Rapamycin inhibits proliferation and apoptosis of retinoblastoma cells through PI3K/AKT signaling pathway

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Abstract. Effects of Rapamycin on the proliferation and apoptosis of retinoblastoma cells through the phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT) signaling pathway were studied. The retinoblastoma Y79 cells were selected and divided into negative control group (NC group), 0.2 µM Rapamycin group and 0.4 µM Rapamycin group. Then the proliferative activity of Y79 cells was detected using Cell Counting Kit-8 (CCK8) assay, the content of reactive oxygen species (ROS), malondialdehyde (MDA) and superoxide dismutase (SOD) in cells in each group was detected using enzyme-linked immunosorbent assay (ELISA), and the apoptosis of Y79 cells was detected via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Moreover, the changes in Y79 cell cycle and apoptosis were determined through flow cytometry, and apoptosis and PI3K/AKT pathway were detected using reverse transcription-polymerase chain reaction (RT-PCR) and western blotting. It was found that the number of cells and the proliferative activity were significantly reduced in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group. In 0.2 µM Rapamycin group and 0.4 µM Rapamycin group, the content of ROS and MDA was significantly decreased, while that of SOD was notably increased. TUNEL assay and flow cytometry showed that in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group, the number of apoptotic cells was obviously increased, and the cell cycle was basically arrested in S phase. The expression levels of Bcl-2, PI3K and AKT declined in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group, whereas the expression of Caspase 8 increased. Similar results were also obtained in the protein assay. The above results were significantly superior in 0.4 µM Rapamycin group to those in 0.2 µM Rapamycin group. Rapamycin inhibits proliferation and promotes apoptosis of retinoblastoma cells through inhibiting the PI3K/AKT signaling pathway.

Introduction

Retinoblastoma is the most common primary intraocular malignant tumor in infants and young children, and it ranks 3rd among all tumors only following leukemia and neuroblastoma (1,2). Despite some progress made in the treatment of retinoblastoma, some major problems have not been resolved yet. Traditional external beam radiation is used to control large tumors, but there are complications, including the secondary malignant tumors, such as osteosarcoma, which leads to a higher incidence rate of hereditary retinoblastoma in patients (3,4). Although external therapies such as chemotherapy have become indispensable treatment for retinoblastoma currently, it results in noticeable complications (5). Therefore, search for new therapeutic strategies to improve the clinical efficacy of patients with retinoblastoma is urgently needed.

Rapamycin is a kind of macrolide produced by Streptomyces hygroscopicus, which was originally developed as an antifungal agent (6). However, it has been used more for other purposes when its strong immunosuppressive and antiproliferative properties were found (7). Nowadays, Rapamycin contributes to the treatment of some cancers through inhibiting the Rapamycin target pathway in mammals (8). Over the years, it has been proven that apoptosis is the primary mechanism for eliminating cancer cells, including intrinsic or mitochondrial pathway and extrinsic or death receptor pathway (9). Therefore, it is of great significance to study the proliferation and apoptosis of retinoblastoma cells.

The phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B (AKT) pathway is an important signaling pathway that affects the cellular energy metabolism, cell size, cell cycle, cell proliferation, survival and apoptosis, which is closely related...
to other important signal transduction pathways (10-12). This pathway is composed of three major driving molecules: PI3K, AKT and the mammalian target of rapamycin (mTOR) (13-15). However, the role of the PI3K/AKT signaling pathway in retinoblastoma still needs further research. The aim of the present study was to enrich and improve the theoretical basis of the effects of Rapamycin on proliferation and apoptosis of retinoblastoma cells through the PI3K/AKT pathway.

