Research Article

Anticancer Effects of Cyclocarya paliurus Polysaccharide (CPP) on Thyroid Carcinoma In Vitro and In Vivo

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In this study, we explored the role and mechanisms of Cyclocarya paliurus polysaccharide on cell apoptosis in thyroid cancer (TC) cells. The apoptosis of thyroid cancer cells in vitro and tumor tissues in vivo induced by Cyclocarya paliurus polysaccharide was determined by MTT assay and flow cytometric assay. The downstream molecules including phosphoprotein kinase B (p-Akt), Akt, B-cell lymphoma 2 (Bcl-2), and Bcl-2-associated X protein (Bax) in tumor tissue were evaluated by western blotting. MTT and flow cytometry assay in vitro revealed Cyclocarya paliurus polysaccharide-induced apoptosis of thyroid cancer cell line in a manner of time-dependent and dose-dependent. In vivo assay showed 50 mg/kg and 100 mg/kg Cyclocarya paliurus polysaccharide significantly suppressed the proliferation of thyroid cancer in mice. Western blotting showed downregulation of p-Akt, Akt, and Bcl-2 and upregulation of Bax. These results suggest that Cyclocarya paliurus polysaccharide may enhance thyroid cancer cell apoptosis by suppressing the activation of p-Akt, Akt, and Bcl-2 and activating Bax, which provide a novel use of CPP as a thyroid cancer treatment.

1. Introduction

Plant polysaccharides are natural polymeric macromolecules generally composed of more than ten monosaccharide units joined by glycosidic bonds, playing an important role in life-support activities. Biological activities of polysaccharides have been broadly investigated since the 1940s. Plant polysaccharides have biological effects such as immunoregulation, anticancer, antiaging, serum glucose-depressing, and lipid-depressing [1–5]. And recently a growing number of studies have demonstrated the significant advantages of polysaccharides in the prevention and treatment of critical or chronic diseases in human such as cancers, cardiovascular diseases, and diabetes [6–8].

Moreover, there are some limitations of present therapy against thyroid cancer. Patients with different types of thyroid cancer exhibited poor or modest response to traditional chemotherapy [9]. Till date, present goals are to identify novel agents to improve response and progression-free survival. Researches show that most of the plant polysaccharides perform anticancer effect through strengthening immune function against cancer cells by regulating the levels of cytokines secreted by the host cells [10]. On the other hand, cytotoxic polysaccharides possess tumor cytotoxicity, which showed direct interactions with cancer cells [11]. However, the effects of Cyclocarya paliurus polysaccharide on thyroid carcinoma are still largely unknown.

In the past decade, progress has been made to understand the molecular mechanisms of thyroid cancer. The best represented advances in genetic and epigenetic events occurring in thyroid cancer was Akt, which was reported to be involved in several major pathways [12]. Hyperactivation of Akt promotes cell transformation and tumorigenesis in multiple tumors. In addition, Bcl-2 family of proteins is a major intracellular modulator of apoptotic signaling, which exist in normal and neoplastic thyroid tissue [13]. It was reported
that overexpression of Bcl-2 in thyroid carcinoma cells increased oncogenic addiction of cells. Another molecule influencing the apoptotic balance in cancer tissues is Bax. It has been assumed that the ratio of Bcl-2/Bax determines cell death [14].

In the present study, we investigate the mechanism by which polysaccharides isolated by Cyclocarya paliurus and determine its possible anticancer properties. Furthermore, we established a mouse model to reveal the underlying mechanism of anticancer effects in vitro and in vivo.

2. Materials and Methods

2.1. Reagents. Plant materials and preparation of C. paliurus polysaccharides were obtained previously [15]. The leaves of C. paliurus were collected from a planting base in Enshi, Hubei Province, China. Sugar content was measured by the phenol-sulfuric colorimetric method with D-glucose as a standard at 490 nm. Phospho-Akt1 (S246) polyclonal antibody was purchased from R&D. AKT antibody was purchased from Rockland. Bcl-2 monoclonal antibody, Bax polyclonal antibody, GAPDH, and HRP Mouse mAb were purchased from MultiSciences.

2.2. Cell Lines. Thyroid carcinoma cell lines (FRO, ARO, 8505C, SW579, K1, FTC133, and BCPAP) were purchased from FuHeng Cell Center, Shanghai, China. The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 100 units/mL penicillin (Sigma-Aldrich, St. Louis, USA), and 0.1 mg/mL streptomycin (Sigma-Aldrich, St. Louis, USA). All cells were maintained in a humidified incubator containing 5% humidified CO₂ at 37°C.

