MicroRNA-101 inhibits the metastasis of osteosarcoma cells by downregulation of EZH2 expression

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Abstract. MicroRNAs (miRNAs) are a class of small non-coding RNA molecules, which play regulatory roles at the post-transcriptional level by suppressing the translation of protein-coding genes or inducing mRNA cleavage. Dysregulated expression of miRNAs is involved in multiple types of cancers and plays important roles in regulating various biological processes including metastasis. miR-101 is downregulated in various types of cancer and functions as a suppressor of cell migration and invasion. Meanwhile, enhancer of zeste homolog 2 (EZH2) is associated with the metastatic potential of several aggressive tumors. In the present study, we reported that ectopic overexpression of miR-101 downregulated the expression level of EZH2 and significantly inhibited migration and invasion of osteosarcoma cells. In addition, knockdown of EZH2 by siRNA showed the same effect of miR-101 on migration and invasion. To conclude, these results indicate that miR-101 may act as a tumor suppressor in osteosarcoma, as it has a suppressive role in cell migration and invasion by targeting EZH2.

Introduction

MicroRNAs (miRNAs) are small non-coding regulatory RNA molecules, ~22 nucleotides in length, which suppress gene expression based on post-transcriptional suppression of target mRNAs by affecting mRNA translation or stability (1). It has been widely demonstrated that mRNAs are involved in the regulation of many cellular processes, such as cell differentiation, proliferation and apoptosis (2,3). Recently, increasing evidence indicates that miRNAs are closely associated with cancer, and aberrant expression of miRNAs is often reported in cancers (4,5). miRNAs can function as oncogenes or tumor suppressors, and play significant roles in tumorigenesis (6,7). Those miRNAs, such as miR-21 and miR-155, whose expression levels tend to be upregulated in tumors, may be considered as oncogenes (8,9). Meanwhile, some miRNAs, such as miR-143, miR-126 and miR-138, whose expression levels are usually downregulated in tumors, may be considered as tumor suppressors (10-12). These data indicate that miRNAs may be promising targets for tumor treatment.

Osteosarcoma is one of the most common primary malignant bone tumors and accounts for approximately 60% of all malignant bone tumors in childhood and adolescence (13). It is characterized by a high potential of lung metastasis (14), and for patients who develop lung metastasis, the survival rate is less than 30% (15). Therefore, to achieve a better prognosis, it is essential to identify the molecular mechanisms of osteosarcoma metastasis and to identify new therapeutic targets. Recent studies have confirmed the altered expression profile of miRNAs in osteosarcoma (16). Dysregulation of miRNAs, such as miR-183, miR-34a, miR-145 and miR-143, has been shown to be involved in the migration and invasion of osteosarcoma cells (17-20), which undoubtedly highlights the significance of miRNAs in osteosarcoma development and may reveal new insights into the mechanisms of metastasis.

MicroRNA-101 (miR-101) is a tumor-suppressor miRNA. It has been proven that miR-101 is downregulated in various types of cancer cells including lung cancer, gastric cancer, breast cancer and glioblastoma, and functions as a suppressor of cell migration and invasion (21-24). However, little is known concerning the functions of miR-101 in osteosarcoma. Therefore, it is of great importance to study the biological role and mechanism of miR-101 in osteosarcoma metastasis.

In the present study, we found that miR-101 expression in high-metastatic human osteosarcoma F5M2 cells was lower than that in low-metastatic human osteosarcoma F4 cells. Furthermore, ectopic overexpression of miR-101 inhibited migration and invasion of osteosarcoma cells by downregulating the expression level of EZH2, which was
closely correlated with tumor metastatic potential (25-27). Overall, miR-101 may play a suppressive role in osteosarcoma metastasis through EZH2 downregulation, indicating that it may be a novel promising therapeutic target for osteosarcoma.

**Materials and methods**

**Cell culture.** The human osteosarcoma F5M2 and F4 sublines, which are respectively high-metastatic and low-metastatic cell lines and originate from the same human osteosarcoma SOSP-9607 cell line, were established and reserved by our laboratory (28). F5M2 and F4 cells were maintained in RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (Sijiqing Co., China) and incubated at 37°C with 5% CO₂. The human osteosarcoma MG63 cell line was maintained under the same conditions except that MEM was used.

**Transfection.** Cells were transfected with miR-101 mimics and the NC mimics (negative control) (GenePharma Co., China) at a concentration of 100 nM, using Lipofectamine™ 2000 reagent (Invitrogen Life Technologies, USA) according to the manufacturer’s instructions. Efficiency of miR-101 transfection was detected by quantitative real-time PCR (qRT-PCR).

