MicroRNA-381 suppresses the proliferation of osteosarcoma cells through LRH-1/Wnt/β-catenin signaling pathway

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Abstract. We explored the function of microRNA-381 on osteosarcoma cell growth and anticancer mechanism for clinic treatment. MicroRNA-381 of osteosarcoma patients was decreased, microRNA-381 levels were more downregulated at stages III/IV than those at stage I/II in osteosarcoma patients. Downregulation of microRNA-381 using anti-microRNA-381 mimics increased cell proliferation, decreased LDH activity and apoptosis, and inhibited caspase-3/9 activities, Bax/Bcl-2 and p53 protein expression in vitro osteosarcoma cells through upregulation of LRH-1/Wnt/β-catenin signaling pathway. Overexpression of microRNA-381 using microRNA-381 mimics inhibited cell proliferation, induced apoptosis and increased LDH activity, caspase-3/9 activities, expression of Bax/Bcl-2 and p53 protein in osteosarcoma of in vitro model through downregulation of LRH-1/Wnt/β-catenin signaling pathway. Si-LRH-1 promoted the anticancer effects of microRNA-381 on osteosarcoma cell growth through Wnt/β-catenin signaling pathway. Thus, our data suggested that microRNA-381 suppresses the proliferation of osteosarcoma cells through upregulation of LRH-1/Wnt/β-catenin signaling pathway.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor deriving from mesenchymal tissue, which is characterized by the production of fusiform stromal cells of osteoid tissue (1). It frequently occurs in metaphysis of long bone in the adolescent, especially around the knee joint, with the cause being unclear so far (2). A majority of cases show sporadic features without a definitely known genetic or environmental factor (2). A majority of cases show sporadic feature and have no definitely known genetic or environmental factor. Various laboratories are now probing into the possibility that tumor stem cells may be involved in OS formation (3). Under normal cell state, a part of genes participate in DNA repair and tumor inhibitory pathways, which contributes to maintaining integrity of cellular processes. Defects of these genes may participate in the pathogenesis of OS (4).

MicroRNA is a kind of non-coding single-strand small molecule, which specifically binds with its target gene and thus regulates its expression (5). A large amount of microRNAs have been discovered and shown to play important roles in the genesis, differentiation, proliferation and apoptosis of cells since the discovery of the first microRNA (6). According to reports, microRNAs regulate expression of human protein-coding genes, bind with the 3' untranslated region of target gene mRNA, and thus result in mRNA degradation or translation inhibition (7). Apart from the silencing effect, some microRNAs are verified to activate gene expression. MicroRNAs play two distinct roles in various types of tumors, namely, tumor suppressor gene and oncogene (8).

LRH1 is also named NR5A2, which is involved in the differentiation of liver and pancreas during early embryonic development (9). LRH1 regulates the balance between cholesterol and bile acid, as well as the production of steroid in adults. In the field of tumor research, LRH1 is reported in literature to participate in the genesis and development of breast cancer, gastric cancer, colon cancer and pancreatic cancer as an oncogene (10). LRH1 can promote proliferation of breast cancer cells through upregulating Erα transcription that involves GREB1. In addition, it plays an important role in promoting the invasion and migration of breast cancer cells through activating remodeling of protein cytoskeleton and decomposition of E-cadherin (11). Furthermore, LRH1 gene mutation is related to susceptibility of cancer, which acceler-
ates OS cell proliferation and tumor growth in nude mice through upregulation of cyclin D1/E1 (12).

As reported, activation of Wnt/β-catenin pathway is an important inducing factor for the metastasis of OS cells (13). Expression of β-catenin gene is upregulated in highly metastatic OS cell lines. Apoptotic resistance, which is a key molecular mechanism guaranteeing survival of the metastatic tumor cells, plays an important role during the entire metastasis process. Metastasis can only take place in case the tumor cells survive during metastasis. Wnt/β-catenin pathway also plays an important resisting role during apoptotic resistance, which is considered to be a key signaling pathway for the invasion and metastasis of tumor cells. In the present study, we aimed to systematically detect the function of microRNA-381 on osteosarcoma cell growth and anticancer mechanism for clinic treatment.

