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Effects of rapamycin on osteosarcoma cell proliferation and apoptosis by inducing autophagy

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Abstract. – OBJECTIVE: To investigate the influences of rapamycin on proliferation and apoptosis of human osteosarcoma MG-63 cells and the mechanisms of action.

MATERIALS AND METHODS: The human osteosarcoma MG-63 cells were randomly divided into Control group, Rapamycin group, and Rapamycin + Beclin-1 plasmid transfection group. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was adopted to detect the viability of MG-63 cells in each group, and the 5-Ethynyl-2'-deoxyuridine (EdU) staining and Hoechst staining were applied to determine the proliferation and apoptosis, respectively, of MG-63 cells in each group. The levels of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) were measured using enzyme-linked immunosorbent assay (ELISA) kits, and the protein expression levels of Beclin-1 and Vps34 in each group of MG-63 cells were tested using the Western blotting.

RESULTS: Compared with the Control group, Rapamycin group, and Rapamycin + Beclin-1 plasmid transfection group had markedly weakened the viability of MG-63 cells, inhibited cell proliferation, remarkably increased cell apoptosis rate, elevated Bax level, notably declined Bcl-2 level, and significantly raised the levels of Beclin-1 and Vps34 proteins in MG-63 cells. Besides, the effects in Beclin-1 plasmid transfection group were stronger.

CONCLUSIONS: Rapamycin may decrease the viability, inhibit the proliferation, and promote the apoptosis of MG-63 cells by activating autophagy.

Key Words:

Rapamycin, Autophagy, Osteosarcoma cells, Proliferation, Apoptosis.

Introduction

Osteosarcoma, a type of primary malignant tumor originating in primitive mesenchymal cells, frequently occurs in the metaphysis of the long bone in children and adolescents, with pain and swelling as the clinical manifestations. It is characterized by rapid onset and tumor growth as well as easy relapse and metastasis[1-3]. Clinically, osteosarcoma is mainly treated by means of surgical resection, during which adjunctive therapy with chemotherapy drugs such as cisplatin, Adriamycin, and methotrexate are applied to the patients. However, no prominent improvement in the overall treatment effect on osteosarcoma has been achieved over the past decades, so seeking novel treatment methods or researching and developing new drugs is a hotspot of the research on the disease[4,5].

Autophagy is widespread in eukaryotic cells and serves as one of the pathways accelerating the degradation of impaired, denatured, or senescent proteins in cells, thus maintaining cellular homeostasis[6]. When the cells are stimulated by stress, the autophagy can effectively prevent the intracellular accumulation of toxic or carcinogenic proteins. In recent years, a large amount of literature[7,8] has reported that autophagy is closely associated with the occurrence and development of tumors, and it is involved in the regulation of the apoptosis and drug resistance of tumor cells and also in the proliferation and differentiation of tumor stem cells. Autophagy and apoptosis are two overlapping modes of programmed cell death. With the deepening of some studies, frizzled-related protein Beclin-1 is discovered by researchers for the first time. Known as the BECN1 gene and located in human chromosome 17q21, Beclin-1 is the first target found to be related to autophagy. It can form autophagy-associated complexes by binding to Vps34 through the evolutionarily conserved district, thereby triggering autophagy[9,10]. It suggests that the cell autophagy, activated by appropriate drugs or intervention means, can repress the proliferation and promote the apoptosis of tumor cells.

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Rapamycin, also known as "sirolimus", is extracted from *Streptomyces hygroscopicus* and belongs to the 36-membered triene macrolide antibiotics[11]. Donckier et al[12] have revealed that rapamycin has anti-tumor, immunosuppressive, and other pharmacological actions, which are extensively applied to prevent organ rejection after transplantation in the clinic. Some investigations have discovered that rapamycin manifests a favorable anti-tumor effect, but its regulatory role in human osteosarcoma cells is rarely reported. Therefore, this paper aims to investigate the influences of rapamycin on the proliferation and apoptosis of osteosarcoma cells and explore the mechanisms of action, so as to provide a new basis for the clinical treatment of osteosarcoma with rapamycin.

