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Expression and function of microRNA-497 in human osteosarcoma

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Abstract. The expression and function of microRNA-497 (miR-497) has previously been reported in various types of human cancer; however, miR-497 has not previously been investigated in human osteosarcoma (OS). In the present study, the expression levels of miR-497 were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in OS tissues and cell lines. In addition, post-transfection with miR-497, RT-qPCR, cell proliferation, migration and invasion assays, western blot analysis, and luciferase assays were performed in OS cell lines. The results of the present study demonstrated that miR-497 was downregulated in OS tissues and cells compared with normal controls. Furthermore, upregulation of miR-497 inhibited cell proliferation, migration and invasion in osteosarcoma cell lines compared with the negative control group. In addition, the present study demonstrated that miR-497 may function by directly targeting insulin-like growth factor 1 receptor in OS cells. These findings indicated that miR-497 may be useful as a therapeutic target for the treatment of OS.

Introduction

Osteosarcoma (OS), which is the most common primary malignancy of the bone and joints, is the leading cause of cancer-associated mortality among children and adolescents (1). The estimated OS morbidity is 4.4 per million worldwide, with a peak incidence at 15-19 years old (2). The pathogenesis of OS is complex, and numerous genetic and environmental factors contribute to the disease (3). The well-known environmental risk factors of OS include ionizing radiation, alkylating agents and hereditary retinoblastoma. In addition, increasing evidence has suggested that genetic susceptibility is an important factor in the etiology of OS (4,5). Despite advances in modern therapeutic strategies, including surgery, chemotherapy, and the use of combinational chemotherapy and advanced surgery, the five-year survival rate of patients diagnosed with OS is ~65% (6), and the majority of patients eventually develop fatal metastases (7). Therefore, it is crucial to explore the molecular mechanisms underlying OS, and develop novel prognostic biomarkers and targeted therapies for patients with OS.

Previous studies have focused on the expression and function of microRNAs (miRNAs) in carcinogenesis and cancer progression, including OS (8,9). miRNAs are a class of short, non-coding, single-stranded, endogenous RNAs 16-27 nucleotides in size (usually 21-23 nucleotides) (10,11). miRNAs are highly conserved in the genome of the majority of species, including humans, animals, plants and DNA viruses (12). miRNAs regulate gene expression by base pairing to complementary sites in the 3'-untranslated region (3'-UTR) of mRNA transcripts, resulting in translational repression or silencing of target genes (13,14). Numerous miRNAs, >50%, are located in cancer-associated regions or in fragile sites of the genome, thus indicating that they may be involved in carcinogenesis and cancer progression (15). miRNAs have previously been demonstrated to have important roles in various biological processes, including cell proliferation, development, apoptosis, differentiation and cancer tumorigenesis (16). Increasing evidence has demonstrated that miRNAs may act as oncogenes or tumor suppressors (17). Upregulated miRNAs in cancer may function as oncogenes, whereas, downregulated miRNAs may function as tumor suppressors. Therefore, identification of novel miRNAs involved in OS carcinogenesis and progression may be useful to develop future strategies for the diagnosis, treatment and prognosis of OS (18).

miRNA-497 (miR-497) has previously been investigated in various types of human cancer (19-21). The present study initially determined the expression levels of miR-497 in OS tissues and cell lines. Subsequently, OS cells were transfected with miR-497 mimics to identify the function of miR-497 in OS. The present study demonstrated that miR-497 was downregulated in human OS tissues and cell lines. Upregulation of miR-497 inhibited cell proliferation, migration and invasion via the negative regulation of insulin-like growth factor 1 receptor (IGF-1R) expression. These results suggested that miR-497 may be associated with a novel molecular mechanism underlying OS tumorigenesis and progression.

Materials and methods

Clinical specimens. OS tissue specimens and matched normal adjacent tissues (NATs) (n=24) were collected from

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the Department of Orthopaedic Surgery, Shengjing Hospital, China Medical University (Shenyang, China). All specimens were histologically and clinically diagnosed. The present study was approved by the Medical Ethics Committee of Shengjing Hospital, and written informed consent was obtained from all patients with OS who had been treated by surgical resection at the Department of Orthopaedic Surgery, Shengjing Hospital, China Medical University (Shenyang, China). None of these patients with OS had received chemotherapy or other treatments prior to surgery. Tissues were snap-frozen using liquid nitrogen and stored at -80˚C.

