

Target therapy of TRIM-14 inhibits osteosarcoma aggressiveness through the nuclear factor- κ B signaling pathway

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Abstract. Osteosarcoma is the most common cause of cancer-associated mortality and the prognosis is yet to be fully elucidated due to the paucity of effective therapeutic targets that significantly influence the quality of life and mean survival rates of patients with osteosarcoma. Studies have showed that tripartite motif-containing (TRIM)-14 is a member of the TRIM protein family that has a vital role in tumor progression and metastasis and promotes angiogenesis, invasion and apoptotic resistance of bone cancer. In this study, a chimeric antibody targeting TRIM-14 (Chanti-TRIM) was constructed and the molecular mechanism of target therapy for TRIM-14 was investigated in osteosarcoma cells and xenograft mice. The growth, migration and invasion properties of U-2OS cells were analyzed following incubation with 10-160 mg/ml Chanti-TRIM. Apoptosis of U-2OS cells was detected after Chanti-TRIM treatment. Matrix metalloproteinase (MMP)-9-mediated nuclear factor- κ B (NF- κ B) signal pathway was analyzed in U-2OS cells treated with Chanti-TRIM. The inhibitory efficacy of Chanti-TRIM was studied in U-2OS-bearing xenograft mice. Our results demonstrated that neutralizing TRIM-14 expression markedly inhibited the growth, migration and invasion of osteosarcoma cells, *in vitro* and *in vivo*. We found that TRIM-14 depletion decreased cell viability and induced cells apoptosis *in vitro*. In addition, we identified Chanti-TRIM inhibited growth and promoted apoptosis induced by cisplatin through MMP-9-mediated NF- κ B signal pathway. Furthermore, we observed that Chanti-TRIM treatment inhibited osteosarcoma growth *in vivo*. Histological analysis indicated that apoptotic bodies were increased and NF- κ B nuclear translocation factors, including I κ k β , p65 and I κ B α , were decreased in

tumors treated by Chanti-TRIM. In conclusion, these results showed that Chanti-TRIM markedly inhibited the progression of osteosarcoma, suggesting Chanti-TRIM may be a potential anti-cancer agent that functions via the activation of the NF- κ B pathway for osteosarcoma.

Introduction

Bone cancer is a disease of cells that occur in the skeleton and presents via aberrant growth and migration (1,2). Osteosarcoma is a typical systemic malignant disease, which predominantly leads to characteristic symptoms of bone and joint pain and fatigue in patients (3,4). In recent years, novel strategies have been proposed; however, the overall survival for patients with osteosarcoma has remained limited due to the stubborn resistance of osteosarcoma cells to these strategies (5,6). The resistance of osteosarcoma cells to apoptosis contributes to the growth and invasion of tumor cells (7-9). Apoptotic resistance has become the greatest challenge in cancer therapy due to the fierce resistance of tumor cells via various mechanisms (10,11). Furthermore, although the emergence of adjuvant and neoadjuvant chemotherapy has improved the survival rate of patients with osteosarcoma, the morbidity and mortality rates of patients with osteosarcoma are steadily increasing (12). Hence, elucidating the underlying mechanism of apoptotic resistance is urgently required in order to identify novel efficacious target therapies that may improve the overall survival rate of patients with osteosarcoma.

The superfamily of tripartite motif-containing (TRIM) proteins, which includes >60 types of TRIM proteins, is evolutionarily conserved with a highly conserved order of the domains in the Ring, B-box, Coiled-Coil motif (13). A large number of reports have suggested that TRIM proteins may be novel markers for human cancer metastasis, including gastric cancer, liver cancer and colorectal cancer (14). Recently, TRIM-14 was identified as an important member of the TRIM family of proteins; TRIM-14 promotes growth, invasiveness and resistance to cisplatin-induced apoptosis (15). In addition, increased expression of TRIM-14 has been identified in monkey lymphomas caused by human immunodeficiency virus and Simian immunodeficiency virus (16). TRIM-14 gene expression has a mediator role in the immune response and is associated with the transcription of various genes involved

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in innate immunity by regulating nuclear factor (NF)- κ B signaling pathways (15). Therefore, these reports suggest that TRIM-14 may be a potential target for the treatment of human cancer via the regulation of the NF- κ B signaling pathway.

Aberrant activation of NF- κ B has been observed in various types of human cancer (17,18). Previous reports have indicated that poor survival of bone cancer patients is associated with aberrantly activation of NF- κ B nuclear translocation factors (19,20). Indicators of NF- κ B activation, including inhibitor of nuclear factor- κ B kinase subunit β (Ikk β), p65 and NF- κ B inhibitor α (Ikb α), also exhibited increased activity in clinical specimens of bone cancer tissues (21). In addition, the relationship between the ubiquitin-proteasome system and activation of NF- κ B has been studied in human cancer cells, which demonstrated that NF- κ B activation may stimulate the ubiquitin-proteasome system (22). Furthermore, previous investigations have demonstrated that the NF- κ B pathway is involved in apoptosis resistance induced by chemotherapy and enhances tumor cell survival, invasion and angiogenesis (23). Nevertheless, exploring novel molecules that regulate aberrant activation of the NF- κ B signaling pathway may be beneficial for the treatment of clinical osteosarcoma.

The present study investigated TRIM-14 expression in osteosarcoma cells and studied the efficacy of targeted therapy for TRIM-14 on osteosarcoma growth and aggressiveness, *in vitro* and *in vivo*. The findings demonstrated that antibody targeting of TRIM-14 significantly inhibited the invasive phenotype via the inactivation of the NF- κ B pathway through inhibited MMP-9 expression levels in U-2OS-bearing xenograft mice. The results of the present study provide new evidence that targeted therapy for TRIM-14 may contribute to the inhibition of osteosarcoma progression, suggesting that TRIM-14 may represent a potential target for the treatment of patients with osteosarcoma.