Materials and Methods

Reagents

The main reagents used were: rapamycin (Sangon Biotechnology, Shanghai, China); Beclin-1 plasmid (Wuhan Miaoling Bioscience & Technology Co., Ltd., Wuhan, China); Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA); MG-63 cells lines (Shanghai Xin Yu Biotechnology Co., Ltd., Shanghai, China); high glucose Dulbecco's Modified Eagle's Medium (H-DMEM, Hyclone, South Logan, UT, USA); fetal bovine serum (FBS, Hyclone, South Logan, UT, USA); penicillin/streptomycin (P/S; Gibco, Grand Island, NY, USA); trypsin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China); cell culture plate and flask (Corning, Corning, NY, USA); kits of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 5-Ethynyl-2'-deoxyuridine (EdU) staining and Hoechst staining (Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China); enzyme-linked immunosorbent assay (ELISA) kits for B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax; Abcam, Cambridge, MA, USA); rabbit anti-human Beclin-1 and Vps34 primary antibodies (Sigma-Aldrich, St. Louis, MO, USA); horseradish peroxidase (HRP-labeled secondary antibodies; Beijing Bioss Biological Technology Co., Ltd., Beijing, China).

Instruments

The main instruments used were: Inverted fluorescence microscope (Nikon, Tokyo, Japan); gel electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) and microplate reader (Shanghai Flash Spectrum Biotechnology Co., Ltd., Shanghai, China).

MG-63 Cell Culture and Transfection

MG-63 cells were cultured in the H-DMEM containing 10% FBS and 1% P/S for 3 d and then washed using high-temperature sterilized 1× Phosphate-Buffered Saline (PBS) solution. Later, the cells were reacted with trypsin for 5 min, and the complete medium was added to terminate the digestion. The cell suspension was collected and centrifuged at 1,500 rpm for 7 min. After the medium was discarded, another complete medium was added to resuspend the cell pellets, and the cells were paved on a 6-well plate overnight. When the cell fusion reached 70-80%, 2 μg of Beclin-1 plasmid was dissolved in 150 μL of Opti-Medium and placed for 5 min according to the instructions of Lipofectamine 2000. Next, 6 μL of Lipofectamine 2000 was dissolved in 150 μL of Opti-Medium, followed by standing for 5 min. Subsequently, the two solutions were mixed and let stand for 20 min, and then the mixture was added dropwise into the culture medium of MG-63 cells. 6 h later, the medium was replaced, and the transfection was observed 24 h later.

Detection of MG-63 Cell Viability in Each Group Via MTT Assay

MTT kit is a very typical tool to detect cell viability, in which the stronger the cell viability means the higher the absorbance. The MG-63 cells were paved on a 96-well plate at a density of $5×10^3$ cells/well and cultured overnight. Later, the MTT solution (5 mg/mL) was prepared and stored away from light. At 24 h after the reaction with rapamycin (50 nM), 10 μL of MTT solution was added into each well and cultured for other 4 h. After that, 100 μL of formazan solution was added into each well, mixed, and cultured again for 4 h. Finally, the absorbance at the wavelength of 570 nm was measured using the microplate reader.

Detection of MG-63 Cell Proliferation in Each Group Via Edu Staining

The Edu-488 cell proliferation detection kit was used for cell proliferation detection, in which the thymidine analog Edu was incorporated and labeled in the process of DNA synthesis. 100 μL of Edu working solution (10 μM) was added into MG-63 cells in the 96-well plate for incubation for 2 h. Subsequently, the cells were fixed in 4% paraformaldehyde for 15 min, sealed in PBS solution containing 3% bovine serum albumin (BSA), permeabilized in 0.3% Triton X-100 solution for

15 min, and washed with PBS (5 min/time). Finally, the staining was observed at the wavelength of 495 nm under a microscope.

Detection of MG-63 Cell Apoptosis in Each Group Via Hoechst 33258 Staining

Hoechst 33258 (molecular formula: $C_{25}H_{24}N_6O{\cdot}3HCl$, molecular weight: 533.88) is a kind of blue fluorescent dye capable of penetrating the cell membrane, with relatively low cytotoxicity. MG-63 cells in the 96-well plate were fixed with 4% paraformaldehyde and then permeabilized with 0.3% Triton X-100 solution for 15 min, followed by reaction with Hoechst 33258 staining solution (1 μg/mL) for 15 min, washing with PBS solution and observation of staining under the microscope.

