTRIM28 and shctrl cells. The results showed that knockdown of TRIM28 could significantly affect the cell abilities of proliferation (Figure 4A), colony formation (Figure 4B, C) and migration in U2OS (Figure 4D). In addition, the same results were found in MG63 cell line, silencing TRIM28 not only could repress cell proliferation (Figure 5A), but also could decrease the number of both two-dimension colony formation (Figure 5B) and soft agar suspension colony formation (Figure 5C). And silencing TRIM28 could repress cell migration in MG63 cell line (Figure 5D). All these results suggested that higher TRIM28 levels promoted OS cell malignancy.

**Figure 3** Silencing TRIM28 in OS cells by shRNA. A, silencing efficiency of shTRIM28in U2OS cell line detected by western blot. B, silencing efficiency of shTRIM28in U2OS cell line detected by PCR. C, silencing efficiency of shTRIM28in MG63 cell line detected by western blot. D, silencing efficiency of shTRIM28in MG63 cell line detected by PCR.
Figure 4 TRIM28 silencing significantly affect cell proliferation and migration in U2OS cell line. 
A, MTT assay was used to detect cell proliferation of shctrl and shTRIM28 cells. 
B, representative images and histogram of two-dimensional colony of shctrl and shTRIM28 cells. 
C, representative images and histogram of soft agar colony of shctrl and shTRIM28 cells. 
D, photographs representing the cells migrated into the wounded area and histogram showing the relative migration distance of cells in the wound-healing assay. (** p < 0.01)

2.4. The effect of TRIM28 in OS cells in vivo

To confirm the role of TRIM28, in vivo animal experiment was also performed, shctrl and 
shTRIM28 cells were injected subcutaneously to dorsal midline in nu/nu mice to generate murine 
subcutaneous tumors. Then tumor volumes were measured, and the survival days of mice with 
tumor were accounted. We found that there was a marked difference in the tumor growth rate 
between the mice with shctrl and shTRIM28 cells, and the tumor growth rate (mean tumor volume) 
was much slower in shTRIM28 tumors (Figure 6B). The smallest tumor in the shctrl group was still 
bigger than the biggest one in the shTRIM28 group (Figure 6A). Meanwhile, the survival rate of 
shctrl mice decreased to 20% in 30 day after injected tumor cells, but the survival rate of shTRIM28 
only decreased to 80% (Figure 6C). In addition, compared with shctrl group, the area ratio of 
necrosis in U2OS xenografts was insignificantly increased in shTRIM28 group (Figure 6D). The 
levels of caspase3 expression in tumor tissues of shTRIM28 group were higher than that of shctrl 
group (Figure 6E), which suggested that TRIM28 could inhibit cell apoptosis in vivo. As higher 
TRIM28 expression was significantly associated with tumor distant metastasis in clinical OS, we 
investigated the relationship between TRIM28 expression and distant metastasis in mice. The results 
showed that the number of metastasis focuses both in lung and liver was significantly increased in 
shTRIM28 group (Figure 6F). Meanwhile, the expression levels of E-cadherin in shTRIM28 group 
were significantly increased than that of E-cadherin in shctrl group (Figure 6G).
Figure 5 TRIM28 silencing significantly affect cell proliferation and migration in MG63 cell line. A, MTT assay was used to detect cell proliferation of shctrl and shTRIM28 cells. B, representative images and histogram of two-dimensional colony of shctrl and shTRIM28 cells. C, representative images and histogram of soft agar colony of shctrl and shTRIM28 cells. D, photographs representing the cells migrated into the wounded area and histogram showing the relative migration distance of cells in the wound-healing assay. (** p < 0.01)
The effect of TRIM28 in OS cells in vivo animal experiment. A, the smallest tumor in the shctrl group was bigger than the biggest one in the shTRIM28 group. B, the difference in tumor growth rate between shctrl and shTRIM28 cells. C, the survival rate of mice with shctrl and shTRIM28 cells. D, the area ratio of necrosis in tissues of shctrl and shTRIM28. E, the representative images of caspase3 expression in tumor tissues of shctrl and shTRIM28. F, the number of metastasis in tumor tissues of shctrl and shTRIM28. G, the representative images of E-cadherin expression in tumor tissues of shctrl and shTRIM28.

