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Lung Dysbiosis in Airway Diseases

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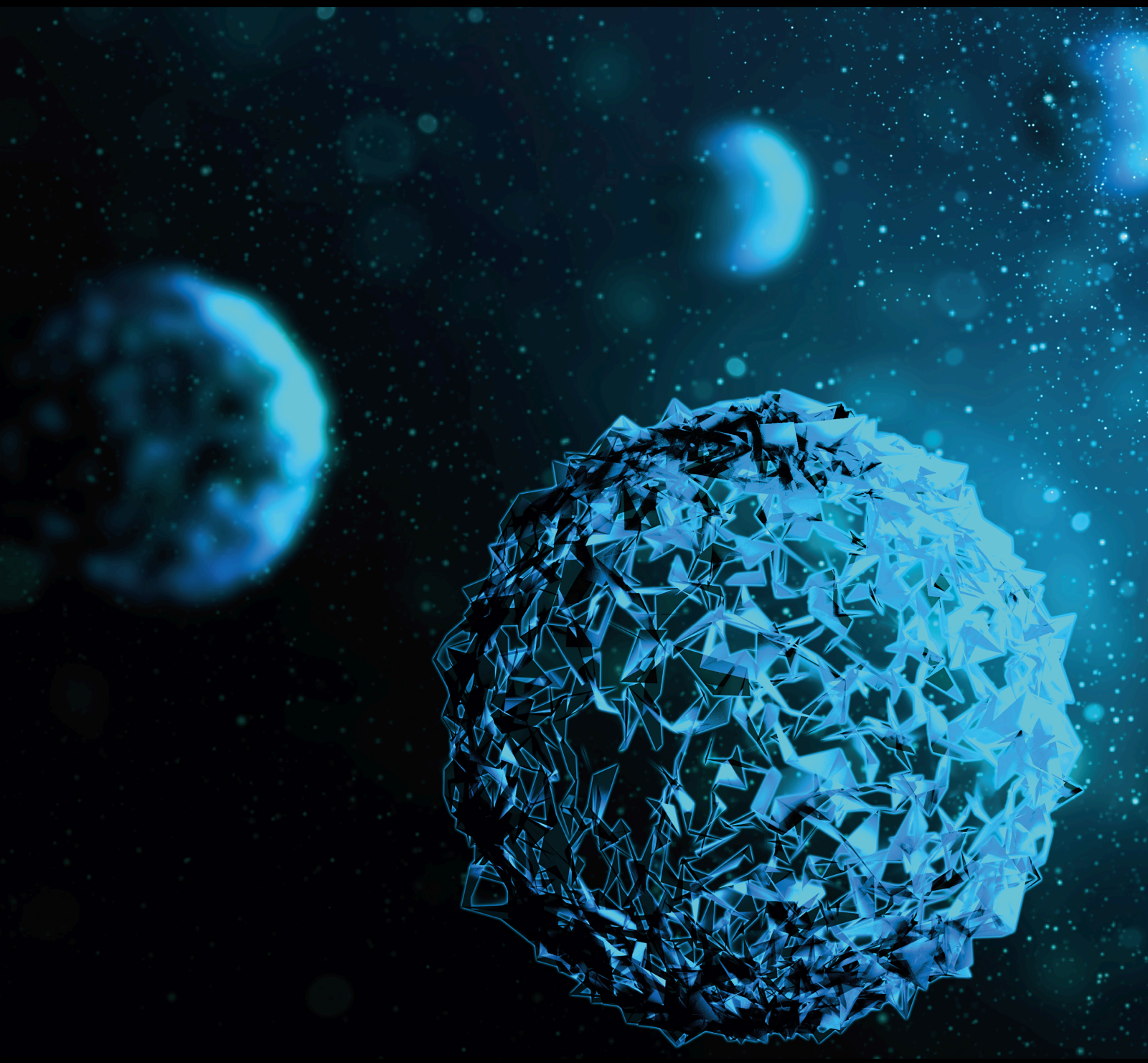

