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Original Research Article

Expression of mTOR conduction pathway in human osteosarcoma MG-63 cells and their stem cells, and the inhibitory effect of different doses of rapamycin

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Abstract

Purpose: *To investigate the expressions of rapamycin target protein (mTOR) conduction pathway in human osteosarcoma MG-63 cells and their stem cells, and to examine the inhibitory effect of different doses of rapamycin.*

Methods: *mTOR mRNA in osteosarcoma stem-like cells and human osteosarcoma MG-63 cells were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The cells were treated with different doses of rapamycin and divided into low dose group (0.5 mg), medium dose group (1.0 mg), high dose group (2.0 mg) and blank (control) group. Apoptosis and cell cycle of MG-63 cells were determined by flow cytometry, while proliferation of MG-63 cells up was assessed by CCK-8 kit.*

Results: *mTOR in human osteosarcoma MG-63 cells was significantly lower than that in osteosarcoma stem-like cells. Compared with the control group, mRNA expression levels of mTOR in MG-63 cells and osteosarcoma stem-like cells were significantly decreased after treatment with different concentrations of rapamycin ($p < 0.05$). MG-63 cells treated with various doses of rapamycin exhibited a significant decrease in their proliferation, compared with control group, while only the high rapamycin concentration group exhibited a significant decrease in osteosarcoma stem-like cell proliferation ($p < 0.05$). Treatment with rapamycin in MG-63 cells and osteosarcoma stem-like cells resulted in a significant increase in apoptosis, prolonged G0/G1 phase and shortened S phase ($p < 0.05$).*

Conclusion: *Rapamycin inhibits the expression of mTOR mRNA in osteosarcoma stem-like and MG-63 cells. It also inhibits the proliferation and cell cycle formation of osteosarcoma stem-like cells and MG-63 cells via mTOR signal pathway. These findings may provide a new target for the treatment of osteosarcoma.*

Keywords: *Rapamycin, MTOR signal pathway, Human osteosarcoma MG-63, Osteosarcoma stem like cells*

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INTRODUCTION

Osteosarcoma is a common malignant bone tumor disease in clinical oncology. It is more common in young people, and it occurs in the metaphysis of long bones. The lesion is highly malignant and has a poor prognosis [1,2]. At present, the medical field is still unable to fully

explain the mechanism and causes of the disease. There are many clinical treatment methods for the disease, and they are surgery, radiotherapy and chemotherapy. For most patients, surgery combined with radiotherapy and chemotherapy can achieve relatively good results. The 5- year survival span of patients has significantly improved [3,4].

However, some patients have lung metastases, and their prognosis is poor, which is also the main risk factor in the death of patients [5,6]. For patients with metastases, chemotherapy is the only effective treatment. However, a large number of studies have shown that the emergence of chemotherapy resistance has a serious impact on the prognosis and long-term survival of such patients. It is, therefore, important to urgently find a more effective drug with low resistance and good sensitivity for use in clinical practice.

EXPERIMENTAL

Equipment and reagents

Vertical electrophoresis instrument was purchased from Beijing Liuyi Company (Beijing, China), the PCR instrument was purchased from ABI Company (Waltham, MA, USA), the Coulter EpicsXL flow cytometer was purchased from Beckman Company (Franklin Lakes, NJ, USA), and the MK3 enzyme reader was purchased from Bio-Rad Company (Hercules, CA, USA). MTOR mRNA primers were designed and synthesized by Shanghai Shenggong Company (Shanghai, China). MTOR protein antibody was purchased from CST Company (Danvers, MA, USA); Reverse transcription kit was purchased from Fermentas Company (Burlington, Canada); VEGF and HIF-1α ELISA kit were purchased from R&D Company (Minneapolis, MN, USA); DNA-prep kit was purchased from Bakman Company (Franklin Lakes, NJ, USA); Cell counting kit-8 (CCK-8) kit was purchased from Suzhou Biyuntian Biological Co. LTD. (Suzhou, China); Gibco (Rockville, MD, USA) provided fetal bovine serum (FBS); Dulbecco's Modified Eagle Medium (DMEM) and double antibody; and Fujian Institute of Biology (Fuzhou, China) provided rapamycin.

