MicroRNA-145 Suppresses Osteosarcoma Metastasis via Targeting MMP16

Bin Chen a Zhonglian Huang a Yuantao Zhang a Youbin Chen a Zhanchun Li b

a Department of Orthopedics, the First Affiliated Hospital, Shantou University Medical College, Shantou, b Department of Orthopedic, Zhejiang Provincial Hospital of TCM, Hangzhou, P.R.China

Key Words
MiR-145 • Osteosarcoma • Metastasis • MMP16

Abstract
Background: Metastasis is a leading cause of mortality for osteosarcoma (OS) patients, and its molecular pathological mechanisms remain to be elucidated. Previous studies have suggested a significant role of microRNAs (miRNAs) in the control of cancer cell migration and invasion.
Methods: Real-time PCR was used to screen the differentially expressed miRNAs between OS with or without metastasis, and miR-145 underexpression was observed in metastatic OS. Luciferase assay was performed to validate the target gene.
Results: Further, we identified three genes, MMP16, ADAM17 and metadherin, as possible targets of miR-145. We identified MMP16 as a target gene of miR-145 and ruled out ADAM17 and metadherin as targets in OS using a dual luciferase reporter system. Subsequently, we determined and compared the expression level of MMP16 in human OS samples and showed that the mRNA and protein levels of MMP16 were significantly up-regulated in primary OS with metastasis compared with those without metastasis. We also altered miR-145 expression by transfecting OS cells with miR-145 mimics or inhibitors. MMP16 expression was similarly downregulated in the cells transfected with miR-145 mimics or MMP16-specific siRNA, and the invasive and migratory capability of those cells was significantly suppressed compared with negative controls. MMP16 expression was consistently significantly upregulated in the cells transfected with miR-145 inhibitors, and the invasive and migratory capability of those cells was significantly promoted compared with negative controls.
Conclusion: Our results suggest that miR-145 functions as a tumor metastasis suppressor gene by down-regulating MMP16 and may be a potential target in osteosarcoma treatment.

Introduction

Osteosarcoma (OS) is the most frequent primary malignant bone tumor that affects children and adolescents. The incidence of this tumor worldwide is estimated to be 4 million/year, with a peak incidence at 15-19 years [1]. Unfortunately, no significant improvements
regarding patient survival have been reported for three decades, especially for those who exhibited metastasis at initial diagnosis [2]. OS is characterized by its highly metastatic features (approximately 15%-20%), particularly in the lung, which is a primary contributor to mortality in OS patients [3, 4]. A previous study indicated that non-metastatic patients have a 5-year survival rate of 60-70%, whereas patients with metastasis have a survival rate of less than 30% [5]. Hence, it is of clinical and academic significance to develop new therapeutic strategy to prevent early-stage metastases in OS and improve the prognosis of OS patients.

MicroRNAs (miRNAs) are major factors in the genetic network that regulates a number of significant pathophysiological processes, including the initiation and progression of cancers [4]. miRNAs are dysregulated in OS, resulting in abnormal expression of target genes. For example, the down-regulation of miRNA-143 caused abnormal up-regulation of its target, Bcl-2, which suppressed cellular apoptosis and promoted the proliferation of OS [6]. Further, the expression of Fas in OS can be down-regulated by overexpressed miRNA-20a, which facilitated the formation of metastasis in OS by alternating the relevant phenotype [7]. MiR-206 may play a therapeutic role in the management of OS by decreasing cellular viability, promoting apoptosis, and suppressing cell invasiveness and migration [8].

To better understand the mechanisms that underlie the role of miRNAs in OS formation, we performed real-time PCR to screen candidate miRNAs (miR-145, -143, 193b, -28, -149 and -99b) that previously reported to be differentially expressed in metastatic OS [9] and confirmed the downregulation of miR-145 in metastatic OS compared with OS without metastasis. Further, we identified three potential targets of miRNA and validated MMP16 as an miR-145 target. We also assessed the effect of miR-145 upregulation and downregulation on MMP16 expression and its influence on the invasive and migratory ability of OS cells.

