Ubiquitin-specific protease 7 promotes osteosarcoma cell metastasis by inducing epithelial-mesenchymal transition

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Abstract. Osteosarcoma (OS) is the most common primary malignant bone tumour among adolescents and young adults; however, its molecular pathogenesis has not been completely elucidated. Ubiquitin-specific protease 7 (USP7), a member of the deubiquitinating enzyme family, plays a role in the malignancy process of various cancer types by targeting the key oncoprotein; however, its biological function and mechanism in OS have not been elucidated. The present study demonstrated that USP7 expression in OS tumour tissues was markedly higher than that in the paired surrounding tissues, and high USP7 expression was positively correlated with the TNM stage and metastasis in patients with OS. Next, biological function assays demonstrated that USP7 knockdown markedly inhibited OS cell migration and invasion, whereas USP7 overexpression enhanced it. Notably, USP7 can directly bind with β-catenin to activate the Wnt/β-catenin signalling pathway and induce epithelial-mesenchymal transition (EMT) of OS cells. Overall, USP7 overexpression could promote OS cell metastasis by activating the Wnt/β-catenin signalling pathway by inducing EMT, suggesting that USP7 is a potential therapeutic target for OS.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumour among adolescents and young adults (1) and has a high metastatic rate and poor prognosis. With advances in the treatment modality for OS, such as chemotherapy or surgery combined with chemotherapy, the survival of patients with OS has markedly improved in recent years (2), and the 5-year survival rate in patients with non-metastatic OS has reached 60-70% (3). However, the survival of patients with distant metastasis remains poor with the 5-year survival rate rarely reaches 20% (4). To date, the molecular mechanisms underlying OS metastasis remain partially elucidated.

The ubiquitin-proteasome system, comprising ubiquitin, ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, ubiquitin-ligating enzyme, proteasome and deubiquitinating enzymes (DUBs), regulates most intracellular protein degradation cellular functions and maintains protein homeostasis (5). Previously, a body of research has revealed the involvement of the dysregulation of this system in oncogenesis (6); however, most studies have focused on the ubiquitination process rather than the deubiquitination process. It is now becoming apparent that reversible ubiquitination also plays a crucial role in the intracellular signalling pathway; hence, a disorder of this process could lead to the development of many diseases, including cancer (7). DUBs catalyse the removal of ubiquitin and polyubiquitin chains from the target protein (8). Ubiquitin-specific proteases (USPs) constitute the largest subgroup of DUBs and are involved in the development of many cancers by upregulating various key oncoproteins (9-11).

USP7, a member of the USPs, is required for cell growth control, cell cycle regulation, receptor function, development and stress response (12). However, abnormal USP7 expression contributes to tumorigenesis (13,14). In addition, USP7 knockdown inhibits the proliferation of colon cancer cells *in vitro* and tumour growth *in vivo*, resulting in a constitutively high p53 level (15). Furthermore, USP7 is overexpressed in hepatocellular carcinoma (HCC) and promotes HCC progression by accelerating p14ARF degradation by deubiquitinating TRIP12 (16). However, the biological function and molecular mechanisms of USP7 in OS tumorigenesis and development remain unclear. Therefore, the present study aimed to elucidate the function and potential mechanism of USP7 in OS and to provide novel insights into therapeutic targets for the treatment of OS.

Materials and methods

Patient specimens. We obtained 45 pairs of fresh specimens containing tumour tissues and paired adjacent tissues from patients who underwent radical resection at the Department

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of Orthopaedics of University-Town Hospital of Chongqing Medical University (Chongqing, China) from September 2013 to July 2017. The patients were diagnosed according to the diagnosis of bone cancer of the NCCN (National Comprehensive Cancer Network) guidelines (https://www.nccn.org/) and the pathology of the tumour tissues was evaluated by pathologist. Patients with other sarcomas including chondrosarcoma, osteochondroma and Ewing's sarcoma were excluded from this study. Patients who received prior radiotherapy or chemotherapy were also excluded. The patient clinical data including age, sex, tumour size, stage and metastasis were obtained. The age range of the patients was from 11 to 30 years and the average age was 18, the ratio of men to women was 1.812. Detailed information is listed in Table I. All tissue samples were obtained following surgical resection and stored in liquid nitrogen immediately. Written informed consent was obtained from all tissue donors, and this study was approved by the Ethics Committee of Chongqing Medical University (Chongqing, China) prior to surgery.

Cell lines. We purchased human OS cell lines (U2OS, Saos-2, MG-63, 143B and HOS) and normal human osteoblastic (NHOst) cell line) from the American Type Culture Collection (ATCC; Manassas, VA, USA) and Chinese Academy of Medical Science (Beijing, China). All cells were maintained in HyClone™ Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cultured in an incubator maintained at 37°C with 5% CO_2.

Immunostaining. We embedded OS tumour tissues into paraffin to perform the immunohistochemical analysis. First, tissue sections were dewaxed, antigen repaired with citrate solution and blocked in goat serum. Subsequently, we incubated the sections with the USP7 antibody (dilution 1:200; cat. no. ab4080; Abcam, Cambridge, UK) overnight at 4°C, following incubation with a secondary of goat anti-rabbit antibody carrying horseradish peroxidase (HRP) for 30 min at room temperature. Finally, we dyed sections with DAB dyestuff for 30 sec. This assay was performed with the immunohistochemistry kit (cat. no. SP9001; ZSGB-BIO, Beijing, China).

Quantitative real-time polymerase chain reaction. We used TRIzol reagent (Takara, Biotechnology Co., Ltd., Dalian, China) to extract the total RNA from OS tumour tissues or cells. Subsequently, cDNA was synthesised from the RNA samples using the PrimeScript™ RT Master Mix (Takara Biotechnology Co., Ltd.), according to the manufacturer's instructions. The following primer sequences were used: USP7 forward, 5'-AATCATTGGTGTTCATCA-3' and reverse, 5'-CAAGCATCTCATTCTCTT-3'; β-actin forward, 5'-TCC TTTGGAACTCTGCAGGT-3' and reverse, 5'-GACCTACTG TGCGCCTACTT-3'. In addition, we performed quantitative real-time polymerase chain reaction (qRT-PCR) assays with the SYBR Green Master Mix kit (Takara Biotechnology Co., Ltd.). Of note, β-actin was an internal control. Furthermore, we used the $2^{-\Delta Cq}$ or $2^{-\Delta\Delta Cq}$ method (17) to analyse the relative USP7 expression in OS tumour tissues and cells.

