Research Article

The Anticancer Activity of *Lycium barbarum* Polysaccharide by Inhibiting Autophagy in Human Skin Squamous Cell Carcinoma Cells In Vitro and In Vivo

Meihua Zeng,1 Qingtao Kong,2 Fang Liu,2 Jun Chen,2 and Hong Sang1

1Jinling Hospital Department of Dermatology, South Medical University, Nanjing 210002, China
2Jinling Hospital Department of Dermatology, Nanjing 210002, China

Correspondence should be addressed to Hong Sang; sanghong@nju.edu.cn

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Objective. This study is aimed at investigating the effects of *Lycium barbarum* polysaccharide (LBP) on the proliferation and apoptosis of human cutaneous squamous cell carcinoma A431 cells in vitro and in vivo via its regulation on autophagy.

Methods. In vitro experiment: A431 cells were treated with different concentrations of LBP, and cell viability was measured by the CCK8 method. Flow cytometry was used to detect the cell apoptosis rate. The expression of Ki67, PCNA, cl-caspase-3, Bcl-2, and LC3II and the phosphorylation status of JNK and ERK1/2, as well as the effect of SP600125 cotreatment on the expression of autophagy and apoptosis-associated proteins, were determined via Western blot. In vivo experiment: a transplanted tumor model was established by subcutaneous injection of A431 cells to the nude mice. 50 mg/kg LBP was injected into the mice intraperitoneally; the survival rate of mice, volume, and weight of tumor were determined on the 30th day. The expression of Ki67 and MMP-2 proteins was measured by immunohistochemistry.

Results. LBP at concentrations of 400 μg/ml and above was significantly cytotoxic to A431 cells, whereas, within the dose range of 50 μg/ml–200 μg/ml, LBP significantly inhibited the expression of Ki67 and PCNA proteins, promoted the expression of cl-caspase-3, inhibited the expression of Bcl-2 protein, downregulated the expression of autophagy marker LC3II, and reduced the phosphorylation of ERK1/2, whereas the level of JNK phosphorylation was upregulated. At the same time, the regulation of Beclin1, LC3II, Bcl-2, and cl-caspase-3 by LBP was effectively reversed by the cotreatment of SP600125. In addition, LBP increased the survival rate of transplanted nude mice, reduced tumor volume and weight, and downregulated the expression of Ki67 and MMP-2.

Conclusion. LBP can induce apoptosis of A431 cells by inhibiting autophagy and can inhibit tumor growth in vivo.

1. Introduction

Cutaneous squamous cell carcinoma (CSCC) is one of the most common skin malignancies, only second to basal cell carcinoma (BCC), which is developed in keratinocytes in epi-dermal or adnexal structures. It is more common in male patients and preferentially occurs in the elderly. It often happens in the patient’s face, head, and parts where light exposure is more frequent; advanced patients are prone to lymph node metastasis, posing a serious threat to their lives [1–3]. At present, surgical resection is the main treatment for early-stage CSCC patients for whom the prognosis is typically good; however, for the middle- and late-stage patients, they often need to receive a combination of various treatments such as surgery, radiotherapy, and chemotherapy, and the prognosis is usually less desirable. In recent years, targeted therapy for cancer patients has made great progress [4, 5] and finding a suitable drug combined with targeted delivery is one of the approaches for cancer therapy. Along with that, many plant extracts with antitumor activity have begun to be used as an adjunct medication to cancer treatment. Studies have shown that *Lycium barbarum* polysaccharides (LBP), the main bioactive ingredient of Chinese herbal medicines and food supplements, have antioxidation, antiaging, and neuroprotective effects [6]. It is also worth noting that LBP has been shown to exhibit significant
biological activity in inhibiting tumor growth both in vitro and in vivo, including bladder, cervical, colon, liver, and kidney cancers [6–10]. Autophagy is an evolutionarily conserved catabolic process that recycles proteins and damaged organelles and plays an important regulatory role in the balance of cell survival and death. It is also involved in the development of various diseases, including cancer [11, 12]. However, the main focus has been on the mechanism of inhibition of tumor cells by LBP through its effects on cell growth, apoptosis, and autophagy; the relationship between LBP on human CSCC A431 cells and autophagy has not been reported. Therefore, this study preliminarily focuses on the effects of LBP on proliferation and apoptosis of A431 cells in vitro and in vivo and its associated mechanism.

