

Original Article

Effect of miR-208b on the proliferation, migration and apoptosis of osteosarcoma cells

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Received July 15, 2020; Accepted August 15, 2020; Epub February 15, 2021; Published February 28, 2021

Abstract: Objective: To explore the expression of miR-208b in human osteosarcoma tissues and its effects on proliferation, migration, and apoptosis of osteosarcoma cells line U2OS, and to investigate the possible mechanisms. Methods: The expression of miR-208b in osteosarcoma cells line U2OS and osteosarcoma tissues were examined by qPCR; miR-208b mimics was transfected into U2OS cells using Lipo2000 reagents. The effects of miR-208b mimics on the proliferation, migration, and apoptosis were determined by CCK-8 assay, Transwell assay and TUNEL assay, respectively. The expression level of C-myc in U2OS cells influenced by miR-208b mimics was examined by Western blot. Results: qPCR analysis showed that the expression levels of miR-208b in U2OS cells or osteosarcoma tissues were significantly lower than that in the control group ($P < 0.001$). The up-regulation of miR-208b expression remarkably inhibited the proliferation and migration ability of U2OS cells and promoted cell apoptosis, compared with those in miRNA control group ($P < 0.001$). The up-regulation of miR-208b expression up-regulated the expression of C-myc. Conclusions: miR-208b overexpression could significantly inhibit proliferation and migration ability of osteosarcoma cells, which may be related to the down-regulation of C-myc.

Keywords: miR-208b, proliferation, migration, apoptosis, osteosarcoma

Introduction

Osteosarcoma is a common kind of primary bone tumor associated with high disability and mortality rate. According to epidemiological investigation, the incidence of osteosarcoma in the population is about 3 per one million [1, 2]. With the development of science and technology, the work of osteosarcoma diagnosis and treatment has made significant progress. However, the overall 5-year survival rate of patients with osteosarcoma remains low [3]. It was reported that the main cause of death was a high recurrence rate and metastasis rate [4]. Thus, the discovery of new therapeutic targets is very important for clinical and basic research.

Recently, miRNAs have been considered as one of the hot biomolecules in cancer research [5]. Previous research shows that miRNA is intimately associated with growth, metastasis, and differentiation of tumors [6, 7]. Many studies have reported that miRNAs play an important role in the development of many tumors such as osteosarcoma [8, 9]. It was reported that miR-208b was down-regulated by curcum-

in, which inhibited the growth of prostate cancer [10]. Ma et al reported that miR-208b-5p could inhibit the migration and invasion of non-small cell lung cancer cells by regulating the STAT-3 and IL-9 pathway [11]. However, the exact function of miR-208b in the progression of osteosarcoma is still unknown.

C-myc as a proto-oncogene belongs to a transforming member of the Myc family. Previous studies have shown that the abnormal regulation of C-myc was very crucial for tumorigenesis [12]. The up-regulation of C-myc was closely associated with proliferation, apoptosis and differentiation in cellular processes [13, 14]. It was reported that C-myc was an important oncogene for the proliferation of osteosarcoma cells [15]. As we can see, C-myc is a potential treatment target for osteosarcoma.

In this research, we detected the expression level of miR-208b in osteosarcoma tissues and U2OS cells, and its function in proliferation, migration and apoptosis of U2OS cells. In addition, we also investigated the possible mechanisms. The results of this research can provide

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a better understanding of miR-208b in the development of osteosarcoma.

Materials and methods

Cell lines and tissues specimens

Human osteosarcoma cell line U2OS and human osteoblastic cell line hFOB1.19 were purchased from the American Type Culture Collection (ATCC), America. In this study, U2OS and hFOB1.19 cells were cultured and kept in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS).

The fresh osteosarcoma tissue specimens and para-carcinoma tissues were obtained from patients who underwent osteosarcoma operations, without any chemotherapy or radiotherapy. All of the cases were confirmed as osteosarcoma by the method of pathological diagnosis. Before the experiment, patients gave their informed consent to use the clinical tissue samples and this study was approved by the hospital ethic committee.

Reagents and instruments

DMEM and fetal bovine serum were purchased from Gibco, USA; miRNA extraction kits, TaqMan microRNA reverse transcription kits, and PCR kits were obtained from TaKaRa, Japan; Lipo2000 transfection reagent was purchased from Invitrogen, USA; CCK-8 was obtained from Dojindo, Japan; TUNEL kits were purchased from Roche, Swiss; Rabbit-anti-human C-myc antibody was obtained from Santa Cruz, USA; Transwell was obtained from Corning, USA; Inverted microscope was from Japanese Nikon; Real-time PCR instruments was obtained from Applied Biosystems, USA.

