High expression of TRIM29 (ATDC) contributes to poor prognosis and tumor metastasis by inducing epithelial-mesenchymal transition in osteosarcoma

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Abstract. The association of TRIM29 overexpression with cancer progression and poor clinical prognosis has been reported in the context of several types of cancers. In the present study, we investigated the prognostic relevance of *TRIM29* and its involvement in the progression of human osteosarcoma. To the best of our knowledge, this is the first study to demonstrate a major role of *TRIM29* in osteosarcoma. Our results showed that the expression of TRIM29 in osteosarcoma tissues was much higher than that in normal bone tissues. Furthermore, TRIM29 expression was significantly correlated with tumor size, recurrence, metastasis and overall survival time. High expression of TRIM29 and presence of metastasis were independent predictors of poor prognosis in these patients. Both protein and mRNA expression of TRIM29 in osteosarcoma cell lines were significantly higher than those in osteoblast cell line, hFOB1.19. Moreover, the results indicated that TRIM29 promoted migration and invasive growth of osteosarcoma cells by inducing epithelial-mesenchymal transition. Therefore, ectopic expression of TRIM29 potentially contributes to metastasis and poor prognosis in patients with osteosarcoma. In summary, TRIM29 is a potential prognostic biomarker and a therapeutic target for patients with osteosarcoma.

Introduction

Osteosarcoma is the most common type of primary bone cancer (1,2), which typically occurs in children and young adults. It is characterized by aggressive growth and a tendency for early metastasis. The most common sites of osteosarcoma are long bones of the limbs (3), particularly the distal ends of femur and proximal tibia (4). Despite advances in multimodal treatment strategies, including neoadjuvant chemotherapy and surgery, the survival rate of these patients remains abysmal (5). The five-year survival rate for localized osteosarcoma is ~65-70%, while that for metastatic disease is a lowly 20% (6-9). Approximately one-third of all patients with osteosarcoma experience recurrent or metastatic disease; the average survival time after development of metastasis or recurrence is less than 1 year (10). The development of targeted therapies over the last decade has brought a paradigm change in cancer treatment. However, gaps in knowledge pertaining to the complex molecular biology of osteosarcoma has been a barrier to the development of targeted therapies for these patients. Identification of genetic therapeutic targets to counter metastatic and invasive growth characteristics of osteosarcoma is a potential approach to new therapeutics for osteosarcoma.

TRIM29, also known as ataxia-telangiectasia group D complementing gene (*ATDC*), is located at chromosome 11q23. It encodes a 588-amino acid protein which is a member of the tripartite motif (TRIM) protein family (11). The TRIM family comprises multidomain ubiquitin E3 ligases, which are characterized by a conserved RING-finger domain, one or two B-box zinc-finger motifs and a coiled-coil domain (12-14). Unlike most other TRIM family proteins, there is no RING domain in TRIM29, which was earlier considered indicative of its lack of E3 ubiquitin ligase activity. However, a weak E3 ligase activity of TRIM29 mediated via B-box domain has recently been reported (15). In addition, following DNA damage, TRIM29 is phosphorylated and interacts with RNF8, an E3 ubiquitin ligase, which promotes DNA repair and cell survival (16). The TRIM family proteins are involved in a series of biological and physiological processes. However, when altered they are implicated in several pathological processes, including carcinogenesis (11,17). Several studies have revealed a role of TRIM29 in carcinogenesis, which suggests its potential as a therapeutic target for cancer therapy (18). Recent studies have revealed that TRIM29 overexpression exerts an oncogenic function in several types of cancers, including lung cancer (19), hepatocellular carcinoma (20), pancreatic adenocarcinoma (21), gastric cancer (22), esophageal

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carcinoma (23), colorectal (24) and bladder cancer (25), nasopharyngeal carcinoma (26) and ovarian serous papillary tumors (27). Upregulation of TRIM29 in these cancers shows a significant correlation with pathological grade, tumor invasion, metastasis, and poor prognosis. TRIM29 has also been shown to act as a tumor suppressor in several types of cancers, such as breast cancer (28,29). However, the significance and prognostic value of TRIM29 expression in osteosarcoma remain unclear. In the present study, we evaluated the TRIM29 expression pattern and its relevance in osteosarcoma.

Materials and methods

Patients and tissue samples. The present study was approved by the Research Ethics Committee at The First Affiliated Hospital of Xi'an Jiaotong University (Shaanxi, China). All patients or their guardians provided informed consent for participation in the present study. All specimens were handled and rendered anonymous according to ethical and legal standards. All pathological specimens were obtained from the Department of Pathology at The First Affiliated Hospital of Xi'an Jiaotong University. Retrospective analysis of data pertaining to 64 patients (40 men, 24 women; mean age ± SD: 23.6±10.9 years; range, 6-59 years) with osteosarcoma who underwent surgery at our hospital from January 2005 to December 2014 was conducted. Normal bone samples (femur, tibia, humerus, radius and ulna) obtained from 30 patients who underwent surgery (internal fixation of fracture, amputation, knee arthroplasty or hip arthroplasty) at our hospital between January 2010 and December 2014 were used as controls. The diagnosis of osteosarcoma was based on histopathological and radiographic findings. Data on clinical variables such as age at diagnosis, sex, tumor size, tumor location, tumor recurrence, visceral metastasis and survival time after surgery were collected and analyzed. Overall survival time was defined as the interval between initial surgery and death or the end of follow-up (follow-up was terminated on September 1, 2016). The sites of osteosarcoma were femur (n=34), tibia (n=18), humerus (n=4), radius and ulna (n=3), fibula (n=2), pelvis (n=2) and sacrum (n=1). Tumor size was found to be >5 cm in 39 cases. Visceral metastasis was present in 26 cases and absent in 38 cases. Lung was the most common site of metastasis. Post-surgery tumor recurrence occurred in 16 cases.

Immunohistochemical staining. Immunohistochemical staining for TRIM29 was performed for all 64 samples of osteosarcoma and 30 samples of normal bone. All biopsy specimens were embedded in paraffin and 4-μm thick sections were prepared. These sections were first dehydrated by heating, dewaxed and rehydrated using a graded series of xylene and ethanol, and blocked with 3% H_2O_2 for 20 min in order to quench endogenous peroxidase activity. Microwave antigen retrieval procedure was performed in 10 mM citrate buffer. The sections were incubated overnight at 4˚C with TRIM29 primary antibody (GTX115749; 1:200; GeneTex, San Antonio, TX, USA). Immunostaining was conducted using the 3,3'-diaminobenzidine (DAB) kit. The sections were then stained with hematoxylin, dehydrated, cleared and mounted. Phosphate-buffered saline (PBS) was used as the negative control (NC).

Table I. Primer sequences for RT-qPCR analysis.

