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Research Article

Effects of Laminaria Japonica Polysaccharides on the Survival of Non-Small-Cell Lung Cancer A549 Cells

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Objective. To investigate the effect of Laminaria japonica polysaccharides (LJP) on the survival of non-small-cell lung cancer (NSCLC) A549 cells and its mechanism. *Methods*. In vitro: the cells were randomly divided into control group, LJP (5 mg/ml) group, LJP (10 mg/ml) group, and LJP (20 mg/ml) group. After corresponding treatment, the survival rate and the expression of proteins related to proliferation, apoptosis, epithelial-mesenchymal transition (EMT), and signaling pathway were detected by CCK8 assay and Western blot, respectively. In vivo: a xenograft model was established to detect the tumor volume and mass and the expression of the above pathway proteins. *Results*. Compared with the control group, LJP decreased the survival rate of A549 cells ($P < 0.05$), inhibited the protein expression of Ki67 and PCNA ($P < 0.05$), downregulated the expression of Bcl-2 while upregulated the expression of Bax, cl-caspase-3, and cl-caspase-9 ($P < 0.05$), upregulated the expression of E-cadherin, downregulated the expression of vascular endothelial growth factor (VEGF) and N-cadherin ($P < 0.05$), and downregulated β-catenin, transcription factor-4 (TCF4), and c-Myc protein expression levels ($P < 0.05$). In vivo: LJP decreased the volume and mass of the xenograft tumors and downregulated β-catenin, TCF4, and c-Myc protein expression levels compared with the control group ($P < 0.05$). *Conclusion*. LJP can inhibit the survival of non-small-cell lung cancer A549 cells in vitro, and its mechanism is related to the inhibition of activation of β-catenin/TCF4 pathway activation.

1. Introduction

Lung cancer ranks among the leading types of malignant tumors in the world, whose incidence rate ranks the first in male malignant tumors and the second in female in China with its increasing trend year by year. Among them, non-small-cell lung cancer (NSCLC) accounts for about 80% of the total number of lung cancers. Although progression has been made in new technologies for prevention, screening, diagnosis, and treatment, the 5-year survival rate is still very low, imposing a heavy burden on patients and society [1, 2]. At present, the treatment methods for lung cancer are relatively limited, mainly including surgery, radiotherapy, chemotherapy, and targeted drug therapy with poor prognosis commonly seen in patients with lung cancer [3]. Therefore, it is vital to strengthen the research on this disease in medical and medicine field. In recent years, many natural extracts have been widely used in the adjuvant treatment of cancers. Laminaria japonica polysaccharides (LJP) isolated from Laminaria is a general term for polysaccharides including laminarin, seaweed gel, alginic acid, and fustian. Laminaria japonica polysaccharides show an average degree of polymerization between 26 and 31 with an average chain length between 7 and 10 and 2 to 3 branches to each chain. Figure 1 shows repeated structural units of LJP. Studies have found that Laminaria japonica polysaccharides have a variety of biological activities, including promoting fertility [4], alleviating metabolic syndrome [5], preventing vascular calcification [6], resisting virus [7, 8], immunoregulating [9], and antioxidating [10]. In addition, Laminaria japonica polysaccharides have inhibitory effect on a variety of tumor cells, such as liver cancer cells, nasopharyngeal carcinoma cells, cervical cancer cells, and leukemia cells [11–14], but there are no reports on the effect of Laminaria japonica polysaccharides on human non-small-cell lung cancer A549 cells. In this study, we investigated the effects of Laminaria japonica

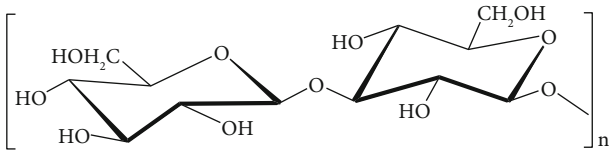

Figure 1: Repeated structural units of LJP.

polysaccharides on the survival of A549 cells in vitro and in vivo and its possible mechanism.

2. Materials and Methods

2.1. Reagents and Instruments. DMEM cell culture solution, 0.25% pancreatin, and fetal bovine serum were all purchased from Gibco Company of the United States. Streptomycin sulfate for injection was purchased from Huabei Pharmaceutical Company. Penicillin sodium for injection was purchased from Shanghai New Pioneer Pharmaceutical Company. Both primary and secondary antibodies were purchased from British Abcam Company. RIPA lysate was purchased from Sigma Company of the United States. BCA kit was purchased from Bi Yun Tian Biotechnology Company.

