

Original Article

mir-497 inhibits cell migration and invasion by targeting FASN in human osteosarcoma cells

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Abstract: Objective: This study is to investigate the expression of miR-497 and its roles in the tumorigenesis of osteosarcoma. Methods: The osteosarcoma and adjacent tissues were obtained from 30 cases of osteosarcoma. The expression of miR-497 and fatty acid synthase (FASN) mRNA were detected with real-time quantitative PCR. Expression of FASN was transfected with miR-497 mimics or miR-497 inhibitor, FASN mRNA and protein expression were detected using qRT-PCR and Western blot respectively. The regulatory effects of miR-497 on migration and invasion of U2OS osteosarcoma cells were evaluated using Transwell migration assay and Matrigel invasion assay respectively. Result: There were decreased expression of miR-497 and increased mRNA and protein expression of FASN in osteosarcoma tissues compared with that in adjacent tissues. Compared with normal control group, the cell numbers of migration and invasion, as well as FASN protein expression were significantly decreased in miR-497 mimic group; on the contrary, the cell numbers of migration and invasion and FASN protein expression were significantly increased in miR-497 inhibitor group. Conclusion: Our preliminary findings demonstrated that miR-497, functions as a tumor suppressor gene, inhibits cell migration and invasion by regulating FASN in human osteosarcoma cells. miR-497 could be considered as a potential therapeutic target and novel prognostic marker in osteosarcoma.

Keywords: Osteosarcoma, migration, invasion, MiR-497, FASN

Introduction

Osteosarcoma is the most common diagnosed malignant tumor of bone in children and adolescents, accounting for about 35% of malignancies of bone [1]. Osteosarcoma often exhibits high invasive and metastatic ability. Distant metastases, especially pulmonary metastasis, were detected in about 80%-90% of patients with the initial diagnosis of osteosarcoma [2]. Although we have made great progress in neoadjuvant chemotherapy and surgical treatment for osteosarcoma in the last decades, the prognosis of osteosarcoma with lung metastasis remains very poor [3]. The five-year survival rate is about 80% in patients with localized osteosarcoma but only 28%-33% in those with metastatic disease especially pulmonary metastasis [3]. Therefore, the investigation of the molecular mechanisms of osteosarcoma metastasis has been a hotspot in osteosarcoma research.

Increased expression of fatty acid synthase (FASN), a pivotal enzyme in production of fatty acids from dietary carbohydrates, has been reported in a wide range of malignancies [4]. In healthy subjects on a normal diet, the expression and activity of FASN is low and plays only a minor role in lipogenesis [5]. However, in patients with malignancies, FASN expression could not be inhibited by general regulatory mechanisms, which results in over-production of lipid materials and supplies for tumor cell proliferation [4-6]. In contrast, synthetic FASN inhibitors can effectively induces tumor cell apoptosis and inhibits tumor cell proliferation [7-9]. In recent years, the roles of FASN in tumor invasion and metastasis have attracted more and more attentions. Animal study suggested that FASN played key roles in proliferation and survival of melanoma cells while its inactivation significantly reduced the metastasis of tumor cells [10]; the liver metastasis of colorectal cancer were significantly suppressed

by treating with FASN inhibitor in mice model [11]; in head and neck squamous cell carcinomas, high expression of FASN was one of the most important features of lung metastasis [12]. These findings show that FASN overexpression may play an important role in tumor invasion and metastasis.

MicroRNAs (miRNAs, miR) are small noncoding RNAs with a length of 20-22 nucleotides (nt) and widely exist in eukaryotes [13]. miRNAs are involved in the regulation of a variety of biological processes and have important functions in cell development, differentiation, and metabolism [13]. Current studies have found that dysregulation of miRNAs is one of the important pathogenic factors in tumorigenesis and tumor metastasis [14, 15]. miR-497 is a potential tumor suppressor gene located on the miRNA cluster site at chromosome 17p13.1 [16]. Downregulation of miR-497 was found in many malignancies including primary peritoneal carcinoma, prostate cancer and breast cancer [16-18]. However, the association between the miR-497 and osteosarcoma especially patients with pulmonary metastasis are rarely studied. The purpose of this study was to examine the expression of miR-497 and FASN in osteosarcoma. Furthermore, in order to assess the effects of miR-497 on osteosarcoma cell migration and invasion, U2OS osteosarcoma cells were transfected with miR-497 mimics and miR-497 inhibitors to upregulate and downregulate the expression of miR-497 respectively.