Genes		Primer sequences
TRIM29	F	5'-CATGTACCTGACACCCAAAG-3'
	R	5'-CCGGGAGGTGTAGTTACCTT-3'
E-cadherin	F	5'-ATGAGTGTCCCCCGGTATCT-3'
	R	5'-CAAACACGAGCAGAGAATCA-3'
N-cadherin	F	5'-ACAGTGGCCACCTACAAAGG-3'
	R	5'-CCGAGATGGGGTTGATAATG-3'
Vimentin	F	5'-CGCCAGATGCGTGAAATGG-3'
	R	5'-ACCAGAGGGAGTGAATCCAGA-3'
ZEB1	F	5'-ACTTAAAGTGGCGGTAGATGGTA-3'
	R	5'-CAACTGTTTGTAGCGACTGGATT-3'
Snail	F	5'-TTTACCTTCCAGCAGCCCTACGA-3'
	R	5'-CAACTGTTTGTAGCGACTGGATT-3'
GAPDH	F	5'-GGGAAACTGTGGCGTGAT-3'
	R	5'-GAGTGGGTGTCGCTGTTGA-3'

F, forward; R, reverse.

Evaluation of immunohistochemical staining, immunohistochemical score (IHS). The staining results were evaluated by 3 independent pathologists. The intensity of staining was graded as score 0 (negative), 1 (pale yellow), 2 (dark yellow), and 3 (brown). The staining extent was scored by the percentage of positive cells as 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The final IHS was obtained by multiplying the score of intensity and extent. Based on the IHS, specimens were categorized into 1 of 4 groups: 0-1 (negative, -); 2-4 (weak positive, +); 5-8 (moderate positive, ++); 9-12 (strong positive, +++). Scores of 0-4 were designated as low expression (-/+), while scores of 5-12 were designated as high expression (++/+++).

Cell culture. Human osteoblast cell line hFOB1.19 and human osteosarcoma cell lines MG-63, Saos-2, 143B and U-2OS were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). hFOB1.19 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 34˚C in 5% CO_2. MG-63, Saos-2, 143B and U-2OS cells were maintained in DMEM supplemented with 10% FBS at 37˚C in 5% CO_2.

Cell transfection. The effects of TRIM29 knockdown was studied in the 143B cell line, while overexpression of TRIM29 was studied in the Saos-2 cell line. *In vitro* transfections were achieved by Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) by following the manufacturer's protocols. Transfection efficiency was estimated by western blotting and RT-qPCR. Small interfering RNAs (siRNAs) to knock down TRIM29 were designed and synthesized, and the scramble nonsense sequence was used as the negative control (NC); a final concentration of 50 nM of siRNA and NC-siRNA was used. The target siRNA sequence for TRIM29 was: 5'-CUGU GUUGUUUCUGCAGGAdTdT-3';

Table II. Immunohistochemical expression of TRIM29 in osteosarcoma and normal bone tissues.

		Expression of TRIM29					
Tissues	N	Negative (-)	Weak positive (+)	Moderate positive (++)	Strong positive (+++)	Positive rate (%)	P-value
Osteosarcoma	64	18	13	19	14	71.88	0.000[a]
Normal bone	30	23	7	0	0	23.33	

[a]Significant difference.

NC siRNA sequence was: 5'-UUCUCCGAACGUGUCACG UTT-3'. For overexpression of TRIM29, the pcDNA3.1-HA expression vector containing a full-length human TRIM29 sequence (pcDNA3.1-HA-TRIM29) was synthesized and the empty pcDNA3.1-HA plasmid was used as NC.

Reverse transcriptase quantitative-PCR (RT-qPCR). Total RNA from the cell lines was extracted using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Concentration and purity of total RNA were evaluated by UV-Vis spectroscopy with the Bio-Rad SmartSpec 3000 system (Bio-Rad, Hercules, CA, USA) by optical density (OD) readings at 260 nm and the ratio of 260/280, respectively. Then, first-strand cDNA was synthesized using a PrimeScript RT reagent kit (Takara, Tokyo, Japan). RT-qPCR was performed using SYBR-Green Quantitative Real-Time Polymerase Chain Reaction Master Mix on the ABI PRISM® 7500 Sequence Detection System (both from Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. Each sample was measured in triplicate. Relative expression level of mRNA was quantified by the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table I.

Western blot analysis. Total protein was collected from cells using RIPA lysis buffer with protease inhibitors (Roche Diagnostics, Basel, Switzerland) and phenylmethylsulfonyl fluoride (PMSF) on ice. The insoluble debris was removed by centrifugation at 12,000 rpm for 30 min at 4˚C. The concentration of each sample was quantified using the Pierce BCA protein detection kit (Thermo Scientific, Hudson, NH, USA). Protein samples were separated on SDS-PAGE gels, and then electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% fat-free milk in PBS + Tween-20 (PBST) buffer, and then incubated with primary antibodies against TRIM29 (GTX115749; 1:1,000; GeneTex), HA tag (ab9110; 1:4,000; Abcam, Cambridge, MA, USA), E-cadherin (3195T; 1:1,000), N-cadherin (13116T; 1:1,000), vimentin (5741T; 1:1,000) [all from Cell Signaling Technology (CST), Danvers, MA, USA], Snail (GTX100754; 1:1,000; GeneTex), ZEB1 (GTX105278; 1:1,000; GeneTex), and GAPDH (5174S; 1:1,000; CST) overnight at 4˚C. Then, the membranes were washed and incubated with the HRP-conjugated secondary antibody for 2 h [OB4050-05; 1:20,000; goat anti-rabbit secondary antibody (Southern Biotech, Birmingham, AL, USA)] at room temperature. The visual bands were obtained using enhanced chemiluminescence (ECL) detection reagents (Pierce, Rockford, IL, USA). GAPDH was used as an internal control.

Wound healing assays. Cells were seeded into 6-well plates. After transfection, cells were cultured for 24 h until 95-100% confluence. Then, pipette tips were used to make straight scratches on the surfaces of confluent cell monolayers. After that, cells were gently washed with PBS for 3 times to remove the detached cells. Then, cells were cultured in serum-free medium with 10 μg/ml mitomycin to inhibit cell proliferation. Images were obtained at 0, 24 and 48 h using an inverted microscope. Cell migration rates were calculated from all the images using Image-Pro Plus 6.0 software (version 6.0; Media Cybernetics, Rockville, MD, USA).

Transwell invasion assays. Cell invasion assays were performed on Transwell chambers. The Transwell membrane was coated with 1:3 diluted Matrigel (BD Biosciences, San Jose, CA, USA). Cells were resuspended and trypsinized in serum-free medium, 100 μl of cell suspension ($1x10^5$ cells) was added into the upper chamber and 600 μl medium with 10% FBS was added into the lower chamber. Then, cells were cultured at 5% CO_2 at 37˚C for 24 h. After that, the cells on the upper surface of the membrane were wiped with a cotton swab, and the attached cells below the membrane were fixed with paraformaldehyde for 15 min and stained with crystal violet for 10 min. After washing with PBS, the number of stained cells was calculated under a microscope.

Statistical analysis. All statistical analyses were performed using SPSS software (version 20.0; SPSS, Inc., Chicago, IL, USA). Between-group differences with respect to categorical variables (frequencies) were assessed using Chi-squared test. Those with respect to continuous variables were assessed with t-test. Survival analysis was performed using the Kaplan-Meier method, and differences in survival were evaluated using the log-rank test. Cox regression model was applied for multivariate survival analysis. Differences were considered to be statistically significant at $P<0.05$ or highly significant at $P<0.01$.

