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Original Article

MicroRNA-494 inhibits proliferation and metastasis of osteosarcoma through repressing insulin receptor substrate-1

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Abstract: Despite microRNA-494 (miR-494) has a well-established role in many types of cancer; the biological function and potential mechanism of miR-494 in human osteosarcoma (OS) has not been elucidated. The aim of this study was therefore to investigate the role and underlying mechanism of miR-494 expression in osteosarcoma. Here, we found that miR-494 was significantly decreased in OS tissues and cell lines compared to the adjacent noncancerous bone tissues (P<0.01) and human normal osteoblast cells (NHOst) (P<0.05), respectively. Functional assays demonstrated that ectopic overexpression of miR-494 could significantly inhibit cell proliferation, colony formation, migration and invasion in vitro, as well as suppress tumor growth in nude mice model. Further integrative and functional studies suggested insulin receptor substrate 1 (IRS1) as a target gene of miR-494 in OS cells. IRS1 expression was upregulated, and inversely correlated with miR-494 expression in clinical OS tissues (r=-0.589, P=0.001). Moreover, downregulation of IRS1 had similar the inhibition effect on cell proliferation, colony formation, migration and invasion of miR-494 overexpression. Overexpresion of miR-494 obviously decreased AKT signal pathway activation. These findings suggested that miR-494 functioned as a tumor suppressor in OS, at least in part, by targeting IRS1.

Keywords: miR-494, osteosarcoma, proliferation, metastasis

Introduction

Osteosarcoma is the most common primary bone malignancy in young adults and adolescents, with high morbidity [1]. Despite the improvement of currently available treatment strategies, including wide tumor excision, adjuvant chemotherapy and radiotherapy, has significantly improved the prognosis of patients with malignancy OS [2], for patients who present with metastatic disease or whose tumor recurs, the survival of five years was less than 30% [3, 4]. Therefore, there is an urgent need to understand the molecular mechanisms that involved in development and metastasis of OS, which may contribute to finding novel diagnosis marker and novel therapeutic targets for this disease.

MicroRNAs (miRNA) are small endogenous noncoding RNAs composed of about 19-25 nucleotides that negatively regulate mRNA stability and/or repress mRNA translation by binding to the 3'-untranslated region (UTR) of their target mRNAs [5]. miRNAs have been proven to play crucial roles in many biological progresses, including cell proliferation, differentiation and death [6]. Accumulating evidence suggested that miRNAs functioned as oncogene or tumor suppressor by regulating initiation and progression of cancer, including differentiation, apoptosis, proliferation, cell cycle, and metastasis [7, 8]. To date, a larger number of miRNAs has been identified to involve in OS initiation and development, and act as potent therapeutic targets or diagnosis marker for OS [9, 10].

MiR-494, located on chromosome 14q32.31, has been widely reported to play crucial roles in initiation and development in a variety of cancers including colorectal cancer [11], breast cancer [12], hepatocellular carcinoma [13],

ovarian cancer [14], prostate cancer [15], and non-small lung cancer [16] etc. However, whether miR-494 is involved in OS progression remains unclear. In this study, we investigate the biological function and underlying mechanism of miR-494 in osteosarcoma by *in vitro* and *in vivo* study. Here, we show that miR-494 was downregulated in OS tissues and cell lines, and that restoration of miR-494 expression in OS cells inhibited cell proliferation, colony formation, migration and invasion *in vitro*, and suppressed tumor growth *in vivo* by direct targeting of IRS1. Through both *in vitro* and *in vivo* analyses, we revealed the mechanisms of miR-494 downregulation and its tumor suppressive role in OS pathogenesis.

Materials and methods

Clinical samples

The collection and the use of all tissue samples in the present study were approved by the Research Ethics Committee of the First affiliate hospital of Liaoning medical university (Jinzhou, China). Written informed consent was obtained from all the patients. A total of 30 OS tissues and self-matched noncancerous bone tissues collected from 30 patients with osteosarcoma were retrospectively enrolled according to the surgical pathology records of the First affiliate hospital of Liaoning medical university, from September 2013 to September 2015. All OS tissues were confirmed pathologically from the specimens obtained from surgery. Those patients who received chemo- or radio-therapy, or other therapy were excluded from this study. All tissues were snap-frozen in liquid nitrogen following surgery, and stored at -80°C until use for further analysis.