Materials and methods

Ethics statement. This preclinical work was performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of The First Hospital of Hebei Medical University (Shijiazhuang, China). All experimental protocols and animals were approved by Committee on the Ethics of Animal Experiments Defence Research. All surgery and euthanasia were made to minimize suffering.

Cells and reagents. Osteosarcoma cell line U-2OS and human normal osteoblast MC3T3-E1 cells were purchased from American Type Culture Collection (Manassas, VA, USA). U-2OS cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.). MC3T3-E1 cells were cultured in RPMI-1640 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) medium supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich; Merck KGaA). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

MTT assay. A total of 1x10³ U-2OS cells (1x10³) were incubated with Chanti-TRIM (10-160 mg/ml) or PBS (Control) in

96-well plates for 24, 48 or 72 h in triplicate. Subsequently, 20 μ l MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to cells and incubated for 2 h at 37°C. The entire medium was removed and 100 μ l of DMSO was added into the wells to solubilize the crystals. Mitochondrial activity was assessed by measuring the optical density at 570 nm with a light microscope.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In order to investigate the expression levels of matrix metalloproteinase-9 (MMP-9), cyclin D1 gene (CCND1) and NF- κ B target genes, including B-cell lymphoma-extra-large (Bcl-XL), vascular endothelial growth factor (VEGF)-C and Myc proto-oncogene protein (c-Myc), total RNA (2 μ g) from U-2OS and MC3T3-E1 cells was extracted using an RNeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA). Total RNA (2 μ g) was used to synthesize cDNA with the SuperScript II First-strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The PCR comprised of the following thermocycling conditions: Initial denaturation at 96°C for 1 min, 45 amplification cycles consisting of denaturation at 95°C for 30 sec, primer annealing at 66°C for 45 sec and then 54°C for 50 sec, and applicant extension at 72°C for 60 sec. Gene expression levels were measured by RT-qPCR. All the forward and reverse primers (Table I) were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). All mRNA levels were quantified using Power SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative mRNA expression levels were calculated by 2^{- $\Delta\Delta$ Cq} (24). Results were analyzed in triplicate according to the $\Delta\Delta$ Cq method and were presented as n-fold relative to the control.

ELISA. Affinity of Chanti-TRIM with TRIM-14 was analyzed using a commercial ELISA kit (cat. no. E5020h; Beijing Huaxia Ocean Technology Co., Ltd., Beijing, China). Operational procedures were performed as outlined by the manufacturer's instructions. Results were assessed via an ELISA reader system (Bio-Rad Laboratories, Inc.).

Tissue specimens. Osteosarcoma tissues from 12 patients with stage I-IV cancer (n=5) were sliced into 4- μ m-thick sections and paraffin-embedded to achieve osteosarcoma sections. Clinical staging (I-IV) of the patients was categorized as previously reported (25). Osteosarcoma sections were prepared to analyze the expression of TRIM-14. Osteosarcoma sections were collected from 12 patients admitted to The First Hospital of Hebei Medical University between February 2001 and October 2015. The analysis of these clinical osteosarcoma specimens was approved by the Institutional Review Board of The First Hospital of Hebei Medical University and written informed consent was obtained from all patients.

Construction of Chanti-TRIM. Single chain variable fragments of the mouse anti-human TRIM-14 antibody (cat. no. ab50941; dilution 1:1,000; Abcam, Cambridge, MA, USA) were cloned and linked with the pET-27b vector (pET-27b-TRIM-14; Takara Biotechnology Co., Ltd., Dalian, China). The constant domain heavy chain and light chain of the mouse anti-human TRIM-14 antibody were inserted

Table I. Sequences of primers were used in this study.

Gene	Sequence (5'-3')	
	Reverse	Forward
c-Myc	GAAATGTCCTGAGCAATCACCT	TGAGGCAGTTTACATTATGGCT
BcL-XL	CACCATGTCTCAGAGCAACCGGGAGCTGGTGGTT	TGGTCATTTCCGACTGAAGAGTGAGCCCAG
VEGF-C	TGCATTCACATTGTGCTGCTGTAG	GCAGATTATGCGGATCAAACC
TRIM-14	CACCATGGCGTCTCCCAGTGGGAA	TCACTTATCGGAACTCCTGCGC
β-actin	CGGAGTCAACGGATTTGGTC	AGCCTTCTCCATGGTTCGTGA

c-Myc, Myc proto-oncogene protein; BcL-XL, B-cell lymphoma-extra-large; VEGF-C, vascular endothelial growth factor-C; TRIM-14, tripartite motif-containing protein-14.

into the pET-27b-TRIM-14 vector (pET-27b-Chanti-TRIM). Vector of pET-27b-Chanti-TRIM was transfected into the *E. coli* Rossetta (DE3; Merck KGaA, Darmstadt, Germany) using electrotransformation and induced by isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich; Merck KGaA) at a concentration of 0.5 mM and a wavelength of 600 nm. Cells were harvested, disrupted, and dissolved in PBS. Protein was purified by gel filtration chromatography (26) and termed Chanti-TRIM.

Apoptosis analysis. U-2OS cells were cultured until 90% confluence. A total of 1x10⁶ Cells were subsequently incubated with Chanti-TRIM (80.0 mg/ml) for 12 h at 37°C. Cells were washed three times using PBS and treated with cisplatin (4.0 mg/ml) for 12 h at 37°C. Subsequently, cells were trypsinized and underwent apoptosis analysis using an annexin V-fluorescein isothiocyanate and propidium iodide kit (BD Biosciences, Franklin Lakes, NJ, USA). Results were analyzed using a FACScan flow cytometer (BD Biosciences).