2.5. The effect of TRIM28 in the expression of E-cadherin

We have found that there was a close relationship between TRIM28 and E-cadherin expression in U2OS xenografts, and some study have reported that TRIM28 contributed to regulation of E-Cadherin in breast cancer and lung cancer cells[3, 19]. To investigate if there is any relationship between TRIM28 and E-cadherin expression in OS cell line in vitro, the expression level of E-cadherin was detected by western blot and PCR in shTRIM28 and shctrl cells. Both the protein level (Figure 7A) and mRNA (Figure 7B) level of E-cadherin were increased in shTRIM28 cells. To explore the main mechanism of TRIM28 regulating E-cadherin, a transcription blocker, 1uM actinomycin D (ActD) was used to detect the stability of E-cadherin mRNA. At different time points after actinomycin D treatment, the cells were collected and analyzed for E-cadherin mRNA expression. We observed that the E-cadherin mRNA expression was significantly reduced (>60% reduction) at 6 h and 12h in shTRIM28 cells, compared with shctrl cells (Figure 7C, D). Our data suggested that TRIM28 regulated E-cadherin at the post-transcriptional level by reducing its stability. To further confirm the effect of E-cadherin in regulating E-cadherin expression, TRIM28 was overexpressed in U2OS using lentiviral transduction system. We found that the expression level of E-cadherin was significantly decreased in TRIM28 overexpression cells (Figure 7E). To exclude the effect of protein degradation in E-cadherin expression, 100uM CHX was used to treat shTRIM28 and shctrl cells. The results demonstrated that the expression levels of E-cadherin were all decreased after treatment with
CHX, but there was no any significant deference in E-cadherin expression between shTRIM28 and shctrl cells (Figure 7F), which suggested that the role of TRIM28 in regulating E-cadherin was not through protein degradation system. Meanwhile, we found that there was a significantly negative correlation between TRIM28 and E-cadherin expression in OS tissues, and the OS tissues with TRIM28 higher expression always had E-cadherin lower expression (Figure 7G).

Figure 7: The effect of TRIM28 in the expression of E-cadherin. A, the effect of TRIM28 silencing in the expression of E-cadherin in U2OS detected by western blot. B, the effect of TRIM28 silencing in the expression of E-cadherin in U2OS detected by PCR. C, the expression level of E-cadherin mRNA in shctrl and shTRIM28 after treatment with ActD detected by PCR. D, normalized E-cadherin mRNA level in shctrl and shTRIM28 after treatment with ActD. E, the expression levels of E-cadherin after treatment with CHX in shctrl and shTRIM28 cells. G, the correlation of TRIM28 and E-cadherin expression all OS tissues.

3. Discussion

In this study, we found that TRIM28 was overexpressed in OS tissues, and its expression was correlated with malignant progression of OS and poor survival of OS patients. And silencing TRIM28 inhibited cell growth and migration ability in OS cells. Meanwhile, TRIM28 played a crucial role in regulating E-cadherin expression both in cell experiments and animal experiment, and there was a significantly negative correlation between TRIM28 and E-cadherin expression in OS tissues.

As we all know, OS is the leading cause of cancer-relevant death for children in the worldwide. Although the advanced diagnostic techniques and treatment for OS have been applied over the past few decades, 5-year postoperative survival rate is still very low, because of its rapid proliferation and strong tendency for invasion and metastasis[23, 24]. Therefore, it is urgent to find new biomarkers, which are able to predict the prognosis of OS, and to exploit the in-depth molecular mechanism and finding novel treatment strategies are crucial for OS therapy.
TRIM28 has been considered as a transcriptional co-factor by interacting with KRAB-containing zinc finger proteins (KRAB-ZFP). Meanwhile, TRIM28 has pleiotropic biological activities, and regulates its target genes at both transcriptional and posttranscriptional levels\cite{5, 8, 25}. TRIM28 could affect the transcriptional activity of KRAB-ZFP-specific loci, trans-repression or epigenetic modulation of chromatin structure, and then regulate many genes expression\cite{26}. Many studies have investigated the correlation between TRIM28 and tumor malignance and progression. Several studies demonstrated that TRIM28 overexpression was always associated with clinicopathological features, which gives prominence to the importance of TRIM28 in tumor progression. High levels of TRIM28 correlated with a significantly lower survival rate in patients with ovarian, gastric and pancreatic cancer\cite{10, 16, 27, 28}. There was a positive correlation between TRIM28 expression and glioma malignancy\cite{29, 30}. Recently, it has been reported that TRIM28 could promote proliferation and metastatic progression in breast cancer cell\cite{12}. Meanwhile, TRIM28 was involved in EMT in lung cancer\cite{20}. In this study, we found that TRIM28 was overexpressed in OS tissues, and higher expression of TRIM28 led to a worse prognosis via promoting the cell proliferation ability and migration of OS cells both in vitro and in vivo.