Western blot assay. We extracted the total protein of OS tumour tissues and cells using RIPA lysate (Beyotime Institute of Biotechnology, Shanghai, China). In addition, the protein concentration was tested using a BCA kit (Beyotime Institute of Biotechnology) and standardised using RIPA lysate. Overall, a 40-µg protein sample was separated using 10% SDS-PAGE gel for 2 h. These protein samples were transferred into polyvinylidene fluoride membranes, which were blocked in 5% skimmed milk for 2 h at room temperature. Subsequently, we incubated the membranes with primary antibodies against USP7 (dilution 1:1,000; cat. no. ab4080; Abcam), β-catenin (dilution 1:1,000; cat. no. ab32572; Abcam), Snail (dilution 1:1,000; cat. no. ab53519; Abcam), E-cadherin (dilution 1:1,000; cat. no. 14472; Cell Signaling Technology, Danvers, MA, USA), N-cadherin (dilution 1:1,000; cat. no. 13116; Cell Signaling Technology), vimentin (dilution 1:1,000; cat. no. 5741; Cell Signaling Technology), MMP9 (dilution 1:1,000; cat. no. 13667; Cell Signaling Technology) and β-actin (dilution 1:2,000; cat. no. 3700; Cell Signaling Technology) at 4°C overnight. The membranes were further incubated in secondary antibodies conjugated with horseradish peroxidase (HRP) (dilution 1:3,000; cat. nos. 7074 and 7076; Cell Signaling Technology) at room temperature for 2 h. Finally, we visualised images of proteins using ECL reagent (Beyotime Institute of Biotechnology). The gray value of protein expression was quantified with Quantity One software (Bio-Rad, Shanghai, China).

Cell transfection. A lentivirus carrying a USP7-specific small-hairpin RNA (sh/USP7) and USP7 overexpression sequence were constructed at Shanghai Genechem Company (Shanghai, China). The sh/USP7 sequences were as follows: shRNA1, 5'-TGTATCTATTGACTGCCCTTT-3'; shRNA2, 5'-CGTGGTGTCAAGGTGTACTA-3'; shRNA3, 5'-CTCAGA ACCCTGTGATCAA-3'. After 48 h, OS cells were transfected with the lentiviral diluent, according to the manufacturer's instructions, and selected with puromycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) after 2 weeks.

Transwell migration and invasion assays. The migration and invasion abilities of the OS cells were assayed in 24-well Transwell™ Chambers (Corning Inc., Corning, NY, USA). Briefly, for the migration assay, cells were digested and adjusted to $2x10^5$ cells/ml using serum-free medium; 100 µl of this cell suspension was added to the upper chamber, and 700 µl of medium containing 10% FBS was added to the bottom chamber. After a 24-h incubation, the chamber was carefully taken out and washed with PBS buffer. Subsequently, the migrated cells were fixed with 4% paraformaldehyde for 30 min, stained with crystal violet for 30 min and photographed using an Leica DMi1 inverted microscope (Leica Microsystems, Solms, Germany) at x200 magnification. During optical microscope observation, three random regions were selected for the statistical analysis.

For the invasion assay, Matrigel (BD Biosciences, San Diego, CA, USA) was diluted with the serum-free medium at an appropriate ratio. Overall, 100 µl of diluent Matrigel was added to the upper chamber and placed in an incubator at 37°C until the Matrigel was completely solidified. The next steps were performed in accordance with the migration

Table I. Association between USP7 expression and the clinico-pathological factors of the osteosarcoma cases.

| Factor | N | USP7 expression | | Chi-square | P-value |
		Low	High		
Age (years)					0.5262
<18	21	8	13	0.4018	
≥18	24	7	17		
Sex					0.3784
Male	29	11	18	0.7759	
Female	16	4	12		
Tumor size (cm)					0.5186
<8	27	10	17	0.4617	
≥8	18	5	13		
TNM stage					0.0031[b]
I	14	9	5	8.762	
II-IV	31	6	25		
Metastasis					0.0277[a]
Yes	16	2	14	4.849	
No	29	13	16		

[a]P<0.05, [b]P<0.01; P<0.05 was considered to indicate a significant difference. USP7, ubiquitin-specific protease 7; TNM, Tumor-Node-Metastasis.

assay. After 48 h, the chamber was carefully taken out and washed with PBS buffer. Subsequently, the migrated cells were fixed with paraformaldehyde, stained with crystal violet and photographed with the Leica DMi1 inverted microscope (Leica Microsystems) at x200 magnification. Three random regions were selected for the statistical analysis.

Co-immunoprecipitation (Co-IP). The total protein of OS cells was isolated using RIPA lysate (Beyotime Institute of Biotechnology). Protein A/G magnetic beads (Millipore Corporation, Billerica, MA, USA) were added and incubated at 4°C for 2 h to discard the non-specific protein in the cell lysates. The cell lysates were incubated with the primary antibodies (USP7, cat. no. ab4080; Abcam; Use at an assay dependent concentration) (β-catenin, dilution 1:200; cat. no. ab32572; Abcam) at 4°C overnight. The magnetic beads were re-added to the cell lysates and incubated at 4°C for 3 h to pull down the protein complex. Tube was placed on the magnetic stand to remove the supernatant. Then, the beads were carefully washed with PBS buffer and were added to 1X loading buffer and boiled in 100°C water for 10 min. Finally, we removed the magnetic beads using a magnetic stand to obtain the immunoprecipitated protein complex. This protein complex was further analysed using western blot assays.

Statistical analysis. All statistical analyses were performed using SPSS version 22.0 statistical software (SPSS, Chicago, IL, USA). Data are presented as mean ± SD from independent triplicate experiments. We used Student's t-test or Mann-Whitney test to analyse the difference between two independent groups. Variance (ANOVA) followed by Dunnett or Bonferroni post hoc test was used to analyse the difference of multiple comparisons. We analysed the correlation between USP7 expression and clinicopathological features with χ^2 statistical testing. Furthermore, Spearman correlation analysis was performed to test the correlation between USP7 and β-catenin expression. For all tests, P<0.05 was considered statistically significant.