Cell migration and invasion assays. U-2OS cells were cultured in DMEM medium for 48 h. PBS or Chanti-TRIM-treated (80.0 mg/ml) cells were suspended as a density of 1x10⁵ in 500 μl serum-free DMEM for 24 h at 37°C. U-2OS cells were then inserted into the tops of BD BioCoat Matrigel Invasion Chambers (BD Biosciences) according to the manufacturer's instructions. U-2OS cells were incubated with Chanti-TRIM or PBS for 72 h at 37°C using a Matrigel Migration Chamber (BD Biosciences) to analyze the migration of tumors cells. For the invasion assay, a Matrigel Invasion Chamber (BD Biosciences) was used to instead of a Matrigel Migration Chamber. U-2OS tumor cell invasion and migration was measured using a stain-field microscope.

MMP-9 overexpression. A total of 1x10⁶ U-2OS cells were cultured in DMEM in a 6-well plate until 90% confluence. The media was then removed from culture plate and the cells were washed with PBS three time. U-2OS cells were transfected with plentivirus-MMP-9 (100 pmol; Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 (Sigma-Aldrich; Merck KGaA) according to manufacturer's protocol. A total of 48 h after transfection, subsequent experimentations were performed in MMP-9-overexpressed U-2OS cells.

Western blot analysis. TRIM protein expression levels in U-2OS and MC3T3-E1 cells were analyzed via western blotting. U-2OS cells were treated by PBS or Chanti-TRIM, homogenized in lysate buffer containing protease-inhibitor and subsequently centrifuged at 5,000 x g for 10 min (4°C). The supernatant of the mixture was used to analyze the target protein. To detect the target protein, transmembrane proteins were extracted via a transmembrane protein extraction kit (Qiagen Sciences, Inc.) according to the manufacturer's instructions. Proteins were separated by 12% SDS-PAGE as previously described (27). For western blotting, primary antibodies: TRIM-14 (cat. no. ab50941), MMP-9 (cat. no. ab38898), CCND1 (cat. no. ab134175) and β-actin (cat. no. ab8226) (all 1:1,000; Abcam) were added after blocking (5% skimmed milk) for 1 h at 37°C. Following washing three times with PBS, the membrane was incubated with HRP-conjugated IgG mAb secondary antibodies (1:5,000; cat. no. PV-6001; OriGene Technologies, Inc., Beijing, China) for 24 h at 4°C. Finally, protein bands were visualized using Advansta WesternBright enhanced chemiluminescent HRP substrate (Menlo Park, CA, USA).

Animal experiments. To further evaluate the therapeutic efficacy of Chanti-TRIM on osteosarcoma growth, a murine xenograft model of osteosarcoma was established. A total of 68 female specific pathogen-free BALB/c nude mice were purchased from Orient Bio Inc., (Seoul, Korea). All mice were free to access food and water, and were housed under an artificial 12-h light-dark cycle. In total, 1x10⁵ U-2OS cells were subcutaneously injected into the backs of the BALB/c nude mice. Mice bearing osteosarcoma were randomly divided into two groups (n=10 per group) and subsequently received treatment with Chanti-TRIM (10 mg/kg) or PBS. Treatments for tumor-bearing mice were initiated when tumor diameters reached 5 to 7 mm on day 5 after tumor inoculation. Full details of the procedures have been outlined in a previous report (28). Treatments were administered seven times with 2-day intervals. Tumor diameters were recorded once every 2 days and tumor volumes were calculated using the following formula: Tumor volume = 0.52 x smallest diameter² x largest diameter.

Immunohistochemical staining. Osteosarcoma sections from experimental mice were analyzed via an

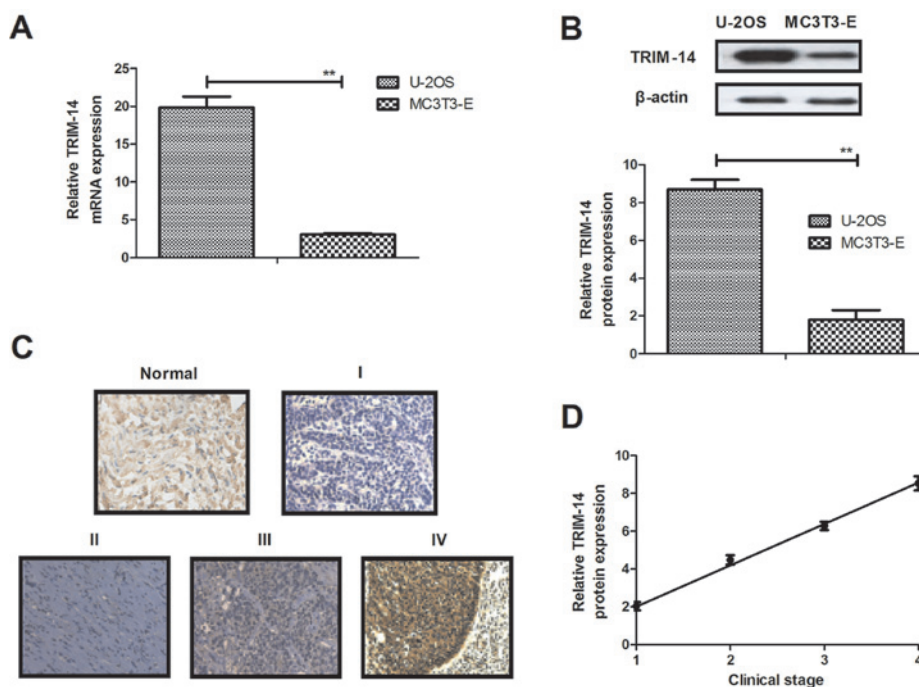


Figure 1. TRIM-14 expression in bone cancer cells and clinical osteosarcoma tissues. (A) mRNA and (B) protein expression levels of TRIM-14 in osteosarcoma cells. (C) TRIM-14 expression levels in osteosarcoma tissues (clinical stages I-IV), as determined by immunohistochemical staining. (D) Association between TRIM-14 protein expression levels and the clinical stage of osteosarcoma. Data are presented as the mean \pm standard error of the mean of three independent experiments. ** $P < 0.01$. TRIM-14, tripartite motif-containing protein-14.

