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Rhizoma Paridis Saponins Induces Cell Cycle Arrest and Apoptosis in Non-Small Cell Lung Carcinoma A549 Cells

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Manuscript Preparation E
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Background: As a traditional Chinese medicine herb, Chonglou (*Paris polyphylla var. chinensis*) has been used as anticancer medicine in China in recent decades, as it can induce cell cycle arrest and apoptosis in numerous cancer cells. The saponins extract from the rhizoma of Chonglou [*Rhizoma Paridis* saponins (RPS)] is known as the main active component for anticancer treatment. However, the molecular mechanism of the anticancer effect of RPS is unknown.

Material/Methods: The present study evaluated the effect of RPS in non-small-cell lung cancer (NSCLC) A549 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and flow cytometry. Subsequently, the expression of several genes associated with cell cycle and apoptosis were detected by reverse transcription-quantitative polymerase chain reaction (qRT-PCR) and Western blotting.

Results: RPS was revealed to inhibit cell growth, causing a number of cells to accumulate in the G1 phase of the cell cycle, leading to apoptosis. In addition, the effect was dose-dependent. Moreover, the results of qRT-PCR and Western blotting showed that p53 and cyclin-dependent kinase 2 (CDK2) were significantly downregulated, and that BCL2, BAX, and p21 were upregulated, by RPS treatment.

Conclusions: We speculated that the RPS could act on a pathway, including p53, p21, BCL2, BAX, and CDK2, and results in G1 cell cycle arrest and apoptosis in NSCLC cells.

MeSH Keywords: **Apoptosis • Carcinoma, Non-Small-Cell Lung • Cell Cycle Checkpoints • Saponins**

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Background

Lung cancer occurs frequently in males and females, with ~520 000 new cases generated and 450 000 fatalities from lung cancer/year in China [1]. Over 80% of lung cancer patients are diagnosed as having non-small-cell lung cancer (NSCLC) at advanced stages of the disease [2]. Despite significant progress toward understanding lung cancer in the past 2 decades, NSCLC has an extremely poor 5-year survival rate [3,4], as evidenced by the finding that the epidermal growth factor receptor (EGFR) tyrosine kinase and anaplastic lymphoma kinase (ALK) inhibitors, including gefitinib, erlotinib, and crizotinib, have shown superior improvement of survival time and life quality in the subset of patients harboring EGFR or ALK mutations [5–8]. Regardless, gefitinib and crizotinib has improved the progression-free and overall survival in patients; however, drug resistance has remained a significant problem affecting patient survival [9]. Thus, new, longer-lasting, targeted therapeutic strategies are required.

Natural products have been the mainstay of cancer chemotherapy for the past 30 years, and their mechanisms of action require in-depth analysis [10,11]. Chonglou (*Paris polyphylla var. chinensis*), a traditional Chinese medicine herb, has been applied in the treatment of various types of cancer, including lung cancer, breast cancer, brain tumors, and digestive system carcinomas in recent decades [12,13]. The main constituents of the saponins extract from the rhizoma of Chonglou, known as *Rhizoma Paridis* saponins (RPS), were identified as polyphyllin D, formosanin C, dioscin, Paris H, and Paris VII. Numerous studies have proved that RPS was the main active ingredient for anticancer treatments [14–16]. These extracts could induce apoptosis, affect cell cycle distribution, inhibit angiogenesis, and improve the immune function in cancer cells [12–16]. However, due to the complexity and various actions of herbal components, the explicit antitumor mechanisms of RPS remain unknown.

In the present study, the antitumor effect and mechanism of RPS were examined on NSCLC A549 cells, and cell proliferation, cell cycle, and apoptosis were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and flow cytometry, and the expression level of the genes and proteins associated with the cell cycle and apoptosis were detected by reverse transcription-quantitative polymerase chain reaction (qPCR) and Western blotting. Finally, a pathway was identified that could be affected by RPS and results in G1 cell cycle arrest and apoptosis in NSCLC cells.

Material and Methods

RPS extraction

Chonglou rhizomes were ground to powder and 20 g of the powder was extracted twice with 30 ml of 80.0% alcohol under reflux in a water bath for 1 h. The combined extracts were filtered and concentrated by a rotary evaporator (De Hua Materials Testing Co., Ltd., Chengdu, China). Distilled water (250 ml) was added to the crude extract and the sample was extracted by water-saturated butanol (500 ml) for 12 h. Finally, the water-saturated butanol was concentrated to sediment, which was the RPS.