Materials and methods

Specimen collection

The fresh specimens of tumor and adjacent tissues were obtained from 30 patients with osteosarcoma (17 males and 13 females; age 15-51 years, mean age 28 years) who had underwent surgery from January 2015 to August 2015 in the Department of Spine Surgery, the First People's Hospital of Jining. Before surgery, all patients were examined by X-ray and computed tomography (CT) for the tumor sites, lung and whole body bone scan; all patients did not receive any radiotherapy or chemotherapy. The tissues were quickly frozen in liquid nitrogen in 10 min after collecting. Prior written and informed consent were obtained from every patient and the study was

approved by the ethics review board of the First People's Hospital of Jining.

Reagents

TRIzol kit for RNA extraction was purchased from Invitrogen (CA, USA). U2OS cell line was purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). miR-497 mimics/inhibitors were purchased from GenePharma (Shanghai, China). Rabbit anti-human FASN polyclonal antibodies were purchased from Abcam (Boston, USA). Reverse Transcription System and SYBR® PrimeScript™ miRNA RT-PCR kit were purchased from Takara (Dalian, China). mRNA SYBR Green RT-PCR reagents were purchased from Kapa Biosystems (Boston, USA). Lipofectamine 2000 (Lipo2000) was purchased from Invitrogen (NY, USA).

Cell culture and transfection

U2OS cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). U2OS cells in logarithmic growth phase were seeded in 24-well plates at 2×10^5 /well and divided into miR-497 mimics group, miR-497 inhibitors group, negative control group (NC), and blank group (Blank). Cell transfection was conducted using liposome-mediated transfection methods. DMEM (125 μ l, serum-free medium) were added to EP tubes. A cocktail of Lipo2000 (7.5 μ l) with human hsa-miR-497 (7.5 μ l), hsa-miR-497 inhibitors (7.5 μ l) or negative control (7.5 μ l) was added to above-mentioned EP tubes. Then, the tubes were mixed and incubated for 15 min at room temperature. Finally, cells were added with the cocktails and transfected for 48 h in 6-well plates.

RNA extraction and reverse transcription

Frozen tissues (100 mg) were pulverized and homogenized in liquid nitrogen and lysed thoroughly by 1 ml TRIzol reagent. Then, the samples were digested by trypsin to harvest cells. The harvested cells were lysed again by TRIzol reagent (2×10^5 cells/ml). The total RNA was extracted using phenol chloroform methods. The gel electrophoresis and UV spectrophotometry were performed to measure the OD-260/280 ratio and detect the relative expression of RNA. Total RNA (1 μ g) was used for reverse transcription reaction. The mRNA was reverse transcribed into cDNA and the cDNA products were stored at -20°C . The miRNA was

reverse transcribed using Poly (A) tailing method. miRNA reverse transcription was carried out in a 20.0 µl reaction volume, including 6 µl RNA template, 10 µl miRNA Reaction Buffer Mix (2×), 2 µl 0.1% BSA, and 2 µl miRNA PrimeScript RT Enzyme Mix. To perform PolyA tailing reaction and reverse transcription reaction, the samples were incubated at 37°C for 60 min. Then, the samples were diluted by RNase Free H₂O to 100 µl, and 2 µl of it was taken for the subsequent quantitative detection.