Results

High USP7 expression in OS tissues and cells. We detected USP7 expression in 45 pairs of OS specimens using immuno-histochemical assays; USP7 was highly expressed in tumour tissues compared with that noted in the paired adjacent tissues (Fig. 1A). In addition, qRT-PCR assays revealed that USP7 mRNA expression was upregulated in 30/45 (66.6%) OS tumour tissues compared with the paired adjacent tissues (Fig. 1B). Similarly, western blot assay revealed that the protein level of USP7 was markedly upregulated in the OS tumour tissues compared with the level noted in the adjacent normal tissues (Fig. 1C). We examined USP7 expression in five OS cell lines and normal human osteoblastic cells and found that USP7 mRNA and protein levels in OS cell lines (U2OS, Saos-2 and MG-63) were markedly higher than these levels in the NHOst cell line (Fig. 1D and E). However, USP7 expression was lower in the HOS cell line when compared to that noted in the NHOst cells. These findings indicated that USP7 was highly expressed in OS tissues and most OS cell lines when compared with the level in the paired adjacent tissues or the NHOst cell line.

Correlation between USP7 expression and clinicopathological characteristics of the OS cases. Since USP7 was revealed to be upregulated in OS tumour tissues, we assessed the clinical association of USP7 expression. USP7 expression in OS tumour tissues, detected beforehand by qRT-PCR, revealed that USP7 expression in patients with advanced stages, including stages II-IV, was higher than that in patients with early stage OS (Fig. 1F). In addition, patients with metastasis exhibited high USP7 expression, compared with those without metastasis (Fig. 1G). Furthermore, χ^2 test revealed that USP7 expression was positively correlated with TNM stage and metastasis in patients with OS. However, we observed no marked correlation between its expression and other clinicopathological features, including patient age, sex and tumour size (Table I).

USP7 knockdown and inhibition of OS cell migration and invasion. In this study, U2OS cells, which presented high USP7 expression, were stably transfected with a lentivirus carrying the USP7 shRNA target sequence (sh/USP7) or negative control sequence (NC/shRNA). We extracted the total RNA and protein of cells to assess the efficiency of USP7 knockdown. qRT-PCR results indicated that USP7 mRNA expression in U2OS cells transfected with the sh/USP7 target sequence was significantly less compared with that in cells transfected with the control sequence (Fig. 2A). As anticipated, the protein level of USP7 in sh/USP7-transfected cells was decreased compared to that in NC/shRNA cells (Fig. 2B).
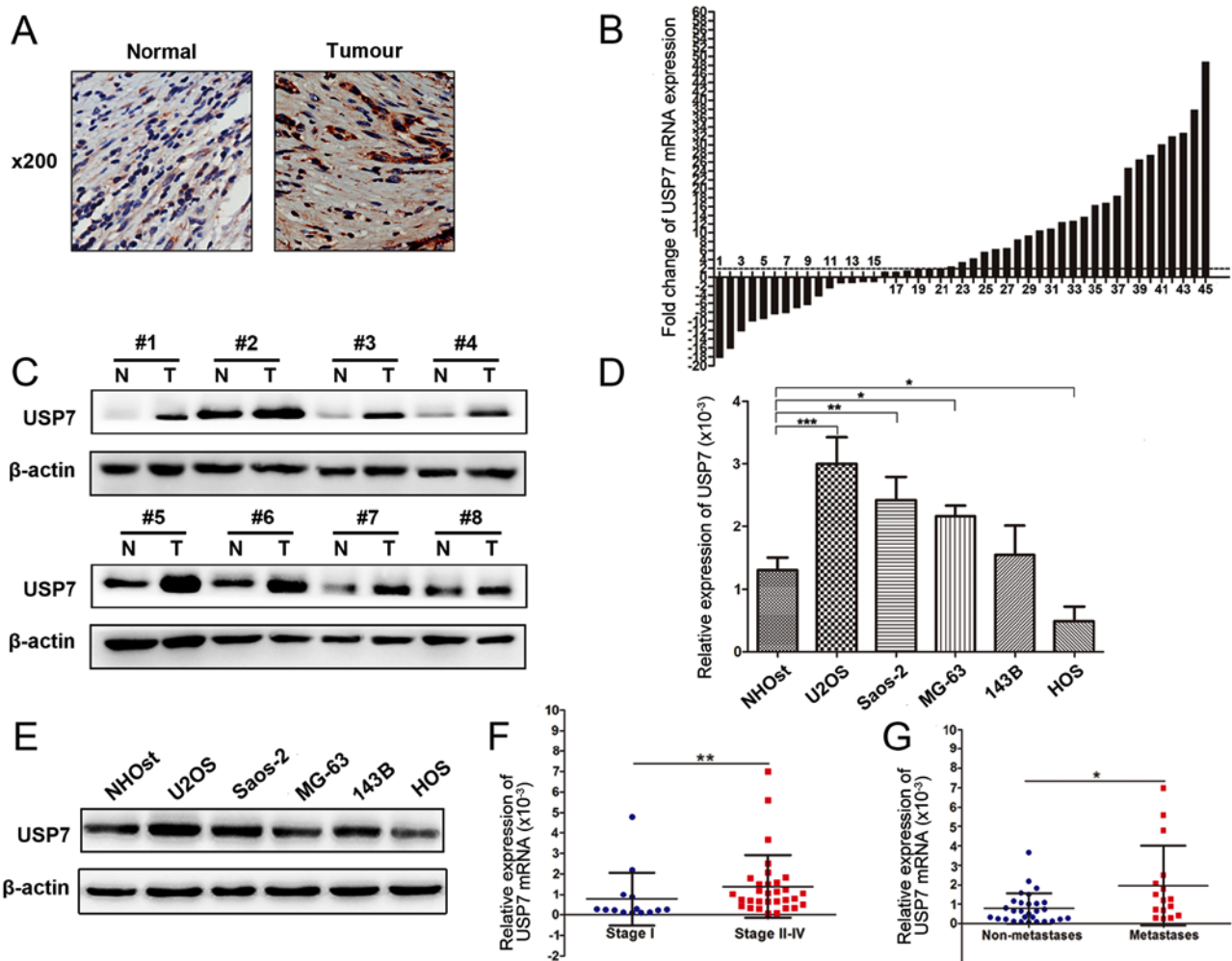
Figure 1. USP7 is overexpressed in osteosarcoma (OS) tissues and cells, and is positively correlated with the clinicopathological characteristics of the OS patients. (A) USP7 expression in OS tissues as detected by immunohistochemistry (magnification, x200). (B) USP7 mRNA expression in OS tissues as detected by qRT-PCR. The results are presented as fold change (cancer tissue compared to paired adjacent tissues). (C) The protein level of USP7 in OS tissues was examined by western blot analysis. T, OS tissue; N, normal adjacent tissue. (D) The relative expression of USP7 in OS cells and normal human osteoplastic cells was detected by qRT-PCR. $^{***}P<0.0001$, $^{**}P<0.01$, $^{*}P<0.05$. (E) The protein level of USP7 in OS cells and normal human osteoplastic cells was examined by western blot analysis. (F) The relative expression of USP7 in OS patients with different stage tumours. $^{**}P<0.01$. (G) The relative expression of USP7 in OS patients with or without metastasis. $^{*}P<0.05$. USP7, ubiquitin-specific protease 7.