Cell culture and transfection

The osteosarcoma cell lines U2OS, MG-63, and SAOS-2 and human osteoblastic cell line hFOB were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and maintained in Dulbecco's Modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified 5% CO_2 atmosphere.

miR-494 mimic and negative control (NC) were purchased from GenePharma (Shanghai, China). siRNA against IRS1 (si-IRS1) and siRNA against negative control (si-NC) RiboBio (Guangzhou, China). Transfection was carried out using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For miR-494 detection, RT-qPCR assays were performed using the TaqMan miRNA Assay (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. The expression level of miR-494 was normalized to the expression level of U6. Primers for miR-494 and U6 in this study were obtained from Applied Biosystems (Foster City, USA). For the detection of IRS1 mRNA level, 0.5 μg of total RNA was reverse transcribed into cDNA using PrimeScript RT reagent Kit (Takara, Dalian, China) following the manufacturer's instructions, followed by quantitative PCR (qPCR) with SYBR Premix Ex Taq (Takara) under 7900 Real-Time PCR System (Applied Biosystems). IRS1 mRNA level was normalized to the β-actin mRNA level. Primers for IRS1 and β-actin were used in this study as previous described [17]. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation and colony formation assays

Cell proliferation was assessed by MTT assay. In brief, the cells were seeded in 6-well plates at the density of 2×10^4 cells per well at 24 h post-transfection. At the indicated time (24 h, 48 h and 72 h), 20 μl MTT reagent (5 mg/ml) was added to each well and further incubated at 37°C for 4 h. Then the culture medium was removed from each well, and 150 μl dimethyl sulfoxide (DMSO, Sigma) was added to each well to dissolve the MTT-formazan crystals for 10 min at 37°C. The absorbance of each well at 570 nm was determined using a microplate reader (Thermo Fisher Scientific, Pittsburgh, PA, USA).

For colony formation assay, 1,000 transfected cells/well were seeded in 6-well plate and cultured for 2 weeks to allow colony formation. Then colonies were washed three times with PBS, and fixed with 4% paraformaldehyde for

20 min and stained with 1% crystal violet (Sigma). Images were captured digitally and colonies were counted under a light microscope (Olympus, Tokyo, Japan).

Cell migration and invasion assay

The migration and invasion assays were performed using Transwell insert chambers (8-μm pore size, Corning Costar Corp, USA). For migration assay, 1×10^5 transfected cells were seeded into upper transwell chamber in free-serum medium. 600 μl DMEM medium containing 10% FBS was added to the lower chamber. After incubating for 24 h at 37°C with 5% CO_2, cells migrated to the lower surface of filter were fixed in 70% ethanol for 30 min and stained with 2% crystal violet for 10 min. Invasion assays were applied similarly with coating the upper chamber with Matrigel. The number of migration or invasion cells was imaged and counted in five randomly selected fields under microscope (Olympus).

Luciferase assays

The 3'-untranslated regions (3'-UTRs) of the IRS1 gene containing predicted miR-494 target sites were amplified by PCR, and were inserted into downstream of the firefly luciferase coding region into the NheI and XhoI restriction sites of the pGL3-control plasmid (Promega Corporation, Madison, WI, USA), named as Wt-IRS1. Mutant of IRS1 3'-UTR was constructed from wild-type 3'-UTR of IRS1 using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol, refereed as Mut-IRS1. All constructs were verified by sequencing.

For luciferase assays, The U2OS cells were plated in 24-well plates at the density of 5×10^5/well, and were cotransfected with 100 ng Wt-IRS1 or Mut-IRS1 luciferase reporter plasmid, and 100 nM of miR-494 mimic or miR-NC, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were harvested at 48 h post-transfection, and firefly luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA), and normalized to Renilla signals.