Real-time quantitative PCR

The expression of miR-497 and FASN mRNA in osteosarcoma tissues and U2OS cells were detected using qRT-PCR (SYBR Green). U6 and GAPDH were used as internal control respectively. All detections were carried out in triplicate. For detection of miR-497, qRT-PCR was carried out in a 25 µl reaction volume, including 12.5 µl SYBR Premix Ex Taq, 1 µl PCR Forward Primer (miR-497 5'-GCAGCACACTGTGGT-TTGTA AAA-3'), 1 µl Uni-miR qPCR Primer, 2 µl template, and 8.5 µl ddH₂O. The qRT-PCR program was as follows: 30 s at 95°C for pre-denaturation, followed by 40 cycles of amplification: 5 s at 95°C, 20 s at 60°C. For detection of FASN mRNA, qRT-PCR was carried out in a 20 µl reaction volume including 10 µl SYBR EX Taq-Mix, 0.5 µl upstream primer (5'-AAGC-AGGCACACACGATGG-3'), 0.5 µl downstream primer (5'-TCGGAGTGAATCTGGGTTGATG-3'), 1 µl cDNA and 8 µl ddH₂O. The qRT-PCR program was as follows: 10 min at 95°C for pre-denaturation, followed by 40 cycles of amplification: 1 min at 95°C for denaturation, 40 s at 60°C for annealing, 30 s at 72°C for extension and 1 min at 72°C for a final extension. The relative expression of miR-497 and FASN mRNA was calculated as the ratio of miR-497/U6 and FASN/GAPDH respectively using 2^{-ΔΔCt} method.

Bioinformatics predictions of miRNA target

The binding site for miR-497 in 3'UTR of FASN mRNA was analysed and determined using TargetScan (www.targetscan.org) and miRanda (www.microrna.org).

Western blot

Homogenized tissues (50 mg) were lysed by pre-cooled RIPA lysis solution (600 µl, 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1%

SDS, 1% TritonX-100, 1% Sodium deoxycholate) for 50 min at 4°C and centrifuged at 12,000 g/min for 5 min for protein extraction. Cells were harvested from tissue specimen after trypsin digestion, and protein was extracted with RIPA lysis buffer. FASN protein concentrations in tissues and U2OS cells were measured using the BCA protein assay kit (Thermo Scientific, CA, USA). The proteins were analysed using 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. The membrane was then blocked with 50 g/L non-fat milk for 1 hour at room temperature followed by incubation overnight with primary antibodies (FASN 1:10000, GAPDH 1:2000) at 4°C. The membrane was rinsed three times with PBS containing 0.6% Triton-X 100 (PBST) and incubated with horseradish peroxidase (HRP)-labeled secondary antibody (goat anti-rabbit, 1:1000) for 1 hour at room temperature. The membrane was washed three times with PBST and followed by addition of Enhanced Chemiluminescence (ECL) reagent. Imaging signals of protein bands was acquired and analyzed using Image Lab Software (Bio-rad, USA). The relative expression of target protein was defined as the ratio of gray values between target protein and GAPDH.

Transwell migration assay and Matrigel invasion assay

Transwell assay was conducted to evaluate the migration ability of U2OS cells. The transfected U2OS cells were re-suspended in DMEM medium containing 0.1% BSA (5×10⁵ cells/ml). Cells (200 µl) were then seeded in the upper chambers of the transwell (Corning, NY, USA). The lower chambers were filled with DMEM medium containing 20% serum (750 µl). After 4 hours of incubation, the cells that migrated to the lower chamber were fixed in 100% methanol and stained by 0.1% crystal violet, followed by counting under microscope.

