MicroRNA-192 inhibits the proliferation, migration and invasion of osteosarcoma cells and promotes apoptosis by targeting matrix metalloproteinase-11

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Abstract. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression during stem cell growth, proliferation and differentiation. miRNAs are also involved in the development and progression of a number of cancer types, including osteosarcoma (OS). miR-192 is significantly down-regulated in various tumors, including lung, bladder and rectal cancer. miR-192 expression is associated with the migration and invasion of OS cells. However, the expression of miR-192 and its effects on the development of OS have not been reported. In the present study, the involvement of miR-192 and its molecular mechanisms in the development of OS was investigated. The results indicate that miR-192 expression was significantly downregulated in OS tissues compared with non-tumor tissues (P<0.05). Next, a miR-192 agomir was transfected into the OS cell line MG-63 to upregulate miR-192. The effects of miR-192 overexpression were then investigated by examining cell proliferation, apoptosis, migration and invasion. Matrix metalloproteinase (MMP)-11 belongs to a family of nine or more highly homologous Zn²⁺-endopeptidases. It was demonstrated that the mRNA and protein expression of MMP-11 were upregulated in OS tissues compared with non-tumor tissues (P<0.05). MMP-11 was predicted by TargetScan and miRanda as a miR-192 target, which was confirmed by western blotting and dual-luciferase assays. Finally, it was demonstrated that the overexpression of miR-192 was able to downregulate MMP-11 expression and reduce proliferation, migration and invasion, and promote apoptosis in OS cells. Together, these data indicate that miR-192 may be a tumor suppressor that inhibits the progression and invasion of OS by targeting MMP-11. Therefore, miR-192 may be useful for the diagnosis and treatment of OS.

Introduction

Osteosarcoma (OS) is a highly aggressive bone malignancy that is the sixth most common cancer in children and adolescents (1). OS has two peaks of onset: 15-19 years and ≥70 years (2,3). The 5-year survival rate of primary OS is ~65%. However, OS has a substantially poorer prognosis when associated with fatal lung metastases. The 5-year survival rate of patients with OS metastases is <20% despite the use of aggressive chemotherapy and radiotherapy (4), or even when physicians resort to radical surgery (5). As current efforts have failed to obtain impressive achievements in treatment outcomes and quality of life, it is imperative to find new treatment and diagnostic strategies for early OS, discover new therapeutic targets and develop new techniques to improve disease prognosis.

MicroRNAs (miRNAs) have been found to be involved in multiple biological processes, including differentiation, proliferation, developmental timing, apoptosis, transposon silencing and antiviral defense (6). Previous research has confirmed that >1,000 miRNAs may be involved in regulating >50% of human genes (7). One characteristic of miRNAs is their ability to interact with multiple targets. Each miRNA can regulate hundreds of protein-encoding genes and vice versa, as each structural gene can be a target for multiple miRNAs (8). The incorporation of miRNAs in the study of tumor progression and pathogenesis has reached new levels in recent years for various malignancies, including breast, colon, bladder, pancreatic and lung cancer (9-12). However, the field of miRNAs in OS is still in its infancy. To date, miR-RNAs, including miR-124, miR-30a, miR-144, miR-202 and miR-300 have...
been demonstrated to be closely associated with the initiation and progression of OS (13-17). A recent study reported that miR-192 was abnormally expressed in a variety of carcinomas, including OS (18). However, the exact role of miR-192 in OS remains unknown.

Matrix metalloproteinase (MMP)-11 is a member of the MMP family that have important roles in tumor invasion and metastasis, participating in all aspects of the formation and progression of digestive tract, breast and ovarian tumors (19-21). MMP-11 overexpression in primary tumors is associated with poor prognoses (22). Previous studies have shown that MMP-11 is associated with the pathogenesis of various types of cancer, and therefore it is a potential therapeutic target and/or tumor biomarker.

In the present study, the potential association between miR-192 expression, MMP-11 expression and the grade of malignancy in OS cells were investigated. The data indicated that the upregulation of miR-192 inhibited the proliferation, migration and invasion of the OS cell line MG63. Furthermore, it was demonstrated that MMP-11 was downregulated in MG63 cells. Bioinformatic analysis, reverse transcription-quantitative polymerase chain reaction, western blotting and luciferase reporter assays were performed to elucidate the underlying mechanism through which miR-192 targets MMP-11 in OS cells.

