

ORIGINAL ARTICLE

MiR-212 promotes proliferation and inhibits apoptosis of osteosarcoma cells via regulating hedgehog signaling pathway

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Summary

Purpose: To explore the effects of micro ribonucleic acid 212 (miR-212) on proliferation and apoptosis of osteosarcoma cells via the Hedgehog signaling pathway.

Methods: hFOB1.19 cells were enrolled as normal group, and osteosarcoma MG63 cells were divided into osteosarcoma group and miR-212 mimics group. The cells in the normal and osteosarcoma group were cultured normally, while those in miR-212 mimics group were cultured in medium containing miR-212 mimics. After 24 h of culture, the cells were collected for detection. Quantitative polymerase chain reaction (qPCR) assay was performed to determine the expression level of miR-212. The Gli1 expression level was measured by immunohistochemistry and Western blotting. Additionally, cell proliferation and apoptosis were detected by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively.

Results: Based on immunohistochemistry, the average optical density of Gli1 positive expression was evidently in-

creased in osteosarcoma group and miR-212 mimics group compared with that in normal group. The results of Western blotting and qRT-PCR showed that compared with those in normal group, the relative protein expression level of Gli1 and relative expression level of miR-212 were notably raised in the other two groups, and these levels were remarkably higher in miR-212 mimics group than those in osteosarcoma group. According to CCK-8 assay, the proliferation rate of cells was overtly higher in osteosarcoma group and miR-212 mimics group than that in normal group, and also distinctly higher in miR-212 mimics group than that in osteosarcoma group.

Conclusions: MiR-212 promotes the proliferation and inhibits the apoptosis of osteosarcoma cells by up-regulating the Hedgehog signaling pathway.

Key words: miR-212, osteosarcoma, Hedgehog signaling pathway, cell proliferation, cell apoptosis

Introduction

Osteosarcoma is a neoplastic disease with high grade of malignancy and poor prognosis [1,2]. It is a primary malignant tumor common in children and adolescents in the clinic. As to its treatment, there is no ideal method at present. Besides, the 5-year survival rate of patients with non-metastatic osteosarcoma is about 70%, and that of patients with metastatic osteosarcoma is less than 20% [3,4]. Therefore, further studying the pathogenesis

and related pathological mechanisms of osteosarcoma is of great importance.

The proliferation and apoptosis of osteosarcoma cells are important factors affecting the development and progression of the disease, and it is a challenge to maintain their proliferative ability at a low level and their apoptotic capacity at a high level. In addition, the proliferation and apoptosis of osteosarcoma cells are affected by many factors,

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especially the Hedgehog signaling pathway that plays a vital role therein [5,6]. The Hedgehog signaling pathway can be activated by abnormally high expression of Gli1, a key molecule of this pathway, thus promoting the proliferation of osteosarcoma cells and suppressing their apoptosis.

This study aimed to evaluate the effect of miR-212 on the proliferation and apoptosis of osteosarcoma cells by modulating the Hedgehog signaling pathway, so as to further clarify the pathogenesis of osteosarcoma and the important role of miR-212 in it.

Methods

Materials and reagents

Normal human osteoblast cell line hFOB1.19 (ATCC, USA), osteosarcoma MG63 cell line (American Type Culture Collection (ATCC) (Manassas, VA, USA), micro ribonucleic acid 212 (miR-212) mimics (MCE, Monmouth Junction, NJ, USA), anti-Gli1 antibody and secondary antibody (Abcam, Cambridge, MA, USA), RPMI 1640 medium and Dulbecco's modified Eagle medium (DMEM)-Nutrient Mixture F-12 (DMED-F12) (Hyclone, South Logan, UT, USA), fetal bovine serum (FBS) and trypsin (Gibco, Rockville, MD, USA), penicillin and streptomycin (Sigma, St. Louis, MO, USA), cell counting kit-8 (CCK-8) kit (Beyotime, Shanghai, China), Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit, and kits for quantitative polymerase chain reaction (qPCR) (Vazyme, Nanjing, China).

