Long non-coding RNA phosphatase and tensin homolog pseudogene 1 suppresses osteosarcoma cell growth via the phosphoinositide 3-kinase/protein kinase B signaling pathway

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Abstract. Osteosarcoma is a common type of human carcinoma, which exhibits a high metastasis and recurrence rate. Previous studies have indicated that long non-coding RNA phosphatase and tensin homolog pseudogene 1 (lnPTENP1) has tumor suppressive action by modulating PTEN expression in different types of tumor cells. However, the potential mechanism by which lnPTENP1 has an effect in osteosarcoma cells remains elusive. In the present study, the role of lnPTENP1 in osteosarcoma cells was investigated and the possible mechanisms by which it functions were explored. It was revealed that lnPTENP1 transfection significantly inhibited osteosarcoma cell growth, proliferation, migration and invasion. LnPTENP1 transfection also significantly promoted apoptosis in Mg63 cells treated with tunicamycin. Further analysis revealed that lnPTENP1 transfection regulated osteosarcoma cell growth via the PI3K/AKT signaling pathway. In vivo assays revealed that lnPTENP1 transfection significantly inhibited osteosarcoma cell growth and significantly increased the protein expression and phosphorylation levels of PI3K and AKT. In conclusion, the results of the present study indicated that lnPTENP1 may inhibit osteosarcoma cell growth via the PI3K/AKT signaling pathway, which may be a potential novel target for human osteosarcoma therapy.

Introduction

Osteosarcoma is a type of cancer, and 50% of patients who develop it exhibit the common symptoms of bone and joint pain and fatigue in patients in the world (1). It has been observed that osteosarcoma tumors are highly metastatic and have a high recurrence rate (2). Despite a number of proposed clinical strategies, the prognosis for patients with osteosarcoma remains poor as there is limited understanding of the disease and few effective therapeutic targets have been identified (3,4). Osteosarcoma cells also have a high degree of apoptotic resistance (5,6), therefore, it is necessary to investigate the underlying mechanisms behind their angiogenesis and migration to better understand the pathological processes of the disease.

Long non-coding (Inc)RNAs are endogenous cellular non-coding RNA molecules longer than 200 nucleotides, which perform specific functions within tumor cells, but not in normal cells (7-9). Recently, specific IncRNAs, including IncRNA MALAT1 and IncRNA-AK123072, have been reported as associated with human cancer growth, migration and metastasis (10,11). A previous study has indicated that IncRNA phosphatase and tensin homolog pseudogene 1 (lnPTENP1) may act as a competing endogenous RNA to modulate the PTEN protein level by decoying microRNA (miR)-106b and miR-93 in gastric cancer (12). PTENP1 is a pseudogene of PTEN and is regarded as tumor suppressor and contains a highly homologous region upstream of the 3'-untranslated region (UTR) of PTEN (13). Chen et al (14) have recently reported that lnPTENP1 delivered by baculovirus effectively mitigated tumor growth, inhibited angiogenesis, suppressed cell proliferation and elicited apoptosis and autophagy. In addition, a previous study has demonstrated that PTEN may regulate angiogenesis through the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/vascular endothelial growth factor signaling pathway in human pancreatic cancer cells (15). Furthermore, PTEN may enhance the enzymatic activity of glutathione peroxidase, superoxide dismutase and catalase by suppressing the PI3K/AKT signaling pathway in lung cancer cells (16). However, the role and molecular mechanisms of lnPTENP1 in osteosarcoma cells is not fully understood.
In the present study, the tumor suppressive role of lnPTENP1 in osteosarcoma cells was investigated and the possible mechanisms by which it functions were explored. The role of lnPTENP1 in apoptotic resistance and in vivo anti-cancer efficacy were also investigated.

Materials and methods

Cell lines and cell culture. Mg63 and SAOS2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium ( Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Normal bone cell line hFOB1.19 was supplied by the Biochemistry Laboratory, Shandong University (Jinan, China) and was also cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS in a 6-well plate. Mg63 cells were treated with PI3K inhibitor (PI3KIR; LY-294,002) or tunicamycin (both 10 µg/ml; 20 µg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h. All cells were cultured at 37°C in 5% CO₂.