2.3. Animals. All animal procedures were approved by the Animal Care and Use Committee of the institution in compliance with the Guide for the Care and Use of Laboratory Animals. Forty male BALB/c nude mice (18–20 g, 5–6 week age) were obtained from a commercial vendor (Beijing Huafukang Bioscience Co. Inc., Beijing, China, permit number: SCXK-JING 2014-0004). All the mice were housed under pathogen-free conditions in accordance with laboratory animal care and allowed free access to sterilized food and water. After FRO cells were harvested, tumor inoculation was performed in the right foreleg (5 × 10⁶ cells/mice). Mice in NC and TC groups were orally administered with distilled water. Tumor tissues isolated from mice and collected by surgical manipulation were quickly frozen in liquid nitrogen and sliced to a thickness of 8 μm using a microtome (Leica CM1900, Berlin, Germany). Total protein was extracted from cells and tumor tissues using ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). The concentration was calculated after protein was isolated using a bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China). 30 μg of proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, CA, USA) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocked with 5% nonfat milk for 1h, samples were incubated with primary antibodies against p-Akt (1:1000) polyclonal antibody, Akt (1:1000), Bcl-2 (1:1000), Bax (1:1000), and GAPDH (1:1000) at 4°C overnight. After washed with Tris-buffered saline/Tween 20 (TBST), the membrane was incubated with HRP-conjugated secondary antibody (1:5000) for 1h at room temperature. Washed three times with TBST, the protein was detected with an enhanced chemiluminescence (ESL) western blot detection system (Millipore, Bedford, MA, USA). GAPDH was considered to be a control for the relative expression.

2.4. MTT Assay. The inhibition effect of CPP on seven human thyroid carcinoma cell lines (FRO, ARO, 8505C, SW579, K1, FTC133, and BCPAP cells) was measured by MTT assay in vitro. Briefly, the thyroid carcinoma cells (2 × 10⁴ cells/well) were incubated in 96-well plates with 0.2 mL cell culture medium at 37°C. Cells were allowed to adhere for 24h, then were incubated with the culture containing CPP with different concentration (0, 30, and 100 μg/mL) for extra 48h. After the exposure to CPP, 20 μL of MTT assay reagent were added to each well, and the cells were incubated for 1h. After the medium was removed, 50 μL of DMSO were added to each well and were incubated for 10 min. The absorbance value of the plate was read at 570 nm by the microplate reader. The inhibition rate was calculated as follows: growth inhibitory rate (%) = (1 - absorbance of the experimental group/absorbance of the blank control group) × 100%.

2.5. Flow Cytometry. Flow cytometry was performed to identify cell apoptosis by using Annexin V-FITC/PI kit (Qiangen, China), followed by the manufacturer’s instructions. After incubation with Cyclocarya paliurus polysaccharide, cells were collected and washed with phosphate-buffered saline (PBS) and stained with Annexin V-FITC/PI. Binding buffer (150 μL) and Annexin-V-FITC (5 μL) were added into each tube and incubated at room temperature for 15 min. Subsequently, binding buffer (100 μL) and PI (5 μL) were also added and incubated for 5 min. At least 10000 cells in each sample were acquired by flow cytometry FC500 (Beckman Coulter, USA) and analyzed with CXP analysis software 2.2 (Beckman Coulter, Inc.).

2.6. Western Blotting. Tumor tissues isolated from mice and collected by surgical manipulation were quickly frozen in liquid nitrogen and sliced to a thickness of 8 μm using a microtome (Leica CM1900, Berlin, Germany). Total protein was extracted from cells and tumor tissues using ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). The concentration was calculated after protein was isolated using a bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China). 30 μg of proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, CA, USA) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocked with 5% nonfat milk for 1h, samples were incubated with primary antibodies against p-Akt (1:1000) polyclonal antibody, Akt (1:1000), Bcl-2 (1:1000), Bax (1:1000), and GAPDH (1:1000) at 4°C overnight. After washed with Tris-buffered saline/Tween 20 (TBST), the membrane was incubated with HRP-conjugated secondary antibody (1:5000) for 1h at room temperature. Washed three times with TBST, the protein was detected with an enhanced chemiluminescence (ESL) western blot detection system (Millipore, Bedford, MA, USA). GAPDH was considered to be a control for the relative expression.