Western blot assay

Total proteins were extracted from cultured cells by using lysis buffer (0.2% SDS, 0.2% sodium deoxycholate, 1% Triton X-100, 1 mmol/l EDTA and 50 mmol/l Tris-HCl at pH 7.4). The protein concentration was quantified by BCA kit (Beyotime Institute of Biotechnology, Guangzhou, China). Equal amounts of protein (30 ug) was separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then electrotransferred to nitrocellulose membranes (Bio-Rad, Munich, Germany). The membranes were blocked in 5% non-fat dry milk diluted with Tri-buffered saline Tween-20 (TBST) (in mmol/l: Tris-HCl 20, NaCl 150, pH 7.5, 0.1% Tween-20) at room temperature for 2 h and incubated overnight at 4°C with primary antibody against IRS1, Akt, p-Akt (Ser473) and β-actin (all from Santa Cruz Biotechnology Inc., California, USA). After the membrane was washed three times with TBST, was further probed with horseradish peroxidase (HRP)-conjugated second antibody (Santa Cruz, USA) for 2 h at room temperature. The protein bland was detected with using Supersignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL, USA).

Tumor growth assay in mice

Male BALB/c nude mice (4-6 weeks old) were brought from Experiment Animal Center of Liaoning Medical University (Jinzhou, China), and maintained under specific pathogen-free (SPF) conditions. Animal studies were performed according to Institutional Animal Care and Use Committee guidelines of Liaoning Medical University. U2OS cells (2×10^6) stably expressing miR-494 or miR-NC were harvested by trypsin, washed with PBS, and resuspended in Matrigel: DMEM medium (1:1), and were s.c. injected into the flanks of nude mice (n=10). Tumor volume was measured every week, and was calculated from the length (a) and the width (b) by using the following formula: Volume $=ab^2/2$. Five weeks after inoculation, the mice were killed, and tumor tissues were striped and weighted.

Statistical analysis

Data from each group were expressed as mean ± standard error of the mean (mean ± SEM) and statistically analyzed by Student's t-test. Pearson correlation analysis was conducted to determine the correlation between expression of miR-494 and IRS1. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences were

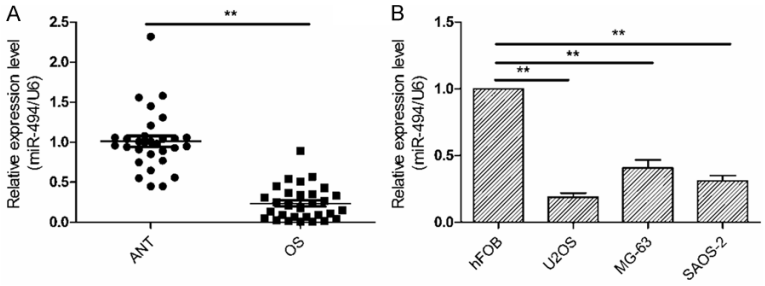

Figure 1. miR-494 was downregulated in osteosarcoma tissues and cell lines. A. The expression level of miR-494 in 30 OS tissues and corresponding adjacent noncancerous tissues (ANT) were detected by RT-qPCR. ***P*<0.01 compared to ANT. B. The levels of miR-494 in three cell lines U2OS, MG-63, and SAOS-2 and human osteoblastic cell line hFOB were detected by RT-qPCR. **P*<0.05, ***P*<0.01 compared to hFOB.

