Original Article
microRNA-486 suppressed proliferation, migration and invasion of osteosarcoma cells via directly targeting SIRT1

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Abstract: Significant amount of studies demonstrated that the abnormal expression of microRNAs were involved in the carcinogenesis and progression of various kinds of human cancers, also including osteosarcoma. Therefore, understanding the physiological and molecular mechanisms of miRNAs in osteosarcoma may provide novel therapeutic strategies for treatments of patients with osteosarcoma. In this study, we investigated the expression, biological roles of microRNA-486 (miR-486) on osteosarcoma and its underlying mechanism. The results showed that miR-486 was significantly down-regulated in osteosarcoma tissues and osteosarcoma cell lines. In addition, over-expression of miR-486 inhibited cell proliferation, migration and invasion of osteosarcoma. Moreover, SIRT1 was identified as a direct target gene of miR-486 in osteosarcoma. SIRT1 knockdown could mimics the functions of miR-486 over-expression in osteosarcoma cells. In conclusion, the data of the present study provides compelling evidences that miR-486 acted as a tumor suppressor in osteosarcoma through targeting SIRT1 and could serve as a novel therapeutic target.

Keywords: microRNA-486, SIRT1, proliferation, migration, invasion, osteosarcoma

Introduction
Osteosarcoma (OS), the most common form of primary bone tumor in children and adolescents, accounts for approximately 19% of all malignant bone tumors and 5% of all childhood tumors [1, 2]. It occurs mainly around regions with active bone growth and repairation [3]. A great deal of studies demonstrated that OS is caused by genetic and epigenetic changes, and environmental factors which block mesenchymal stem cells differentiating into osteoblast [4]. Currently, the effective standard treatments for patients with OS include surgery, radiotherapy and chemotherapy [5, 6]. Due to progress of therapeutic treatments for OS, the 5-year survival rate of the patients without metastasis has improved to approximately 60%-70% [7]. However, for OS patients with metastasis or recurrence, the overall clinical outcomes remain poor with a 5-year survival rate of 5%-20%. This predominantly mainly results from the low efficacy of standard therapeutic strategies [8]. Therefore, a better understanding of the mechanisms underlying carcinogenesis and progression of OS is important for exploring novel therapeutic strategies for this disease.

microRNAs (miRNAs) are a large family of non-protein-coding, single-stranded and short RNA molecules with 19-25 nucleotides in length [9]. They negatively regulate the expression of multiple target genes through binding to their 3'UTR in a base-pairing manner and thereby causing translational repression and/or mRNAs degradation [10, 11]. Numerous studies showed that miRNAs play significant roles in a wide range of biological processes, such as cell proliferation, differentiation, apoptosis, cycle, metastasis, and metabolism [12, 13]. Abnormal expression of miRNAs has been reported in many different types of human cancers, such as bladder cancer [14], breast cancer [15], gastric cancer [16],...
These dysregulated miRNAs may act as either oncogenes or tumor suppressors in carcinogenesis and progression of human cancers [17]. Downregulated miRNAs in cancer may normally function as tumor suppressor genes and inhibit cancer by regulating oncogenes. On the contrary, upregulated miRNAs in cancer may function as oncogenes by negatively regulating tumor suppressors [18-20]. Taken together, miRNAs could be investigated as anti-cancer therapies as a result of its close association with tumorigenesis and tumor progression.

In this study, we showed that the expression levels of miR-486 were reduced in OS tissues and cell lines. In addition, restoration of miR-486 significantly inhibited OS cells proliferation, migration, and invasion in vitro. Furthermore, computational prediction, luciferase report assay, qRT-PCR and western blot analysis demonstrated that the tumor suppressive roles of miR-486 on OS cells were mediated through the inhibition of Sirtuin1 (SIRT1). Taken together, miR-486 may play antitumor roles in osteosarcoma growth and metastasis by directly targeting SIRT1, suggesting that it may be a novel therapeutic target for the OS treatments.

Material and methods

OS tissues, cell lines and transfection

OS tissues (n=9) and matched normal non-tumor tissue samples (n=9) were obtained from OS patients who had been treated by surgical resection at Department of Orthopaedic Surgery, The Second Affiliated Hospital of Zhejiang University school of Medicine. All tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C refrigerator until use. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University school of Medicine.