Carbon dioxide incubator was purchased from Thermo-Forma Company of the United States. Clean bench was purchased from Suzhou Zhongya Purification Equipment Co., Ltd. Electrophoresis apparatus and semidry film transfer apparatus were both purchased from Bio-Rad Laboratories, Inc. of the United States. Gel View 6000 chemiluminescent gel imager was purchased from Guangzhou Yunxing Instrument Co., Ltd. Optical microscope was purchased from Olympus Corporation of Japan. Multifunctional microplate reader was purchased from Bio-Rad Laboratories, Inc. of the United States.

2.2. Cell Culture. Human non-small-cell lung cancer cell line A549 was purchased from FuHeng Cell Center, Shanghai, China. The cells were cultured in a 5% CO_2 incubator at 37°C with DMEM medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. The cell growth was observed daily, and subculture could be carried out when the fusion rate reached 80%. The logarithmic growth phase cells were taken for subsequent experiments.

2.3. Experimental Methods

2.3.1. Preparation of Laminaria Japonica Polysaccharides and Molecular Weight Distribution Analysis. The Laminaria japonica polysaccharides were extracted by acid process. The Laminaria japonica dry powder was mixed with hydrochloric acid solution at 1 : 20 (W/V), and the mixed solution was heated at 75°C for 4 hours. After the waste residue was filtered, the filtrate was centrifuged. The precipitate was then dried to obtain Laminaria japonica polysaccharides after acetone washing, TCA precipitating, dialyzing, and centrifuging. Laminaria japonica polysaccharides with compete composition were obtained for subsequent experimental research after the filtrate was precipitated and extracted three times. The molecular weight distribution of Laminaria japonica polysaccharide was detected by high-performance liquid chromatography (HPLC). The HPLC calibration curve was prepared by using the standard dextran. TSK-GEL G4000PW columns and a refractive index detector (RID) were used for detection (mobile phase: double distilled water, injection volume 20 μl, flow rate 0.15 ml/min at column temperature of 70°C).

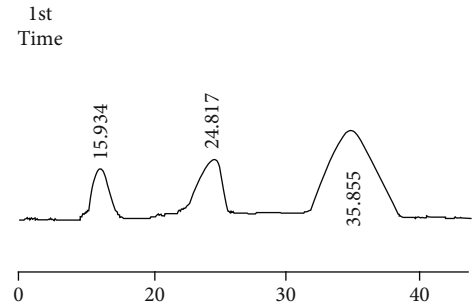

Figure 2: HPLC graph of the LJP.

2.3.2. Cell Proliferation Detected by CCK 8 Test. Human non-small-cell lung cancer cell line A549 and NCI-H292 cells were seeded into a 96-well plate at 100 μl per well, respectively. After attachment, the cells were randomly divided into 4 groups: the control group, the LJP (5 mg/ml) group, the LJP (10 mg/ml) group, and the LJP (20 mg/ml) group, each group having 5 duplicates and receiving corresponding treatment. After 24 h of culture, 10 μl of CCK-8 solution was added to each well, and incubation was continued for 4 h. The OD value of A549 cells and NCI-H292 cells was detected at 450 nm absorbance by a microplate reader.

2.3.3. Cell Migration Measured by Transwell Assay. The A549 cells were seeded into a 6-well plate with 1×10^6 cells/well. The cells, at 80% confluence, were then grouped in the same way as in Section 2.3.2 and the corresponding final concentration of the solutions was added for treatment for 24 h. The cells in each group were collected, adjusted to a density of 2×10^5 cells, seeded on the upper chamber of a Matrigel-coated Transwell, and cultured in fetal bovine serum-free medium. While normal cell culture medium was added in the lower chamber for another 48 h of incubation. After incubation, the cells underwent crystal violet staining, and five fields of view under the microscope were used to count the stained cells.

2.3.4. Establishment of Transplanted Tumor Models. Five-week-old SCID nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and the animal quality certificate number was SCXK (Beijing) 2014-0001. Mice were kept in sterile environment at temperature (26-28°C) and humidity (50%-60%) and with free access to food and water. After a week of acclimation, the mice were used for experiments.

The cells were adjusted to a density of 2×10^7/ml, and 0.2 ml of the cell suspension was administered subcutaneously to the mice at the same position in the dorsum of their necks. The next day, the mice were randomly divided into 4 groups: control group, LJP (5 mg/kg) group, LJP (10 mg/kg)

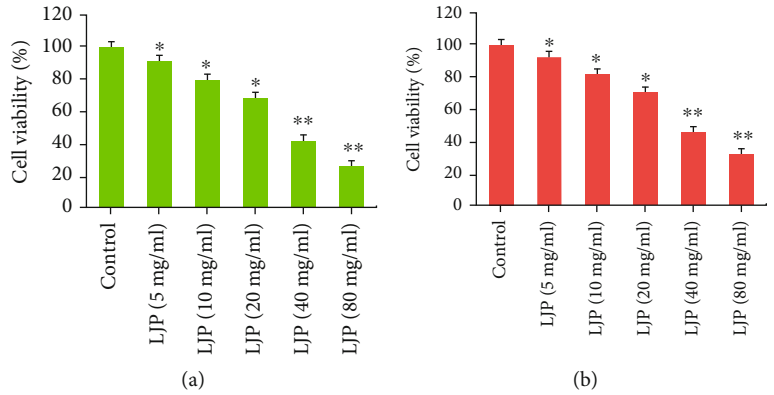

Figure 3: Proliferation of A549 and NCI-H292 cells. $^*P < 0.05$ versus control group. CCK8 assay was used to detect cell viability of A549 (a) and NCI-H292 (b) after treated with different concentrations of LJP for 24 h.

