

Suppressive effect of microRNA-138 on the proliferation and invasion of osteosarcoma cells via targeting SIRT1

ZHENCHAO YUAN, HAO MO, LIGEN MO, JULIANG HE, ZHENJIE WU and XIANG LIN

Department of Bone and Soft Tissue Neurosurgery, Affiliated Tumor Hospital of Guangxi Medical University, Nanning, Guangxi 530021, P.R. China

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Abstract. MicroRNAs (miRs), a class of small non-coding RNAs, function as key regulators in gene expression through binding to the 3'-untranslated region (UTR) of their target mRNA, which further leads to translational repression or mRNA degradation. Recently, miR-138 has been found to have a tumor suppressive role in a variety of human malignancies. However, the exact role of miR-138 in regulating the malignant phenotypes of osteosarcoma (OS) has remained to be elucidated. In the present study, reverse-transcription PCR analysis showed that the expression of miR-138 was markedly reduced in OS tissues compared to that in matched adjacent non-tumorous tissues. Furthermore, it was also downregulated in several common OS cell lines, when compared with that in a normal human osteoblast cell line. Overexpression of miR-138 suppressed cell proliferation and invasion and led to a significant decrease in the protein expression of sirtuin 1 (SIRT1), which was further identified as a direct target gene of miR-138 in MG63 cells. Moreover, restoration of SIRT1 expression reversed the suppressive effects of miR-138 on MG63 cell proliferation and invasion. Finally, the expression of SIRT1 was found to be significantly upregulated in OS tissues compared to that in matched adjacent tissues, and SIRT1 levels were inversely correlated with the miR-138 levels in OS tissues. Therefore, the present study demonstrated that miR-138 has a role in inhibiting OS cell proliferation and invasion via directly targeting SIRT1, and suggested that the miR-138/SIRT1 axis may become a promising therapeutic target for OS.

Introduction

Osteosarcoma (OS) is the most common cancer type primarily present around regions with active bone growth and repair (1,2). Dysfunction of tumor suppressors or oncogenes participates

in the development and malignant progression of OS (1,3). Therefore, exploration of the underlying mechanisms is urgently required for identifying potential therapeutic targets for OS.

Certain microRNAs (miRs) are known to have oncogenic or tumor suppressive roles (4). They are a class of non-coding small RNA, which negatively regulate the gene expression at a post-transcriptional level (5). Mature miRs bind to specific complementary sites within the 3'untranslated region (UTR) of their target mRNAs, which leads to the inhibition of translation or to mRNA degradation (6). Through negatively mediating the protein expression of their targets, miRs are involved in the regulation of various cellular processes, such as cell proliferation, survival, differentiation, apoptosis, autophagy and motility (7). In recent years, deregulation of miRs has been observed in various types of human cancer (4). Moreover, miRs may function as tumor suppressors or oncogenes depending on the target genes and tumor type (8). However, the underlying mechanism by which certain specific miRs regulate the malignant phenotypes of OS cells still remains to be fully elucidated.

Among the cancer-associated miRs, miR-138 generally has a tumor suppressive role in certain common cancer types (9,10). For instance, miR-138 was found to be downregulated in ovarian cancer and to suppress the invasion, migration and metastasis of ovarian cancer cells (11,12). Besides, miR-138 suppressed invasion and epithelial-mesenchymal transition (EMT), while it promoted apoptosis in head and neck squamous cell carcinoma cells (13,14). Wang *et al* (15) reported that miR-138 inhibited tumor cell proliferation by inducing cell cycle arrest in hepatocellular carcinoma (HCC). Ye *et al* (16) found that miR-138 inhibited the proliferation of non-small cell lung cancer (NSCLC) cells by targeting 3-phosphoinositide-dependent protein kinase-1. miR-138 was found to inhibit histone H2AX expression and induce chromosomal instability after DNA damage in OS cells (17). Moreover, overexpression of miR-138 enhanced cellular sensitivity of OS cells to cisplatin and camptothecin (17). A systems biological study by Poos *et al* (18) suggested that miR-138 is involved in the regulation of OS cell proliferation through interaction with certain transcription factors associated with proliferation. However, the roles of miR-138 in OS as well as the underlying mechanisms have remained to be elucidated.

