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Research Article

Effects of *Polygonatum sibiricum* Polysaccharides (PSP) on Human Esophageal Squamous Cell Carcinoma (ESCC) via NF-κB Signaling Pathway

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Objective. To explore the effects of different concentrations of *Polygonatum sibiricum* polysaccharides (PSP) on human esophageal squamous cell carcinoma (ESCC) cell line Eca109 and explore the new approach for the treatment of ESCC. *Methods*. Eca109 cells were divided into 5 groups, including one control group and 4 experimental groups where the concentrations of PSP used were 50, 100, 200, and 400 μg/mL. The proliferation rate of Eca109 cells in each group was measured with the CCK8 assay, and the apoptosis rate in each group was analyzed by flow cytometry; the in vitro scratch assay was used to determine the migration ability of Eca109 cells after PSP treatment; the expression levels of IL-1, IL-6, IL-10, TNF-α, and TGF-β were measured by RT-PCR, and the expression levels of TLR4 and proteins that are related to NF-κB signaling pathways were determined by Western blot. *Results*. PSP significantly inhibited the proliferation of Eca109 cells ($p < 0.05$) on a time- and dose-dependent manner; the apoptosis rates of Eca109 cells in experimental groups were significantly increased after 48 h of culture ($p < 0.05$); PSP significantly reduced the migration and invasion ability of Eca109 cells ($p < 0.05$); RT-PCR results showed that the expression of IL-10 in Eca109 cells increased significantly after treatment with PSP ($p < 0.05$), while the expression of IL-1, IL-6, TNF-α, and TGF-β decreased significantly ($p < 0.05$). Compared with the control group, the expression level of TLR4, NF-κB/p50, and NF-κB/p65 protein in each experimental group was significantly lower than that in the control group ($p < 0.05$). *Conclusions*. PSP significantly inhibited the proliferation, invasion, and migration of Eca109 cells and promoted cell apoptosis. These observed effects were probably due to the PSP's inhibition on the NF-κB signaling pathway in Eca109 cells via the regulation of the TLR4 expression.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors in the world, accounting for 80% of esophageal cancer (EC) in China [1]. ESCC is characterized by strong invasiveness, poor prognosis, and rapid clinical progression, is often accompanied by lymph node metastasis, and is also highly prone to recurrence. Although the diagnostic and therapeutic techniques have been greatly advanced, the pathological mechanism of ESCC is still poorly understood, and the current five-year survival rate of ESCC is only 20-30% [2]. Therefore, the exploration of the mechanism for the occurrence, invasion, and metastasis of ESCC has become one of the topical issues in the current research field.

The NF-κB signaling pathway is involved in the regulation of gene expression related to immune response, inflammatory response, and apoptosis and is an important signaling pathway affecting tumorigenesis and tumor development. It is also of great significance in cell stress response. The most common form of NF-κB is a heterodimer consisting of p50 and p65, where p50 is the site for DNA binding and p65 is involved in transcriptional regulation of genes and promotes p50 binding to DNA [3]. In quiescent cells, NF-κB typically binds to repressor IkBα and is present in the cytoplasm in an inactive form. This inactive form can be degraded by a

number of stimuli via phosphorylation of IkBα, allowing dissociated NF-κB to enter the nucleus and expose its nuclear recognition site, thereby promoting transcription of the target gene. Toll-like receptors (TLRs) are a class of transmembrane receptors expressed on the surface of immune cells, which are involved in innate immunity and indirect activation of acquired immunity [4]; it can also activate transcription factor NF-κB by recognizing invading pathogens and mediate the massive release of inflammatory cytokines and chemokines. Studies have shown that the TLR/NF-κB signaling pathway is involved in the development of various malignant tumors including prostate cancer, non-small cell carcinoma, and colorectal cancer and is also associated with tumor proliferation, apoptosis resistance, invasion, and metastasis [5–7]. At present, there are few reports on the connections between TLR/NF-κB and ESCC.

As a traditional Chinese medicinal material, *Polygonatum sibiricum* has been intensively researched in recent years. *Polygonatum sibiricum* polysaccharides (PSP) are the main active ingredient extracted from *Polygonatum*. Because of its low toxicity, it can be used for clinical practice in the long term. Studies have shown that PSP plays various important roles including antioxidation, antiaging, neuroprotection, immune regulation, and antitumor [8]. Especially in human breast cancer cells (MCF-27), colorectal cancer cells (HCT-8), and gastric cancer cells (HGC-27), PSP can significantly inhibit tumor growth and promote tumor cell apoptosis [9]. Han et al. found that PSP can inhibit the proliferation of cancer-associated fibroblasts (CAFs) in prostate cancer cells but had no effect on the proliferation of normal fibroblasts [10]. At present, the therapeutic effects of PSP on human esophageal squamous cell carcinoma have not been reported, and the associated mechanism is still unclear. Therefore, it is of clinical significance to study the effect of PSP on esophageal squamous cell carcinoma. In this study, we investigated the effects of PSP on the proliferation, apoptosis, migration, and TLR4/NF-κB signaling pathway-related proteins in human ESCC cell line Eca109 and explored the modes of action of PSP on ESCC, hoping to explore new approaches for the treatment of human ESCC.