Wound-healing assay that was performed to assess the effect of USP7 knockdown on OS cell migration revealed that the wound-healing ability of the U2OS cells transfected with sh/USP7 was substantially weaker than that of the control cells (Fig. 2C). In addition, Transwell migration assays further suggested that the migratory ability of the sh/USP7-transfected U2OS cells was markedly inhibited compared with that in the control cells (Fig. 2D and F). These findings suggest that USP7 knockdown impaired the migratory ability of OS cells. Furthermore, the invasion assays revealed that the U2OS cell invasive capability was markedly blocked after USP7 knockdown (Fig. 2E and F).

USP7 overexpression and promotion of OS cell migration and invasion. We selected HOS cells, which exhibited low USP7 expression, to further identify the regulatory role of USP7 on OS cell migration and invasion; these cells were stably transfected with a lentivirus carrying the USP7 overexpression sequence (USP7). qRT-PCR revealed that USP7 mRNA expression in the HOS cells transfected with the USP7 overexpression sequence was significantly higher than that in the negative control cells (NC/USP7; Fig. 3A). Moreover, the protein level of USP7 in HOS cells transfected with the USP7 overexpression sequence was increased compared with that in the control cells (Fig. 3B).

Wound-healing assay revealed that the healing ability of HOS cells transfected with the USP7 overexpression sequence was considerably better than that of the control cells (Fig. 3C). Likewise, Transwell migration assay revealed that the migratory ability of the USP7 overexpression sequence-transfected cells was substantially enhanced compared with that in the control cells (Fig. 3D and F). As expected, the invasion assay presented that USP7 overexpression significantly promoted OS cell invasive capability (Fig. 3E and F). These findings revealed that USP7 plays a crucial role in contributing to OS cell migration and invasion.

Activation of the Wnt/β-catenin signalling pathway by USP7 to induce epithelial-mesenchymal transition of OS cells. USP7 was able to promote OS cell migration and invasion;
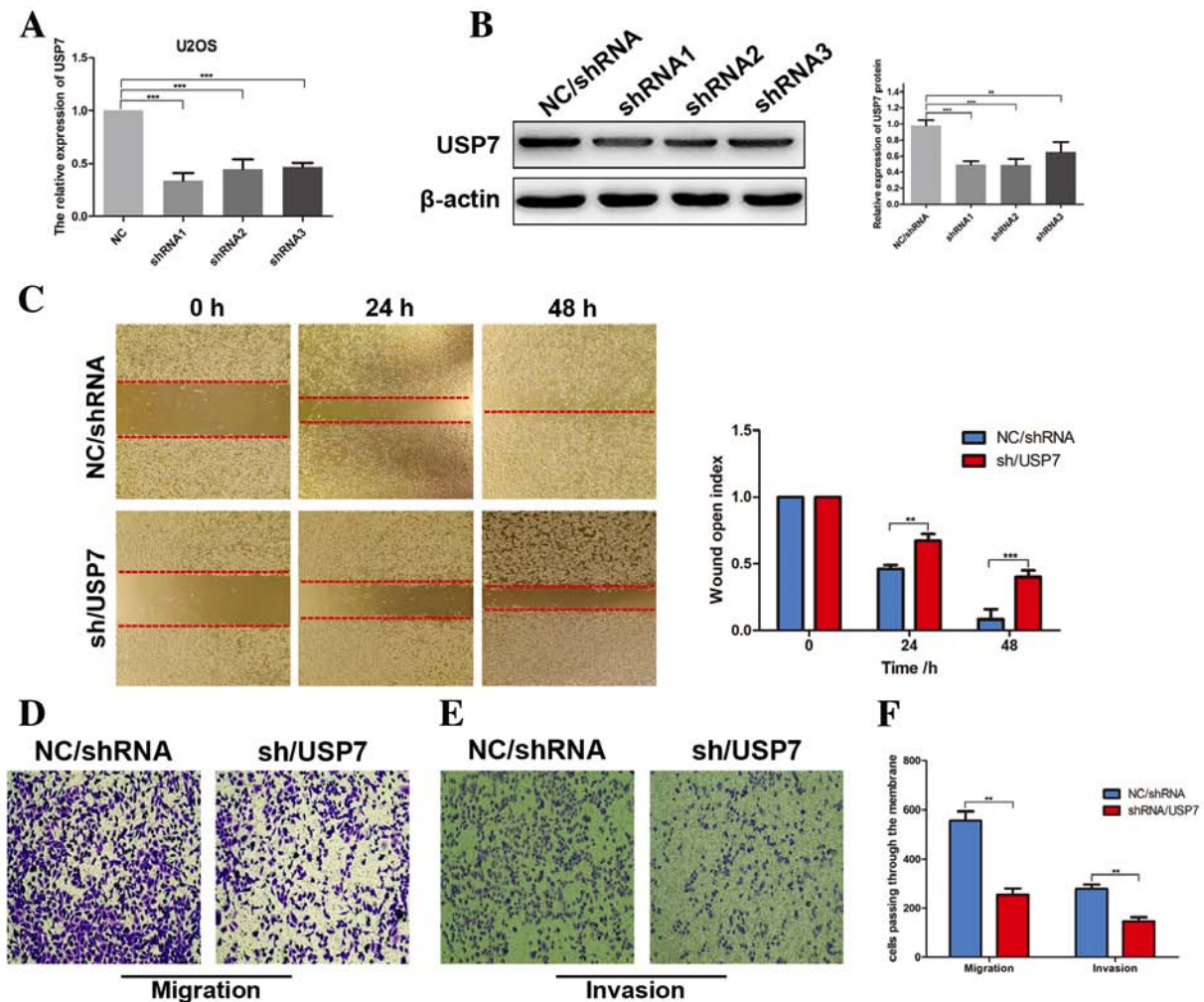
Figure 2. USP7 knockdown inhibits osteosarcoma (OS) cells migration and invasion. (A) The relative expression of USP7 mRNA in U2OS cell transfected with the USP7 shRNA target sequence (sh/USP7) or the negative control sequence (NC/shRNA) as assessed by qRT-PCR. ***P<0.0001. (B) The relative expression of USP7 protein in U2OS cells transfected with USP7 shRNA target sequence or negative control sequence, as assessed by western blot analysis. **P<0.01, ***P<0.0001. (C) Wound healing assay to detect the effect of USP7 knockdown on U2OS cell healing ability. **P<0.01, ***P<0.0001. (D) Transwell migration assays examined the effect of USP7 knockdown on U2OS cell migratory ability. (E) Transwell invasive assays examined the effect of USP7 knockdown on U2OS cell invasive ability. (F) Quantitative analysis of migratory and invasive cells. **P<0.01. USP7, ubiquitin-specific protease 7.