Materials and methods

Commonly-used reagents and consumables. Y79 cells (American Type Culture Collection (ATCC) (Manassas), Rapamycin (Pfizer), enzyme-linked immunosorbert assay (ELISA) kits of reactive oxygen species (ROS) and malondialdehyde (MDA) (Nanjing Jiancheng Bioengineering Institute), radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime), loading buffer, protease inhibitor and bicinechonic acid (BCA) protein concentration assay kit (Biorad), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) secondary antibodies (ImmunoWay), primary antibodies (Cell Signaling Technology, Inc.), TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), diethyl pyrocarbonate (DEPC)-treated water, SuperScript III RT kit and SYBR qPCR Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), electrophoresis apparatus (Bio-Rad Laboratories, Inc.), microplate reader (Thermo Fisher Scientific, Inc.), 2500 gel imager (Bio-Rad Laboratories, Inc.) and quantitative polymerase chain reaction (qPCR) instrument (7900 Fast, Applied Biosystems; Thermo Fisher Scientific, Inc.).

Cell culture and grouping. The retinoblastoma Y79 cells purchased from ATCC were quickly taken out from the liquid nitrogen container, and rapidly thawed in 65°C sterile water prepared in advance, followed by centrifugation at 2,500 x g at 20°C for 10 min. The supernatant was discarded. After the above operation was repeated several times, the cells were resuspended with medium and inoculated into a 6-well plate at an appropriate density, followed by incubation in a thermostatic incubator. The medium was replaced every other day. Then the cells in good growth status were divided into negative control group (NC group), 0.2 µM Rapamycin group and 0.4 µM Rapamycin group (the dose of Rapamycin was determined by preliminary experiments (not shown)). After stimulation for 24 h, the cell samples were collected.

Cell proliferation assay using Cell Counting Kit-8 (CCK-8). The cells in the logarithmic growth phase in each group were inoculated into a 96-well plate and cultured in the thermostatic incubator at 37°C in the incubator with 5% CO₂. The cells in the logarithmic growth status in the three groups were selected from the incubator, and the medium was discarded. The cells were collected using a cell scraper and lysed using the RIPA lysis buffer (strong), followed by centrifugal separation at 2,500 x g at 20°C for 10 min. Then the supernatant was collected to detect the levels of ROS, MDA and SOD using the ELISA kits (HMI0870, Bio-Swamp; HMI0250, Bio-Swamp; CSB-E17044h, Cusabio Biotech Co., Ltd.) according to the actual situations and instructions. Finally, the absorbance in each group was measured using a microplate reader.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay. Apoptosis of Y79 cells was detected using the apoptosis assay kit (Roche), as follows: The cells in each group were collected, and the supernatant was discarded. The cells were washed with phosphate buffered saline (PBS), added with proteinase K working solution, immersed in blocking buffer, fixed, rinsed and infiltrated with 0.1% Triton X-100, followed by fluorescein isothiocyanate (FITC) end labeling of apoptotic DNA fragment using the TUNEL assay kit. The FITC-labeled TUNEL-positive cells were observed under a fluorescence microscope, and the TUNEL-positive cells were counted in 10 fields of view.

Detection of cell cycle using flow cytometry. The cells were treated for 24 h and washed twice with PBS, and the cell sediment at the bottom of tube was collected. Then the cell sediment was suspended with 0.1% Triton X-100, followed by centrifugation at 1,750 x g at 4°C overnight, followed by centrifugation at 1,750 x g at 20°C for 5 min. The supernatant was collected. With 3 replicates in each group, the cell sediment was suspended with 500 µl of binding buffer according to the instructions, added with 5 µl of propidium iodide (PI) dye and placed at room temperature for 30 min in the dark. Finally, the cell cycle was detected using the flow cytometer according to the programmed operation. Flow cytometer (FACScalibur; BD Biosciences) was used for analysis. Data were obtained and analyzed using the CellQuest professional software (version 3.3; BD Biosciences).

Detection of apoptosis via flow cytometry. The cells were treated for 24 h and washed twice with PBS, and the cell sediment at the bottom of tube was collected. With 3 replicates in each group, the cell sediment was suspended with 500 µl of binding buffer according to the instructions, added with 5 µl of Annexin V-binding buffer and 5 µl of PI dye and placed at room temperature for 10 min. Finally, the apoptosis rate was measured using the flow cytometer according to the programmed operation.

