Original Article

MicroRNA-145 targets vascular endothelial growth factor and inhibits invasion and metastasis of osteosarcoma cells

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MicroRNAs are important gene regulators that play a profound role in tumorigenesis. MicroRNA-145 (miR-145), an important member in the family of microRNAs, is under-expressed in several types of tumors and acts as a tumor suppressor. The role and probable pathways of miR-145 in osteosarcoma carcinogenesis are still unknown. In this study, we found that miR-145 was significantly under-expressed in osteosarcoma tissues, and the over-expression of miR-145 could inhibit invasion and angiopoiesis of osteosarcoma cells. Furthermore, the results showed that vascular endothelial growth factor (VEGF) expression was down-regulated in osteosarcoma cells after miR-145 transfection. On the basis of these results, we performed the luciferase assay and verified that miR-145 could down-regulate VEGF at the translational level by partially binding to VEGF 3’ untranslated region (3’UTR). Therefore, it can be concluded that miR-145 can inhibit invasion and metastasis of osteosarcoma cells. One of the mechanisms is the down-regulation of VEGF expression by miR-145 by binding to the 3’UTR of VEGF mRNA specifically. These novel findings may have extensive implications for an effective gene therapy of osteosarcoma.

Keywords miR-145; vascular endothelial growth factor; osteosarcoma; invasion; metastasis

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Introduction

As the most common osteogenic malignant tumor, osteosarcoma is characterized by a high level of malignancy, relapse, metastasis, and poor prognosis. About 5.6 per million children below 15 years of age suffer from osteosarcoma annually [1]. Despite recent advancements, such as wide tumor excision, adjuvant chemotherapy, and radiotherapy in therapeutic strategies, the survival rate of osteosarcoma patients is less optimistic with the 5-year survival rate being only 55%–68% [2]. Invasion and metastasis of carcinoma cells are predictive in the prognosis of osteosarcoma patients that always plays critical roles in the pathogenesis of this disease [3].

MicroRNAs are small non-coding RNA molecules that exhibit a high-degree conservation of structure and function in metazoa. They regulate gene expression mainly through affecting productive translation and mRNA stability [4,5]. MicroRNAs exist in two forms of pre-microRNAs and mature microRNAs. Only the mature microRNAs mediated by two RNaseIII endonucleases Dicer and Drosha [6] play a key biological role. Mature microRNAs, which contain about 22 nucleotides, are complementary to genomic regions and incorporate into the RNA-induced silencing complex. The microRNA complex binds to the partially complementary binding sites located in the 3’ untranslated region (3’UTR) of target mRNA to inhibit protein translation [6–8]. They can also function by cleaving a target mRNA, in which case the microRNAs may target sequences outside the 3’ UTR [8,9]. Increasing evidence indicated that microRNAs are implicated in the pathogenesis of a variety of diseases, notably neoplasms. A conclusion can be inferred that microRNAs can act as oncogenes or tumor suppressors, which exerts a key function in tumorigenesis [10,11]. It is predicted that each microRNA can have more than 100 target genes [12]. MicroRNA-145 (miR-145), an important member in the family of microRNAs, is under-expressed in several types of tumors. Expression of miR-145 can inhibit cell growth, as observed in gastric cancer, breast cancer, prostate cancer colon cancer, etc. [13–16]. It has been proved that miR-145 affects the proliferation, differentiation, and apoptosis of cells by targeting c-Myc, IRS-1, BNIP3, and pluripotency factors including OCT4, SOX2, and KLF4 [14,16–18]. Nevertheless, the role and probable pathway of miR-145 in osteosarcoma carcinogenesis, especially the effect in invasion and metastasis of cancer cells, still need further study.
In this study, we found miR-145 was under-expressed in osteosarcoma tissues, and confirmed that the over-expression of miR-145 could inhibit invasion, metastasis, and angiogenesis of osteosarcoma cells. Furthermore, we found that vascular endothelial growth factor (VEGF) which involved both vasculogenesis and angiogenesis was a direct target gene of miR-145.

Materials and Methods

Human tissues and cell lines
Human osteosarcoma tissues and related normal bone tissues were surgically obtained from patients in Union Hospital (Wuhan, China) and diagnosed by an independent pathologist. Human osteosarcoma cell line MG63 was purchased from China Center for Type Culture Collection (Wuhan, China). Human umbilical vein endothelial cell line ECV304 was kindly provided by Molecular Biology Laboratory of Huazhong University of Science and Technology (Wuhan, China). Cells were cultured in RPMI 1640 medium (Boshide Biology, Wuhan, China), which was supplemented with 105 U/l penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (Gibco, Grand Island, USA), and replaced every other day in a humidified 37°C incubator with 5% CO2.

