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EXPERIMENTAL STUDY

MicroRNA-497 suppress osteosarcoma by targeting MAPK/Erk pathway

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ABSTRACT
BACKGROUND: The aim of this study was to study the mechanism of miRNA-497 in the apoptosis of osteosarcoma cells.
METHODS: MG-63 cells were divided into the three groups: NC, BL and miRNA groups, NC group were treated with nothing; BL group were transfected with blank vector; miRNA group were transfected with miRNA-497. Cell proliferation rate was detected by MTT method; Apoptosis rate was detected by flow cytometry and measuring the gene and protein expression of MAPK, Erk and P 21 by RT-PCR and Western blot.
RESULTS: The cell proliferation rate of miRNA group was significantly lower compared to NC group and BL group ($p < 0.05$); while the apoptosis rate of miRNA group (32.17 ± 3.23 %) was significantly higher than that of NC group (8.40 ± 1.78 %) and BL group (8.83 ± 0.99 %) ($p < 0.05$, respectively). Regarding the gene expression detection, we found that gene and protein expressions of MAPK, Erk and P21 of miRNA group were significantly different compared to NC and BL groups ($p < 0.05$, respectively).
CONCLUSION: MiR-497 can activate P21 expression by inhibiting the expression of MAPK/Erk signaling pathway, thus promoting the apoptosis of osteosarcoma cells *(Fig. 5, Ref. 18)*. Text in PDF *www.elis.sk.*
KEY WORDS: miRNA-497, MG-63, apoptosis, MAPK/Erk, P21.

Introduction

Some previous studies found that microRNAs (miRNAs) were involved in many kinds of pathological and physiological processes, such as cell proliferation, growth and tumor inhibition, and have a wide range of gene regulatory functions (1–3). MicroRNA-497 (miR-497), as an important member of the microRNA family, had been demonstrated in a variety of tumor cells with low expression, affecting the proliferation and apoptosis of tumor cells (4–6). However, the reports, miR-497 mechanism in osteosarcoma, has been relatively limited. In this study, miR-497 was transfected into MG-63 cells by transfection, compared to untreated group (NC group) and blank vector treated group (BL group), observing effects of miR-497 overexpression on proliferation and apoptosis in MG-63 cells, and to explore the mechanism of miR-497 inhibiting the growth of osteosarcoma MG-63 cells.

Materials and methods

Materials

MG-63, osteosarcoma cells, were purchased from the United States ATCC company, RPMI-1640 medium and fetal calf serum were purchased from the United States Gibco company; LipofectAMINE reagent Lipofectamine™2000 and Trizol were purchased from the United States Invitrogen company, TaqMan-miRNA test kit and PCR Real-Time instrument purchased from the United States AB company, MAPK, Erk and P21 were both Rabbit anti - human, purchased from the British Abcam company, Sheep anti rabbit G Ig two anti purchased from the United States Santa company, MTT purchased from the United States Biomol company, flow cytometry with AV/PI staining and PI staining kit was purchased from Shanghai LEYBOLD cable company.

Cell culture and transfection

RPMI-1640 culture medium containing 10 % fetal bovine serum was used in osteosarcoma cell line MG-63, conditions in 37 °C and 5 % CO_2, liquiding every 2~3 d, passaging when the cell confluence reached 90 %, setting aside keeping cells in logarithmic growth state. MiRNA-497 primer sequences were designed and synthesized according to the literature (6). Log growth phase cells were inoculated in 6 hole plate and divided into blank control group (NC group), negative control group (BL group) and miR-497 group. The transfection procedure was carried out according to the specification of Lipofectamine™2000 kit.

Cell RNA extraction

Trizol method was used to extract the total RNA in the three groups of cells. UV spectrophotometer determination of concentration, agarose gel electrophoresis to determine the integrity of RNA, stored in the –80.

Cell proliferation assay

Adherent cells, joining 50 μl MTT into each wells, culturing

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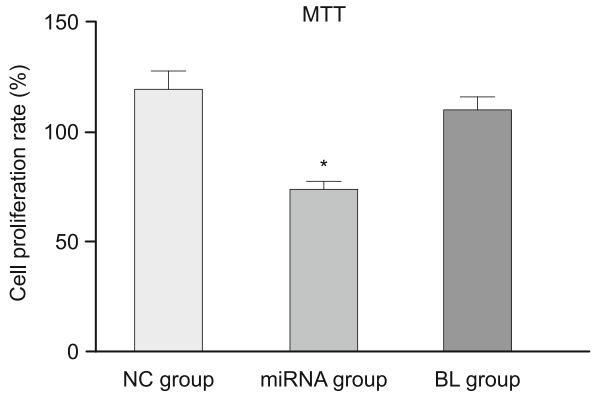

Fig. 1. MTT testing in three groups. * p < 0.05, compared to NC group.

in 37 °C for 4h, sucking out supernatant, each hole joined 150 μL DMSO oscillation 10 min, at 570 nm detection optical density (A), calculated 3 sets of cell proliferation rate.