Materials and methods

Collection of human samples. Osteosarcoma tissues (n=7) and adjacent normal tissues (n=7) were collected from Department of Orthopedics, The Second Hospital of Jilin University, Changchun, Jilin, China. Our study protocol was approved by Research Ethics Committee of The Second Hospital of Jilin University.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was harvested using the RNA Isolation kit (Ambion, USA) from tissue samples and cells. Then cDNA synthesis kit (Qiagen, Germany) was carried out to conduct reverse to cDNA. qRT-PCR was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, USA) and a TaqMan Universal PCR Master Mix (Applied Biosystems). MicroRNA-381 expression was calculated using the 2^-∆∆Ct method.

Cell culture. The human osteosarcoma cancer cell line 143B was purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen) in a humidified incubator containing 5% CO2 at 37°C.

MicroRNA-381 mimics, antisense and transfection. MicroRNA-381 mimics, antisense microRNA-381 mimics, Si-LRH-1 or negative control mimics were obtained from GenePharm Co. (Shanghai). Cells were transfected with microRNA-381 mimics, antisense microRNA-381 mimics or negative control mimics by Lipofectamine™ 2000 reagent (Invitrogen). Cells were transfected with microRNA-381 mimics and Si-LRH-1 or negative control mimics by Lipofectamine 2000 reagent (Invitrogen).

MTT assays and LDH activity. Cells were incubated with 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml, Sigma, St. Louis, MO, USA) for 4 h at 37°C. After incubation for 4 h, medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve the formazan product for 20 min at 37°C. Absorbance was determined by an ELISA reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm. LDH of the cells were incubated with LDH activity kit (Beyotime, Haimen, China) for 1 h at 37°C. Absorbance was determined by an ELISA reader (Bio-Rad) at a wavelength of 450 nm.

Cell apoptosis assay and caspase-3/9 activity. Cells were stained with FITC-labeled Annexin V (Sigma-Aldrich) and propidium iodide (Sigma-Aldrich) at dark for 30 min. Apoptosis rate was analyzed via flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). Cells were harvested, washed with PBS and lysed in a cell lysis buffer (Beyotime). Protein concentration was quantified using a BCA kit (Beyotime). Proteins (10 µg) were incubated with caspase-3/9 activity kits (Beyotime) at 37°C for 2 h. Absorbance was determined by an ELISA reader (Bio-Rad) at a wavelength of 405 nm.

DAPI measuring. Cells were washed with PBS, and stained with DAPI assay for 30 min at 37°C. Then, cells were washed with PBS and observed using a microscope (Olympus, Tokyo, Japan).

Western blotting. Cells were harvested, washed with PBS and lysed in a cell lysis buffer (Beyotime). Protein concentration was quantified using a BCA kit (Beyotime). Proteins (50 µg) were separated by 8-10% SDS-agarose gel, transferred to nitrocellulose membranes (Bio-Rad). After blocking with 5% non-fat milk in PBS, membranes were immunoblotted with the antibodies Bax, Bcl-2, p53, LRH-1, Wt, β-catenin and GAPDH (Santa Cruz, CA, USA) at 4°C, followed by HRP secondary antibodies (Cell Signaling) for 1 h at 37°C. The protein bands were visualized by an ECL Western blotting kit (Millipore, Boston, MA, USA) and analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are expressed as the mean ± SEM. Independent-samples t-test or ANOVA (one-way analysis of variance) was used to determine data. P-value <0.05 was considered statistically significant.

Results

MicroRNA-381 expression of osteosarcoma patients. To determine the function of microRNA-381 in osteosarcoma, its expression levels were analyzed in osteosarcoma tissues and pair-matched adjacent normal. As showed in Fig. 1, microRNA-381 expression of osteosarcoma patients was decreased, microRNA-381 levels were more downregulated at stages III/IV than those at stage I/II in osteosarcoma patients.

