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Original Article

miR-196 acts as a tumor suppressor in osteosarcoma by targeting HOXA9

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Abstract: Abnormal expression of microRNAs (miRNAs) has been found in most cancer types. Therefore, the discovery of miRNAs could help us to understand the mechanism of tumor initiation and development. The purpose of this study was to investigate the significance of miR-196 in osteosarcoma (OS) and to identify its target genes. We found miR-196 expression was significantly reduced in OS tissues and cell lines (Saos-2 and MG-63) as compared to normal tissues and cell line. The OS cell line proliferation and migration abilities were inhibited by miR-196 overexpression but promoted by miR-196 downregulation *in vitro*. Moreover, we revealed that miR-196 could bind to the 3'-untranslated region (3'-UTR) of homeobox A9 (HOXA9) and inhibit HOXA9 expression in OS cell lines. Furthermore, knockdown the expression of HOXA9 resulted in decreased proliferation and migration which was similar to that observed with miR-196 overexpression in OS cell lines. In summary, miR-196 inhibits proliferation and migration of OS cell lines through regulating HOXA9, which might be a useful target for OS treatment.

Keywords: miR-196, HOXA9, osteosarcoma, tumor suppressor

Introduction

The development of combined therapy methods and surgical procedures over the past decades has greatly improved the 5-year overall survival of osteosarcoma (OS) patients [1-3]. However, it is still unfavorable especially for patients with recurrent or metastatic OS [4]. It has been greatly appreciated that the dysregulation of oncogenes or tumor suppressors plays crucial roles in the progression of OS [5, 6]. The most encouraging part is that these studies have shed light on the development of new molecular therapeutic targets, which promise effective therapy of OS [7, 8]. Therefore, further understanding the molecular mechanisms in OS progression could allow targeted therapy, eventually improving the prognosis of OS .

MicroRNAs (miRNAs) are endogenous RNA with the length of 22-25 nucleotides and typically target the sequence in the 3' untranslated region (3'-UTR) of mRNAs, leading to translation repression or mRNA degradation [9, 10]. Until now, more than 7,000 miRNAs have been identified and most of them were found linked to the progression of many human diseases, especially cancer [10-12]. miR-196 is located in the regions of homeobox (HOX) clusters within the genome of vertebrates [13]. It has been reported that miR-196 was overexpressed in glioblastoma and may serve as a new therapeutic target for glioblastoma [14]. Highly elevated levels of miR-196a have also been detected in hepatocellular carcinoma [15]. Although many studies have revealed miR-196 plays an oncogenic function in some types of cancer, there is also a study that implied that miR-196 plays a tumor suppressive role as well. Namely, the miR-196 expression was found strongly reduced in breast cancer and the ratio of miR-196 to HOXC8 mRNA might be an indicator of the metastatic capability of breast cancer [16]. However, the expression of miR-196 in OS remains unclear. Identification of putative mRNA targets is crucial to understand the biological functions of miRNAs in cancers. It was reported that miR-196 could regulate the expression of various genes in the HOX family including HOXC8, HOXB8, HOXD8 and HOXA7 [16-18]. The HOX family contains 39 members that were reported to play an important role in

cellular development [19]. Unfortunately, whether or not HOXA9 is a target of miR-196 remains to be elucidated.

In this study, we found that levels of miR-196 were significantly lower in OS tissues than those in corresponding noncancerous bone tissues. Further, we identified miR-196 could bind to 3'-UTR of HOXA9 to directly down-regulate the expression of HOXA9. Moreover, we found the downregulation on HOXA9 has a similar effect on OS cell proliferation and migration abilities as miR-196 overexpression.

Materials and methods

Patients and tissue samples

This study was approved by the Ethic Committee of The First affiliated hospital of Harbin medical university. A total of 42 OS tissues and noncancer tissues were included from the enrolled patients. The written informed consent was obtained from all the included patients. These tissues were immediately frozen in liquid nitrogen and stored at -80°C.

Cell culture

The normal osteoblast cell line NHOst and OS cell lines (Saos-2 and MG-63) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell cultivation atmosphere was maintained at 37°C in a humidified incubator (5% CO_2/95% air).

Cell transfection

Lipofectamine 2000 was used to perform cell transfection in line with the supplier's recommendations. Scrambled miRNA, miR-196 mimic, miR-196 inhibitor, scrambled siRNA, and HOXA9-specific siRNA (si-HOXA9) were synthesized by GenePharma Co. Ltd. (Shanghai, China).