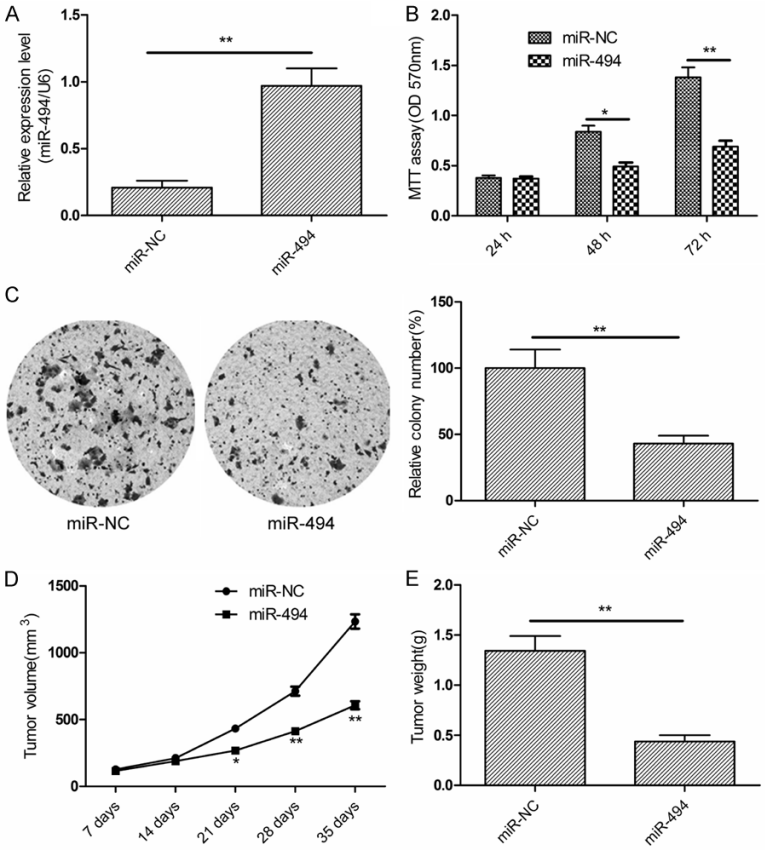

Figure 2. miR-494 suppresses osteosarcoma growth *in vitro* and *in vivo*. (A) The expression level of miR-494 was detected in U2OS cells transfected with miR-494 mimic or miR-NC by RT-qPCR. (B, C) Cell proliferation (B) and colony formation were determined in U2OS cells transfected with miR-494 mimic or miR-NC. (D) Tumor growth curves were established by measurement tumor volume every week until five weeks. (E) Tumor weights of different group. **P*<0.05, ***P*<0.01 compared to miR-NC.

considered statistically significant at a *P*-value of <0.05.

Results

miR-494 was downregulated in osteosarcoma tissues and cell lines

To explore the role played by miR-494 in osteosarcoma development, miR-494 expression levels in 30 paired OS tissues and adjacent noncancerous bone tissues (ANT) were measured. The result of RT-qPCR demonstrated that miR-494 was significantly downregulated in OS tissues compared with adjacent noncancerous bone tissues (**Figure 1A**). We also examined miR-494 expression in the normal human osteoblastic cell line hFOB and three human osteosarcoma cell lines U2OS, MG-63, and SAOS-2 by RT-qPCR. As shown in **Figure 1B**, miR-494 expression was significantly decreased in the osteosarcoma cell lines compared to normal human osteoblastic cell line. These data suggested that miR-494 might play a role in human osteosarcoma development.

miR-494 suppresses osteosarcoma growth in vitro and in vivo

To explore the effect of miR-494 on osteosarcoma growth in vitro, miR-494 was overexpressed using a miR-494 mimic in U2OS cells, which express low levels of endogenous miR-494 (**Figure 1B**). miR-494 overexpression in transfected U2OS cells was confirmed by RT-qPCR (**Figure 2A**). Next, cell proliferation and colony formation were measured using the MTT assay and colony forming assay, respectively. Overexpression of miR-494 in U2OS cells significantly inhibited proliferation (**Figure 2B**) and colony formation (**Figure 2C**).