Cell culture. The human OS cell lines, HOS and U2OS, and a human normal osteoblastic cell line, hFOB 1.19, were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2.

Transfection of HOS and U2OS in vitro. Mature miR-497 mimics and negative control (NC) mimics were synthesized and confirmed by Shanghai GenePharma Co., Ltd. (Shanghai, China). For functional analysis, HOS and U2OS cells were seeded into 6-well plates. When cell density reached 60-70% confluence, cells were transfected with miR-497 mimics or NC mimics using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The sequences were as follows: miR-497 mimics, 5'-CAGCAGCAC ACUGUGGUUUGU-3' and NC mimics, 5'-UUCUCCGAA CGUGUCACGUTT-3'.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The OS tissues and matched NATs were homogenized prior to RNA extraction. Total RNA was isolated from OS tissues, matched NATs, OS cells and normal osteoblastic cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT was performed using the Moloney Murine Leukemia Virus RT system (Promega Corporation, Madison, WI, USA). The protocol for reverse transcription was as follows: 95˚C for 2 min; 20 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 2 min; then 72˚C for 5 min. RT-qPCR was conducted with an Applied Biosystems 7500 Real-time PCR Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a SYBR *Premix Ex Taq* kit (Takara Biotechnology Co., Ltd., Dalian, China) in a final volume of 20 μl according to the manufacturers' protocols. The reaction system contained 10 μl SYBR *Premix Ex Taq II*, 2 μl cDNA, 0.8 μl forward primer, 0.8 μl reverse primer, 0.4 μl ROX Reference Dye and 6 μl double distilled water. Primers were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The cycling conditions were as follows: 95˚C for 30 sec; 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Each sample was measured in triplicate. The relative expression levels of miR-497 were normalized using U6 as a reference gene and were calculated using the $2^{-\Delta\Delta Cq}$ method (22).

Cell proliferation assay. Post-transfection with miR-497 or NC mimics for 24 h, HOS and U2OS cells were seeded in 96-well plates at a density of $3x10^3$ cells/well. At various time points post-transfection, 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) solution was added to each well and incubated for a further 4 h at 37˚C. Subsequently, the MTT solution was removed and the formazan precipitates were dissolved in 200 μl dimethyl sulfoxide. The absorbance was measured at 490 nm using a 96-well plate reader (ELx800; Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were measured in triplicate.

Cell migration and invasion assays. Migration and invasion analysis of HOS and U2OS cells was performed using 8-μm Transwell chambers (Costar; Corning Incorporated, Corning, NY, USA) coated with (invasion assay) or without (migration assay) Matrigel® (BD Biosciences, San Jose, CA, USA). A total of 48 h post-transfection with miR-497 or NC mimics, $1x10^5$ cells were harvested and resuspended in 200 μl DMEM without FBS. The cells were then seeded into the upper chamber, and 0.5 ml DMEM with 20% FBS was added to the lower chamber as a chemoattractant. Following a 12 h incubation for the migration assay and a 24 h incubation at 37˚C for the invasion assay, the cells remaining on the upper surface of the membranes were scraped off with cotton swabs. The migrated or invasive cells were fixed and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China). Images of the migrated and invasive cells were captured and counted under an inverted microscope (IX53; Olympus Corporation, Tokyo, Japan). All experiments were measured in triplicate.