Cell cycle and apoptosis rate

After transfection with 24 h, the cells were collected and made into single cell suspension, and PBS solution was washed for 2 times, and 70 % ethanol was fixed overnight. According to the operation instructions, the cell cycle was detected by flow cytometry; AV/PI staining was performed according to the specification, and apoptosis was detected by flow cytometry. The experiment was repeated 3 times.

RT-PCR testing

The relative gene primers were following:

MAPK: F:5'-TTCCCCCAACAACACGCACAT-3'; R:

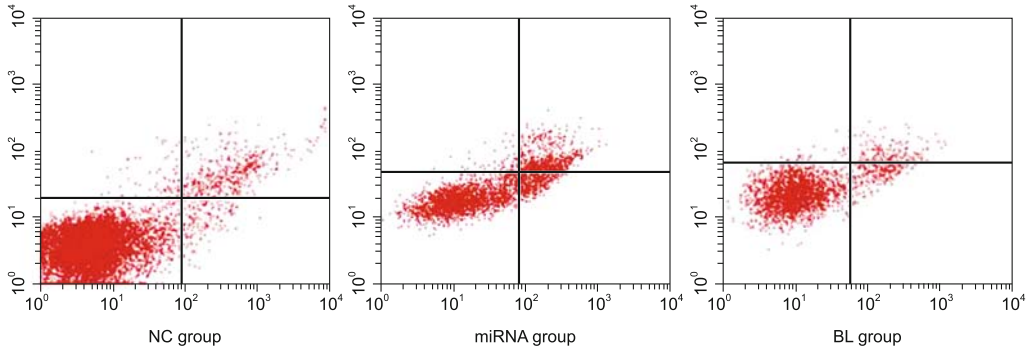

Fig. 2. The cell apoptosis of three groups. * p < 0.05, compared to NC group.

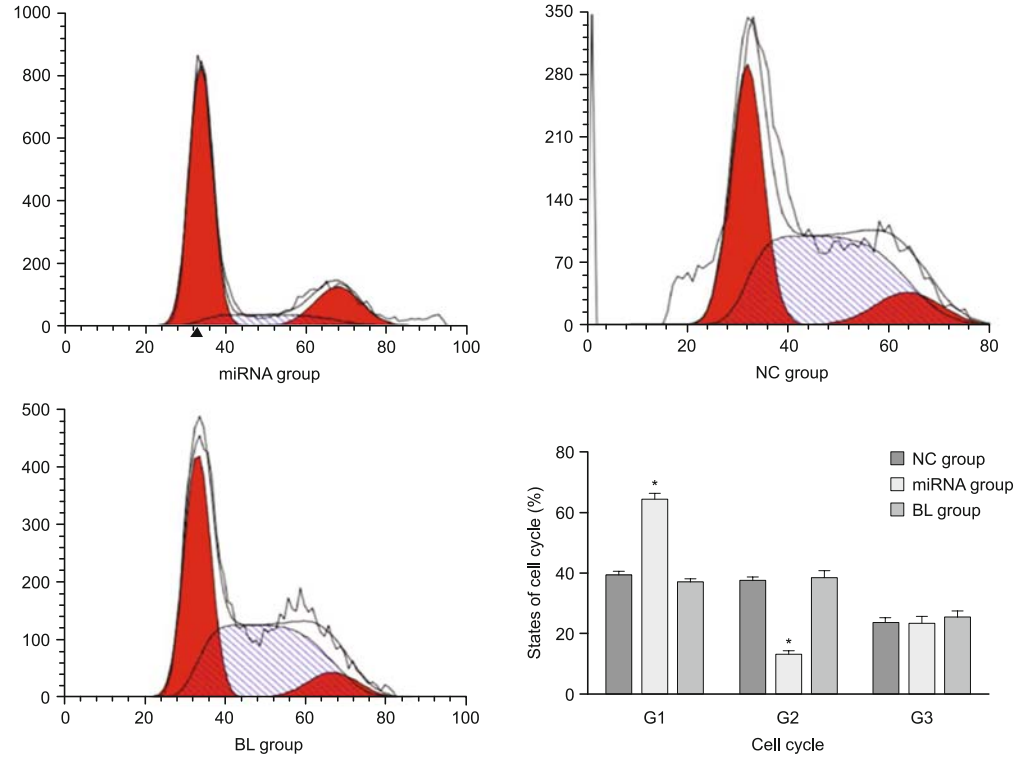

Fig 3. Cell cycles of three groups. * p < 0.05, compared to NC group.