Detection of Bax and Bcl-2 Levels in Each Group of MG-63 Cells Via ELISA

The blank wells were added with 100 μL of samples, while the control wells were added with 100 μL of deionized water. Then, 50 μL of the enzyme-labeled solution was added into each well, and the microplate was sealed using sealing gum, followed by incubation at 37°C for 1 h. Next, the microplate was washed adequately for 3-5 times (5 min/time) and patted thoroughly dry on absorbent paper. Later, 50 μL of color developer A and 50 μL of color developer B were added for incubation at room temperature for 15 min. Finally, 50 μL of stop buffer was added to terminate the reaction. The absorbance at the wavelength of 450 nm in each well was detected using the microplate reader within 30 min, and the levels of Bax and Bcl-2 were calculated.

Detection of Beclin-1 and Vps34 Protein Levels in MG-63 Cells in Each Group Via Western Blotting Assay

The total proteins in each group of MG-63 cells were extracted, and the concentration was determined via the Bradford method. According to the loading of electrophoresis (20 μg), a corresponding volume of protein lysate was added. The voltage was set as 80 V when the proteins were in the spacer gel, which was switched to 120 V when the proteins ran to the dividing line between the separation gel and spacer gel. After the electrophoresis, the proteins were transferred onto a membrane, and the target bands were clipped according to the Marker, followed by incubation, sealing with 5% BSA for 1 h, and incubation with rabbit anti-human Beclin-1 and Vps34 primary antibodies diluted by 1% BSA (1:1000) overnight. The next morning, the proteins were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h, the color was developed using the diaminobenzidine (DAB) color developer, and the optical density of the bands was recorded and analyzed.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) software was employed for statistical analyses and the measurement data were presented as mean ± standard deviation. The *t*-test was used to analyze the measurement data. The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Rapamycin Could Reduce the MG-63 Cell Viability

The impact of rapamycin on MG-63 cell viability was investigated via MTT assay. As shown in Figure 1, the cell viability was markedly reduced in Rapamycin group ($^*p<0.05$) and Rapamycin + Beclin-1 plasmid transfection group ($^\#p<0.05$) compared with that in Control group,. Moreover, the impact in Rapamycin + Beclin-1 plasmid transfection group was stronger than that in the Rapamycin group, suggesting that rapamycin is able to attenuate the viability of MG-63 cells, and the effect of transfection with Beclin-1 plasmid is stronger.

Rapamycin Could Inhibit the MG-63 Cell Proliferation

The Edu staining was conducted to detect the impact of rapamycin on MG-63 cell proliferation, and the results are shown in Figure 2. According to the statistical analysis, in comparison with that in the Control group, the cell proliferative capacity was remarkably decreased in the Rapamycin group ($^*p<0.05$), and it was also significantly weakened in Rapamycin + Beclin-1 plasmid transfection group ($^\#p<0.05$), manifesting stronger impact than that in the Rapamycin group (Figure 3). This indicated that rapamycin

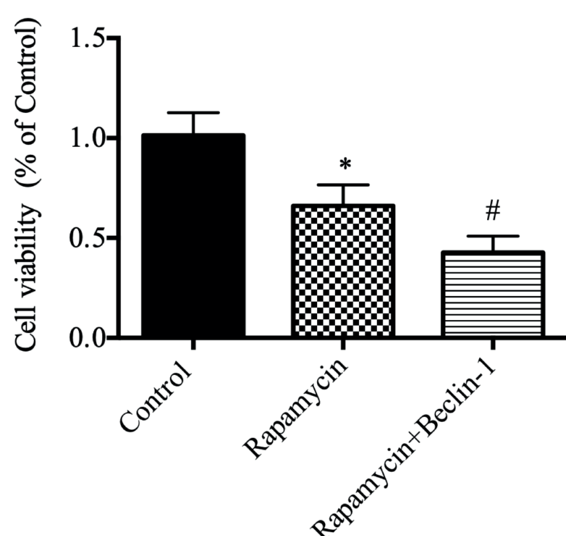

Figure 1. Comparison of MG-63 cell viability among groups. (*$p<0.05$: Rapamycin group *vs.* Control group, #$p<0.05$: Rapamycin + Beclin-1 plasmid transfection group *vs.* Rapamycin group).

can repress the proliferation of MG-63 cells, and that the transfection with Beclin-1 plasmid had a stronger effect.