LncRNA transfection. LncRNA transfection was performed as previously described (17). All lncRNAs were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). In brief, Mg63 cells (1x10⁶) were transfected with 100 nM plentivirus-lnPTENP1 or the plentivirus-lncRNA-vector as the control using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 6 h following transfection the RPMI 1640 medium was removed and fresh media was added. At 48 h following transfection the cells were used for further analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from Mg63 and SAOS2 tumor cells, and hFOB1.19 cells using an RNAeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA) following the manufacturer’s protocol. RNA was reverse transcribed into cDNA at 42°C for 2 h using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Expression levels of PTEN in cells were measured by RT-qPCR with β-actin as the endogenous control as described previously (18). Forward and reverse primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and their sequences were as follows: PTEN forward, 5'-GTT TAC CGG CAG CAT CAA-3' and reverse, 5'-TGG AGT CAA CGG ATT TGG TC-3'; β-actin forward, 5'-TCA GAA CAT GGC ATA CAC CAA-3' and reverse, 5'-TGA TGC AGC TCC GAT TTCA TTA C-3'. 

Western blotting. The lnPTENP1- or vector-transfected Mg63 cells (1x10⁶) were homogenized in a radioimmunoprecipitation assay buffer with protease inhibitors (Sigma-Aldrich; Merck KGaA) and centrifuged at 8,000 x g at 4°C for 10 min. Protein concentration was measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). A total of 10 µg/lane protein was separated in a 15% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked in 5% skimmed milk for 1 h at 37°C. Cell colonies were counted using a light microscope at a magnification of x40 and Image Pro 5.0 software (Media Cybernetics, Inc., Rockville, MD, USA). At least three field of view were selected.

Apoptosis assays. The lnPTENP1-transfected Mg63 cells were seeded in 6-well plates at a density of 1x10⁴ cells/well and cultured in RPMI 1640 at 37°C for 14 days. Following incubation, the medium was removed and the cells were fixed with 100% methanol for 10 min at 37°C and stained with 0.1% (w/v) crystal violet (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C. Cell colonies were counted using a light microscope at a magnification of x40 and Image Pro 5.0 software (Media Cybernetics, Inc., Rockville, MD, USA). At least three field of view were selected.

MTT assay. The lnPTENP1-transfected Mg63 cells were seeded in 96-well plates at a density of 1x10⁴/well for 48 h at 37°C in triplicate. Following incubation, 20 µl MTT (5 mg/ml; Sigma-Aldrich, Merck KGaA) in PBS solution was added to each well and the plates were incubated for a further 4 h. The medium was removed and 100 µl dimethyl sulfoxide was added into the wells to dissolve the crystals. The optical density of purple formazan was measured using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at a wavelength of 490 nm.

Cell proliferation assay. The lnPTENP1-transfected Mg63 cells were seeded in 6-well plates at a density of 1x10⁴ cells/well and cultured in RPMI 1640 at 37°C for 14 days. Following incubation, the medium was removed and the cells were fixed with 100% methanol for 10 min at 37°C and stained with 0.1% (w/v) crystal violet (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C. Cell colonies were counted using a light microscope at a magnification of x40 and Image Pro 5.0 software (Media Cybernetics, Inc., Rockville, MD, USA). At least three field of view were selected.

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Western blotting. The lnPTENP1- or vector-transfected Mg63 cells (1x10⁶) were seeded in 6-well plates at a density of 1x10⁴ cells/well and cultured in RPMI 1640 at 37°C in a humidified incubator with 5% CO₂. Previous studies have showed that tunicamycin could induce human colon cancer (20-22). The lnPTENP1-transfected Mg63 cells were subsequently incubated with tunicamycin (10 mg/ml; 20 µg) or PBS for 24 h at 37°C to identify the role of lnPTENP1 on apoptosis in Mg63 cells. The cells were subsequently removed and washed with PBS three times. They were then incubated with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide, using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) for 2 h at 4°C according to the manufacturer’s protocol. The apoptotic rate and percentage of apoptotic Mg63 cells were measured with a fluorescence-activated cell sorting flow cytometer (BD Biosciences) and analyzed with FCS Express™ 4 IVD (De Novo Software, Glendale, CA, USA). All primary antibodies were used at a dilution of 1:1,000 and purchased from Abcam (Cambridge, UK). The membranes were then incubated with horseradish peroxidase
Osteosarcoma tissues were fixed using 10% formaldehyde for 30 min at 37˚C followed by embedding in paraffin wax. Osteosarcoma tissue sections were cut into 4-µm-thick sections were deparaffinized in xylene and washed with PBS three times at room temperature. Antigen retrieval was performed on the tumor sections using a 0.01 M citrate buffer (pH 6.0) at 95˚C for 15 min. The sections were incubated with 3% hydrogen peroxide for 10 min at room temperature to inactivate endogenous peroxidase activity. The tumor sections were incubated with specific primary antibodies against PI3K, pPI3K, AKT and PTEN (dilution 1:1,000). Western Blotting Detection Reagent was used to detect protein expression in tumor tissues with light microscopy. The staining results were observed using fluorescent microscope (Olympus Corporation, Tokyo, Japan) at a magnification x400 and semi-quantitatively evaluated by multiplying the staining intensity and the percentage of positive staining cells. The density of the tumor tissues was analyzed using Quantity One software version 4.62.