Metastasis is the most common cause of death for patients with OS, and most metastasis happens via spreading to the lungs and other bones, some time to lymph node\cite{31, 32}. It is a remarkable fact that 10-15% of OS will have detectable metastases at the time of diagnosis, but over 90% of clinically significant OS have already micrometastasized when diagnosed\cite{33, 34}. Loss of the epithelial adhesion molecule E-cadherin is thought to enable metastasis by disrupting intercellular contacts, and the significance of E-cadherin for metastasis has been shown in a variety of in vitro and in vivo models\cite{35, 36}. The correlation of TRIM28 and E-cadherin has been valued in some papers. TRIM28 decreased the expression level of E-cadherin, and correlated with increased metastatic ability in pancreatic cancer\cite{13}. Lu Chen et al found that TRIM28 could suppress the expression of E-cadherin in lung cancer via altering histone 3 modification of its promoter, which contributed EMT and lung cancer metastasis\cite{22}. In addition, TRIM28 promoted breast cancer metastasis by stabilizing TWIST1 protein, which indirectly affected E-cadherin expression\cite{21}. In this study, we also found that TRIM28 could suppress the expression of E-cadherin, and the role of TRIM28 in regulating E-cadherin was not through affecting protein expression, but at the post-transcriptional level by reducing its mRNA stability.

4. Materials and Methods

4.1. Antibodies and drug

The primary antibodies in this study were listed as follows: rabbit anti-TRIM28 (Abcam, USA), rabbit anti-E-cadherin (Abcam, USA), rabbit anti-caspase3 (Abcam, USA), rabbit anti-Ki67 (Abcam, USA), and mouse anti-GAPDH (Santa Cruz Biotechnology) for both western blot and immunohistochemistry staining. The anti-mouse secondary antibodies (Santa Cruz Biotechnology), and anti-rabbit secondary antibodies (Zhongshan Goldbridge Biotechnology, Beijing, China) were purchased for western blot. The secondary antibodies PV6001 was obtained from Zhongshan Goldbridge Biotechnology for immunohistochemistry staining. Cycloheximide (CHX) and Actinomycin D (Act D) were obtained from Sigma-Aldrich.

4.2. Patient samples

87 osteosarcoma were obtained from the Department of Pathology, Tianjin Medical University Cancer Institute and Hospital from 2007 to 2010. All tissue sections were confirmed by specialists to make a final diagnosis. Histopathological diagnoses were made using the World Health Organization criteria, and clinical stages were evaluated according to American Joint Committee on Cancer (AJCC). All patients’ clinicopathological data were collected and summarized in Table 1. This study complied with the Declaration of Helsinki and was approved by the Human Ethics and
Research Ethics Committees of the hospital. Written informed consents were obtained from all patients.

4.3. Immunohistochemistry Staining and Evaluation

Paraffin-embedded tissue sections were deparaffinized and rehydrated with xylene and graded alcohol solutions, then endogenous peroxidase activity was quenched by 3% hydrogen peroxide, and sections were boiled in 10 mM citrate buffer (pH 6.0) for 3 min to exposure antigens. After cooling to room temperature, sections were incubated with primary antibodies for 18 h at 4 °C, and incubated with PV6001 for 30 min at 37 °C, and then stained with DAB (Zhongshan Goldbridge Biotechnology) for 1 to 2 min. Control sections were incubated with PBS instead of a primary antibody, and the washing buffer was PBS.

For scoring the stain of tissue sections, we chose five high-power fields from each slices, and estimated the mean percentage of stained cells. For TRIM28, the level ≤ 50% in tissues was assigned to the lower expression group, whereas >50% was the higher expression group (Figure 1A).

4.4. Cell culture and treatment

Human osteosarcoma cell lines (U2OS and MG63) were purchased from the ATCC (Rockville, MD, USA), and cultured in RPMI 1640 medium supplemented with 10% of fetal bovine serum (Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen, USA). The cells were cultured at 37°C in a humidified incubator with 5% CO2. 1μM actinomycin D (ActD) was used to detect the stability of E-cadherin mRNA, and 100uM CHX was used to treat cells.

4.5. Plasmid construction and cell infection

Small hairpin RNAs (shRNA) were constructed into pLKO.1 plasmids. The shRNA sequences were as follows: shTRIM28#1: CCTGGCTCTGTCTCTGTCT; shTRIM28#2: CTGAGACCAAACCTGTGCTAA, and synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). Full-length human TRIM28 cDNA was obtained by PCR and was cloned into pCDH-puro-vector. The day before transfection, 1×105 cells were placed in 35 mm dishes in DMEM supplemented with 10% fetal bovine serum and without antibiotics. The transfected cells were cultured for 48h and expression protein level were confirmed with western blot analysis in cells.

