Downregulation of miR-136 promotes the progression of osteosarcoma and is associated with the prognosis of patients with osteosarcoma

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Abstract. Osteosarcoma (OS) is the most common bone tumor in children and young adults, and is an aggressive tumor with poor prognosis. MicroRNAs (miRNAs) are aberrantly expressed in various types of cancer, and contribute to cancer tumorigenesis and progression. In the present study, the potential prognostic value and biological function of miRNA-136 (miR-136) in OS was investigated. Reverse transcription-quantitative polymerase chain reaction analysis was used to evaluate the expression of miR-136 in OS tissues and cell lines. Kaplan-Meier survival analysis and Cox regression analysis were conducted to investigate the prognostic significance of miR-136. Various in vitro cell based assays were used to evaluate the effects of miR-136 on the biological behavior of OS cells. A luciferase assay was performed to determine the key miR-136 targets associated with OS. The expression of miR-136 was significantly downregulated in osteosarcoma tissues and cells compared with the normal controls (all P<0.05). Decreased miR-136 expression was significantly associated with Enneking staging (P=0.030) and distant metastasis (P=0.016). Decreased miR-136 expression in patients was associated with shorter overall survival compared with patients with increased expression levels (log-rank test; P<0.05). The expression of miR-136 was indicated as an independent prognostic factor for the patients (hazard ratio=0.496; 95% confidence interval=0.250-0.987; P=0.046). MTT, transwell and Matrigel assays demonstrated that upregulation of miR-136 decreased proliferation, migration and invasion of OS cells. Bioinformatics and luciferase assays demonstrated that migration and invasion enhancer 1 (MIEN1) is a direct target of miR-136. Together, the results suggested that miR-136 functions as a tumor suppressor gene to regulate proliferation, migration and invasion of OS cells. MIEN1 was a potential target of miR-136. Additionally, miR-136 may serve as a prognostic biomarker for OS.

Introduction

Osteosarcoma (OS) is the most common primary malignant type of bone malignancy in adolescence and is characterized by the formation of immature bone or osteoid tissues from spindle matrix cells (1). Due to the high degree of malignancy of OS and early metastasis to the lungs, the majority of the patients present pulmonary micrometastasis at the primary diagnosis (2). At present, the combination of surgical resection and chemotherapy is the most effective treatment (3,4). Despite a significant improvement in the 5-year overall survival for patients, the cure rate for patients with OS has not improved and patients with metastatic or relapsed disease have a poor prognosis (5,6). Therefore, identifying more precise prognostic biomarkers and novel approaches to the treatment of OS is necessary to improve the outcome of patients with OS.

MicroRNAs (miRNAs) are a group of short non-coding RNAs (18-25 nucleotides in length) that regulate post-transcriptional gene expression by targeting the 3’ untranslated region (3’-UTR) of mRNA (7). An increasing number of studies have demonstrated that miRNAs serve crucial roles in various biological processes, including inflammation, cell proliferation, migration, invasion, apoptosis and differentiation (8-10). Depending on their dysregulation, miRNAs may serve as tumor suppressors or oncogenes in tumorigenesis processes, which contribute to cancer metastasis by influencing cell proliferation and invasion (11,12). Overexpression of miRNA-34a inhibited the migratory and invasive ability of OS cells by repressing the expression of CD44 antigen, serving as a tumor suppressor in the metastasis of OS cells (13). A previous study by Mosakhani et al (14) identified miRNA-136 (miR-136) and its target gene, nuclear factor 1 B-type, as novel biomarkers that may aid in distinguishing primary giant cell tumors of bone with an increased risk for metastasis. Previous studies have additionally demonstrated that miR-136 is involved in the progression

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of a number of different types of cancer, including cervical carcinoma, hepatocellular carcinoma, and colon cancer (15-17). However, the role of miR-136 in OS remains unclear.

In the present study, the expression patterns and prognostic significance of miR-136 in patients with OS was investigated. Additionally, the effects of miR-136 on the biological behaviors of cancer cells were assessed.