Cell migration and invasion assay. For the migration and invasion assays the lnPTENP1- or vector-transfected Mg63 cells were placed into the upper chamber of Transwell plates with non-coated membranes at a density of 1x10^4 cells/well with 150 µl serum-free Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.). Matrigel-coated and uncoated Transwell inserts (8 µm pore size; Merck KGAA) were used to evaluate cell invasion and migration, respectively. The cells were incubated in DMEM with 5% FBS (both Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37˚C and then the Mg63 cells were fixed in 4% paraformaldehyde for 15 min at 37˚C and stained with 0.1% crystal violet dye (Sigma-Aldrich; Merck KGAA) for 20 min at 37˚C. The cells were removed with a cotton swab and counted at three randomly selected views using a light microscope (BX51; Olympus Corporation, Tokyo, Japan) at a magnification of x40.

Animal study. A total of 40 old female Balb/c mice (age, 8 weeks; weight, 25-32 g) were purchased from Shanghai SLAC Experimental Animals Co., Ltd. (Shanghai, China). The mice were maintained in a 12 h light/dark cycle with ad libitum access to food and water. All animals were housed in a temperature-controlled facility at 23±1˚C with a relative humidity of 50±5%. lnPTENP1- or vector-transfected Mg63 cells (1x10^5) in 200 µl PBS were subcutaneously injected into a single side of the posterior flank of the mice (n=20/group). On day 30, the mice were anesthetized with intravenous pentobarbital sodium (37 mg/kg) prior to the tumor removal. The tumor weight was calculated as previously described (24). When tumor diameter reached 18 mm the mice were sacrificed. Multiple tumors were not observed in individual mice.

The present study was approved by the Institutional Review Board of the Second Affiliated Hospital of Xinjiang Medical University (Urumchi, China). The protocols used were approved by Ethical Committee of the Second Affiliated Hospital of Xinjiang Medical University.

Immunohistochemistry analysis. Osteosarcoma tissues were fixed using 10% formaldehyde for 30 min at 37˚C followed by embedding in paraffin wax. Osteosarcoma tissue sections (4-µm-thick) were deparaffinized in xylene and washed with PBS-Tween-20 three times at room temperature. Antigen retrieval was performed on the tumor sections using a microwave to heat the sections in a graded series of ethanol, followed by blocking of endogenous peroxidase activity with 3% hydrogen peroxide for 10 min at room temperature as previously described (25). Tumor sections were incubated with specific primary antibodies against PI3K, pPI3K, AKT and pAKT for 12 h at 4˚C. All antibodies were used at a dilution of 1:1,000. The tumor tissues were subsequently incubated with HRP-conjugated goat anti-rabbit IgG monoclonal secondary antibodies (dilution 1:5,000). Amersham™ ECL Select™ Western Blotting Detection Reagent was used to detect protein expression in tumor tissues with light microscopy. The staining results were observed using fluorescent microscope (Olympus Corporation, Tokyo, Japan) at a magnification x400 and semi-quantitatively evaluated by multiplying the staining intensity and the percentage of positive staining cells. The density of the tumor tissues was analyzed using Quantity One software version 4.62.

Statistical analysis. Data are expressed as the mean ± standard deviation and a minimum of three independent repeats were performed. All data were analyzed with SPSS software version 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) using one-way analysis of variance followed by Tukey's multiple comparison post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

LnPTENP1 and PTEN expression in osteosarcoma cells. PTEN and lnPTENP1 expression was evaluated in Mg63 and SAOS2 osteosarcoma cells and hFOB1.19 normal osteocytes. The PTEN and lnPTENP1 mRNA expression levels were significantly downregulated in osteosarcoma cells compared with normal osteocytes (Fig. 1A and B). The results revealed that lnPTENP1 transfection significantly increased the mRNA and protein expression levels of PTEN in Mg63 and SAOS2 cells (Fig. 1C and D). These findings suggest that lnPTENP1 may regulate PTEN expression in osteosarcoma cells.

LnPTENP1 transfection inhibits osteosarcoma cell growth, proliferation, migration and invasion in vitro. The effects of lnPTENP1 transfection on osteosarcoma cell growth, proliferation migration and invasion were investigated in vitro. The results demonstrated that lnPTENP1 transfection significantly inhibited Mg63 cell growth and proliferation compared with the control (Fig. 2A and B). It was also observed that lnPTENP1 transfection significantly inhibited the migration and invasion of Mg63 cells compared with the control (Fig. 2C and D). These results suggest that LnPTENP1 transfection may inhibit osteosarcoma cell growth, proliferation, migration and invasion in vitro.