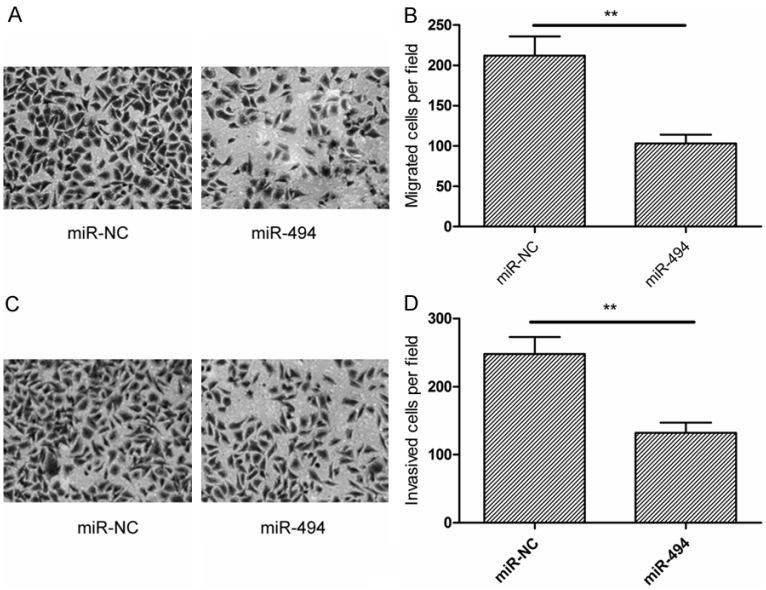

Figure 3. miR-494 suppresses osteosarcoma cell migration and invasion. A, B. Representative images of and quantitative data on the migration of U2OS cells transfected with miR-494 mimic or miR-NC. C, D. Representative images of and quantitative data on the invasion of U2OS cells transfected with miR-494 mimic or miR-NC. *P<0.05, **P<0.01 compared to miR-NC.

To investigate the effects of miR-494 on tumor growth in vivo, U2OS cells stably expressing miR-494 or miR-NC were s.c. injected into the flanks of nude mice, tumor volume was measured every weeks until five weeks. It was found that tumor growth was slower in U2OS/miR-494 group than that of U2OS/miR-NC group (**Figure 2D**). Five weeks after inoculation, the mice were killed, and tumor tissues were striped and weighted, and found that average tumor weight was also markedly reduced in U2OS/miR-494 compared with U2OS/miR-NC group (**Figure 2E**). These data suggested that miR-494 inhibited OS growth *in vitro* and *in vivo*.

miR-494 suppresses osteosarcoma cell migration and invasion

To explore the effect of miR-494 on osteosarcoma metastasis in vitro, cell migration and invasion were determined in U2OS cells transfected with miR-494 mimic or miR-NC by transwell chamber assay. It was found that overexpression of miR-494 induced a significant decrease in migration (**Figure 3A** and **3B**) and invasion (**Figure 3C** and **3D**) abilities of U2OS cells. These data suggested that miR-494 inhibited OS metastasis.

IRS1 is a direct target of miR-494 in osteosarcoma cells

To investigate the underlying mechanism the growth inhibition by miR-494 in OS, we used two publicly available algorithms (Targetscan 6.2 and miRanda) to identify target gene of miR-494. miR-494 was predicted to bind to one site within the 3'-UTR of IRS1 mRNA at position (4748-4754 bp) (**Figure 4A**). To verify whether IRS1 direct target of miR-494, we cotransfected Wt/Mut-IRS1 report plasmid and miR-494 mimic or miR-NC in U2OS cells, then luciferase assay was performed. U2OS cells transfected with miR-494 mimic decrease wild-type IRS1-3'UTR reporter activity (P<0.01), while had no inhibition effect on the mutant IRS1-3'UTR reporter activity in U2OS cells (**Figure 4B**), indicting the direct regulation of miR-494 in the 3'UTR of IRS1 mRNA. Next, we examined the effect of miR-494 on IRS1 expression on mRNA level and proteins level by RT-qPCR and western blot, respectively. We found that overexpression of miR-494 markedly reduced mRNA and protein expressions of IRS1 in U2OS cells (**Figure 4C** and **4D**). In addition, IRS1 mRNA expression level was measured in OS specimens and adjacent noncancerous tissues. qRT-PCR analysis showed significantly higher mRNA levels of IRS1 in OS tissues, compared with adjacent noncancerous tissues (**Figure 4E**). Pearson's correlation analysis disclosed an inverse correlation between miR-494 expression and IRS1 expression (r=-0.589, P=0.001) (**Figure 4F**).

Inhibition of IRS1 performed similar effect with miR-494 overexpression in osteosarcoma cells

To investigate the roles played by IRS1 in the miR-449a inhibition effect in OS cells, we explored whether knockdown of endogenous IRS1 mimicked the effect of miR-494 overexpression in OS cells. We found transfection of si-IRS1 significantly suppressed IRS1 expression both on mRNA level and protein level in