Materials and methods

Patients and tissue samples. OS tissue and matched adjacent normal tissue specimens (located ≥3 cm away from the tumors) were obtained between January 2007 and February 2012 from 116 patients with OS who underwent surgery at The Affiliated Hospital of Qingdao University (Qingdao, China). All the tissues were verified and experienced pathologists confirmed the tissue stages, according to the National Comprehensive Cancer Network guidelines (18,19). The OS tissue and normal tissue specimens were snap-frozen in liquid nitrogen following surgery and subsequently stored at -80˚C until use. The inclusion criteria were: All patients were pathologically diagnosed with OS, none of the enrolled patients received any therapy prior to surgery, and all patients had complete clinical, pathological, and follow-up information. All patients agreed to participate in the present study and the Ethics Committee of The Affiliated Hospital of Qingdao University approved the protocol. The characteristics of the patients are summarized in Table I. The 5-year follow-up information was updated for the subsequent analysis.

Cell lines and transfection. Human OS cell lines HOS and U2OS and the normal osteoblast cell line NHOst were obtained from The American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with 5% CO2. The miR-136 mimic (5'-ACUCAUCAUUUGUUGCAGU AUGC-3'), miR-136 inhibitor (5'-CCAUCAUCCAAACAAAUGGGAGU-3'), mimic negative control (NC) (5'-TTTCTCCGAGGCTGTCACGT-3') or inhibitor NC (5'-UUUCUCGGACGUGUCACGUTT-3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). A total of 5x104 cells were seeded into the wells of a 6-well plate. On the subsequent day, the cells were transfected with miR-136 mimic, miR-136 inhibitor or the respective miR-negative controls were transfected. After 48 h, 10 µl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well and the cells were incubated at 37˚C for 4 h. Subsequent to incubation, the medium was removed and 100 µl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to the wells to dissolve the formazan crystals. A wavelength of 490 nm was used for the colorimetric analysis with a Multiskan MK3 (Thermo Fisher Scientific, Inc.). Experiments were repeated in triplicate.

Cell proliferation assay. The cell proliferation of HOS or U2OS cells transfected with NC, miR-136 mimics or miR-136 inhibitor was measured using the colorimetric MTT method. HOS and U2OS cells were seeded into 96-well plates at a density of 5x103 cells/well and transfected. After 48 h, 10 µl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well and the cells were incubated at 37˚C for 4 h. Subsequent to incubation, the medium was removed and 100 µl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to the wells to dissolve the formazan crystals. A wavelength of 490 nm was used for the colorimetric analysis with a Multiskan MK3 (Thermo Fisher Scientific, Inc.). Experiments were repeated in triplicate.

Colony-forming assay. Following transfection, cells were plated at a density of 300 cells/well in a 6-well plate and incubated at 37˚C. The culture medium was replaced according to the change of pH of the medium, as determined by the phenol red indicator in the culture medium. After 14 days, cells in each well were washed carefully with PBS, fixed with methanol for 15 min and stained for 10 min with Giemsa at room temperature. Subsequently, cell colonies (groups >50 cells) were counted under a light microscope (magnification, x40). The test was independently conducted three times.

Cell migration and invasion analysis. To examine the effects of miR-136 on cell migration and invasion, cell assays were performed using a 24-well transwell chamber (8 µm pore size). For the transwell migration assays, the cells transfected with mimics, inhibitor or respective miR-negative controls were added to the top chamber at a density of 1x105 cells/well. For the invasion assays, 2x104 cells were plated in the upper compartment with Matrigel-coated membranes (Corning Life Sciences, Bedford, MA, USA). The cells were subsequently incubated in serum-free RPMI-1640 medium at 37˚C for 24 h. The lower compartment contained 300 µl RPMI-1640 medium supplemented with 20% FBS, which was used as the chemotactic factor. Following incubation for 24 h, the cells that had migrated into the lower compartment were fixed in 3.7% formaldehyde for 5 min and stained with 0.1% crystal violet for 15 min at room temperature. The number of cells was counted using a light microscope (magnification, x200). Each experiment was repeated in triplicate.