avidin-biotin-peroxidase technique. Paraffin-embedded tumor tissue sections were prepared and epitope retrieval was performed for further analysis. Paraffin sections were subjected to hydrogen peroxide (3%) for 10-15 min, and subsequently blocked by a regular blocking solution for 10-15 min at 37°C. Finally, the sections were incubated with anti-p65 (cat. no. ab16502; Abcam), anti-IKK β (cat. no. IMG-129A; Novus Biologicals, LLC, Littleton, CO, USA) and anti-I κ B α (cat. no. 9242; Cell Signaling Technology, Inc., Danvers, MA, USA) (all 1:1,000) at 4°C for 12 h. To analyze TRIM-14 expression, tumor sections were stained with DAPI for 60 min at 37°C and incubated with anti-TRIM-14 after washing with PBS three times for 60 min at 37°C. All sections were washed three times with PBS and incubated with peroxidase-labeled antibodies (1:5,000; PV-6013; OriGene Technologies, Inc.) at 37°C for 60 min. From the sections, six random fields of view were observed under a light microscope.

Histological assay. Tumor sections (4- μ m-thick) from experimental mice were prepared and fixed in 4% paraformaldehyde. Tumor sections then were embedded in paraffin and stained with hematoxylin and eosin (Sigma-Aldrich; Merck KGaA) for 60 min at 37°C. Total numbers of TUNEL-positive cells were counted in 6 randomly views to calculate the apoptotic tumor cells.

Statistical analysis. Statistical tests for data analysis included Fisher's exact test, log-rank test, Chi-square test, and two-tailed Student's t-test. Multivariate statistical analysis was performed using a Cox regression model. Statistical analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Data were present as the mean and standard error

of the mean. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TRIM-14 expression levels are upregulated in osteosarcoma cells and clinical tumors. Expression levels of TRIM-14 were investigated in osteosarcoma cells and clinical tumor tissues. As shown in Fig. 1A and B, respectively, TRIM-14 mRNA and protein expression levels were significantly upregulated in U-2OS cells. TRIM-14 expression levels were relatively increased in osteosarcoma tissues when compared to normal adjacent tissues (Fig. 1C). Furthermore, TRIM-14 expression levels were positively related with the clinical stage of the patients with osteosarcoma (Fig. 1D). These results showed that TRIM-14 is overexpressed in osteosarcoma cells and tumor tissues, suggesting TRIM-14 may be a potential target for the treatment of osteosarcoma.

Construction of an antibody targeting TRIM-14 and analysis of its characteristic β -actin. In order to study the efficacy of TRIM-14 on osteosarcoma cells, a chimeric antibody targeting TRIM linked with cell-penetrating peptide (Chanti-TRIM) was constructed. The affinity of Chanti-TRIM for TRIM-14 was analyzed and presented high affinity binding with TRIM, as determined by ELISA (Fig. 2A). Western blot analysis also showed that Chanti-TRIM was able to specially bind with TRIM-14 (Fig. 2B). In addition, TRIM-14 expression levels in U-2OS cells were analyzed after treatment with Chanti-TRIM. As illustrated in Fig. 2C, TRIM-14 expression levels were significantly decreased by Chanti-TRIM treatment. Furthermore, immunofluorescence staining assay showed that

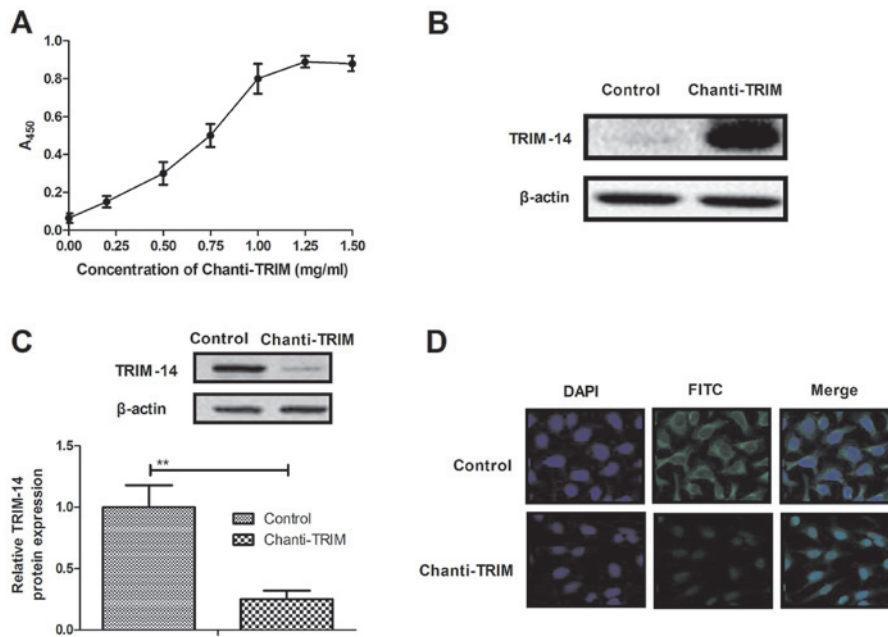


Figure 2. Analysis of the affinity of Chanti-TRIM for TRIM-14 in U-2OS cells *in vitro*. (A) ELISA analyses of the affinity of Chanti-TRIM and TRIM-14. (B) Western blot analysis of the specificity of Chanti-TRIM for TRIM-14. (C) mRNA expression levels of TRIM-14 in osteosarcoma cells after treatment with Chanti-TRIM. (D) Immunofluorescence staining assay analysis of TRIM-14 content in Chanti-TRIM-treated U-2OS cells. Data are presented as the mean \pm standard error of the mean of three independent experiments. **P<0.01. TRIM-14, tripartite motif-containing protein-14; FITC, fluorescein isothiocyanate.