2.7. Statistical Analysis. All values were expressed as mean ± standard deviation (SD). All experimental data were analyzed using SPSS 21.0 software (IBM Corp., USA). Comparisons between groups were conducted using a paired t-test. Comparisons between two groups were performed using an independent sample t-test. Comparisons among multiple groups were assessed by one-way analysis of variance (ANOVA). Enumeration data were expressed as percentage and analyzed by chi-square test. A value of p < 0.05 was indicative of statistical significance.

3. Results

3.1. Extraction and Content Determination of CPP. In this study, we isolated polysaccharide from C. paliurus by water extraction and ethanol precipitation. To confirm purity of
polysaccharide was determined to be 89.5%.

3.2. Growth Inhibitory Effect of CPP on Thyroid Carcinoma Cell Lines. The growth inhibitory rate of CPP with different levels on seven human thyroid carcinoma cell lines was evaluated to characterize the antithyroid cancer effect of CPP and screen for the cell line with the most effective inhibition induced by CPP. Cell viability of cell lines was analyzed by MTT assay. The cell growth was significantly inhibited in all of the seven thyroid carcinoma cell lines after incubated with 30 and 100 μg/mL of CPP (p < 0.001, Figure 1(a)). And the effect of CPP in 100 μg/mL on growth inhibition of thyroid carcinoma cells was more significant than that in 30 μg/mL.

The inhibitory rate on anaplastic thyroid carcinoma-derived FRO cells was approximately 50% at the CPP concentration of 30 μg/mL, only slightly lower than that on differentiated thyroid carcinoma-derived BCPAP cells (%). However, the inhibitory rate on FRO was up to 90% at the concentration of 100 μg/mL, which is higher than all other thyroid carcinoma cell lines tested including BCPAP cells. Therefore, anaplastic thyroid carcinoma-derived FRO cells were selected and used for further investigation.

We further investigated the effect of 100 μg/mL CPP on FRO for 24 h, 48 h, and 72 h. As shown in Figure 1(b), FRO cells treated for 72 h presented higher inhibitory effect, when compared to those of 48 h and 24 h, with no significance. These results indicating that the inhibitory effect of CPP possessed dose-dependent and time-dependent.

3.3. Apoptosis Assay of Tumor Tissues Induced by CPP. Mice treated with PBS were served as control. As shown in Figure 2(a), 50 mg/kg and 100 mg/kg for 30 days significantly decreased the tumor weight, when compared with PBS group. During the period of dosing with CPP, tumor volume in mice increased (Figure 2(b)). Annexin V-FITC double staining was performed to detect tumor cell apoptosis in tumor tissues of mice (Figures 2(c)–2(f)). Treatment of 50 mg/kg and 100 mg/kg CPP resulted in a significant apoptosis of tumor cells in mice by 17.7 ± 2.3% and 21.3 ± 2.6% (p < 0.001).

3.4. Expressions of p-Akt, Akt, Bcl-2, and Bax Proteins in Tumor Tissues Regulated by CPP. Protein (Figure 3) expressions of p-Akt, Akt, Bcl-2, and Bax after the mice were sacrificed were evaluated in all three treatment groups. 50 mg/kg and 100 mg/kg treatment of CPP significantly raised p-Akt/Akt protein expressions by 77.6% and 48.1% (Figure 3(b), p < 0.01). The Bcl-2 protein expressions decreased by 78.5% and 69.2% (Figure 3(c), p < 0.01). The Bax protein expression had elevated to 1.67-fold and 2.23-fold (Figure 3(d), p < 0.001). CPP had a strong promoting effect on apoptosis, indicating functional involvement of p-Akt, Akt, Bcl-2, and Bax in apoptosis induced by CPP.

4. Discussion
The infiltration of inflammatory cells usually could be found in thyroid cancer, including lymphocytes, macrophages, and mast cells. Although the role of these cells in cancer and function mechanism is not entirely clear, most of the experiments show that these cells promote the development of tumor. Therefore, inflammatory molecules can be a potential thyroid cancer treatment target, while immunotherapy gradually become the focus in the treatment of thyroid cancer in recent years. As reported, qing qian liu polysaccharide has been proven to have the function of immune regulation and can inhibit the release of IL-6 and TNF-β by macrophages stimulated by LPS [16]. Cyclocarya palurus polysaccharide is a kind of natural plant polysaccharide extracted from Cyclocarya palurus. Because of its special physical and chemical effects and small side effects, it has become a...
Figure 2: Effect on tumor weight and apoptosis induced by CPP. (a) and (b) presented the tumor weight of mice with specific treatment. In (c), (d), (e), and (f), apoptotic cells were quantified by flow cytometry after stained with Annexin V and PI. Data were presented as mean ± SD. *p < 0.05 compared with control. **p < 0.01 compared with control. ***p < 0.001 compared with control. All experiments were performed triplicate.
potential active compound. Currently, studies on the anti-cancer capacities of polysaccharides focused on the apoptotic effect. The cytotoxicity of the components from Inonotus obliquus was identified in human prostatic carcinoma cell PC3 and breast carcinoma MCF-7 cell [17, 18]. In vivo and in vitro studies have confirmed polysaccharides from P. However, the signal pathways involved in the apoptosis of cancer cells are complex, and the signal mechanisms of apoptosis are often different in different tumor cell types.