Dual-luciferase reporter assay. The putative targeting gene, migration and invasion enhancer 1 (MIEN1), of miR-136 was predicted using web-based miRNA databases, Targetscan (http://www.microrna.org) and
miRNA-PicTar (https://dorina.mdc-berlin.de/), all of which used the 3'-UTR as the target region to determine miRNA recognition elements, and subsequently verified by a dual-luciferase reporter assay. The 3'-UTR sequence of MIEN1 was amplified and subcloned into the pGL3 luciferase reporter vector (Promega Corporation). Cells were cotransfected with wild-type (WT) or mutant (MUT) 3'‑UTR vectors and miR-136 mimics, inhibitors or controls using Lipofectamine® 2000. After 36 h, the luciferase activities of the cells were determined with the Dual-Luciferase Assay System (Promega Corporation) according to the manufacturer's protocol. The firefly luciferase activities were normalized to Renilla luciferase activity. All the experiments were performed in triplicate.

Statistical analysis. Statistical analysis was conducted using SPSS 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA), and the data are presented as the mean ± standard deviation. One-way analysis of variance with Tukey’s post hoc test was used for multiple comparisons. The association between the clinicopathological characteristics of the patients and miR-136 was analyzed by the χ² test. The association between overall survival and miR-136 expression was estimated using the Kaplan-Meier method with a log-rank test. The prognostic effects of each clinical characteristic were determined using a Cox regression analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-136 in tissue specimens and cells. The expression of miR-136 in 116 paired OS tissue specimens and
cell lines were detected by RT-qPCR. As presented in Fig. 1A, the expression of miR-136 was significantly decreased in OS tissues compared with matched adjacent non-tumorous tissues (P<0.001). Furthermore, the expression of miR-136 in tumor tissues at different Enneking stages (19), including stage I (n=30), stage II (n=38), stage III (n=48), was analyzed and compared with the expression in matched normal tissues. The expression of miR-136 in tumor tissues at each stage was significantly decreased compared with matched normal tissues (all P<0.05; Fig. 1B). The expression of miR-136 was consistently decreased in the OS cell lines, HOS and U2OS, compared with the normal human OS cell line NHOst (both P<0.05; Fig. 1C). These results suggested that miR-136 may be a tumor suppressor in OS.

**Association of miR-136 expression with clinical characteristics of patients with OS.** Relative miR-136 expression in patients with OS was associated with specific clinicopathological characteristics. According to the mean expression level of miR-136 (1.329), the patients were divided into a low miR-136 expression group (n=66; 0.46-1.31) and a high expression group (n=50; 1.34-2.48). The analysis results are presented in Table I. The expression of miR-136 was associated with Enneking stage (P=0.030) and distant metastasis (P=0.016). There was no association between miR-136 expression levels and other clinicopathological characteristics, including sex, age and tumor site (P>0.05; Table I).

**Association between miR-136 expression and overall survival time of patients with OS.** To investigate the prognostic factor of miR-136 expression in OS, Kaplan-Meier and Cox proportional hazard regression model analyses were performed. As presented in Fig. 2, the survival time of patients with low miR-136 expression was shorter compared with patients with high miR-136 expression (log-rank test; P=0.039). Univariate and multivariate Cox regression analysis results demonstrated that miR-136 expression (hazard ratio=0.496; 95% confidence interval=0.250-0.987; P=0.046; Table II) may be an important prognostic factor and may thus be an independent biomarker in patients with OS.

**Overexpression of miR-136 inhibits proliferation of OS cells.** As miR-136 expression was negatively associated with the survival time of the patients, the functional role of miR-136 and its effects on tumor cell proliferation, migration and invasion were investigated. The normal osteoblast cell line NHOst and two OS cell lines HOS and U2OS were transfected with miR-136 mimics, inhibitor or their respective miR-negative controls to regulate the expression of miR-136 in the tumor cells. The RT-qPCR results demonstrated that the expression of miR-136 in OS cells transfected with the miR-136 mimics was significantly increased compared with the cells transfected with the negative control and mock (P<0.05; Fig. 3A). The results additionally demonstrated that the expression of miR-136 in OS cells transfected with miR-136 inhibitor was decreased compared with miR-136 expression in the cells with the negative control and mock (P<0.05; Fig. 3A). The expression of miR-136 in the normal osteoblast cell line NHOst was increased in cells transfected with miR-136 mimics and decreased in cells transfected with miR-136 inhibitor, although these differences were not considered statistically significant (P>0.05; Fig. 3A).