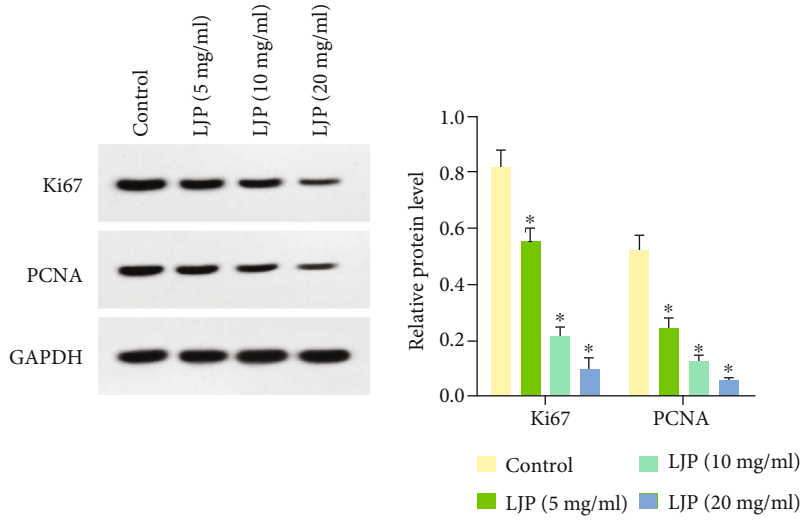

Figure 4: Expressions of Ki67 and PCNA. $^*P < 0.05$ versus control group. Western blot assay was used to detect expressions of Ki67 and PCNA after treated with different concentrations of LJP for 24 h.

group, and LJP (20 mg/kg) group. Each group was intraperitoneally injected with PBS and 5, 10, and 20 mg/kg LJP drugs, respectively, once a day for 1 week. Tumor volume was measured every 5 days. The mice were sacrificed on the 30th day, and the tumor tissue weight was weighed. Tumor volume: $V = \text{length} \times \text{width}^2/2$.

2.3.5. Detection of Expressions of Proliferation, Apoptosis, EMT, and Signaling Pathway-Related Proteins in Lung Cancer A549 Cells Detected by Western Blot. A549 cells were seeded in a 96-well plate. After attachment, the cells were randomly divided into four groups: control group, LJP (5 mg/ml) group, LJP (10 mg/ml) group, and LJP (20 mg/ml) group. Each group had five duplicates and were treated with the corresponding final concentration of solutions for 24 h. The cells washed with PBS were collected after trypsin digestion. The cells of each group or incubated tumor tissues as mentioned in Section 2.3.3 were collected, and the total proteins of each group were extracted using RIPA protein lysate on ice. Then, the protein concentrations of each group were measured by BCA kit and adjusted. Equal amounts of proteins of each group were separated by 12% SDS-PAGE and were then transferred to PVDF membranes. The membranes were blocked with 5% skim milk at room temperature for 2 h. After the blocking process, the membranes were incubated at 4°C overnight with primary antibodies of Ki67, PCNA, Bax, Bcl-2, cl-caspase-3, cl-caspase-9, VEGF, E-cadherin, N-cadherin, β-catenin, TCF-4, and c-Myc. The primary antibodies were discarded the next day. After being washed with buffer solution, the membranes were added with the corresponding second antibodies, blocked at room temperature for 1 h, and then added with ECL dropwise for exposing and developing in the darkroom. GAPDH was used as an internal reference.

2.4. Statistical Methods. All experimental data were statistically analyzed by statistical software SPSS 20.0. The experimental results were expressed as mean ± standard deviation. Between-group differences conformed to normal distribution were tested by one-way ANOVA. The difference was considered statistically significant when $P < 0.05$.