2. Materials and Methods

2.1. Main Materials. Human cutaneous squamous cell carcinoma cell line A431 was purchased from the American model culture pool; DMEM from Sigma; DMSO from Shanghai Biotech; fetal bovine serum, 0.2% trypsin, from Gibco; CCK8 kit from Jiangsu Kaji Biotechnology Co., Ltd.; Annexin V-FITC/PI Apoptosis Detection Kit from Beijing Solarbio Science & Technology Co., Ltd.; BCA kit from Biyuntian Biotechnology Co., Ltd.; and Ki67 antibody (Cat. No.: ab16667), PCNA antibody (Cat. No.: ab92552), cl-caspase-3 antibody (Cat. No.: ab32042), Bcl-2 antibody (Cat. No.: ab32124), JNK antibody (Cat. No.: ab76125), p-JNK antibody (Cat. No.: ab4821), ERK1/2 antibody (Cat. No.: ab17942), p-ERK1/2 antibody (Cat. No.: ab214362), LC3B antibody (Cat. No.: ab51520), Beclin1 antibody (Cat. No.: ab210498), MMP-2 antibody (Cat. No.: ab92536), and goat anti-rabbit IgG-HRP secondary antibody (Cat. No.: ab6721) from Abcam; the immunohistochemistry kit was purchased from Zhongshan Jinqiao, Beijing. Mannose, glucose, galactose, xylose, rhamnose, and arabinose were purchased from the China National Pharmaceutical Group Co., Ltd. (Sinopharm). 2.2. Methods

2.2.1. Cell Culture. Human CSCC A431 cells were inoculated in DMEM medium containing 10% fetal bovine serum and 1% penicillin-streptomycin and cultured in an incubator at 37°C and 5% CO2 at 5% O2. The microscope was used to observe the growth of cell adherence. When the cell fusion rate reached 80% or more, it was digested and passaged with 0.25% trypsin. Cells in the logarithmic growth phase were selected for subsequent experiments.

2.2.2. Separation and Purification of LBP. The Lycium barbarum was dried at room temperature. Then, it was refluxed with 95% ethanol at 75°C for 5 hours and repeated 3 times to remove the lipid and the supernatant in order to extract the dried fruit residue. The dried fruit residue was washed with 10 volumes of distilled water at room temperature for 3 hours each time and repeated 4 times. It was then centrifuged at 5000 rpm for 10 minutes at 20°C, and the supernatant was collected and concentrated 10 times, followed by precipitation with 95% ethanol at a volume ratio of 1:5 at 4°C for 12 hours. The precipitate was then washed with absolute ethanol, acetone, and ether, through which the crude polysaccharide (CLBP) was obtained. CLBP (100 mg) was dissolved in 0.1 M NaCl (10 mg/ml) solution, and 2 ml of this solution was then placed in a DEAE cellulose-52 column (2.6 × 30 cm) and then gradually eluted with 0.1, 0.3, and 0.5 M NaCl solutions at a flow rate of 60 ml/h. The eluate (5 ml/tube) was collected automatically, and the carbohydrate was determined by the phenolsulfuric method using glucose as a standard. These polysaccharides were lyophilized for further study after dehydration and dialysis.

2.2.3. Cell Proliferation Measured with CCK8 Assay. The A431 cell suspension was seeded at a concentration of 2.0 × 10^5 cells/ml in a 96-well plate at 100 μl per well and randomly divided into 10 groups where LBP solutions of different final concentrations were given to each group (0, 0.1, 0.3, 1, and 10 μM). 6 duplicate wells were set up for each group. After 24 hours of culture, 10 μl of the CCK8 solution was added to each well, and incubation was continued for another 4 h. After that, the absorbance value of each well was measured at 450 nm by a microplate reader, and the experiment was repeated 3 times. The survival rate of human CSCC A431 cells is equal to the average value of absorbance in the treatment group divided by the average value of absorbance in the control group multiplied by 100%.