Human OS cell lines (MG63, HOS, SAOS2, U2OS, KHOS) and human normal osteoblastic cell line (hFOB 1.19) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). HEK293T cell line, used for luciferase report assay, was also obtained from ATCC. hFOB 1.19 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) containing 10% FBS, 100 U/ml penicillin (Gibco, Grand Island, NY) and 100 U/ml streptomycin (Gibco, Grand Island, NY) in conditions of 95% air and 5% CO₂ at 37°C.

miR-486 mimics and negative control (NC), purchased from GenePharma (Shanghai, China), was used to enforce miR-486 expression. For depletion of SIRT1, the SIRT1 siRNA was synthesized and purified by Guangzhou RiboBio Co., Ltd (Guangzhou, China). Transfection of mimics and siRNA was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions.

RNA isolation and quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from tissues and cells by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) under the guidance of operation instructions. For miR-486 expression, qRT-PCR was performed in Applied Biosystems 7500 Real-time PCR System with TaqMan MicroRNA assay kits (Applied Biosystems, Foster City, CA, USA). U6 was used as the internal control for miR-486 expression. Expression of SIRT1 mRNA was quantified using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. GAPDH was used as an internal reference for SIRT1 mRNA expression.

Cell viability assay

Cell viability was evaluated using the Cell Counting Kit 8 (CCK8; Dojindo, Kumamoto, Japan) assay. Cells were seeded into 96-well plates at a density of 3000 cells per well. After incubation overnight, cells were transfected with miRNA or siRNA and incubated for 24, 48, 72 and 96 h. At above time points, 10 ul CCK8 solution was added into each well, and incubated for additional 4 h at 37°C. Cell viability was determined by absorbance at 450 nm using microplate reader (Bio-Rad, Richmond, CA, USA).

Cell migration and invasion assay

Migration and invasion assays were performed in 24-well plates using transwell chambers with 8mm pore filter (BD Bioscience, San Jose, CA,
For cell migration assay, $1 \times 10^5$ transfected cells (miRNA or siRNA) in 300 μL FBS-free medium were plated in the upper chambers. For cell invasion assays, $1 \times 10^5$ transfected cells (miRNA or siRNA) were seeded into the upper chambers precoated with Matrigel (BD Biosciences, San Jose, CA, USA). 500 μL culture medium containing 20% FBS was added into the lower chambers as a chemoattractant. After incubation 24 h, the migrated or invaded cells were fixed, stained with 0.5% crystal violet and washed with phosphate-buffered saline (PBS, Gibco, Grand Island, NY). The migrated or invaded cells were photographed and counted under an inverted microscope.

**Bioinformatic predication and luciferase report assay**

TargetScan (http://www.targetscan.org/index.html) was used to predicate the potential target genes of miR-486.

The luciferase reporter vectors (pGL3-SIRT1-3'UTR Wt and pGL3-SIRT1-3'UTR Mut) were synthesized by GenePharma. HEK293T cells were seeded into 24-well plates and transfected with pGL3-SIRT1-3'UTR Wt or pGL3-SIRT1-3'UTR Mut, along with miR-486 mimics or NC using Lipofectamine 2000, following to the manufacturer’s instructions. At time after transfection 48 h, luciferase reporter assays were performed with Dual-Luciferase Reporter Assay System (Promega, Madison, WI, Germany). The relative firefly luciferase activities were measured by normalizing to renilla luciferase activities.

**Western blot analysis**

Transfected cells were solubilized with radioimmunoprecipitation buffer (RIPA) lysis buffer (Beyotime, China). Protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% non-fat milk in Tris-buffered saline (TBS) for 2 h at room temperature, followed by incubation with primary antibodies for overnight at 4°C. In this study, SIRT1 (1:1000 dilution; sc-74504) and β-actin (1:1000 dilution; sc-47778) primary antibodies were purchased from Santa Cruz Biotechnology. Subsequently, the membranes were washed with TBST for three times and probed with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature. ECL reagent (Pierce, Rockford, IL, USA) was used to detect the signals on the membranes.

**Statistical analysis**

The data were presented as mean ± S.D., and compared using Student’s t test and analysis of variance. Results were analyzed using SPSS software (version 13.0; IBM SPSS, Armonk, NY, USA). Differences were considered significant at $P$ value less than 0.05.
miR-486 was down-regulated in OS tissues and cell lines

Firstly, we measured the expression levels of miR-486 in OS tissues (n=9) and matched normal non-tumor tissue samples (n=9) using qRT-PCR. The results showed that miR-486 was obviously down-regulated in OS tissues compared with that in matched normal non-tumor tissues (Figure 1A, P<0.05). Meanwhile, miR-486 expression levels in five OS cell lines and human normal osteoblastic cell line hFOB 1.19 were also measured. The results showed that miR-486 expression levels in all examined OS cell lines were lower than in hFOB 1.19 (Figure 1B, P<0.05). HOS and U2OS, expressed relatively lower expression of miR-486, were selected for functional studies.