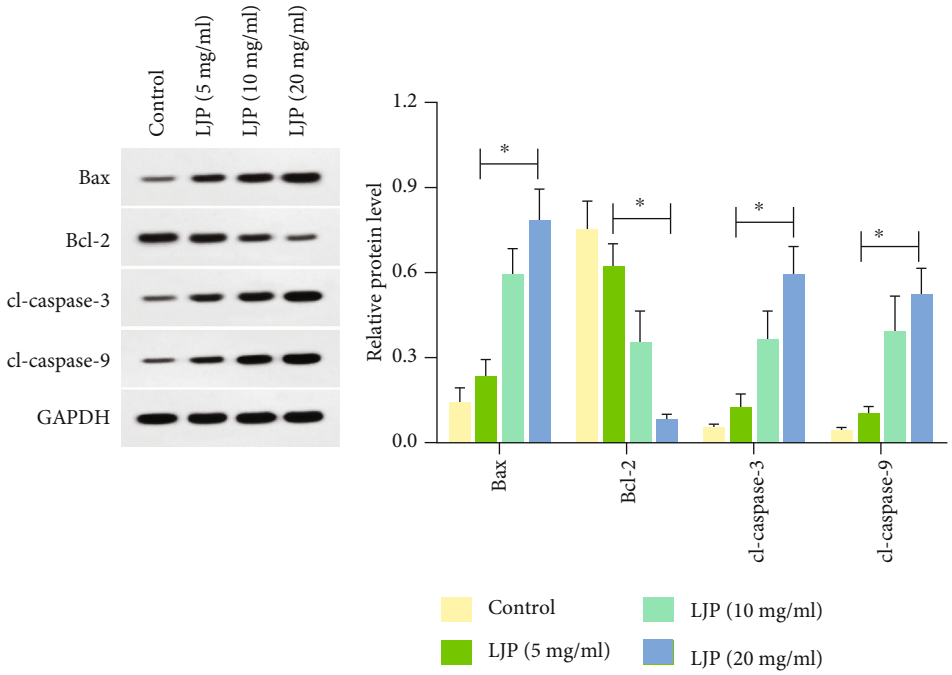

Figure 5: Expression of apoptosis-related proteins. $^*P < 0.05$ versus control group. Western blot assay was used to detect expression of apoptosis-related proteins after treated with different concentrations of LJP for 24 h.

3. Results

3.1. Molecular Weight Distribution of LJP Measured by HPLC. Standard dextran (T-2000, T-500, T-70, T-40, T-10) was prepared for a mixture. A calibration curve ($y = -0.1789x + 9.3810$, $R^2 = 0.994$) was developed based on HPLC detection of elution time (x, min) and the corresponding logarithm of molecular weight (y, lg (MW)). Laminaria japonica polysaccharides extracted by acid process were detected by HPLC to obtain the LJP profile (see Figure 2), which showed three major peaks at 15.934 min, 24.817 min, and 35.855 min, respectively. Calculation by standard curve showed the LJP molecular weight of 3391621, 87345, and 926.

3.2. Effect of Laminaria Japonica Polysaccharides on the Survival Rate of Lung Cancer Cells. The survival rate of lung cancer cells was detected by a CCK 8 test. As shown in Figure 3, with the increase of LJP administration concentration, the survival rates of A549 and NCI-H292 cells were significantly lower in the LJP treatment group compared with the control group ($P < 0.05$, Figures 3(a) and 3(b)), and the 50% inhibitory concentration (IC50) of LJP on A549 and NCI-H292 cells was 33.21 mg/ml and 39.02 mg/ml, respectively. Therefore, 5, 10, and 20 mg/ml were selected for treatment in subsequent experiments.

3.3. Effect of Laminaria Japonica Polysaccharides on the Expressions of Proliferation-Related Proteins in A549 Cells. The expressions of proliferation-related proteins were detected by Western blot. As shown in Figure 4, the expression levels of Ki67 and PCNA proteins in the LJP treatment groups were significantly downregulated with the increase of LJP concentrations as compared with the control group ($P < 0.05$).

3.4. Effect of Laminaria Japonica Polysaccharides on the Expressions of Apoptosis-Related Proteins in A549 Cells. The expressions of apoptosis-related proteins were detected by Western blot. As shown in Figure 5, the expression levels of Bax, cl-caspase-3, and cl-caspase-9 proteins in the LJP treatment groups were significantly upregulated with the increase of LJP concentrations as compared with the control group ($P < 0.05$, Figure 5), while the expression of Bcl-2 protein was significantly downregulated.

3.5. Effect of Laminaria Japonica Polysaccharides on Invasion and Migration of A549 Cells and on Expressions of EMT-Related Proteins in A549 Cells. The invasion and migration of A549 cells were detected by Transwell assay, and the expressions of EMT-related proteins in A549 cells were detected by Western blot. As shown in Figure 6, the number of invasive A549 cells was significantly reduced ($P < 0.05$, Figure 6(a)) and the expression levels of VEGF and N-cadherin proteins were significantly downregulated ($P < 0.05$, Figure 6(b)) in the LJP treatment groups as compared with the control group, while the expression of E-cadherin protein was significantly upregulated ($P < 0.05$, Figure 6(b)), which was also associated with LJP concentrations.