2. Material and Methods

2.1. PSP Extraction and HPLC Analysis. The hot water leaching method was used to extract the PSP. The *Polygonatum sibiricum* pieces (purchased from Golden Leaf Pharmaceutical Co., Ltd., China) were mixed with water at the volume ratio of 1 : 8, which was then boiled for 2 h and repeated 3 times. After that, the aqueous extract was precipitated with 80% ethanol (Sinopharm Group, China) to remove the bioactive protein substance. PSP was obtained after precipitation and evaporation of ethanol. 0.25 g of PSP was then mixed with 0.1 L of 2 mol/L TFA (Sinopharm Group, China) solution before drying in a DHG-9070A oven (Yiheng Technology, China) at 100°C. 0.1 L of methanol (Sinopharm Group, China) was then added to remove TFA. After being repeated for 3 times, the resulting product was subjected to (1-phenyl-3-methyl-5-pyrazolone) PMP for derivatization. The product was then mixed with 0.1 L of 0.3 mol/L NaOH (Sinopharm Group, China), and together with 0.12 L of 0.5 mol/L methanol solution, the mixed solution was then incubated in a 70°C water bath for 30 min. After incubation, 0.1 L of 0.3 mol/L HCL (Sinopharm Group, China) was added and the solution was then extracted by an equal volume of chloroform to obtain an aqueous layer. And the aqueous layer was dried afterward. Finally, this extracted product was dissolved with 0.2 L of methanol and the filtrate was taken for the test. The mixed monosaccharide (consisting of mannose, glucose, galactose, glucuronic acid, galacturonic acid, and trehalose purchased from Sinopharm Group, China) solution was used as a reference for HPLC analysis.

2.2. Cell Culture and Treatment Grouping. The human ESCC cell line Eca109 (purchased from China Center for Type Culture Collection) was cultured in RPMI 1640 (Gibco, USA) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C with 5% CO_2. A blank control group and 4 PSP experimental groups with final concentrations of 50, 100, 200, and 400 μg/mL were set up in this study.

2.3. Cell Proliferation Measured with CCK8 Assay. Cell counting kit-8 (CCK8) (Dojindo, Japan) was used to determine the proliferation of human ESCC cells. Eca109 cells in the logarithmic growth phase were seeded in 96-well plates at a concentration of 1×10^4 cells/well. The culture was grouped as described above, and 4 replicate wells were set for each group. After being cultured for 0, 24, 48, and 72 h in an incubator at 37°C, with 5% CO_2, 10 μL of CCK8 working solution was added to each well, and incubation was continued at 37°C for another 2 h. The optical density (OD) was measured at 450 nm afterward.

2.4. Apoptosis Determined with Flow Cytometry. Annexin V-FITC (Sigma-Aldrich, USA) was used to measure the apoptosis of Eca109 cells with flow cytometry. After 48 h of culture, cell suspensions of the control group and the experimental group were collected and centrifuged at 1000 rpm for 5 min. Following that, the cells within the supernatant were washed with PBS buffer. Centrifuge again at 1000 rpm for 5 min, and a 500 μL of annexin binding buffer was added to the pellet and mixed by pipetting. Then, 5 μL of Annexin V-FITC reagent was added, and the solution was kept at room temperature for 10 min in the dark. 10 μL of PI reagent was added as well, and the solution was incubated at room temperature in the dark for another 5 min before the apoptosis rate of cells in each group was analyzed using a flow cytometer.

2.5. Cell Migration and Invasion Measured by In Vitro Scratch Assay. Eca109 cells in a logarithmic growth phase were taken to prepare a cell suspension with a density of 2×10^5 cell/mL. 2 mL of cell suspension was inoculated into a 6-well plate and cultured for 12 h. Then, a 200 μL micropipette tip was used to draw a straight line along the diameter of the tip's hole for the control group and experimental group, and the scratches were recorded under the microscope at 0 h and 48 h after scratching. PS software was used to calculate the relative ratio of cell scratching space between the

Table 1: The primers used in this study.