Detection of gene expression using RT-PCR. The RNA was extracted from cells and synthesized into DNA using the kit (Takara) in accordance with the instructions. The primer amplification system (20 µl) was constructed using 2 µl of complementary deoxyribosenucleic acid (cDNA), 10 µl of qPCR mix, 2 µl of primers and 6 µl of ddH₂O. Then, PCR amplification was performed: pre-denaturation at 95°C for 2 min, 94°C for 20 sec, 60°C for 20 sec and 72°C for 30 sec, for a total of 40 cycles. The primer sequences of target genes and the internal reference GAPDH were designed according to those in the GenBank (Table I). The expression levels of target genes were detected via RT-PCR.
Western blotting. The cells at an appropriate density in each group were collected, from which the protein was extracted according to the instructions, and the protein concentration was calculated. After that, the protein was subjected to water bath and centrifugation at 10,500 x g at 4˚C for 10 min. Then western blotting was performed: 12% separation gel and 5% spacer gel were prepared for protein loading and electrophoresis, and the protein was transferred onto a membrane using the semi-dry method, sealed, incubated with the primary antibodies overnight and incubated again with the secondary antibodies. The protein band was scanned and quantified using the Odyssey scanner, and the level of protein to be detected was corrected using GAPDH. Finally, the protein expression was calculated through gray scan.

Statistical analysis. All raw data obtained in the experiments were statistically analyzed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM Corp.), the validity of raw data was retained, and multiple comparisons were performed. The experimental results were expressed as mean ± standard deviation (mean ± SD). Analysis of variance followed by post hoc test (least significant difference) was applied for the comparison among groups. P<0.05 suggested that the difference was statistically significant. The bar graph was plotted using GraphPad Prism 8.0.

Results

Results of cell proliferation assay using CCK8. The absorbance in each group at different time points was measured through proliferation assay using CCK8. As shown in Fig. 1, the proliferation ability of Y79 cells was significantly stronger in NC group than that in other groups at 24, 48, 72 and 96 h (P<0.05), and it was the weakest in 0.4 µM Rapamycin group (P<0.05). CCK8, Cell Counting Kit-8; NC, negative control; OD, optical density.

Detection results of oxidative stress cytokines in each group. As shown in Table II, in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group, the content of ROS and MDA is significantly decreased (P<0.05), while that of SOD is significantly increased (P<0.05), all of which are remarkably superior in 0.4 µM Rapamycin group to those in 0.2 µM Rapamycin group. P<0.05 vs. NC group, P<0.05 vs. 0.2 µM Rapamycin group. NC, negative control.

TUNEL apoptosis assay results. The level of apoptosis in each group was determined using TUNEL staining. As shown in Fig. 2, there were fewer TUNEL-positive cells in NC group, and they could hardly be observed. The number of TUNEL-positive cells in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group was obviously higher than that in NC group, and it was the highest in 0.4 µM Rapamycin group (P<0.05). The above results suggest that Rapamycin can promote apoptosis of Y79 cells.

Cell cycle detection via flow cytometry. The cell cycle in each group was detected using flow cytometry. The results manifested that there were more cells in S phase and fewer...
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Table III. Cell cycle detection (%).

<table>
<thead>
<tr>
<th>Group/Cycle</th>
<th>G1</th>
<th>S</th>
<th>G2</th>
</tr>
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<tbody>
<tr>
<td>NC</td>
<td>28.4±0.5</td>
<td>49.0±0.4</td>
<td>22.6±0.6</td>
</tr>
<tr>
<td>0.2 µM Rapamycin</td>
<td>28.6±0.4</td>
<td>60.5±0.1</td>
<td>11.9±0.8</td>
</tr>
<tr>
<td>0.4 µM Rapamycin</td>
<td>29.0±0.5</td>
<td>66.5±0.7</td>
<td>4.5±0.9</td>
</tr>
</tbody>
</table>

There are more cells in S phase and fewer cells in G2 phase in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group. The cell cycle in 0.4 µM Rapamycin group is obviously superior to that in 0.2 µM Rapamycin group (P<0.05). NC, negative control.

cells in G2 phase in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group. The cell cycle in 0.4 µM Rapamycin group was obviously superior to that in 0.2 µM Rapamycin group (P<0.05) (Table III). The above findings demonstrate that 0.4 µM of Rapamycin can inhibit the proliferation of retinoblastoma cells, and arrest the cell cycle in S phase.