Materials and Methods

Ethics statement
Research that involved human tissue samples was approved by the Ethics Review Committee of Zhejiang Chinese Medicine University, Hangzhou, Zhejiang, China, and all participants provided written informed consent.

Human tissue samples
A total of 31 osteosarcoma (OS) cases with metastasis and 35 osteosarcoma cases without metastasis were included from patients who received surgical removal at the first affiliated hospital of Shandong University, Medical College, and the first affiliated hospital of Zhejiang Chinese Medicine University. After resection, biopsies were immediately snap-frozen in liquid nitrogen and stored at -80°C. Hematoxylin-eosin (H&E) was used to stain the slide sections of each sample for histopathological assessment. Those patients who received chemo- or radio-therapy were excluded from this study. The classification of clinical stage was performed for these OS patients in compliance with the sixth version of the tumor–node–metastases (TNM) classification designed by the International Union Against Cancer (UICC). The clinicopathological features are described in Table 1.

RNA extraction and quantitative real-time PCR of miRNAs
The mirVana miRNA Isolation kit (Ambion, Austin, TX) was used to extract total RNA from cell lines and clinical samples according to manufacturer’s instructions. Total RNA (20 ng) was used to generate cDNA using the specific RT-primer from the TaqMan gene expression assay kit (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed to determine the expression levels of miR-145, -143, 193b, -28, -149 and -99b and MMP16 in an ABI 7500 (Applied Biosystems, Foster City, CA). U6 was used as an internal control, and each experiment was repeated at least three times. The sequences of each primer was summarized in Table 1. 2\(^{-\Delta\Delta CT}\) method was used to analyze the results of real-time PCR analysis with one in non-metastatic samples as calibrator.

Western blot analysis
Culture cells or tissue samples were homogenized and lysed. The BCA Assay kit (Thermo Fisher Scientific, DE, USA) was used to measure protein concentrations. After separation of protein samples using SDS-PAGE, the proteins were electrotransferred onto PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% milk without fat, cultured at 4°C overnight with the primary antibody and then incubated with secondary antibody coupled horseradish peroxydase. Enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA) was performed with the following antibodies: anti-β-actin (Sigma-Aldrich, St.Louis, MO, USA) and anti-MMP16 (SCBT, Santa Cruz, CA). The bands of target protein obtained in the western blot analysis were densitometrically analyzed and the relative density were grouped and compared between those with and without metastasis.

**Cell line**

Human OS cell lines (MG-63 and Saos-2) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MG-63 and Saos-2 cells were cultured in MEM/EBSS (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), 50
U/ml penicillin and 50 mg/ml streptomycin in a humidified incubator with 5% CO\textsubscript{2} at 37°C.

Cell transfection

MiR-145 mimics (50 nM) and inhibitors (50 nM) and MMP16-specific siRNA (50 nM) were purchased from Ambion (Austin, TX). Oligonucleotides with scramble sequence without any known target in human genome was used a control of miRNAs or siRNA. Scramble sequence with fluorescence was used to determine the transfection efficiency. The empty pmirGLO vector without any insert was used as a control of plasmid transfection. The sequences of each was summarized in Table 1. Lipofectamin 2000 (Invitrogen, Carlsbad, CA) was used for the transfection.

Wound healing assay

Prior to wounding, the conditions for incubation and transfection of MG-63 and Saos-2 were optimized to guarantee a homogeneous and viable cell monolayer. Equivalent amounts of MG-63 and Saos-2 cells (5.0×10\textsuperscript{4}) were plated onto 24-well plates without antibiotics one day prior to transfection. Subsequently, the cells were transfected with MiR-145 mimics and inhibitors and MMP16-specific siRNA. When the cells grew to confluence of approximately 90% at 48 h after transfection, a sterilized plastic 100 μL micropipette tip was used to make an artificial homogenous wound onto the monolayer. Next, the cells were washed with serum-free medium to remove debris. The cells that had migrated into the wound were determined at different time points. Under an inverted microscope (40× objective) (Leica, Solms, Germany), the cells with extended protrusions from the border of the wound or that had migrated into the wounded area were observed and photographed. A total of three areas were randomly included from each well, and each experiment was performed with triplicate wells of cells.