Western blot analysis. A total of 72 h post-transfection with miR-497 or NC mimics, HOS and U2OS cells were collected and homogenized in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was measured using a bicinchoninic acid protein assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Equal amounts of protein (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnology) and were then transferred onto polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology), which were blocked for 2 h with 5% non-fat milk. Subsequently, the membranes were incubated with primary antibodies overnight at 4˚C. The mouse anti-human monoclonal primary antibodies used in the present study were as follows: Anti-IGF-1R (ab54274; 1:1,000) and anti-β-actin (ab8226; 1:1,000) (Abcam, Cambridge, MA, USA). The membranes were then incubated for 1 h with the goat anti-mouse horseradish peroxidase-conjugated secondary antibody (ab97023; 1:2,000; Abcam) in Tris-buffered saline containing 0.1% Tween 20 (Beyotime Institute of Biotechnology). The blots were detected using enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc., Rockford, IL, USA) and the FluorChem imaging system, version 4.1.0 (ProteinSimple, San Jose, CA, USA).

Luciferase assay. The pMIR-IGF-1R-3'UTR wild-type and pMIR-IGF-1R-3'UTR mutant-type reporter vectors were synthesized and purified by Shanghai GenePharma Co., Ltd.

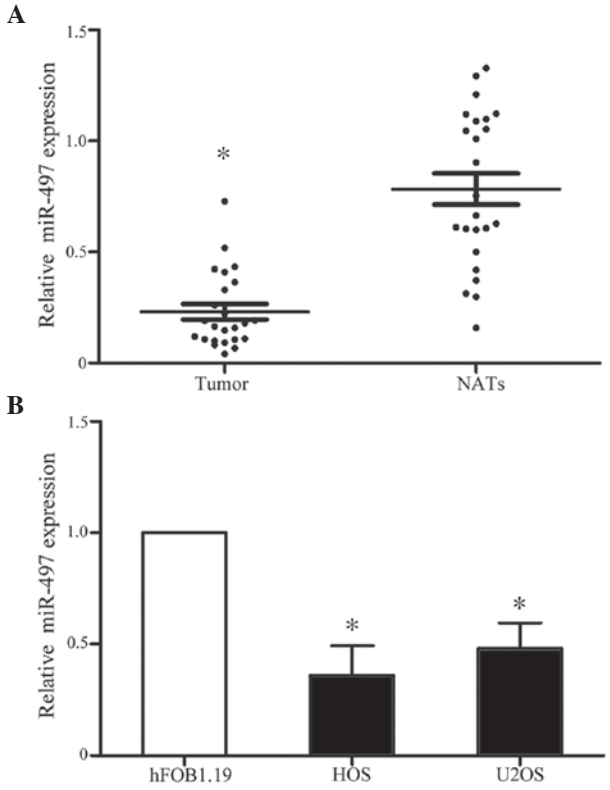

Figure 1. Expression levels of miR-497 in OS primary tumor tissues and OS cell lines. (A) miR-497 expression was significantly lower in OS tissues compared with NATs; *P<0.05. (B) miR-497 expression was also downregulated in OS cell lines HOS and U2OS compared with in the human normal osteoblastic cell line hFOB 1.19; *P<0.05. Data are presented as the mean ± standard deviation. miR, microRNA; NATs, normal adjacent tissues; OS, osteosarcoma.

The HOS and U2OS cells were seeded into 12-well plates at ~90% confluence. Subsequent to incubation overnight, luciferase reporter vectors and miR-497 mimics or NC were transfected into cells using Lipofectamine 2000, according to the manufacturer's instructions. Post-transfection, relative luciferase activity was measured at 48 h using the Dual-Luciferase Reporter Assay system (Promega Corporation) and absorbance was measured using the spectrophotometer (xMark™; Bio-Rad Laboratories, Inc.). All experiments were performed in triplicate.

Statistical analysis. Data are presented as the mean ± standard deviation and were analyzed using SPSS software version 11.5 (SPSS, Inc., Chicago, IL, USA) using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-497 expression is decreased in OS primary tumor samples and OS cell lines. The expression levels of miR-497 in OS tissues, NATs, OS cell lines and the human normal osteoblastic cells were measured by RT-qPCR. As demonstrated in Fig. 1A, miR-497 was significantly downregulated in OS tissues compared with matched NATs (P<0.05). As demonstrated in Fig. 1B, miR-497 expression was also decreased in OS cell lines compared with hFOB 1.19 cells (P<0.05). These results indicate that miR-497 may be involved in OS.