Cell lines and cell culture

The NSCLC A549 cell line was provided by West China-Frontier Pharma Tech Co., Ltd. (Chengdu, China). The cells were maintained as monolayers at 37°C in an atmosphere containing 5% CO₂/O₂ in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, Rockville, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco Life Technologies) and 1% penicillin/streptomycin (Gibco Life Technologies). For RPS treatment, the cells were plated for 48 h in DMEM containing 10% FBS. The medium was subsequently changed to DMEM containing 5% charcoal-dextran-treated FBS with various concentrations of RPS.

MTT assay

The cytotoxicity of RPS in the A549 cell line was assessed using the MTT assay. Cells (5×10^3 cells/well) were plated in 96-well plates in 100 μ l DMEM with various concentrations of RPS (0.5, 1.0, and 2.0 mg/ml) for 24, 48, 72, 96, and 120 h, and subsequently, an equivalent volume of MTT (0.5 mg/ml) was added to each well. After 4-h incubation at 37°C, the cells were centrifuged at 2000 rpm for 5 min, followed by the addition of 100 μ l dimethylsulfoxide to each well to dissolve the formed formazan crystals by agitation for 10 min. The absorbance at 490 nm was measured using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry

A549 cells were treated with various concentrations of RPS (0.5, 1.0, and 2.0 mg/ml) for 24 h. A total of 3×10^5 – 5×10^5 cells were washed with chilled phosphate-buffered saline and resuspended in 1X binding buffer (100 ml). A total of 5 μ l of annexin V (AV)-fluorescein isothiocyanate solution and 1 μ l of dissolved propidium iodide (PI) were added to the cell suspensions to investigate whether the growth inhibition of RPS was caused by apoptosis. Subsequently, the cells were gently vortexed and incubated at room temperature in the dark for 15 min. Following this, 400 μ l of chilled binding buffer was added and mixed gently prior

to the examination of the cell preparations by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

RT-qPCR

Genes were validated by RT-qPCR assays. The RT-qPCR reactions were performed on a Roche LightCycler Instrument 1.5, using a LightCycler® FastStart DNA Master PLUS SYBR-Green I kit (Roche cat. no. 03515885001; Roche Diagnostics Australia Pty Limited, Castle Hill, Australia). Briefly, the reactions had a 15 µl volume: 7.5 µl Master mix, 0.1 µl forward primer and reverse primer, 1 µl cDNA sample and 6.3 µl ddH₂O were prepared. Each sample was run in triplicate. The RT-qPCR program was set to 95°C for 5 min and subsequently 45 cycles of 95°C for 10 s, 55–60°C for 35 s and 72°C for 40 s. At the end of each program, a melting curve analysis was performed. In addition, the data were automatically analyzed by the system and an amplification plot was generated for each cDNA sample at the end of each qPCR run. The reference gene was β-actin. The primers of each gene used are shown in Table 1.

Western blotting

Cell lysates were separated by SDS-PAGE in 8% Tris-glycine gels (Invitrogen Life Technologies, Carlsbad, CA, USA) and transferred to a nitrocellulose membrane. To determine the expression levels of p53, cyclin-dependent kinase 2 (CDK2), BCL2, BAX and p21, blots were probed with their specific antibodies [diluted with 5% bovine serum albumin (BSA) to 1:1,000]. Membranes were probed with horseradish peroxidase-labeled anti-rabbit secondary antibody (diluted with 5% BSA to 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA). Antibody binding was detected by enhanced chemiluminescence detection kit (Amersham International PLC, Buckinghamshire, UK)

Results

RPS inhibits the proliferation of A549 cells

As shown in Figure 1, accompanied with the increasing incubation times, the optical density (OD) values of the RPS-treated cells were significantly lower compared with the control cells and showed an inverse correlation between OD values and concentrations of RPS, which indicate that RPS could inhibit the proliferation of A549 cells in a dose-dependent manner. Additionally, the A549 cells almost stop proliferation when concentrations of RPS were >1.0 mg/ml.