Gene	Primers
IL-1	F: 5′-CCACAGACCTTCCAGGAG-3′
	R: 5′-GCGTGCAGTTCAGTGATC-3′
IL-6	F: 5′-CCTCCAGAACAGATTTGAG-3′
	R: 5′-ATTTGTGGTTGGGTCAGG-3′
IL-10	F: 5′-AGTGTCTCGGAGGGATTC-3′
	R: 5′-TTCACCATGTTGACCAGG-3′
TNF-α	F: 5′-TCTGCCTGCTGCACTTTG-3′
	R: 5′-AACATGGGCTACAGGCTT-3′
TGF-β	F: 5′-AGAGCTGCGTCTGCTGAG-3′
	R: 5′-ACCACTGCCGCACAACTC-3′
β-Actin	F: 5′-GCAAATGCTTCTAGGCGGAC-3′
	R: 5′-GCTGTCACCTTCACCGTTCC-3′

control group and the experimental group. The experiment was repeated 3 times and averaged. The scratch recovery rate was calculated with the following formula: (width at 0 h − width at 48 h)/width at 0 h.

2.6. Cytokine Expression Detected with RT-PCR. The mRNA expression level of IL-1, IL-6, IL-10, TNF-α, and TGF-β was measured with RT-PCR, and the β-actin was used as the reference gene. Eca109 cells cultured for 48 h were collected, and the total RNA was extracted according to the instructions of TRIzol (Invitrogen, USA). The concentration of RNA was quantified based on the O.D. value and subsequently subjected to reverse transcription with the help of the PrimeScipt™ RT Master (Takara, Japan) kit. The resulting cDNA was stored at -20°C. RT-PCR experiments were then performed using cDNA as a template according to the instructions of the SYBR Green Realtime PCR Master Mix (Toyobo, Japan). $2^{-\Delta\Delta ct}$ was used for quantitative analysis. The primers used in this are shown in Table 1.

2.7. Expression of TLR4 and Proteins Involved in the NF-κB Signaling Pathway Determined with Western Blot. Total protein was extracted from the control group and each experimental group after 48 h of culture. The protein concentration was determined by BCA kit (Beyotime, China). After protein quantification, an appropriate amount of protein was taken for electrophoresis and to ensure that the total amount of protein added to each well is approximately the same. Protein was separated by 12% polyacrylamide gel electrophoresis and then transferred to the PVDF membrane. 5% BSA was then used for blocking for 1 h, followed by addition of primary antibodies (rabbit anti-human TLR4 polyclonal antibody (Boster, China 1 : 50), rabbit anti-human NF-κB/p50 monoclonal antibody (Santa Cruz, USA 1 : 100), NF-κB/p65 polyclonal antibody (Santa Cruz, USA 1 : 100), and β-actin, after which it was then incubated overnight at 4°C. The next day, the second antibody was added and incubated for 2 h at room temperature. The PVDF membrane was then washed with TBST 3 times, and Image Lab 3.0 was used for imaging. ImageJ software was used to quantitatively analyze the differential expression of proteins in each group, and β-actin was used as an internal reference for the relative quantification.

2.7.1. Statistical Analysis. The data were collated and analyzed using Origin 8.0. The results for each group were expressed as mean ± SEM, and one-way ANOVA was used for comparison between groups. $p < 0.05$ represents a statistical difference.

3. Results

3.1. PSP Extraction Analysis by HPLC. In order to detect the monosaccharide component in the PSP extract, the PSP extract (test sample) and the mixed monosaccharide solution (control) were separately subjected to HPLC separation. As shown in Figure 1, monosaccharide mixture control was well separated, with each monosaccharide showing a single peak, and there were 4 single peaks in the test sample. Compared with the control curve, the monosaccharides with higher content in the PSP extract were mannose, glucose, galactose, and trehalose, among which the glucose is the major component of PSP.

3.2. Effect of PSP on the Proliferation of Human ESCC. PSP of 50, 100, 200, and 400 μg/mL were added to Eca109 cells and cultured for 48 h. CCK8 was then used to measure the effects of different concentrations of PSP on the proliferation of human ESCC. As shown in Figure 2(a), after 48 h of culture, compared with the control group, the cell survival rate of Eca109 in each experimental group decreased significantly, and the proliferation ability of Eca109 cells was significantly inhibited ($p < 0.05$). The half-inhibitory concentration (IC50) of PSP against Eca109 cells was 262 μg/mL. The cell viability of Eca109 after 0, 24, 48, and 72 h of PSP culture at 200 μg/mL was further explored as well. The results showed that the viability of Eca109 cells decreased significantly with time (Figure 2(b)), and the difference was statistically significant ($p < 0.05$). Thus, these results indicated that PSP significantly inhibited the proliferation of Eca109 cells in a dose- and time-dependent manner.

3.3. Effect of PSP on Apoptosis of Human ESCC. Flow cytometry was used to detect the apoptosis of Eca109 cells in the control group and the experimental group 48 h after culture. The results showed that the apoptosis rates of Eca109 cells cultured at 50, 100, 200, and 400 μg/mL PSP were 23.07%, 33.09%, 41.20%, 63.15%, and 69.18%, respectively (Figure 3). Compared with the control group, the apoptosis rate of Eca109 was significantly higher in the experimental group, and the difference was statistically significant ($p < 0.05$), which indicates the apoptosis-promoting effect of PSP on Eca109 cells.