Detection of qPCR

According to the instructions of the one-step method miRNA Kits, total miRNA of osteosarcoma tissue, U2OS cells and hFOB1.19 cells were isolated and reversely transcribed into cDNA with TaqMan microRNA reverse transcription kits. Internal reference U6 was applied for normalization. miR-208b forward primer: 5'-GTCG TATCCAGTGC GTGTCGTC-3' and reverse primer, 5'-CACACTCTTGATGTTCCA GGA-3'; U6 forward primer, 5'-CTC GCT TCGGCAGCA CA-3' and reverse primer, 5'-AACGCTTCACGAATTTGCGT-3'. The reaction system was as follows:

each primer was 0.5 μ L, SYBR Green Real-time PCR Master Mix 10 μ L, cDNA 1.8 μ L, ddH₂O 7.2 μ L. PCR amplification was performed at the following reaction conditions: 95°C for 2 min, denaturation at 95°C for 20 s, annealing at 60°C for 30 s, extension at 72°C for 20 s and a total of 35 cycles. The corresponding Ct values of targeted genes were obtained using ABI 7300 System software. U6 served as an internal reference, and the relative miR-208b expression level was calculated by 2- $\Delta\Delta$ Ct method.

CCK-8 assay

U2OS cells in each group were cultured in 96-well plates with the density of 1×10^3 /ml and kept in an incubator with the conditions of 5% CO₂ and 37°C for 12 h, 24 h, and 48 h, respectively. The cells were divided into miR-208b mimics group and miRNA control group. After adding 10 μ L CCK-8 in each well, these cells were continually incubated for 2 hours. The optical density values were measured using the microplate reader.

Transwell assay

After cells were transfected with miR-208b or miRNA control using Lipo2000 reagent, 1×10^5 cell in FBS-free DMEM were seeded in the upper chamber, and the lower chamber was filled with DMEM with 20% FBS. After culturing for 24 h, the upper un-migrated cells were swabbed and removed. The migrated cells were fixed with 4% paraformaldehyde for 20 min, and then stained by 0.1% crystal violet for 10 min. Under the light microscope, the values for migration were calculated by counting 10 randomly selected observation fields per membrane.

TUNEL assay

Cell apoptosis was performed with TUNEL assay as previously described [16]. Briefly, after transfection, U2OS cells on coverslips were fixed with 4% paraformaldehyde for 20 min, followed by permeabilizing using 0.1% Triton X-100 for 1 min on ice. According to the manufacturer's instruction of TUNEL kits, these cells were labeled. The apoptotic index was obtained according to the following formula: Apoptotic index = (Number of apoptotic cells/Total number of cells) \times 100%.

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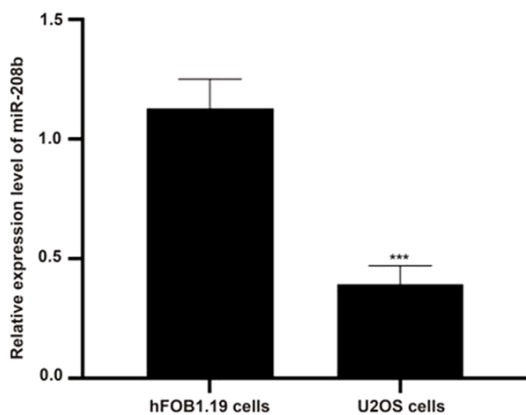


Figure 1. Comparison of miR-208b expression between U2OS cells and hFOB1.19 cells. Compared with hFOB1.19 cells, *** $P < 0.001$.

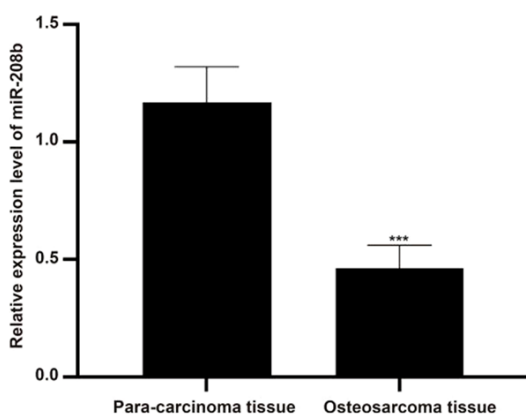


Figure 2. Comparison of miR-208b expression between osteosarcoma tissue and para-carcinoma tissue. Compared with para-carcinoma tissue, *** $P < 0.001$.