Cell culture

The Cell Bank of the Type Culture Collection Committee of the Chinese Academy of Sciences (Shanghai, China) provides human osteosarcoma MG-63 cell lines and osteosarcoma stem-like cells. DMEM/F12 medium (containing 15 % fetal bovine serum) and DMEM medium (10 % fetal bovine serum) were used to culture osteosarcoma stem-like cells and MG-63 cell lines, and they were placed in an incubator with 5 % CO_2 at a temperature of 37 °C. Trypsin (0.25 %) was added when the cells were at 80 % of the bottom of the flask. After the cells are digested, the cells were centrifuged at 3000 rpm and then resuspended. Subculture was carried out at a ratio of 1:3; and Osteosarcoma stem cells and MG-63 cells were treated with rapamycin at different effective concentrations of 1×10^{-6} mol/L and divided into low dose group (0.5 MG), medium dose group (1.0 MG), high dose group (2.0 MG) and blank control group.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Osteosarcoma stem cells and MG-63 cells at the logarithmic growth phase were collected for the preparation of a single cell suspension and the inoculation of 5×10^4 cells/well in a 6- well culture plate for culture. The total RNA of the cells was extracted by the TRIzol method and reverse transcribed. mTOR upstream primer: . 5'-TTGGAGACGGTTTGGTGA-3', downstream primer: 5'-GTTTGTTCGCTGTAGGGT-3', 157 bp; p70S6K amplified fragment length. Using qRT-PCR was analyzed osteosarcoma stem cells. PCR conditions were described in a previous report [7].

Western blotting

Osteosarcoma stem cells and MG-63 cells in logarithmic growth phase were collected. After being cultured for 24 h, total protein was extracted from each group for electrophoresis with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then the proteins were transferred onto membranes and sealed overnight at 4 °C.

Human rabbit anti-mouse mTOR and phosphorylated mTOR primary antibody (1:3000) were added, incubated for 2 h at room temperature, followed by incubation with secondary antibodies (1: 5000) at room temperature for 1 h. Finally, the membranes were exposed according to the relevant instructions of ECL luminescence kit. Quantity One software was used for quantitative analysis of gray values [8].

CCK-8

Osteosarcoma stem cells and MG-63 cells were taken from each group and made into single cell suspension at logarithmic growth stage. 200 μL

was taken and inoculated in 2 × 10^3 / well 96-well culture plates. Blank control group was set up and 6 multiple Wells were set up. After the cells were attached to the wall, different doses of rapamycin were added, and cultured for 72 h. After the cells were washed with PBS twice, 10 μL CCK-8 reagent and 90 μL culture medium were added to each well, and cultured for 2 h. After 72 h of medication, the absorbance (A) of each well was measured at the wavelength of 450 nm [9].

Flow cytometry

The osteosarcoma stem cells and MG-63 cells in each group were inoculated on 5×10^4 mol/L 6-well culture plate to set up negative control. After 24 h culture, 0.25 % trypsin was added into the well for 20 min digestion treatment, and single cell suspension was prepared.

The cells were washed twice by PBS, and then the cell suspension was centrifuged at 300 g for 5 min, and the cells were collected. The cell density was adjusted to 1 × 10^5 cells/mL, and 300 mesh sieve was used for filtering. Flow cytometry was performed for 5 × 10^3 – 1 × 10^4 cell cycles in strict accordance with the instructions of DNA detection kit [10].

Statistical analysis

SPSS statistical analysis software (version 26.0) was used for analysis and the data are presented as mean ± SD while t-test was used for comparison between groups. Chi squared test or Fisher's exact probability method was used to compare count data, which was expressed as rate (%). Logistic multifactor regression model was used to analyze the difference. $P < 0.05$ was considered statistically significant.

RESULTS

mTOR mRNA and mTOR protein expressions in osteosarcoma stem-like cells and human osteosarcoma MG-63 cells

The qRT-PCR results showed that mTOR mRNA in human osteosarcoma MG-63 cells (0.407 ± 0.092) and Osteosarcoma stem cells (0.759 ± 0.078) were significantly different (t = 7.721, $p < 0.05$). Western Blot showed that mTOR/β-actin gray level in human osteosarcoma MG-63 cells (0.374 ± 0.056) was significantly lower than that in Osteosarcoma stem cells (0.514 ± 0.081), and the difference was significant (t = 3.761, $p < 0.05$), as shown in Figure 1.

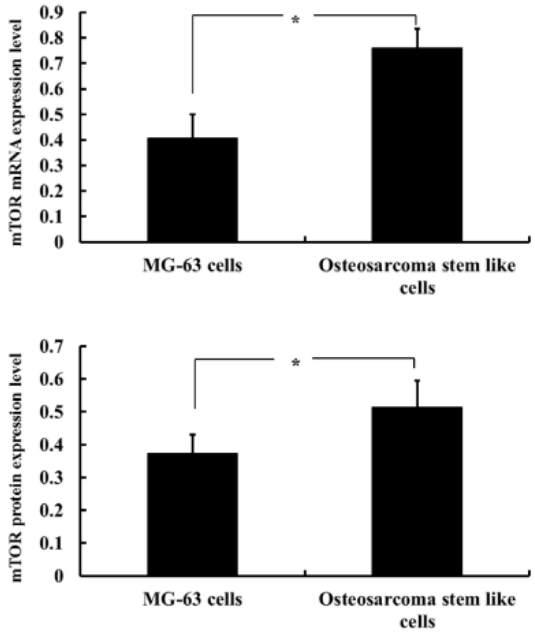

Figure 1: mTOR mRNA and mTOR protein expressions in osteosarcoma stem-like cells, human osteosarcoma MG-63 cells. (A) mToR mRNA expression level; (B) mTOR protein expression level; *$p < 0.05$ compared with control group