5'-TGACTTCTCTTTGAGGCG-3'. Erk: F: 5'-CCAGACCATGATCACACAGG-3';R: 5'-CTCGTCACTCGGGTCGTAAT-3'. P21:F:5'-ATGTAAGCTTATGTCAGAACCGGCTGGG-3';R:5'-AAGEGAATTCTTAGGGCTTCCTCTTGGAG-3'. GAPDH: F: 5'-GTACGACTCACTATAGGGA AGGTCCACCACCCTGTTGCTGT-3'; R:5'-AGGTGACACTATAGAATA AACAGCGACACCCACTCCTCCA-3'.

PCR amplification reaction conditions were 95 10 s, 60 20 s, 72 10 s, 40 cycles, each experiment was repeated 3 times. GAPDH was selected as the reference in this study, depending on $2^{-\Delta\Delta Ct}$ value to measure the relative expression of MAPK, Erk and P21.

Western blot testing

Extracting the total protein from three cell groups and controlling. Western blotting was deployed to MAPK, Erk and P21 as previous studies (7).

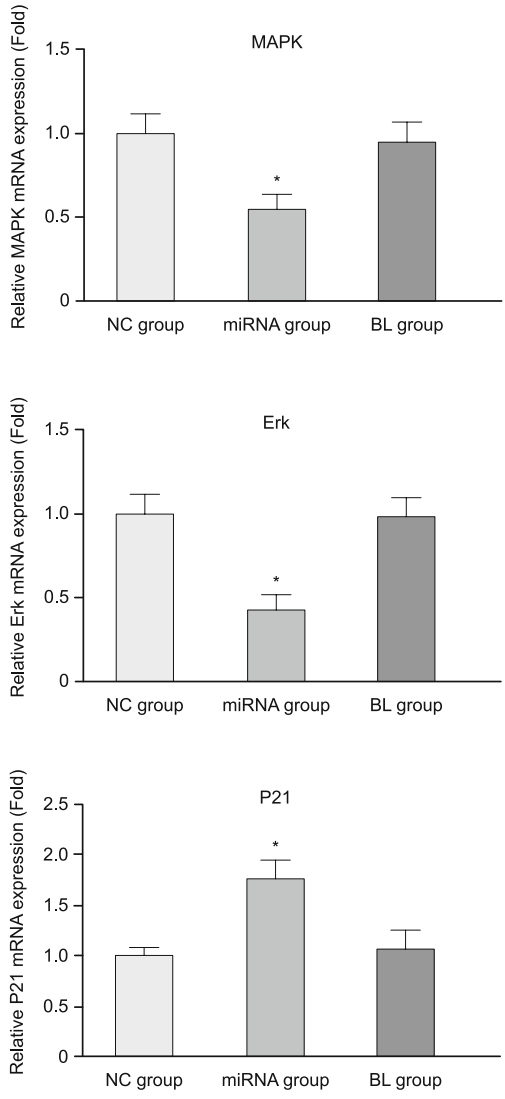

Fig 4. The relative gene expression in three groups. * p < 0.05, compared to NC group.

Statistical methods

This study was used by SPSS 19.0 soft (SPSS Inc., Chicago, USA). All data were expressed as the mean ± standard deviation (SD) values, using one way *ANOVA* with LSD analysis methods in differences groups, $p < 0.05$ considered as statistical significant.

Results

Effects of miR-497 overexpression on cell proliferation

MTT assay showed that the proliferation rate of miRNA group was significantly lower than that of NC group and BL group ($p < 0.05$, respectively). There was no significant difference between the two groups in the NC group and the BL group ($p > 0.05$). The data are shown in Figure 1.