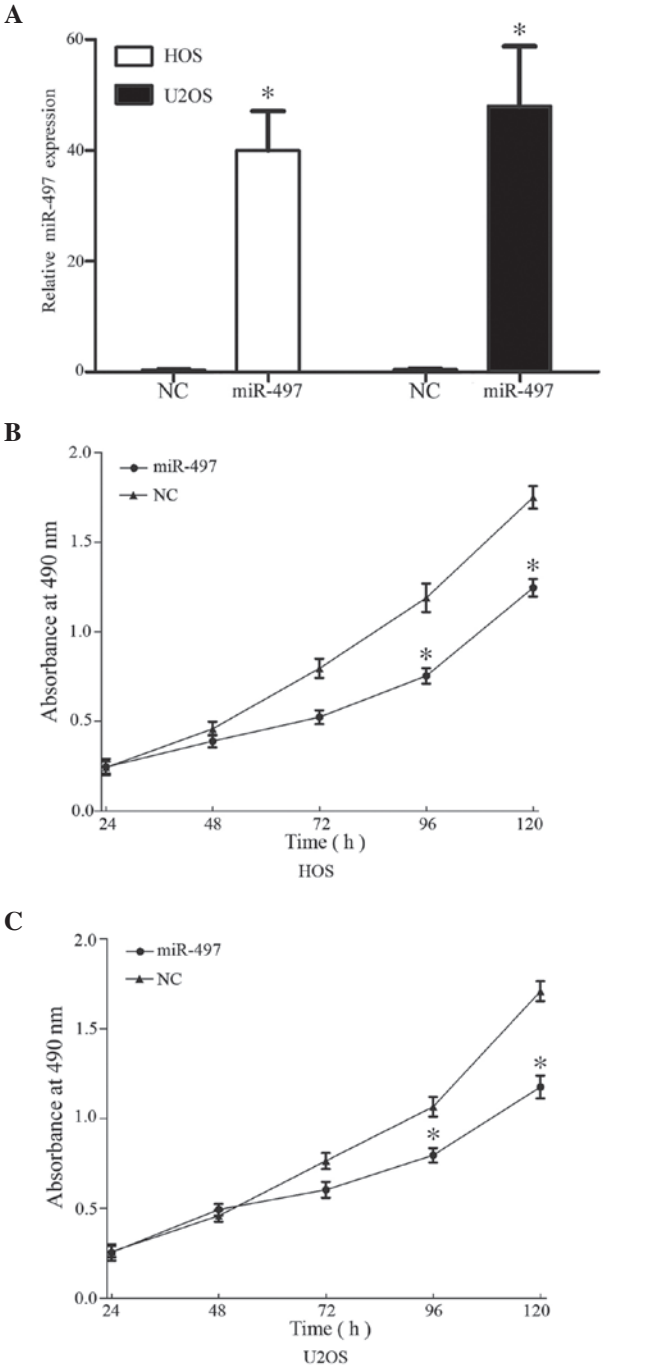

Figure 2. Overexpression of miR-497 inhibits OS cell proliferation. (A) Successful overexpression of miR-497 in HOS and U2OS cell lines was confirmed by reverse transcription-quantitative polymerase chain reaction following transfection with miR-497 or NC mimics. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay revealed that upregulation of miR-497 significantly inhibited cell proliferation in (B) HOS and (C) U2OS OS cell lines. Data are presented as the mean ± standard deviation; *P<0.05 vs. NC. miR, microRNA; NC, negative control; OS, osteosarcoma.

miR-497 suppresses cell proliferation in OS cell lines. To assess the effects of miR-497 on OS cell proliferation, HOS and U2OS cells were transfected with miR-497 mimics. As demonstrated in Fig. 2A, RT-qPCR confirmed that miR-497 expression levels