RPS induces cell apoptosis in A549 cells

As shown in Table 2 and Figure 2A, the percentages of apoptotic cells were significantly increased in RPS treatment groups

Table 1. The PCR primers of genes.

Gene	Sequence (5'→3')	Length
β-actin	Forward primer	CATGTACGTTGCTATCCAGGC
	Reverse primer	CTCCTTAATGTCACGCACGAT
P53	Forward primer	GAGGTTGGCTCTGACTGTACC
	Reverse primer	TCCGTCACAGTAGATTACCAC
BCL2	Forward primer	GGTGGGGTCATGTGTGTGG
	Reverse primer	CGGTTACAGTACTCAGTCATCC
p21	Forward primer	CGATGGAAGTCTGACTTTGTCA
	Reverse primer	GCACAAGGGTACAAGACAGTG
BAX	Forward primer	CCGAGAGGTCTTTTCCGAG
	Reverse primer	CCAGCCCATGATGTTTCTGAT
Cyclin A	Forward primer	TGAAAGCAAACAGTAAACAGCC
	Reverse primer	GGGCATCTTCACGCTCTATT
Cyclin B1	Forward primer	TTGGGGACATTGGTAAACAAAGTC
	Reverse primer	ATAGGCTCAGGCGAAAGTTTTT
Cyclin D1	Forward primer	TGGAGCCCGTAAAAAGAGC
	Reverse primer	TCTCCTCATCTTAGAGGCCAC
Cyclin E	Forward primer	GCCAGCCTTGGGACAATAATG
	Reverse primer	CTTGACCGTTGAGTTGGGT
CDK2	Forward primer	GTACCTCCCCTGGATGAAGAT
	Reverse primer	CGAATCCGCTTGTAGGGTC
CDK4	Forward primer	CTGGTGTGAGCATGTAGACC
	Reverse primer	GATCCTTGATCGTTCCGGCTG
CDK6	Forward primer	CCAGATGGCTCTAACCTCAGT
	Reverse primer	AACTCCACGAAAAAGAGGCTT

and showed a positive correlation with concentrations of RPS, indicating that RPS could induce the apoptosis of A549 cells in a dose-dependent manner.

RPS induces the G1 cell cycle arrest in A549 cells

As shown in the results in Figure 2B and Table 3, the treatment of A549 cells with RPS caused the accumulation of cells at the G1 phase of the cell cycle, in addition to a significant decrease in the number of cells in the S phase. These trends were enhanced with increased concentrations of RPS. However, there was no significant change for the number of cells in the G2 phase, which indicated that RPS could induce cell cycle arrest of A549 cells in G1 phase.

Effect of RPS on the expression of genes associated with cell cycle and apoptosis

As shown in Figure 3, the expression of p53, BCL2 and CDK2 were significantly downregulated by RPS treatment

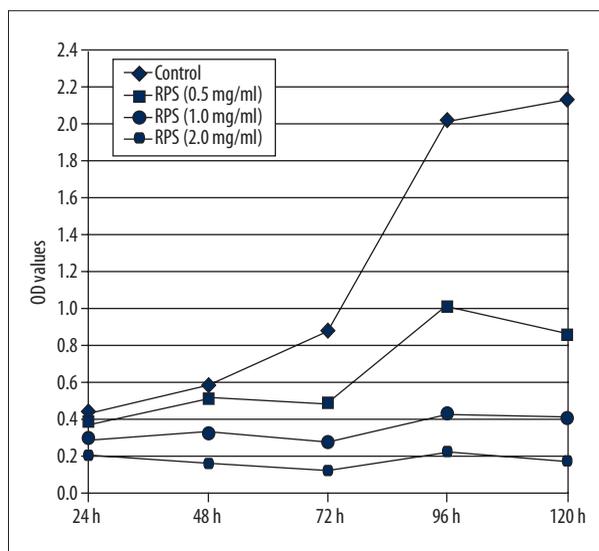


Figure 1. Effect of *Rhizoma Paridis* saponins (RPS) on the proliferation of A549 cells. OD, optical density.

(Figure 3A, 3D, 3I). In comparison, the expression of p21 and BAX were significantly upregulated by RPS treatment (Figure 3B, 3C). However, the expression of cyclins A/B1/D1/E and CDK4/6 did not show any clear changes (Figure 3E–3H, 3J, 3K). Subsequently, Western blotting was used to detect the protein expression of p53, p21, BAX, BCL2, and CDK2, and the results verified that the expression of the 5 proteins changed in accordance with the qPCR results (Figure 3L).