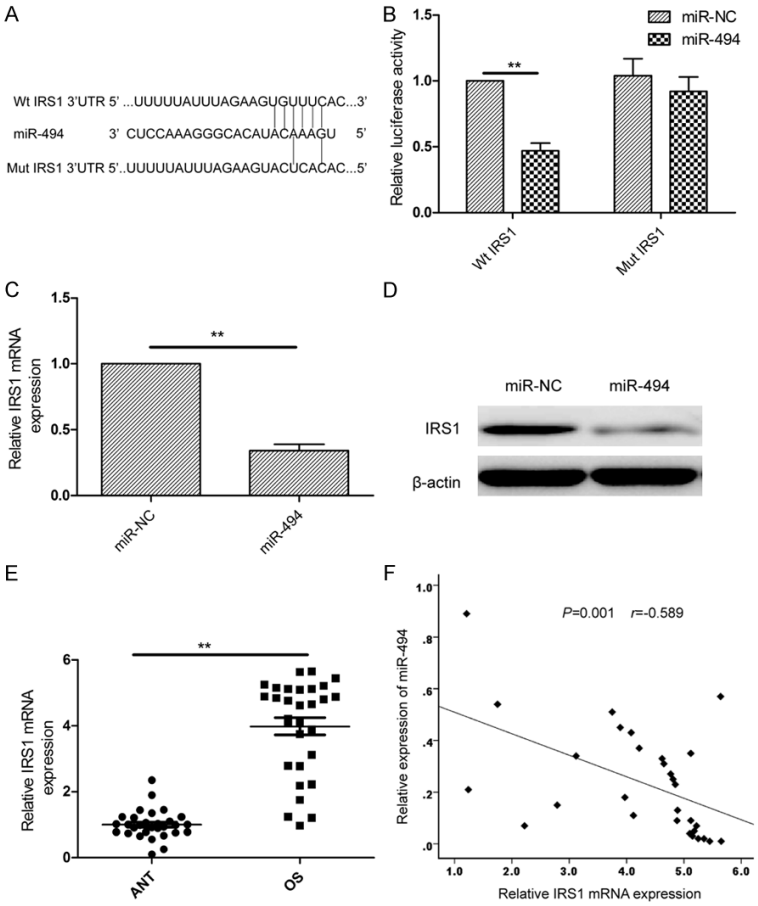

Figure 4. IRS1 is a direct target of miR-494 in osteosarcoma cells. (A) Putative binding sites of miR-494 within the 3'-UTR region of IRS1 mRNA, and the sequences of wild-type and mutant-type vector were shown. Wt: wild-type; Mut: mutant-type. (B) Luciferase reporter assays were measured in U2OS cells co-transfected with Wt or Mut 3'UTR of IRS1 and miR-494 or miR-NC. WT: wide-type, Mut: Mutant-type. (C, D) IRS1 expression on mRNA level (C) and protein level (D) were detected in U2OS cells transfected with miR-494 mimic or miR-NC. β-actin was used as internal control. *P<0.05, **P<0.01 compared to miR-NC. (E) IRS1 expression on mRNA level was detected in 50 OS tissues and corresponding adjacent noncancerous tissues (ANT) were detected by RT-qPCR. β-actin was used as internal control. **P<0.01 compared to ANT. (F) Perason's correlation analysis between miR-494 expression and IRS1 mRNA level (r=-0.589, P=0.001).

U2OS cells (**Figure 5A** and **5B**). In addition, we also showed that downregulation of IRS1 by transfection of si-IRS1 in U2OS cells significantly inhibited cell proliferation, colony formation, migration and invasion (**Figure 5C-F**), suggesting that the tumor suppressor functions of miR-494 in OS cells may be exerted via repressing IRS1.

miR-494 targets IRS1 in osteosarcoma cells via inhibiting activation AKT signaling pathway

IRS1, a key mediator in oncogenic insulin-like growth factor (IGF) signaling, has been reported to play crucial roles in the growth, cell cycle, metastasis and apoptosis of human tumors by activating AKT signaling pathways [18, 19]. Therefore, we hypothesized that by targeting IRS1, miR-494 might affect the AKT signaling pathway in osteosarcoma cells. IRS1, AKT and pAKT protein levels were determined in U2OS cells after transfection with the miR-494 mimic or si-IRS1. The results showed that overexpression of miR-494 in U2OS cells obviously decreased IRS1 and p-AKT protein expression, but the total AKT levels did not significantly change (**Figure 6**). Similarly, downregulation of IRS1 by transfection of si-IRS1 in U2OS cells reduced IRS1 and p-AKT protein expression, without effect total AKT expression (**Figure 6**). These results indicate that miR-494 exerts tumor suppressor role in OS cells by repressing IRS1, and thus indirectly regulating the AKT signaling pathway.