Upregulation of microRNA-381 on cell proliferation and LDH activity of osteosarcoma. To determine the function of microRNA-381 in osteosarcoma, we used microRNA-381 mimics to inhibit microRNA-381 expression. As shown in Fig. 2, results from ELISA reader showed that microRNA-381 upregulation significantly inhibited cell proliferation and increased LDH activity of osteosarcoma.
Upregulation of microRNA-381 on apoptosis of osteosarcoma. Then, we determined the function of microRNA-381 on apoptosis rate, caspase-3/9 activities and DAPI assay of osteosarcoma. As showed in Fig. 3A and B, significant increases of apoptosis rate, caspase-3/9 activities, and nucleus apoptosis were remarkably observed in osteosarcoma by microRNA-381 upregulation.

Upregulation of microRNA-381 on Bax/Bcl-2 and p53 protein expression of osteosarcoma. Therefore, we aimed to identify its regulated target and reveal the underlying molecular mechanisms of microRNA-381 on Bax/Bcl-2 and p53 protein expression of osteosarcoma. As Fig. 4 shows, upregulation of microRNA-381 significantly induced Bax/Bcl-2 and p53 protein expression of osteosarcoma.
Upregulation of microRNA-381 on LRH-1/Wnt/β-catenin signaling pathway of osteosarcoma. To determine if LRH-1 is targeted by microRNA-381 in osteosarcoma, LRH-1, Wnt and β-catenin protein expression were measured in this study. As shown in Fig. 5, upregulation of microRNA-381 significantly suppressed LRH-1/Wnt/β-catenin signaling pathway of osteosarcoma.

Downregulation of microRNA-381 on cell proliferation and LDH activity of osteosarcoma. Then, we also upregulated microRNA-381 using anti-microRNA-381 mimics, cell proliferation and LDH activity of osteosarcoma were measured. Besides, downregulation of microRNA-381 significantly increased cell proliferation and inhibited LDH activity of osteosarcoma (Fig. 6).

Downregulation of microRNA-381 on apoptosis of osteosarcoma. There was significant inhibition of the apoptosis rate and caspase-3/9 activities in osteosarcoma by microRNA-381 upregulation (Fig. 7A and B), whereas, microRNA-381 downregulation reduced nucleus apoptosis of osteosarcoma (Fig. 7C).

Downregulation of microRNA-381 on Bax/Bcl-2 and p53 protein expression of osteosarcoma. We determined the potential tumor suppressing effects of microRNA-381 on Bax/Bcl-2 and p53 protein expression of osteosarcoma. As shown in Fig. 8, expression of Bax/Bcl-2 and p53 protein of osteosarcoma was also reduced by microRNA-381 downregulation.

Downregulation of microRNA-381 on LRH-1/Wnt/β-catenin signaling pathway of osteosarcoma. Next, to clarify the roles of deregulated microRNA-381 in osteosarcoma, LRH-1/Wnt/β-catenin signaling pathway of osteosarcoma by microRNA-381 upregulation was measured. As a result, microRNA-381 downregulation significantly induced LRH-1/Wnt/β-catenin signaling pathway of osteosarcoma (Fig. 9).

The inhibition of LRH-1 on cell proliferation and LDH activity of osteosarcoma by microRNA-381. Therefore, LRH-1 regulation of the function of microRNA-381 on osteosarcoma was
investigated to clarify the underlying molecular mechanisms. As showed in Fig. 11, the inhibition of LRH-1 significantly inhibited cell proliferation and increased LDH activity of osteosarcoma by microRNA-381 (Fig. 11).

The inhibition of LRH-1 on apoptosis of osteosarcoma by microRNA-381. On the other hand, the inhibition of LRH-1 significantly induced apoptosis and caspase-3/9 activities of osteosarcoma by microRNA-381 (Fig. 12).