Matrigel assay was conducted to evaluate the invasive ability of U2OS cells. Serum-free DMEM medium (500 µl) was added to the growth-factor reduced Matrigel transwell chambers (BD Biosciences, CA, USA) and incubated at room temperature for 1 hour. Lower chambers of the transwell were filled with 750 µl of DMEM medium containing 20% serum. Cells (4×10⁵ cells/ml, 500 µl) were added to the Matrigel chambers. The transwell chambers were

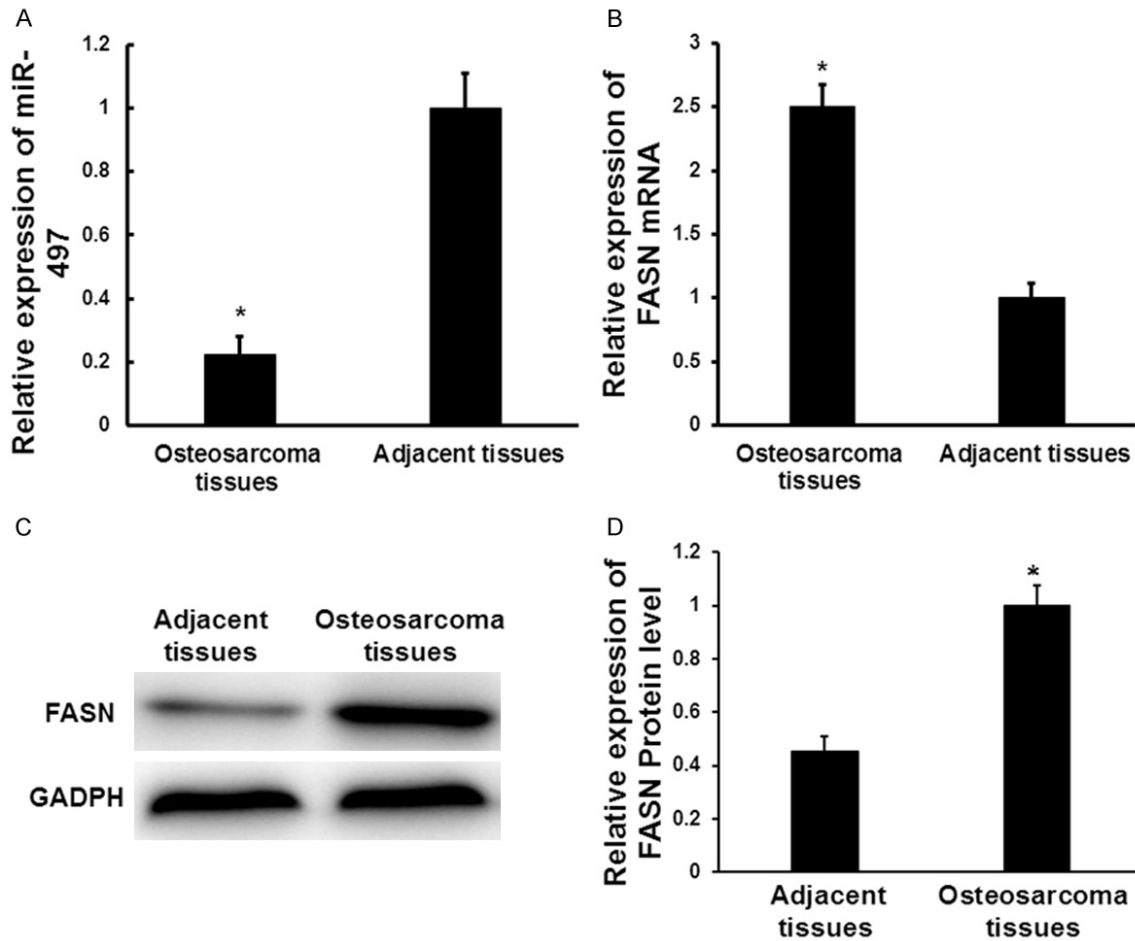


Figure 1. Analysis of miR-497 and FASN mRNA expression. The expression of miR-497 and FASN mRNA expression was detected with qRT-PCR analysis. A. The miR-497 expression in osteosarcoma tissues and adjacent tissues was measured. B. FASN mRNA expression in osteosarcoma tissues and adjacent tissues was measured. Compared with adjacent tissues, * $P < 0.05$. The expression of FASN protein was detected by Western blot. C. The protein bands of FASN and GADPH were showed. D. The quantitative comparison of FASN protein between different tissues was demonstrated. Compared with adjacent tissues, * $P < 0.05$.

incubated in a humidified 5% CO₂-containing atmosphere at 37°C for 18 hours. The non-invaded cells on the top of the transwell were scraped off with a cotton swab; the invaded cells on the transwell were visualized by staining with 0.1% crystal violet and counted under a light microscope.