Rapamycin Could Promote the MG-63 Cell Apoptosis

The Hoechst 33258 staining was utilized to detect the impact of rapamycin on MG-63 cell apoptosis. It was shown in the results (Figure 4) that the apoptotic cells were stained bright blue and had shrunk nuclei. The results of the statistical analysis indicated that both the Rapamycin group (*$p<0.05$) and the Rapamycin + Beclin-1 plasmid transfection group (#$p<0.05$) had notably higher cell apoptosis rates than the Control group. Besides, the impact in the Rapamycin + Beclin-1 plasmid transfection group was stronger than that in the Rapamycin group (Figure 5), illustrating that rapamycin is capable of promoting the apoptosis of MG-63 cells, and that the transfection with Beclin-1 plasmid has a stronger effect.

Rapamycin Could Increase Bax Level and Decrease Bcl-2 Level in MG-63 Cells

The impacts of rapamycin on the expression levels of Bax and Bcl-2 in MG-63 cells were explored via ELISA. Compared with those in the Control group, the Bax level was elevated (*$p<0.05$), while the Bcl-2 level was lowered (*$p<0.05$) in Rapamycin group (Table I). Moreover, the raised Bax level (#$p<0.05$) and declined Bcl-2 level (#$p<0.05$) were also observed in the Rapamycin + Beclin-1 plasmid transfection group, and the effects were stronger than those in the Rapamycin group.

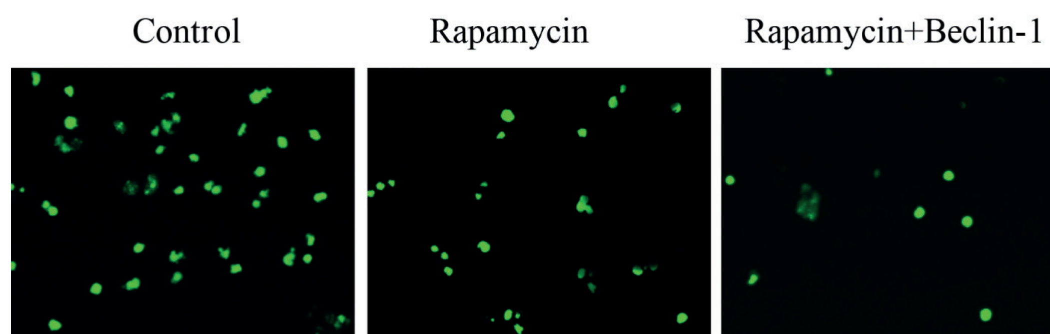

Figure 2. Comparison of MG-63 cell proliferative capacity among groups via Edu staining (20×).

Table I. Bax and Bcl-2 levels in each group of MG-63 cells detected via ELISA.

Group	Bax (pg/mL)	Bcl-2 (pg/mL)
Control	9.36 ± 1.02	23.45 ± 2.84
Rapamycin	23.48 ± 2.73*	14.26 ± 2.05*
Rapamycin + Beclin-1	35.39 ± 2.96#	10.33 ± 2.17#

Note (*$p<0.05$: Rapamycin group *vs.* Control group, #$p<0.05$: Rapamycin + Beclin-1 plasmid transfection group *vs.* Rapamycin group).

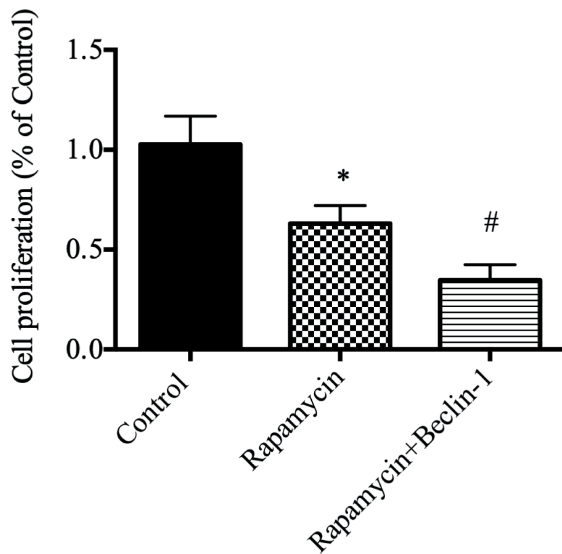

Figure 3. Comparison of MG-63 cell proliferative capacity among groups. (*p<0.05: Rapamycin group *vs*. Control group, #p<0.05: Rapamycin + Beclin-1 plasmid transfection group *vs*. Rapamycin group).