Over-expression of miR-486 inhibited proliferation, migration and invasion of OS cells

We investigated the effects of miR-486 on proliferation, migration and invasion of OS cells using cell viability assay, migration and invasion assay, respectively. After transfection with miR-486 mimics or NC, qRT-PCR was performed to detect miR-486 expression. The results showed that miR-486 was markedly upregulated in both HOS and U2OS cells (Figure 2A). The expression levels of miR-486 in HOS and U2OS cells transfected with miR-486 mimics or NC were significantly different compared with their respective controls (P<0.05). Over-expression of miR-486 inhibited proliferation, migration and invasion of OS cells (Figure 2B, C).
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Figure 3. miR-486 negatively regulated SIRT1 expression by binding to its 3'UTR. A. The predicted wild-type and mutant binding sites of miR-486 in SIRT1 3'UTR are shown. B. Luciferase reporter assays in HEK293T cells co-transfected with miR-486 mimics or NC and pGL3-SIRT1-3'UTR Wt or pGL3-SIRT1-3'UTR Mut. C. SIRT1 mRNA levels were measured by using qRT-PCR in HOS and U2OS cells transfected with miR-486 mimics or NC. D. SIRT1 protein levels were determined by using western blot analysis in HOS and U2OS cells transfected with miR-486 mimics or NC. *P<0.05 compared with their respective controls.

Cell viability assays revealed that over-expression of miR-486 inhibited proliferation of HOS and U2OS cells (Figure 2B, P<0.05). Similarly, migration and invasion assays demonstrated that miR-486 significantly inhibited migration and invasion capacities of HOS and U2OS cells (Figure 2C, P<0.05). These data collectively suggested that miR-486 may act as a tumor suppressor in OS.

miR-486 negatively regulated SIRT1 expression by binding to its 3'UTR

Potential targets of miR-486 were predicted by using TargetScan. Bioinformatics analysis showed that SIRT1 contained a miR-486 seed match at 3'UTR of SIRT1 (Figure 3A). To explore whether miR-486 directly binds to the 3'UTR of SIRT1, pGL3-SIRT1-3'UTR Wt or pGL3-SIRT1-3'UTR Mut were co-transfected with miR-486 mimics or NC into HEK293T cells. The luciferase reporter assays showed that up-regulation of miR-486 reduced luciferase activities of pGL3-SIRT1-3'UTR Wt in HEK293T cells. However, the suppressive effects of miR-486 mimics on luciferase activities were completely deprived upon introduction of the pGL3-SIRT1-3'UTR Mut, indicating its identification as a true miR-486 target site (Figure 3B, P<0.05).

To verify whether SIRT1 was regulated by miR-486, miR-486 mimics or NC was transfected into HOS and U2OS cells. qRT-PCR and western blot showed that miR-486 reduced SIRT1 mRNA (Figure 3C, P<0.05) and protein (Figure 3D, P<0.05) expression in HOS and U2OS cells, respectively. These results suggested that miR-486 negatively regulated SIRT1 expression by binding to its 3'UTR.

SIRT1 was involved in miR-486-induced suppressive functions in OS cells

To determine whether the suppressive functions of miR-486 were mediated by SIRT1, SIRT1 siRNA or NC siRNA was injected into HOS and U2OS cells. The efficiency of SIRT1 siRNA transfection was evaluated using western blot.
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As shown in Figure 4A, SIRT1 was significantly downregulated in HOS and U2OS cells transfected with SIRT1 siRNA (P<0.05). Consistent with our earlier results, knockdown of SIRT1 significantly suppressed proliferation (Figure 4B, P<0.05), migration and invasion (Figure 4C, P<0.05) of HOS and U2OS cells. These findings suggested that miR-486 inhibited the proliferation, migration and invasion of OS cells via suppression of SIRT1 expression.