The present study aimed to reveal the exact role of miR-138 in the regulation of proliferation and invasion of OS cells. Furthermore, the underlying mechanism was investigated.

Correspondence to: Dr Hao Mo, Department of Bone and Soft Tissue Neurosurgery, Affiliated Tumor Hospital of Guangxi Medical University, 71 He Di Road, Nanning, Guangxi 530021, P.R. China
E-mail: mohaodoctor@qq.com

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Materials and methods

Tissue samples. The present study was approved by the Ethic Committee of the Affiliated Tumor Hospital of Guangxi Medical University (Nanning, China). Primary OS (n=12) and their matched adjacent non-tumorous tissues were collected from OS patients who underwent surgical resection at the Hospital from June 2013 to June 2014. None of the OS patients had received any radiation therapy or chemotherapy prior to surgery. Tissues were snap-frozen in liquid nitrogen and then stored at -70°C prior to use.

Cell culture and transfection. Human OS cell lines, including Saos-2, U2OS, MG63 and SW1353, as well as the human osteoblast cell line hFOB, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 IU/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. For miR-138 overexpression, miR-138 mimics (Genepharma, Shanghai, China) were used to transfect MG63 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. For restoration of Sirtuin 1 (SIRT1) expression, SIRT1 open reading frame (ORF) expression plasmid (GeneCopoeia, Guangzhou, China) and miR-138 mimics were used to co-transfect MG63 cells using Lipofectamine® 2000. After transfection for 48 h, reverse-transcription quantitative polymerase chain reaction (RT-PCR) or western blot analysis were used to examine the expression levels of miR-138 or SIRT1.

RNA extraction and RT-qPCR. Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Relative mRNA expression was detected using the standard SYBR-Green RT-PCR kit (Takara, Otsu, Japan), in accordance with the manufacturer's instructions. The specific primers for SIRT1 were as follows: Forward, 5'-TAGCCTTGTCAGATAAGGAAGGA-3' and reverse, 5'-ACAGCTTCACAGTCAACTTTGT-3'. GAPDH was used as the internal reference. The specific primers for GAPDH were forward, 5'-CTGGGCTACACTGAGCAC C-3' and reverse, 5'-AAGTGGTCGTTGAGGGCAATG-3'. Primers for U6 and miR-138 were purchased from GeneCopoeia (Rockville, MD, USA). For miRNA analysis, real-time PCR was performed using the PrimeScript® miRNA RT-PCR kit (Takara), according to the manufacturer's instructions. U6 small nuclear RNA was used as the internal reference. The reaction conditions were 95°C for 5 min, and 40 cycles of denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 30 sec. The relative expression was analyzed by the 2^{-ΔΔC_t} method (19). The experiments were repeated three times.

Cell proliferation assay. MG63 cells in each group were seeded into a 96-well plate and cultured at 37°C with 5% CO₂ for 0, 12, 24, 48 or 72 h. Subsequently, 20 μl MTT (5 mg/ml; Thermo Fisher Scientific, Inc.) was added. After incubation at 37°C for 4 h, 150 μl of dimethylsulfoxide was added. After incubation at room temperature for 10 min, the amount of formazan product formed was detected by determining the

optical density (OD) at 570 nm using a Multiskan FC enzyme immunoassay analyzer (Thermo Fisher Scientific, Inc.).

Cell invasion assay. The invasive capacity of MG63 cells in each group was evaluated using Transwell inserts with 8-μm pores (Corning, Inc., Corning, NY, USA). In brief, 300 μl serum-free cell suspension containing 1x10⁵ MG63 cells was added to the upper insert pre-coated with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA) and 400 μl of medium containing 10% FBS was added to the lower chamber. After 48 h of incubation, non-invaded cells were removed from the upper surface of the Transwell membrane with a cotton swab. Invaded cells on the lower surface of the Transwell membrane were fixed in methanol, stained with 0.1% crystal violet and images were captured under a microscope. Cells in five randomly selected fields were counted.