Materials and methods

Collection of patient samples. OS specimens (n=22) were obtained from eligible patients treated at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between June 2014 and May 2016. Tumor and matched adjacent non-cancerous tissue samples were collected and characterized based on histopathological examination. All tissues are snap frozen in liquid nitrogen immediately following surgery and stored at -80°C until use. Prior to the use of these clinical materials for research purposes, written informed consent was obtained from all patients, and the present study was approved by the Institute Research Medical Ethics Committee of Zhengzhou University.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from tissue samples and cultured cells was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. cDNA was synthesized using the RevertAid First Strand cDNA kit (Thermo Fisher Scientific, Inc.). RT-qPCR was performed using SYBR-Green Premix Ex Taq (Takara Bio, Inc.) to quantify miR-192 and MMP-11 expression by ABI 7500 fast apparatus, thermocycling conditions: 95°C, 5 min; 95°C, 5 min; 40 cycles, 95°C, 15 sec; 60°C, 30 sec. U6 small nuclear RNA and GADPH were used as endogenous controls. The primer sequences was has-miR-192: Forward 5'-CCTAAA GGTATGCGAGATGT-3' and reverse 5'-CGATATTCC AGGTTCATCATCAT-3'. The RT-qPCR results were compared using the 2^ΔΔCq method (23). The experiments were repeated in three times.

Cell culture. The human OS cell line MG63 was obtained from the Chinese Academy of Medical Sciences (Beijing, China). MG63 cells were cultured in RPMI-1640 medium that was supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO2 humidified atmosphere. Human osteoblast hFOB1.19 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO2 humidified atmosphere.

Transfection of miRNA. MG-63 cells were seeded into each well of a 6-well plate (2x10^4 cells/well) and then transfected with either miR-192 agomir or negative control (NC), or miR-192 antagonir or NC (Shanghai GenePharma Co., Ltd., Shanghai, China) (50 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) when the cell confluence was ~90%. The cells were harvested for further experiments 24-48 h following transfection.

Cell proliferation and apoptosis assays. Cell proliferation was analyzed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The transfected MG63 cells (5x10^4 cells/well) were seeded into 96-well plates in a final volume of 100 µl and cultured for 24, 48, 72, 96 and 120 h. The CCK-8 solution (10 µl) was added into each well, incubated for 4 h, and the optical density at 450 nm was measured to calculate the number of viable cells. A total of five parallel wells were analyzed for each group. The apoptosis assay was conducted using caspase-3 analysis. The cells from each group were harvested 36-h post-transfection by trypsinization. The cells were then seeded into 96-well plates at a total density of 2x10^4 cells/well in a mixed Ac-DEVD-PNA buffer and incubated at 37°C overnight. On the following day, absorbance values were measured at 415 nm.

Transwell assays. The cells from each group were collected 36-h post-transfection and resuspended in medium at 2x10^5 cells/ml. The upper chamber of a 24-well Transwell Permeable Support device (Corning, Corning, NY, USA) with 8-µm pores was loaded with 200 µl cell suspension, and the lower chamber was supplied with 500 µl complete medium (RPMI-1640) containing 10% FBS. The loaded device was incubated at 37°C in a humidified incubator with 5% CO2 for 36 h. After incubation, the medium was removed from the upper chamber and the cells in this chamber were scrapped off with a cotton swab. The cells that had migrated to the other side of the membrane were fixed with methanol. The cells were then stained with hematoxylin, mounted and dried at 80°C for 30 min. The number of invaded cells in three randomly selected fields was counted using an inverted microscope at a magnification of x400. The experiments were performed in triplicate.
Wound-healing assays. The cells from each group were collected 24-h post-transfection, seeded in 6-well plates (2x10⁴ cells/well) and allowed to adhere for a further 24 h. An artificial wound was made using a 10-µl pipette tip across the cell monolayer. The cells were then rinsed with phosphate-buffered saline (PBS) three times to clear cell debris and cells remaining in suspension, and then cultured in serum-free RPMI-1640 medium. Micrographs (taken using an inverted microscope) of the same position relative to the wound were taken at 0 and 36 h. Each assay was performed in triplicate.