Instruments

A CO₂ cell incubator (Thermo Fisher, Waltham, MA, USA), a light microscope (Leica DMI 4000B/DFC425C, Wetzlar, Germany), a NanoDrop ND-000 spectrophotometer (NanoDrop Tech, Waltham, MA, USA), a fluorescence qPCR instrument (ABI 7500, Foster City, CA, USA), and a microplate reader and a flow cytometer (Bio-Rad, Hercules, CA, USA).

Cell culture

hFOB1.19 (normal human osteoblasts) and MG63 cells were cultured with DMEM-F12 complete medium containing 10% FBS and 1% penicillin and streptomycin in 5% CO₂ incubator at 37°C, with the medium replaced every 3 days. When the confluence of the cultured cells reached 80%, they were sub-cultured.

Cell subculture

When the confluence rate of the cells cultured was 80%, the medium was removed, and the cells were added with 0.125% trypsin for 1 min of digestion that was terminated with the addition of DMEM-F12 complete medium containing 10% FBS and 1% penicillin and streptomycin, during which the adherent cells were gently pipetted for full digestion. Then, the cells were centrifuged at 1600 rpm for 5 min. Next, the supernatant was discarded, and the sediment was re-suspended with

DMEM-F12 medium, cultured, and collected for experiments when they had passaged to the third generation.

Cell treatment

The normal human osteoblast cell line hFOB1.19 was enrolled as normal group (with normal cultivation and no treatment), and MG63 cells were randomly divided into the osteosarcoma group (with normal cultivation and no treatment) and the miR-212 mimics group (with culture in DMEM-F12 complete medium containing 0.3 μM miR-212 mimics, 10% FBS, and 1% penicillin and streptomycin). In each group, the cells cultured for 24 h were collected for detection.

Immunohistochemistry

The cells collected were fixed with 4% paraformaldehyde for 30 min, rinsed with phosphate-buffered saline (PBS), and dropwise added with endogenous peroxidase blocker for reaction for 10 min, followed by rinsing. Then, the cells were blocked with goat serum added in drops for 20 min. Subsequently, the goat serum blocking solution was removed, and the cells were incubated with anti-Gli1 primary antibody (1:200) in a refrigerator at 4°C overnight. On the next day, the cells were washed with PBS, incubated with secondary antibody solution dropwise added for 10 min, thoroughly rinsed and reacted with streptavidin-peroxidase solution for 10 min, followed by color development with dropwise addition of diaminobenzidine (DAB) (Solarbio, Beijing, China). Lastly, the nuclei were counterstained with hematoxylin, mounted and observed.

Western blotting (WB)

The cells transfected were collected from each group, added with RIPA lysis buffer, and subjected to ice bath for 60 min. Next, they were centrifuged at 14000 g for 10 min, and then the protein was quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The standard curve and absorbance were obtained using microplate reader, based on which the protein concentration was calculated. After protein denaturation, the samples were separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at the corresponding concentration, which was terminated when the Marker protein ran to the bottom of the glass plate and the sample protein was in a straight line in the bottom. Thereafter, the samples were transferred to

Table 1. Primer sequences

Name	Primer sequence
MiR-212	Forward primer: 5'TTGGGGTTAGGTTTGGGTTTTC 3'
	Reverse primer: 5'CGTAAACGAAATTCGACCCCACTA 3'
GADPH	Forward primer: 5'ACGGCAAGTTCAACGGCACAG 3'
	Reverse primer: 5'GAAGACGCCAGTAGACTCCACGAC 3'

a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), blocked and washed with phosphate buffered saline-tween (PBST) for 3 times. After that, the membrane was blocked with blocking solution for 1.5 h and incubated with anti-Gli1 primary antibodies (1:1000) and secondary antibodies (1:1000) successively, with washing with Tris-buffered saline with Tween®20 (TBST) after each step. Afterwards, the secondary antibodies were washed away with TBST, and the membrane was placed in chemiluminescent reagent for 1 min of development in the dark, followed by analysis using a gel imaging system.