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Research Article

Polyacetylenes from *Codonopsis lanceolata* Root Induced Apoptosis of Human Lung Adenocarcinoma Cells and Improved Lung Dysbiosis

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Codonopsis lanceolata is a perennial smelly herbaceous plant and widely employed for the treatment of various lung cancer and inflammation. However, the anticancer substances in *C. lanceolata* and their underlying mechanisms had not been well clarified. In this study, six compounds were obtained from the water extracts of *C. lanceolata* polyacetylenes (CLP) and then identified as syringin, codonopilodiynoside A, lobetyol, isolariciresinol, lobetyolin, and atractylenolide III. Treatment with CLP remarkably suppressed the cell proliferation, colony formation, migration, and invasion of A549 cells. Synergistic effects of lobetyolin and lobetyol were equivalent to the antiproliferative activities of CLP, while other compounds did not have any inhibition on the viabilities of A549 cells. CLP also reduced the expression of Ras, PI3K, p-AKT, Bcl-2, cyclin D1, and CDK4 but increased the expression of Bax, GSK-3β, clv-caspase-3, and clv-caspase-9, which could be reversed by the PI3K activator 740YP. Furthermore, CLP retarded the growths of tumor and lung pathogenic bacteria in mice. It demonstrated that lobetyolin and lobetyol were the main antitumor compounds in *C. lanceolata*. CLP induced cell apoptosis of lung cancer cells via inactivation of the Ras/PI3K/AKT pathway and ameliorated lung dysbiosis, suggesting the therapeutic potentials for treating human lung cancer.

1. Introduction

Lung cancer is presently one of the most harmful human diseases with the highest morbidity throughout the world, which has an incidence of 20.9 million new cases and 18.0 million deaths in 2018 [1]. The occurrence and development of lung cancer involve various complex pathological mechanisms and are prone to metastasis to the bone, brain, liver, and lymph node, resulting in the trick reversion in clinic [2–4]. Although chemotherapy has been a major means for lung cancer therapy, prolonged, repeated intravenous chemotherapy causes drug resistance and several side effects [5, 6].

In recent years, traditional Chinese medicines have played a prominent part in tumor growth, immune function, and life expectancy for the treatment of lung cancer [7, 8]. *Codonopsis lanceolata* (family *Campanulaceae*) is a dicotyledonous herbaceous perennial plant mainly distributed in the northern parts of China, Russian, and North Korea. The roots of *C. lanceolata* are used as a folk medicine for treating various lung diseases, including cough, bronchitis, edema, asthma, and lung cancer over thousands of years [9–12]. It contains a wide variety of distinctive metabolites (e.g., polysaccharides, saponins, and polyacetylenes) [9, 13, 14]. It has been reported that the n-butanol extract of *C. lanceolata* root induced the apoptosis of human colon cancer HT-29 cells via ROS accumulation and polyamine depletion [15]. Its methanol extract also induced the apoptosis of human oral cancer HSC-2 cells through activation of the Bak pathway [16]. Codonoposide 1c, an echinocystic acid derivative obtained from the root of *C. lanceolate*, triggered caspases-

dependent apoptosis in acute myeloid leukemia HL-60 cells [17]. In addition, the water extract of *C. lanceolata* attenuated various stimuli-induced lung inflammation by inhibiting alveolar macrophage and Th2 cell activation, indicating its anti-inflammatory potential on respiratory inflammatory diseases [12, 18, 19]. However, previous studies mainly focused on the quality control and structure analysis of various chemical components from *C. lanceolata*. The ingredients responsible for the anticancer effects of *C. lanceolata* and their underlying mechanisms remain unknown. In the present study, the polyacetylenes of *C. lanceolata* (CLP) loaded onto the chromatographic column to the bioactive polyacetylenes of *C. lanceolata* (CLP) were yielded through silica gel chromatography separation, and then, their antitumor potentials were assessed in A549 cells and in tumor-bearing mice.