An MTT assay was used to measure cell proliferation. The results demonstrated that upregulation of miR-136 inhibited OS cell proliferation (P<0.05; Fig. 3B). In contrast, an inhibitor of miR-136 had the opposite effect, increasing OS cell proliferation compared with cells transfected with respective negative controls or mock (P<0.05; Fig. 3B). Although cell proliferation was inhibited in NHOst cells transfected with miR-136 mimics and promoted in cells transfected with miR-136 inhibitor, the differences were not statistically significant compared with the controls (P>0.05; Fig. 3B). Colony formation assays demonstrated that overexpression of miR-136 by miR-136 mimic inhibited colony formation, while inhibition of miR-136 promoted colony formation (P<0.001; Fig. 3C); however, the differences in colony count, relative to NHOst cells, was not considered statistically significant (P>0.05; Fig. 3C).

**Overexpression of miR-136 decreases the migratory and invasive capacities of OS cells.** In addition to proliferation, cell migration and invasion were measured using transwell and Matrigel assays, respectively. The assay results demonstrated that OS cells transfected with miR-136 mimics exhibited significantly decreased migratory and invasive capacity compared with the negative control and mock groups (P<0.05; Fig. 4). The OS cells transfected with miR-136 inhibitor promoted the capacity of migration and invasion, compared with cells transfected with negative control and mock (P<0.05, Fig. 4). Similar to cell proliferation, cell migration and invasion in NHOst cells transfected with the miR-136 mimics or inhibitor demonstrated similar trends to the OS cells, although the differences were not considered statistically significant (P>0.05; Fig. 4).

**MIEN1 is a direct target of miR-136 in OS cells.** MIEN1 was predicted to be a target of miR-136 (Fig. 5A). A luciferase reporter assay was used to investigate this hypothesis. As
presented in Fig. 5B, the luciferase activity assay results demonstrated that cotransfection of the miR-136 mimics inhibited the luciferase activity of the reporter containing the WT MIEN1 3'-UTR sequence; however, failed to suppress that containing the MUT MIEN1 3'UTR. In contrast, the luciferase activity of the reporter with the WT 3'-UTR of MIEN1 was increased in the cells transfected with the miR-136 inhibitor; however, the luciferase activity did not alter with the MUT 3'-UTR (Fig. 5B). These results suggested that MIEN1 may be a direct functional target of miR-136 in OS.

Table II. Univariate and multivariate Cox analysis of miR-136 expression in patients with osteosarcoma.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Univariate analysis</th>
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<th>Multivariate analysis</th>
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<td></td>
<td>HR</td>
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<td>P-value</td>
<td>HR</td>
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<td>0.250-0.987</td>
<td>0.046</td>
<td>0.496</td>
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<td>Age</td>
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<td>-</td>
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<tr>
<td>Tumor site</td>
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<td>-</td>
<td>0.390</td>
<td>-</td>
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<td>Tumor site (2)</td>
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<td>-</td>
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<td>Enneking staging (19)</td>
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<td>Distant metastasis</td>
<td>0.766</td>
<td>0.425-1.380</td>
<td>0.375</td>
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-, no associated data; miR-136, microRNA-136; HR, hazard ratio; CI, confidence interval.

Figure 3. Effects of miR-136 expression on proliferation of the OS cells HOS and U2OS compared with the normal osteoblast cell line NHOst. Each treatment group was measured at least three times. (A) Expression of miR-136 in NHOst, HOS and U2OS cells transfected with miR-136 mimics, miR-136 inhibitor or their respective negative controls. (B) Cell viability was measured in OS cells that were transfected with miR-136 mimics, inhibitor or negative controls by an MTT assay. The cell viability in NHOst was not significantly different. (C) Proliferation of OS cells and the normal osteoblast cell line NHOst was detected by a colony formation assay (magnification, x40). Quantification of the colony count. *P<0.05, **P<0.01, ***P<0.001 vs. mimic-NC; #P<0.05, ###P<0.001 vs. inhibitor-NC; $P<0.05, $$$P<0.001 vs. mock. miR-136, microRNA-136; NC, negative control; OD, optical density; ns, not significant.
Discussion