Results

Immunohistochemical expression of TRIM29 in osteosarcoma and normal bone tissues. Fig. 1 shows representative immunohistochemical staining images of TRIM29 in human

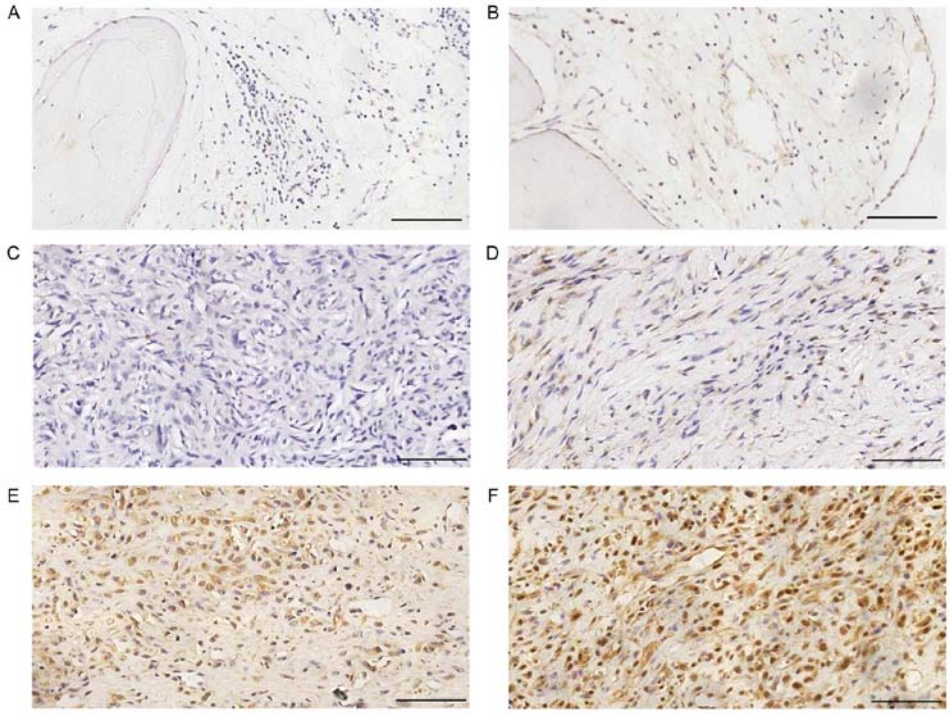

Figure 1. Representative images of immunohistochemical staining for TRIM29 in human normal bone and osteosarcoma tissues. Staining intensity in normal bone tissues was graded as: (A) negative, (B) weak positive. Staining intensity in osteosarcoma tissues was graded as: (C) negative, (D) weak positive, (E) moderate positive and (F) strong positive. Scale bar, 100 μm.

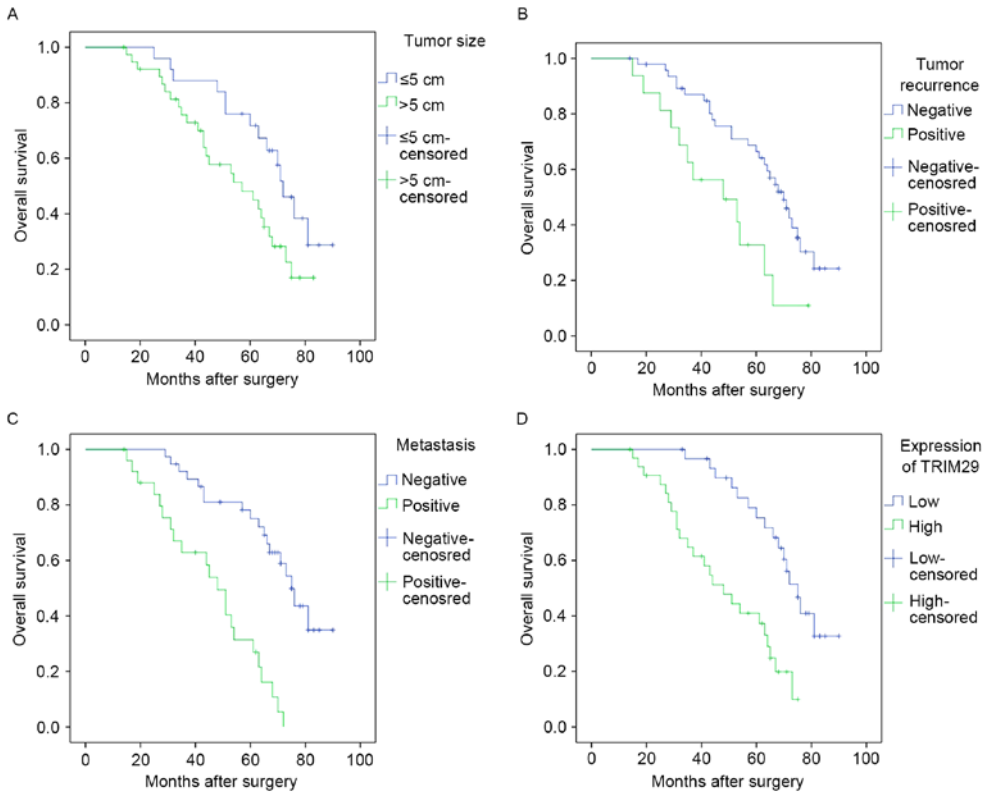

Figure 2. Univariate analysis of overall survival in patients with osteosarcoma was performed by the Kaplan-Meier method. (A) Tumor size (>5 vs. ≤5 cm; P<0.05). (B) Tumor recurrence (positive vs. negative; P<0.01). (C) Metastasis (positive vs. negative; P<0.01). (D) Expression of TRIM29 (high vs. low; P<0.01).

normal bone and osteosarcoma tissues. Forty-six out of 64 (71.88%) osteosarcoma specimens and 7 out of 30 (23.33%) normal bone specimens stained positive for TRIM29. The between group difference in this respect was statistically significant (Table II; P<0.01). None of the normal bone tissue specimens showed high expression of TRIM29, while 33 out

Table III. Correlation between TRIM29 expression and clinical parameters.

Parameters	N	Expression of TRIM29 Low (-/+)	High (++/+++)	χ^2	P-value
Age (years)					
>18	31	14	17	0.258	0.627
≤18	33	17	16		
Sex					
Male	40	18	22	0.505	0.607
Female	24	13	11		
Tumor location					
Femur	34	15	19	0.631	0.729
Tibia	18	10	8		
Other	12	6	6		
Tumor size (cm)					
>5	39	14	25	6.286	0.020[a]
≤5	25	17	8		
Tumor recurrence					
Positive	16	4	12	4.692	0.043[a]
Negative	48	27	21		
Visceral metastasis					
Positive	26	7	19	8.115	0.006[b]
Negative	38	24	14		
Overall survival time (years)					
>5	32	21	11	7.570	0.012[a]
≤5	32	10	22		

[a]P<0.05; [b]P<0.01.

Table IV. Univariate analysis of overall survival time in patients with osteosarcoma.