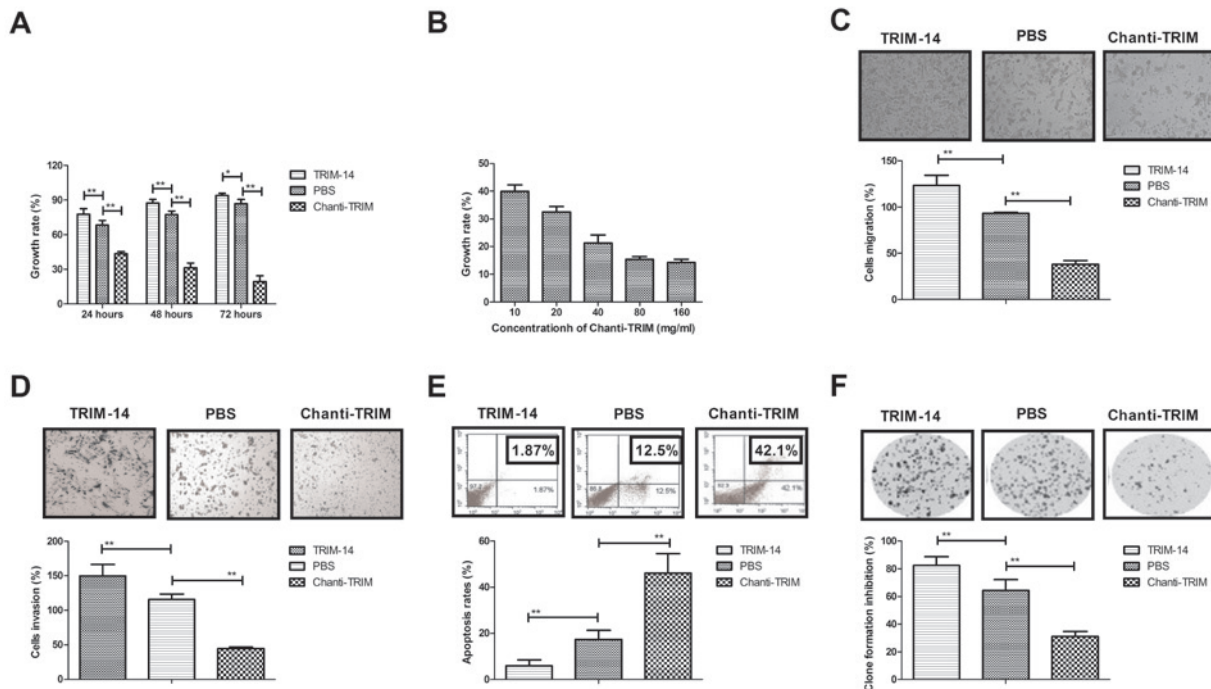


Figure 3. Chanti-TRIM inhibits growth and aggressiveness and promotes the apoptosis of osteosarcoma cells. (A) Growth of Chanti-TRIM-treated and TRIM-14-treated U-2OS cells after treatment for 24, 48 and 72 h. (B) Inhibitory effects of Chanti-TRIM are dose-dependent. Analysis of U-2OS cell (C) migration and (D) invasion after treatment with Chanti-TRIM, TRIM-14 or PBS. (E) Evaluation of the cisplatin-induced apoptosis of U-2OS cells after treatment with Chanti-TRIM, TRIM-14 or PBS. (F) Formation of U-2OS cell colonies after treatment with Chanti-TRIM, TRIM-14 or PBS. Data are presented as the mean \pm standard error of the mean of three independent experiments. **P<0.01. TRIM-14, tripartite motif-containing protein-14.

Chanti-TRIM inhibited TRIM-14 expression in U-2OS cells, as determined by fluorescence intensity (Fig. 2D). These findings suggest that Chanti-TRIM is able to specially bind with TRIM-14 to neutralize TRIM-14 expression in U-2OS cells.

Chanti-TRIM inhibits growth and aggressiveness and promotes apoptosis in osteosarcoma cells. We further analyzed the efficacy of Chanti-TRIM on the growth, aggressiveness and apoptosis of osteosarcoma cells. The results in