3.6. Effect of Laminaria Japonica Polysaccharides on Expression of Signaling Pathway Proteins in A549 Cells. The expression of signaling pathway proteins in A549 cells cultured in vitro was detected by Western blot, and the results shown in Figure 7 indicated that the expressions of β-catenin, TCF4, and c-Myc proteins were significantly downregulated

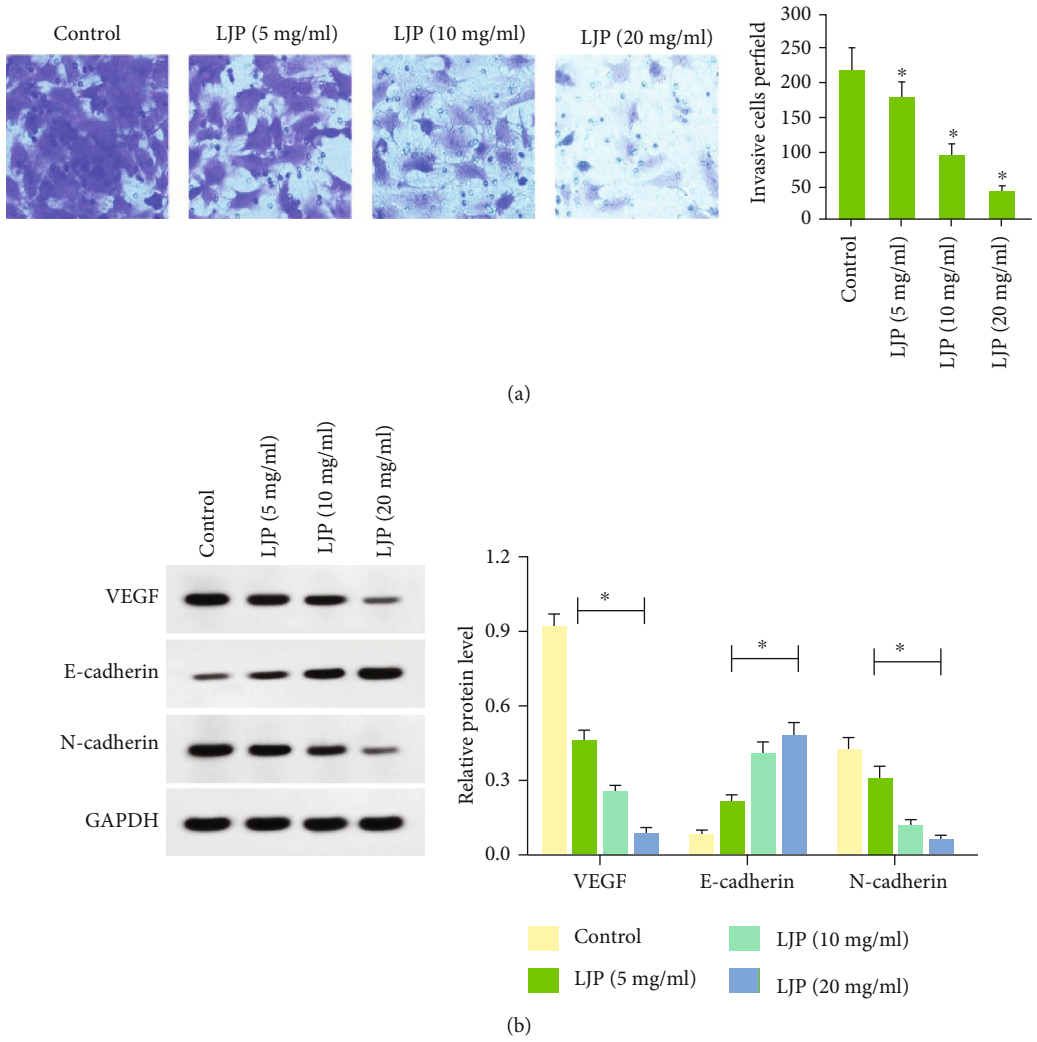

Figure 6: Expressions of EMT-related proteins. $^*P < 0.05$ versus control group. Transwell assay was used to detect invasive ability of A549 cells (a) and Western blot was used to detect expressions of EMT-related proteins (b) after treated with different concentrations of LJP for 24 h.

with increasing concentrations of LJP administration in the LJP treatment groups as compared with the control group ($P < 0.05$, Figure 7).