Western blot analysis. Cells were lysed in cold radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). The protein concentration was determined using the BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein (50 μg per lane) was separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Inc.), and then blocked in 5% nonfat dried milk in PBS (Thermo Fisher Scientific, Inc.) for 4 h. The PVDF membrane was then incubated with mouse anti-SIRT1 monoclonal antibody (1:200 dilution; ab110304, Abcam, Cambridge, MA, USA), or mouse anti-GAPDH monoclonal antibody (1:500 dilution; ab8245, Abcam) as an internal reference for 3 h at room temperature, and then washed with PBS for 10 min. Subsequently, the PVDF membrane was incubated with rabbit anti-mouse secondary antibody (1:10,000 dilution; ab46540, Abcam) for 1 h at room temperature. After washing with PBS for 15 min, the immune complexes on the PVDF membrane were detected using the ECL Western Blotting kit (Pierce; Thermo Fisher Scientific, Inc.). Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze the relative protein expression, which was represented as the density ratio vs. GAPDH.

Luciferase reporter assay. The fragment of wild-type (WT) SIRT1 3'untranslated region (3'UTR) containing the putative binding sites of miR-138 was amplified by PCR. The specific primers used were as follows: Forward, 5'-CCGCTCGAGCAC CAGTAAACAAGGAAGTGTG-3' and reverse, 5'-GAATGC GGCCGCTTTACAGAAACAAATGCAATGTTAC-3'. The SIRT1 3'UTR was then subcloned into the psiCHECK-2 vector (Promega Corp., Madison, WI, USA), downstream of the luciferase gene sequence. The mutant type (MUT) of SIRT1 3'UTR containing mutant binding sites of miR-138 was generated using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) in accordance with the manufacturer's protocol, which was also subcloned into the psiCHECK-2 vector downstream of the luciferase gene sequence. MG63 cells were co-transfected with 100 ng SIRT1 3'UTR WT vector or SIRT1 3'UTR MUT vector, and 50 nM of miR-138 mimics or scramble miR (miR-NC), respectively, by using Lipofectamine® 2000, in accordance with the manufacturer's protocol. After transfection for 48 h, a dual-luciferase reporter assay system (Promega Corp.) was used to determine the activities of *Renilla* luciferase and

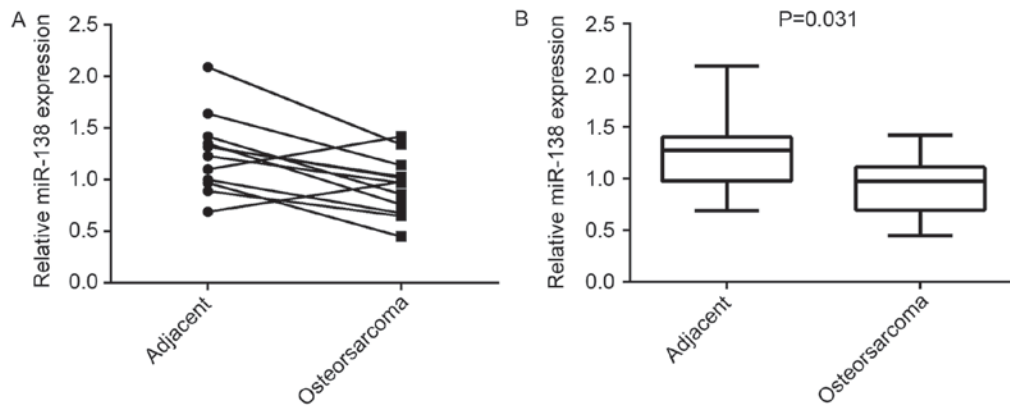


Figure 1. The expression levels of miR-138 in a total of 12 osteosarcoma tissues and their matched adjacent non-tumor tissues were examined by reverse-transcription quantitative polymerase chain reaction analysis. (A) Individual data-points within groups and (B) quantified box plots are shown. miR, microRNA.