Parameters	N	Overall survival (months) Mean ± SD	Median	χ^2	P-value
Sex					
Male	40	54.5±19	58	0.016	0.900
Female	24	55.3±22.3	60.5		
Age (years)					
>18	31	55.6±19.7	60	0.256	0.613
≤18	33	54±20.9	61		
Tumor location					
Femur	34	54.1±21.7	64	0.126	0.939
Tibia	18	54.6±19.9	59.5		
Others	12	56.9±17.4	58.5		
Tumor size (cm)					
>5	39	48.7±19.9	45	4.974	0.026[a]
≤5	25	64.2±16.9	68		
Tumor recurrence					
Positive	16	43.8±17.9	44	7.555	0.006[b]
Negative	48	58.4±19.7	65		
Visceral metastasis					
Positive	26	42.7±18.6	44.5	25.592	0.000[b]
Negative	38	63±16.9	67.5		
Expression of TRIM29					
High	33	44.6±19.1	43	15.022	0.000[b]
Low	31	65.6±15.2	70		

[a]P<0.05; [b]P<0.01.

of 64 (51.56%) osteosarcoma specimens showed high expression (Table II).

Correlation between TRIM29 expression and clinical parameters. Overall, there was no significant correlation between TRIM29 expression and age at diagnosis (P=0.627), sex (P=0.607) or tumor location (P=0.729) (Table III). However, a significant correlation was found between TRIM29 expression and tumor size (P<0.05), tumor recurrence (P<0.05), visceral metastasis (P<0.01) and overall survival time after surgery (P<0.05).

Prognostic value of TRIM29 expression. Upon Kaplan-Meier survival analysis, tumor size (P<0.05), recurrence (P<0.01) and metastasis (P<0.01) showed a significant direct correlation with overall survival (Fig. 2A-C; Table IV). Additionally, high expression of TRIM29 in osteosarcoma specimens showed a significant inverse correlation with overall survival (P<0.01) (Fig. 2D; Table IV). Nevertheless, no significant correlation was noted between overall survival time and other clinical parameters, such as age at diagnosis, sex and tumor location (Table IV). Variables that showed significant association upon univariate analysis were included in the multivariate analysis using Cox proportional hazard model. High expression of TRIM29 and presence of metastasis were found to be independent predictors of poor prognosis in patients with osteosarcoma (Table V).

Expression of TRIM29 in human osteoblast and osteosarcoma cell lines. At the cellular level, both protein and mRNA expression of TRIM29 were significantly higher in osteosarcoma cell lines as compared to that in the osteoblast cell line hFOB1.19 (Fig. 3A). Moreover, in these 4 osteosarcoma cell lines, the protein and mRNA expression of TRIM29 were highest in 143B, and lowest in Saos-2. Coincidentally, invasion and proliferation capability of 143B was much higher than that of Saos-2. These findings indicate that *TRIM29* may

Table V. Multivariate analysis of overall survival time in patients with osteosarcoma.

Parameters	HR	95% CI of HR	P-value
Tumor size (>5 vs. ≤5 cm)	1.661	0.816-3.379	0.162
Tumor recurrence (positive vs. negative)	2.112	0.994-4.484	0.052
Visceral metastasis (positive vs. negative)	4.911	2.339-10.310	0.000[b]
Expression of TRIM29 (high vs. low)	2.485	1.157-5.334	0.020[a]

HR, hazard ratio; CI, confidence interval. [a]P<0.05; [b]P<0.01.

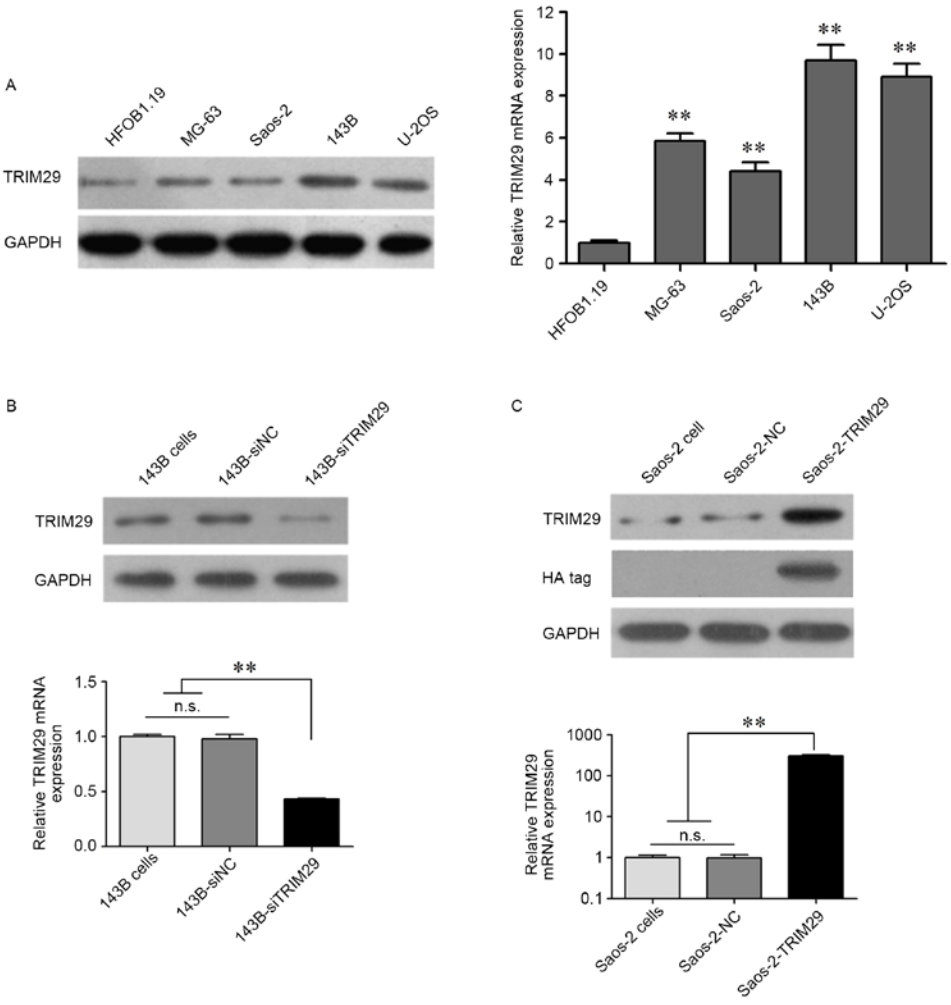

Figure 3. (A) Expression of TRIM29 in osteosarcoma cell lines was significantly higher than that in the hFOB1.19 cell line; **P<0.01, compared to hFOB1.19. (B) Interference of TRIM29 expression in 143B cells was analyzed by western blotting and RT-qPCR; **P<0.01, compared to 143B cells and 143B-siNC (negative control) groups. (C) Overexpression of TRIM29 in Saos-2 cells was analyzed by western blotting and RT-qPCR. Proteins from HA-TRIM29-expressing cells (Saos-2-TRIM29, transfected with pcDNA3.1-HA-TRIM29 plasmid), as well as from Saos-2-NC (negative control, transfected with empty pcDNA3.1-HA plasmid) and Saos-2 cells (untreated), were analyzed by western blotting using anti-HA tag antibody; **P<0.01, compared to Saos-2 cells and Saos-2-NC groups. GAPDH was used as an internal control. n.s., not significant.

play an important role in the progression of osteosarcoma. Knockdown of TRIM29 in 143B cells and overexpression of TRIM29 in Saos-2 cells were detected using western blotting and RT-qPCR (Fig. 3B and C). For confirmation of exogenous TRIM29 expression, anti-HA tag antibody was used (Fig. 3C). HA-TRIM29 protein was detected only in Saos-2-TRIM29 cells (transfected with pcDNA3.1-HA-TRIM29 plasmid).