Rapamycin Could Increase Protein Expressions of Beclin-1 and Vps34 in MG-63 Cells

The Western blotting was performed to determine the impacts of rapamycin on the protein expressions of Beclin-1 and Vps34 in MG-63 cells. According to the bands (Figure 6), the protein levels of Beclin-1 and Vps34 were increased in the Rapamycin group ($^*p$<0.05) and Rapamycin + Beclin-1 plasmid transfection group ($^\#p$<0.05) compared with those in Control group, while the Rapamycin + Beclin-1 plasmid transfection group manifested stronger effects than Rapamycin group (Figure 7).

Discussion

Osteosarcoma, as the most common primary malignant bone tumor, mainly occurs in adolescents aged 15-25 years old, and males have a prominently higher incidence rate than females[13,14]. In previous clinical practices, the treatment protocol for osteosarcoma was dominated by the life-saving amputation, but unfortunately, the 5-year survival rate of the patients was only 20%. Moreover, the imperfect overall appearance due to amputation will cause tremendous psychological damage to the patients. Lugowska et al[15], however, showed that the rapid development in medicine has made limb salvage treatment possible for osteosarcoma patients. Later, combined with the chemotherapy drugs, the survival time of the patients is extended greatly, and the quality of life is also improved. The pathogenesis of osteosarcoma is still a task to be solved by researchers, and the understanding and mastering the pathogenesis is of important significance for the prevention and treatment of the disease.

Autophagy is a cell protective mechanism and a lysosome-mediated intracellular protein degradation process[16,17]. The autophagy at the cellular level is conducive to maintaining cellular homeostasis, while its destruction can easily trigger tumors. More attention and importance have been paid to the exploration of autophagy by researchers. The cell autophagy is suppressed in the case of tumor, so the normal autophagy cannot be exerted to promote cell apoptosis. Tsai et al[18] discovered that autophagy plays a positive regulatory role in the epithelial-mesenchymal transition in non-small cell lung cancer cells, suggesting that the development of non-small cell lung cancer can be

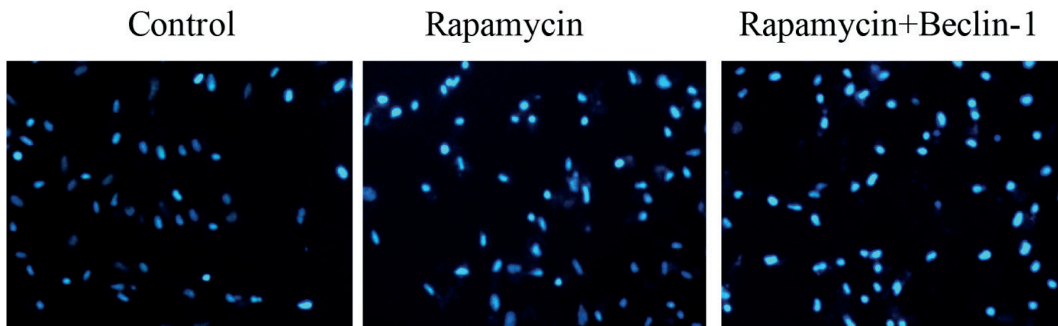

Figure 4. Comparison of MG-63 cell apoptosis among groups *via* Hoechst 33258 staining (20×).

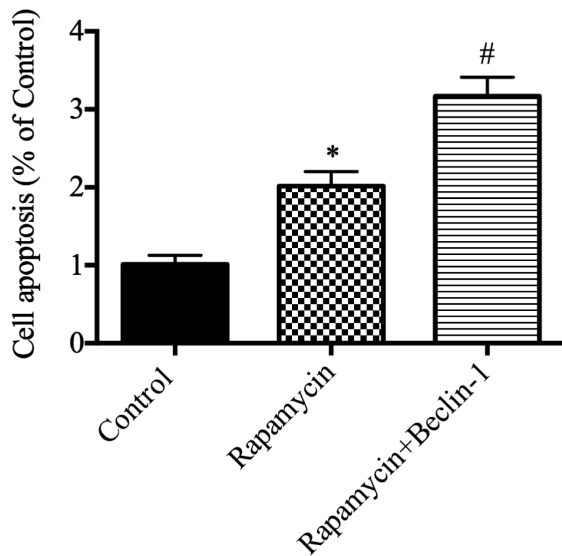

Figure 5. Comparison of MG-63 cell apoptosis rate among groups. (*p<0.05: Rapamycin group *vs.* Control group, #p<0.05: Rapamycin + Beclin-1 plasmid transfection group *vs.* Rapamycin group).