however, the mechanism for this regulatory process is unclear. Currently, it is widely recognised that epithelial-mesenchymal transition (EMT) is the key process for promoting tumour cell invasion and metastasis (18). Several studies have revealed that EMT is involved in OS malignancy process (19,20). Thus, the effect of USP7 on EMT was evaluated. The results revealed that the epithelial marker (E-cadherin) was significantly increased and mesenchymal markers (N-cadherin, vimentin and MMP9) were significantly decreased in the U2OS cells after USP7 knockdown (Fig. 4A). In contrast, the epithelial marker was significantly decreased and the mesenchymal markers were significantly increased in the HOS cells after USP7 overexpression (Fig. 4B). Therefore, USP7 may induce EMT of OS cells.

A previous study demonstrated that when the Wnt/β-catenin signalling pathway is activated, β-catenin accumulates abnormally in the cytoplasm and transfers into the nucleus; subsequently, the increment of β-catenin can recruit the transcription factor Snail1, which is a key upstream regulating factor of EMT (21). Surprisingly, we observed that USP7 knockdown markedly decreased the expression of β-catenin and Snail1 in the U2OS cells (Fig. 4C) and USP7 overexpression increased their expression in the HOS cells (Fig. 4D). Overall, these findings revealed that USP7 could activate the Wnt/β-catenin signalling pathway to induce EMT of OS cells.

USP7-regulated β-catenin expression by direct combination. USP7 can regulate various oncoproteins by directly binding with the targets and increasing their stability. In the present study, significant efforts were made to determine the correlation between USP7 and β-catenin in OS cells. Thus, CO-IP assays were performed using the total endogenous protein of U2OS cells. Remarkably, the results revealed that USP7 and β-catenin interact directly (Fig. 5A and B). The re-examination of USP7 and β-catenin expression in OS tumour tissues demonstrated that β-catenin exhibited strong positive staining in tumour tissues that presented high USP7 expression (Fig. 5C), and the correlation analysis revealed that their expression levels were markedly positively correlated (Fig. 5D).
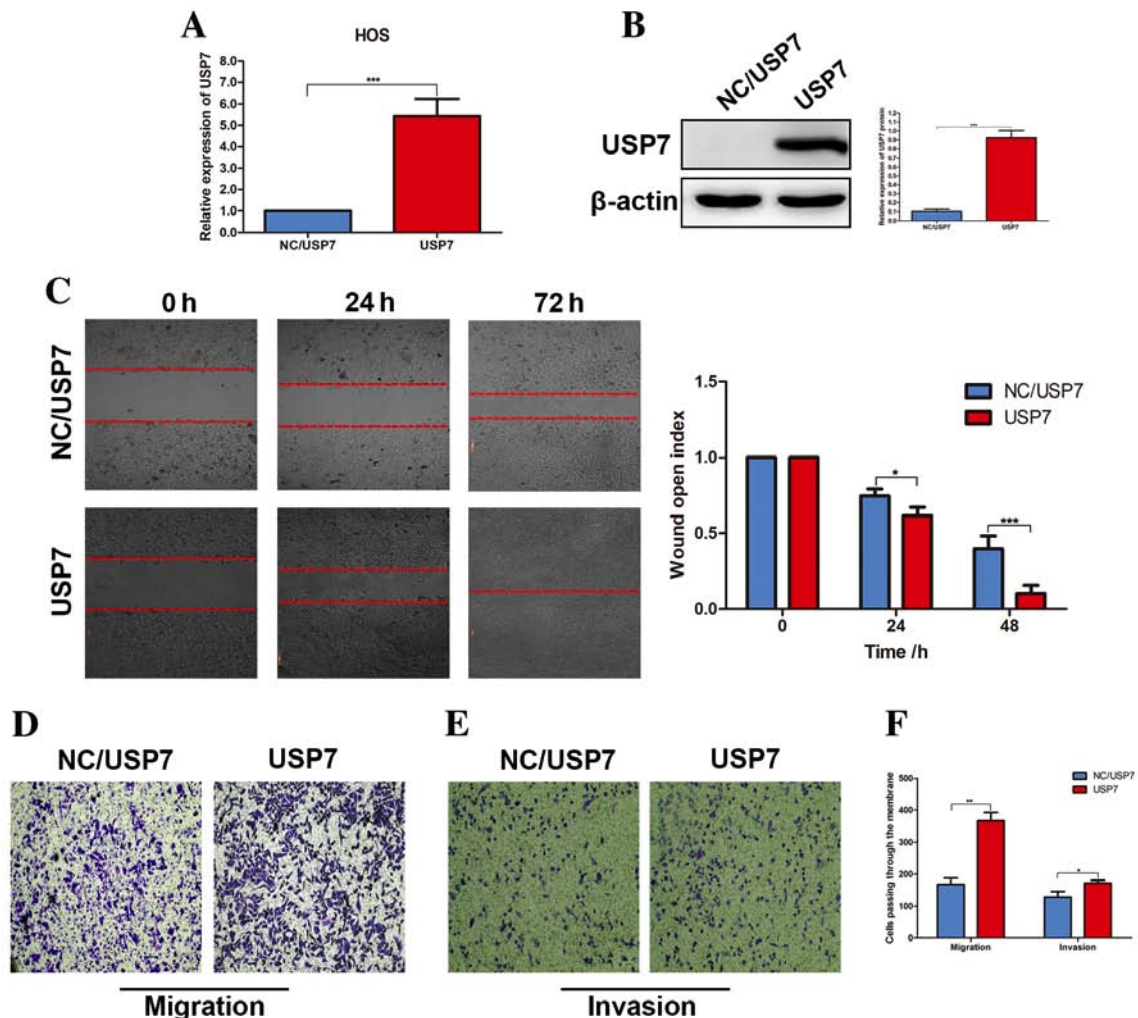
Figure 3. USP7 overexpression promotes the migration and invasion of osteosarcoma (OS) cells. (A) The relative expression of USP7 mRNA in HOS cells transfected with the USP7 overexpression sequence (USP7) or negative control sequence (NC/USP7), as assessed by qRT-PCR. ***P<0.0001. (B) The relative expression of USP7 protein in HOS cells transfected with the USP7 overexpression sequence or negative control sequence, as assessed by western blot analysis. ***P<0.0001. (C) Wound healing assay to detect the effect of USP7 overexpression on HOS cell healing ability. *P<0.05, ***P<0.0001. (D) Transwell migration assays examined the effect of USP7 overexpression on HOS cell migratory ability. (E) Transwell invasive assays examined the effect of USP7 overexpression on HOS cell invasive ability. (F) Quantitative analysis of migratory and invasive cells. *P<0.05, **P<0.01. USP7, ubiquitin-specific protease 7.