Discussion

RPS has extensive medicinal value, including antimicrobial, anthelmintic, hemostasis, immunoregulation, analgesia, and anticancer effects [17–20]. However, the complexity and varying action of components of RPS has limited its extensive application and hindered the study of their underlying molecular mechanism(s). A number of studies have focused on identifying

Table 2. Effect of RPS on the apoptosis of A549 cells.

Groups	UL (%) (monocyte)	UR (%) (necrotic cells, late stage apoptotic cells)	LL (%) (live cells)	LR (%) (early apoptotic cells)
Control	0.01	8.79	87.03	4.17
RPS (0.5 mg/ml)	0.08	28.9	48.89	22.13
RPS (1.0 mg/ml)	0.21	25.8	35.17	38.82
RPS (2.0 mg/ml)	1.16	28.56	23.79	46.49

UL – upper left; LL – lower left; UR – upper right; LR – lower right.

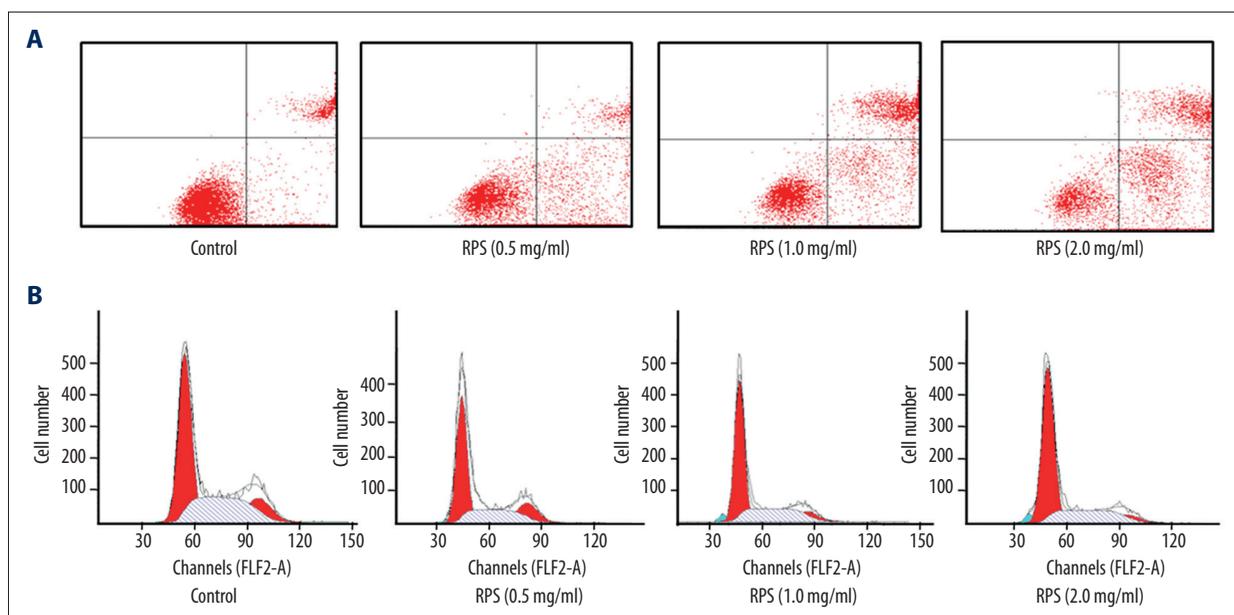


Figure 2. Effect of *Rhizoma Paridis* saponins (RPS) on the apoptosis and cell cycle of A549 cells. Effect of RPS on the (A) apoptosis and (B) cell cycle of A549 cells.

Table 3. Effect of RPS on the cell cycle of the A549 cells.

Groups	G1 (%)	S (%)	G2 (%)
Control	50.84±0.03	35.35±2.89	13.81±3.34
RPS (0.5 mg/ml)	63.57±4.93*	24.11±5.17*	12.32±2.19
RPS (1.0 mg/ml)	70.79±6.07*	17.13±4.87*	12.08±5.12
RPS (2.0 mg/ml)	74.05±3.61*	14.19±2.85*	11.76±1.17*

* P<0.05 as determined by a student's t-test compared with the control group.