In this study, we isolated polysaccharide from C. paliurus by water extraction and ethanol precipitation. To confirm the contents of polysaccharide, we further measured the sugar contents by the phenol-sulfuric acid colorimetric method. The total content of polysaccharide was determined to be 89.5%. Previous study showed that CPP exerts antiproliferation effect on various tumor cell lines [17–19]. We observed CPP inhibited cell proliferation and induced apoptosis in a pair of thyroid carcinoma cell lines including FRO, ARO, 8505C, SW579, K1, FTC133, and BCPAP. Also, a similar trend was observed in a mouse model. Tumor weight of mice was significantly reduced after treated with CPP.

In our previous study, we explored the potential mechanism of apoptosis of human colon cancer cell HT29 induced by CPP. MTT colorimetric assay was used to detect that Cyclocarya paliurus polysaccharides could significantly inhibit the proliferation of human colon cancer cell HT29. Our previous results suggested that apoptosis induction may be the main mechanism of antitumor effect of CPP. The results of flow cytometry showed that the polysaccharide could induce apoptosis of human colon cancer cell line HT29, and the typical morphological characteristics of apoptotic cells could be observed. As a major strategy for cancer therapeutics, Annexin V/PI staining was conducted to determine whether the cytotoxic effect was related to the apoptotic process. We found that after dosing with CPP, the percentages of early and late apoptotic tumor cells were markedly increased. All these data indicated that CPP induced apoptosis in thyroid tumor.

**Figure 3:** Effect on p-Akt, Akt, Bcl-2, and Bax protein levels induced by CPP. (a) The expression of p-Akt, Akt, Bcl-2, and Bax in tumor tissues are detected by immunoblotting. GAPDH is served as loading control. (b), (c), and (d) presented the relative expression of p-AKT, Bcl-2, and Bax proteins. Data were presented as mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with control. All experiments were performed triplicate.
To understand the possible molecular mechanisms of thyroid cancer cytotoxicity, we explored the related mechanisms. The PI3K/AKT pathway is the most studied frequently mutated network in human cancer [20]. Overactivation of this protein is associated with tumor growth, invasion, and drug resistance. Our results confirmed that CPP markedly downregulated p-Akt and Akt protein levels. Considering p-Akt is the active form of Akt, our data suggested that CPP inhibited active form of Akt directly and indirectly.

In general, cellular life and death are regulated by the Bcl-2 family proteins and the balance between Bcl-2 and Bax. Abnormal expression of Bcl-2 and Bax contribute to tumor survival and growth. Furthermore, Bcl-2 and Bax regulate cell apoptosis process in an independent way [21]. Mitochondrial membrane associated Bcl-2 modulates the expression of some apoptotic factors that usually sequestered in the mitochondrial intermembrane space. Bax plays an essential role in rescuing the sensitivity of apoptosis [22]. If Bcl-2 failed to exert the antiapoptosis role, Bax will form a channel in the mitochondrial outer membrane that facilitate apoptosis [23]. Here, we revealed that CPP markedly reduced Bcl-2 protein levels and elevated Bax protein levels.

To summarize, CPP has a remarkable inhibition in thyroid tumor growth both in vitro and in vivo, making it of significance in studies. CPP exerts an anticancer effect on thyroid cancer cells through p-Akt, Akt, Bcl-2, and Bax pathways. Our data suggest a potential strategy as a promising therapy for thyroid cancer.

Data Availability
The data used to support the findings of this study are included within the article.

Ethical Approval
Approval for the present study was obtained by the Ethics Committee of the Shanghai Post and Telecommunication Hospital (Shanghai, China).

Consent
All patients admitted to the study provided informed consent for their participation of the present study and the publication of this data.

Conflicts of Interest
The authors declare that they have no competing interests.

Authors’ Contributions
TQ was responsible for the study conception and design and revised the manuscript; ZH and FL performed the experiments and drafted the manuscript; YG and JG analyzed the data. All authors read and approved the final manuscript.

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