The Total Flavonoids of Clerodendrum bungei Suppress A549 Cells Proliferation, Migration, and Invasion by Impacting Wnt/β-Catenin Signaling

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Abstract

Objectives: The objective of this study is to evaluate the effect of the total flavonoids of Clerodendrum bungei (TFCB) on the proliferation, invasion, and metastasis of A549 lung cancer cells through the Wnt signaling pathway. Materials and Methods: A549 cells were transfected with a β-catenin overexpression plasmid and the empty vector pcDNA3.1. The A549 cells were divided into six groups: normal A549 cell group, normal A549 cells with TFCB group, vector control group, vector with TFCB group, β-catenin overexpression group, and β-catenin with TFCB group. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to detect cell proliferation, a scratch test was used to observe cell migration, and a transwell experiment was employed to evaluate cell invasion. Proteins related to the Wnt pathway were detected with Western blot analysis, including β-catenin, GSK-3β, P-GSK-3β, c-Myc, and CyclinD1. Results: The proliferation, invasion, and metastasis of A549 cells were significantly enhanced after being transfected with the β-catenin overexpression plasmid (P < 0.05 or 0.01), accompanied by increased expression of β-catenin, C-Myc, CyclinD1 and reduced expression of Gsk-3β and P-GSK-3β. Treatment of cells with TFCB resulted in inhibition of cell proliferation, migration, and invasion; downregulated expression of β-catenin, C-Myc, and CyclinD1; and upregulated expression of GSK-3β and P-GSK-3β, especially in the β-catenin overexpression group. Conclusion: TFCB has the potential to inhibit the Wnt/β-catenin pathway by prohibiting the overexpression of β-catenin and regulating its downstream factors.

Keywords: Migration and invasion, total flavonoids of Clerodendrum bungei, β-catenin overexpression plasmid, β-catenin signaling pathway

Introduction

Primary bronchogenic carcinoma, commonly referred to as lung cancer, is a type of malignant tumor that originates from the bronchial mucosa and gland. This disease falls under the domain of “Fei Ji, Fei Yan, Xi ben, Xu Lao” in traditional Chinese medicine. The morbidity and mortality rates of lung cancer are the highest of all cancer types worldwide according to the World Cancer Report of 2014, including China. Local recurrence and distant metastasis are the main causes of death related to lung cancer. Over 80% of patients with lung cancer already have systemic metastases when diagnosed. Clerodendrum bungei was screened among Hunan folk antitumor prescriptions through repeated experimental and clinical studies. The medicinal properties of C. bungei are pungent, bitter, and neutral. Its primary medicinal effects are to relieve wind and dispel dampness, relieve toxicity and remove blood stasis, reduce swelling, and alleviate pain. Some studies have shown that the compounds or extract of C. bungei can suppress several types of tumors, especially lung cancer.¹⁻⁶ We previously reported that C. bungei can reduce the incidence rate and metastasis of lung cancer.⁶⁻⁸ The Wnt/β-catenin pathway is a highly conserved signaling pathway that participates in tumor genesis through signal...
transduction, and regulation of the cell cycle, cell proliferation, apoptosis, and adhesion when abnormally activated in mature animals. The Wnt/β-catenin pathway is also closely related to the occurrence and development of lung cancer. In the present study, we transfected the lung cancer cell line A549 with a β-catenin overexpression plasmid to activate the Wnt/β-catenin pathway and determined the additional effect of treatment with the total flavonoids of C. bungei (TFCB). We evaluated the effect of TFCB on the expression of molecules involved in the Wnt/β-catenin pathway and on the proliferation, invasion, and metastasis of A549 cells.

**Materials and Methods**

**Preparation of total flavonoids of Clerodendrum bungei**

C. bungei crude herb was extracted twice with 95% ethanol refluxing, followed by filtering, ethanol recovery, concentrating to a relative density of approximately 1.30, and vacuum drying. The content of total flavones was approximately 40% in the dry extract as determined by ultraviolet spectrophotometry with rutin as the reference standard. The original dry extract was dissolved with 95% ethanol, and macroporous resin was used to attach the solution, which was then eluted with water, followed by elution with 80% ethanol. The water elution was then discarded. The eluent was collected, and the ethanol was recycled. The elution was concentrated, and the purified dry paste was obtained. The content of total flavones was approximately 65% in the dry paste as determined by ultraviolet spectrophotometry.