Discussion

Although USP7, a crucial member of the DUBs, has been extensively reported to act as an oncogene or tumour-suppressor gene in various tumours (22,23), its impact on OS remains unclear. To the best of our knowledge, this is the first study to demonstrate that USP7 was upregulated in OS tumour tissues and cell lines compared with the paired adjacent tissues or NHOst cells. In addition, the correlation analysis indicated that its high expression was positively associated with TNM stage and metastasis in patients with OS; this demonstrated that USP7 exhibits abnormal expression in OS and is associated with clinicopathological characteristics, which is consistent with the finding of USP7 in epithelial tumours. Therefore, USP7 is extensively involved in tumorigenesis in humans. Due to the low incidence of OS, the sample size of tumour tissues was insufficient in this study and there may be a significant sample heterogeneity, which may have resulted in a limitation to our conclusion. However, we maintained that our results were valuable as the crucial role of USP7 in OS

cells was found. Certainly, we will continue to collect samples to identify the results in this study.

Moreover, this study revealed that USP7 expression was dysregulated and was correlated with TNM stage and OS metastasis. However, its bio-function remained unclear. Thus, wound-healing and Transwell migration assays were performed to assess the effect of USP7 expression on the migration and invasion abilities of the OS cells; the results revealed that USP7 knockdown markedly weakened OS cell migration and USP7 overexpression markedly enhanced it. Notably, Transwell invasion assay revealed that USP7 knockdown markedly inhibited OS cell invasion and USP7 overexpression displayed contrasting results. Our findings suggest that USP7 plays a crucial role in OS cell migration and invasion; this critical finding may explain why high USP7 expression is positively associated with advanced stage OS.

EMT and its intermediate states have recently been determined as crucial drivers of tumour progression, particularly, its contribution to tumour metastasis (18). The hallmarks of EMT include the loss of epithelial markers and increase in
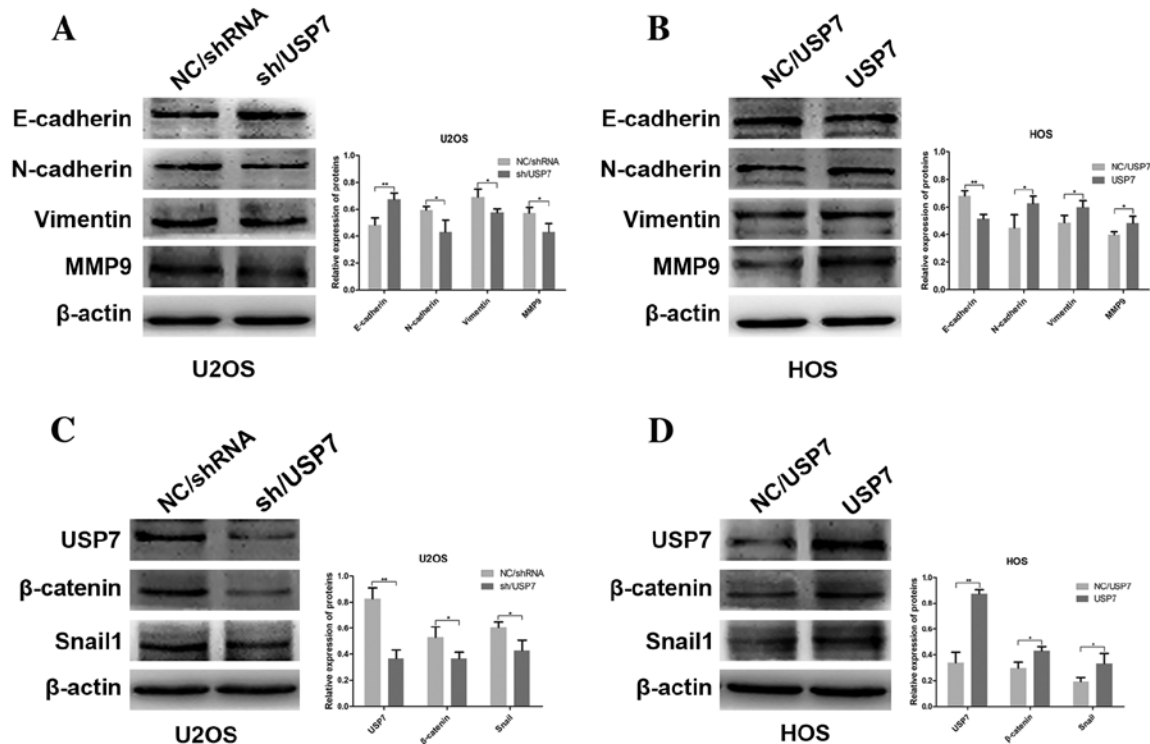
Figure 4. USP7 activates the Wnt/β-catenin signalling pathway to induce epithelial-mesenchymal transition (EMT) of osteosarcoma (OS) cells. (A) The expression of epithelial marker (E-cadherin) and mesenchymal markers (N-cadherin, vimentin and MMP9) in U2OS cells transfected with the USP7 shRNA target sequence (sh/USP7) or the negative control sequence (NC/shRNA). *P<0.05, **P<0.01. (B) The expression of epithelial marker (E-cadherin) and mesen-chymal markers (N-cadherin, vimentin and MMP9) in HOS cells transfected with USP7 overexpression sequence (USP7) or negative control sequence (NC/USP7). *P<0.05, **P<0.01. (C) The expression of β-catenin and Snail1 in U2OS cells transfected with the USP7 shRNA target sequence or negative control sequence. *P<0.05, **P<0.01. (D) The expression of β-catenin and Snail1 in HOS cells transfected with the USP7 overexpression sequence or negative control sequence. *P<0.05, **P<0.01. USP7, ubiquitin-specific protease 7.
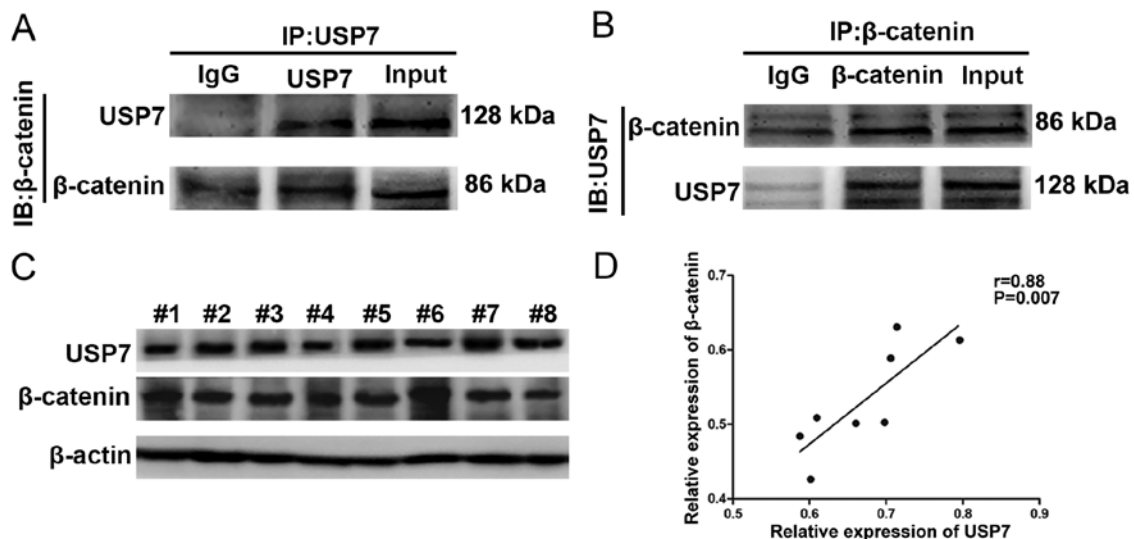