3.4. Effect of PSP on Migration and Invasion of Human ESCC. After 48 h of Eca109 cells cultured with different concentrations of PSP, the cell migration and invasion ability was

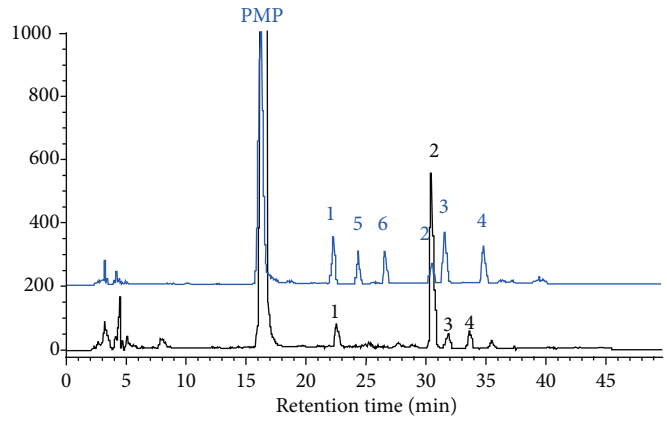


Figure 1: PSP component analysis by HPLC. 1: mannose; 2: glucose; 3: galactose; 4: trehalose; 5: glucuronic acid; 6: galacturonic acid.

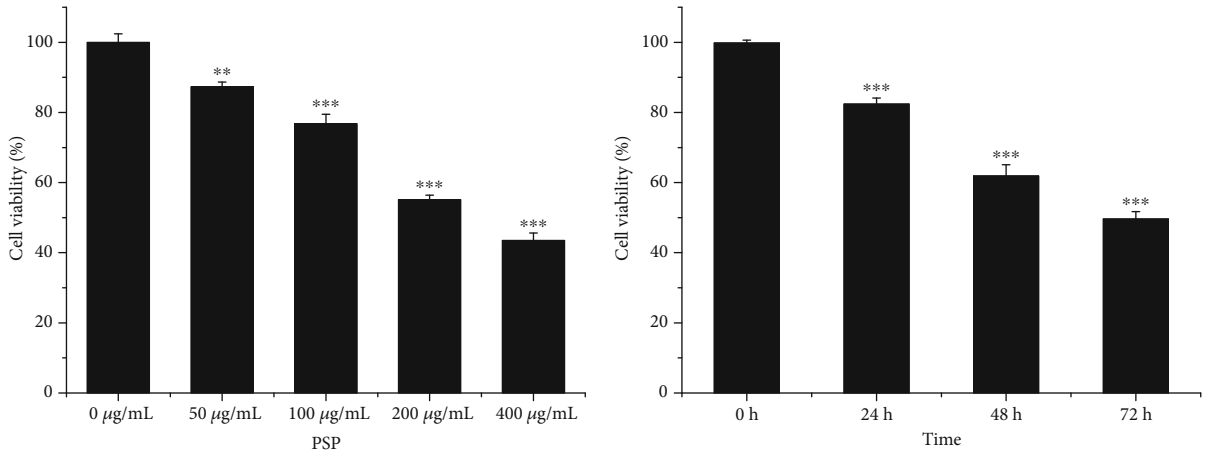


Figure 2: The proliferation of esophageal squamous cell carcinoma Eca109 cells was inhibited by PSP. (a) The toxicity of different concentrations of PSP on Eca109 cells (culture for 48 h) measured with CCK8, compared with the control group, $^{**}p < 0.01$; $^{***}p < 0.001$. (b) The toxicity of PSP on Eca109 cells after different durations (200 μg/mL PSP) measured with CCK8, compared with the control group; $^{***}p < 0.001$. The proliferation of Eca109 cells in each group measured by CCK8 assay. Compared with the control group: $^{**}p < 0.01$ and $^{***}p < 0.001$.

detected by in vitro scratch assay. The results showed that the cell scratch recovery rate of the experimental group was significantly lower than that of the control group (Figure 4, $p < 0.05$). Specifically, the recovery rates of the control group and the experimental group were 80.37%, 76.63%, 68.77%, 50.47%, and 43.63%, respectively, which indicated that PSP decreased the ability of Eca109 cells to migrate and invade.

3.5. Effect of PSP on Cytokine in Human ESCC. Furthermore, the expression of various cytokines (IL-1, IL-6, IL-10, TNF-α, and TGF-β) affected by PSP in Eca109 cells was measured by RT-PCR. As shown in Figure 5, the mRNA expression levels of IL-I and IL-6 in Eca109 cells were significantly downregulated compared with those in the control group after 48 h of culture with PSP at 200 μg/mL ($p < 0.05$). This decreasing trend was heightened with the increasing concentration of PSP. However, as the concentration of PSP increased, the expression level of IL-10 was significantly upregulated ($p < 0.05$) while there was a significant decrease in the expression level of TNF-α and TGF-β ($p < 0.05$).

