**Autophagic inhibitor attenuates rapamycin-induced inhibition of proliferation in cultured A549 lung cancer cells**


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**Abstract.** – BACKGROUND: Increasing studies have suggested that rapamycin has inhibitory effect for cancer cell proliferation.

AIM: The present study aimed to investigate the effect of rapamycin on the proliferation of A549 lung cancer cells and try to elucidate its probable mechanism.

MATERIALS AND METHODS: A549 cells were randomly divided into the following 3 groups (n=6): the Dulbecco’s modified Eagle’s medium (DMEM) culture solution administered alone group (C group), the 10 nmol/l rapamycin administered alone group (R group) and the 5 mmol/l 3-methyladenine (3-MA) plus 10 nmol/l rapamycin administered group (MR group). Death percentage of A549 cells was observed and the levels of caspase-3, Beclin-1, and microtubule-associated protein 1 light chain 3-II (LC3-II) were determined.

RESULTS: Compared with C group, percentage of cell death, caspase-3, Beclin-1 and LC3-II both showed a significant increase in R group (p < 0.05). On the contrary, as compared with R group, percentage of cell death, caspase-3, Beclin-1 and LC3-II both showed a significant decrease in MR group (p < 0.05).

CONCLUSIONS: Rapamycin has an inhibitory effect for the proliferation of A549 cells, and its mechanism is likely related to the activation of autophagic pathway.

Key Words: 
Rapamycin, Autophagic activator, Proliferation, A549 lung cancer cells, 3-methyladenine.

**Introduction**

Rapamycin, a novel macrolide antibiotic, has potent immunosuppressant effect and has been widely used for anti-rejection reaction after organ transplantation and treatment for patients with autoimmune diseases. Increasing studies have suggested that rapamycin has inhibitory effect for cancer cell proliferation. National Cancer Institute (NCI) analyzed the effects of rapamycin on various kinds of malignant cells, and the results showed that rapamycin has an inhibitory effect for the growth of breast cancer, ovarian cancer, leukemia, and lung cancer. Unfortunately, the precise mechanism underlies rapamycin-induced inhibitory effect for malignant cell growth is still not clear.

It is widely known that mammalian target of rapamycin (mTOR) acts as a target for rapamycin is a 289 KDa serine/threonine kinase. Previous studies have demonstrated that rapamycin has a potent effect to inhibit cell cycle progression, which suggest that mTOR regulating the cell growth and proliferation. More importantly, mTOR is also a potent suppressor for the autophagic pathway. This implies that rapamycin can stimulate autophagic pathway via inhibiting mTOR levels, which can be called mTOR-dependent autophagy by regulation of rapamycin. Autophagic pathway is a catabolic process which is utilized by all cells to clear damaged proteins. Although mounting studies have pointed that rapamycin-stimulated mTOR-dependent autophagy would be a probable mechanism to elucidate rapamycin exerting therapeutic effects for neurodegenerative diseases, there is little literature reporting rapamycin for the treatment of cancer by mTOR-dependent autophagic pathway. Thus, in the present work, we used A549 lung cancer cells to investigate the effect of rapamycin on its growth and try to observe whether autophagic pathway play a critical role in its mechanism.

**Materials and Methods**

**Cell Culture**

A549 lung cancer cells were obtained from Jinling Hospital, School of Medicine, Nanjing
Autophagy and rapamycin-induced effect

University, Nanjing, China. The A549 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; HyClone, UT, USA) culture solution containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere of 5% CO₂ at 37°C. A549 cells were randomly divided into 3 groups (6 duplicates): The DMEM culture solution administered alone group (C group), the 10 nmol/l rapamycin (Sigma-Aldrich Inc., St. Louis, MO, USA) administered alone group (R group) and the 5 nmol/l 3-methyladenine (3-MA, Sigma-Aldrich Inc., St. Louis, MO, USA) plus 10 nmol/l rapamycin administered group (MR group). Forty-eight hours after incubation, A549 cells were prepared for analysis.

Cell Viability Analysis
According to previous studies²⁸⁻²⁹, at the end of the incubation, culture solution in each well was discarded and 100 µl media containing 200 g/ml MTT was added in each well and the plates were incubated at 37°C for 2 h. Subsequently, the culture solution containing MTT was discarded and 200 µl dimethyl sulfoxide (DMSO) was added to dissolve the insoluble purple formazan product to colored solution. The absorbance was measured at 570 nm, the 50% inhibitory concentration (IC₅₀) value was determined using Easyplot software version 4.0.4. Percentage of cell death = 100 – (Absorbance of treated/Absorbance of control x100).

Real-time PCR Analysis
Real time polymerase chain reaction (PCR) was performed with an ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR reagents (Tiangen, China). Reaction mixtures were incubated for 2 min at 94°C, and were amplified for 40 cycles (94°C for 15s, 55°C for 20s, and 68°C for 35s). For each sample, gene expression was corrected against β-actin mRNA levels and the comparative threshold cycle number (CT) method was used to assess the relative quantification of gene expression. The fold changes of the target genes were calculated by using the 2⁻∆∆CT. The caspase-3 primers used for PCR reactions were: forward sequence, 5′-TGTCAAGCAGCAACCT-3′; reverse sequence, 5′-CATCCAGTCGTTGTGC-3′. The Beclin-1 primers used for PCR reactions were: forward sequence, 5′-AGCTGCCGTATACTGTTCTG-3′; reverse sequence, 5′-ACTGCCCTCGTGTCTTCAATCTT-3′. The LC3-II primers used for PCR reactions were: forward sequence, 5′-CTACAATGACGTGCTGTTGCC-3′; reverse sequence, 5′-CAGGCTCA-GACGCGATTGCGC-3′.