RNA extraction and quantitative real-time PCR (RT-qPCR) assay

Total RNA from the collected tissues and cell lines was extracted using Trizol regent (Life Technologies, Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. For detection of miR-196 expression, the MicroRNA reverse transcription kit (Life Technologies, Thermo Fisher Scientific, Inc.) was used. RT-qPCR was performed with an ABI 7500 instrument (Applied Biosystems, Thermo Fisher Scientific, Inc.) using SYBR Green Premix Ex Taq (TaKaRa, Dalian, China). The PCR reaction was conducted at 95°C for 30 s and followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Primers were designed as follows: miR-196 F: 5'-CGTCAGAAGGAATGATGCACAG-3', R: 5'-ACCTGCGTAGGTAGTTTCATGT-3'; U6 snRNA F: 5'-CTCGGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'.

Western blot assay

Total protein from the collected tissues and cell lines was extracted using RIPA lysis buffer (Beyotime) according to the manufacturer's instruction. Protein samples were separated using 10% SDS-PAGE and transferred to PVDF membrane. After blocked by 5% fat-free milk, the membrane was incubated with primary antibodies at 4°C for overnight (anti-HOXA9: ab140631 or anti-GAPDH: ab181602, both from Abcam, Cambridge, MA, USA). Then, the membrane was incubated with the mouse anti-rabbit secondary antibody (ab6728, Abcam) for 40 min at room temperature. Following, the BeyoECL Plus kit (Beyotime) was used to visualize the protein bands.

Luciferase activity assay

The full-length 3'-UTR of HOXA9 (WT) contains the putative binding site for miR-196 was amplified using PCR and then cloned into pmirGLO vector (Promega, Beijing, China). The site-direct mutagenesis was conducted to obtain the mutant type 3'-UTR of HOXA9 (MUT). Then the cells were co-transfected with scramble miRNA or miR-196 mimic, and WT or MUT of 3'-UTR HOXA9 luciferase reporter vector using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). At 48 h post-transfection, the Luciferase Assay System (Promega) was used to determine luciferase activity according to the manufacturer's protocols.

Cell proliferation assay

Cells were seeded into a 96-well plate and cultured in the aforementioned atmosphere. After incubation 0, 24, 48, or 72 h, MTT (Beyotime)

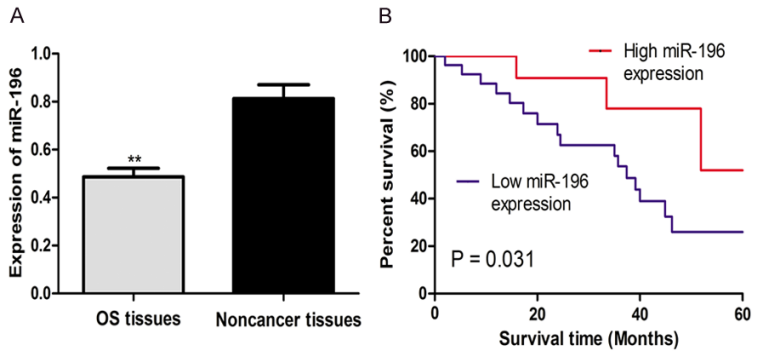

Figure 1. miR-196 was decreased in OS tissues. A. RT-qPCR to detect the expression level of miR-196 in OS tissues and noncancer tissues. B. Overall survival of patients with high or low miR-196 expression. (**P < 0.01). miR-196, microRNA-196; OS, osteosarcoma; RT-qPCR, quantitative Real Time PCR.

was added into the medium. After incubation for another 4 h, dimethyl sulfoxide was added to each well and cultured at 37°C for 10 min. The optical density (OD) at 570 nm was measured using the Absorbance microplate reader (BioTek, Winooski, VT, USA).

Cell migration assay

Cells were seeded in a 6-well plate at a density of 2×10^5 cells/well and incubated in the aforementioned atmosphere for 24 h at 37°C. A wound was created with a sterile 200 μL pipette tip at cell surface in each well. Following, the cells were observed and the wound closure was quantified at 0 h and 48 h after the wound was created using Image J 1.42 (NIH, Bethesda, MA, USA).

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (SPSS Inc, Chicago, IL). Significance among the groups was determined using Student's t-test and one-way ANOVA analysis and Tukey test. Kaplan-Meier curve and log-rank test were used to analyze the effect of miR-196 on patient survival. Whether miR-196 and HOXA9 expression was correlated was analyzed by Pearson's correlation. A significant difference was recognized as $P < 0.05$.