2.2.4. Apoptosis Detected by Flow Cytometry. A431 cells (density, 1 × 10^6 cells/well) were seeded on 6-well plates, and cells at 80% confluence were divided into four groups: Ctrl group, LBP (50 μg/ml) group, LBP (100 μg/ml) group, and LBP (200 μg/ml) group, and were incubated with medium containing the final concentrations of LBP for 24 h. Cells from each group were collected and adjusted to a density of 1 × 10^6 cells/ml. According to kit instructions, 100 μl of cells were added with 5 μl of Annexin V-FITC and 10 μl of PI staining solution and incubated at room temperature in the dark for 15 min for detection.

2.2.5. Autophagy, Apoptosis, and Pathway-Related Protein Expression Measured by Western Blot. The cells cultured for 24 h in each experiment were collected, and the total protein was extracted on an icebox by using the RIPA protein lysate. After centrifugation at 4°C, the supernatant was taken for SDS-PAGE and transferred to a PVDF membrane. The resulting protein was blocked with 5% skim milk powder for 2 h at room temperature. After that, the primary antibody was added and incubated overnight at 4°C. The primary antibody was then discarded, and the corresponding secondary antibody labeled with horseradish peroxidase was added and incubated for another 1 h at room temperature. The ECL was then added dropwise in the darkroom for exposure development. The GAPDH was used as an internal control.

2.2.6. Preparation of the Transplanted Tumor Model. 6-8-week-old BALB/c nude mice were purchased from Beijing...
Charles River Laboratory Animal Technology Company, and the experiment was carried out after one week of adaptive feeding. A431 cells in the logarithmic growth phase were adjusted to a density of $1 \times 10^7$ cells/ml, and 0.2 ml of the cell suspension was injected at the nape subcutaneously. Nude mice were randomly divided into the control group and the LBP (50 mg/kg) group. Animals in the LBP (50 mg/kg) group were given 10 mg/kg of 50 mg/kg LBP solution intraperitoneally, and the control group was given an equal amount of matrix solution. The drug was administered everyday consecutively for a week. The survival data of the mice was recorded, and the tumor volume was measured every 5 days. The mice were sacrificed on the 30th day, and the tumor was separated and weighed. Tumor volume $= 0.5 \times \text{length} \times \text{width}^2$.

2.2.7. The Expression of Ki67 and MMP-2 in Tumor Tissues Detected by Immunohistochemistry. The tumor tissue obtained in Section 2.2.4 was routinely prepared and sectioned, and immunohistochemical staining was performed according to instructions. In brief, the sections were immersed in a 3% H$_2$O$_2$ solution at 37°C for 30 min, then washed with PBS for 5 min for 3 times. It was then blocked with 10% BSA, after which, the primary antibody was added and incubated overnight at 4°C. It was then rinsed with PBS for 5 min and repeated 3 times. After that, the secondary antibody was added and incubated at 37°C for 1 h, then rinsed with the PBS for 5 min for 3 times. DAB was used to color it for 10 s and fully rinsed with the running water before being counterstained with hematoxylin for 30 s. After that, it was fully rinsed with the running water again and neutral gum was added after the conventional dehydration and finally covered with a coverslip. The expression of Ki67 and MMP-2 was observed under the microscope.

2.2.8. Statistics. All the obtained data were analyzed with SPSS 17.0. Differences conformed to a normal distribution between groups were tested by one-way ANOVA. The results were denoted as mean ± standard deviation. The difference was considered statistically significant when $P < 0.01$.