QPCR assay

Total RNA was extracted from cells using an RNA extraction kit and then reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit, and the reaction system was 20 μ L. Reaction conditions: reaction at 51°C for 2 min, pre-denaturation at 96°C for 10 min, denaturation at 96°C for 10 s, and annealing at 60°C for 30 s, for 40 cycles. The relative mRNA expression level of miR-212 was calculated. The primer sequences are shown in Table 1.

Determination of cell proliferation via CCK-8 assay

After transfection, the cells in each group were seeded in 96-well plates at a density of 3×10^3 cells/mL, with three replicates, incubated with complete medium (100 μ L/well) in 5% CO₂ incubator at 37°C for 24 h and then with CCK-8 solution (10 μ L/well) for 1 h under the above conditions.

Next, the optical density (OD) at 450 nm was measured with microplate reader (Thermo Fisher Scientific, Waltham, USA) and the cell proliferation rate was calculated.

Detection of apoptosis through flow cytometry

The cells in each group were collected and rinsed with PBS, followed by discarding the supernatant. Next, the cells were re-suspended with binding buffer, and the cell concentration was adjusted to 1×10^5 cells/mL. Afterwards, the cells were added with 10 μ L of Annexin V-FITC solution and 5 μ L of PI solution, mixed and reacted in the dark at room temperature for 15 min. Then, they were put in the flow cytometer for detection, and the apoptosis rate was calculated.

Statistics

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Measurement data were expressed as mean \pm standard deviation. T-test was utilized for data with normal distribution and homogeneity of variance, corrected t-test for those with normal distribution and heterogeneity of variance, and non-parametric test for those without normal distribution and homogeneity of variance. Rank sum test was applied for ranked data. For enumeration data, χ^2 test was employed. Kaplan-Meier method was used to plot survival curves and log-rank test to compare survival between groups.

Results

Results of immunohistochemistry

As shown in Figure 1A, the Gli1 positive expression was brown and it was less in the normal group and more in the miR-212 mimics group. Compared with that in the normal group, the average optical density of Gli1 positive expression was evidently increased in the osteosarcoma group and miR-212 mimics group, showing a statistically significant difference ($p < 0.05$), and it was distinctly higher in the miR-212 mimics group than that in the osteosarcoma group, with a statistically significant difference ($p < 0.05$) (Figure 1B).

Related protein expression determined through WB

The protein expression level of Gli1 was low in the normal group and highest in the miR-212 mimics group (Figure 2A, 2B). The relative protein expression level of Gli1 was raised in the other two groups compared with that in the normal group, and the difference was statistically significant ($p < 0.05$), while it was also elevated in the miR-212 mimics group compared with that in the osteosarcoma group, and the difference was statistically significant ($p < 0.05$).