4.6. Western blot analysis

All agents were purchased from Santa Cruz Biotechnology. Cell lysates were prepared with cell lysis buffer (20 mmol Tris-HCl, pH 7.5, 150 mmol NaCl, 1% Triton, 2.5 mmol sodium pyrophosphate; 1 mmol/L b-glycerophosphate; 1 mmol/L Na3VO4; 1 mg/mL leupeptin; 1 mmol/L phenylmethylsulfonylfluoride). The protein concentration was measured with Bradford method, and proteins were resolved on a denaturing 10% SDS-PAGE gel, subsequently transferred to polyvinylidene fluoride membranes via semidry transfer. The membrane was then blocked in 5% dried milk in Tris-buffered saline and Tween 20, incubated with primary antibodies for overnight at 4 °C, and then with secondary antibodies for 1h at 37 °C. GAPDH protein was used as a loading control.

4.7. RNA extraction and PCR

Cellular total-RNA was extracted by using RNeasy® mini kit (QIAGEN) according to the manufacturer’s instructions. RNA was quantified using the NanoDrop 1000 (Thermo Fisher) and quality assessed by gel electrophoresis. cDNA was synthesized using a Quantitext Reverse Transcription kit (QIAGEN) according to the manufacturer’s instructions. The cDNA were used as templates for PCR, and the primers as following: TRIM28, forward 5’-GATCATGAAGGAGCTGAACAAGCG-3’ and reverse:
5'-TGGATCTTAGTCTAGCCAGTC-3'; E-cadherin: forward 5'-GTCACTGACACCAACGATAATCCT-3' and reverse 5'-TTTCAGTGTGGTGATTACGACGTTA-3'; GAPDH: forward 5'-AAGGTGAAGGTCGGAGTCAA -3', reverse 5'- AATGAAGGGGTACGTGATGG -3'.

4.8. MTT assay for cell viability and proliferation

Cell viability and proliferation were analyzed via MTT assay using a Roche Cell Proliferation Kit I according to its protocol. Cells were seeded in sextuplicate in 96 well plates at a density of 3000 cells/well, and incubated for 0, 24, 48, 72 and 96 h. At the end of incubation, 20 μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma, St. Louis, MO, USA) were added to each well. The plates were incubated in a humidified incubator at 37 °C, under 5% CO2 for 4 h, following which 150 μL dimethyl sulfoxide was added. The plates were gently agitated until the formazan was completely dissolved, and the absorbance was measured at 490 nm wavelength.

4.9. Clonogenic Survival Assay

Cells (500/well) were seeded on 6-well plates and incubated for 7-14 days. Then the cells were fixed with methanol for 6min in -20 °C and stained with gentian violet. Colonies containing more than 50 cells were scored as surviving cells. The colony number and colony formation fraction were detected.

4.10. Soft Agar Colony Formation Assay

1 × 104 cells were plated in 0.4% agarose on top of a 1% agarose base supplemented with complete medium in 6-well plates. A further 1 mL of 1× media without agarose was added on top of the growth layer on day 0 and again on day 14 of growth. Cells were allowed to grow at 37 °C for 4 weeks and total colonies were counted. The pictures were taken by digital camera or microscope and the number of colonies was counted by Quantity One software.

4.11. Wound-Healing Assay

About 5×105 cells were seeded in 6-well plates and grown overnight to reach 90% confluence. Cell monolayer was wounded by scratching with a 20 μL pipette tip, followed by washing three times with PBS. Then cells were incubated in serum-free culture medium. For each well, images of the scratch were taken at 0 and 24 h. The distances of cell migration were calculated by subtracting the distance between the lesions edges at 24 h from the distance measured at 0 h.

4.12. Mice xenograft models

2*106 U2OS of shctrl and shTRIM28 cells were injected subcutaneously to the right of the dorsal midline in nu/nu mice to generate murine subcutaneous tumors. When the subcutaneous tumor size had reached the size of approximately 60-80 mm3, tumor volumes were calculated by the following formula: length*width * width/2, and the survival days were accounted. All animal studies were followed an approved protocol by Tianjin Medical University Cancer Institute and Hospital, in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

4.13. Statistical analysis

SPSS 16.0 was used to evaluate the data. The χ2 test was used to assess the correlation of TRIM28 expression and pathological and clinical factors. Kaplan-Meier analysis was used to analyze the patient survival, and Cox’s proportional hazard regression model was used for multivariate survival analysis of prognostic factors. Other data were analyzed by 2-tailed Student t
test or ANOVA least significant difference test. The significance level was defined as p< 0.05. All experiments were performed in triplicate.

5. Conclusions

In summary, we found that TRIM28 was overexpressed in OS tissues, and its expression was correlated with malignant progression of OS and poor survival of OS patients. Meanwhile, this study reveals a mechanism for transcriptional inactivation of E-cadherin by TRIM28, and this pathway may be a therapeutic target for OS.

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Author Contributions: Xin Fu and Yanfen Cui conceived and designed the experiments; Yanfen Cui and Zhaosong Wang performed the experiments; Shaobin Yang analyzed the data; Yanfen Cui wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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