The inhibition of LRH-1 on Bax/Bcl-2 and p53 protein expression of osteosarcoma by microRNA-381. To further verify the functional connection between LRH-1 and Bax/Bcl-2 and p53 protein expression of osteosarcoma by microRNA-381, osteosarcoma cells were transfected with Si-LRH-1 and microRNA-381. As shown in Fig. 13, the inhibition of LRH-1 significantly increased the function of microRNA-381 on Bax/Bcl-2 and p53 protein expression of osteosarcoma (Fig. 13).
Figure 10. The inhibition of LRH-1 on LRH-1/Wnt/β-catenin signaling pathway of osteosarcoma by microRNA-381. The inhibition of LRH-1 LRH-1/ Wnt/β-catenin signaling pathway statistical analysis (A-C) and western blot assay of LRH-1, Wnt and β-catenin (D) of osteosarcoma by microRNA-381. Control, negative control group; miRNA-381, miRNA-381 group; Si-LRH-1+miRNA-381, Si-LRH-1+miRNA-381 group. ##p<0.01 compared with negative group; **p<0.01 compared with miRNA-381 group.

Figure 11. The inhibition of LRH-1 on cell proliferation and LDH activity of osteosarcoma by microRNA-381. The inhibition of LRH-1 on cell prolifera -tion (A) and LDH activity (B) of osteosarcoma by microRNA-381. Control, negative control group; miRNA-381, miRNA-381 group; Si-LRH-1+miRNA-381, Si-LRH-1+miRNA-381 group. ##p<0.01 compared with negative group; **p<0.01 compared with miRNA-381 group.

Figure 12. The inhibition of LRH-1 on apoptosis of osteosarcoma by microRNA-381. The inhibition of LRH-1 on apoptosis rate (A), caspase-3/9 activities (B), and nucleus apoptosis using DAPI staining (C) by microRNA-381. Control, negative control group; miRNA-381, miRNA-381 group; Si-LRH-1+miRNA-381, Si-LRH-1+miRNA-381 group. ##p<0.01 compared with negative group; **p<0.01 compared with miRNA-381 group.

Figure 13. The inhibition of LRH-1 on Bax/Bcl-2 and p53 protein expression of osteosarcoma by microRNA-381. The inhibition of LRH-1 on Bax/Bcl-2 and p53 protein expression by statistical analysis (A and B) and western blot assays Bax/Bcl-2 and p53 protein expression (C) of osteosarcoma by microRNA-381. Control, negative control group; miRNA-381, miRNA-381 group; Si-LRH-1+miRNA-381, Si-LRH-1+miRNA-381 group. ##p<0.01 compared with negative group; **p<0.01 compared with miRNA-381 group.
**Discussion**

OS is the most common primary malignant bone tumor, 75% of which occur at the age of 10-30 years (14). Morbidity of OS is relatively low compared with other malignant tumors; however, it frequently occurs in adolescent and is associated with the clinical manifestations of rapid growth and short course of disease, which severely affects the physical and mental health of adolescent (15). The comprehensive application of neoadjuvant chemotherapy, limb salvage surgery and pulmonary metastasis dissection in recent years has greatly improved the prognosis for OS; however, its major pathogenesis remains unclear (16). Therefore, in-depth investigation of the molecular and biological characteristics of OS is of great significance to the diagnosis and treatment of OS (17). The roles of microRNAs in genesis and development of tumors are increasingly prominent, mainly exerting their functions through specifically regulating expression of tumor-related genes (6). In our study, we for the first time determined that microRNA-381 of osteosarcoma patients was decreased, microRNA-381 levels were more downregulated at stages III/IV than those at stage I/II in osteosarcoma patients. Zhang et al showed that microRNA-381 suppresses cell growth and invasion of liver cancer through LRH-1 (18).