3. Results

3.1. Separation and Purification of LBP. In this paper, CLBP was isolated by ethanol precipitation, and the UV spectrum showed no absorption peak at 280 nm, indicating that there was no protein in CLBP, as shown in Figure 1. The content of polysaccharides purified by column chromatography was measured by phenol-sulfuric acid method. Six elution peaks of the polysaccharides were designated as LBP-1, LBP-2, LBP-3, LBP-4, LBP-5, and LBP6 according to the sequence of the elution column. The polysaccharide contents at the six peaks are shown in Table 1. A mixed monosaccharide solution containing mannose, glucose, xylose, rhamnose, and arabinose was used as a control for HPLC detection. By comparing the mixed monosaccharide solution curves, the composition at each peak was different (see Table 1). LBP-5

![Figure 1: UV spectrum for CLBP.](image)

<table>
<thead>
<tr>
<th>Designations</th>
<th>Content</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBP-1</td>
<td>2.23%</td>
<td>Xylose, glucose, and rhamnose</td>
</tr>
<tr>
<td>LBP-2</td>
<td>0.88%</td>
<td>Arabinose, xylose, and glucose</td>
</tr>
<tr>
<td>LBP-3</td>
<td>3.11%</td>
<td>Mannose, xylose, and glucose</td>
</tr>
<tr>
<td>LBP-4</td>
<td>2.43%</td>
<td>Arabinose, and glucose</td>
</tr>
<tr>
<td>LBP-5</td>
<td>83.68%</td>
<td>Arabinose, mannose, xylose, glucose, and rhamnose</td>
</tr>
<tr>
<td>LBP-6</td>
<td>5.36%</td>
<td>Xylose, glucose, and rhamnose</td>
</tr>
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having the highest content of polysaccharides consisted of arabinose, mannose, xylose, glucose, and rhamnose, among which glucose showed much higher content than the other monosaccharides. Therefore, we chose LBP-5 for follow-up experiments and study, which was referred to as LBP in the following experiments.

3.2. The Effects of LBP on the Survival Rate of A431 Cells In Vitro. The CCK8 method was used to measure the effect of LBP on the survival rate of A431 cells in vitro. The results in Figure 2 showed that the survival rate of A431 cells in each LBP treatment group decreased gradually. Compared with the control group, the survival rate of the LBP-treated group at 400, 800, and 1000 μg/ml decreased significantly (P < 0.01), and the half inhibitory concentration of LBP was 873.7 μg/ml. In other words, when the concentration of LBP exceeds 400 μg/ml, it caused significant cytotoxicity to A431 cells in vitro, and the survival rate of A431 cells was less than 80%. Therefore, subsequent experiments were performed using three maximum treatment concentrations of LBP without causing significant cytotoxicity, namely, 50, 100, and 200 μg/ml.

3.3. Effect of LBP on Apoptosis Rate of A431 Cells and the Expression of Proliferation and Apoptosis-Related Proteins in A431 Cells Cultured In Vitro. The apoptotic rate of A431 cells was detected by flow cytometry, and the results shown in Figure 3(a) indicated that the proportion of apoptotic A431 cells was increased in each LBP-treated group compared with the control group (P < 0.01), which was in a LBP dose-dependent manner.

The expression of proliferation and apoptosis-related proteins in A431 cells was determined by Western blot. The results are shown in Figure 3(b). Compared with the control group, the expression levels of proliferation-related proteins (Ki67 and PCNA) in A431 cells treated with LBP of different concentrations were significantly lower than those in the control group (P < 0.01). The expression level of apoptosis-related protein cl-caspase-3 was significantly increased (P < 0.01), while the expression level of Bcl-2 was significantly decreased (P < 0.01). Additionally, the effects of LBP on the expression of these proteins are dose-dependent.