3.6. Effect of PSP on the Expression of TLR4 and Proteins Involved in the NF-κB Signaling Pathway in Human ESCC. The expression of TLR4 and proteins involved in the NF-κB signaling pathway in Eca109 cells was determined after 48 h of cell culture (Figure 6). Compared with the control group, the expression of TLR4 showed a significant decrease with the increase of PSP concentration, and the difference was statistically significant (Figure 6(b), $p < 0.05$); the expression levels of proteins related to the NF-κB signaling pathway (NF-κB/p50 and NF-κB/p65) were also significantly downregulated (Figure 6(c), $p < 0.05$). This result indicated that in Eca109 cells, PSP may decrease the expression of NF-κB signaling pathway-related proteins by inhibiting the TLR4 protein expression.

4. Discussion

In China, the majority of esophageal cancers are squamous cell carcinoma and the incidence of ESCC ranks fifth in all malignant tumor types [11]. At present, the research on

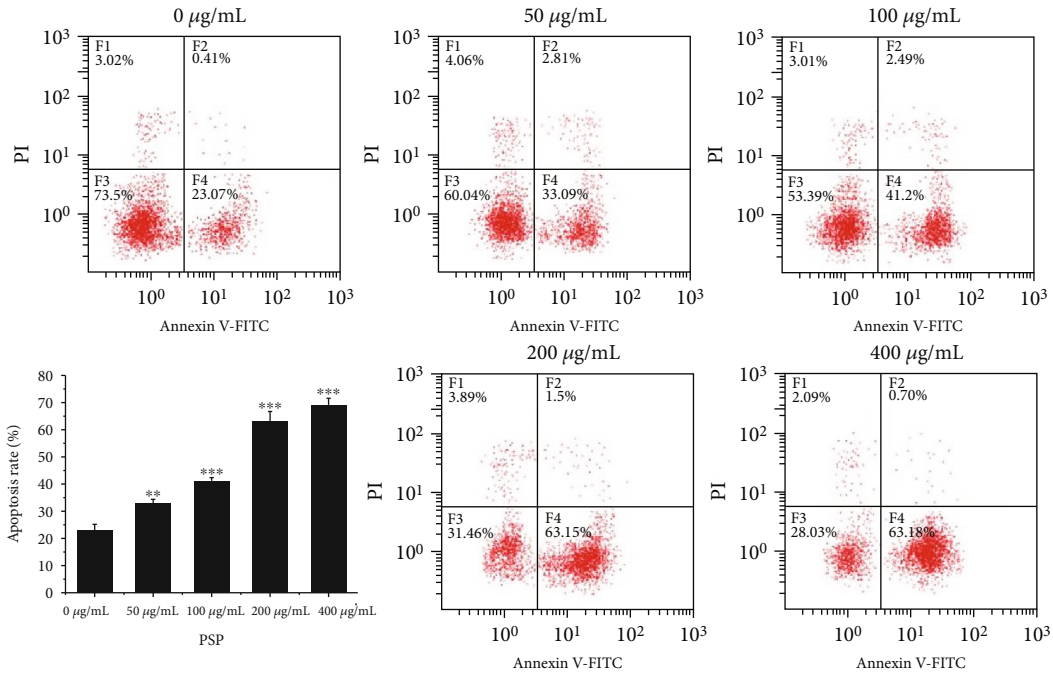


Figure 3: Flow cytometry used to detect apoptosis of Eca109 cells. Compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