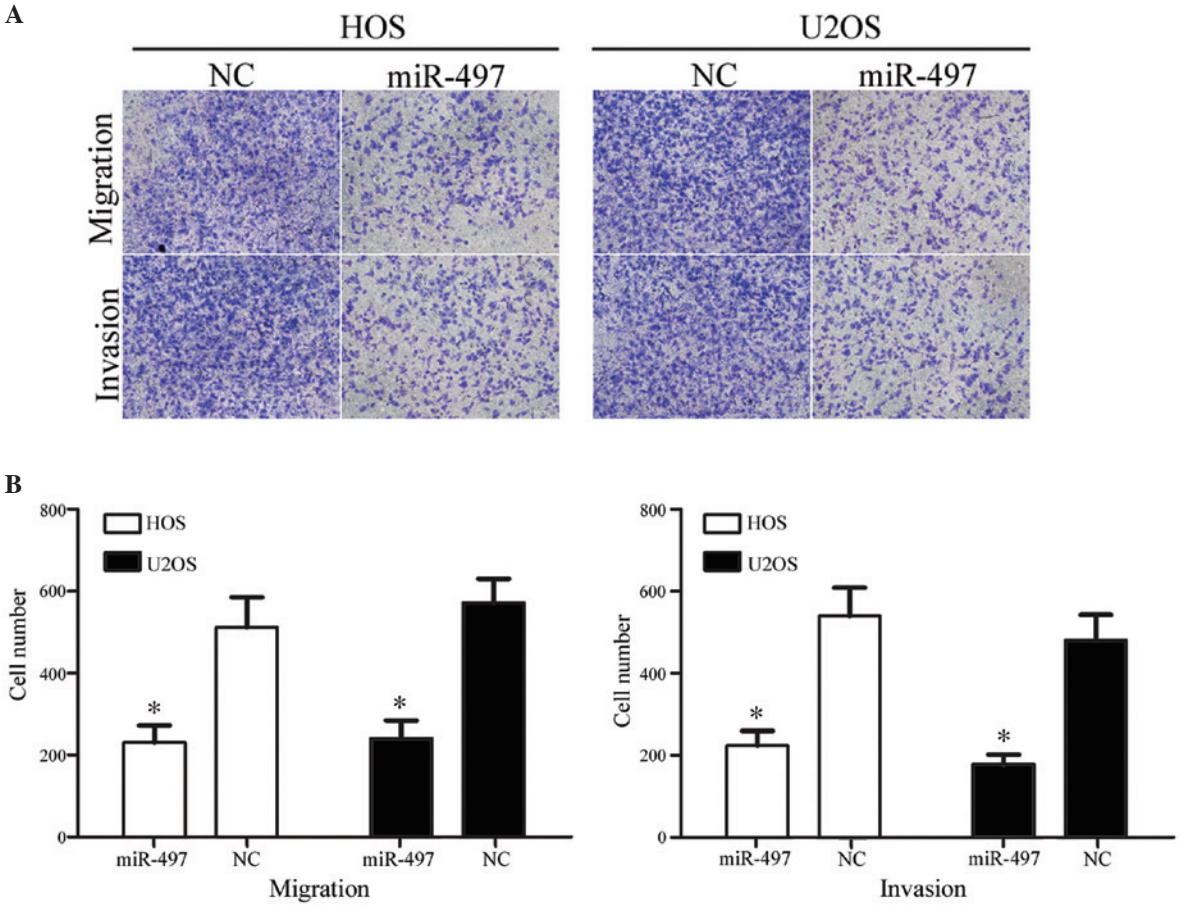

Figure 3. Effects of miR-497 overexpression on HOS and U2OS migration and invasion. The migration and invasion assays were performed using Transwell chambers. (A) Representative images (magnification, x200) and (B) quantification are presented. miR-497 overexpression inhibited HOS and U2OS migration and invasion *in vitro* (P<0.05). Data are presented as the mean ± standard deviation; *P<0.05 vs. NC. NC, negative control; miR, microRNA.

were increased in cells transfected with miR-497 mimics compared with those transfected with NC mimics (P<0.05).