3.4. Effect of LBP on the Expression of Autophagy Markers and Phosphorylation of JNK1/2 and ERK1/2 in Cultured A431 Cells. Similarly, the expression of LC3II and phosphorylation status of JNK1/2 and ERK1/2 in A431 cells were measured by Western blot. The results are shown in Figure 4. Compared with the control group, the expression of LC3II and p-ERK1/2/ERK1/2 protein in A431 cells of each LBP treatment group was significantly lower (P < 0.01), while the expression of p-JNK/JNK protein was significantly upregulated (P < 0.01). And these regulations by LBP were all in a dose-dependent manner.

3.5. Effect of LBP on the Expression of Autophagy Marker and Apoptosis Proteins Mediated by the MAPK Pathway in Cultured A431 Cells. The effect of JNK inhibitor SP600125 on the expression of autophagy markers and apoptotic proteins in A431 cells was determined by Western blot. As shown in Figure 5, compared with the control group, Beclin1, LC3II, and Bcl-2 protein were significantly downregulated in A431 cells after treatment with 50 μg/ml LBP (P < 0.01) and the expression of cl-caspase-3 protein was upregulated (P < 0.01). When A431 cells were cotreated with LBP (50 μg/ml) and SP600125 (10 μM), compared with LBP (50 μg/ml), the expression of these proteins was reversely regulated (P < 0.01).

3.6. Effect of LBP on the Survival of Transplanted Tumor Mice and Their Tumor Growth. The survival rate of transplanted tumor mice, volume, and weight of the tumor in the two groups are shown in Figures 6(a)–6(c). The survival rate of transplanted tumor mice in LBP treatment groups was significantly higher than that in the control group (P < 0.001), and the tumor volume and weight were significantly lower than those in the control group (P < 0.001). At the same time, immunohistochemistry performed on the tumor tissues of nude mice demonstrated that the expression levels of Ki67 and MMP-2 were significantly lower in the LBP-treated group than in the control group (see Figure 6(d)) (P < 0.01).

4. Discussion

The occurrence and development of human cutaneous squamous cell carcinoma is an extremely complex process, which is controlled by a variety of genes and various factors and is associated with abnormal proliferation, differentiation, and apoptosis of tumor cells. Previous studies have shown that LBP, the main effective component of Lycium barbarum, has shown in antitumor studies some efficacy that it can inhibit the growth of a variety of tumor cells and has the advantages of low toxicity, fewer side effects, and availability [6–10]. In recent years, autophagy is emerging as a new strategy for cancer prevention and treatment and has been proven to be closely related to tumor cell growth and proliferation, as well as malignant metastasis [13]. In addition, studies have shown that LBP can protect against cell injury by regulating autophagy and apoptosis [14–19]. The study was aimed at investigating the regulatory effect of LBP on autophagy, and its effect on the proliferation, apoptosis of squamous cells.
developing carcinoma in vitro and in vivo, and survival rate of tumor-xenografted nude mice.

The occurrence of cancer is often associated with abnormal proliferation and apoptosis resistance of tumor cells. Therefore, in this study, human skin cutaneous squamous cell carcinoma A431 cells were first cultured in vitro and treated with LBP at concentrations of 0, 5, 10, 20, 50, 100, 200, 400, 800, and 1000 μg/ml. And the survival rate of A431 cells was determined by the CCK8 method. The results showed that the survival rate of A431 cells in culture was significantly reduced when the LBP concentration reached 400 μg/ml and above. Studies have shown that the proliferative activity of tumor cells is an important prognostic indicator for tumor diagnosis. Ki67 is a widely used proliferation marker, and PCNA is a proliferating cell-specific expression protein, which is also an important participant in DNA replication and repair process [20]. Caspase-3 is the major executive protein for protein degradation during apoptosis; when cells are abnormally stimulated, cytochrome enzyme C together with procaspase-9 forms an apoptosome to activate caspase-9, which further activates caspase-3 to form cl-caspase-3 (cleaved caspase-3), thereby triggering apoptosis [21], whereas Bcl-2 is a class of anti-apoptotic protein that plays a key role in promoting cell survival [22]. Therefore, the expression levels of proliferating and apoptotic key proteins were measured by Western blot in this study. It was found that LBP treatment induced cl-caspase-3 protein expression in A431 cells in a dose-dependent manner within the concentration range of 50 μg/ml~200 μg/ml. In parallel, inhibition of Ki67, PCNA, and Bcl-2 expression was observed at the same time. The above results indicate that LBP can induce the apoptosis of A431 cells in vitro.