Apoptosis detection using flow cytometry. Apoptosis level in each group was determined through flow cytometry. It was found that the apoptosis rate in NC group was lower, and
the apoptotic cells could hardly be observed. The number of apoptotic cells was obviously increased in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group compared with that in NC group, and it was the highest in 0.4 µM Rapamycin group (P<0.05) (Fig. 3), indicating that Rapamycin can promote apoptosis of Y79 cells.

**RT-PCR results.** The results of RT-PCR revealed that 0.2 µM Rapamycin group and 0.4 µM Rapamycin group had evidently lower expression levels of Bcl-2, PI3K and AKT, and evidently higher expression of Caspase 8 (P<0.05), while the above expression levels were the opposite in NC group (Fig. 4), which suggests that Rapamycin suppresses cell proliferation and promotes apoptosis, further inhibiting the occurrence of retinal diseases.

**Western blot results.** According to the results of western blotting, 0.2 µM Rapamycin group and 0.4 µM Rapamycin group had remarkably lower protein levels of PI3K and AKT (P<0.05) and a remarkably higher protein level of Caspase 8 (P<0.05), while the above expression levels were the opposite in NC group (Fig. 5), which suggests that Rapamycin suppresses cell proliferation and promotes apoptosis through inhibiting the PI3K/AKT signaling pathway. Rabbit polyclonal Akt antibody (dilution: 1:500; cat. no.: ab8805); rabbit monoclonal; Caspase 8 antibody (dilution: 1:500; cat. no.: ab32397); rabbit polyclonal PI3K antibody (dilution: 1:500; cat. no.: ab70912); rabbit polyclonal GAPDH antibody (dilution: 1:500, cat. no.: ab37168) and secondary goat anti-rabbit (HRP) IgG antibody (dilution: 1/2000; cat. no.: ab6721) were all purchased from Abcam.

**Discussion**

Retinoblastoma is the most common primary intraocular malignant tumor in infants and young children. Despite some progress made in the treatment of retinoblastoma, some major problems have not been resolved yet, including the secondary malignant tumors, such as osteosarcoma. Therefore, it is urgent to search for new therapeutic strategies to improve the clinical efficacy of patients with retinoblastoma. Rapamycin is a kind of macrolide produced by Streptomyces, which can contribute to the treatment of some cancers through inhibiting the Rapamycin target pathway in mammals (16). In the present study, the effects of Rapamycin on the proliferation and apoptosis of retinoblastoma cells through the PI3K/AKT pathway were explored. It was found through CCK-8 assay that the proliferation ability of Y79 cells was significantly stronger in NC group than that in other groups at 24, 48, 72 and 96 h, and it was the weakest in 0.4 µM Rapamycin group. In addition, the cell cycle was detected using flow cytometry. The results manifested that there were more cells in S phase and fewer cells in G2 phase in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group. The cell cycle in 0.4 µM Rapamycin group was obviously superior to that in 0.2 µM Rapamycin group. The above findings demonstrate that 0.4 µM of Rapamycin can inhibit the proliferation of retinoblastoma cells, and arrest the cell cycle in S phase, consistent with previous studies (17). The level of ROS in cancer cells is higher than that in normal cells due to oncogene stimulation, increased metabolic activity and mitochondrial dysfunction. Under the condition of continuous oxidative stress, cancer cells are adapted through a series of mechanisms, which not only activate ROS scavenging system, but also inhibit cell apoptosis. Therefore, understanding the ROS adaptation mechanism is very important for killing cancer cells and solving the problem of drug resistance. SOD is ubiquitous and prevents the optic nerve conduction abnormality. MDA can resist the effect of SOD, with cytotoxicity (18). In this study, we chose the dose of Rapamycin (0.2 and 0.4 µM) based on our preliminary experimental results, which was the basis of this study. Results showed that in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group, the content of ROS and MDA was significantly decreased, while that of SOD was significantly increased, all of which were markedly superior in 0.4 µM Rapamycin group to those in 0.2 µM Rapamycin group, indicating that 0.4 µM of Rapamycin can inhibit the oxidative stress, further suppressing the occurrence of retinoblastoma. The mitochondrial function should be further examined to more accurately account for this effects.