Results

Downregulation of miR-196 in OS tissues

We investigated the expression of miR-196 in 42 paired OS tissues and noncancer tissues by RT-qPCR. Our results revealed that miR-196 was significantly downregulated in OS tissues compared with noncancer tissues (**Figure 1A**). Based on the expression level of miR-196, we divided the enrolled patients into two groups: namely high miR-196 group and low miR-196 group. Then, we further evaluated the clinical significance of miR-196 expression through analyzing the effect of miR-196 expression on the overall survival of OS patients. We found patients with low expression of miR-196 tended to have poorer survival than those with high expression of miR-196 (**Figure 1B**).

Overexpression of miR-196 reduced cell proliferation and migration of OS cell lines

To explore the biological function of miR-196 in OS, we examined the expression of miR-196 in OS cell lines. The expression of miR-196 was significantly reduced in OS cell lines compared with normal osteoblast cell line (**Figure 2A**). Then, we transfected the scrambled miRNA, miR-196 mimic, or miR-196 inhibitor into OS cell lines to manipulate the expression of miR-196. Not surprisingly, the transfection of miR-196 mimic enhanced the expression level of miR-196 (**Figure 2B**). On the contrary, the miR-196 inhibitor could effectively reduce the expression level of miR-196 (**Figure 2B**). MTT assay revealed the cell growth was promoted in cells with miR-196 inhibitor transfection (**Figure 2C**). In contrast, cell growth was inhibited in cells transfected with miR-196 mimic (**Figure 2C**). Furthermore, we found the transfection of miR-196 mimic significantly decreased cell migration of OS cell lines (**Figure 2D**). These findings suggest miR-196 inhibits OS cell proliferation and migration and acts as a tumor suppressor.

Overexpression of miR-196 reduced cell proliferation and migration by decreasing HOXA9 expression

To investigate the mechanism of miR-196 in OS progression, we predicted its potential targets using bioinformatics software tools. We found HOXA9 was a target gene of miR-196 (**Figure**