Detection of Western blot

After transfection, total proteins in each group were extracted with protein lysis buffer RIPA containing phosphatase inhibitors and protease inhibitors, and quantified by BCA method. Then, 20 μg of lysate was isolated by SDS-PAGE gel electrophoresis, and transferred to the PVDF membrane. The membrane was blocked in the TBST with 5% non-fat milk powder at room temperature 1 h. C-myc primary antibody (dilution 1:500) was incubated in the shaker at 4°C overnight. After washing with TBST, the PVDF membrane was placed into HRP secondary antibody (dilution 1:1000) to incubate for 1 h at room temperature. ECL reagent was added to examine the signal on the membrane, and the bands were analyzed

through densitometry with Bio-Rad image software. GAPDH served as the internal control.

Statistical methods

SPSS 21.0 software was applied for conducting statistical analysis for data in this study. The mean \pm standard deviation (SD) was used to express measurement data and the comparison between two groups was performed by T test. Enumeration data was presented by percentage or cases and the comparison between two groups was conducted by chi-squared test. $P < 0.05$ indicates significantly statistical differences.

Results

The expression of miR-208b in U2OS cells

qPCR results showed that the expression level of miR-208b in U2OS cells was significantly lower than that in hFOB1.19 cells, and there was significant statistical differences (1.13 ± 0.12 vs 0.39 ± 0.08 , $t = P < 0.001$), as seen in **Figure 1**.

The expression of miR-208b in osteosarcoma tissue

According to qPCR test, the expression of miR-208b in osteosarcoma tissue was significantly lower than that in para-carcinoma tissue, and there were remarkably statistical differences (1.17 ± 0.15 vs 0.46 ± 0.10 , t , $P < 0.001$), as seen in **Figure 2**.

Effects of miR-208b mimics on the proliferation of U2OS cells

CCK-8 assay showed that there were no significant differences for OD values at 12 h and 24 h after incubation between miR-208b mimics group and miRNA control group. The OD value in miR-208b mimics group at 48 h after incubation was significantly lower than that in miRNA control group, and a significant difference was found ($P = 0.006$), as seen in **Table 1**.

Effects of miR-208b mimics on the migration of U2OS cells

At 24 h after miR-208b mimics transfection, compared with miRNA control group, miR-208b mimics obviously inhibited the migration ability of U2OS cells, and there were significant differences ($P < 0.001$), as seen in **Figure 3**.

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Table 1. Comparison of proliferation ability of U2OS cells between two groups

| Groups | OD values | | |
|-----------------------|-----------|-----------|-----------|
| | 12 h | 24 h | 48 h |
| miR-208b mimics group | 0.35±0.04 | 0.41±0.07 | 0.46±0.05 |
| miRNA control group | 0.37±0.06 | 0.47±0.08 | 0.63±0.09 |
| t value | 0.620 | 1.262 | 3.692 |
| P value | 0.552 | 0.243 | 0.006 |

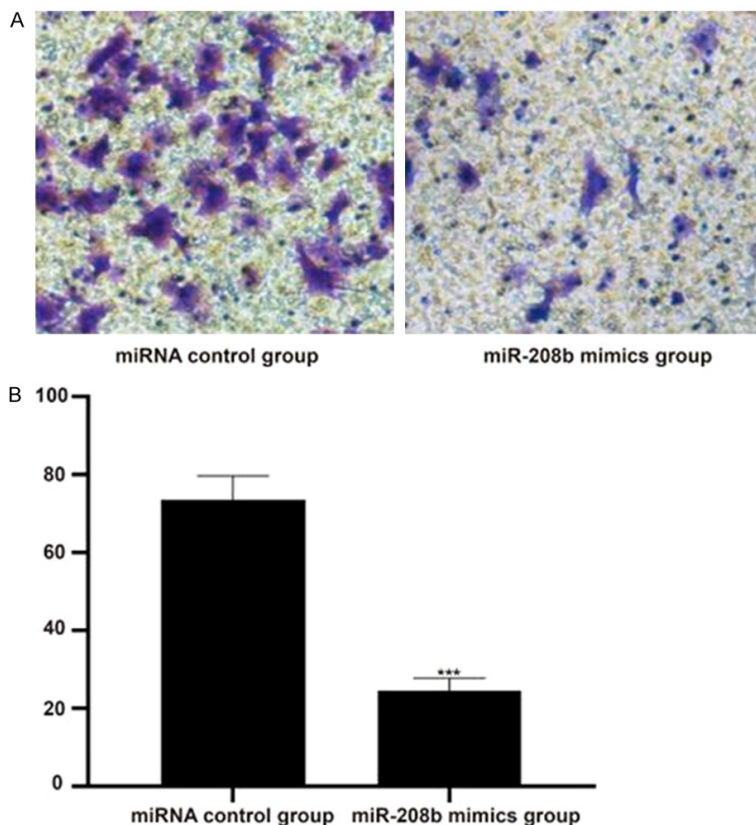


Figure 3. Comparison of migration ability of U2OS cells between two groups. Compared with miRNA control group, ***P<0.001.