Figure 5. USP7 regulates β-catenin expression via direct combination. (A) USP7 was found to interact with β-catenin at exogenous levels. (B) β-catenin was found to interact with USP7 at endogenous levels. Cell lysates from U2OS cells were immunoprecipitated with anti-USP7 or anti-β-catenin antibody, respec-tively. Western blot analysis of lysates after immunoprecipitation; IgG was used as a control. (C) The expression of USP7 and β-catenin in OS tumour tissues was detected by western blot analysis. (D) The correlation between USP7 and β-catenin expression in osteosarcoma (OS) tumour tissues (r=0.88, P=0.007). USP7, ubiquitin-specific protease 7.

mesenchymal markers (24). This study revealed that USP7 knockdown markedly suppressed EMT and its overexpression evidently promoted the EMT process, suggesting that USP7 regulates EMT of OS cells. Various upstream signalling

pathways can regulate EMT in cancer (21); however, the pathway through which USP7 regulates EMT needs to be elucidated. Reportedly, the Wnt/β-catenin signalling pathway can promote EMT in cancer (25). It is a canonical Wnt

pathway that is responsible for the accumulation of β-catenin and its translocation into the nucleus. Additionally, β-catenin in the nucleus interacts with the TCF/LEF family to regulate the downstream gene expression (26,27). Moreover, β-catenin can recruit the transcription factor Snail, which acts as a key promoter in EMT by downregulating epithelial genes and upregulating mesenchymal genes (28,29). Reportedly, aberrant Wnt/β-catenin signalling plays a vital role in the progression of OS and the frequent elevation of cytoplasmic and nuclear β-catenin protein (30,31). Remarkably, this study revealed that USP7 knockdown negatively regulated the Wnt/β-catenin signalling pathway and that USP7 overexpression activated this signalling pathway. In addition, a previous study reported that USP7 is a Wnt activator for APC-mutated colorectal cancer by mediating β-catenin deubiquitination (32). Therefore, we infer that USP7 can bind and deubiquitinate β-catenin, preventing the degradation of β-catenin by ubiquitin-proteasome in OS cells. In this study, the CO-IP assays revealed that USP7 and β-catenin interacted directly, and the correlation analysis revealed that their expression levels were positively correlated in OS tumour tissues. Thus, we determined that USP7 can directly bind with β-catenin to activate the Wnt/β-catenin signalling pathway in OS cells. Together with the results that USP7 regulates Snail1 expression, we inferred that USP7 could regulate β-catenin to activate the Wnt/β-catenin signalling pathway and induce the EMT of OS cells, which could be the primary reason for the USP7 promotion of OS metastasis.

In conclusion, the present study revealed that USP7 acts as an oncoprotein that promotes OS metastasis by activating the Wnt/β-catenin signalling pathway consequently inducing EMT, suggesting that USP7 could act as a potential therapeutic target for the treatment of OS.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