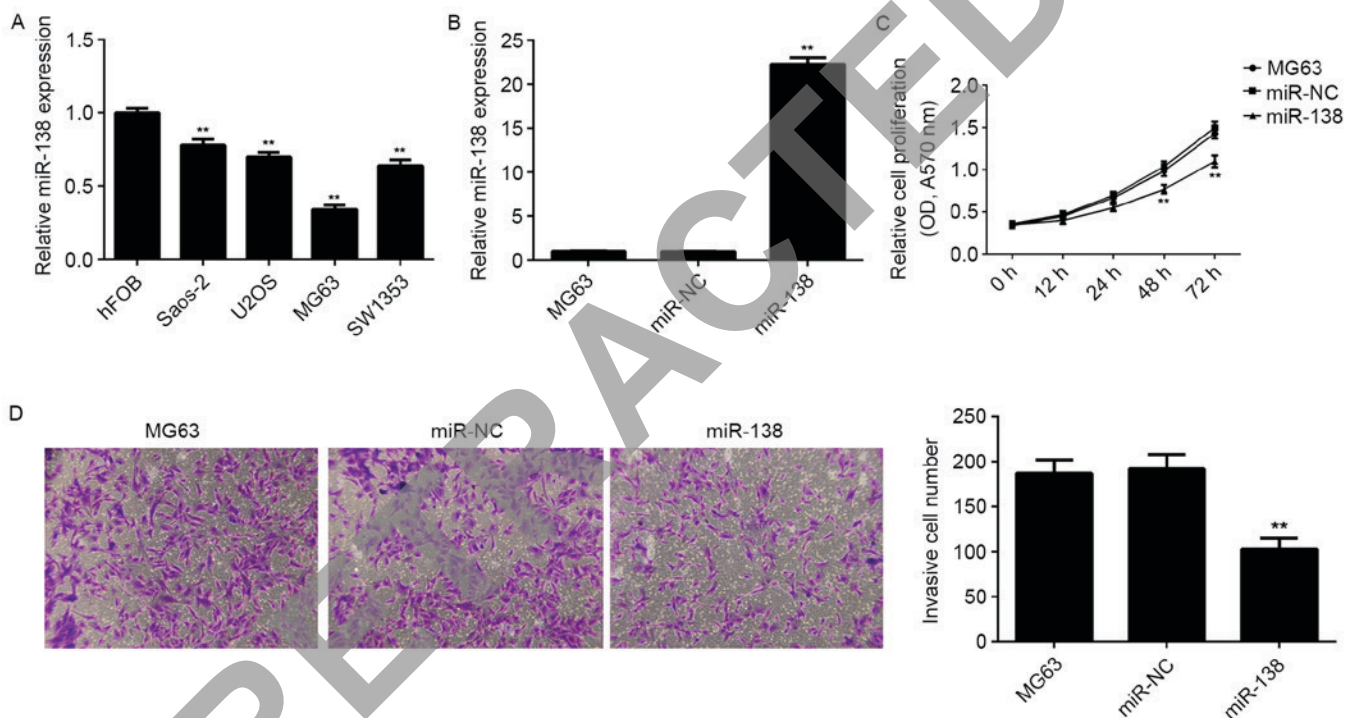


Figure 2. (A) The expression levels of miR-138 in osteosarcoma cell lines (Saos-2, U2OS, MG63 and SW1353) as well as in the human osteoblast cell line hFOB were examined by RT-qPCR. ** $P < 0.01$ vs. hFOB. (B) RT-qPCR analysis was performed to examine the miR-138 levels in MG63 cells transfected with miR-NC or miR-138 mimics. Non-transfected MG63 cells were used as controls. (C) An MTT assay and (D) a Transwell assay were used to examine the cell proliferation and invasion, respectively. Magnification, $\times 200$. ** $P < 0.01$ vs. MG63. RT-qPCR, reverse-transcription quantitative polymerase chain reaction analysis; miR, micro RNA; miR-NC, scramble miR; OD, optical density.

firefly luciferase. The activity of *Renilla* luciferase was normalized to that of firefly luciferase.