The low prevalence and large heterogeneity of OS make it difficult to improve patient survival (23). The specific tumor markers and prognostic factors of OS have important clinical significance. A number of previous studies demonstrated a critical role for molecular biomarkers in tumor pathogenesis (24-26). In OS, specific prognostic biomarkers have additionally been identified. Hou et al (27) demonstrated that cyclin-dependent kinase-1 gene expression was increased in patients with OS and may thus serve as a biomarker to predict the occurrence, development and prognosis of OS. Fernanda Amary et al (28) demonstrated that
fibroblastic growth factor receptor 1 gene amplification in OS was associated with a poor response to neoadjuvant chemotherapy. Liang et al (29) demonstrated that Phospholipase A2 Group XVI expression was increased in OS and may thus serve as a prognostic factor in patients with primary OS for predicting the development of metastases and poor survival. Together, these previous studies suggested that identification of cancer-associated biomarkers for tumor progression and outcome may help to predict patient prognosis and treatment strategies.

A number of miRNAs have been demonstrated to contribute to the development of cancer and to serve as biomarkers for the diagnosis, prognosis or treatment of different types of cancer (30-32). Taheriazam et al (33) demonstrated that miRNA-130b expression was increased in OS tissues, whereas, the level of miRNA-218 expression was downregulated; these miRNAs may serve as potential biomarkers in the early detection of OS. In this study, it was indicated that the expression of miR-136 was downregulated in the patients with OS, which is consistent with the results in previous studies (34,35). For instance, miR-136 was downregulated and acted as a tumor suppressor in colon cancer (34). In renal cell carcinoma, miR-136 was reported to be downregulated and associated with renal cell carcinoma cellular functions (35). To improve the understanding of the role of miR-136 in OS, its expression patterns and association with the clinicopathological features of the patients with OS were investigated. In the present study, the expression of miR-136 was decreased in OS tissues and cells compared with the corresponding normal controls. Furthermore, the expression level of miR-136 was associated with Enneking staging and distant metastasis in patients with OS. These results indicated that miR-136 expression is negatively associated with tumor progression and OS tumorigenesis.

To evaluate the association of miR-136 with the overall survival of patients with OS, Kaplan-Meier and Cox regression analyses were used. Kaplan-Meier analysis demonstrated that patients in the low miR-136 expression group had shorter survival times compared with patients with high miR-136 expression. According to Cox regression analysis, miR-136 expression was associated with the prognosis of OS. Additionally, it may be an independent prognostic marker in OS.

Previous studies demonstrated the effects of miR-136 on biological behaviors during cancer progression (15,21). miR-136 inhibited colon cancer cell proliferation and invasion, which may serve as a potential therapeutic target for colon cancer (15). However, the functions of miR-136 in OS cells have not yet been studied, to the best of the authors' knowledge. In the present study, the effects of miR-136 on the biological behavior of OS cells were assessed to demonstrate a functional involvement of miR-136 during OS progression. The results demonstrated that overexpression of miR-136 may inhibit tumor cell proliferation, migration and invasion, which suggested a potential tumor suppressor role for miR-136.

Figure 5. MIEN1 is a target of miR-136 in osteosarcoma cells. (A) Bioinformatics prediction of miR-136 binding sites in the 3'-UTR of the human MIEN1 gene. (B) Luciferase reporter plasmid containing the WT or MUT MIEN1 3'-UTR was cotransfected into HOS and U2OS cells with miR-136 mimics or inhibitors. Luciferase activity of the cells was assayed at 36 h after transfection and the values were normalized to the controls. *P<0.05 vs. respective MUT. miR-136, microRNA-136; NC, negative control; WT, wild-type; MUT, mutant; MIEN1, migration and invasion enhancer 1; 3'-UTR, 3' untranslated region.
In conclusion, the present findings demonstrated that miR-136 was downregulated in OS cells and tissues of patients with OS. Furthermore, overexpression of miR-136 resulted in the inhibition of cell proliferation, migration and invasion in OS cells. Further investigation of miR-136 identified that MIEN1 was a potential target of miR-136. Together, these data suggested that miR-136 may be a prognostic biomarker and potential therapeutic target for patients with OS.

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Availability of data and materials

The analyzed datasets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YC and YW initiated and designed the work, analyzed the data, and wrote the manuscript. XH and GW collected clinical tissues, performed RNA extraction and RT-qPCR assays, and corresponding data analysis. ZW performed cell experiments. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

All patients agreed to participate in the present study and Ethics approval and consent to participate manuscript. All authors have read and approved the final version of this manuscript.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