**Migration and invasion assays.** A total of 1x10⁶ cells in low-serum media containing 1% FBS was added to the upper chamber of a Transwell system (24-well insert; pore size, 6 µm; Corning, USA). Six hundred microliters of complete media containing 20% FBS was added to the lower chamber. In the migration assay, cells that migrated to the outer surface of the membrane were fixed with 95% ethanol and stained with crystal violet after 24 h of incubation. In the invasion assay, the upper chamber was precoated with Matrigel (1:8 dilution; BD Biosciences, USA). After 48 h incubation, the invaded cells were fixed and stained as described above. The cell numbers in 5 random fields were counted for each insert under a microscope (magnification, x200).

**Wound healing assay.** Adherent cell monolayers in 6-well plates were scratched with a 200-µl pipette tip. The cells were then cultured in complete culture media at 37°C with 5% CO₂. Wound healing ability was observed under microscopy after 0, 24 and 48 h (magnification, x200).

**MTT assay.** Cell proliferation capacity was detected using the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] procedure, which was performed in 96-well plates. In brief, at 48 h after transfection, F5M2 cells were harvested and seeded at a density of 4,000 per well with 200 µl complete culture medium. Each group contained 8 wells, and the wells without cells were used as blanks. Cell proliferation was documented every 24 h for 4 days; 20 µl of 5 g/1 MTT (Sigma, USA) was added into each well. After 4 h of incubation at 37°C, the media were removed and 150 µl dimethyl sulfoxide (DMSO; Sigma) was added into each well. The optical density (OD) was evaluated according to the absorbance value detected at 490 nm with a reference of 630 nm.

**Western blot analysis.** The western blot assay was carried out to detect the protein expression level of EZH2 following the standard method and this assay was performed with the EZH2 rabbit monoclonal antibody and the anti-β-actin mouse monoclonal antibody (Abcam, UK).

**Statistical analysis.** All experiments were performed at least in triplicate. The results are expressed as the mean values ± SD and performed using SPSS 19.0 software. The Student’s t-test was performed to determine the differences between groups, and P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression of miR-101 in the F5M2 cells is lower than that in the F4 cells.** To investigate the potential role of miR-101 in osteosarcoma metastasis, qRT-PCR analysis was carried out to compare miR-101 expression between the F5M2 and F4 cell lines. miR-101 expression in the F5M2 cells with high-metastatic potential was lower than that in the F4 cells with low-metastatic potential. The difference was statistically significant (P<0.05, Fig. 1A).

**Osteosarcoma cells transfected with miR-101 mimics show significant overexpression of miR-101.** SOSP-9607 and MG63 cells were separately transfected with the miR-101 mimics at a concentration of 100 nM. qRT-PCR analysis was performed to detect the expression level of miR-101 at 48 h after transfection. The expression level of miR-101 was significantly upregulated in both the F5M2 and MG63 cells after treatment with the miR-101 mimics, compared with the F5M2 or MG63 cells treated with the NC mimics, respectively (P<0.01, Fig. 1A and B). Meanwhile, there was no significant difference in miR-101 expression between the F5M2 cells treated with the NC mimics and untreated cells (Fig. 1A).

**miR-101 significantly inhibits the migratory and invasive capacities of osteosarcoma cells.** We used the Transwell assay to measure the migratory and invasive capacities of SOSP-9607 and MG63 cells. The results showed that osteosarcoma cells treated with the miR-101 mimics displayed significantly lower Transwell migration capacity.
Figure 1. Efficiency of miR-101 transfection is verified by qRT-PCR. (A) miR-101 expression in the F5M2 cells was significantly lower than that in the F4 cells. Moreover, F5M2 cells transfected with the miR-101 mimics (F5M2 101) expressed a higher level of miR-101 when compared with the non-transfected cells (F5M2). Moreover, there was no difference between cells transfected with NC (F5M2 NC) and the non-transfected cells. (B) Compared with the cells transfected with NC (MG63,NC), MG63 cells following transfection with miR-101 mimics (MG63,101) expressed a higher level of miR-101. *P<0.05, **P<0.01, compared with F5M2 or MG63 with NC; #P>0.05, compared with F5M2.