**Main reagents and instruments**

Human lung adenocarcinoma A549 cells were purchased from the Chinese Academy of Sciences cell bank. The β-catenin plasmid (pCDNA3.1+ vector, ampicillin [AMP]-resistant), and empty vector (pCDNA3.1+, AMP-resistant) were provided by Professor Wei-Lin Jin from Shanghai Jiaotong University. The transwell chamber was purchased from Corning (USA). β-catenin rabbit monoclonal antibody (mAb), phospho-GSK-3β rabbit mAb, and GS‑K‑3β antibody sampler kit were obtained from Cell Signaling Technology (USA). C-Myc and CyclinD1 rabbit mAbs were purchased from Beijing Biosynthesis Biotechnology (China). The β-actin murine mAb was purchased from Sigma Aldrich (USA). Lipofectamine™ 2000 was obtained from Life Technologies (USA). Fetal bovine serum (FBS) was purchased from Gibco (USA). The laminar flow bench and carbon dioxide cell incubator were obtained from Thermo Fisher Scientific (USA). The enzyme-linked immunosorbent assay instrument was obtained from Huisong (China).

**Cell culture and plasmid transfection**

**Cell culture**

A549 cells were cultivated in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS. The culture solution was replaced every 2–3 days. The cells were passaged with 0.25% pancreatin with ethylenediaminetetraacetic acid when covering 80% of the medium.

**Plasmid transformation, expansion, and extraction**

Competent *Escherichia coli* cells were prepared and mixed with 50 µL of the β-catenin overexpression plasmid or empty vector. The mixture solution was placed on ice for 30 min, followed by heat shock in 42°C water for 90 s and then, cooled in ice water immediately for 3–5 min afterward. Luria-Berti culture solution without AMP was added to the prepared mixture solution and oscillated for 1 h at 37°C. Two-hundred microliters of the bacteria suspension were smeared on the culture board with AMP and cultured at 37°C for 24 h. The plasmid was extracted according to the instructions in E. Z. N. A. Endo-free Plasmid Mini Kit I. The plasmid concentration was detected with a Nanodrop 2000 ultramicro UV/Vis spectrophotometer.

**Plasmid transfection in A549 cells**

The A549 cells were plated in 6-well plates. DMEM culture solution with 10% FBS without antibiotics was added when the cells covered 80%–90% of the bottom of the well. Four micrograms of the empty vector or β-catenin overexpression plasmid were used to transfect A549 cells according to the steps in the Lipofectamine™ 2000 kit.

**Cell grouping**

A549 cells were divided into six groups: Group A (normal control), Group B (normal cells with TFCB), Group C (empty vector control), Group D (empty vector control with TFCB), Group E (β-catenin overexpression), and Group F (β-catenin overexpression with TFCB).

**Cell proliferation assay**

The nontransfected A549 cell lines were plated on a 96-well culture plate with 5000 cells/well. The TFCB mother solution was prepared at a concentration of 10 mg/mL. The mother solution was diluted with 4% FBS-contained DMEM to various concentrations (0, 0.2, 0.6, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg/mL). The former culture solution was replaced with 200 µL of different concentrations of TFCB solution for 48 h. Control wells were set with culture solution without...
cells. Inhibition of cell proliferation was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The experiment was repeated three times. The 50% inhibitory concentration (IC50) of TFCB was chosen for the following steps.

The A549 cells were divided into six groups as described in Section 2.4. Cells were seeded at a density of 5000 cells/well in a 96-well plate overnight and then treated with 2 mg/mL of TFCB solution for 48 h. Cell proliferation inhibition was tested with the MTT assay.

**Migration and invasion assays**

Cell migration and invasion were determined using wound-healing and transwell migration assays. The cells were divided into the six groups as described above in Section 2.4 and treated with 2 mg/mL of TFCB solution for 48 h. The cells were grown to confluence in 6-well plates, and the medium was replaced with serum-free medium for an additional 24 h of culture. Cell monolayers were scraped with a 100-µL micropipette tip. The wound area was photographed with microscopy at 0 h, 24 h, and 48 h after the treatment and the percentage of wound closure was determined as follows: \((\text{initial width of scratch area} - \text{final width of scratch area}) / \text{initial width of scratch area} \times 100\%\). The experiment was repeated three times, and the average value was calculated.