The effect of miR-497 on cell apoptosis

The results of flow cytometry showed that the apoptosis rate of miRNA group (32.17 ± 3.23 %) was significantly higher than that of NC group (8.40 ± 1.78 %) and BL group (8.83 ± 0.99 %) ($p < 0.05$, respectively), however, there were no significantly differences between NC and BL groups. The data are shown in Figure 2.

The effect of miR-497 on cell cycle.

Compared to the G1 and S phase cell ratios of the NC and BL groups, miRNA group was significantly different ($p < 0.05$, respectively). There were no significantly differences among three groups in G2 phase ($p > 0.05$). The data are shown in Figure 3.

Effect of miRNA in related genes expression

MAPK and Erk gene expression of miRNA group were significantly decreased compared to NC group and BL group ($p < 0.05$, respectively); However, the P 21 gene expression of miRNA group was significantly lower than NC and BL groups ($p < 0.05$, respectively). There was no significant difference between NC group and BL group in MAPK, Erk and P21 gene expression ($p > 0.05$, rescpetively). The data are shown in Figure 4.

Effect of miRNA in protein expression

Detection of apoptosis related protein MAPK, Erk and P21 by Western blot, the results showed that the expression of MAPK and Erk in miRNA group was significantly lower than that in NC group and BL group, while the expression of P21 was significantly higher. The data are shown in Figure 5.

Discussion

Osteosarcoma is one of the common malignant bone tumors in children and adolescents. The prognosis is poor, the survival rate is low, and the treatment is difficult. The development of tumor biology therapy opens up a new way for tumor therapy, especially for tumor gene therapy. At present, studies show that the microRNA (miRNA) has a wide range of gene regulation function, all aspects of gene activity regulation, to participate in a series of biological processes such as growth and development, cell proliferation, apoptosis and tumor suppression and so on (1–3,

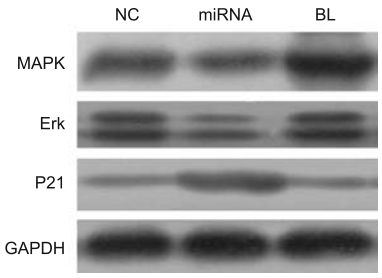

Fig 5. The relative protein expression in three groups.

8, 9). MiR-497, as one of the important members of the miRNA family, has been proved to be low in many kinds of solid tumor cells, which have an effect on the proliferation and apoptosis of tumor cells (4–6). However, the correlation between miR-497 and osteosarcoma is relatively limited. This study confirmed that miR-497 could inhibit the proliferation of osteosarcoma by promoting the apoptosis of osteosarcoma MG-63 cells.

In this study, we observed the effect of miR-497 on the proliferation of osteosarcoma cells by transfection with miR-497, investigating the possible mechanism of miR-497 on the proliferation and apoptosis of MG-63 cells. MAPK is a kind of serine / threonine protein kinase, which is widely expressed in eukaryotic cells. In mammalian, MAPK can be activated by physical stress, cytokines and bacterial complexes extracellular signal or stimulus and with this costimulatory signal via the kinase cascade reaction transfer to the nucleus, and the regulation of cell proliferation, differentiation, transformation, invasion and apoptosis of biological effects (10). MAPK signaling pathway plays an important role in the growth and proliferation of tumor cells (11–13), and can affect the growth and proliferation of cells by ERK expression (14–16). P21 protein is a kind of widely used cell cycle regulatory factor, which can inhibit CDKl, and its specific binding to make it lose its activity, so that the cell cycle is blocked, so as to promote cell apoptosis (17, 18). Through this study, we found that the rate of cell proliferation in miR-497 over expression group was significantly lower than that in NC group and BL group. The results of flow cytometry showed that the apoptosis rate of miRNA group was significantly increased, and the number of G1 phase cells increased significantly. The results suggested that the overexpression of miR-497 could promote the apoptosis of osteosarcoma cells by affecting the growth cycle of osteosarcoma cells. Meanwhile, we found that the gene and protein expression levels of MAPK and Erk were significantly lower in the miR-497 group, while the gene and protein expression level of P21 was significantly increased in miRNA group. We hypothesized that miR-497 might promote P21 expression by reducing the expression of MAPK/Erk signaling pathway, thereby inhibiting cell proliferation and promoting apoptosis.

In summary, miR-497 can effectively inhibit osteosarcoma cell proliferation and promote apoptosis. The mechanism may reduce the MAPK / ERK signaling pathway and promote the expression of P21 and thereby osteosarcoma cells remain in G1 phase and promote cell apoptosis.

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