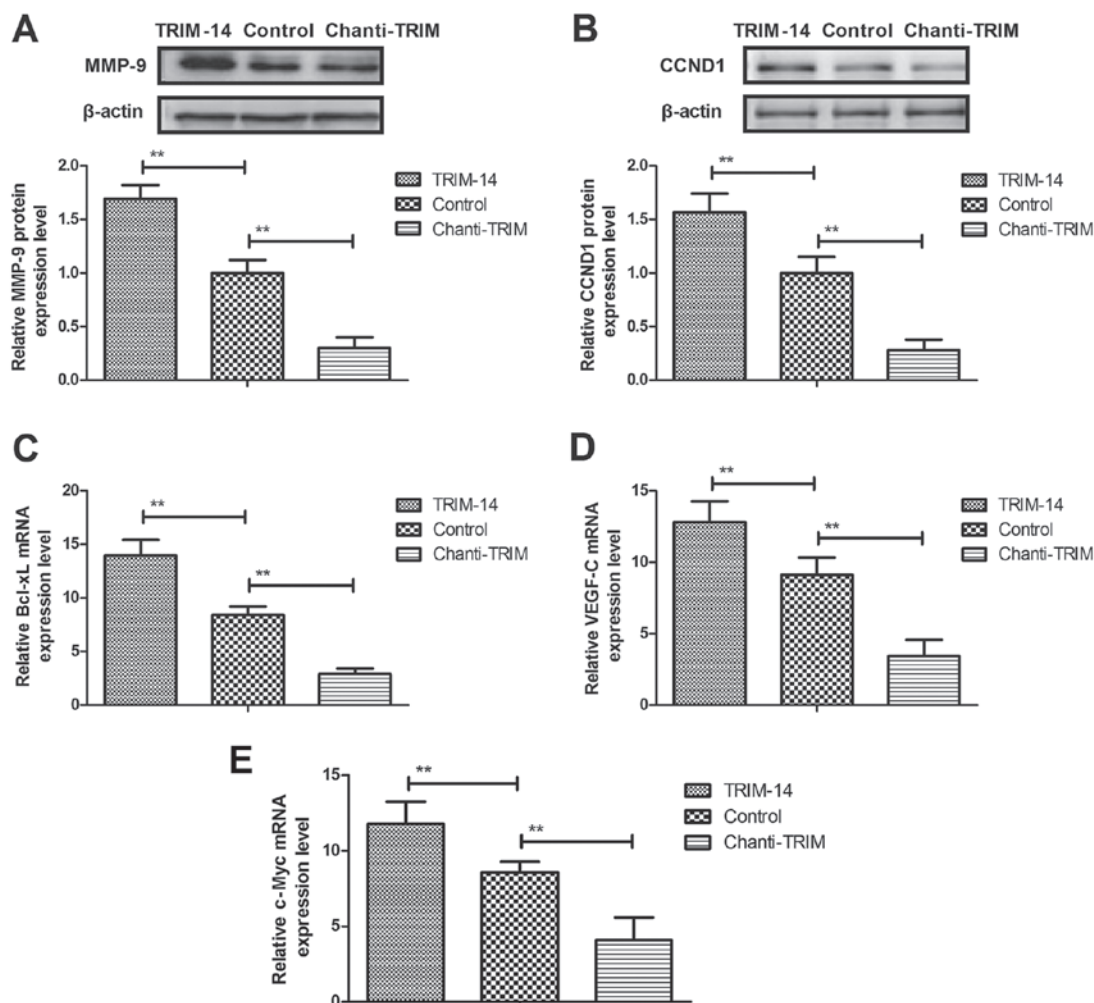


Figure 4. TRIM-14 regulates osteosarcoma cells growth and aggressiveness through the NF- κ B signaling pathway. (A) MMP-9 and (B) and CCND1 protein expression levels in U-2OS cells after treatment with Chanti-TRIM or TRIM-14. Analysis of the expression levels of (C) Bcl-XL, (D) VEGF-C and (E) c-Myc in Chanti-TRIM- or TRIM-14-treated U-2OS cells. ** $P < 0.01$. TRIM-14, tripartite motif-containing protein-14; MMP-9, matrix metalloproteinase-9; CCND1, cyclin D1 gene; Bcl-XL, B-cell lymphoma-extra-large; VEGF-C, vascular endothelial growth factor-C; c-Myc, Myc proto-oncogene protein.

showed that Chanti-TRIM significantly inhibited the growth of U-2OS cells compared with the control cells (Fig. 3A). These inhibitory effects were demonstrated to be dose-dependent (10, 20, 40, 80 and 160 mg/ml Chanti-TRIM; Fig. 3B). It was also observed that TRIM-14 enhanced migration, whereas Chanti-TRIM treatment significantly suppressed U-2OS cells growth migration compared with the control group (Fig. 3C). In addition, the results showed that the invasion of U-2OS cells was significantly inhibited by Chanti-TRIM treatment compared with the TRIM-14-treated cells; whereas TRIM-14 significantly promoted the invasion of U-2OS cells compared with the control (Fig. 3D). Furthermore, the results of apoptosis analysis indicated that Chanti-TRIM significantly promoted the apoptosis of osteosarcoma cells induced by cisplatin when compared with the control group; whereas TRIM-14 significantly promoted apoptotic resistance in U-2OS cells induced by cisplatin when compared with the control group (Fig. 3E). Formation of U-2OS colonies was significantly promoted by TRIM-14 and was significantly inhibited by Chanti-TRIM, as compared with the control (Fig. 3F). These results suggest that Chanti-TRIM not only inhibits the growth and aggressiveness of osteosarcoma cells, but also promotes apoptosis induced by cisplatin.

Chanti-TRIM regulates the growth of osteosarcoma cells through the NF- κ B signaling pathway. To elucidate the mechanisms underlying TRIM-14-mediated osteosarcoma progression, the NF- κ B signaling pathway was investigated in U-2OS cells. The results in Fig. 4A and B show that TRIM-14 treatment significantly increased MMP-9 and CCND1 expression levels, whereas Chanti-TRIM significantly down-regulated MMP-9 and CCND1 expression in U-2OS cells, as compared with the control group. Further analysis indicated that Chanti-TRIM inhibited the expression of NF- κ B target genes, including Bcl-XL, VEGF-C and c-Myc, in U-2OS cells (Fig. 4C-E). Western blotting assays demonstrated that the protein expression levels of p65, IKK- β and I κ B α were markedly decreased in U-2OS cells after treatment with Chanti-TRIM, whereas TRIM increased expression levels of p65, IKK- β and I κ B α in U-2OS cells (Fig. 5A). Furthermore, MMP-9 overexpression abrogated Chanti-TRIM-mediated (MMP-9/ChTRIM) inhibitory effects on NF- κ B activity and expression levels in U-2OS cells (Fig. 5B and C). These results indicated that Chanti-TRIM may be able to inhibit the aggressive phenotype in osteosarcoma cells via the MMP-9-induced NF- κ B signaling pathway.