The transwell inserts were precoated with Matrigel. The cells grown in serum-free medium were plated into the upper chambers. DMEM containing 10% FBS was added to the lower chambers. The chambers were then incubated for 48 h. Nonmigratory cells were removed, and migrated cells were fixed, stained, and observed with an inverted microscope. Five fields were randomly selected, and the cell number was counted. The experiment was repeated three times, and the average value was calculated.

**Western blot analysis**

The cells were divided into the following groups: group A (empty vector control), Group B (empty vector control with TFCB), Group C (β-catenin overexpression), and Group D (β-catenin overexpression with TFCB). The cells were treated with 2 mg/mL of TFCB solution for 48 h when they covered 70%–80% of the medium.

The cells were cleaned with phosphate-buffered saline, 100 µL RIPA lysis buffer was added, and the cells were placed on ice for 30 min. After disruption, they were centrifuged under 4°C at 12,000 rpm for 15 min, and the supernatant was collected in a 0.5-mL centrifuge tube. The protein standard curve was drawn, and the protein concentration was determined according to the BCA protein assay kit. The chamber was filled with gel and loaded with the sample for electrophoresis. Five microliters of denatured protein were added and then transferred to the membrane after electrophoresis. The gel was cut according to the positions of GSK-3β (46 kDa), P-GSK-β (46 kDa), C-Myc (49 kDa), CyclinD1 (32 kDa), and β-actin (42 kDa). After transferring to the polyvinylidene fluoride membrane, the membrane was sealed for 1 h, the diluted primary antibodies were added (β-catenin 1:1000, GSK-3β 1:1000, P-GSK-3β 1:1000, C-Myc 1:500, CyclinD1 1:500, β-actin 1:4000), and the membrane was incubated with shaking overnight. The membrane was then incubated with diluted secondary

**Figure 2:** The OD value of each group after treatment with total flavonoids of *Clerodendrum bungei* for 48 h

**Figure 3:** Image of the migration of cells in each group after 0, 24, and 48 h in a scratch test. (a) Normal control, (b) normal control with total flavonoids of *Clerodendrum bungei*, (c) vector control, (d) vector control with total flavonoids of *Clerodendrum bungei*, (e) β-catenin overexpression, (f) β-catenin overexpression with total flavonoids of *Clerodendrum bungei*
antibodies (1:3000 dilution) for 60 min. Specific immune complexes were detected using the Western Blotting Plus Chemiluminescence Reagent.

**Statistical analysis**

All data were analyzed with Statistical Product and Service Solutions (SPSS), SPSS 17.0 software and are presented as the mean ± standard deviation one-way analysis of variance was used to determine the differences among multiple groups. Comparison of two groups was performed by two-sided Student’s t-tests. 

**RESULTS**

**Effects of total flavonoids of Clerodendrum bungei and β-catenin overexpression on A549 cell proliferation**

The MTT assay was used to test the effect of different concentrations of TFCB on the proliferation of A549 cells. The results indicated that TFCB decreased the proliferation of A549 cells in a dose-dependent manner [Figure 1]. The IC50 value was calculated to be approximately 2.0 mg/mL, which was used as the TFCB concentration in subsequent experiments.

Compared with the empty vector control, the cells transfected with the β-catenin overexpression vector showed significantly increased proliferation (P < 0.05). TFCB decreased the proliferation of A549 cells in each group (P < 0.05), with outstanding inhibition detected in the β-catenin overexpression group (P < 0.01) [Figure 2].

**Effects of total flavonoids of Clerodendrum bungei on A549 cell migration**

The β-catenin overexpression plasmid enhanced the invasiveness of A549 cells. TFCB significantly inhibited cell migration in the normal group (P < 0.05), vector control group (P < 0.01), and β-catenin overexpression group (P < 0.01) [Figures 3 and 4].