In vitro Matrigel invasion assay

Cell invasiveness in vitro was evaluated by the capacity of cells to cross a layer of extracellular matrix in BioCoat Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA). At 48 h after transfection, the cells were trypsinized and seeded at a density of 5.0×10\textsuperscript{4}/well. Medium containing 10% FBS, which was used as a chemo-attractant, was transferred to the lower chamber. After culture for 20 h, a cotton-tipped swab was used to discard non-invading cells from the top of the Matrigel, and the invasive cells were fixed, stained and counted. Data were collected from three chambers in each experiment.

Luciferase reporter assay

PCR was used to amplify the full-length 3′UTRs of MMP16, ADAM17 and metadherin, which contained putative miR-145 binding sites, into pmirGLO vector (Promega, Fitchburg, WI, USA) to produce the wild-type constructs. An overlap extension PCR assay was performed to introduce mutations using a site-directed mutagenesis kit (Stratagene, Heidelberg, Germany).

After incubation in 96-well plates, the cells were transfected with 100 ng of constructs containing either wild-type or mutant 3′UTR of MMP16, ADAM17 and metadherin. At 24 h post-transfection, the Dual-Glo Luciferase Assay System (Promega, Fitchburg, WI, USA) was used to determine luciferase activity. Renilla luciferase activity was transferred to relevant firefly luciferase activity and prepared as a plot of percentage.

Statistics analysis

Statistical analysis was conducted using SPSS V20.0 software (SPSS Inc, Chicago, IL). Significance among the groups was determined using a Student’s t-test and one-way ANOVA analysis, and Chi square test and logistic regression analysis were used to compare the clinicopathological data between the two patient groups. Statistical significance was set at P < 0.05.

Results

To study the effect of miRNAs on metastasis in OS in the second peak of incidence of the disease, we collected tumor tissue samples from 31 osteosarcoma cases with metastasis and 35 osteosarcoma cases without metastasis. Clinicopathological features are described in Table 1. In this study, the expression levels of miR-145, -143, 193b, -28, -149 and -99b were
examined by qRT-PCR in 31 osteosarcoma cases with metastasis and 35 osteosarcoma cases without metastasis, and miR-145 underexpression was identified in metastatic OS compared with non-metastatic OS (* P < 0.05, ** P < 0.01).

To identify the target of miR-145 in osteosarcoma, we searched the online microRNA database (www.mirdb.org) and obtained several candidate target genes. Through additional analysis of the physiological and pathological function of each candidate gene, we narrowed down the candidate genes to MMP16, ADAM17 and metadherin, and the potential binding sequence in the 3’ UTR of each possible target gene is presented.

Fig. 1. The expression levels of miR-145, -143, -193b, -28, -149 and -99b were determined by qRT-PCR in 31 osteosarcoma cases with metastasis and 35 osteosarcoma cases without metastasis, and miR-145 underexpression was identified in metastatic OS compared with non-metastatic OS (* P < 0.05, ** P < 0.01).

Fig. 2. Three genes, MMP16, ADAM17 and metadherin, were predicted to be possible target of miR-145, and the potential binding sequence in the 3’ UTR of each possible target gene is presented.

Fig. 3. (A) The luciferase activity of the cells cotransfected with miR-145 mimics and wild-type MMP16 3’UTR was lower than those transfected with negative controls, whereas the introduction of the mutant with the potential “seed sequence” in the 3’UTR of MMP16 nearly completely abolished this effect; (B) No significant difference was observed between the wild-type or mutant ADAM17 and negative control; (C) No significant difference was observed between the wild-type or mutant metadherin and negative control (* P < 0.05, ** P < 0.01).
Fig. 4. (A) The mRNA levels of MMP16 were significantly upregulated in primary OS with metastasis compared with those without metastasis (0.99 ± 0.16 versus 3.23 ± 0.52); (B) The protein expression of MMP16 was increased in the OS with metastasis (* P < 0.05, ** P < 0.01).