Apoptosis can be induced by internal and external signals, and the release of cytochrome c due to the loss of mitochondrial membrane potential is the key to the activation of Caspase 9, causing intracellular damage. The...
apoptosis activator transmits signals to the cytoplasm, leading to the activation of Caspase 8, and results in apoptosis through the in vitro pathway (19,20). The cleaved Caspase 8 and 9 accompanied by the cascade activation of Caspase have been observed in studies, and Rapamycin can trigger the intrinsic and extrinsic PI3K/AKT apoptotic pathways of human retinoblastoma Y79 cells, thus facilitating apoptosis (21). In this study, the apoptosis level in each group was determined using TUNEL staining. It was found that there were fewer TUNEL-positive cells in NC group, and they could hardly be seen. The number of TUNEL-positive cells in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group was obviously larger than that in NC group, and it was the largest in 0.4 µM Rapamycin group. Moreover, the results of flow cytometry showed that the apoptosis rate in NC group was lower, and the apoptotic cells could hardly be observed. The number of apoptotic cells was obviously increased in 0.2 µM Rapamycin group and 0.4 µM Rapamycin group compared with that in NC group, and it was the largest in 0.4 µM Rapamycin group, indicating that Rapamycin can promote apoptosis of Y79 cells. The PI3K/AKT pathway is a central regulator for cancer proliferation, tumorigenesis and metastasis. PI3K is a lipid kinase family that phosphorylates the phosphate-3-hydroxyl. Both AKT and mTOR are downstream targets of PI3K, and they can stimulate protein synthesis, cell growth and proliferation. mTOR is an important component of the network, as well as a PI3K-associated serine-threonine kinase that can regulate anti-apoptosis and survival mechanisms through phosphorylating AKT (22,23). In this study, the results of RT-PCR revealed that 0.2 µM Rapamycin group and 0.4 µM Rapamycin group had evidently lower expression levels of Bcl-2, PI3K and AKT, and evidently higher expression of Caspase 8, while the above expression levels were the opposite in NC group. According to the results of western blotting, 0.2 µM Rapamycin group and 0.4 µM Rapamycin group had remarkably lower protein levels of PI3K and AKT and a remarkably higher protein level of Caspase 8, while the above levels were the opposite in NC group, which suggests that Rapamycin suppresses proliferation and promotes apoptosis of retinoblastoma cells through inhibiting the PI3K/AKT signaling pathway, thereby further inhibiting the occurrence of retinal diseases. Differently, Wang et al (21) found that Rapamycin disturbed mitochondrial membrane potential and subsequently helped cytochrome c release from mitochondria to cytosol and activated Caspase 8, inducing apoptosis in human retinoblastoma Y79 cells. In subsequent research, animal experiments need to be introduced to further explore the deeper regulatory mechanism of PI3K/AKT signaling pathway from in vivo and in vitro levels.

In conclusion, it was found that Rapamycin may regulate the proliferation and apoptosis of retinoblastoma cells through inhibiting the PI3K/AKT signaling pathway, so Rapamycin may be used as a therapeutic drug for patients with retinoblastoma.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
JY, MX and ZL designed the study and performed the experiments; JY and ZL collected the data; MX and ZL analyzed the data; JY, MX and ZL prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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