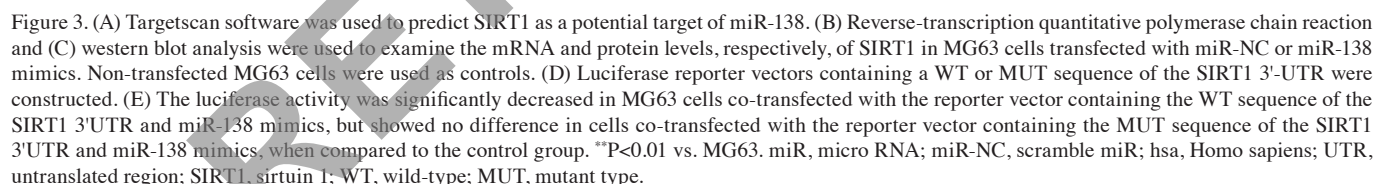
Statistical analysis. Values are expressed as the mean \pm standard deviation. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data were analyzed by using a Student's t-test for two-group comparison and one-way analysis of variance for multiple-group comparison. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-138 is frequently downregulated in OS tissues and cell lines. To reveal the role of miR-138 in OS, quantitative RT-PCR

analysis was performed to determine its expression levels in OS tissues and their matched adjacent non-tumor tissues. The results showed that the expression of miR-138 was decreased in 10 of OS tissues (83.3%), when compared with that in matched adjacent normal tissues (Fig. 1A). Moreover, its expression was significantly downregulated in OS tissues compared to that in their matched adjacent normal tissues (Fig. 1B).

miR-138 suppresses MG63 cell proliferation and invasion. The expression levels of miR-138 were further examined in several common human OS cell lines, including Saos-2, U2OS, MG63 and SW1353, as well as in the human osteoblast cell line hFOB. RT-qPCR analysis revealed that miR-138 was also downregulated in OS cell lines compared to hFOB cells (Fig. 2A). As



MG63 cell invasion. These findings suggested that miR-138 acts as a tumor suppressor in OS.

SIRT1 is a direct target of miR-138 in MG63 cells. SIRT1 has been found to have an oncogenic role in OS (20). In the present study, Targetscan (www.targetscan.org) was used to analyze the putative target genes of miR-138 and SIRT1 was predicated to be a direct target of miR-138 (Fig. 3A). Therefore, the expression levels of SIRT1 in MG63 cells with or without miR-138 overexpression were examined. RT-qPCR revealed showed no difference in the SIRT1 mRNA levels after miR-138 overexpression (Fig. 3B). However, western blot analysis indicated that overexpression of miR-138 significantly repressed the SIRT1 protein expression compared to that in the control group (Fig. 3C). To further clarify their association, the SIRT1 3'UTR WT and SIRT1 3'UTR MUT luciferase reporter vectors were constructed (Fig. 3D). MG63 cells were