Target prediction. Bioinformatic analysis was conducted using TargetScan (http://www.targetscan.org/vert_71/) and miRanda (http://www.targetscan.org/mirrenasa/home.do) to predict the target gene of miR-192 and to verify MMP-11 3'UTR containing the seed region of miR-192.

Luciferase reporter assays. Regions of the human MMP-11 gene containing putative miR-192 binding sites were amplified from human genomic DNA by RT-PCR, performed as previously mentioned. These fragments were cloned into the pmirGLO vector (Promega Corporation, Madison, WI, USA) downstream of the luciferase gene to generate the recombinant vectors pGL3-MMP-11-wt and pGL3-MMP-11-mut. For luciferase reporter assays, a Dual-Luciferase Reporter Assay system (Promega Corporation) was used. MG63 cells were cultured in 96-well plates and co-transfected using 0.8 pmol Pmir-Glo with 50 nM miRNA and wild-type or mutant reporter vectors using Lipofectamine® 2000. Luciferase values were determined using the Dual-Luciferase Reporter Assay system (Promega Corporation) 24 h post-transfection.

Western blotting. RIPA buffer was prepared for protein isolation, and the cell lysis solution was centrifuged at 13,000 x g for 5 min at 4˚C, and then the supernatants were collected. Protein concentration was determined using the BCA assay. Proteins were loaded for SDS-PAGE on 10% polyacrylamide gels followed by transfer to polyvinylidene difluoride membranes. The membrane was blocked for 0.5 h at room temperature in 5% skimmed milk, followed by incubation with primary antibodies (1:300; Ruiqi Biotechnology, Shanghai, China) at 4˚C overnight. After washing three times with TBST (5 min each), the membranes were incubated with a 1:2,500 dilution of goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) secondary antibody for 1 h at room temperature and washed again. Immunoblotting was performed using a chemiluminescence detection kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The signals from target proteins were normalized to those of GAPDH. The software used for densitometric analysis was Image J (version 1.80_112; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All analyses were performed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. The differences were evaluated using a paired Student's t-test or one-way analysis of variance as appropriate. A value of P<0.05 was used to indicate a statistically significant difference.

Results

Inverse correlation between miR-192 and MMP-11 expression in human OS tissues and cell lines. The levels of miR-192 and MMP-11 expression were detected by RT-qPCR in human OS and matched adjacent non-tumor tissues as well as a normal human osteoblastic cell line (hFOB1.19) and an OS cell line (MG63) in order to investigate the role of miR-192 in the development of human OS. These results showed that miR-192 expression was significantly down-regulated in OS tissues compared with matched adjacent non-tumor tissues (P<0.05; Fig. 1A). By contrast, the levels of MMP-11 mRNA were higher in OS tissues compared with matched adjacent non-tumor tissue (P<0.05; Fig. 1A). Similarly, the level of miR-192 expression in MG63 cells was lower compared with hFOB1.19 cells. Conversely, MMP-11 mRNA was upregulated in MG63 cells compared with hFOB1.19 cells (P<0.05; Fig. 1B). Furthermore, western blotting showed that MMP-11 protein levels were also markedly upregulated in OS tissues compared with matched adjacent non-tumor tissues, and in MG63 cells compared with hFOB1.19 cells (Fig. 1C). These data revealed that there is an inverse correlation between the expression of miR192 and MMP-11 in OS tissues and matched adjacent non-tumor tissues (Fig. 1D).

Upregulation of miR-192 inhibits proliferation and induces apoptosis in MG63 cells. To investigate the functional roles of miR-192, miR-192 agonor or NC constructs were transfected into MG63 cells and miR-192 expression was detected after 36 h. Subsequently, CCK-8 and caspase-3 assays were conducted to evaluate the changes in proliferation and apoptosis, respectively. RT-qPCR analysis revealed that miR-192 expression was increased by transfection of miR-192 agonor compared with the control group (P<0.01; Fig. 2A). The CCK-8 assay showed that transfection of miR-192 agonor was able to significantly suppress the proliferation of MG63 cells at five consecutive time points compared with the NC and control groups (Fig. 2B). Moreover, the caspase-3 assay indicated that the upregulation of miR-192 in MG-63 cells was able to promote apoptosis compared with the NC and control groups (P<0.05; Fig. 2C).