**Expression of Wnt pathway marker proteins**

Expression analysis of the Wnt pathway marker proteins by Western blotting revealed that the β-catenin overexpression plasmid increased the protein levels of β-catenin (P < 0.01), C-Myc (P < 0.01), and CyclinD1 (P < 0.01) and decreased the levels of GSK-3β (P < 0.05) and P-GSK-3β (P < 0.01). TFCB inhibited β-catenin (P < 0.05) and C-Myc (P < 0.05) expression and enhanced GSK-3β (P < 0.05) and P-GSK-3β (P < 0.05) expression in normal A549 cells. However, TFCB inhibited β-catenin (P < 0.05), C-Myc (P < 0.05), and CyclinD1 (P < 0.05) expression in β-catenin overexpressing cells [Figure 7].

**DISCUSSION**

*C. bungei* is cold in property. However, it has the efficacy of “dispelling wind and dampness, detoxifying and dispersing
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blood stasis, and relieving swelling and pain”, while on the other hand, it also can tonify deficiencies of the lung, spleen, and kidney to treat a consumptive chronic cough. These effects of C. bungei conform to the lung cancer therapeutic principle in Chinese medicine, which is “detoxification, dispersing blood stasis, and tonifying the deficiency”. Modern pharmacological research of C. bungei has demonstrated its sedative hypnosis, local anesthesia, analgesia, anti-inflammation, bacteriostasis, and antineoplasm effects.[11,12] We conducted research on four types of samples extracted from C. bungei, including the flavonoids, alkaloids, water extract without flavonoids, and water extract and alcohol precipitate. We found that all four components have direct cytotoxicity in vitro but with different intensities. Therefore, we decided to further focus on the flavonoids after considering the antineoplastic intensity and extraction process of the four components.

The Wnt/β-catenin pathway participates in tumorigenesis through signal transduction and regulates the cell cycle, migration, apoptosis, and adhesion. The Wnt signaling pathway plays its role by regulating β-catenin. When the Wnt/β-catenin signaling pathway is not activated, the axin complex in the cytoplasm is in a stable state, and β-catenin is maintained at a low level. Once the pathway is activated, β-catenin will break away from the degrading complex, composed of axin/APC/GSK-3β, which gathers and transfers to the nucleus where it combines with T-cell factor (TCF)/lymphoid enhancer factor and activates the transcription of the downstream target genes C-Myc, CyclinD1, slug, among others. Activation of the typical Wnt signaling pathway is closely related to the tumorigenesis and development of lung cancer.[13] One study showed that Wnt-1 was overexpressed in the tumor issues of patients with lung cancer.[14] By contrast, when the Wnt signaling pathway was downregulated, the apoptosis of lung cancer cells was promoted, the cell activity and invasion were reduced, tumor diffusion and transplanted tumor proliferation were inhibited, and phenotypic differentiation was induced while the expression of β-catenin in the cytoplasm and the transcriptional activity of TCF decreased.[15,16]

Our experiments revealed that the β-catenin overexpression plasmid could be transiently transfected in A549 cells and could promote cell proliferation, migration, and invasion. The
β-catenin overexpression plasmid significantly influenced the Wnt/β-catenin signaling pathway marker proteins by increasing the expression levels of β-catenin, C-Myc, and CyclinD1, and decreasing the levels of GSK-3β and P-GSK-3β. Our study also showed that the high expression of β-catenin was the initiator for activating the Wnt pathway. TFCB was expected to have the potential to suppress cell proliferation, migration, and invasion, especially in β-catenin overexpression cells. Indeed, we found that TFCB could suppress the Wnt/β-catenin signaling pathway by decreasing the levels of β-catenin, C-Myc, and CyclinD1 and increasing the levels of GSK-3β and P-GSK-3β. Based on these results, we have concluded that TFCB, as an antineoplastic herbal extract, has good potential to suppress the Wnt pathway by inhibiting the high expression of β-catenin and regulating downstream factors. In addition, a previous study showed that GSK-3β exhibited dual-directional regulation of tumors. Therefore, it will be important to further explore the role of GSK-3β in the Wnt/β-catenin pathway, and how it is affected by TFCB.

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Conflicts of interest

There are no conflicts of interest.

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