Discussion

Accumulated evidences have indicated that the abnormal expression of miRNAs contributed to the carcinogenesis and progression of various kinds of human cancers, including OS, by negatively regulating expression of multiple target mRNAs related to the carcinogenesis and progression [21-23]. Therefore, understanding the physiological and molecular mechanisms of miRNAs in cancer may provide novel therapeutic strategies for treatments of various human cancers [24]. In this study, our results showed that miR-486 was markedly downregulated in OS tissues and cell lines. In addition, overexpression of miR-486 inhibited cell proliferation, migration and invasion of OS. Furthermore, SIRT1 was identified as a direct and functional target gene of miR-486 in OS. OS is characterized by rapidly growth and metastasis. Our findings provides new tools to investigate the mechanisms underlying rapidly growth and metastasis of OS. More importantly, it may be a therapeutic target in the treatments of patients with OS.

Dysregulation of miR-486 expression has been found in many human cancers. For example, miR-486 was downregulated in gastric adenocarcinoma, and low miR-486 expression was associated with TNM stage. Furthermore, low miR-486 expression was correlated with a poor prognosis compared with neighboring normal tissues, while high expression was associated with a good prognosis in gastric adenocarcinoma [25]. Wang et al. reported that expression level of miR-486 was
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lower in lung tumors compared with that in corresponding normal tissues. Low miR-486 expression level was associated with stage and lymph node metastasis of non-small-cell lung cancer [26]. The down-regulation of miR-486 was also found in hepatocellular carcinoma [27] and breast cancer [28]. However, miR-486 also has been proved to be frequently up-regulated in many human cancers, such as chronic myeloid leukemia [29], renal cell carcinoma [30] and pancreatic ductal adenocarcinomas [31]. These conflicting studies suggested that expression of miR-486 in cancers has tissue specificity.

Accumulated studies have shown significant roles of miR-486 in cancer progression. In breast cancer, enforced miR-486 expression inhibited cell growth in vitro and in vivo, improved G0/G1 arrest, and induced apoptosis by directly targeting PIM-1 [28]. Huang et al. reported that over-expression of miR-486 significantly decreased hepatocellular carcinoma cells proliferation, migration and invasion in vitro, and growth in vivo. Mechanistically, PIK3R1 was identified as a direct target gene of miR-486 in hepatocellular carcinoma [27]. Sun and his colleagues showed that restoration of miR-486 resulted in a markedly reduction the ability of cell proliferation, colony formation and migration in vitro through blockade of CITRON and CLDN10 [32]. miR-486 played tumor suppressor roles in progression of gastric cancer by directly targeting OLFM4 [33]. These findings suggested that miR-486 may play important functions in these cancers, and could be investigated as a potential therapeutic treatments for these cancers.

SIRT1 was subsequently identified as a candidate target of miR-486 in OS. Firstly, Bioinformatic analysis showed that SIRT1 contained a miR-486 seed match at 3’UTR of SIRT1. Secondly, luciferase reporter assays showed that miR-486 directly targeted 3’UTR of SIRT1. qRT-PCR and western blot analysis indicated that expression levels of SIRT1 was down-regulated at both mRNA and protein levels after transfection with miR-486 mimics. Finally, knockdown of SIRT1 inhibited cell proliferation, migration and invasion of OS, also suggesting that SIRT1 was a functional target gene of miR-486 in OS. All these results demonstrated that miR-486 acted as a tumor suppressor in OS via directly targeting SIRT1. Identification of miR-486 target gene is essential for understanding its functions in carcinogenesis and progression of OS.

Sirtuins are nicotinamide adenine dinucleotide (NAD)+-dependent protein deacetylase class III histone deacetylases [34]. The sirtuin family includes seven members, designated SIRT1 through SIRT7, which are characterized by a conserved 275-amino acid catalytic core and unique additional N-terminal and/or C-terminal sequences of variable length [35]. Further studies demonstrated that expression of SIRT1 is up-regulated in various kinds of cancers, such as human epithelial cancer, breast cancer, colon cancer and prostate cancer, suggesting that SIRT1 plays important roles in these cancers [36-39]. Moreover, SIRT1 was proved to involve in cellular survival, migration, invasion and apoptosis [40-43]. All these findings indicated that SIRT1 functions as an oncogene in cancer, and is worthwhile to explore new therapeutic treatments to against SIRT1.

In conclusion, this study have demonstrated that miR-486 was down-regulated in OS tissues and cell lines. In addition, over-expression of miR-486 suppressed OS cells proliferation, migration and invasion through directly targeting SIRT1. The present study therefore provided a potential therapeutic target for OS treatments.

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Disclosure of conflict of interest

None.

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