TRIM29 enhances osteosarcoma cell migration and invasion. To study the functional relevance of *TRIM29* in human osteosarcoma cells, wound healing assays were performed by calculation of cell migration rates into the wounded area after scratching, performed under serum-free conditions. Since these assays were performed in the absence of serum or growth factors, healing of the wounds occurred by cell migration and

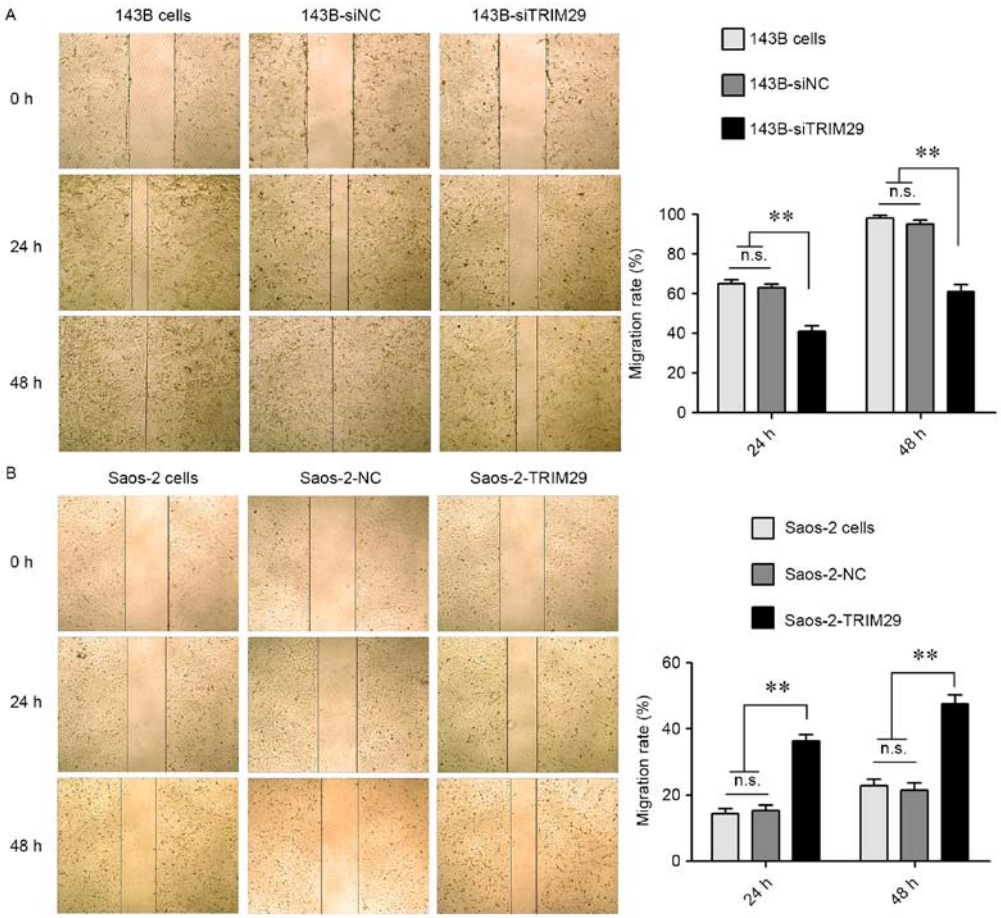

Figure 4. Wound healing assays were performed in 143B and Saos-2 cells. Representative images visualized at 0, 24 and 48 h are shown. (A) Migration rates of 143B-siTRIM29 at both 24 and 48 h were significantly lower than that in the other two control groups. (B) Migration rates of Saos-2-TRIM29 at both 24 and 48 h were significantly higher than that in the other two control groups. **P<0.01; n.s., not significant.

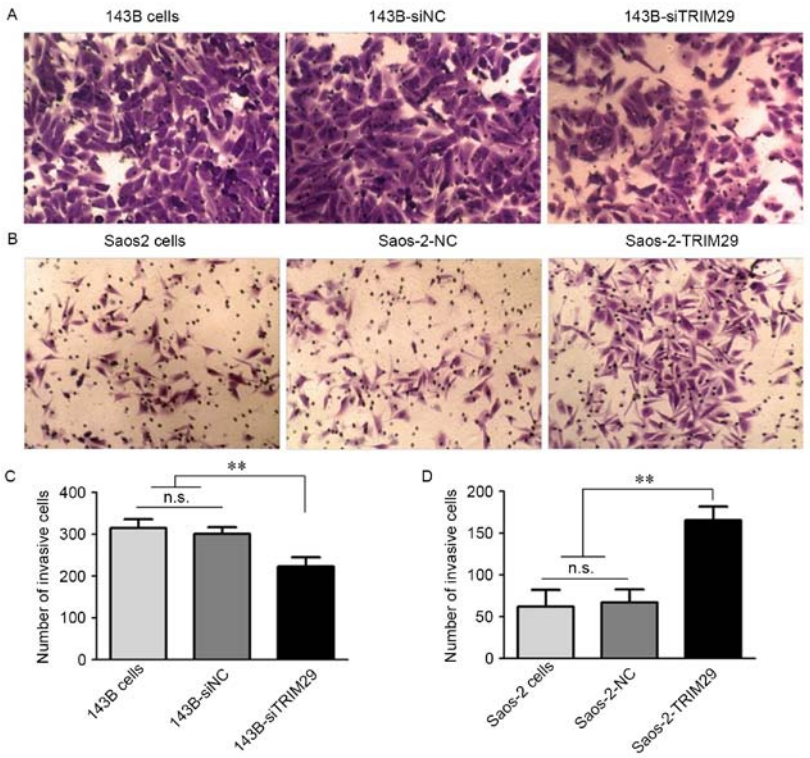

Figure 5. (A and B) Transwell invasion assays were performed in 143B and Saos-2 cells. (C) The number of invasive cells in 143B-siTRIM29 group was significantly lower than that in 143B cells and 143B-siNC groups. (D) The number of invasive cells in Saos-2-TRIM29 group was significantly higher than that in Saos-2 cells and Saos-2-NC groups. **P<0.01; n.s., not significant.