2. Materials and Methods

2.1. Materials and Reagents. Fetal bovine serum (FBS) was acquired from Tianhang Biotech. Co. Ltd., Hangzhou, China. MTT was obtained from Sigma-Aldrich (China). Antibodies used in the study were obtained from Cell Signaling Technology (Danvers, USA) or Abcam (Cambridge, UK). Other regents were all purchased from Hangzhou Bozan Biotech. Co. Ltd., China.

2.2. Extraction and Isolation of Compounds in CLP. The isolation process was based on the related articles with minor changes [20–22]. The herb was purchased from the Zhejiang Traditional Chinese medicine factory, appraised by Dr. Xiong-Ning Wu in our college, and the rest of the samples were stored in a specimen room (no. 2019W0512). The air-dried plants (20 kg) were extracted 2 times (boiling 1 h with 200 L of water each time). The water extract was concentrated to be 30 kg of weight and then mixed with 45 L of 95% ethanol overnight. The supernatant was concentrated and then purified by AB-8 macroreticular resin. After being eluted with 80% alcohol, the eluent was collected and then dried by hypobaric drying to yield the purified extract (CLP, 2.6 kg). This extract CLP was subsequently subjected to column chromatography over HP-20 resin (100–200 mesh) and separated with a gradient elution [H_2O–EtOH (4 : 1–1 : 1, *v*/*v*)]. Compounds 1 (10.2 mg), 2 (8.8 mg), 3 (10.6 mg), 4 (20.5 mg), 5 (94.3 mg), and 6 (37.9 mg) were found from 50% EtOH– elution by the preparative HPLC system. The purities of these compounds were analyzed by using HPLC.

2.3. Quality Control of CLP. The CLP or each isolated compound was prepared by dissolving in methanol. After filtering through a 0.22 μm membrane, 20 μL of the sample was subjected to the HPLC system. The HPLC column was a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm). The temperature was kept at 30°C during the whole analytical process. The detection wavelength was 220 nm. The flow rate of the mobile phase (acetonitrile (A) and 0.1% phosphoric acid (B)) was 1.0 mL/min. The conditions of elution were set as follows: 0–20 min, 10% A; 20–30 min, 10% A ⟶ 30 % A; 30–40 min, and 30% A ⟶ 70% A. The resolution of each compound was not less than 1.5. The theoretical plate numbers of syringin should be larger than 5000.

2.4. Cell Viability and Colony Formation Assays. The cells were seeded into the 96-well plates (each well had 5000 cells) and then treated with CLP (2.5, 5, and 10 μg/mL) or DDP (5 μg/mL) for 24, 48, and 72 h. Twenty microliters of PBS solution containing 5 mg/mL of MTT was assigned to each well and incubated at 37°C for 4 h. At last, each well was mixed with 150 μL of dimethyl sulfoxide. The absorbance was measured at 490 nm.

Cells were seeded into six-well plates (10^3 cells/well) and then treated with CLP (2.5, 5, and 10 μg/mL) or DDP (5 μg/mL). When a clearly visible colony appeared in the culture dish, the cells were fixated with methanol and subsequently dyed with 10% Giemsa for 10–30 min. Colonies were counted under an optical microscope (OLYMPUS, Japan).

2.5. Transwell Invasion and Wound Healing Assays. The serum-free medium containing 1×10^5 cells were added into the upper chambers of the transwell chambers (8 μm pore size) with Matrigel (BD Biosciences, USA), while 500 μL of 20% FBS medium was presented into the matched lower chambers. Forty-eight hours after incubation with test drugs, only the lower chambers were collected, fixated with methanol for 30 min, and stained with 0.1% crystal violet for 15–30 min. The cells on the lower surfaces of the chambers were counted under the optical microscope (OLYMPUS, Japan).

The cells were seeded into six-well plates (each well had 2.5×10^5 cells). The cell layer of each well was wounded by using the tip of a 200 μL pipette. The wells were carefully washed with PBS to remove the detached cells, and then, the remaining cells were cultured at 37°C for 48 h. Images were captured at 0 and 48 h after scratching, and the wound width in each well was measured with a ruler under the microscope (OLYMPUS, Japan).