Reagents
miR-145 mimics, mimics negative control (NC), and Cy3-labeled mimics NC were purchased from Ribobio Biology, Inc. (Guangzhou, China). Lipofectamine 2000 and Trizol were bought from Invitrogen, Inc. (Carlsbad, USA). Mouse anti-human VEGF monoclonal antibody and GAPDH monoclonal antibody were purchased from Boshide Biology, Inc. (Wuhan, China). TaqMan microRNA isolation kit, TaqMan microRNA reverse transcription kit, TaqMan universal PCR master mix, and TaqMan microRNA assay kit were purchased from Applied Biosystems, Inc. (Foster city, USA). Matrigel and transwell chamber were from BD, Inc. (Franklin, USA). pMIR-Report vector was purchased from Ambion, Inc. (Austen, USA). Restriction enzyme SpeI and HindIII were purchased from TaKaRa (Dalian, China). Dual-Glo luciferase assay kit was purchased from Promega (Sunnyvale, USA).

Quantitative real-time reverse transcriptase-polymerase chain reaction analysis of miR-145 expression in tissues
Twenty-eight osteosarcoma tissue samples diagnosed by pathological examination and 28 normal bone tissue samples from the same patients were investigated. The edge of osteosarcoma tissues were the tissues between the osteosarcoma and adjacent normal tissues. It was proved histologically that the normal tissue samples contained no tumor cells. Total RNA was extracted from the center of osteosarcoma tissues, the edge of osteosarcoma tissues, and normal bone tissues, respectively, using TaqMan microRNA isolation kit. After reverse transcription, mature miR-145 expression was quantified using TaqMan microRNA assay kit as well as TaqMan universal PCR master mix. The polymerase chain reactions (PCRs) were carried out at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 40 s, and 70°C for 1 s. All reactions were run in triplicate. The expression level of miR-145 in each sample was measured in terms of threshold cycle value and normalized to U6, which was used as the internal control [19].

Transfection
MG63 cells were seeded in 6-well plates during the exponential phase of growth, then randomly allocated into three groups: non-transfected group (blank group), miR-145 transfected group, and NC transfected group. The cells in miR-145 transfected and NC transfected groups were transfected with the miR-145 mimics and mimics NC, respectively, using Lipofectamine 2000 according to the manufacturer’s instruction. The Cy3-labeled mimics NC was transfected to MG63 cells under the same conditions and transfection efficiency was evaluated according to the level of Cy3 fluorescence labeling.

Measuring the expression level of miR-145 and VEGF in transfected cells
The cells in each group were harvested 48 h after transfection. Total RNA and protein were extracted, respectively. The miR-145 expression was evaluated by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) as described previously [19]. The VEGF protein expression level was evaluated by western blot. After being quantified, protein (50 μg) from each group was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. According to the protocol, the membranes were blotted with the primary antibody and horseradish peroxidase-conjugated secondary antibody, the protein bands were visualized using an enhanced chemiluminescence kit. The expression level of VEGF was normalized to the expression of GAPDH, which was used as the internal control. Each sample was measured in triplicate.

Cell adhesion assay
The cells transfected for 48 h in each group were suspended in complete medium, and then seeded into a 96-well plate which has been coated with 100 ml of 20 mg/ml matrigel at a density of 5 × 104 cells per well. The cells were incubated at 37°C in serum-free complete medium for 2 h. Then, the wells were washed three times with phosphate-buffered saline (PBS), and the remaining cells...
were fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were stained with 0.1% crystal violet and washed three times with PBS to remove the free dye. After being extracted with 10% acetic acid, the absorbance (A value) of the samples was read at 570 nm. The experiment was repeated in triplicate.

**Transwell invasion assay**
The upper transwell chambers were coated with 50 ml of 20-mg/ml matrigel for filtering; the lower chambers were added with culture medium. The primed transwell chambers were placed in 24-well plates. The cells in each group were suspended in serum-free medium and placed into the upper chambers. After a 24-h incubation, non-migrated cells were removed from the upper surface of the filter with a cotton swab. Fixation and staining of migrated cells were performed using 0.1% crystal violet. Cells were quantified in six different fields under a light microscope. The experiment was repeated in triplicate.

**Endothelial cell tube formation assay**
The endothelial cell tube formation assay was done as described previously [20]. Fifty microliters of growth factor-reduced matrigel was polymerized on 96-well plates. Human umbilical vein endothelial cell lines were serum starved in medium for 2 h. The cells were suspended in medium which was extracted from each group, then added to the matrigel-coated wells at the density of 5×10^4 cells/well and incubated at 37°C for 24 h. Quantification of anti-angiogenic activity was calculated by measuring the number of branch points under a light microscope.