Downregulation of miR-192 promotes proliferation and suppresses apoptosis in MG63 cells. Next, the NC or miR-192 antagonor was transfected into MG63 cells. Following incubation for 36 h, miR-192 expression, cell proliferation and apoptosis were detected. miR192 expression was sharply decreased by miR-192 antagonist transfection compared with the control (P<0.01; Fig. 3A). The miR-192 antagonor was also able to markedly promote proliferation (Fig. 3B) and significantly block apoptosis in MG63 cells (P<0.05; Fig. 3C). These data indicated that miR-192 had a strong effect on the proliferation and apoptosis of OS cells.

Effects of miR-192 on the migration and invasion of MG63 cells. Next, the effects of miR-192 on the migration and invasion of MG63 cells were investigated. In wound healing assays, the upregulation of miR-192 was able to suppress the
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migration of MG63 cells to a significant extent, as observed by the degree of wound closure after 36 h under a microscope (Fig. 4A). Similarly, Transwell assays indicated that the overexpression of miR-192 led to a significantly decreased invasive ability of MG63 cells compared with the NC and control groups (Fig. 4B). Then, miR-192 expression was decreased by transfection of miR-192 antagomir, and wound healing and Transwell assays were performed. After 36 h, the
Figure 3. miR-192 downregulation increases proliferation and suppresses apoptosis in MG63 cells. (A) The miR-192 antagonomer was transfected into MG63 cells. (B) Cell proliferation was measured via Cell-Counting Kit 8 assay following transfection in MG63 cells. (C) Apoptosis was detected by caspase-3 assay following transfection in MG63 cells. *P<0.05, **P<0.01 vs. control. NC, negative control; miR, microRNA.

Figure 4. miR-192 agonior and antagonomer modulate the migration and invasion of MG63 cells. The miR-192 agonior or NC and miR-192 antagonomer or NC were transfected into MG63 cells. (A) Effect of miR-192 on the migration of MG63 cells as assessed by wound-healing assay. Representative images for each group were taken using a light microscope. Magnification, x200. (B) Effect of miR-192 on the invasion of MG63 cells as assessed by Transwell assay. Representative images for each group were evaluated by light microscopy. Magnification, x400. (C) The migrated cells in each field were counted and analyzed. (D) The invaded cells in each field were counted and analyzed. *P<0.05 vs. control. miRNA, microRNA; NC, negative control.
downregulation of miR-192 was able to promote the migration and invasion of MG63 cells, which was on the contrary to the miR-192 overexpression results (Fig. 4A and B). Moreover, statistical analyses demonstrated that the overexpression of miR-192 was able to suppress the migration and invasion of MG63 cells. By contrast, the inhibition of miR-192 enhanced migration and invasion (P<0.05; Fig. 4C and D).

**MMP-11 is a potential target of miR-192.** To gain further insight into the molecular mechanism through which miR-192 modulates the behavior of OS cells, target genes of miR-192 were searched using two commonly used target prediction programs, TargetScan (http://www.targetscan.org/vert_71/) and miRanda (http://34.236.212.39/microrna/home.do). The results showed that MMP-11 was a potential miR-192 target gene (Fig. 5A). To confirm that MMP-11 is a direct miR-192 target, dual-luciferase reporter plasmids containing the 3'-UTR of MMP-11 with either a wild-type or mutant putative miR-192 binding site were constructed. Following the overexpression of miR-192 in MG63 cells, the relative luciferase activity of the construct containing the wild-type 3'-UTR of MMP-11 was significantly downregulated compared with the mutant MMP-11 3'-UTR (Fig. 5B). In subsequent western blot assays, MMP-11 protein levels were decreased in MG63 cells when miR-192 expression was upregulated via transfection of agomir (Fig. 5C-E). These results suggested that miR-192 suppressed MMP-11 expression by directly targeting its 3'-UTR.