Results of qPCR assay

The results (Figure 3) showed that the rela-

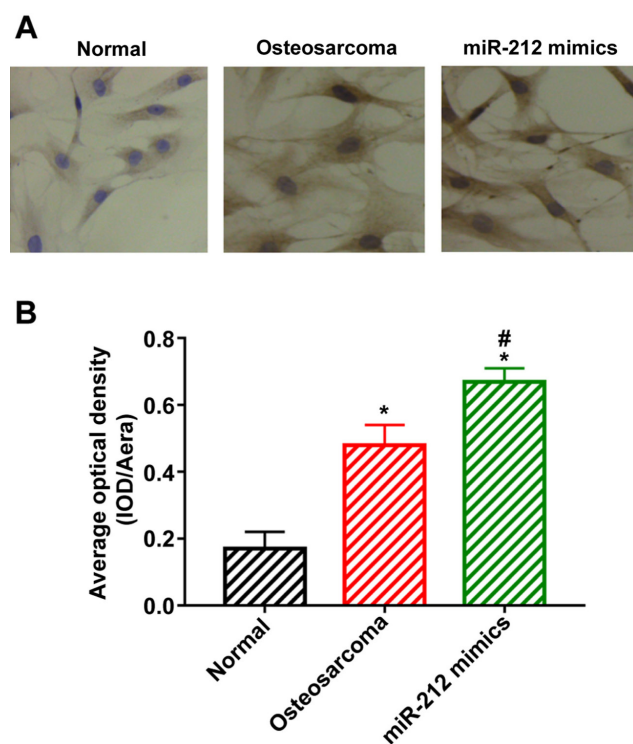


Figure 1. Immunohistochemistry. **A:** Gli1 positive expression detected via immunohistochemistry (magnification: 400 \times). **B:** Average optical density of Gli1 positive expression in each group. * $p < 0.05$ vs. normal group, # $p < 0.05$ vs. osteosarcoma group.

tive expression level of miR-212 was higher in the other two groups than that in the normal group, displaying a statistically significant difference ($p < 0.05$), and it was also higher in the miR-212 mimics group than that in the osteosarcoma group, and there was a statistically significant difference ($p < 0.05$).

Cell proliferation determined via CCK-8 assay

The results (Figure 4) demonstrated that the proliferation rate was dramatically higher in the miR-212 mimics group and the osteosarcoma group than that in the normal group, and the difference was statistically significant ($p < 0.05$), and it rose markedly in the miR-212 mimics group compared with that in the osteosarcoma group, with a statistically significant difference ($p < 0.05$).

Apoptosis detected through flow cytometry

The results showed that there were more apoptotic cells in the normal group and less apoptotic cells in the miR-212 mimics group (Figure 5A), and the apoptosis rate was prominently lower in the other two groups than that in the normal group, with a statistically significant difference ($p < 0.05$),

and reduced clearly in the miR-212 mimics group compared with the osteosarcoma group, showing a statistically significant difference ($p < 0.05$) (Figure 5B).

Discussion

As a primary malignant tumor frequently occurring in children and adolescents, osteosarcoma is mainly derived from mesenchymal tissue cells. The tumor is extremely malignant and often detected in the metaphysis of patients. Currently, with further understanding of osteosarcoma, its clinical characteristics are summarized as common and long disease course in children and adolescents, high malignancy, and high mortality and disability rates [7,8]. Surgery combined with postoperative adjuvant chemotherapy is the main treatment method for osteosarcoma at present, but its clinical efficacy is unsatisfactory, and the 5-year survival rate of osteosarcoma remains low. Studies have reported that osteosarcoma cells are extremely ma-