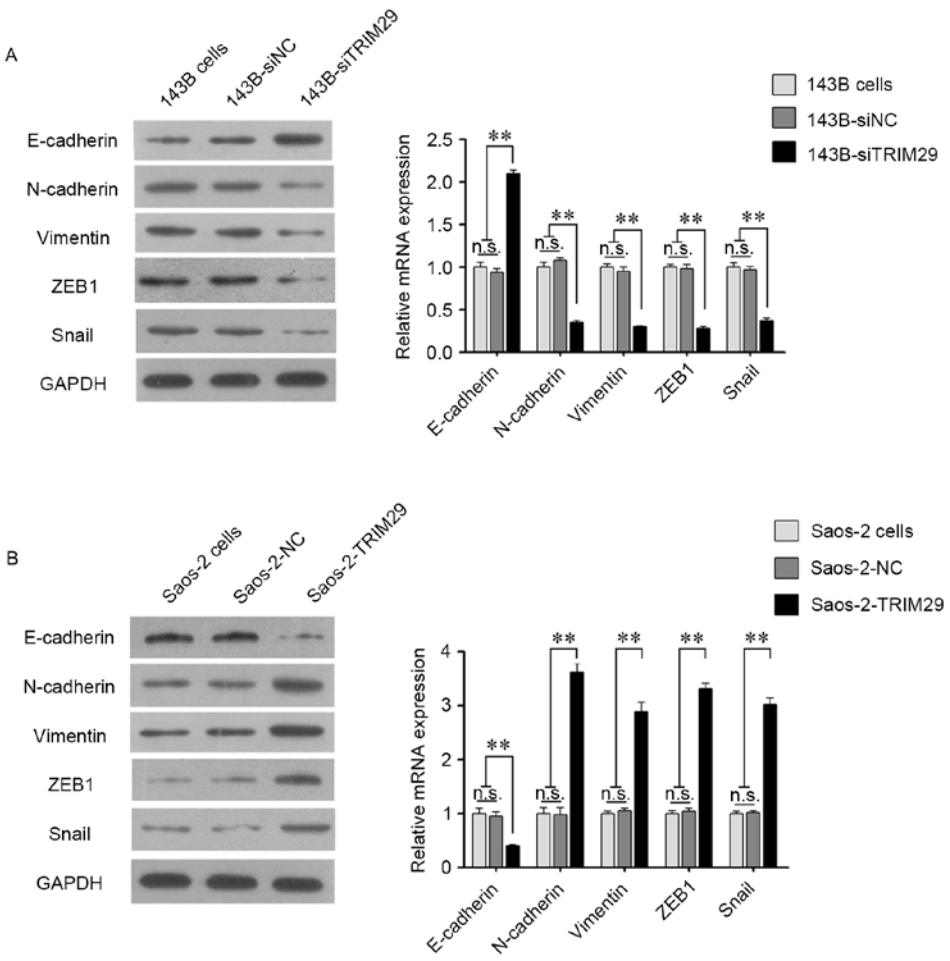

Figure 6. Western blot and RT-qPCR analyses of EMT markers in 143B and Saos-2 cells. (A) Knockdown of TRIM29 in 143B cells resulted in a significant upregulation of the epithelial marker E-cadherin and downregulation of N-cadherin, vimentin, ZEB1 and Snail. (B) Overexpression of TRIM29 in Saos-2 cells showed a significant downregulation of E-cadherin and upregulation of N-cadherin, vimentin, ZEB1 and Snail. **P<0.01; n.s., not significant.

not as a result of proliferation of cells. Fig. 4 depicts representative images obtained at 0, 24 and 48 h. As shown in Fig. 4A, the migration rates of 143B-siTRIM29 cells were significantly lower than that of 143B and 143B-siNC cells. The P-values for 143B-siTRIM29 group vs. 143B cells or 143B-siNC groups at both 24 and 48 h were all <0.01. Moreover, overexpression of TRIM29 enhanced the migration of the Saos-2 cells (Fig. 4B). P-values for the Saos-2-TRIM29 group vs. Saos-2 cells or Saos-2-NC groups at 24 and 48 h were also <0.01. Transwell invasion assays were also performed under serum-free conditions. As shown in Fig. 5, TRIM29 knockdown in 143B cells resulted in a significant reduction in invasive growth as compared to that in the control groups (P=0.000 <0.01, P=0.004 <0.01, respectively). Furthermore, overexpression of TRIM29 in Saos-2 cells significantly enhanced the invasion ability (P=0.001<0.01, P=0.000<0.01, respectively). These results suggest that TRIM29 markedly stimulates human osteosarcoma cell motility.

TRIM29 promotes the migration and invasive growth of osteosarcoma cells by inducing EMT. In cancer biology, cellular migration and invasion properties are frequently correlated with EMT. To explore the potential mechanisms of TRIM29-related cancer cell motility, we investigated the expression of key markers of EMT. As shown in Fig. 6, knockdown of TRIM29 resulted in a significant upregulation of the epithelial marker E-cadherin, and downregulation of vimentin, N-cadherin, ZEB1 and Snail at both the mRNA and protein levels. Furthermore, opposite results were observed in the Saos-2 cell line with overexpression of TRIM29.

Discussion

Osteosarcoma is the most common type of primary bone cancer with a high rate of mortality and distant metastasis (30). Its complex biological and molecular mechanisms make identification of an effective therapeutic target difficult, hence it is a challenge to improve clinical outcome of treatment (31). Therefore, it is critical to identify the metastasis-associated and prognostic biomarkers of osteosarcoma.

Invasive growth is a hallmark of malignant cancer cells. It includes translocation of cancer cells from the primary site into anatomically contiguous tissues as well as to distant metastatic lesions via blood circulation or lymphatic system (32). The association of EMT with cancer metastasis is well-established (33-36). The essential feature of EMT is the loss of polarized epithelial traits and acquisition of mesenchymal characteristics, which contributes to enhanced cell invasion

and motility and release of cells from the primary cancer site. EMT is characterized by the downregulation of E-cadherin, a protein that provides physical adhesion to adjacent epithelial cells that is critical for the maintenance and establishment of the structural integrity and polarity of epithelium. The transition is also marked by accumulation of mesenchymal markers, such as N-cadherin and vimentin (37,38). The process of EMT is governed by a cohort of transcription factors, such as the ZEB, Snail and Twist families (37,39,40). These transcription factors regulate extensive gene expression and cellular phenotypic switch. As core drivers of EMT, Snail and zinc-finger E-box binding homeobox 1 (ZEB1) are transcriptional repressors that directly inhibit the expression of E-cadherin and several other intercellular adhesion components (39,41).