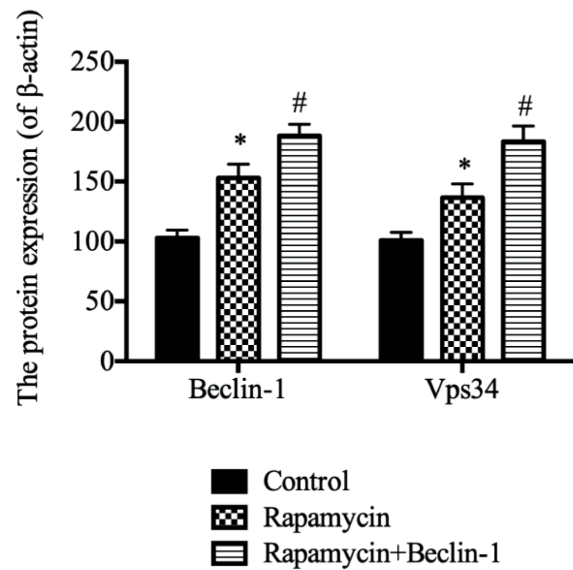

Figure 7. Comparisons of Beclin-1 and Vps34 protein levels in MG-63 cells among groups. (*p<0.05: Rapamycin group *vs.* Control group, #p<0.05: Rapamycin + Beclin-1 plasmid transfection group *vs.* Rapamycin group).

affected by controlling the cell autophagy. Jiao et al[19] revealed that the cell apoptosis in liver cancer is associated with glycolysis level. The total RNA was extracted from human liver cancer tissues, and the specifically expressed target hexokinase 2 (HK2) was screened via microarray assay. It was found that the regulation of the autophagy targeting HK2 can intervene in the glycolytic pathway in liver cancer, providing a new method for the treatment of liver cancer with impaired autophagy[19]. The above research findings demonstrate that cell autophagy is closely related to the occurrence and development of tumor diseases. In addition, autophagy has been regarded as a crucial factor for tumor cell migration and invasion. Xu et al[20] found that the overexpressed miR-30a is able to significantly accelerate the apoptosis of the osteosarcoma cells, whose mechanism is probably associated with the targeted regulation of Beclin-1 level, thus implying that the regulation of the cell autophagy by proper drugs or intervention means have vital effects on the prevention and treatment of osteosarcoma.

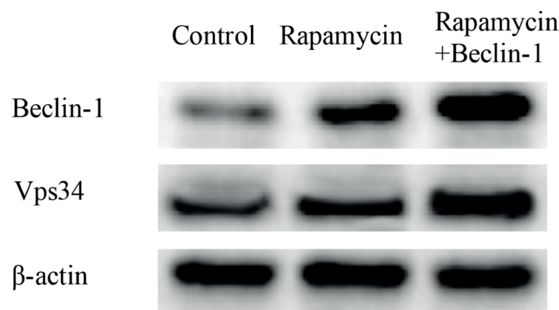

Figure 6. Comparisons of protein expressions of Beclin-1 and Vps34 in MG-63 cells among groups *via* Western blotting assay.

Hence, the impact of rapamycin on MG-63 cell viability was detected via MTT assay in this experiment, and it was shown that rapamycin could markedly weaken the MG-63 cell viability (*p<0.05). Later, the results of Edu and Hoechst 33258 staining manifested that rapamycin could inhibit the proliferation but promote the apoptosis of MG-63 cells, and the effects in Beclin-1 plasmid transfection group were more apparent after the intervention with rapamycin. Subsequently, the Bax and Bcl-2 levels in MG-63 cells were measured using ELISA kits. The results displayed that rapamycin could remarkably increase the level of the pro-apoptotic factor Bax and decrease that of the anti-apoptotic factor Bcl-2. Moreover, Beclin-1 plasmid transfection group exhibited more significant effects after the intervention with rapamycin. To further explore the regulatory role of rapamycin in the MG-63 cell proliferation, and apoptosis, the Western blotting assay was adopted to determine the protein expression levels of Beclin-1, Vps34, and the key target proteins in the autophagy-related

signaling pathways. It was revealed that rapamycin could exert its effect by up-regulating the protein levels of Beclin-1 and Vps34 and activating the autophagy. The results of the present experiment provide an experimental basis for the treatment of osteosarcoma with rapamycin.

Conclusions

Rapamycin may decrease the viability, inhibit the proliferation, and promote the apoptosis of MG-63 cells by activating the autophagy.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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