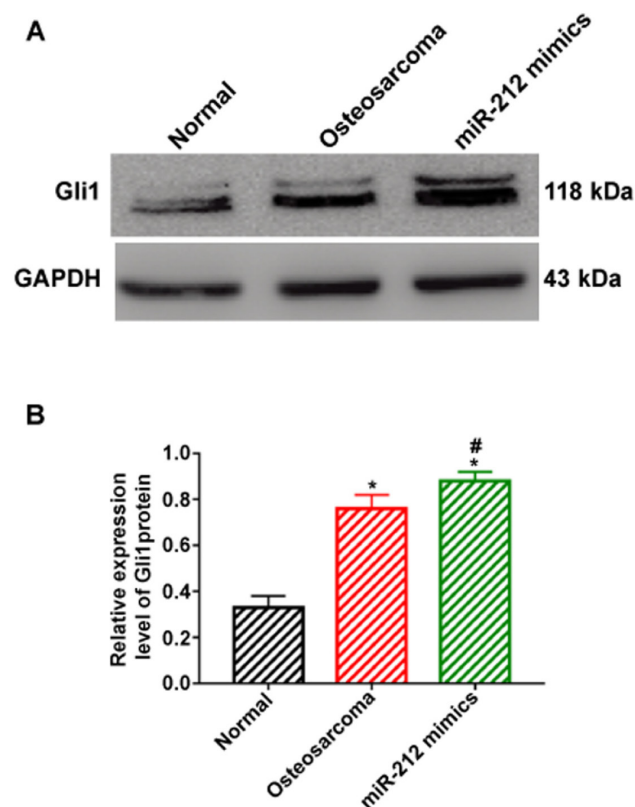


Figure 2. A: Protein expression level measured through western blotting and **B:** Relative expression level of proteins in each group. * $p < 0.05$ vs. normal group, # $p < 0.05$ vs. osteosarcoma group.

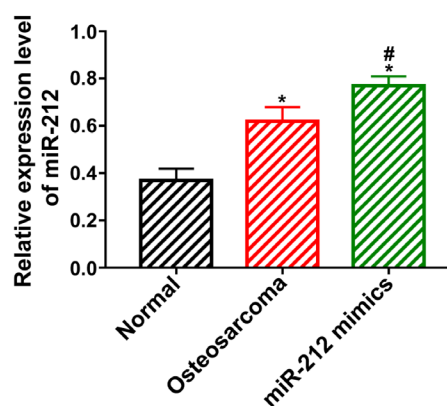


Figure 3. Relative expression level of miR-212 in each group. * $p < 0.05$ vs. normal group, # $p < 0.05$ vs. osteosarcoma group.

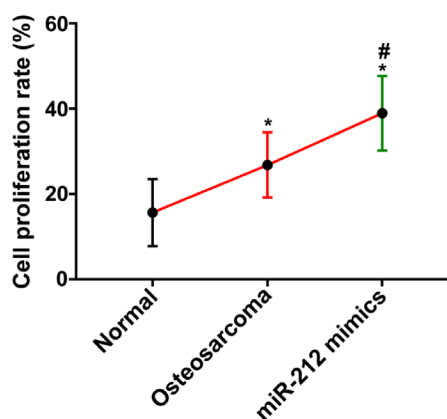


Figure 4. Cell proliferation rate in each group. * $p < 0.05$ vs. normal group, # $p < 0.05$ vs. osteosarcoma group.