Given the role of *TRIM29* in cancer progression, it is a promising target gene for cancer therapy. Recent studies have linked TRIM29 overexpression with carcinogenesis and cancer progression. Increased TRIM29 expression was associated with shorter overall survival and disease-free survival; and was shown to be an independent prognostic factor in patients with non-small cell lung cancer (19). Similarly, TRIM29 expression showed a strong correlation with aggressive malignant behavior and was an independent predictor of poor survival in patients with colorectal cancer (24). Overexpression of TRIM29 in gastric cancer cells was associated with histological differentiation, lymph node metastasis, large tumor size, tumor invasion and poor prognosis (42). TRIM29 knockdown in gastric cancer cells downregulated the Wnt/β-catenin pathway (22). TRIM29 was also found to stabilize β-catenin in pancreatic cancer via DVL-2, a negative regulator of GSK3β (43). This finding indicates that TRIM29 promotes tumor progression via activation of the Wnt/β-catenin signaling pathway in pancreatic cancer. Furthermore, TRIM29 promotes cancer cell proliferation by inhibiting the nuclear activities of p53 (44,45). Furthermore, TRIM29 was shown to bind with p53 and repress the expression of p53-mediated genes, including *NOXA* and *p21* (44,46). Recent studies have shown that TRIM29 is involved in EMT regulation. Overexpression of TRIM29 was shown to promote EMT, metastasis and proliferation of nasopharyngeal carcinoma cells via the PTEN/AKT/mTOR pathway (26). In mouse and human PanIN lesions, TRIM29 was found to upregulate CD44 via activation of β-catenin signaling, which leads to induction of the EMT phenotype characterized by expression of ZEB1 and Snail (47). In cervical cancer cells, knockdown of TRIM29 was shown to increase E-cadherin expression but decrease N-cadherin and β-catenin expression, which also indicates that TRIM29 promotes EMT (48).

The present study focused on *TRIM29* and investigated its potential role in osteosarcoma. To the best of our knowledge, this is the first research to demonstrate a major role of *TRIM29* in osteosarcoma. The results of IHC showed that TRIM29 expression in osteosarcoma tissues was significantly higher than that in the normal bone tissues ($P<0.01$). Furthermore, we observed that TRIM29 expression was significantly correlated with tumor size, recurrence, metastasis, and overall survival time after surgery, which suggests that *TRIM29* may play an important role in the malignant potential of osteosarcoma. Subsequently, we determined the expression levels of TRIM29 in human osteoblast and osteosarcoma cell lines. Results of RT-qPCR and western blot analysis were consistent with those of immunohistochemical analysis. Both mRNA and protein expression of TRIM29 was significantly higher in osteosarcoma cell lines as compared to that in the osteoblast cell line hFOB1.19. Moreover, in these 4 osteosarcoma cell lines, the expression levels of TRIM29 protein and mRNA were highest in 143B, and lowest in Saos-2. Since the invasion and proliferation capability of 143B was much higher than that of Saos-2, these results showed that high expression of TRIM29 may have an adverse influence on the progression of osteosarcoma. Then, we found that knockdown of TRIM29 in 143B cells significantly reduced the migration rate in wound healing assays, and overexpression of TRIM29 enhanced Saos-2 cell migration. In accordance with the above data, TRIM29 also correlated well with the invasive growth of osteosarcoma cells observed in Transwell assays. Furthermore, we observed a significant upregulation of E-cadherin and downregulation of vimentin, N-cadherin, ZEB1 and Snail at both the mRNA and protein levels following knockdown of TRIM29 in 143B cells; furthermore, overexpression of TRIM29 in Saos-2 cells had an opposite effect. These results indicate that TRIM29 promotes osteosarcoma cell migration and invasion via the EMT process.

In conclusion, we demonstrated significantly higher expression of TRIM29 in osteosarcoma tissues and cells as compared to that in normal bone tissues and osteoblast cells. Expression of TRIM29 was significantly associated with tumor size, recurrence, metastasis, and overall survival in osteosarcoma patients. In addition, the TRIM29 expression level was an independent prognostic factor in patients with osteosarcoma. Moreover, TRIM29 promoted migration and invasion of osteosarcoma cells by inducing EMT. TRIM29 may serve as a useful prognostic biomarker in osteosarcoma patients and a potential therapeutic target for osteosarcoma treatment. However, the precise molecular mechanisms by which TRIM29 contributes to tumorigenesis and progression of osteosarcoma warrant further investigations.

References

1. Luetke A, Meyers PA, Lewis I and Juergens H: Osteosarcoma treatment - where do we stand? A state of the art review. Cancer Treat Rev 40: 523-532, 2014.
2. Aung L, Tin AS, Quah TC and Pho RW: Osteogenic sarcoma in children and young adults. Ann Acad Med Singapore 43: 305-313, 2014.
3. Mirabello L, Troisi RJ and Savage SA: Osteosarcoma incidence and survival rates from 1973 to 2004: Data from the Surveillance, Epidemiology, and End Results Program. Cancer 115: 1531-1543, 2009.
4. Ottaviani G and Jaffe N: The epidemiology of osteosarcoma. Cancer Treat Res 152: 3-13, 2009.
5. Dobrenkov K and Cheung NK: GD2-targeted immunotherapy and radioimmunotherapy. Semin Oncol 41: 589-612, 2014.
6. Lv YF, Dai H, Yan GN, Meng G, Zhang X and Guo QN: Downregulation of tumor suppressing STF cDNA 3 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by the Wnt/GSK-3β/β-catenin/Snail signaling pathway. Cancer Lett 373: 164-173, 2016.
7. Bacci G, Longhi A, Versari M, Mercuri M, Briccoli A and Picci P: Prognostic factors for osteosarcoma of the extremity treated with neoadjuvant chemotherapy: 15-Year experience in 789 patients treated at a single institution. Cancer 106: 1154-1161, 2006.
8. Strauss SJ, Ng T, Mendoza-Naranjo A, Whelan J and Sorensen PH: Understanding micrometastatic disease and *Anoikis* resistance in Ewing family of tumors and osteosarcoma. Oncologist 15: 627-635, 2010.