QZ performed the experiments and wrote the manuscript, with the contribution of ZL, XZ, LG, CY, JQ, SZ and YZ. XY designed the study and revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Written informed consent was obtained from all tissue donors, and this study was approved by the Ethics Committee of Chongqing Medical University before surgery.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Rainusso N, Wang LL and Yustein JT: The adolescent and young adult with cancer: state of the art-bone tumors. Curr Oncol Rep 15: 296-307, 2013.
2. Omer N, Le Deley MC, Piperno-Neumann S, Marec-Berard P, Italiano A, Corradini N, Bellera C, Brugières L and Gaspar N: Phase-II trials in osteosarcoma recurrences: A systematic review of past experience. Eur J Cancer 75: 98-108, 2017.
3. Goedhart LM, Gerbers JG, Ploegmakers JJ and Jutte PC: Delay in diagnosis and its effect on clinical outcome in high-grade sarcoma of bone: A referral oncological centre study. Orthop Surg 8: 122-128, 2016.
4. Li Z, Tang Y, Xing W, Dong W and Wang Z: LncRNA, CRNDE promotes osteosarcoma cell proliferation, invasion and migration by regulating Notch1 signaling and epithelial-mesenchymal transition. Exp Mol Pathol 104: 19-25, 2018.
5. Chen YJ, Wu H and Shen XZ: The ubiquitin-proteasome system and its potential application in hepatocellular carcinoma therapy. Cancer Lett 379: 245-252, 2016.
6. Pickart CM: Mechanisms underlying ubiquitination. Annu Rev Biochem 70: 503-533, 2001.
7. Sacco JJ, Coulson JM, Clague MJ and Urbé S: Emerging roles of deubiquitinases in cancer-associated pathways. IUBMB Life 62: 140-157, 2010.
8. Komander D, Clague MJ and Urbé S: Breaking the chains: Structure and function of the deubiquitinases. Nat Rev Mol Cell Biol 10: 550-563, 2009.
9. Zhou F, Xie F, Jin K, Zhang Z, Clerici M, Gao R, van Dinther M, Sixma TK, Huang H, Zhang L and Ten Dijke P: USP4 inhibits SMAD4 monoubiquitination and promotes activin and BMP signaling. EMBO J 36: 1623-1639, 2017.
10. Huang Y, Pan XW, Li L, Chen L, Liu X, Lu JL, Zhu XM, Huang H, Yang QW, Ye JQ, *et al*: Overexpression of USP39 predicts poor prognosis and promotes tumorigenesis of prostate cancer via promoting EGFR mRNA maturation and transcription elongation. Oncotarget 7: 22016-22030, 2016.
11. Wang S, Juan J, Zhang Z, Du Y, Xu Y, Tong J, Cao B, Moran MF, Zeng Y and Mao X: Inhibition of the deubiquitinase USP5 leads to c-Maf protein degradation and myeloma cell apoptosis. Cell Death Dis 8: e3058, 2017.
12. Meulmeester E, Maurice MM, Boutell C, Teunisse AF, Ovaa H, Abraham TE, Dirks RW and Jochemsen AG: Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. Mol Cell 18: 565-576, 2005.
13. Meulmeester E, Pereg Y, Shiloh Y and Jochemsen AG: ATM-mediated phosphorylations inhibit Mdmx/Mdm2 stabilization by HAUSP in favor of p53 activation. Cell Cycle 4: 1166-1170, 2005.
14. Cummins JM, Rago C, Kohli M, Kinzler KW, Lengauer C and Vogelstein B: Tumour suppression: Disruption of HAUSP gene stabilizes p53. Nature 428: 1 p following 486, 2004.
15. Colland F, Formstecher E, Jacq X, Reverdy C, Planquette C, Conrath S, Trouplin V, Bianchi J, Aushev VN, Camonis J, *et al*: Small-molecule inhibitor of USP7/HAUSP ubiquitin protease stabilizes and activates p53 in cells. Mol Cancer Ther 8: 2286-2295, 2009.
16. Cai JB, Shi GM, Dong ZR, Ke AW, Ma HH, Gao Q, Shen ZZ, Huang XY, Chen H, Yu DD, *et al*: Ubiquitin-specific protease 7 accelerates p14(ARF) degradation by deubiquitinating thyroid hormone receptor-interacting protein 12 and promotes hepatocellular carcinoma progression. Hepatology 61: 1603-1614, 2015.
17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
18. Turley EA, Veiseh M, Radisky DC and Bissell MJ: Mechanisms of disease: Epithelial-mesenchymal transition-does cellular plasticity fuel neoplastic progression? Nat Clin Pract Oncol 5: 280-290, 2008.

19. Zhang Y, Liu Y, Zou J, Yan L, Du W, Zhang Y, Sun H, Lu P, Geng S, Gu R, et al: Tetrahydrocurcumin induces mesenchymal-epithelial transition and suppresses angiogenesis by targeting HIF-1α and autophagy in human osteosarcoma. Oncotarget 8: 91134-91149, 2017.
20. Shen S, Huang K, Wu Y, Ma Y, Wang J, Qin F and Ma J: A miR-135b-TAZ positive feedback loop promotes epithelial-mesenchymal transition (EMT) and tumorigenesis in osteosarcoma. Cancer Lett 407: 32-44, 2017.
21. Nieto MA, Huang RY, Jackson RA and Thiery JP. EMT: 2016. Cell 166: 21-45, 2016.
22. Tavana O, Li D, Dai C, Lopez G, Banerjee D, Kon N, Chen C, Califano A, Yamashiro DJ, Sun H and Gu W: HAUSP deubiquitinates and stabilizes N-Myc in neuroblastoma. Nature Med 22: 1180-1186, 2016.
23. Wang Q, Ma S, Song N, Li X, Liu L, Yang S, Ding X, Shan L, Zhou X, Su D, et al: Stabilization of histone demethylase PHF8 by USP7 promotes breast carcinogenesis. J Clin Invest 126: 2205-2220, 2016.
24. Hazan RB, Qiao R, Keren R, Badano I and Suyama K: Cadherin switch in tumor progression. Ann N Y Acad Sci 1014: 155-163, 2004.
25. Ghahhari NM and Babashah S: Interplay between microRNAs and WNT/β-catenin signalling pathway regulates epithelial-mesenchymal transition in cancer. Eur J Cancer 51: 1638-1649, 2015.
26. Angers S and Moon RT: Proximal events in Wnt signal transduction. Nat Rev Mol Cell Biol 10: 468-477, 2009.
27. Cadigan KM and Waterman ML: TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harb Perspect Biol 4: pii: a007906, 2012.
28. Valenta T, Hausmann G and Basler K: The many faces and functions of β-catenin. EMBO J 31: 2714-2736, 2012.
29. Wang Y, Shi J, Chai K, Ying X and Zhou BP: The role of snail in EMT and tumorigenesis. Curr Cancer Drug Targets 13: 963-972, 2013.
30. Jiang Z, Jiang C and Fang J: Up-regulated lnc-SNHG1 contributes to osteosarcoma progression through sequestration of miR-577 and activation of WNT2B/Wnt/β-catenin pathway. Biochem Biophys Res Commun 495: 238-245, 2018.
31. 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.
32. Novellasdemunt L, Foglizzo V, Cuadrado L, Antas P, Kucharska A, Encheva V, Snijders AP and Li VSW: USP7 Is a tumor-specific WNT activator for APC-mutated colorectal cancer by mediating β-catenin deubiquitination. Cell Rep 21: 612-627, 2017.