To determine the effects of miR-497 on OS cell proliferation, an MTT assay was performed. As demonstrated in Fig. 2B and C, upregulation of miR-497 significantly inhibited cell proliferation in HOS and U2OS cells compared with cells transfected with NC mimics. The MTT assay demonstrated that 120 h post-transfection with miR-497 mimics, the proliferation rate was suppressed by 26.54±3.8% in HOS cells and 33.72±3.9% in U2OS cells, compared with the NC group (P<0.05).

miR-497 inhibits cell migration and invasion of OS cells. To determine whether miR-497 may regulate human OS cell migration and invasion, migration and invasion assays were performed using OS cell lines. HOS and U2OS cells were transfected with miR-497 and NC mimics. As demonstrated in Fig. 3A and B, overexpression of miR-497 significantly decreased the migratory and invasive capability of HOS and U2OS cells compared with in the NC groups (P<0.05). These results indicate that miR-497 negatively regulates OS migration and invasion.

miR-497 suppresses the expression of IGF-1R in OS cell lines. A public database (TargetScan; www.targetscan.org) predicted IGF-1R as a direct target gene of miR-497. As demonstrated in Fig. 4A, the IGF-1R mRNA 3'-UTR contains a miR-497 six-nucleotide seed match at position 1269-1275.

Western blot analysis was performed to determine whether IGF-1R was downregulated following transfection of OS cells with miR-497 mimics. As demonstrated in Fig. 4B, compared with cells transfected with NC mimics, IGF-1R protein levels were significantly downregulated in OS cell lines following transfection with miR-497 mimics (P<0.05).

IGF-1R is a direct target gene of miR-497 in OS. To further analyze whether IGF-1R is a direct target of miR-497, a luciferase reporter assay was performed in the OS cell lines. As demonstrated in Fig. 5, compared with NC mimics, miR-497 mimics significantly decreased luciferase activity in OS cells transfected with the wild type IGF-1R construct, but did not affect those transfected with the mutant IGF-1R (P<0.05). These results indicate that IGF-1R is a target gene of miR-497 *in vitro*.

Discussion

The expression and function of miRNAs in tumor development has recently become a subject of intense investigation. Mounting evidence has demonstrated that the expression of miRNAs is commonly dysregulated in various types of human cancer, including OS. Alterations to miRNA expression have previously been demonstrated to be involved in the