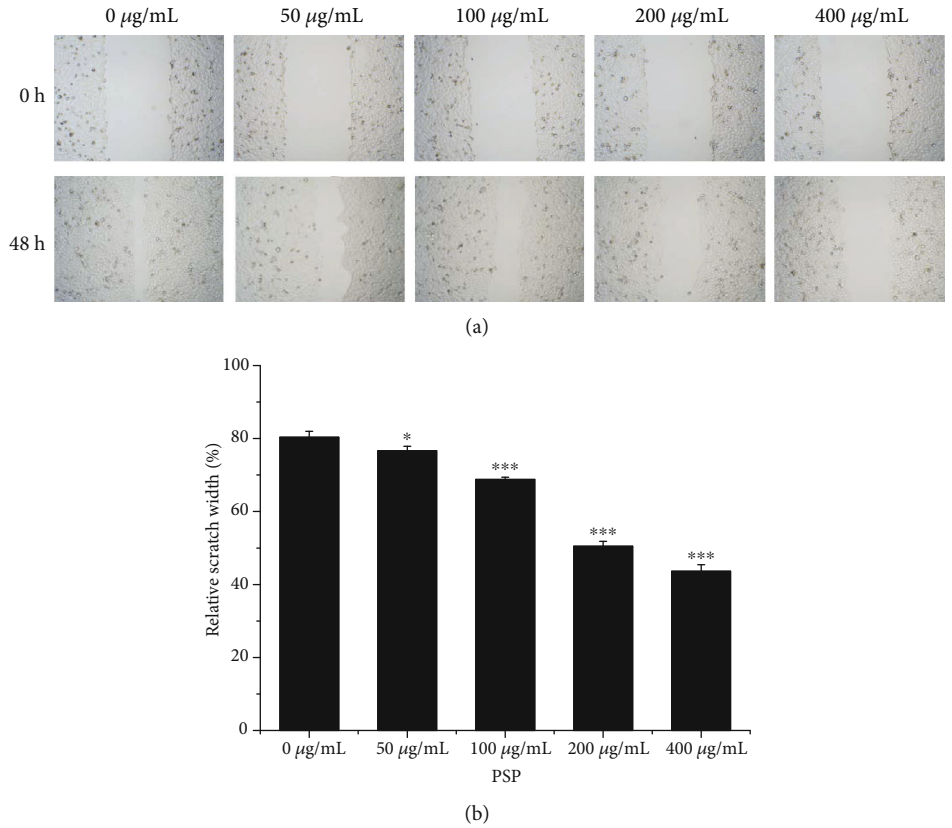


Figure 4: PSP inhibited migration and invasion of Eca109 cells. (a) The migration and invasion of Eca109 cells in each group determined by in vitro scratch assay. (b) Quantitative results of (a), compared with the control group, $^{*}p < 0.05$ and $^{***}p < 0.001$.

ESCC mainly focuses on the excessive activation of key signaling pathways involved in disease occurrence and the abnormal expression of some proteins and cellular inflammatory factors. Although the survival of ESCC patients has been effectively prolonged with the progress of various treatments such as early diagnosis, surgery, and chemotherapy