2.6. Cell Apoptosis and Cell Cycle Assay. The cells in 6-well plates were treated with CLP at the concentrations of 2.5–10 μg/mL for 24 h and then mixed with 500 μL of buffer, 5 μL of annexin V FITC (20 μg/mL), and 10 μL of PI (50 μg/mL). The apoptotic rates of CLP-treated A549 cells were detected by using flow cytometry (BD, USA). On the other hand, the cells were also collected for examining cell cycle distribution according to the commercial kit (Multi-Sciences Biotech Co. Ltd., Hangzhou, China). Cells were treated with 500 μL of buffer and 5 μL of permeabilization solution and then kept for 20 min at room temperature and no-light conditions. Finally, the cell cycles of stained cells were assayed by flow cytometry.

2.7. Western Blot Assay. The total proteins of cells or tumor samples were extracted with 0.2 mL of RIPA, 1 μL of PMSF, and 1 μL of the phosphorylation protease inhibitor. Then, the supernatant of the protein extracts was collected and its quality was controlled by the BCA detection kits (KeyGEN BioTECH Co. Ltd., Nanjing, China). The total proteins were diffused on 12% SDS-PAGE electrophoresis and transferred onto the polyvinylidene difluoride membranes. These

membranes were soaked in 5% nonfat milk for 2 h and then treated with primary antibodies for 10 h at 4°C. After pretreatment with TBST for 3 times, the membranes were treated with secondary antibodies for 1 h. The expression of the target proteins was measured by using chemiluminescence (Beyotime, China). GAPDH was considered as the control for Western blot analysis.

2.8. Animals and Experimental Procedure. Thirty male nude mice (six weeks old) were provided from Shanghai SLAC Co. Ltd., China. Those mice were fed in the specific pathogen-free conditions (room temperature, 22–24°C; humidity, 45–55%). The operational process followed the guidelines of our college and was approved by the ethics committee of the college (no. 2020R0505).

Approximately 5.0×10^5 cells of luciferase-overexpressing A549 cells were injected into the left lung of each nude mouse to prepare a xenograft mouse model as recent reports [23–25]. The tumor-bearing mice after one-week postinjection were randomly divided into the five groups: (1) model group, intragastrical administration (i.g.) with 10 mg/kg of saline; (2) CLP-L group, i.g. 10 mg/kg of CLP; (3) CLP-M group, i.g. 20 mg/kg of CLP; (4) CLP-H group, i.g. 40 mg/kg of CLP; and (5) DDP group, i.g. 5 mg/kg of DPP. Each group had six mice. The mice in the model and CLP groups received oral administration once a day for 15 days, while the mice in DDP-treated group were intraperitoneally injected once every 3 days. The weighs of mice were recorded every 3 days.

The growth of orthotopic tumor was monitored every 5 days by using the IVIS Lumina LT imaging system (PerkinElmer, USA). Briefly, the mice were anesthetized by isoflurane and then intravenously injected with 1.5 mg D-luciferin (Yeasen, China) 10 min prior to imaging.

2.9. HE and TUNEL Assays. Tumor tissues were soaked in 4% formaldehyde for more than 96 h, and then, the cured samples were pretreated with gradient ethanol and finally put into the paraffin. Tumor samples were stained by hematoxylin and eosin (HE) solution. Furthermore, the apoptotic cells in tumor tissues were marked by using an in situ apoptosis detection kit (Roche, USA). The images of positive cells (presented green fluorescence) were captured under fluorescence microscopy (Zeiss, Germany).

2.10. Immunohistochemistry. Tumor tissues were prepared as in Section 2.9. The paraffin-embedded samples excised from A549 nude mice were stained by using Ki-67 and pAKT antibodies for immunohistochemistry. Images of the tumor tissues were captured using a light microscope (Leica DM2500, Germany).