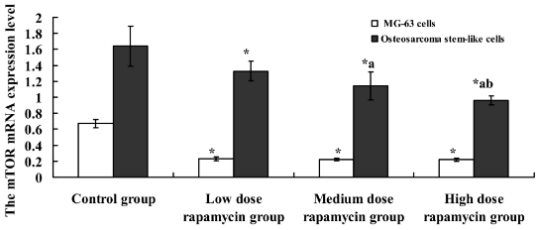

Figure 2: mTOR mRNA expression in osteosarcoma stem like cells, MG-63 cells treated with different doses of rapamycin. Compared with the control group, *$p < 0.05$; Compared with the low dose group, $^a p < 0.05$; compared with the medium dose group, $^b p < 0.05$

mTOR mRNA expression in osteosarcoma stem-like cells and MG-63 cells after treatment with different doses of rapamycin

Results showed that mTOR mRNA expression in MG-63 cells was significantly reduced after treatment with different concentrations of rapamycin compared with the control group ($p < 0.05$), but there was no significant difference in mTOR mRNA expression in the low-dose, middle-dose, and high-dose groups ($p > 0.05$). The mTOR mRNA expression of osteosarcoma stem-like cells decreased after treatment with different concentrations of rapamycin. Compared with the control group ($p < 0.05$), the mTOR

mRNA expression in the high-dose group was lower than that of the middle-dose and low-dose groups. The middle-dose group is lower than the low-dose group ($p < 0.05$, Figure 2).

Proliferation of sarcoma stem-like cells and MG-63 cells treated with rapamycin

Compared with the blank control group, rapamycin significantly inhibited the proliferation of MG-63 cells ($p < 0.05$), while osteosarcoma stem-like cells were treated with different doses of rapamycin. After treatment with mycin, only the high-dose group was compared with the blank control group, and the difference was significant ($p < 0.05$). The other doses were not statistically different from the blank control group ($p > 0.05$).

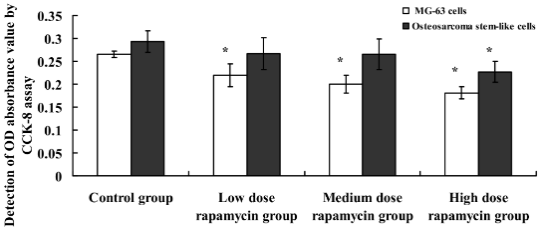

Figure 3: CCK-8 was used to determine the effects of different doses of rapamycin on the proliferation of osteosarcoma stem-like cells and MG-63 cells group; *$p < 0.05$ compared with control

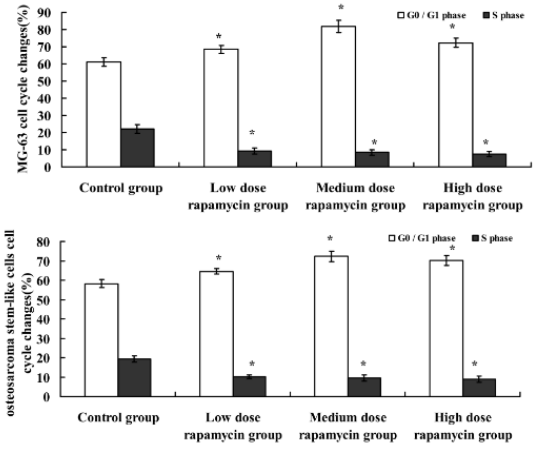

Figure 4: Cell cycle of osteosarcoma stem-like cells and MG-63 cells treated with different doses of rapamycin, *$P < 0.05$, compared with the control group

Cell cycle data for osteosarcoma stem-like cells and MG-63 cells treated with rapamycin

According to flow cytometry analysis, after treatment of MG-63 cells and osteosarcoma stem-like cells with rapamycin, the G0/G1 phase of the low-dose, medium-dose, and high-dose G0/G1 phase was significantly prolonged, while the S phase was significantly shortened, compared with the control group ($p < 0.05$, Figure 4).