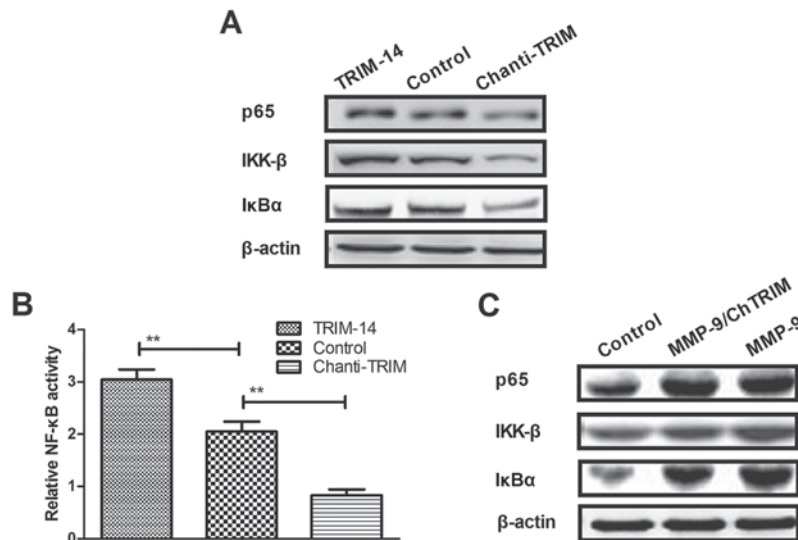


Figure 5. (A) Western blotting analyses of the expression levels of p65, IKK-β and IκBα in U-2OS cells after treatment with Chanti-TRIM or TRIM-14. (B) NF-κB activity in U-2OS cells after treatment with Chanti-TRIM or TRIM-14. (C) Effects of MMP-9 overexpression on Chanti-TRIM-inhibited expression levels of p65, IKK-β and IκBα in U-2OS cells. Data are presented as the mean ± standard error of the mean of three independent experiments. **P<0.01. TRIM-14, tripartite motif-containing protein-14; NF-κB, nuclear factor-κB; MMP-9, matrix metalloproteinase-9; Ikkβ, nuclear factor κ-B kinase subunit β; IκBα, NF-κB inhibitor α.

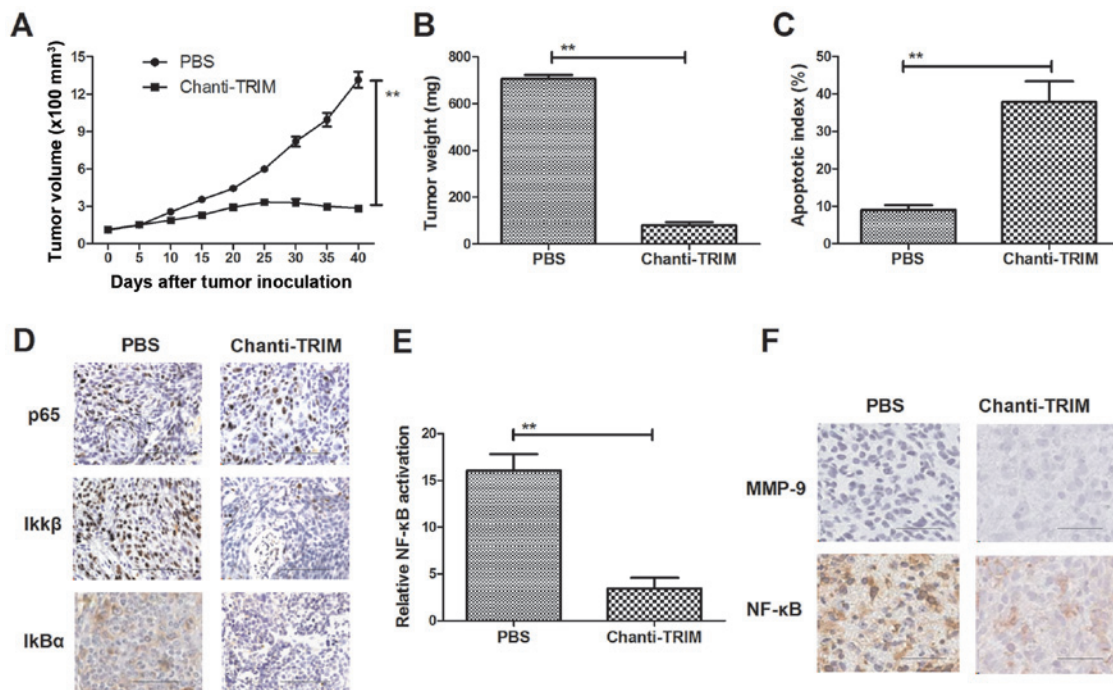


Figure 6. Inhibition of TRIM-14 expression by Chanti-TRIM contributes to osteosarcoma regression *in vivo*. (A) Mean tumor volume was measured after treatment with Chanti-TRIM or control. (B) Tumor weight from experimental mice. (C) Analysis of TUNEL-positive apoptotic cells in Chanti-TRIM-treated and control tumors. (D) Expression levels of p65, Ikkβ and IκBα in Chanti-TRIM-treated and control tumors, as determined by immunohistochemical staining. (E) NF-κB luciferase activity in Chanti-TRIM-treated and control tumors. (F) Representative images of MMP-9 and NF-κB expression levels in Chanti-TRIM-treated and control tumors. Data are presented as the mean ± standard error of the mean of three independent experiments. **P<0.01. TRIM-14, tripartite motif-containing protein-14; NF-κB, nuclear factor-κB; MMP-9, matrix metalloproteinase-9.