Figure 2. miR-101 inhibits migration and invasion of osteosarcoma cells in vitro. (A and B) Representative images of migrated and invaded SOSP-9607 cells under the microscope (magnification, x200). a, F4; b, F5M2 with miR-101; c, F5M2 with NC; d, F5M2. (C and D) F4 and F5M2 cells following transfection with miR-101 mimics (F5M2,101) showed less migratory and invasive ability than non-transfected F5M2 cells. (E and F) Representative images of migrated and invaded MG63 cells under a microscope (magnification, x200). a, MG63 with NC; b, MG63 with miR-101. (G and H) MG63 cells following transfection with miR-101 mimics (MG63,101) showed less migratory and invasive ability than cells transfected with NC (MG63,NC). *P<0.01, compared with F5M2 or MG63 with NC; #P>0.05, compared with F5M2.
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with the cells untreated or treated with the NC mimics (P<0.01, Fig. 2A and E). In the invasion assay, ectopic expression of miR-101 led to significantly decreased invasion of the osteosarcoma cells (P<0.01, Fig. 2B and F). These results indicate a functional role for miR-101 in downregulating the migration and invasion of osteosarcoma cells.

miR-101 significantly inhibits the wound healing ability of osteosarcoma cells. We performed a wound healing assay and found that F5M2 cells closed the wounds faster than F4 cells (P<0.01, Fig. 3). Meanwhile, the migratory potential of the F5M2 cells treated with miR-101 mimics was significantly decreased when compared with that of the cells untreated or treated with the NC mimics (P<0.01, Fig. 3).

miR-101 does not affect the proliferation of osteosarcoma cells. MTT assay was conducted to detect the potential role of miR-101 in the proliferation of osteosarcoma cells. The results indicated that there was no statistically significant difference between the F5M2 cells transfected with the miR-101 mimics and the F5M2 cells transfected with the NC mimics. Therefore, miR-101 had little effect on the proliferation of osteosarcoma cells (P>0.05, Fig. 4).

Figure 3. Wound healing assay. (A) Wound healing ability was observed under a microscope after 0, 24 and 48 h. (B) The data were analyzed and are summarized as width ratio (0, 24 and 48 h width/0 h width). The wound closure rates of the F5M2 cells transfected with the miR-101 mimics (F5M2,101) and the F4 cells were significantly lower than that of the F5M2 cells transfected with NC (F5M2,NC). Meantime, there was no statistically significant difference between the non-transfected F5M2 cells and F5M2 cells transfected with NC. *P<0.01, #P>0.05, compared with F5M2.

Figure 4. miR-101 has little effect on the proliferation of osteosarcoma cells in vitro. Every 24 h, the MTT assay was performed on two groups of F5M2 cells [F5M2 cells transfected with miR-101 mimics (F5M2,101); F5M2 cells transfected with NC mimics (F5M2,NC)]. The cell number was analyzed as the value of the absorbance at 490 nm with a reference wavelength of 630 nm. *P>0.05 is not considered statistically significant.
EZH2 is significantly downregulated by miR-101 and regulates the migration and invasion of osteosarcoma cells. To analyze the expression of EZH2 in osteosarcoma, western blotting was performed on SOSP-9607 and MG63 cells. The results showed that the protein expression level of EZH2 in the F5M2 cells was higher than that in the F4 cells (Fig. 5A-a). Moreover, EZH2 expression was downregulated in the F5M2 cells treated with the miR-101 mimics compared to cells treated with the NC mimics or the untreated cells (Fig. 5A-a). MG63 cells treated with the miR-101 mimics also exhibited a lower expression level of EZH2 than cells treated with the NC mimics (Fig. 5A-a). In addition, qRT-PCR analysis displayed the same tendency at the mRNA expression level in both osteosarcoma cell lines (Fig. 5B). As expected, the F4 cells expressed a higher level of miR-101 than that in the F5M2 cells (Fig. 1A). Furthermore, wound healing and Transwell assays were performed to assess the suppressive role of miR-101 in the migration and invasion of SOSP-9607 and MG63 cells. As expected, the F4 cells expressed a higher level of miR-101 than that in the F5M2 cells (Fig. 1A). Additionally, miR-101 was significantly upregulated after transfection with the miR-101 mimics in numerous biological processes of a wide range of cancers, including metastasis. Aggressive tumor cell metastasis is a key step during cancer progression, and it contributes to secondary tumor formation at distant sites. Studies have reported that several miRNAs, such as miR-126, miR-335 and miR-145, are metastasis suppressors (29,30). These data bring new insights into novel treatments of high-metastatic tumors including osteosarcoma, in which pulmonary metastasis is the leading cause of death (31).