Statistical Analysis
Data are expressed as mean ± SD. Statistical analyses were made by one-way analysis of variance (ANOVA) and post hoc analyses were performed by Least Significant Difference (LSD) tests. These statistical analyses were conducted by Statistical Product for Social Sciences (SPSS Inc., version 17.0, Chicago, IL USA). p < 0.05 was considered statistically significant.

Results
Effects of Rapamycin on the Percentage of Cell Death and the Levels of Caspase-3, Beclin-1 and LC3-II in A549 Cells
Compared with C group, the administration of rapamycin significantly increased the percentage of cell death and increased the expression levels of caspase-3, Beclin-1 and LC3-II (p < 0.05; Table I).

<table>
<thead>
<tr>
<th></th>
<th>C group</th>
<th>R group</th>
<th>MR group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell death (%)</td>
<td>7.5 ± 2.3</td>
<td>26.6 ± 9.3*</td>
<td>14.5 ± 6.4*</td>
</tr>
<tr>
<td>Caspase-3 (%)</td>
<td>100 ± 11.3</td>
<td>163.4 ± 6.6*</td>
<td>127.5 ± 10.5*</td>
</tr>
<tr>
<td>Beclin-1 (%)</td>
<td>100 ± 8.7</td>
<td>147.3 ± 9.4*</td>
<td>119.7 ± 11.2*</td>
</tr>
<tr>
<td>LC3-II (%)</td>
<td>100 ± 7.9</td>
<td>131.2 ± 11.5*</td>
<td>111.3 ± 7.2*</td>
</tr>
</tbody>
</table>

*p < 0.05, compared with C group; *p < 0.05, compared with R group. C group, control group; R group, rapamycin group; MR group, 3-methyladenine+rapamycin group.
Effects of 3-MA on the Rapamycin-Induced Changes in A549 Cells. Beclin-1 and LC3-II in A549 cells

Compare with R group, the administration of 3-MA significantly decreased the percentage of cell death and decreased the expression levels of caspase-3, Beclin-1 and LC3-II ($p < 0.05$; Table I).

Discussion

In the present study, our findings demonstrated that rapamycin could induce high death percent-

age of A549 lung cancer cells, which implying that it has significantly inhibitory effect for the growth of cancer cell. Besides, the results also showed that rapamycin eliciting inhibitory effect for the growth of A549 cells could be attenuated by administration of 3-MA, a specific inhibitor of autophagy, suggesting that autophagic pathway would be likely to be involved in the mechanism of anti-cancer effect of rapamycin. Subsequently, we measured the expression levels of Beclin-1 and LC3-II, two typical marker of autophagic pathway, and the results validated that autophagic pathway probably participate in the mechanism of rapamycin’s anti-cancer effect.
A549 lung cancer cells are adenocarcinomic human alveolar basal epithelial cells. We used A549 cells to investigate whether autophagic pathway is involved in the action of mechanism of rapamycin’s anti-cancer effect. 3-MA, as above mentioned, is a specific inhibitor for autophagic pathway. Liu et al20 have shown that 3-MA has a potent inhibitory effect for autophagic pathway in A549 cells. In this study, we got the result that rapamycin inhibiting the growth of A549 cells was associated with the activation of Beclin-1 and LC3-II, and 3-MA could attenuate rapamycin-induced death of A 549 cells and that was correlated with the inactivation of Beclin-1 and LC3-II. Our result was consistent with previous studies.

Caspase-3 is a member of the cysteine-aspartic acid protease family21. Its activation plays a central role in the execution-phase of cell apoptosis. The results of this study demonstrated that rapamycin induced up-regulated expression of caspase-3, which indicates that rapamycin has potential to induce activation of apoptotic pathway in A549 cells. In other words, rapamycin is capable of killing cancer cells. This validated again that rapamycin could inhibit the growth of A549 cells. On the contrary, caspase-3 levels was showed a significant decrease after administration of 3-MA, suggesting that 3-MA attenuated rapamycin-elicited increase of caspase-1 level.

Beclin-1 is protein that participates in the regulation of autophagic pathway and has a critical role in development and tumorigenesis22. LC3-II is also an important regulator of autophagic pathway22. In this study, our results demonstrated that administration of rapamycin activated the expression of Beclin-1 and LC3-II, indicating that the activation of autophagic pathway many contribute to the increased percentage of A549 cell death. At present, large number of studies considered that rapamycin is a natural stimulator for autophagic pathway via inhibiting mTOR, which is a most negative autophagic regulator23,24. An important regulator of autophagic induction is mTOR, which when activated, inhibits autophagy and when not activated stimulates it25. After administration of autophagic inhibitor 3-MA, rapamycin-induced cancer cell death was significantly attenuated.

Conclusions

Rapamycin is capable of inhibitory the growth of A540 lung cells, and its mechanism is likely related to the activation of autophagic pathway that is negatively regulated by mTOR.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References