Statistical analysis

All data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). The measurement data were expressed as mean ± SD. One-way analysis of variance was used to assess the differences between the multiple groups. An independent-sample t-test was used to compare the differences between two groups.

P value < 0.05 was considered statistically significant.

Results

Lower miR-497 and higher FASN mRNA expression in osteosarcoma

qRT-PCR was performed to detect the expression of miR-497 and FASN mRNA in osteosarcoma and adjacent tissues in osteosarcoma patients. The significantly downregulated miR-497 (**Figure 1A**) and upregulated FASN mRNA (**Figure 1B**) were detected in osteosarcoma tissues when compared to those in adjacent tissues. The differences were statistically significant (all $P < 0.05$). Western blot was conducted

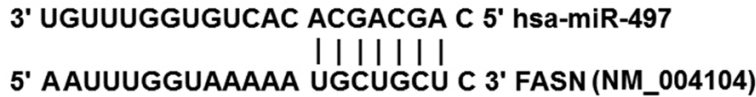


Figure 2. Bioinformatics predictions of target gene of miR-497. The complementary sequences between miR-497 and 3'UTR of FASN mRNA were described.

to determine FASN protein expression in osteosarcoma and adjacent tissues. Similarly, the results demonstrated that FASN protein expression in osteosarcoma tissues were significantly upregulated when compared to those in adjacent tissues ($P < 0.05$) (**Figure 1C** and **1D**). Predicted putative target of miR-497 was determined by target programs of TargetScans and miRanda. Results showed that miR-497 could bind to 3'UTR of FASN mRNA by binding to the complementary sequences in 3'UTR (**Figure 2**).

FASN expression in transfected U2OS cells

Cell transfection assay was conducted in U2OS cells to assess the regulatory effects of miR-497 on FASN expression. After cell transfection, qRT-PCR was performed to assess the transfection efficacies of miR-497 mimics and miR-497 inhibitors. The qRT-PCR results demonstrated that miR-497 expression was 70% higher in miR-497 mimic group than that in NC group and Blank group; on the contrary, miR-497 expression in miR-497 inhibitor group was 30% lower than that in NC group and Blank group (**Figure 3A**). Then, qRT-PCR and Western blot were used to detect the expression of FASN mRNA and protein. The results showed that, compared with NC group and Blank group, FASN mRNA (**Figure 3B**) and protein (**Figure 3C** and **3D**) were significantly downregulated in U2OS cells transfected with miR-497 mimics; on the contrary, FASN mRNA (**Figure 3B**) and protein (**Figure 3C** and **3D**) were significantly upregulated in U2OS cells transfected with miR-497 inhibitors. The differences were statistically significant (all $P < 0.05$). These findings revealed that FASN gene transcription and translation were inhibited by upregulated miR-497.

mir-497 inhibits U2OS cell migration and invasion

Furthermore, Transwell assay and Matrigel assay were conducted to evaluate the effects of

miR-497 on migration and invasion of U2OS cells. The results showed that, compared with NC group and Blank group, migration (**Figure 4A**) and invasion (**Figure 4B**) of U2OS cells were significantly reduced after overexpressing

of miR-497; on the contrary, migration (**Figure 4A**) and invasion (**Figure 4B**) of U2OS cells were significantly increased after inhibiting of miR-497. These findings suggested miR-497 may have the potential to inhibit U2OS cell migration and invasion.