Effects of miR-208b mimics on the apoptosis of U2OS cells

TUNEL assay showed that the apoptotic index in miR-208b mimics group was significantly higher than that in the miRNA control group. There was significant statistical difference between the two groups ($\chi^2=10.010$, $P=0.002$), as seen **Figure 4**.

Comparison of C-myc protein level between two groups

Western Blot analysis showed that the expression level of C-myc protein in miR-208b mimics

group was significantly lower than that in miRNA control group, and there was a remarkably statistical difference ($P<0.001$), as seen in **Figure 5**.

Discussion

Osteosarcoma is a malignant bone tumor, which seriously threatens the life quality and physical and psychological health of patients. Currently, the overall survival of patients with osteosarcoma has been improved due to the application of multi-agent chemotherapy and other treatment interventions. However, the prognosis of these patients is unfavorable. It was reported that targeted therapy had been adopted successfully in breast cancer and liver cancer. Thus, understanding the mechanism of osteosarcoma development might contribute to developing targeted therapies for osteosarcoma [17].

MiRNAs are highly conserved, short non-coding nucleic acid chains. It was reported that miRNAs participate in growth and metastasis of tumor via post-transcriptional inhibition of target gene expression [18]. Some studies reported that abnormal expression of miRNA could be found in most

of the malignant tumors [19]. The discovery of miRNA could provide a new insight for the treatment of osteosarcoma. Increasing evidence indicates that several types of miRNAs such as miR-181a, miR-152, miR-138-5p, and miR-30e-5p play important roles in osteosarcoma regulation [18, 20]. miR-208b is reported to be significantly expressed in cardiac tissue and is deregulated in some cardiovascular diseases such as acute myocardial infarction and cardiac ischemia reperfusion injury [21, 22]. However, the expression and function of miR-208b in tumors, especially in osteosarcoma are still unknown. Therefore, this research aimed to

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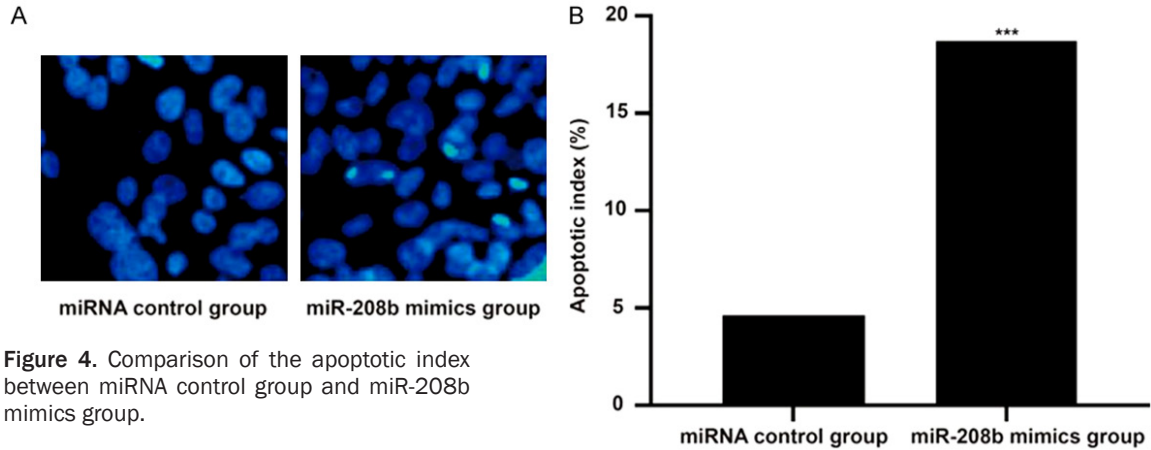


Figure 4. Comparison of the apoptotic index between miRNA control group and miR-208b mimics group.