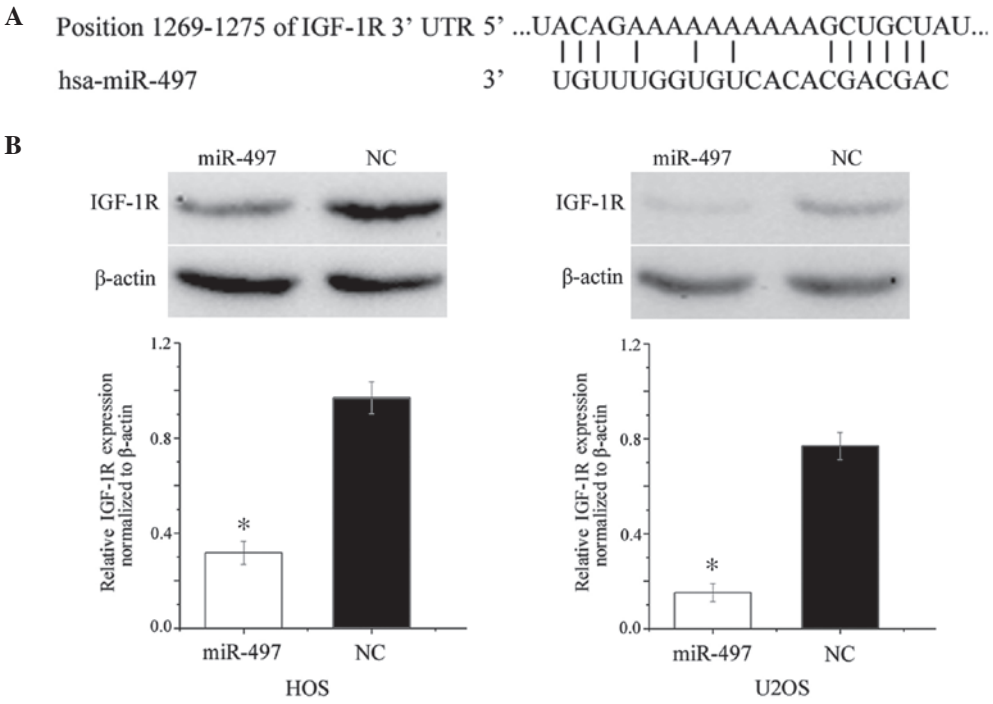

Figure 4. miR-497 decreases the expression of IGF-1R in OS cell lines. (A) TargetScan demonstrated that IGF-1R contained a miR-497 seed match at position 1269-1275 of the IGF-1R 3'-UTR. (B) Western blot analysis demonstrated that IGF-1R was significantly downregulated in osteosarcoma cell lines post-transfection with miR-497 mimics. Data are presented as the mean ± standard deviation; *P<0.05 vs. NC. IGF-1R, insulin-like growth factor 1 receptor; 3'-UTR, 3'-untranslated region; miR, microRNA; NC, negative control.

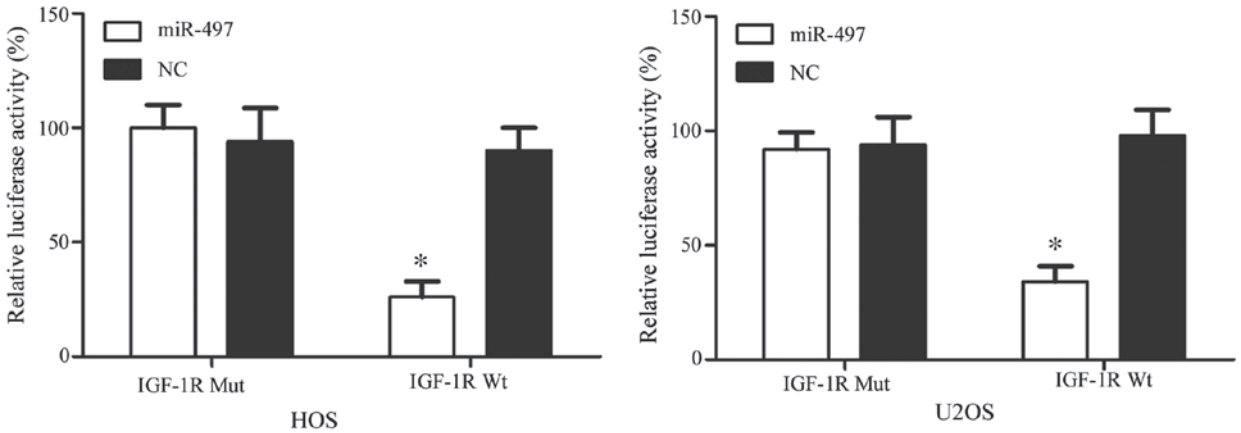

Figure 5. IGF-1R is a direct target gene of miR-497 in osteosarcoma. Overexpression of miR-497 significant inhibited the luciferase activity of IGF-1R Wt but not IGF-1R Mut in HOS and U2OS cell lines. Data are presented as the mean ± standard deviation; *P<0.05 vs. NC. IGF-1R, insulin-like growth factor 1 receptor; miR, microRNA; NC, negative control; Mut, mutant; Wt, wild type.

proliferation, metastasis and tumorigenesis of cancer (23-25). The present study demonstrated that miR-497 was downregulated in OS, and the downregulation of miR-497 may contribute to carcinogenesis and progression in OS.