Discussion

One of the major functions of miRNA is, as an oncogene or tumor suppressor gene, to negatively regulate post-transcriptional gene expression by binding to the 3'UTR of target mRNA [13, 19]. In our study, we have demonstrated the downregulation of miR-497 expression and upregulation of FASN expression in osteosarcoma. Previous study demonstrated miR-497 had regulatory effects on insulin-like growth factor 1 receptor (IGF1-R), thereby inhibiting the proliferation and inducing the apoptosis of colon cancer cells such as HCT28, LoVo or SW480 cells [20]. Furthermore, miR-497 had potential to induce apoptosis of cervical cancer cells by targeting IGF-1R gene expression [21]. In breast cancer, miR-497 inhibited the proliferation of tumor cells via targeting let-7c expression and forming a regulation network with other breast cancer-related miRNAs [22, 23]. However, miR-497 expression and its role in osteosarcoma were rarely described.

Our results demonstrated that, miR-497 expression was significantly downregulated in osteosarcoma tissues compared with that in adjacent tissues. Bioinformatics analysis revealed FASN was a potential target gene of miR-497. FASN gene has been shown to be closely related to the tumor metastasis in a variety of malignancies including melanoma [10], colorectal cancer [11], and head and neck squamous cell carcinoma [12]. Other studies also demonstrated that FASN is a direct target of many miRNAs (including miR-424, miR-195 and miR-320) in suppressing osteosarcoma cell proliferation, migration and invasion [24-26]. In our study, FASN mRNA and protein expression were significantly increased along with the decrease-