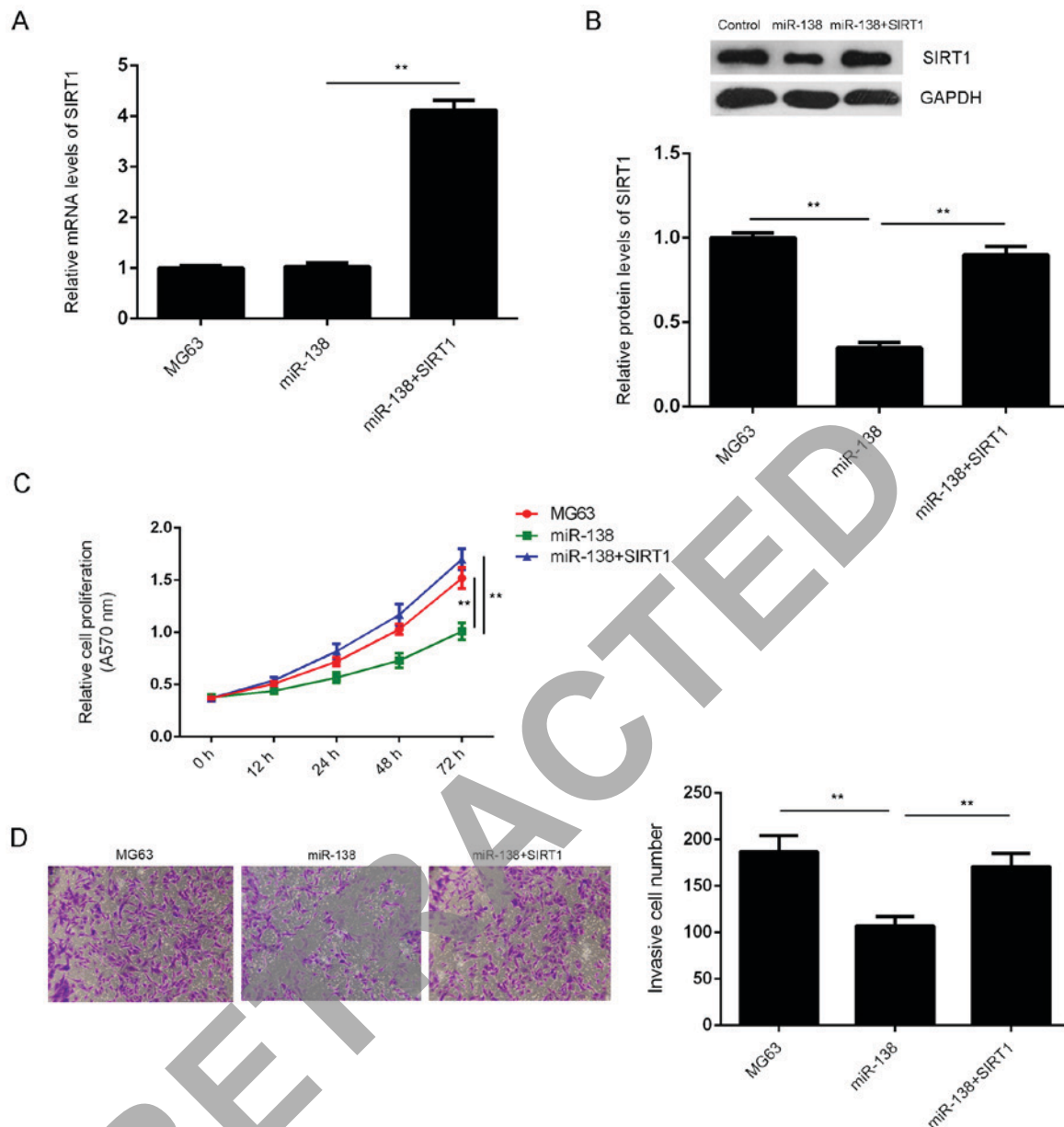


Figure 4. (A) Reverse-transcription quantitative polymerase chain reaction and (B) western blot analysis were used to examine the mRNA and protein levels of SIRT1 in MG63 cells transfected with miR-138 mimics, or co-transfected with miR-138 mimics and SIRT1 open reading frame expression plasmid, respectively. (C) MTT assay and (D) Transwell assay were used to examine the cell proliferation and invasion, respectively. Magnification, x200. **P<0.01. miR, microRNA; SIRT1, sirtuin 1; OD, optical density.

co-transfected with SIRT1 3'UTR WT or SIRT1 3'UTR MUT with miR-138 mimics or miR-NC. After transfection for 48 h, a dual-luciferase reporter assay was performed, revealing that in SIRT1 3'UTR WT-transfected cells, the luciferase activity of miR-138-overexpressing cells was significantly decreased when compared to that of miR-NC-transfected cells, which was abolished when the SIRT1 3'UTR MUT luciferase reporter vector was used (Fig. 3E), suggesting that miR-138 directly binds to the 3'UTR of SIRT1 mRNA. Accordingly, it was demonstrated that SIRT1 is a direct target of miR-138 in OS MG63 cells.