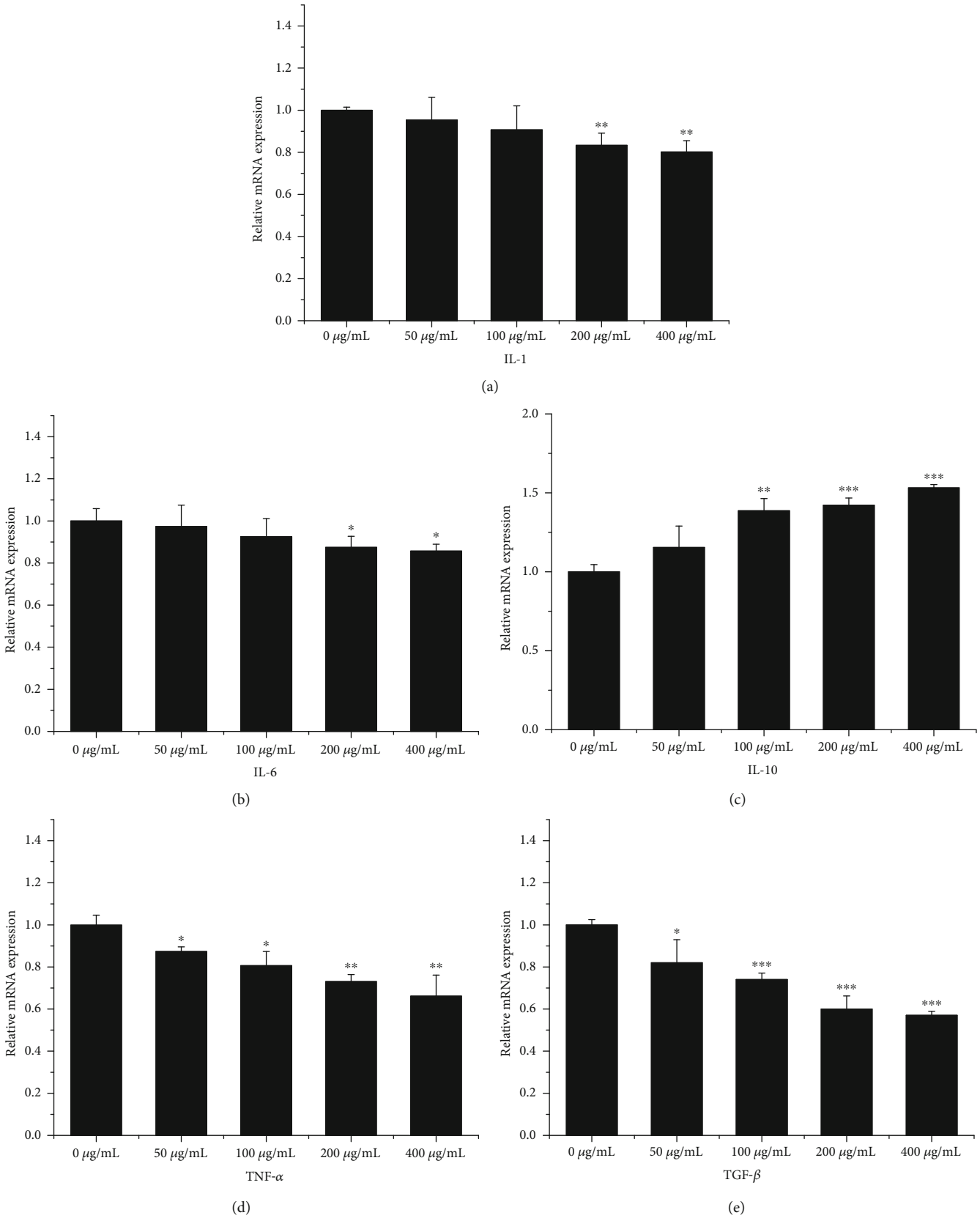


Figure 5: RT-PCR was used to detect the expression of cytokines in Eca109 cells: (a) IL-1; (b) IL-6; (c) IL-10; (d) TNF-α; (e) TGF-β. Compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