**Luciferase assay**
The luciferase-UTR reporter constructs were generated by introducing the VEGF 3’UTR carrying a putative miR-145-binding site into pMiR-report vector. The human VEGF 3’UTR target site was amplified by PCR using the primers 5’-AGAGCTCCCCGGCAGAGAAGAGAC-3’ (forward) and 5’-TCAAGCTTGGAGGGCAGAGCTGAGTGTTA-3’ (reverse) [21]. After digesting of PCR product by SpeI and HindIII, the VEGF 3’UTR was ligated into pMiR-report vector. Then DH5α competent cells were transformed by the products, and positive clones were selected. A mutated 3’UTR of VEGF was introduced in the potential miR-145-binding site using a two-step PCR approach. The DNA sequence of cloned product was confirmed by sequencing. MG63 cells were co-transfected with the pMiR-report vector containing the 3’ UTR variants and miR-145 mimics or mimics NC. Luciferase activity was measured 48 h after transfection using the Dual-Glo luciferase assay system according to the manufacturer’s instructions. β-Galactosidase was used for normalization.

**Statistical analysis**
Data were expressed as the mean ± SD. One-way analysis of variance followed by Dunnett’s t-test was used to determine the significance of the differences between groups. \( P < 0.05 \) was considered statistically significant.

**Results**

**MicroRNA-145 expression is significantly down-regulated in osteosarcoma tissues**
The expression of mature miR-145 in 28 cases of osteosarcoma tissues, edge of osteosarcoma tissues, and normal bone tissues was investigated by qRT-PCR. Underexpression of miR-145 was observed in 25 of 28 osteosarcoma tissues in comparison with the corresponding normal tissues. miR-145 expression in the edge of osteosarcoma tissues was more than that in tumor tissues but less than that in the normal tissues (Fig. 1, \( P < 0.05 \)).

**Transfection efficiency**
The fluorescent signal was observed under fluorescence microscope. The transfection efficiencies were all above 60% in fluorescence-labeled control groups (Fig. 2).

**The over-expression of miR-145 and under-expression of VEGF after transfection**
miR-145 expression in the miR-145 transfected group was significantly up-regulated by 300.1% compared with the blank group [Fig. 3(A), \( P < 0.05 \)]. No significant difference was found in the expression of miR-145 between the NC and the blank groups [Fig. 3(A), \( P > 0.05 \)]. In the meantime, we investigated VEGF protein expression level

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**Figure 1** Relative expression of microRNA in osteosarcoma tissues, matched edge of osteosarcoma tissues, and normal bone tissues
Under-expression of miR-145 was observed in 25 of 28 osteosarcoma tissues in comparison with the corresponding normal tissues. The expression level of miR-145 was normalized by U6 as an internal control. All specimens were analyzed in triplicate. **\( P < 0.05 \).**

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in three groups using western blot, and found that miR-145 and VEGF were inversely expressed. VEGF expression level in the miR-145 transfected group was significantly down-regulated by 49.6% compared with that in the blank group [Fig. 3(B), \( P < 0.05 \)]. No significant difference was found in the level of VEGF between the NC transfected and the blank group [Fig. 3(B), \( P > 0.05 \)].

**miR-145 affects the adhesion and invasion of osteosarcoma cells**

Cell adhesion assay and Transwell-ECM assay were performed to investigate the role of miR-145 on the adhesion and invasion of osteosarcoma cells. The adhesion rate and invasion index of miR-145 transfected group were significantly reduced by 53.6% and 62.2% compared with those...
of the blank groups, respectively (Figs. 4 and 5, P < 0.05). No significant difference in adhesion rate and invasion index of cells was found between the NC and the blank groups (Figs. 4 and 5, P > 0.05).

miR-145 affects the angiopoiesis of osteosarcoma cells
Endothelial cell tube formation assay was performed and the quantification of anti-angiogenic activity was calculated by measuring the number of branch points under a light microscope. The angiopoiesis activity of cells in the miR-145 transfected group was significantly down-regulated by 56.1% compared with that in the blank group. However, no significant difference was observed on the angiopoiesis activity of cells between the NC and the blank group (Fig. 6, P < 0.05).

miR-145 targets VEGF by interacting with 3’ UTR
According to our previous study, the overexpression of miR-145 can down-regulate VEGF expression in osteosarcoma cells. To prove that miR-145 regulates VEGF through interacting with 3’ UTR, we cloned VEGF 3’ UTR and constructed the corresponding mutant with mutation in the predicted microRNA-binding site located in the downstream of the luciferase gene (pMiR-report). After co-transfection with pMiR-report vectors and miR-145 mimics or mimics NC, the cells over-expressing miR-145 were observed with a significant decline in luciferase activity compared with control groups (Fig. 7, P < 0.05). These data demonstrated that miR-145-regulated VEGF expression at the translational level by interacting with the 3’ UTR of VEGF.