**Discussion**

In recent years, it has been widely demonstrated that miRNAs play profound roles in cancer pathogenesis and are implicated in various biological processes. The downregulation of EZH2 by miR-101 suggests that miR-101 is a potential target for inhibiting osteosarcoma metastasis.
both the SOSP-9607 and MG-63 cell lines (Fig. 1), and ectopic miR-101 significantly inhibited the migratory and invasive abilities of the osteosarcoma cells (Figs. 2 and 3). On the basis of these results, this study demonstrated the tumor-suppressive role of miR-101 in osteosarcoma metastasis for the first time, and miR-101 might be proven to be a promising therapeutic target in osteosarcoma.

It has been shown that miR-101 has multiple targets through which it has regulatory roles in the biological behaviors of various cancer cells. A previous study by Cho et al (21) showed that overexpression of miR-101 inhibited the invasive ability of lung cancer cells through suppression of EZH2. Furthermore, Wang et al (22) demonstrated that miR-101 expression in gastric tumor tissues and cells was higher than that in non-tumor gastric tissues and cells, and exogenous miR-101 inhibited the proliferation, migration and invasion of gastric cancer cells by downregulating the expression of EZH2, COX-2, Mcl-1 and FOS.

Predicting and identifying miR-101 target genes is important to further study the regulatory role of miR-101 in tumorigenesis and can offer a potential new therapeutic strategy for osteosarcoma. To investigate the suppressive mechanism of miR-101 in osteosarcoma metastasis, several prediction programs were used to seek the potential targets of miR-101. Markedly, the programs (including Target Scan, Pic Tar, Micro Inspector and Mir Target 2) predicted that EZH2 is one of the targets of miR-101.

Enhancer of zeste homolog 2 (EZH2) functions in a protein complex called the polycomb repressive complex 2 (PRC2), which is involved in the trimethylation of histone3 lysine27 (H3K27) and may lead to epigenetic silencing of target genes implicated in many cellular processes, including cell cycle regulation, cell differentiation and tumorigenesis (32). Enforced expression of EZH2 was initially found in prostate cancer, and EZH2 overexpression is closely correlated to aggressive and metastatic diseases (33). Subsequently, EZH2 was also found to be broadly overexpressed in a wide range of cancer types, such as breast cancer, bladder cancer, colon cancer, lymphomas and osteosarcoma (34-39). EZH2 has properties of oncopgenes, as its overexpression has been correlated with the metastatic potential of several aggressive tumors (40-42). As for the effect of EZH2 in osteosarcoma, it has been reported that osteosarcoma patient biopsy specimens have higher expression levels of EZH2 than normal bone tissues, and overexpression of EZH2 was found in osteosarcoma cells by immunohistochemical assay. However, the aberrant expression of EZH2 is not correlated to osteosarcoma growth in vivo and in vitro (37). In addition, miR-29a has been reported to inhibit cell migration and invasion by targeting the EZH2 gene (43).

miR-101/EZH2 were reported to be deregulated in several types of cancer, including renal cancer (44), prostate cancer (45), gastric cancer (22), invasive squamous cell carcinoma (46), glioblastoma (24) and bladder transitional cell carcinoma (47), and were found to be significantly correlated with migration, invasion, and metastasis (22,24,45).

In our study, we evaluated whether ectopic expression of miR-101 could inhibit the metastasis of osteosarcoma by suppressing the expression level of EZH2 in osteosarcoma cell lines. We revealed that substantial EZH2 suppression by miR-101 was detected at the mRNA and protein levels in SOSP-9607 and MG63 cells (Fig. 5A and B). In addition, knockdown of EZH2 by siRNA showed the same tendency as the effect of miR-101 on migration and invasion (Fig. 5C). We also revealed that overexpression of miR-101 had little role in cell proliferation (Fig. 4), which was consistent with the role of EZH2 in osteosarcoma cells (37). Taken together, we showed that EZH2 expression was inversely correlated with miR-101, and the expression level of EZH2 was positively correlated with the capacity of cellular migration and invasion. It is reasonable to conclude that miR-101 inhibits the migration and invasion of osteosarcoma cells by downregulating EZH2 expression.

In conclusion, this is the first in vitro study to provide new insights into the role of miR-101 in osteosarcoma. It shows that by downregulating the EZH2 expression level, miR-101 plays a suppressive role in cellular migration and invasion of osteosarcoma. Further studies in vivo are being conducted, and miR-101 may be a promising candidate prognostic biomarker and gene therapeutic agent for osteosarcoma treatment.

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References