3.7. Effect of Laminaria Japonica Polysaccharides on the Growth and Expression of Signaling Pathway Proteins in Transplanted Tumors. A xenograft model of small-cell lung cancer was established, and the tumor volumes were measured every 5 days. As shown in Figure 8(a), the results showed that the volumes of mice in the LJP group (5 mg/kg), LJP group (10 mg/kg), and LJP group (20 mg/kg) were all significantly decreased ($P < 0.05$) as compared with the control group. The tumor weights were measured at day 30, and the results shown in Figure 8(b) indicated that the tumor weights in the LJP dose groups were significantly lower than those in the control group ($P < 0.05$). The expressions of signaling pathway proteins in transplanted tumors were detected by Western blot, and the results shown in Figure 8(c) suggested that the expressions of β-catenin, TCF4, and c-Myc proteins in each LJP dose group were significantly decreased ($P < 0.05$) as compared with the control group.

4. Discussion

Non-small-cell lung cancer is a common lung cancer. In recent years, investigations have found that its incidence rate is on the rise, and the main causes leading to death are distant metastasis and local infiltration. The occurrence of non-small-cell lung cancer is extremely complicated, involving many factors such as molecules, cytokines, and genes. Signaling pathway is now an important field of research. Recent years have witnessed increasing attention of many plants due to their wide range of biological activities. Polysaccharides from Laminaria japonica have also been shown to possess a variety of biological functions, including antioxidation, antivirus, and tumor growth inhibition [4–14]. Therefore, we studied the human non-small-cell lung cancer A549 cells and discussed the effects of Laminaria japonica polysaccharides on its growth in vivo and vitro and its possible mechanism.

In this study, we found that 5, 10, and 20 mg/ml of Laminaria japonica polysaccharides could effectively reduce the survival rate of human non-small-cell lung cancer A549 cells in vitro through a CCK8 test, and the effect was dose-

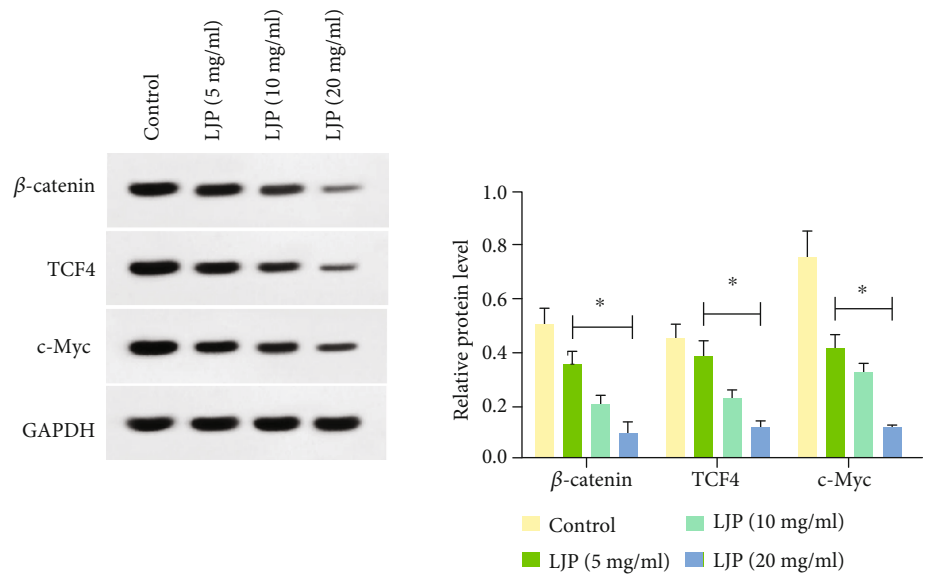

Figure 7: Expressions of signaling pathway proteins in A549 cells. $^*P < 0.05$ versus control group. Western blot was used to detect expressions of pathway-related proteins after treated with different concentrations of LJP for 24 h.

dependent (20 mg/ml of Laminaria japonica polysaccharides had the most obvious inhibitory effect on the survival of tumor cells). In order to study the molecular mechanism of Laminaria japonica polysaccharides inhibiting the survival of A549 cells, we detected the expressions of the proliferation marker Ki67 and PCNA in A549 cells cultured in vitro. The results revealed that Laminaria japonica polysaccharides showed a dose-dependent downregulation of the two proteins within the dose range of 5-20 mg/ml, suggesting that Laminaria japonica polysaccharides could inhibit the proliferation of human non-small-cell lung cancer A549 cells in a dose-dependent manner.

As apoptosis inhibition of tumor cells is one of the important mechanisms of tumor development, the upregulation of apoptosis level of tumor cells is also the target of anticancer drugs to inhibit tumor development. It has been shown that the activation of caspase-3 can trigger a series of events leading to apoptosis, and the activation of caspase-9 can induce apoptosis and growth inhibition in tumor tissues of xenograft mice [15, 16]; additionally, the Bcl-2 family proteins consist of members that either promote (like Bcl-2) or inhibit (like Bax) apoptosis [17]. We further demonstrated by Western blot that Laminaria japonica polysaccharides could inhibit the expressions of Bax, caspase-3, and caspase-9 and promote that of Bcl-2 in a dose-dependent manner, thus resulting in the enhancement of apoptosis of human non-small-cell lung cancer A549 cells at molecular level.