sequence in the 3' UTR of each target gene is presented in Fig. 2. To confirm that miR-145 regulates candidate genes through interacting with the 3' UTR, we cloned the 3' UTR and constructed the corresponding mutant with a mutation in the predicted microRNA-binding site located downstream of the luciferase gene (Fig. 2). Next, we transfected the cells with the construct containing the wild-type or mutant 3'UTR of the candidate gene, together with either miR-145 mimics or negative controls. Luciferase activity of the cells cotransfected with miR-145 mimics and wild-type MMP16 3'UTR was lower than those transfected with negative controls, whereas the introduction of the mutant with the potential "seed sequence" in the 3'UTR of MMP16 nearly completely eliminated this effect (Fig. 3), indicating that miR-145 negatively regulated MMP16 expression at the translational level by binding to the "seed sequence" in the 3' UTR of MMP16. In addition, no differences were identified between the wild-types or mutants of the other two candidate genes (ADAM17 or metadherin) and miR-145 mimics or negative controls (Fig. 3), which ruled out the two genes as the target gene of miR-145.

To confirm this regulatory association between MMP16 and miR-145, we performed real-time PCR and western blot analysis and compared the expression levels of MMP16 in the 31 osteosarcoma cases with metastasis and 35 osteosarcoma cases without metastasis. As shown in Fig. 4A, the mRNA levels of MMP16 were significantly up-regulated in primary OS with metastasis compared with without metastasis (0.96 ± 0.17 vs. 3.24 ± 0.56), and the

Fig. 5. (A) Transfection of miR-145 mimics and anti-MMP16 siRNA similarly suppressed both mRNA and protein expression levels of MMP16 in MG-63 cells; (B) Introduction of miR-145 inhibitors significantly promoted the mRNA and protein expression of MMP16 in MG-63 cells (* P < 0.05, ** P < 0.01); (A) Transfection of miR-145 mimics and anti-MMP16 siRNA similarly suppressed both mRNA and protein expression levels of MMP16 in Saos-2 cells; (B) Introduction of miR-145 inhibitors significantly promoted the mRNA and protein expression of MMP16 in Saos-2 cells (* P < 0.05, ** P < 0.01).
protein expression of MMP16 was consistently increased in the OS with metastasis (Fig. 4B). These data further confirmed the negative regulatory relationship between miR-145 and MMP16.

To investigate the role of miR-145 in the control of OS metastasis, scramble controls, miRs-145 mimics and inhibitors were transfected into OS cells (MG-63 and Saos-2 cells). Transfection of miR-145 mimics and anti-MMP16 siRNA similarly suppressed both mRNA and protein expression levels of MMP16 in both MG-63 and Saos-2 cells (Fig. 5A and C), whereas introduction of miR-145 inhibitors significantly promoted mRNA and protein expression of MMP16 in MG-63 and Saos-2 cells (Fig. 5B and 5D).

To determine and compare the migratory capability of differently treated OS cells (MG-
Chen et al.: MiR-145 Suppresses OS Metastasis via Targeting MMP16

63 and Saos-2), scratch tests were performed in vitro. Migration was much slower in OS cells transfected with miR-145 or anti-MMP16 siRNA compared with those transfected with scramble controls. Introduction of miR-145 inhibitors significantly promoted the migratory capability of the cells (Fig. 6: scratch test performed in MG-63 cells, Fig. 7: scratch test performed in Saos-2 cells). Further, the invasive property of differently treated OS cells (MG-63 and Saos-2) was examined by Transwell-Matrigel penetration assay, which indicated that much fewer cells penetrated through the gel-membrane section when OS cells were transfected with miR-145 or anti-MMP16 siRNA compared with those transfected with the scramble controls, whereas invasive ability was significantly promoted by transfection of miR-145 inhibitors (Fig. 8: transwell test performed in MG-63 cells, Fig. 9: transwell test performed in Saos-2 cells).