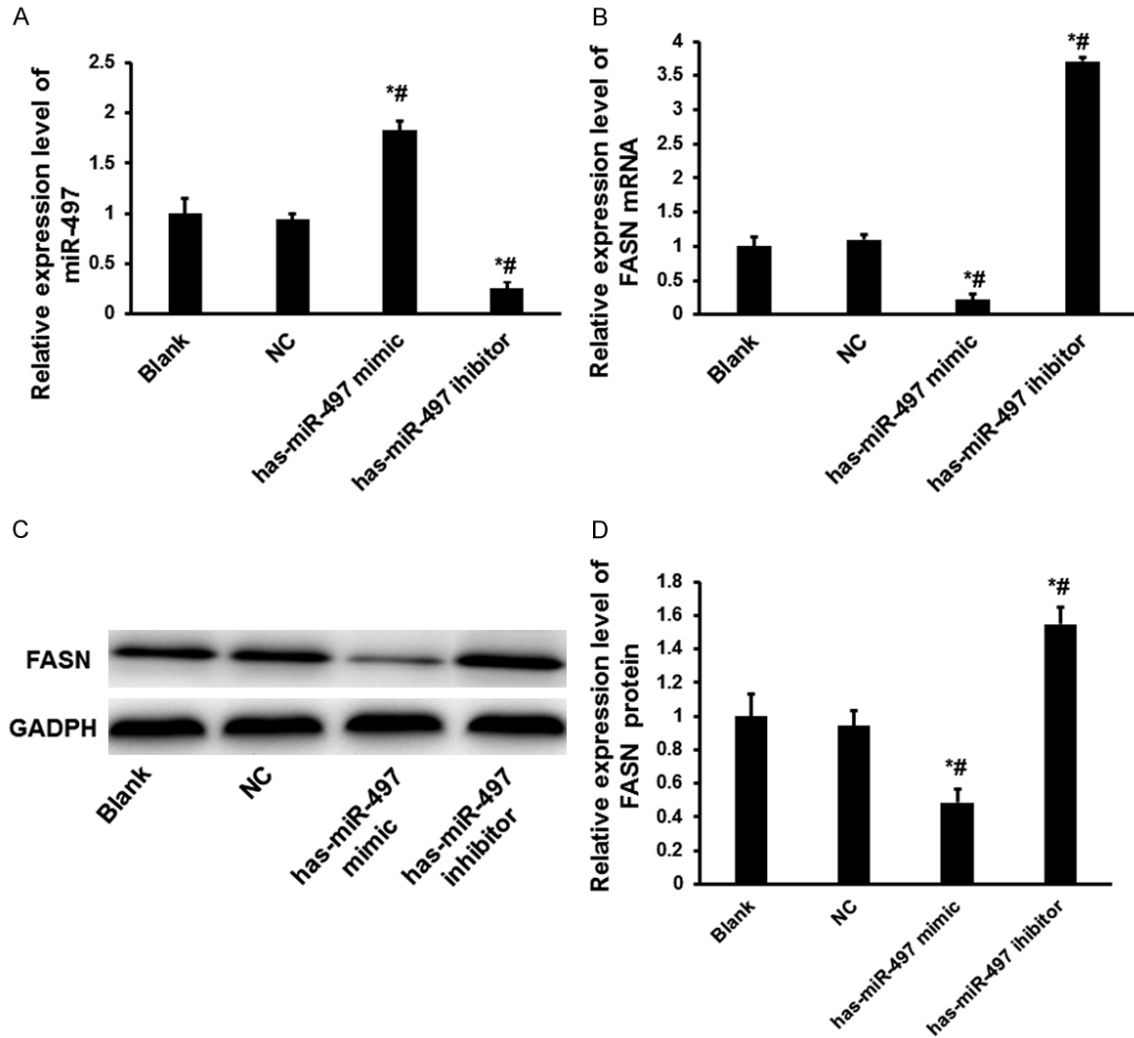


Figure 3. miR-497 and FASN expression in transfected U2OS osteosarcoma cells. Cells were transfected with miR-497 mimics or miR-497 inhibitors. Blank and negative control (NC) was set up. qRT-PCR and Western blot were used to measure miR-497, FASN mRNA and FASN protein expression respectively. A. miR-497 expression in U2OS cells after transfection. B. FASN mRNA expression in U2OS cells after transfection. C. The protein bands of FASN and GADPH were showed. D. The quantitative Western blot results of FASN protein was demonstrated. Compared with Blank, *P < 0.05; Compared with NC, #P < 0.05.

ed expression of miR-497, indicating the association between miR-497 and FASN expression.

Besides, U2OS cell transfection assay was conducted to evaluate miR-497 regulatory effects on FASN expression and tumor cell migration and invasion. The qRT-PCR and Western blot demonstrated that both of FASN protein and mRNA were significantly downregulated in U2OS cells transfected with miR-497 mimics and upregulated in cells transfected with miR-497 inhibitors. Transwell assays showed that migration and invasion of U2OS cells were signifi-

cantly reduced after overexpressing of miR-497 and were significantly promoted after inhibiting of miR-497, indicating inhibitory effects of miR-497 on U2OS cell migration and invasion. In previous studies, a variety of potential target genes of miR-497 has been described in cancers [20, 27-29]. For example, miR-497 modulated ethanol-induced neuronal cell death in neuroblastoma and multidrug resistance of human cancer cell lines by targeting BCL2 [28, 29], promoted tumor growth and angiogenesis by targeting HDGF in non-small cell lung cancer [27], and suppressed proliferation of tumor cells by targeting IGF-1R in human co-