LnPTENP1 transfection promotes the apoptosis of osteosarcoma cells treated with tunicamycin. The effect of lnPTENP1 transfection on the apoptosis of Mg63 osteosarcoma cells was analyzed. Transfection with lnPTENP1 significantly increased lnPTENP1 expression compared with transfection with the vector in Mg63 cells (Fig. 3A). It was observed that lnPTENP1 transfection significantly increased the apoptosis of osteosarcoma cells treated with tunicamycin compared with transfection with the plentivirus-vector (Fig. 3B). Western blot analysis demonstrated that lnPTENP1 transfection significantly inhibited the protein expression of anti-apoptosis protein Bcl-2 (Fig. 3C), whereas it increased the protein expression of pro-apoptosis proteins Bax and Bad in Mg63...
cells (Fig. 3D). However, lnPTENP1 transfection significantly decreased pro-apoptosis protein p53 expression in Mg63 cells (Fig. 3D). These results suggest that lnPTENP1 transfection may promote the apoptosis of osteosarcoma cells treated with the chemotherapy drug tunicamycin.

LnPTENP1 regulates the growth of osteosarcoma cells via the PI3K/AKT signaling pathway. To determine the effect of lnPTENP1-mediated inhibition of osteosarcoma cells, the PI3K/AKT signaling pathway was investigated. It was revealed that lnPTENP1 transfection significantly increased the protein expression and phosphorylation levels of PI3K and AKT in Mg63 cells, compared with controls (Fig. 4A). In addition, PI3KIR significantly reversed the lnPTENP1 inhibition of growth in the Mg63 cells (Fig. 4B). The results also demonstrated that PI3KIR significantly reversed the lnPTENP1-inhibited migration and invasion in Mg63 cells (Fig. 4C and D). These results suggest that PI3KIR may regulate the growth of osteosarcoma cells via the PI3K/AKT signaling pathway.

LnPTENP1 inhibits in vivo growth of osteosarcoma in tumor-bearing mice. To analyze whether lnPTENP1 inhibited osteosarcoma growth in vivo Mg63 cells transfected with lnPTENP1 or an empty vector were subcutaneously injected into a single side of the posterior flank of mice. Transfection with lnPTENP1 significantly inhibited the tumor growth in mice compared with those transfected with the empty vector group following 30 days observation (Fig. 5A). The mean weight of the animals at the time of tumor removal was 34.7 and 32.2 g in the Lncontrol and LnPTENP1 group, respectively (data not shown). Immunohistochemistry assays revealed that lnPTENP1 transfection significantly increased the protein expression and phosphorylation of PI3K and AKT in tumor tissues (Fig. 5B). These findings suggest that endogenetic expression of lnPTENP1 may inhibit osteosarcoma growth in vivo.

Discussion

A number of previous studies have indicated that lncRNAs are associated with tumor cell growth, differentiation, apoptosis and metastasis (26,27). In recent years, several lncRNAs have been implicated as major regulators of cellular phenotypes and oncogenes or tumor suppressors (10,28). In addition, a recent study has demonstrated that pseudogene PTENP1 suppresses gastric cancer growth and metastasis by modulating PTEN (29). In the present study, it was observed that
In PTENP1 transfection significantly upregulated in vitro PTEN expression in osteosarcoma cells, inhibited growth in vivo and promoted apoptosis via the PI3K/AKT signaling pathway.

PTENP1 is a new pseudogene that has been identified as a competitive endogenous RNA that binds with its ancestral gene (30). PTENP1 contains a highly homologous region upstream of the 3'-UTR of PTEN, which has been identified as a tumor suppressor (29,31). In the present study, it was demonstrated that lnPTENP1 was significantly downregulated in osteosarcoma cells compared with normal bone cells. However, transfection of lnPTENP1 significantly increased PTENP1 expression, which led to the inhibition of growth, proliferation, migration and invasion of osteosarcoma cells in vitro.