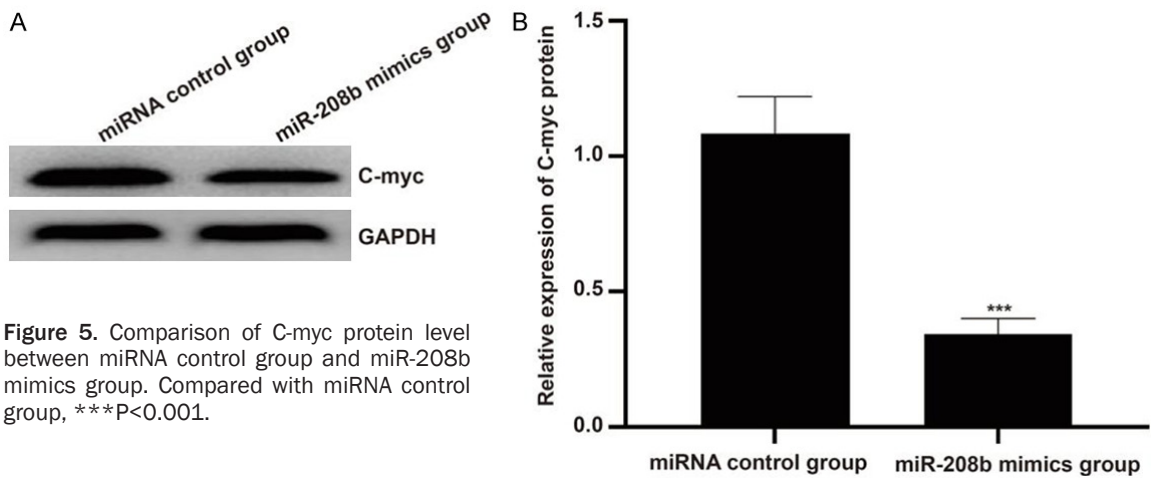


Figure 5. Comparison of C-myc protein level between miRNA control group and miR-208b mimics group. Compared with miRNA control group, *** $P < 0.001$.

explore the potential role of miR-208b in inhibiting osteosarcoma cells proliferation, migration and apoptosis.

The present research demonstrated that the expression level of miR-208b in osteosarcoma tissues was obviously reduced in contrast to para-carcinoma tissues. Furthermore, qPCR results showed that the expression level of miR-208b in osteosarcoma cells lines U2OS was significantly decreased compared with normal osteoblastic cells. The effects of miR-208b overexpression on proliferation, migration, and apoptosis of U2OS were observed by CCK-8, Transwell and TUNEL assays. The results showed that the up-regulation of miR-208b expression remarkably inhibited the proliferation and migration of U2OS cells and promoted cells apoptosis, indicating that miR-208b negatively regulates the proliferation, migration and apoptosis of osteosarcoma cells. These results were consistent with the role of miR-208b in

other types of cancers reported previously [11]. As we can see, miR-208b could be considered as a tumor suppressor gene and applied as a target of treatment and prognostic index in osteosarcoma.

This study also demonstrated that C-myc protein might be a novel target of miR-208b. Previous studies reported that C-myc protein was well associated with cell differentiation, proliferation and metastasis [23]. Also, C-myc protein as a downstream targeted molecule was reported to be involved in the Akt pathway, Notch signaling and Wnt signaling, which were related with proliferation and metastasis of tumors [24]. Targeting C-myc driven super enhancer signaling is considered as a promising and effective strategy for cancer treatment. Zhang et al reported that C-myc could promote the proliferation of breast cancer cells [25]. Tong et al reported that the up-regulation of C-myc could enhance the growth of non-small-

cell lung carcinoma cells [26]. The role of C-myc in osteosarcoma also was confirmed by previous studies [27]. In this study, we found that miR-208b mimics could remarkably down-regulate the expression of C-myc in osteosarcoma cells lines U2OS by Western blot. This indicted that miR-208b may affect the proliferation, migration and apoptosis of osteosarcoma cells via the expression of C-myc protein in the development of osteosarcoma.

In summary, our study found that miR-208b was down-regulated in U2OS cells and osteosarcoma tissues and miR-208b overexpression could significantly inhibit proliferation, migration and apoptosis of osteosarcoma cells, which may be related with down-regulation of C-myc protein. However, further studies are needed to make deeper exploration of miR-208b-regulated target genes and related signaling pathways, so as to provide new insight for the therapy of osteosarcoma.

Acknowledgements

This work was supported by Grants from the Gannan Medical College 2019 University-level project (No. YB201948).

Disclosure of conflict of interest

None.

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