Epithelial-mesenchymal transition (EMT) refers to a biological process by which epithelial cells lose polarity and acquire a migratory, mesenchymal phenotype, thus increasing the ability of cell metastasis and invasion [18, 19]. The process is essential in the occurrence, metastasis, and invasion of a variety of tumors. VEGF is a regulator of tumor angiogenesis, mainly secreted by tumor cells. Through binding to VEGF receptor (VEGFR) on the endothelial cells, it increases the permeability of new blood vessels through paracrine mechanism and promotes the growth of new blood vessels in tumors. Furthermore, VEGF can promote tumor infiltration and metastasis through autocrine mechanism, which may be related to VEGF's regulation of matrix metalloproteinases (MMPs), tumor cytokines, and integrins [20, 21]. Studies have found that the expression level of VEGF has a certain correlation with the prognosis of lung cancer, and its high expression can promote tumor infiltration and metastasis [22]. E-cadherin, an important cell adhesion factor, participates in and mediates adhesion of cells to each other and maintains the adhesion for tissue integrity. It is equivalent to a cancer suppressor and is inextricably linked to invasion and metastasis of various tumors [23]. Its low expression or lack of expression is considered as a key step in epithelial-mesenchymal transformation of tumors. Studies have found that [24] the overexpression of TCF4 in canine renal epithelial cells can increase the invasion ability of cells, thus accelerating the occurrence of tumor EMT, of which the obvious downregulation of epithelial marker protein E-cadherin and the increased expression of interstitial marker protein vimentin can be observed. As for further investigation of Laminaria japonica polysaccharides affecting EMT of A549 cells, we found through experiments that the cells in the LJP treatment group showed high expression of epithelial cell marker protein E-cadherin and low expression of interstitial marker protein N-cadherin and migration marker protein VEGF. The results indicate that Laminaria japonica polysaccharides can inhibit the loss of polarity of non-small-cell lung cancer A549 cultured in vitro, slow down the development of interstitial cells, and reduce the ability of cell invasion and metastasis, thus decreasing the occurrence of EMT in A549 cells.

β-Catenin is a critical component of the Wnt signaling pathway, which is normally inactive. And after being abnormally activated, β-catenin translocates into the nucleus where it binds competitively to TCF4, forming a β-catenin/TCF4 complex which in turn increases the transcription