Restoration of SIRT1 expression reverses the suppressive effect of miR-138 on MG63 cell proliferation and invasion. Based on the above data, it was speculated that the role of miR-138 in

the regulation of the malignant phenotypes of OS cells may be exerted through mediation of SIRT1 expression. To verify this speculation, SIRT1 ORF expression plasmid was used to transfect the miR-138-overexpressing MG63 cells to increase SIRT1 expression. RT-qPCR and western blot analysis demonstrated that the mRNA and protein levels of SIRT1 were significantly higher in the miR-138+SIRT1 group compared to those in the miR-138 group (Fig. 4A and B). Furthermore an MTT assay and a Transwell assay were performed to examine the proliferation and invasion of MG63 cells in each group. As indicated in Fig. 4C and D, the cell proliferation and invasion were significantly increased in the miR-138+SIRT1 group when compared with those in the miR-138 group, indicating that overexpression of SIRT1 reversed the suppressive effects of miR-138 on MG63 cell proliferation and invasion. Taken

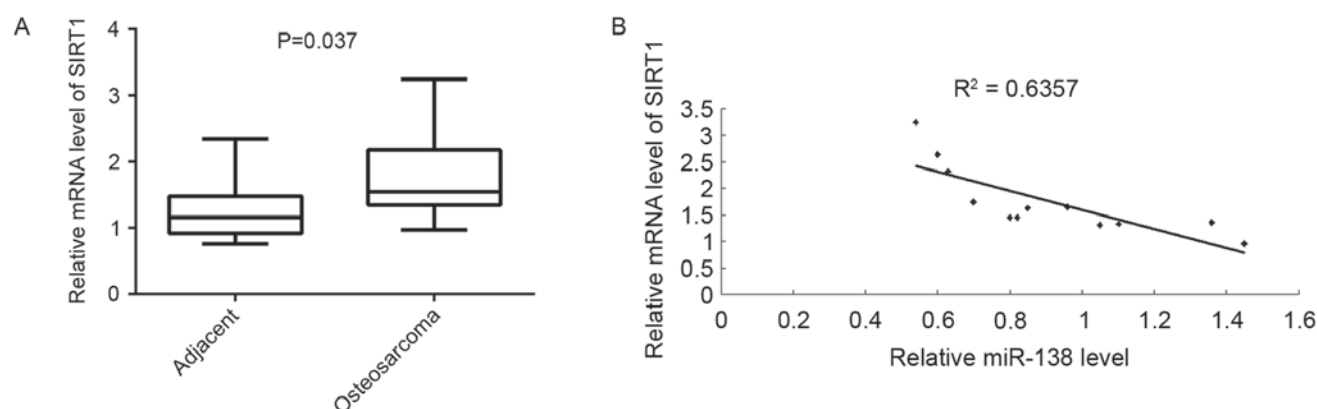


Figure 5. (A) Reverse-transcription quantitative polymerase chain reaction was used to examine the mRNA expression of SIRT1 in a total of 12 osteosarcoma tissues and their matched adjacent non-tumor tissues. (B) SIRT1 levels were inversely correlated with the miR-138 levels in osteosarcoma tissues. miR, microRNA, SIRT1, sirtuin 1.

together, the results suggested that miR-138 has a suppressive role in the regulation of OS cell proliferation and invasion by directly targeting SIRT1.

SIRT1 is upregulated in OS. Finally, the association between miR-138 and SIRT1 in OS was investigated. RT-qPCR results indicated that the mRNA level of SIRT1 was significantly higher in OS tissues compared to that in their matched adjacent tissues (Fig. 5A). Moreover, the SIRT1 levels were inversely correlated to the miR-138 levels in OS tissues (Fig. 5B). Therefore, it was suggested that the upregulation of SIRT1 may be caused by the decreased expression of miR-138 in OS, which further contributes to the growth and metastasis of OS.

Discussion

miRs have key roles in the development and progression of human cancers, including OS (21). However, the underlying mechanism by which miR-138 mediates the proliferation and invasion of OS cells has largely remained elusive. The present study revealed that miR-138 was frequently reduced in OS tissues compared to their matched adjacent normal bone tissues. Besides, it was also downregulated in OS cell lines compared to normal human osteoblast cells. Overexpression of miR-138 suppressed cell proliferation and invasion, and the protein expression of SIRT1, a direct target of miR-138, in MG63 cells. Moreover, restoration of SIRT1 expression reversed the suppressive effects of miR-138 on MG63 cell proliferation and invasion, suggesting that miR-138 inhibits OS cell proliferation and invasion via directly targeting SIRT1.