miRNAs are involved in early embryonic development, cell proliferation, apoptosis, differentiation and metabolism in animals. Furthermore, they can also regulate the differentiation and maturity of stem cells (19). miRNAs play extremely important roles in regulating gene expression of cells, and their abnormal expression is closely related to the genesis of numerous tumors. Recent research has discovered that abnormal expression of miRNAs can be seen in a number of human tumors (20). It is also found through hybridization techniques in some research that expression quantities of miRNAs in tumor tissue are mostly lower than those in normal tissue. In addition, the expression is associated with distinct tissue specificity, which can even be used to judge the histological origin of the undifferentiated tumor (7). It indicates that miRNAs play important roles in tumor differentiation, proliferation and metastasis, but their molecular mechanisms remain to be further investigated (20). Therefore, these results suggest that microRNA-381 downregulation increased cell proliferation, decreased LDH activity and apoptosis, and inhibited caspase-3/9 activities, Bax/Bcl-2 and p53 protein expressions in osteosarcoma cells in vitro.

Many signaling pathway changes are involved in the molecular mechanism of tumor metastasis. Wnt/β-catenin signaling pathway is a pathway in evolution that plays an important role in tumor metastasis-related signaling pathways (13). Wnt signaling pathway is at present the hot research pathway regarding signaling pathways. Abnormal changes of Wnt signaling pathway is found to exist in numerous tumors such as nasopharyngeal carcinoma, breast cancer, gastric cancer, liver cancer and prostate cancer (21). Wnt signaling pathway is closely related to tumor invasion and metastasis-related events; for instance, cytoskeleton formation, migration and adhesion of tumor cells, as well as tumor angiogenesis, plays an important role in tumor invasion and metastasis, as found in research in recent years (22).

In this study, we found that downregulation of microRNA-381 upregulated LRH-1/Wnt/β-catenin signaling pathway. Wnt competitively binds onto a receptor of β-catenin, inhibits the degradation of β-catenin in cytoplasm, and induces increase in β-catenin concentration (23). High concentration of β-catenin can regulate transcription of relevant target genes and cell cycle through nuclear translocation, promotes tumor cell survival and proliferation, thus showing anti-apoptotic effects (23). One of the major characteristics of the metastatic tumor cells is the activity of apoptotic resistance, which is also referred to as apoptosis tolerance (24). It facilitates the survival of metastatic OS cells in the circulatory system; and manifests insensitivity to chemotherapeutics (24). As a result, regulating Wnt/β-catenin signaling pathway, and constructing the balance between pro-apoptotic and anti-apoptotic signals, so as to reduce the activity of apoptotic resistance in tumor cells, contributes to the recovery of sensitivity of tumor cells to chemotherapeutics (25). Our study showed that overexpression of microRNA-381 downregulated LRH-1/Wnt/β-catenin signaling pathway. Zhang et al showed that microRNA-381 suppresses cell growth and invasion of liver cancer through LRH-1/ Wnt/β-catenin signaling pathway (18).

Positive expression of LRH1 in immunohistochemical sections of OS tissue is not related to patient sex, lesion site and pathological type; instead, it is associated with surgical stage and distant metastasis (12). It further reveals that LRH1 can promote genesis, development and metastasis of tumors (12). OS patients with high LRH1 protein expression have remarkably shorter average survival time and average metastasis time compared with those with low LRH1 protein expression, and the difference is of statistical significance (26). It is verified through Cox multifactor survival analysis that LRH1 expression is one of the independent factors influencing prognosis for OS patients (27). Overexpression of LRH1 is an important factor affecting the biological behavior of OS in clinic, which promotes OS progression, and it is closely associated with the role of LRH1 in promoting tumor cell proliferation and metastasis (26). Thus, we provided evidence that downregulation of microRNA-381 upregulated LRH-1/Wnt/β-catenin signaling pathway. Si-LRH-1 promoted the anticancer effects of microRNA-381 on osteosarcoma cell growth through Wnt/β-catenin signaling pathway. Liang et al suggested that downregulation of microRNA-381 promotes cell proliferation and invasion through upregulation of LRH-1 in colon cancer (28).

In conclusion, microRNA-381 suppressed the proliferation and induced apoptosis of osteosarcoma cells through LRH-1/Wnt/β-catenin signaling pathway. Besides, microRNA-381 may be an independent diagnostic and prognostic marker for osteosarcoma.

**References**