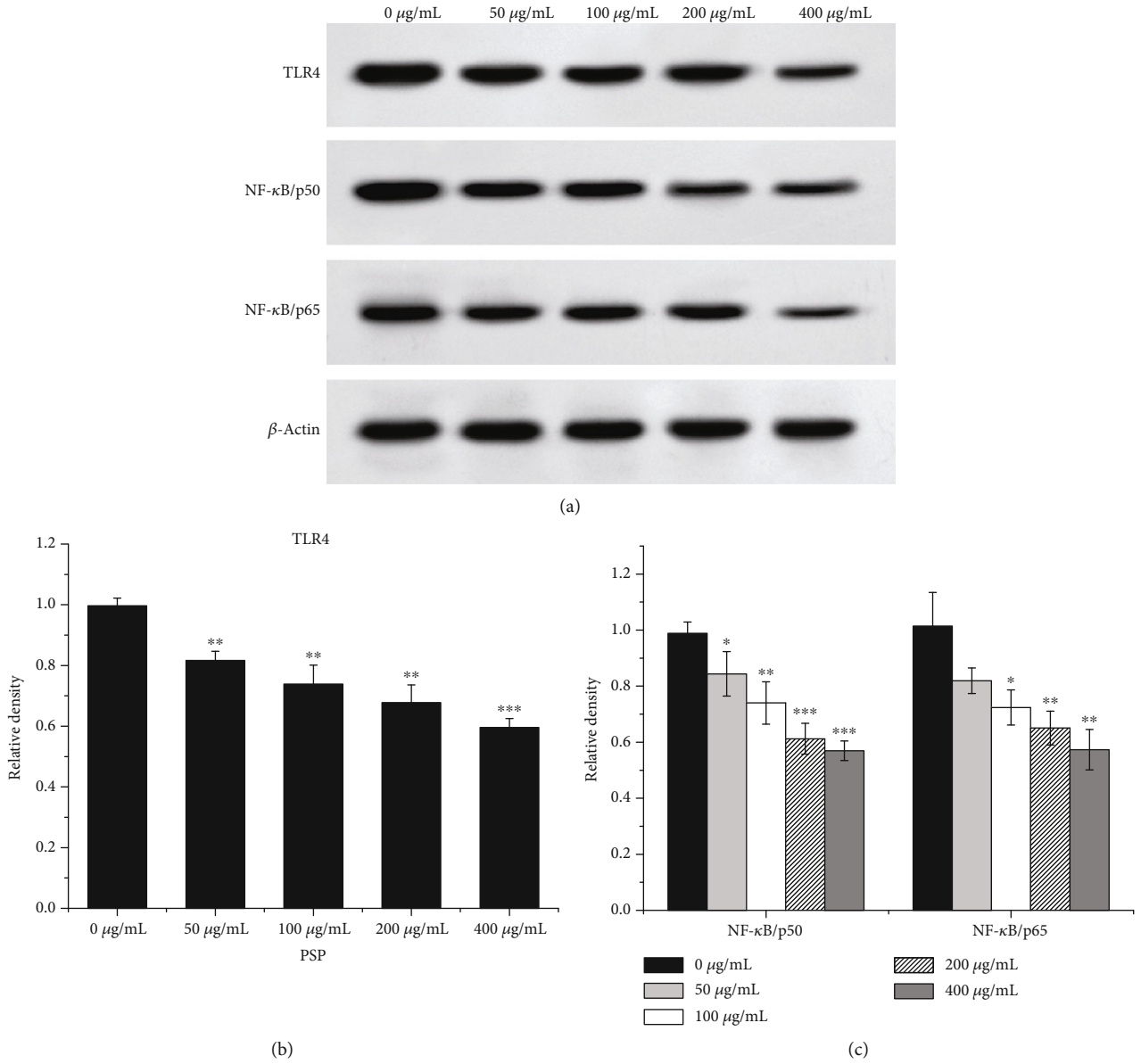


Figure 6: Expression of TLR4/NF-κB signaling pathway protein in Eca109 cells. (a) Western blot analysis of TLR4, NF-κB/p50, and NF-κB/p65 protein expression in Eca109 cells. (b) Quantitative results of (a), compared with the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

drugs, the efficacy of ESCC treatment is still not ideal due to the characteristics of ESCC being easy to relapse and easy to metastasize. Therefore, investigating the pathogenesis, invasion, and metastasis of ESCC and developing more effective therapeutic drugs are of great significance for the treatment of ESCC.

Studies have found the abnormal activation of NF-κB in a variety of malignant tumors, which suggests a certain correlation with tumor proliferation, invasion, and migration [12]. For example, Sovak et al. have reported that NF-κB was in a state of constant activation in ER-negative breast cancer cells and it was associated with poorly differentiated, highly metastatic cancer cells [13]. In this study, we showed that PSP significantly inhibited the proliferation of Eca109 cells and enhanced the apoptosis of Eca109 cells in vitro; in addition, PSP also showed inhibitory effects on the migration and invasion of Eca109 cells. PSP is a class of polysaccharide that is extracted from a traditional Chinese medicine *Polygonatum sibiricum*. In recent years, research has suggested that PSP has certain antitumor and immune-enhancing functions. In this study, we found that the proliferation and migration ability of Eca109 cells were decreased significantly with the increase of PSP concentration and the inhibitory effect of PSP on the proliferation of human ESCC was dose- and time-dependent. Furthermore, we found that PSP significantly increased the apoptosis rate of human ESCC and inhibited the migration ability of ESCC in this study. These results indicated that PSP has a certain antitumor effect on ESCC in vitro. After cultured with various concentrations of PSP, on the one hand, the expression levels of IL-1 and

IL-6 in Eca109 cells showed a decreasing trend, and when the concentration of PSP was 200 μg/mL, the expression levels of IL-1 and IL-6 were significantly reduced. IL-1 is a proinflammatory cytokine whose biological properties are mainly involved in mediating inflammatory responses and regulating immune responses. In the cancer cell development, IL-1 not only provides cancer cells with inflammatory signals, stimulates the production of other proinflammatory cytokines, and mediates changes in the tumor microenvironment, but also directly participates in cancer-related signaling pathways and promotes the occurrence and development of cancer [14]. In fact, one study has found that the level of IL-6 in the serum of patients with gastric cancer is significantly higher than that of the normal control group [15]. To actively play the biological role, IL-6 needs to bind to its specific receptor IL-6R, which subsequently induces proliferation and differentiation of immune cells, regulates immunity, and promotes differentiation and maturation of hematopoietic stem cells. These functions are realized through a complex and delicate regulation within the body. Thus, when the timely and correct regulation of differentiation, proliferation, apoptosis, etc. is out of order, they may cause and promote tumors. On the other hand, the expression of TNF-α and TGF-β in Eca109 cells decreased significantly, whereas the expression of IL-10 significantly increased. TNF-α is one of the key cytokines involved in inflammatory and immune responses, which accelerates tumorigenesis by promoting tumor proliferation and metastasis [16] whereas TGF-β can promote tumorigenesis by inhibiting T cell immune response [17]. As for IL-10, it is a multifunctional cytokine. In melanoma, researchers have found that IL-10 has certain antitumor functions [18]. Not surprisingly, our research has some similarities with those reported in the literature. The effects of PSP on cytokines such as IL-1, IL-6, TNF-α, TGF-β, and IL-10 indicate that in human ESCC, PSP probably exerted a certain immunomodulatory effect and inhibited the development of tumor cells, which is consistent with the effect of PSP in other tumor cells. Our results showed that PSP specifically downregulated the expression of TLR4 protein, thereby inhibiting the expression of NF-κB/p50 and NF-κB/p65 proteins in the NF-κB signaling pathway. Inhibition of p50 and p65 protein expression indicates that PSP may inhibit the NF-κB signaling pathway by regulating the TLR4 protein, thereby exerting immunoregulatory and anticancer effects, and hindering the proliferation, occurrence, and development of ESCC cells. PSP has shown broad promise as an antitumor drug, which also provides perspectives for further research on ESCC.

Data Availability

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

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Weizheng Zhou and Jiang Hong contributed equally to this study.

Acknowledgments

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