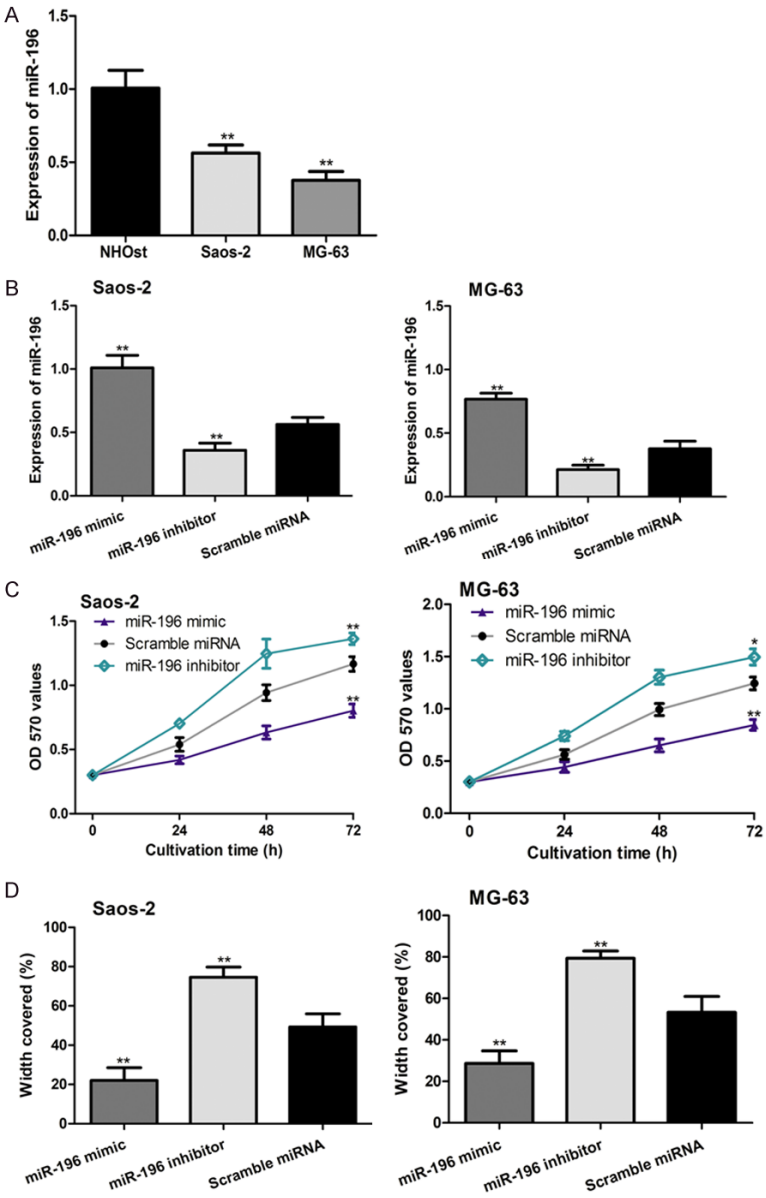

Figure 2. Cell proliferation of OS cells was inhibited by miR-196. A. RT-qPCR to detect the expression level of miR-196 in normal osteoblast cell line NHOst and OS cell lines (Saos-2 and MG-63). B. RT-qPCR to detect the expression level of miR-196 in OS cell lines (Saos-2 and MG-63) with scrambled miRNA, miR-196 mimic, or miR-196 inhibitor transfection. C. MTT assay to detect the cell proliferation and D. Wound healing assay to detect the cell migration of OS cell lines (Saos-2 and MG-63) with scrambled miRNA, miR-196 mimic, or miR-196 inhibitor transfection. (*P < 0.05 and **P < 0.01). miR-196, microRNA-196; OS, osteosarcoma; RT-qPCR, quantitative Real Time PCR.

3A). Therefore, we conducted dual-luciferase assay to validate this finding and we found miR-196 mimic inhibited the luciferase activity in MG-63 cells transfected with WT HOXA9 3'-UTR but did not have any effect on the luciferase activity in MG-63 cells containing MUT HOXA9 3'-UTR (**Figure 3B**). Moreover, we found ectopic expression of miR-196 inhibited the protein expression of HOXA9 in MG63 cells (**Figure 3C**). Interestingly, the expression of miR-196 was inversely correlated with the expression of HOXA9 (**Figure 3D**). Following, the protein expression of HOXA9 in MG-63 cells was reduced by si-HOXA9 (**Figure 3E**). We then co-transfected miR-196 inhibitor and si-HOXA9 into MG-63 cells to validate the hypothesis that HOXA9 was a mediator in the miR-196 induced cell proliferation and migration inhibition process. We found the transfection of si-HOXA9 could attenuate the effect of miR-196 inhibitor on cell proliferation and migration by comparing the rates of cell growth and migration in miR-196 inhibitor and si-HOXA9 transfected groups with the only miR-196 inhibitor transfected group (**Figure 3F**, **3G**). Taken together, the results suggested that miR-196 binds directly to the 3'-UTR of HOXA9 to regulate OS cell proliferation and migration.

Discussion

It has been widely recognized that miRNAs can regulate diverse cellular processes and can function as either tumor suppressors or tumor oncogenes [12]. Indeed, it is possible that one single miRNA may have opposite roles in different cancer types and the reason for that may be the difference in biological function of the miRNA targets [11, 12, 20]. If a miRNA has a dominant effect on oncogenic molecules, the miRNA will play a tumor suppressor role [12, 20]. Otherwise, the miRNA will function as a tumor oncogene [12, 20]. Therefore, investigating the downstream target of a specific miRNA is important to understand the role of miRNA.