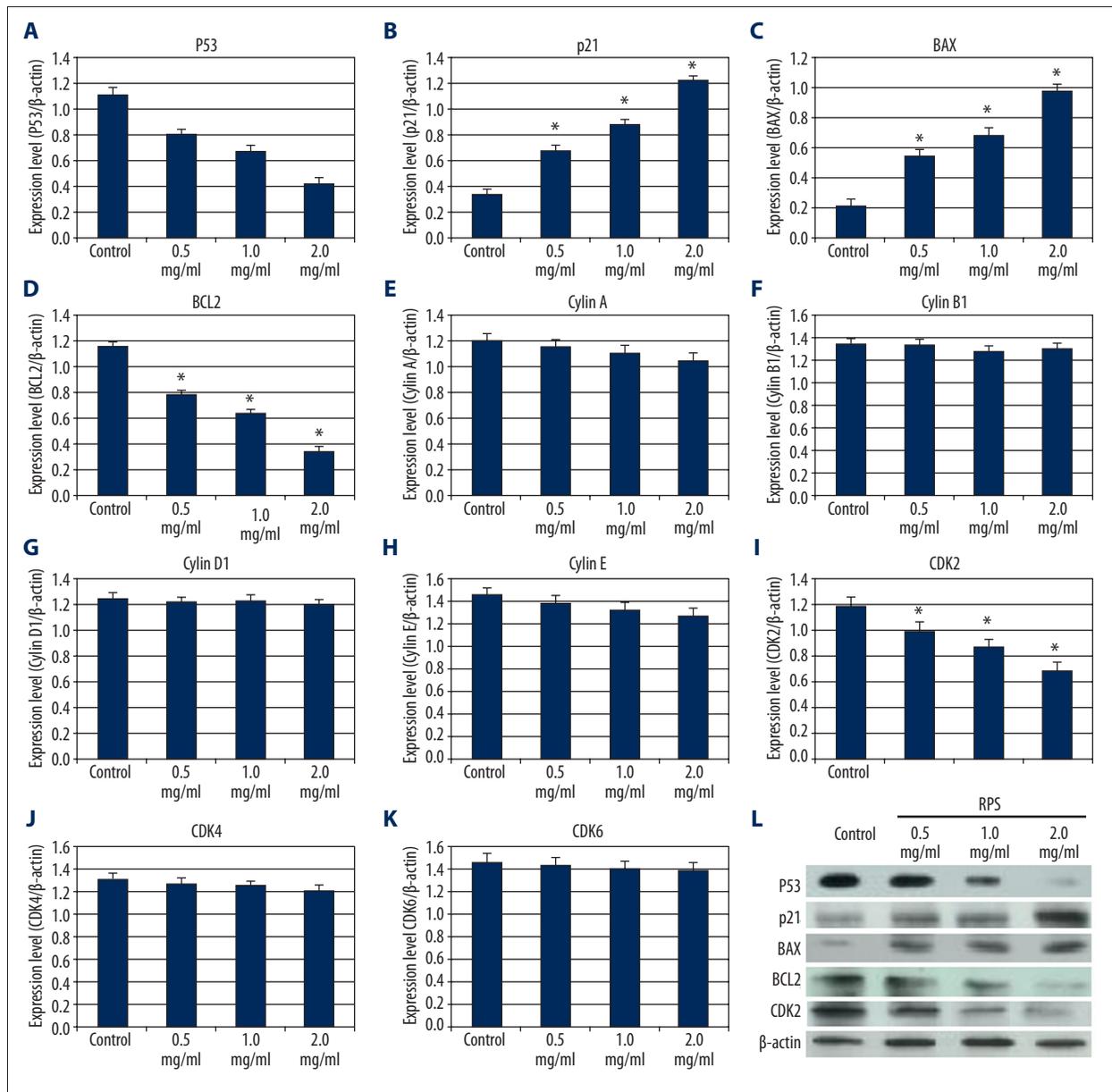


Figure 3. Effect of *Rhizoma Paradisi* saponins (RPS) on expression of cell cycle and apoptosis-related genes. Results of (A–K) quantitative polymerase chain reaction for genes and (L) Western blotting for the proteins. * P<0.05, as determined by a Student's t-test compared with the control group.