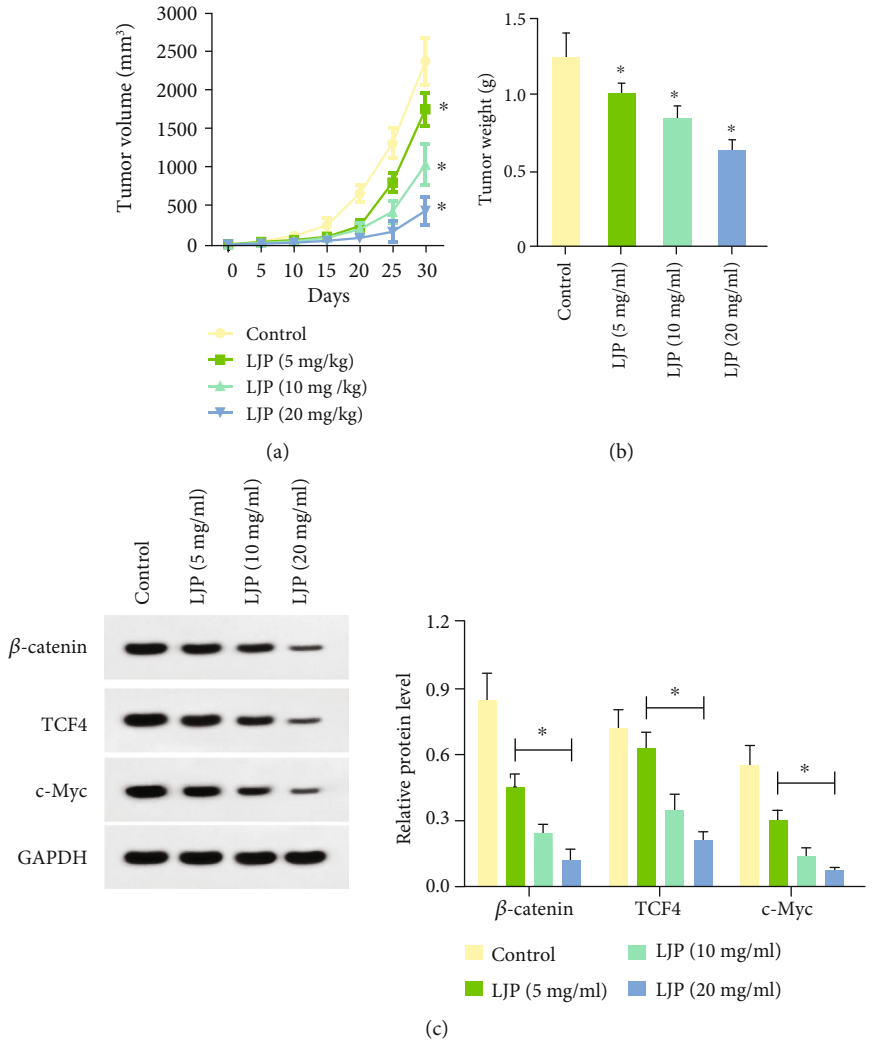

FIGURE 8: Growth and expression of signal pathway protein in transplanted tumor. Mice were randomly divided into 4 groups: control group, LJP (5 mg/kg), LJP (10 mg/kg), and LJP (20 mg/kg) group. Mice were given intraperitoneal injection once a day for a week. Tumor volumes (a) were measured every 5 days, and the mice were sacrificed at the 30th day. Tumor weights (b) and expression of signal pathway proteins (c) in tumors were detected by Western blot. $^{*}P < 0.05$ versus control group.

of downstream oncogenes, including target genes c-Myc, cyclin D1, E-cadherin, and VEGF, thereby contributing to the promotion of development, invasion, and metastasis of such cancers [25, 26]. For example, long noncoding RNA (ENST0000434223) reduces the proliferation, invasion, and migration of gastric cancer cells by inhibiting this pathway [27]. Yan et al. [28] also found that this pathway plays an important role in the proliferation, invasion, and migration of squamous cell carcinoma of the tongue. However, it is still unknown whether Laminaria japonica polysaccharides can affect human non-small-cell lung cancer A549 cells by regulating the β-catenin/TCF4 signaling pathway. In this study, we found that in A549 cells cultured in vitro, Laminaria japonica polysaccharides inhibited the proliferation, invasion, and migration, promoted the apoptosis, and regulated proliferation, invasion, migration, and apoptosis as well as β-catenin/TCF4 pathway-related protein expression. In order to verify whether Laminaria japonica polysaccharides can play a role in affecting the survival of A549 cells in vivo, we performed in vivo experiments and found that Laminaria japonica polysaccharides inhibited the in vivo tumorigenesis of A549 cells (reducing tumor volume and mass), while downregulating the expression of β-catenin/TCF4 as well as its downstream C-Myc protein in tumor tissues. As shown in Figure 9, the effect of Laminaria japonica polysaccharides on A549 cell proliferation, apoptosis, invasion, and migration in vitro and tumorigenesis in vivo was closely related to the downregulation of the β-catenin/TCF4 signaling pathway.

In summary, this study explored the role of Laminaria japonica polysaccharides in survival of human non-small-cell lung cancer A549 cells. We found that Laminaria japonica polysaccharides can inhibit the activation of the β-catenin/TCF4 pathway, so as to regulate the expressions of proliferation-, apoptosis-, and migration-related proteins in human non-small-cell lung cancer A549 cells, thus suppressing the survival rate of A549 cells in vivo and vitro. However, future research is warranted on the effect of Laminaria

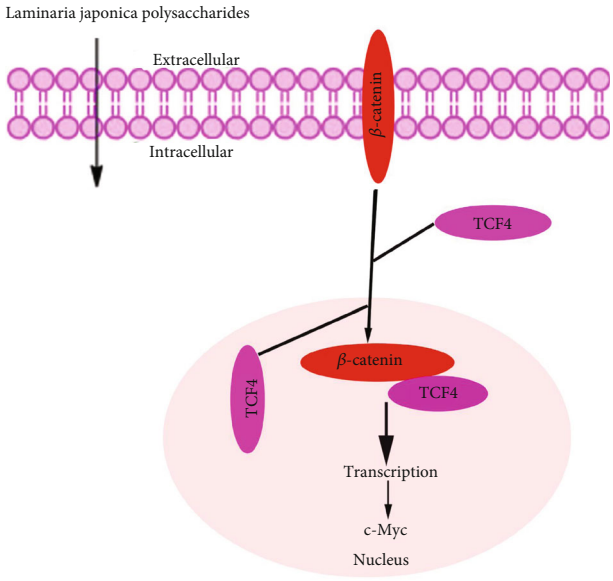

FIGURE 9: Schema of LJP regulating β-catenin/TCF4 signaling pathway.

japonica polysaccharides on the regulation of vital pathways pertaining to growth of other tumors.

Data Availability

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

Conflicts of Interest

The authors confirm that there is no conflict of interest.

Acknowledgments

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