**Discussion**

miRNAs have been shown to be a significant contributor to the growth and migration of tumor cells; however, their possible effects on metastasis in OS are mostly unknown. MiR-145 was first found in mouse heart muscle in 2002 [10]. Subsequently, in 2003, Michael et al. confirmed its expression in humans and identified significant underexpression of miR-145 in neoplastic and precancerous colorectal tissue [11]. MiR-145 has been shown to be involved in the control of cell proliferation and apoptosis, stem cell differentiation and neural development. MiR-145 has been deemed a tumor-suppressive gene, as demonstrated by the observation that miR-145 expression is significantly lower in a variety of human cancers, including breast cancer, colon cancer, hepatocellular carcinoma, lung cancer, prostate cancer and ovary carcinomas [12–20]. MiR-145 acts as a tumor inhibitor by inhibiting the expression of many oncogenic genes. For example, miR-145 inhibits the development and angiogenesis of tumors by acting on insulin receptor substrate-1, c-Myc, and p70S6K1 [12-14, 16]. MiR-145 plays a role in p53-initiated cell cycle arrest by negatively regulating p21 [19-21]. In this study, the expression levels of miR-145, -143, 193b, -28, -149 and -99b were examined by qRT-PCR in 31 osteosarcoma cases with metastasis and 35 osteosarcoma cases without
metastasis, and miR-145 underexpression was identified in metastatic OS compared with non-metastatic OS. To identify the target(s) of miR-145 in osteosarcoma, we identified three genes, MMP16, ADAM17 and metadherin, by searching the online microRNA database as possible target genes of miR-145. Next, we constructed a dual luciferase reporter system to examine the regulatory relationship between miR-145 and the candidate target genes. Luciferase activity of the cells cotransfected with miR-145 mimics and wild type MMP16 3'UTR was lower than those transfected with negative controls, whereas the introduction of the mutant with the potential “seed sequence” in the 3’UTR of MMP16 nearly completely abolished this effect. No differences were identified between the wild-types or mutants of the other two candidate genes (ADAM17 or metadherin) and miR-145 mimics or negative controls (Fig. 3), which ruled out the two genes as target gene of miR-145. These findings identified MMP16 as a target gene of miR-145 in OS.

MMPs have been associated with bone resorption, angiogenesis, rheumatoid arthritis and tumor cell invasion [22, 23]. The enzymatic function of MMPs is rigorously regulated at different levels. MMP16 can directly degrade a few matrix molecules and trigger pro-MMP2 (gelatinase A). MMP2 hydrolyzes collagen type IV and other connective tissue after activation, which facilitates the migration or invasion of the cells. MMP2 is considered to be one of the most significant MMPs in cell migration and tissue remodeling [24–28]. In this study, we determined and compared the expression level of MMP16 in 31 osteosarcoma cases with metastasis and 35 osteosarcoma cases without metastasis and demonstrated that the mRNA levels of MMP16 were significantly up-regulated in primary OS with metastasis compared with those without metastasis, and the protein expression of MMP16 was consistently increased in the OS with metastasis. We also altered miR-145 expression bidirectionally by transfecting the OS cells with miR-145 mimics or inhibitors and found that MMP16 expression was similarly downregulated in the cells transfected with miR-145 mimics or MMP16-specific siRNA. The invasive and migratory capability of those cells was significantly suppressed compared with negative controls [29, 30]. MMP16 expression was consistently significantly upregulated in the cells transfected with miR-145 inhibitors, and the invasive and migratory capability of those cells was significantly promoted compared with negative controls. These results are consistent with a previous report that suggested that lower MMP16 expression significantly reduced the migration of other cancer cell types [31].

In summary, we demonstrated that miR-145 functions as an miRNA in human osteosarcoma cells that inhibits metastasis and that introduction of miR-145 significantly inhibited the metastasis of OS cells. Further in vivo studies are needed to confirm the long-term effectiveness and safety of nucleic acid treatment. We also validated MMP16 as a target gene of miR-145 in OS using a dual luciferase reporter system and demonstrated that miR-145 suppressed OS metastasis via targeting MMP16.

Disclosure Statement

No potential conflicts of interest were disclosed.

References