At present, apoptotic resistance serves a crucial role in the progression of human cancer metastasis (32,33). A previous study has suggested that IncRNAs are associated with human cancer cell apoptosis (34). To identify and characterize the role of lnPTENP1 in osteosarcoma cells, lnPTENP1 was transfected into Mg63 cells; it was demonstrated that the transfection promoted tunicamycin-induced Mg63 cell apoptosis. The upregulation of anti-apoptosis proteins increases the apoptotic resistance of tumor cells (35,36). In the present study, it was demonstrated that lnPTENP1 transfection significantly decreased the protein expression of Bcl-2 in Mg63 cells. Previous studies have revealed that increasing pro-apoptosis protein expression, including Bad and Bax may contribute to the apoptosis of tumor cells (37,38). Notably, PTENP1 repressed the tumorigenic properties of hepatocellular carcinoma cells by regulating the autophagy of genes, including ULK1, ATG7 and p62, which further increased the apoptosis of tumor cells (14). The results of the present study demonstrated that lnPTENP1 transfection increased pro-apoptosis proteins Bax and Bad in osteosarcoma cells. It was also observed that lnPTENP1 transfection significantly increased apoptosis but significantly decreased p53 expression in Mg63 cells. The authors suggest that the increasing pro-apoptosis action is stronger than the anti-apoptosis action following transfection with lnPTENP1. However, further study is required to identify the association between lnPTENP1 and p53 in osteosarcoma cells.

A number of previous studies have proposed various strategies for the treatment of osteosarcoma with the identification of several chemotherapeutic and immunologic agents (39-41). However, the overall survival rate for patients with osteosarcoma has not markedly improved since the introduction of neoadjuvant chemotherapy, radiotherapy and surgery (42). It has been suggested that the PI3K/AKT signaling pathway serves an essential role in human carcinoma cells as it regulates

Figure 2. LnPTENP1 transfection inhibits osteosarcoma cell progression in vitro. LnPTENP1 transfection inhibited the (A) growth, (B) proliferation, (C) migration and (D) invasion of Mg63 cells. The data are presented as the mean ± standard deviation of three independent repeats. *P<0.01. Ln, long non coding RNA; PTENP1, phosphatase and tensin homolog pseudogene 1.
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In the present study, it was revealed that LnPTEINP1 regulates the growth of osteosarcoma cells via the PI3K/AKT signaling pathway. A previous study indicated that PI3K/AKT signaling mediates hexokinase-2-inhibited cell apoptosis and promotes tumor growth in pediatric osteosarcoma (45). In the present study, it was observed that LnPTEINP1 significantly downregulated PI3K/AKT signaling in osteosarcoma cells. Liu et al (46) have recently demonstrated that regulation of the PTEN/PI3K/AKT signaling pathway may inhibit proliferation, apoptosis and migration of Wilms tumor cells. In the present study, it was reported that LnPTEINP1 regulated the growth of osteosarcoma cells in vitro and in tumor-bearing mice through the PI3K/AKT signaling pathway.

In conclusion, the present study analyzed the role and the possible mechanism of LnPTEINP1 in osteosarcoma cells. The results suggest that LnPTEINP1 overexpression may suppress the growth of osteosarcoma cells in vitro and in vivo by regulation of the PI3K/AKT signaling pathway. However, further investigation is required to identify the potential mechanisms mediated by LnPTEINP1 in osteosarcoma cells. The results of the present study may serve as the basis for novel therapy against osteosarcoma in combination with chemotherapy.

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Figure 4. LnPTENP1 regulates the growth of osteosarcoma cells via the PI3K/AKT signaling pathway. (A) LnPTENP1 transfection significantly increased the protein expression and phosphorylation levels of PI3K and AKT in Mg63 cells. (B) PI3KIR reverses the LnPTENP1-inhibited growth of Mg63 cells and the (C) LnPTENP1-inhibited migration and (D) invasion. The data are presented as the mean ± standard deviation of three independent repeats. **P<0.01.

PI3K, phosphoinositide 3-kinase; PI3KIR, PI3K inhibitor; Ln, long non coding RNA; PTENP1, phosphatase and tensin homolog pseudogene 1; AKT, protein kinase B; p, phosphorylated.

Figure 5. LnPTENP1 inhibits in vivo growth of osteosarcoma in tumor-bearing mice. (A) Transfection with LnPTENP1 significantly inhibited tumor growth compared with mice transfected with the empty vector group after 30 days observation. (B) LnPTENP1 transfection significantly increased the protein expression and phosphorylation of PI3K and AKT in tumors tissues. The data are presented as the mean ± standard deviation of three independent repeats. **P<0.01. Magnification, x40. Ln, long non coding RNA; PTENP1, phosphatase and tensin homolog pseudogene 1; AKT, protein kinase B; PI3K, phosphoinositide 3-kinase; p, phosphorylated.
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

BY and AW analyzed and interpreted the data regarding the experiments, and YL contributed in the acquisition of data, did some of the experiments, and was a major contributor in writing the manuscript. XW performed the animal experiments in the present study.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the Second Affiliated Hospital of Xinjiang Medical University (Urumchi, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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