Chanti-TRIM inhibits osteosarcoma growth in U-2OS-bearing xenograft mice. To further evaluate the therapeutic efficacy of Chanti-TRIM on osteosarcoma growth, a murine xenograft model of osteosarcoma was established and treated with Chanti-TRIM or PBS (control). As shown in Fig. 6A and B, tumor growth and tumor weight were significantly reduced in the Chanti-TRIM treated mice, as compared with the control.

Histological analysis revealed that apoptotic bodies were increased in the tumors of mice treated with Chanti-TRIM (Fig. 6C). NF-κB nuclear translocation factors, p65, Ikkβ and IκBα, were decreased in tumors treated with Chanti-TRIM (Fig. 6D). Furthermore, NF-κB luciferase activity in tumors was significantly inhibited by treatment with Chanti-TRIM (Fig. 6E). MMP-9 and NF-κB expression levels were

downregulated after treatment with Chanti-TRIM (Fig. 6F). These results indicate that Chanti-TRIM inhibited osteosarcoma growth *in vivo*, suggesting that Chanti-TRIM may be a potential anti-cancer agent for osteosarcoma.

Discussion

In the present study, the efficacy a targeted strategy of TRIM-14 osteosarcoma suppression was investigated in osteosarcoma cells and osteosarcoma-bearing xenograft mice. Previous studies have suggested that TRIM-14 overexpression may induce an aggressive phenotype in cancer progression through regulation of the NF- κ B signaling pathway (29). Therefore, understanding the role of TRIM-14 is necessary for tumor research and treatment in human tumorigenesis and metastasis. The present study design involved constructing a chimeric antibody target for TRIM-14 and investigating its anti-cancer efficacy *in vitro* and *in vivo*. The findings indicated that Chanti-TRIM decreased the growth, migration and invasion of osteosarcoma cells by inhibiting the MMP-induced NF- κ B signaling pathway; whereas overexpression of TRIM-14 promoted the growth, migration and invasion of osteosarcoma cells. The results demonstrated that overexpression of TRIM-14 increased cisplatin-induced apoptosis resistance by activating the NF- κ B signal pathway. Notably, Chanti-TRIM-treated tumors in xenograft mice were significantly inhibited, as determined via reduced tumor volumes compared with the control group. These results indicate that TRIM-14 may be a potential molecular target and Chanti-TRIM may be a potential anti-cancer agent through the inhibition of the MMP-9-induced NF- κ B pathway for osteosarcoma therapy.

To date, inducing apoptosis in tumor cells is the most efficient clinical regiment for the treatment of patients with cancer (30,31). Resistance to apoptosis is the greatest obstacle to the treatment of human cancer (32,33). Decreasing the apoptosis-resistance of cancer cells and tumors tissues may improve the clinical treatment outcomes of patients with osteosarcoma who have undergone oncotherapy and other comprehensive treatments (34,35). In recent years, TRIM-14 was identified as an oncogene that promotes tumor growth, aggressiveness and tumor angiogenesis; however, knockdown of TRIM-14 expression can significantly inhibit tumor growth, migration, invasion and tumor angiogenesis in human colorectal cancer cells (36). The results of the present study demonstrated that Chanti-TRIM treatment not only inhibits growth, but also enhances the apoptosis of osteosarcoma cells induced by cisplatin. Notably, previous findings have shown that TRIM-14 overexpression promotes cancer cell proliferation and predicts poor survival in patients with colorectal cancer, which is consistent with the present findings (37). The findings of the present study suggest that Chanti-TRIM is able to neutralize TRIM-14 expression, which can lead to opposite outcomes by upregulating MMP-9 through the activation of the NF- κ B signaling pathway.

Notably, different signaling pathways that promote the aggressiveness of osteosarcoma have been associated with the modulation of MMP-9 transcription (38,39). NF- κ B transcription factors may induce the expression and activation of MMP-9 by interacting with binding sites, and may consequently

promote tumor progression (40,41). The results of this study suggest that TRIM-14 may induce MMP-9 expression and promote NF- κ B activity, whereas Chanti-TRIM-mediated blocking of the activity of NF- κ B may significantly downregulate the expression of TRIM-14 and prevent MMP-9 activity in the NF- κ B pathway.

The results of the present study indicate that Chanti-TRIM was able to downregulate MMP-9 expression by inhibiting the NF- κ B signaling pathway in U-2OS cells. Previous studies have demonstrated that targeting CCND1 suppresses osteosarcoma cell metastasis (42-44). In addition, Bcl-XL, VEGF-C, and c-Myc are overexpressed in osteosarcoma cells, which are associated with the apoptosis, growth and aggressiveness of malignant osteosarcoma (45,46). Furthermore, Yu *et al* (47) have suggested that downregulation of the NF- κ B signaling pathway is capable of inhibiting cell invasion and the migration ability of human osteosarcoma *in vitro*. The findings of the present study indicate that Chanti-TRIM suppresses the expression levels of Bcl-XL VEGF-C and c-Myc, which contributes to inhibiting the aggressive phenotype in osteosarcoma cells.

In conclusion, the findings of the present study indicated that TRIM-14 is overexpressed in bone cancer cells and clinical bone cancer tissues. This research suggested that inhibition of TRIM-14 expression by Chanti-TRIM treatment markedly suppressed the growth, aggressiveness, metastasis and apoptosis-resistance in osteosarcoma via MMP-9-induced NF- κ B signaling. According to the molecular and therapeutic study of Chanti-TRIM, TRIM may be a potential target for the treatment of patients with osteosarcoma.

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