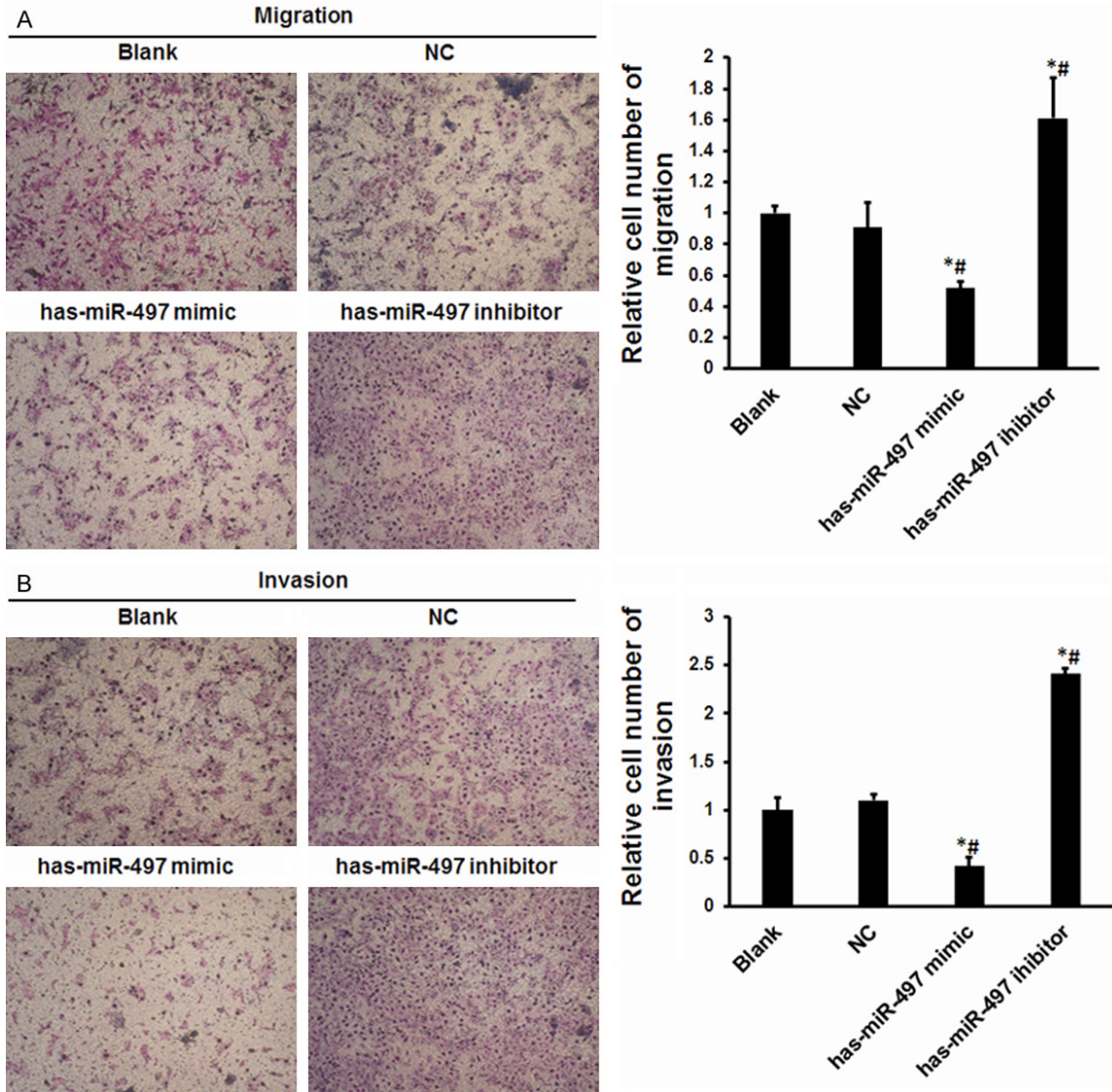


Figure 4. The effects of miR-497 on migration and invasion of U2OS osteosarcoma cells. Cells were transfected with miR-497 mimics or miR-497 inhibitors. Blank and negative control (NC) was set up. A. Cell migration was analyzed by Transwell assay. B. Cell invasion was analyzed by Matrigel assay. Compared with Blank, *P < 0.05; Compared with NC, #P < 0.05.

lorectal cancer [20]. These studies demonstrated the pivotal roles of miR-497 in tumorigenesis, invasion, and therapy of malignancies.

In summary, our preliminary study revealed a negative correlation between miR-497 expression and osteosarcoma cell migration and invasion. miR-497, which functions as a tumor suppressor gene, may play an important role in the invasion and metastasis through targeting FASN gene expression. These findings may provide new insights for research in osteosarcoma-associated miRNAs. Thus, further functional experiments are needed to illustrate the

mechanism of the effect of miR-497 on osteosarcoma cell migration and invasion.

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Disclosure of conflict of interest

None.

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