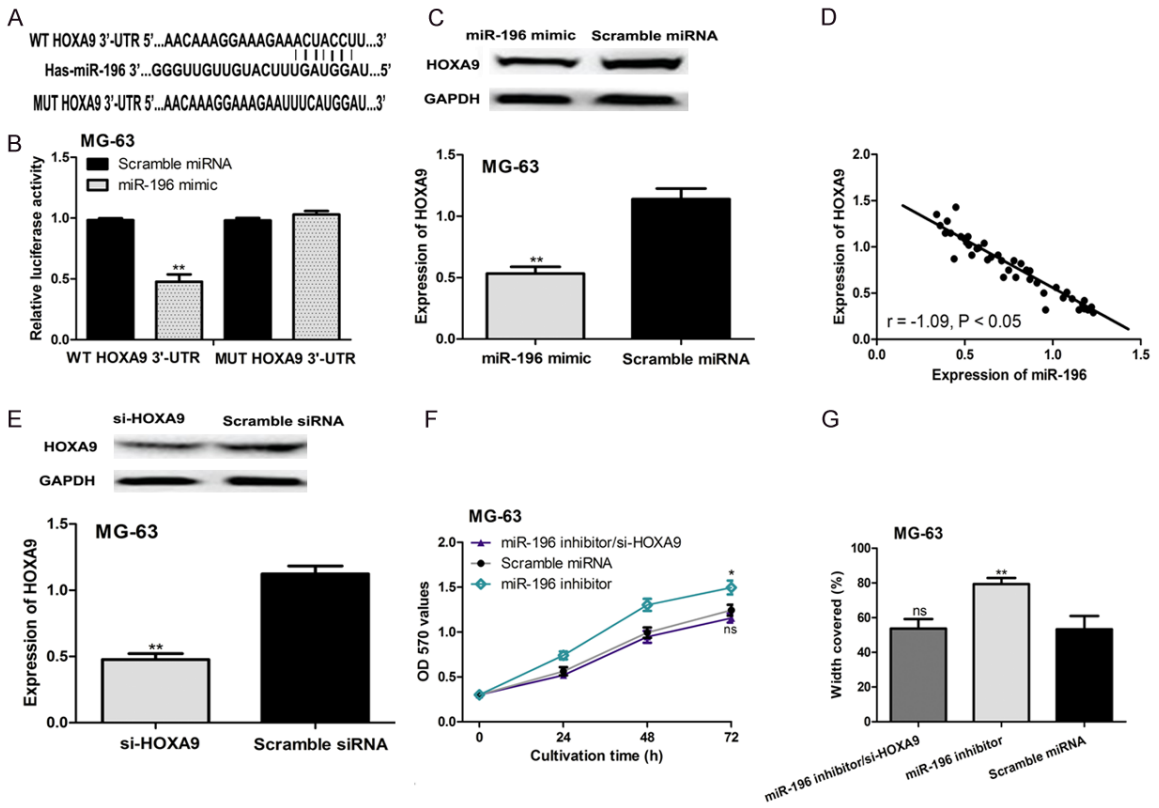

Figure 3. miR-196 directly targets HOXA9 in OS. (A) The putative binding sequences for miR-196 in the 3'-UTR of HOXA9. (B) Luciferase activity of the cells containing miR-196 mimic with WT HOXA9 3'-UTR or MUT HOXA9 3'-UTR. (C) Western blot to detect the expression level of HOXA9 in MG-63 cells with miR-196 mimic or scrambled miRNA transfection. (D) The result of correlation analysis between miR-196 and HOXA9 level in OS tissues. (E) Western blot to detect the expression level of HOXA9 in MG-63 cells with si-HOXA9 or scrambled siRNA transfection. (F) MTT assay to detect the cell proliferation and (G) Wound healing assay to detect the cell migration of MG-63 cells with both miR-196 inhibitor and si-HOXA9 or miR-196 inhibitor. (ns not significant, *P < 0.05 and **P < 0.01). miR-196, microRNA-196; HOXA9, homeobox A9; OS, osteosarcoma; UTR, untranslated region; WT, wild type; MUT, mutant.

The expression patterns of miR-196 have been implicated in various cancer types include glioblastoma, hepatocellular carcinoma, and breast cancer but not in OS [14-16]. Therefore, in this study, we investigated the levels of miR-196 in OS tissues and cell lines and found its expression was significantly reduced when compared with noncancer tissues and normal osteoblast cell line. Interestingly, patients with low miR-196 expression tended to have a worse overall survival compared to those with high miR-196 expression.

HOXA9 is a homeodomain transcription factor that plays an essential role in cancers [21, 22]. A recent study revealed that HOXA9 was overexpressed in OS and its expression could be regulated by miRNA named miR-182 [23]. Since miR-196 was located in the HOX family gene and one target can be regulated by different miRNAs [12, 13], we were interested to investigate the relationship between miR-196 and HOXA9. We found HOXA9 expression can be regulated by miR-196 and was inversely correlated with miR-196 in OS, which provides direct evidence that miR-196 can directly target HOXA9 in OS. Furthermore, we also assessed the effect of miR-196 upregulation and downregulation on the growth and migration abilities of OS cells. We found the cell proliferation and migration abilities of OS cells can be impaired by miR-196 upregulation or HOXA9 downregulation. Further co-transfection of the si-HOXA9 and miR-196 inhibitor into the OS cells revealed that downregulation of HOXA9 antagonized miR-196 downregulation-mediated cell proliferation and migration stimulation. The results suggest that downregulated miR-196 in OS tissue and cell lines may correlate with OS progression.

In conclusion, our study provides novel insight into the molecular mechanism of OS progression, indicating that the miR-196 inhibits HOXA9 to promote proliferation and migration of human OS cells. Meanwhile, our study may provide a new biomarker for ttargeted therapy of OS.

Acknowledgements

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Disclosure of conflict of interest

None.

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