**Discussion**

OS is one of the most prevalent human sarcomas (24), but even with the development of new therapeutic technologies, the overall survival of OS patients remains unsatisfactory (3). The highly complex molecular mechanisms underlying the initiation and progression of OS remain unknown. Recent studies have shown that miRNAs, including miRNA-34a, miRNA-145 and miRNA-206, are involved in the development of OS (25).

miRNAs are small non-coding RNA molecules ranging from 17 to 22 bp (26) that function in mRNA silencing and the post-transcriptional regulation of gene expression (27). Recent studies have demonstrated that miRNAs are involved in diverse biological processes, including tumorigenesis (28,29). Each miRNA can control hundreds of gene targets, indicating that miRNAs may function as tumor suppressors or oncoproteins via cellular pathways (8). A recent study reported that some miRNAs are dysregulated in cancer. The aberrant expression of these miRNAs may promote tumor proliferation or repress tumor development (30). Therefore, it is necessary to elucidate the mechanism of miRNA-mediated OS development. The present study focused on the roles of miRNA-192 in OS. The
dysregulation of miRNA-192 has been detected in man types of cancer. Specifically, miR-192 overexpression has been shown to induce apoptosis in bladder cancer cells, suggesting that it may be a tumor suppressor in bladder cancer by regulating cell cycle progression (31). Uregulated miR-192 has also been demonstrated to inhibit the progression and metastases of colon cancer (32). Moreover, elevated miR-192 expression has been reported to cause cell growth arrest in breast cancer cells (33). However, it has been reported that miR-192 is overexpressed in nasopharyngeal carcinoma (34), esophageal cancer (35) and pancreatic ductal adenocarcinoma (36). To date, it is unclear whether the downregulation or upregulation of miR-192 is associated with the development of OS.

In the present study, it was detected that miR-192 was downregulated in clinical human OS specimens and the OS cell line MG63 compared with adjacent tissues or normal cells. Furthermore, the overexpression of miR-192 was able to decrease proliferation and promote apoptosis in MG63 cells. It was also determined that the upregulation of miR-192 was able to suppress the migration and invasion of MG63 cells. Therefore, miR-192 could be an inhibitor of OS metastasis. Taken together, our data suggest that miR-192 is a tumor suppressor in OS. Using a combination of two publicly available bioinformatic algorithms, putative miR-192 binding sites in the 3'UTR of MMP-11 were detected. Moreover, it was detected that MMP-11 expression was higher in OS tissues compared with matched adjacent normal tissues, and MMP-11 expression was also increased in MG63 cells compared with the normal human osteoblastic cell line hFOB1.19.

MMP-11 is a member of the MMP family, one of the major classes of proteolytic enzymes involved in embryonic development, implantation and organ formation (37). MMP-11 also has a potential correlation with poor prognosis in OS (22). Notably, an inverse correlation between miR-192 expression and MMP-11 in MG63 cells was detected. To further investigate whether MMP-11 is a downstream target of miR-192, dual-luciferase assays were conducted. The overexpression of miR-192 led to a significant decrease in luciferase reporter activity in cells that express wild-type but not mutant MMP-11 3'-UTR constructs. Therefore, MMP-11 may be a direct target of miR-192. These data indicate that the overexpression of miR-192 inhibits the progression and invasion of OS by downregulating MMP-11 expression directly targeting its 3'UTR. In the future, this could be the basis for anti-metastatic therapy for OS. In light of the principle of miRNA-target recognition, a specific miRNA could have >100 targets (38). Therefore, the complex molecular network of miR-192 remains an enormous subject for future investigation.

The present study is the first to demonstrate the crosstalk between miR-192 and MMP-11 in OS. Our findings suggest that miR-192 is downregulated in OS, which plays important roles in mediating the proliferation, apoptosis, migration and invasion of OS cells by allowing the expression of MMP-11. Accordingly, we propose that both miR-192 and MMP-11 could be promising candidate targets for treating OS.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GWS, YSW, GQZ and YBL conceived and designed the experiments; GWS, YM, YWM, GHW and YDW performed the experiments; GWS, YM, YWM, XJL, GHW and YDW analyzed the data; YSW and YBL contributed reagents, materials and apparatus; GWS wrote the paper; YSW and YBL revised this paper; GQZ reviewed this paper.

Ethics approval and consent to participate

The Institute Research Medical Ethics Committee of Zhengzhou University approved the present study and written informed consent was obtained from each patient.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they have no competing interests.

References