Recently, various miRs have been found to be involved in the development and progression of OS. miR-194 was found to suppress OS cell proliferation and metastasis by targeting chromodomain-helicase-DNA-binding protein 2 and insulin-like growth factor 1 receptor (22). miR-33a was reported to promote OS cell resistance to cisplatin by inhibition of TWIST expression (23). However, to the best of our knowledge, the expression and role of miR-138 in OS has not been previously studied. The present study showed that miR-138 was markedly downregulated in OS tissues compared to their matched adjacent non-tumor tissues. Moreover, its expression level was also reduced in four human OS cell lines, including

Saos-2, U2OS, MG63 and SW1353, when compared with that in the human osteoblasts hFOB cells. These results suggested that downregulation of miR-138 may be associated with the development of OS.

In fact, miR-138 was also downregulated in various other cancer types. Qiu *et al* (24) reported that the expression of miR-138 was reduced in glioblastoma (GBM) clinical specimens as well as cell lines, and inhibited the tumorigenesis of GBM by inhibition of the enhancer of zeste homolog 2- cyclin D kinase4/6-retinoblastoma protein-E2F1 signal loop. Besides, miR-138 was also reported to be frequently downregulated in NSCLC tissues and cell lines, and inhibited the proliferation of NSCLC cells *in vitro* and tumor growth *in vivo* (25). Accordingly, it was speculated that miR-138 may also act as a tumor suppressor in OS. To further reveal the role of miR-138 in OS, MG63 cells were transfected with miR-138 mimics, and the results showed that overexpression of miR-138 markedly inhibited the proliferation and invasion of MG63 cells. Therefore, miR-138 may have suppressive effects on OS growth and metastasis, which should be clarified *in vivo* in future studies.

Sirtuins are nicotinamide adenine dinucleotide-dependent class III histone deacetylases. The mammalian sirtuin family consists of seven members (SIRT1-7) and is characterized by a 275-amino acid catalytic core and unique additional N-terminal and/or C-terminal sequences of variable length. As the most well-studied sirtuin, SIRT1 is involved in numerous physiological processes, including senescence, neuroprotection, metabolism and the inflammatory response by acetylating histones and multiple transcription factors (26). Moreover, SIRT1 is associated with tumorigenesis and has an oncogenic role in various types of human malignancy (27,28). For instance, Qu *et al* (29) reported that SIRT1 promoted the proliferation and inhibited the apoptosis of human malignant glioma cells. Furthermore, SIRT1 is also involved in tumorigenesis, metastasis and chemoresistance of HCC (30). Recent studies have reported that SIRT1, as a target gene of several miRs, has an oncogenic role in OS. For instance, Cheng *et al* (31) found that melatonin treatment showed strong antitumor activity in OS cells through inhibition of SIRT1. Xu *et al* (20) reported that miR-126 inhibited OS cell proliferation by targeting SIRT1. Shi *et al* (32) indicated that miR-204

inhibited the proliferation, migration, invasion and EMT in OS cells via targeting SIRT1. However, the knowledge of the regulatory mechanisms of SIRT1 expression in OS remains limited. The present study identified SIRT1 as a direct target gene of miR-138 by using a luciferase reporter assay and its protein levels were negatively regulated by miR-138 in MG63 cells. Further investigation suggested that miR-138 has a suppressive role in cellular proliferation and invasion, at least in part via directly suppressing SIRT1 expression. Therefore, the present study revealed a potential regulatory mechanism by which miR-138 participates in the malignant progression of OS.

To the best of our knowledge, the present study was the first to reveal the suppressive effects of miR-138 on the proliferation and invasion of OS cells via directly targeting SIRT1, expanding the understanding of the function of miRs in the development and progression of OS. The miR-138/SIRT1 axis may become a promising therapeutic target for OS.

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