2.11. Bacterial 16S rDNA Sequencing. The bacterial diversity and abundance of lung samples were analyzed by 16S rDNA sequence analysis [26, 27]. The total DNA was extracted using DNA extraction kit (Tiange, China). The DNA quality and purity were controlled by using the NanoDrop ND-1000 system (Thermo, USA). The 16S rDNA genes of the V3-V4 regions were amplified by using a specific primer (F:5′-ACTCCTACCGAGCAGAGAG-3′, R:5′-GGACTACHgGTWTCTATT-3′) with the barcode. The reaction parameters were set as initial denaturation at 98°C for 30 s, followed by 32 cycles of denaturation at 98°C for 10 s, 54°C for 30 s, and 72°C for 45 s and annealing at 72°C for 10 min. Agilent 2100 Bioanalyzer (Agilent, USA) was used to prepare and evaluate the library, and Illumina's library quantification kit (Kapa Biosystems, USA) was used for quantification. The amplified library was sequenced on Illumina NovaSeq PE250 platform according to the standard steps (2×250) by LC Biotech. Co. Ltd. (China).

2.12. Detection of Antibacterial Activity of CLP. Streptococcus pyogenes (CVCC1882) and *Staphylococcus aureus* (CVCC376) were donated by Dr. Fanwei Dai, Zhejiang Animal Research Center, China. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of CLP on these two tested strains were measured in vitro by the 2-time dilution method and decided visually according to the presence or absence of strains [28–30]. Each concentration was detected 5 times. The plates were cultured at 37°C for 24 h. To detect MBC values, the concentrations of CLP tested in the study were higher than its MIC values. Moreover, the filter paper was made into a circular paper with a diameter of 5 mm and sterilized for further use. The filter papers with the prepared test solution were placed on the solid media and applied evenly with bacteria solution. The diameters of the inhibition zone of samples were determined 24 h after treatment.

2.13. Statistical Analysis. Each assay was performed 3 times. The data were showed as the mean ± standard deviation (SD). Statistical differences were analyzed by one-way ANOVA by using the GraphPad Prism 6 and SPSS 16.0 software. The significant differences between 2 groups were set at $P < 0.05$.

3. Results

3.1. Chemical Analysis of Compounds in CLP. Six compounds were isolated from *C. lanceolata*, and their structures were characterized as syringin (1), codonopilodiynoside A (2), lobetyol (3), (+)-isolariciresinol (4), lobetyolin (5), and atractylenolide III (6) by comparing their physical and spectral data (HR-MS, HPLC, ^{1}H-NMR, and ^{13}C-NMR) with previous reports [20–22, 31, 32]. Among those compounds, compounds 2, 4, and 6 were first found in the plant *C. lanceolata*.

To control the quality of the herbal extract CLP, we determined the contents of the six compounds by HPLC. The representative HPLC chromatograph was presented as shown in Figure 1(a). The contents of syringin, codonopilodiynoside A, lobetyol, (+)-isolariciresinol, lobetyolin, and atractylenolide III in CLP were 46.9%, 5.7%, 9.3%, 12.6%, 10.5%, and 1.1%, respectively.

3.2. Effect of CLP on A549 Cell Proliferation. The results in Figure 1(b) showed that CLP remarkably inhibited the proliferation of A549 cells in time- and concentration-