miR-497 is a member of the miR-15/16/195/424/497 families, which share the same 3'-UTR seed sequence (26). miR-497 is located on chromosome 17p13.1. The loss or deletion of chromosome 17p13.1 has previously been reported in various types of cancer, thus suggesting that reduced miR-497 expression arises from genomic DNA loss or deletion (27-29). miR-497 has previously been demonstrated to be frequently downregulated in various types of human cancer, including breast cancer (19), hepatocellular carcinoma (20), gastric cancer (21), nasopharyngeal carcinoma (30), endometrial cancer (31), colorectal cancer (26) and prostate cancer (32). Previously, miR-497 was demonstrated to be downregulated in the plasma of patients with bladder cancer compared with healthy subjects, which suggests that plasma miR-497 expression may be a promising novel circulating biomarker for clinical detection (33). Xu *et al* (34) demonstrated that miR-497 expression was significantly downregulated in pancreatic cancer tissues and that low expression of miR-497 was an independent adverse prognostic factor for pancreatic cancer. Furthermore, downregulation of miR-497 was correlated with breast cancer progression and may be

considered a potential molecular biomarker for predicting the prognosis of patients with breast cancer (19). Taken together, these results indicate that miR-497 may be a novel therapeutic target and prognostic marker for these types of cancer.

The function of miR-497 has previously been investigated in various types of cancer. In human cervical cancer, overexpression of miR-497 was shown to inhibit cell growth and metastasis, via the negative regulation of IGF-1R and cyclin E1 expression (35,36). In ovarian cancer, exogenous expression of miR-497 decreases cell migration and invasion by directly targeting SMAD specific E3 ubiquitin protein ligase 1 (37). In breast cancer, miR-497 induces cell apoptosis by downregulating BCL2-like 2 at the mRNA and protein levels (38). In addition, overexpression of miR-497 suppresses cell proliferation, migration and invasion by binding the 3'-UTR of cyclin E1 (39). Furthermore, miR-497 reduces cell proliferation in prostate cancer and non-small cell lung cancer by targeting caspase-3 and hepatoma-derived growth factor, respectively (32,40). The present study demonstrated that upregulation of miR-497 decreased cell growth and migration and invasion by targeting IGF-1R. Taken together, these results suggested that miR-497 may be useful for the development of novel molecular markers and therapeutic approaches. Upregulating miR-497, or providing analogous pharmaceutical compounds exogenously, may be effective for the treatment of cancer resulting from overexpression of these oncogenic transcripts.

Previous studies have demonstrated that miRNAs are involved in the regulation of IGF-1R expression. In non-small cell lung cancer, miR-99a decreases the expression of IGF-1R resulting in inhibition of growth, migration and invasion (41). Furthermore, miR-143, miR-145, miR-320a, miR-452 and miR-503 have also been reported to be involved in the regulation of IGF-1R (42-45). The present study demonstrated that upregulation of miR-497 markedly reduced IGF-1R expression. In addition, the present study demonstrated that restoration of miR-497 expression significantly inhibited cell growth, migration and invasion. These results support the hypothesis that IGF-1R is a downstream mediator of miR-497 in OS.

IGF-1R is a transmembrane tyrosine kinase receptor that contains two extracellular α subunits with a ligand-binding site, two transmembrane β subunits, and intracellular tyrosine kinase activity (43). The receptor is activated by insulin-like growth factor (IGF)-1 and IGF-2 (46). IGF-1R has previously been demonstrated to have important roles in various biological processes in tumorigenesis and tumor progression, including malignant transformation, proliferation, apoptosis prevention, vascularization, migration, invasion and distant metastasis (47,48). Upregulation of IGF-1R was previously detected in various types of human cancer, including non-small cell lung cancer, breast cancer, prostate cancer and OS (49,50). In a previous study of OS, increased expression of IGF-1R was associated with poor prognosis, and downregulation of IGF-1R suppressed cell adhesion, motility and metastasis of OS cells, thus indicating that IGF-1R is important in the metastasis of OS (50). Therefore, it is important to investigate IGF-1R as a potential target for inhibition in OS. The results of the present study indicated that miR-497 decreases the proliferation, migration and invasion of OS cells by downregulating IGF-1R, thus suggesting that miR-497 should be investigated as a therapeutic target for the treatment of OS.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that miR-497 is downregulated in OS and contributes to cell proliferation, migration and invasion by negative regulation of IGF-1R. The identification of candidate miR-497 target genes may provide an understanding of potential carcinogenic mechanisms underlying OS. These findings have therapeutic implications and may be exploited for the treatment of OS.

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