Discussion
Recent studies have revealed that miR-145 is involved in the progression of various tumors by regulating the expression of multiple target genes, especially some genes which regulate proliferation and differentiation [14,16–18]. However, the role of miR-145 on genesis and development...
of tumors as well as its multiple target genes are yet to be illuminated. The effects of miR-145 on invasion and metastasis of tumors have rarely been reported. A previous study by Sachdeva and Mo [22] showed the significance of miR-145 as a tumor suppressor in cell invasion and metastasis by targeting MUC1 in vitro and in vivo. Although an optimal molecular target has been found in many kinds of tumors, the function of miR-145 on the invasion and metastasis of osteosarcoma still needs further investigation.

VEGF is a signal protein produced by cells stimulating vasculogenesis and angiogenesis, which locates in the system which supplies oxygen to tissues when blood circulation is inadequate. Overexpression of VEGF could lead to disease. Solid cancers cannot grow beyond a limited size without an adequate blood supply, while VEGF can promote endothelial cell proliferation and play a significant role in tumor angiogenesis and growth [23,24]. VEGF gene has been proved to be closely related to the invasion and metastasis of osteosarcoma and can be used as a clinically significant prognostic factor in osteosarcoma [25–27]. So some anti-VEGF monoclonal antibodies and oral VEGFR inhibitors that specifically bind to VEGF receptor to inhibit its VEGF signal have been under clinical development for the treatment of tumor [28]. However, due to the potential non-specific activities of these inhibitors and its disruption of normal vasculature, genetic approaches have been regarded as a promising alternative, especially physiological regulation of microRNA. miR-126 and miR-200b have been confirmed to regulate the VEGF signal [21,29]. Previous studies on microRNA have suggested that
miR-145 expressed in vascular smooth muscle cells has specific connection with the proliferation of abnormal vascular intima. miR-145 could also regulate VEGF signal by targeting both the ligand and its receptors. All the facts indicated that VEGF might be a target gene of microRNA [30,31]. However, the targeting effects and its receptors still need further investigation.

Riggi et al. [32] reported that miR-145 is one of the key players in differentiation and tumorigenicity of Ewing sarcoma cells. In our study, we found the under-expression of miR-145 in 25 of 28 osteosarcoma tissues comparing it to the corresponding normal tissues. This fact suggested that miR-145 is also involved in the progression of osteosarcoma. On the basis of these results, we transfected miR-145 mimics into osteosarcoma cells and investigated its function by measuring invasion and metastasis of the cells. We performed cell adhesion assay, transwell invasion assay, and endothelial cell tube formation assay and verified that the over-expression of miR-145 could inhibit the invasion and angiopoiesis of osteosarcoma cells. In the meantime, a new question was raised as to how miR-145 impacts on the metastatic potential of osteosarcoma cells. To search for the probable mechanism, we detected the VEGF protein expression level of transfected cells in three groups. The result that VEGF expression in the miR-145 transfected group was significantly down-regulated compared with that in the blank group added to a weight of evidence on the under-expression of miR-145 and VEGF in osteosarcoma cells. Furthermore, luciferase assay was performed to prove whether miR-145 regulates VEGF by the specific mechanism of microRNA. We constructed luciferase-UTR vectors that carried VEGF 3’ UTR as well as its corresponding mutant. The results showed that co-transfection with miR-145 and pMiR-report-3’ UTR resulted in a significant decline of luciferase activity, which indicated that VEGF is a direct target gene of miR-145. The expression of VEGF may be negatively regulated by miR-145 on the translational level through binding to VEGF 3’ UTR partially. According to previous research, VEGF is not the only target gene of miR-145 in osteosarcoma. To have a better idea of miR-145-mediated network, emphasis should be on searching for more target genes and exploring correlations among those genes.

In conclusion, our study suggested that miR-145 might inhibit the invasion and metastasis of osteosarcoma cells. One of the mechanisms is the negative regulation of miR-145 on VEGF expression by binding to the 3’ UTR of VEGF mRNA specifically. Furthermore, these results indicated that miR-145 could regulate the genesis and development of tumor by specific genes. Accordingly, there is a good reason to believe that miR-145 can serve as a target for effective gene therapies of tumors.

References