It has been proved that LC3 proteins are autophagy markers, including LC3I (soluble, cytosolic protein) and LC3II (lipid-soluble, membrane protein), and that LC3I transforms to LC3II and localizes to autophagosomal membranes upon autophagy activation; therefore, LC3II expression can reflect the level of autophagy [13]. Western blot showed that LBP upregulated JNK phosphorylation and downregulated ERK1/2 phosphorylation and LC3II protein expression in A431 cells in vitro in a concentration range of 50 μg/ml~200 μg/ml with a dose-dependent manner. Studies have shown that autophagy is different from apoptosis, and autophagy is related to the balance of intracellular

![Figure 3: Effect of LBP on proliferation- and apoptosis-related protein expression in A431 cells. (a) Apoptosis rate of A431 cells was detected by flow cytometry. (b) Expression of apoptosis-related proteins in A431 cells was detected by Western blot. Compared with the control group, *P < 0.01.](image)
environment and plays an important role in cell survival. In addition, the signaling pathways involved in apoptosis and autophagy actually constitute an interconnected network, including Beclin1 and Bcl-2 [23]. JNK is closely related to autophagy by preventing the phosphorylated Bcl-2 family of proteins from binding to Beclin1, thereby regulating autophagy levels, while activation of the JNK pathway is often caused by MAPK cascades [24]. This study also found that the expression levels of Beclin1, LC3II, and Bcl-2 in A431 cells were upregulated after ANK cells were treated with both JNK inhibitor SP600125 and LBP, while the expression of apoptosis-executing protein cl-caspase-3 was downregulated. Taken together, the above results indicate that the inhibition of A431 autophagy by LBP is achieved by the JNK pathway.

Finally, we established a nude mouse model of A431 cell xenografts in order to further investigate the effect of LBP on the survival rate of transplanted tumor models, in vivo tumor growth, and proliferation, as well as migration protein expression. The results showed that LBP significantly reduced tumor tissue volume and tumor weight, significantly increased the survival rate of tumor-bearing nude mice, and

**Figure 4:** Effect of LBP on expression levels of autophagy markers and signaling pathway proteins in A431 cells. Compared with the control group, *P < 0.01.

**Figure 5:** LBP induced apoptosis and inhibited autophagy in A431 cells. Compared with the control group, *P < 0.01; compared with the LBP (50 μg/ml) group, #P < 0.01.
significantly downregulated the expression of Ki67 and MMP-2, thereby achieving the in vivo inhibition of A431 cells. Combined with these results, it appears that LBP can inhibit autophagy, induce apoptosis, and inhibit cell proliferation in vitro and in vivo, and its mechanism may be related to the upregulation of JNK and downregulation of ERK1/2 phosphorylation.

In summary, LBP at concentrations of 400 μg/ml and above produced significant cytotoxicity in A431 cells cultured in vitro. When within a concentration range of 50 μg/ml–200 μg/ml, LBP dose-dependently regulated proliferation, apoptosis, and autophagy-related protein expression as well as upregulated the JNK and downregulated the ERK1/2 signaling pathway. Furthermore, the regulation of LBP on autophagy and apoptosis-related proteins was reversely regulated by JNK inhibitor SP600125. And LBP can also inhibit the growth and metastasis ability of A431 cells in vivo. This paper firstly explored the effect of LBP on the proliferation and apoptosis of human cutaneous squamous cell carcinoma A431 by inhibiting autophagy; however, there are still many areas to be explored in the future, such as the effect of LBP on the autophagy of other human skin squamous cell carcinoma cell lines and on other apoptotic signaling pathways.

Data Availability
All data generated or analyzed during this study are included in this published article.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Acknowledgments
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References