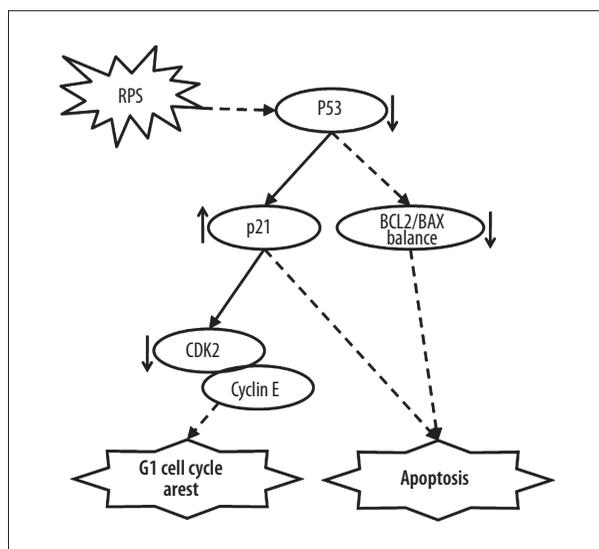


Figure 4. Mechanism of *Rhizoma Paridis* saponins (RPS) treatment for non-small-cell lung cancer. CDK2, cyclin-dependent kinase 2.

and analyzing the compounds in RPS to research the anticancer bioactivators of Chonglou [21–23].

Certain studies have shown that RPS has an anticancer effect by regulation of the cell cycle and apoptosis [14,24,25]. However, the underlying mechanism of RPS remains unknown. The present study investigated the effect of RPS in NSCLC A549 cells by the MTT assay and flow cytometry, and identified that RPS could inhibit cell growth and induce G1 cell cycle arrest and apoptosis in A549 cells in a dose-dependent manner, indicating that RPS has an evident effect in NSCLC treatment. Subsequently, to investigate the molecular mechanism of the effect for RPS treatment, expression of genes (p53, p21, BAX, BCL2, CDK2/4/6, and cyclins A/B1/D1/E) associated with proliferation, cell cycle arrest, and apoptosis were detected using qPCR and Western blotting. Finally, p53, p21, BAX, BCL2 and CDK2 were identified to have significant expression changes. In carcinogenesis, p53 is known as a 'superstar' gene, which can regulate cell growth,

DNA repair, angiogenesis, and apoptosis by participating in various cell signaling pathways [26,27], and p21 is a downstream protein of p53, which is a key inhibitor of the CDKs and can regulate the cell cycle by adjusting the activity of cyclin/CDK complexes [28]. CDK2, a member of the CDK family, is an important kinase in the G₀/G₁ cell cycle [29]. BAX and BCL2 are also downstream proteins of p53, and cell apoptosis depends on the intracellular balance between BCL2 and BAX activity [30]. Evidently, expression changes of those 5 genes can perturb cell growth and apoptosis. NSCLC is a heterogeneity disease that is associated with numerous gene variations and expression imbalance, and disturbance of multiple biological functions, including cell cycle, apoptosis, angiogenesis, and immune escape [31,32].

The present study showed that RPS could suppress proliferation by inducing cell cycle arrest and apoptosis of the NSCLC cells, and regulate the expression of p53, p21, BAX, BCL2, and CDK2. Therefore, in accordance with the cell cycle, apoptosis pathways, and the present experimental results, we speculate that there is a pathway including p53, p21, BAX, BCL2, and CDK2 that can be affected by RPS (Figure 4). In this pathway, RPS could directly or indirectly affect the target-regulated expression of p53, and subsequently change the expression of the downstream genes p21, BAX, BCL2, and CDK2, and lead to cell proliferation inhibition, cell cycle arrest, and apoptosis, finally inhibiting NSCLC progression.

Conclusions

The present study demonstrated that RPS can inhibit NSCLC cell growth by inducing G1 cell cycle arrest and apoptosis in a dose-dependent manner; 5 associated differentially expressed genes (p53, p21, BAX, BCL2, and CDK2) were detected as being involved in these processes. Finally, the study established a pathway for RPS treatment to reveal the molecular mechanism of the anticancer effect of RPS at cellular and molecular levels (Figure 4), which is helpful for improving the medicinal application of the anticancer effect of Chonglou.

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