Discussion

Accumulated evidence has indicated that aberrant expression of miRNAs contributes to the pathogenesis of various human malignancies, including osteosarcoma [9, 10]. A number of miRNAs has been reported to play key roles in OS procession by regulating target genes. For example, Zhao *et al* reported that miR-154 significantly inhibit cell proliferation, colony formation, migration and invasion, as well as induced cell arrest at G1 stage by targeting Wnt5a [20]. Qu *et al* found that miR-150 significantly inhibit OS cell proliferation, migration and invasion, and induced apoptosis *in vitro*, as well as suppressed tumor growth of OS *in vivo* by regulating IGF2 mRNA-binding protein 1 (IGF2BP1) [21]. Chen *et al* showed that miR-133a suppresses osteosarcoma progression and metastasis by targeting IGF1R in human

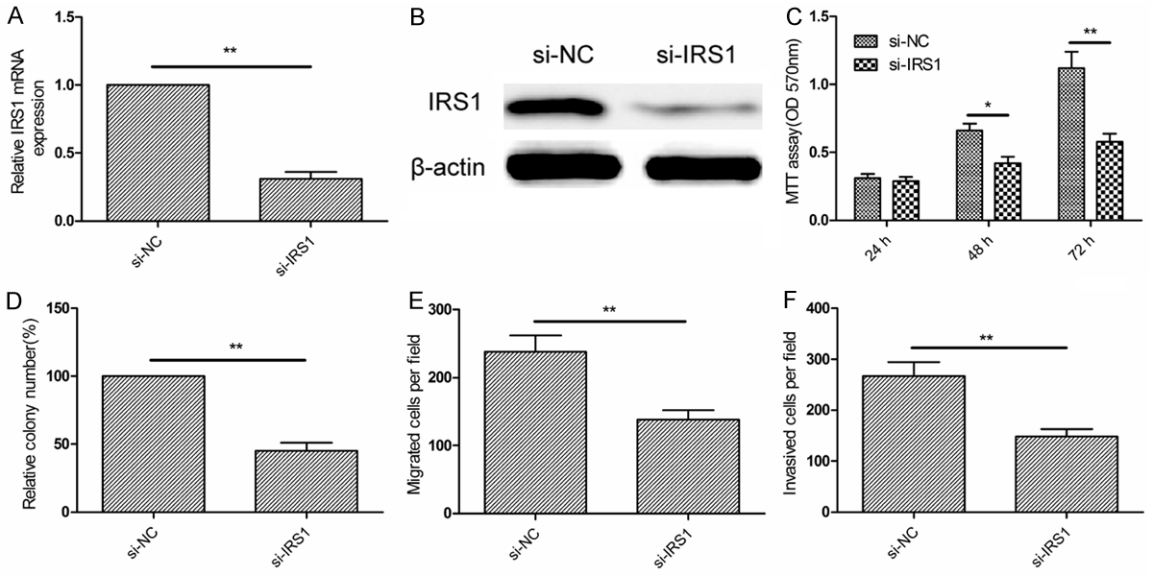

Figure 5. Inhibition of IRS1 performed similar effect with miR-494 overexpression in osteosarcoma cells. (A, B) IRS1 expression on mRNA level (A) and protein level (B) were detected in U2OS cells transfected with si-IRS1 or si-NC. β-actin was used as internal control. (C-F) Cell proliferation (C), colony formation (D), migration (E), and invasion (F) were determined in U2OS cells transfected with si-IRS1 or si-NC. *P<0.05, **P<0.01 compared to si-NC.