Apoptosis of osteosarcoma stem-like cells, MG-63 cells treated with rapamycin

Similarly, flow cytometry analysis revealed that rapamycin treatment of MG-63 cells or osteosarcoma stem-like cells significantly ($p < 0.05$) increased the % apoptotic rate compared with the control cells at low, medium and high doses. For details, see Figure 5.

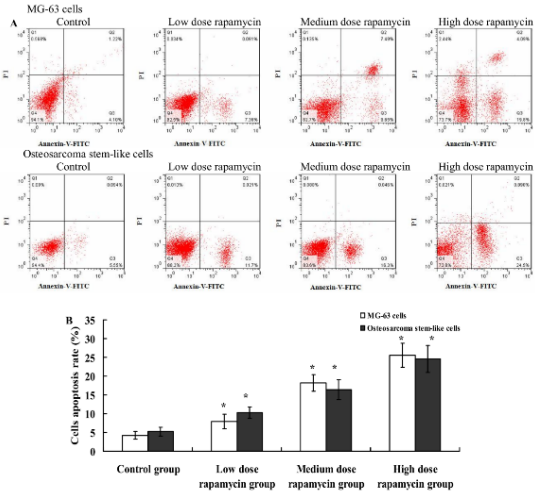

Figure 5: Apoptosis of osteosarcoma stem-like cells and MG-63 cells treated with different doses of rapamycin as flow cytometry. (A) Apoptosis was determined using flow cytometry; (B) Statistics of apoptosis rate in each group *$P < 0.05$, compared with control group

DISCUSSION

The mTOR protein has always been regarded as an important target for the clinical treatment of malignant tumor diseases. It plays a role as the central regulatory point in the process of tumor cell differentiation, growth, and proliferation. Rapamycin specifically binds to the mTOR protein to effectively block the cell mTOR function, and also has an anti-tumor biological effect [11-13]. Studies have shown that ap2357, a derivative of rapamycin, can have a good effect on soft tissue tumors and malignant bone tumors, and inhibits the proliferation of sarcoma cells in intimal cells *in vitro.* At the same time, it inhibited a variety of human transplanted tumor models [14-16].

This study showed that the gray values of mTOR mRNA and mTOR/actin in human osteosarcoma

MG-63 cells were lower than those in osteosarcoma stem-like cells ($p < 0.05$), indicating that the mTOR signaling pathway was involved in the proliferation and growth of human osteosarcoma stem cells and MG-63 cells. The cycle regulation plays an important role; and the higher mTOR expression level of human osteosarcoma stem cells indicated that osteosarcoma stem cells were in a more active cell cycle and proliferate faster, and are closely related to tumor recurrence and metastasis.

In this study, osteosarcoma stem cells and MG-63 cells were treated with different doses of rapamycin. Compared with controls, MG-63 cells treated with different concentrations of rapamycin exhibited significantly decreased mTOR mRNA expression. mTOR mRNA expression in osteosarcoma stem-like cells decreased after treatment with different concentrations of rapamycin. Additionally, mTOR mRNA in the high-dose group was lower than that of the medium-dose and low-dose groups, suggesting that rapamycin inhibited osteosarcoma stem-like cells mTOR mRNA.

In order to further understand the effects of rapamycin on the proliferation and cell cycle of osteosarcoma stem cells, MG-63 cells, CCK-8 testing compared with the blank control group, low-dose, medium-dose, and high-dose rapamycin has an effect on MG-63 cells. Significant inhibition of proliferation suggests that high-dose medication for MG-63 cells reduced cell viability to 69.13 %, effectively inhibiting cell proliferation; while osteosarcoma stem-like cells are treated with different doses of rapamycin,

When compared with the blank control group but the low-dose and middle-dose medication, unlike the high-dose group, had little effect on the activity of osteosarcoma stem cells ($p > 0.05$). mTOR is involved in cell energy metabolism, skeleton construction, protein translation, cell proliferation and other processes, and belongs to the downstream effector of phosphoinositide 3-kinase. Rapamycin inhibits the mTOR signaling pathway, regulates cell number and reduces cell proliferation. High-dose medication can inhibit the proliferation of osteosarcoma stem cells [17-19].

Flow cytometry data showed that MG-63 cells and osteosarcoma stem-like cells treated with low-dose, medium-dose, and high-dose rapamycin, exhibited significantly prolonged G0/G1 phase and shortened S phase. These results indicate that rapamycin has a significant inhibitory effect on the cell cycle of MG-63 cells and osteosarcoma stem-like cells.

CONCLUSION

As a mTOR-targeted drug, rapamycin has significant anti-tumor activity in the treatment of human osteosarcoma. With increase in the dose of the drug, the inhibitory effects on tumor cells become more significant. However, the side effects as well as toxic effects should also be considered.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The authors contributed equally to this research. Hao Wu and Xuelei Wang contributed equally to this work.

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