9. Bielack S, Jürgens H, Jundt G, Kevric M, Kühne T, Reichardt P, Zoubek A, Werner M, Winkelmann W and Kotz R: Osteosarcoma: The COSS experience. Cancer Treat Res 152: 289-308, 2009.
10. Moore DD and Luu HH: Osteosarcoma. Cancer Treat Res 162: 65-92, 2014.
11. Hatakeyama S: TRIM proteins and cancer. Nat Rev Cancer 11: 792-804, 2011.
12. Borden KL: RING fingers and B-boxes: Zinc-binding protein-protein interaction domains. Biochem Cell Biol 76: 351-358, 1998.
13. Reddy BA, Etkin LD and Freemont PS: A novel zinc finger coiled-coil domain in a family of nuclear proteins. Trends Biochem Sci 17: 344-345, 1992.
14. Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, Riganelli D, Zanaria E, Messali S, Cainarca S, *et al*: The tripartite motif family identifies cell compartments. EMBO J 20: 2140-2151, 2001.
15. Xing J, Weng L, Yuan B, Wang Z, Jia L, Jin R, Lu H, Li XC, Liu YJ and Zhang Z: Identification of a role for TRIM29 in the control of innate immunity in the respiratory tract. Nat Immunol 17: 1373-1380, 2016.
16. Yang H, Palmbos PL, Wang L, Kim EH, Ney GM, Liu C, Prasad J, Misek DE, Yu X, Ljungman M, *et al*: ATDC (ataxia telangiectasia group D complementing) promotes radioresistance through an interaction with the RNF8 ubiquitin ligase. J Biol Chem 290: 27146-27157, 2015.
17. Napolitano LM and Meroni G: TRIM family: Pleiotropy and diversification through homomultimer and heteromultimer formation. IUBMB Life 64: 64-71, 2012.
18. Hatakeyama S: Early evidence for the role of TRIM29 in multiple cancer models. Expert Opin Ther Targets 20: 767-770, 2016.
19. Song X, Fu C, Yang X, Sun D, Zhang X and Zhang J: Tripartite motif-containing 29 as a novel biomarker in non-small cell lung cancer. Oncol Lett 10: 2283-2288, 2015.
20. Jiang N, Chen WJ, Zhang JW, Xu C, Zeng XC, Zhang T, Li Y and Wang GY: Downregulation of miR-432 activates Wnt/β-catenin signaling and promotes human hepatocellular carcinoma proliferation. Oncotarget 6: 7866-7879, 2015.
21. Sun H, Dai X and Han B: TRIM29 as a novel biomarker in pancreatic adenocarcinoma. Dis Markers 2014: 317817, 2014.
22. Qiu F, Xiong JP, Deng J and Xiang XJ: TRIM29 functions as an oncogene in gastric cancer and is regulated by miR-185. Int J Clin Exp Pathol 8: 5053-5061, 2015.
23. Lai W, Zheng X, Huang Q, Wu X and Yang M: Down-regulating ATDC inhibits the proliferation of esophageal carcinoma cells. Eur Rev Med Pharmacol Sci 18: 3511-3516, 2014.
24. Jiang T, Tang HM, Lu S, Yan DW, Yang YX and Peng ZH: Up-regulation of tripartite motif-containing 29 promotes cancer cell proliferation and predicts poor survival in colorectal cancer. Med Oncol 30: 715, 2013.
25. Tan ST, Liu SY and Wu B: TRIM29 Overexpression promotes proliferation and survival of bladder cancer cells through NF-κB signaling. Cancer Res Treat 48: 1302-1312, 2016.
26. Zhou XM, Sun R, Luo DH, Sun J, Zhang MY, Wang MH, Yang Y, Wang HY and Mai SJ: Upregulated TRIM29 promotes proliferation and metastasis of nasopharyngeal carcinoma via PTEN/AKT/mTOR signal pathway. Oncotarget 7: 13634-13650, 2016.
27. Santin AD, Zhan F, Bellone S, Palmieri M, Cane S, Bignotti E, Anfossi S, Gokden M, Dunn D, Roman JJ, *et al*: Gene expression profiles in primary ovarian serous papillary tumors and normal ovarian epithelium: Identification of candidate molecular markers for ovarian cancer diagnosis and therapy. Int J Cancer 112: 14-25, 2004.
28. Ai L, Kim WJ, Alpay M, Tang M, Pardo CE, Hatakeyama S, May WS, Kladde MP, Heldermon CD, Siegel EM, *et al*: TRIM29 suppresses TWIST1 and invasive breast cancer behavior. Cancer Res 74: 4875-4887, 2014.
29. Liu J, Welm B, Boucher KM, Ebbert MT and Bernard PS: TRIM29 functions as a tumor suppressor in nontumorigenic breast cells and invasive ER^+ breast cancer. Am J Pathol 180: 839-847, 2012.
30. Anderson ME: Update on Survival in Osteosarcoma. Orthop Clin North Am 47: 283-292, 2016.
31. Zhou W, Hao M, Du X, Chen K, Wang G and Yang J: Advances in targeted therapy for osteosarcoma. Discov Med 17: 301-307, 2014.
32. Guarino M, Rubino B and Ballabio G: The role of epithelial-mesenchymal transition in cancer pathology. Pathology 39: 305-318, 2007.
33. Tsai JH and Yang J: Epithelial-mesenchymal plasticity in carcinoma metastasis. Genes Dev 27: 2192-2206, 2013.
34. Hou CH, Lin FL, Hou SM and Liu JF: Cyr61 promotes epithelial-mesenchymal transition and tumor metastasis of osteosarcoma by Raf-1/MEK/ERK/Elk-1/TWIST-1 signaling pathway. Mol Cancer 13: 236, 2014.
35. Huang L, Wu RL and Xu AM: Epithelial-mesenchymal transition in gastric cancer. Am J Transl Res 7: 2141-2158, 2015.
36. Park SY, Korm S, Chung HJ, Choi SJ, Jang JJ, Cho S, Lim YT, Kim H and Lee JY: RAP80 regulates epithelial-mesenchymal transition related with metastasis and malignancy of cancer. Cancer Sci 107: 267-273, 2016.
37. Lamouille S, Xu J and Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 15: 178-196, 2014.
38. Nantajit D, Lin D and Li JJ: The network of epithelial-mesenchymal transition: Potential new targets for tumor resistance. J Cancer Res Clin Oncol 141: 1697-1713, 2015.
39. Peinado H, Olmeda D and Cano A: Snail, Zeb and bHLH factors in tumour progression: An alliance against the epithelial phenotype? Nat Rev Cancer 7: 415-428, 2007.
40. De Craene B and Berx G: Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 13: 97-110, 2013.
41. Palma CS, Grassi ML, Thomé CH, Ferreira GA, Albuquerque D, Pinto MT, Ferreira Melo FU, Kashima S, Covas DT, Pitteri SJ, *et al*: Proteomic analysis of epithelial to mesenchymal transition (EMT) reveals cross-talk between SNAIL and HDAC1 proteins in breast cancer cells. Mol Cell Proteomics 15: 906-917, 2016.
42. Kosaka Y, Inoue H, Ohmachi T, Yokoe T, Matsumoto T, Mimori K, Tanaka F, Watanabe M and Mori M: Tripartite motif-containing 29 (TRIM29) is a novel marker for lymph node metastasis in gastric cancer. Ann Surg Oncol 14: 2543-2549, 2007.
43. Wang L, Heidt DG, Lee CJ, Yang H, Logsdon CD, Zhang L, Fearon ER, Ljungman M and Simeone DM: Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization. Cancer Cell 15: 207-219, 2009.
44. Yuan Z, Villagra A, Peng L, Coppola D, Glozak M, Sotomayor EM, Chen J, Lane WS and Seto E: The ATDC (TRIM29) protein binds p53 and antagonizes p53-mediated functions. Mol Cell Biol 30: 3004-3015, 2010.
45. Sho T, Tsukiyama T, Sato T, Kondo T, Cheng J, Saku T, Asaka M and Hatakeyama S: TRIM29 negatively regulates p53 via inhibition of Tip60. Biochim Biophys Acta 1813: 1245-1253, 2011.
46. Shibata MA, Yoshidome K, Shibata E, Jorcyk CL and Green JE: Suppression of mammary carcinoma growth in vitro and in vivo by inducible expression of the Cdk inhibitor p21. Cancer Gene Ther 8: 23-35, 2001.
47. Wang L, Yang H, Abel EV, Ney GM, Palmbos PL, Bednar F, Zhang Y, Leflein J, Waghray M, Owens S, *et al*: ATDC induces an invasive switch in KRAS-induced pancreatic tumorigenesis. Genes Dev 29: 171-183, 2015.
48. Xu R, Hu J, Zhang T, Jiang C and Wang HY: TRIM29 overexpression is associated with poor prognosis and promotes tumor progression by activating Wnt/β-catenin pathway in cervical cancer. Oncotarget 7: 28579-28591, 2016.