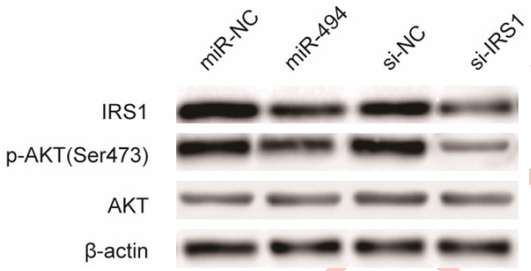

Figure 6. miR-494 targets IRS1 in osteosarcoma cells by regulating the AKT signaling pathway. Western blot analysis of p-AKT, AKT and IRS1 protein levels in U2OS cells transfected with a miR-494 mimic/miR-NC or si-IRS1/si-NC.

osteosarcoma cells [22]. Herein, we found that miR-494 expression is down-regulated in osteosarcoma cells and tissues compared with osteoblastic cell and paired adjacent noncancerous bone tissues, respectively. Moreover, we found that overexpression of miR-494 suppressed osteosarcoma cell proliferation, colony formation, migration and invasion in osteosarcoma cells U2OS. Furthermore, we also identified IRS1 as a direct target of miR-494. Our findings suggest that miR-494 may be a novel potential therapeutic target for OS treatment.

Growing evidence has indicated that miR-494 has dual biological functions: either as tumor suppressor or oncogene dependent on the specific cancer tissue type. It has been reported that miR-494 was up-regulated in several solid tumors, including hepatocellular carcinoma [13], acute myeloblastic leukemia [23], bronchial carcinogenesis [24], retinoblastoma [25], and colorectal cancer [11], plays an oncogenic role by targeting MCC, PTEN. On the contrary, miR-494 has been reported to be downregulate in a number of solid tumors, including pancreatic cancer [26], prostate cancer [15], non-small-cell lung cancer [16], breast cancer [12], ovarian cancer [14], and cholangiocarcinoma [27], and exerts a tumor-suppressive role by targeting IGF1R, Sirt1, C-myc, CXCR4, Bim etc. However, the biological role and underlying mechanism of miR-494 expression in osteosarcoma cells remain unclear. In this study, we identified that miR-494 is an tumor suppressor miRNA in OS cells inhibiting OS cell proliferation, colony formation, migration and invasion by direct targeting of IRS1, suggesting a crucial role of miR-494 in OS progression and metastasis.

It was well known that miRNAs exerted biological activities by suppressing their target genes [7]. To explore the molecular mechanism by which miR-494 suppressed osteosarcoma cell growth, metastasis, we used TargetScan and

miRanda algorithm to search for targets of miR-494. IRS1 was selected as the potential target for further validation since it has been showed to involve in initiation and development of various cancers [28]. IRS1, a key mediator in oncogenic insulin-like growth factor (IGF) signaling, has been showed to be upregulate in various cancers, including osteosarcoma [29]. Recently a reporter showed that osteosarcoma cells over-expressing IRS1 have increased metastatic potential and tumor growth *in vivo* [30]. In addition, it has been reported that abnormal IRS1 could affect several downstream pathways, including AKT signaling pathway [18, 19], which is involved in tumorigenesis and development of osteosarcoma [31, 32]. In the present study, luciferase reporter assays showed that miR-494 overexpression led to a significant decrease in luciferase reporter activities of cells expressing IRS1 with wild-type but not mutant 3'-UTRs. Both mRNA and protein levels of IRS1 was significantly downregulated in miR-494-expressing U2OS cells. IRS1 was also found to be upregulated in osteosarcoma tissues, and be negatively correlated with miR-494 in OS tissues. These results further confirmed that IRS1 was a target of miR-494 in osteosarcoma cells. In addition, downregulation of IRS1 using si-IRS1 inhibited the proliferation, colony formation, migration and invasion of osteosarcoma cells, which had similar effects with miR-494 overexpression. Of note, we found that miR-494 overexpression or downregulation of IRS1 could inhibit activation AKT signaling pathway. These results indicate that miR-494 may function as a tumor suppressor partly mediated by repressing IRS1 expression and regulating AKT signal pathway in osteosarcoma development.

In summary, to our knowledge, the present study first showed that miR-494 was dramatically downregulated in osteosarcoma tissues and cell lines, and that miR-494 overexpression significantly inhibited osteosarcoma cell proliferation, colony formation, migration and invasion *in vitro*, as well as suppressed tumor growth *in vivo* through directly targeting IRS1 and indirectly regulating the AKT signaling pathway. This novel miR-494/IRS1 axis might provide novel insights into the molecular mechanisms underlying progression and metastasis of osteosarcoma.

Acknowledgements

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Disclosure of conflict of interest

None.

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