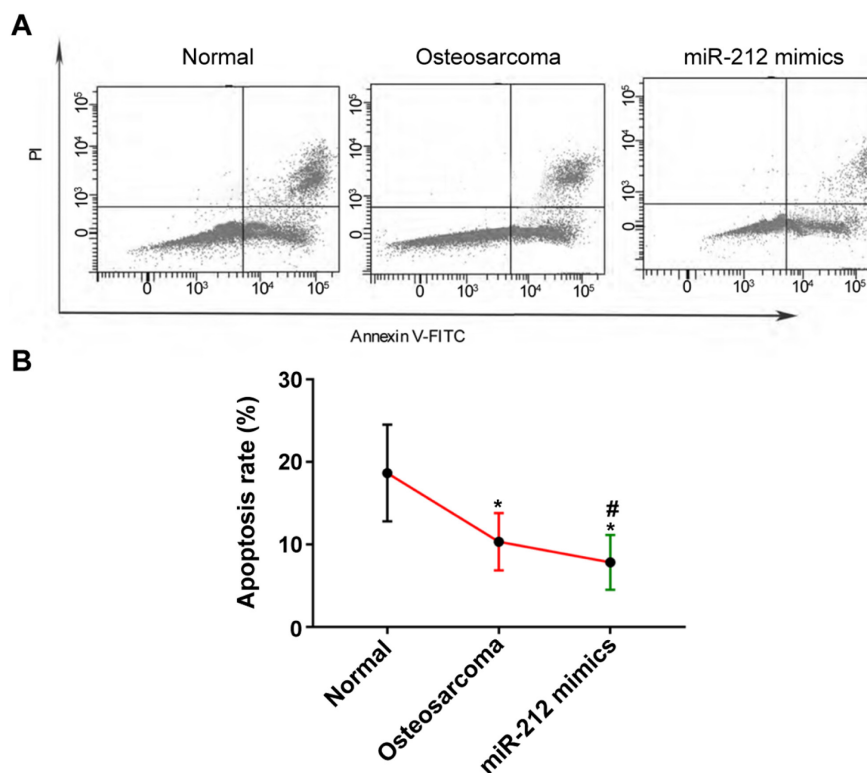


Figure 5. Apoptosis in each group. **A:** Flow cytometry, **B:** Apoptosis rate in each group. * $p < 0.05$ vs. normal group, # $p < 0.05$ vs. osteosarcoma group.

lignant since they have strong proliferation ability but are not prone to apoptosis [9,10]. As a result, the imbalance between proliferation and apoptosis of osteosarcoma cells is currently regarded as one of the important pathogenetic mechanisms of osteosarcoma, despite the unclear and very complicated pathogenesis of this disease [11-13]. Besides, effectively weakening the proliferation ability of osteosarcoma cells and enhancing their apoptosis ability are considered as one of the important ideas for treating osteosarcoma. Research has denoted that the Hedgehog signaling pathway, one of the important signaling pathways in the body, has a close correlation with tumorigenesis and has been proven to participate in the pathogenesis of many malignancies including breast cancer, lung cancer, pancreatic cancer, gastric cancer and colorectal cancer [14,15]. In the Hedgehog signaling pathway, Gli1 is a key molecule, and its abnormally high expression under the effect of stimulating factors is capable of activating the Hedgehog signaling pathway, and the abnormally activated Hedgehog signaling pathway regulates various physiological and pathological responses [16,19]. Moreover, it was confirmed in a study that the abnormal activation of the Hedgehog signaling pathway promotes abnormal proliferation and inhibits apoptosis of cells, which is closely associated with the excessive proliferation and no apoptosis of osteosarcoma

cells [20]. The abnormal activation of the Hedgehog signaling pathway is widely verified in osteosarcoma, indicating that the abnormal activation of the Hedgehog signaling pathway plays a vital role in the pathogenesis of osteosarcoma, and efficaciously repressing the abnormal activation of the Hedgehog signaling pathway is one of the important ideas for the treatment of this disease.

MicroRNAs (miRs) are non-coding RNAs playing crucial roles in the body. In particular, they play a vital role in regulating the proliferation and apoptosis of cells. As an important non-coding RNA, miR-212 exerts an important regulatory effect on many downstream cell signal transduction pathways, thus modulating the development and progression of various diseases. In this study it was uncovered that, compared with that in the normal group, the Gli1 expression was abnormally elevated in the other two groups, suggesting that the Hedgehog signaling pathway is activated in osteosarcoma cells, which may be one of the factors leading to overtly higher proliferation rate and remarkably lower apoptosis rate of osteosarcoma cells in osteosarcoma and the miR-212 mimics groups than those in the normal group. Furthermore, the expression level of miR-212 in the osteosarcoma and miR-212 mimics groups was evidently higher than that in the normal group, implying that miR-212 is abnormally highly expressed during the patho-

genesis of osteosarcoma. What's more, it was discovered that after the intervention of osteosarcoma cells with miR-212 mimics, the proliferation rate of osteosarcoma cells was significantly raised, the apoptosis rate was distinctly reduced, and the expression of Gli1 was abnormally increased, suggesting that miR-212 has a close association with the high proliferation ability and low apoptosis capability of osteosarcoma cells, which is probably achieved by regulating Gli1 expression to modulate the Hedgehog signaling pathway.

Conclusions

This study showed that miR-212 upregulates the Hedgehog signaling pathway to facilitate the proliferation of osteosarcoma cells and repress their apoptosis.

Conflict of interests

The authors declare no conflict of interests.

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