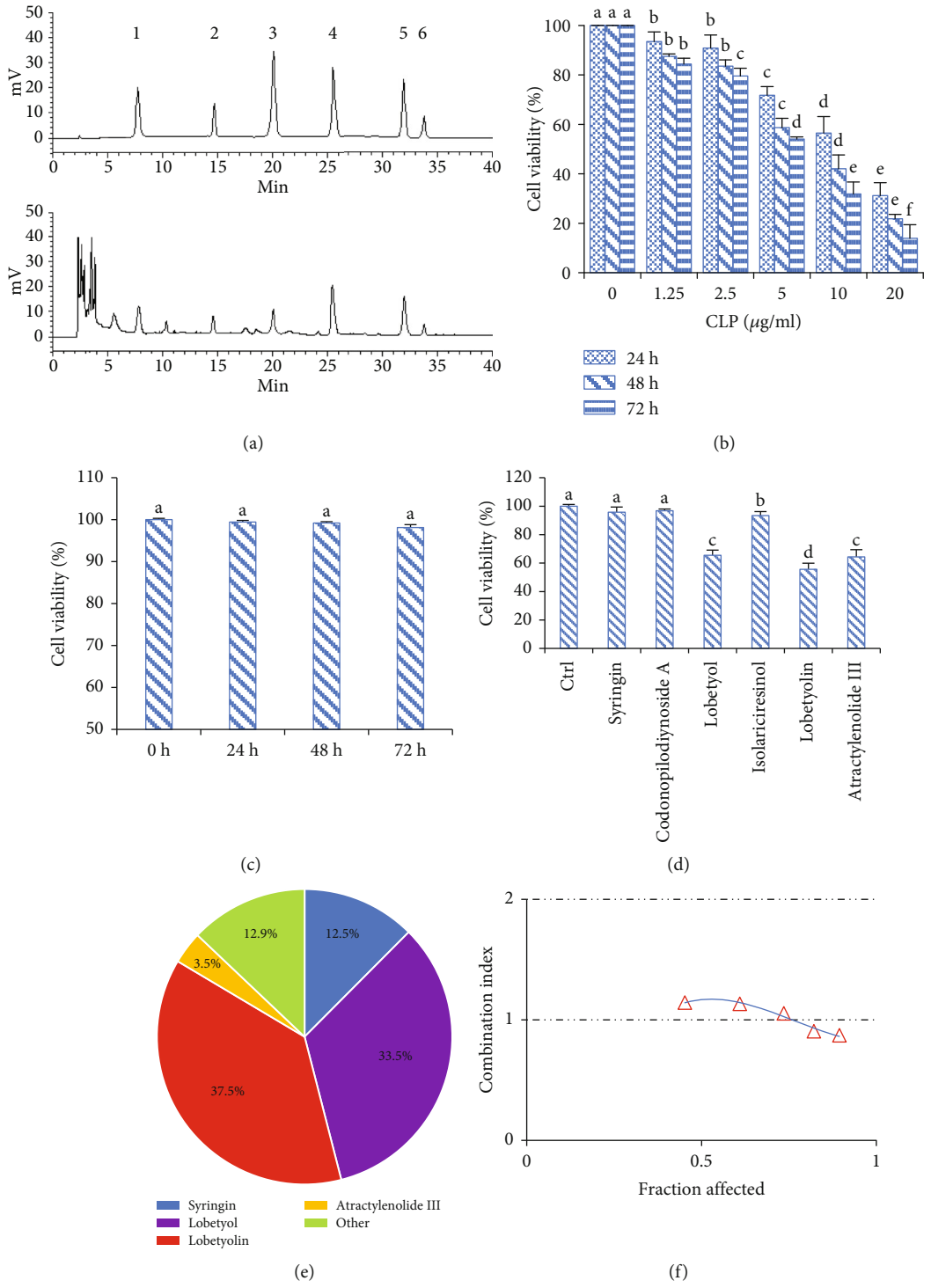


Figure 1: Effects of CLP on the proliferation of A549 cells. (a) HPLC chromatograph of standards (above) and CLP (below). The peaks marked with 1~6 were syringin, codonopilodiynoside A, lobetyol, (+)-isolariciresinol, lobetylin, and atractylenolide III, respectively. (b) Effects of CLP on the viability of A549 cells. The A549 cells were treated with 1.25~20 μg/mL of CLP for 24, 48, and 72 h. (c) Effects of CLP on the viability of normal lung epithelial BEAS-2B cells. Cell viability was determined by MTT assay. The BEAS-2B cells were treated with 20 μg/mL of CLP for 24, 48, and 72 h, respectively. (d) Effects of compounds isolated from CLP on viability of A549 cells. The A549 cells were treated with 20 μg/mL of different compounds for 48 h. (e) The contributions of the main bioactivity compounds to the inhibition rates of CLP on the proliferation of A549 cells. The A549 cells were treated with 9.92 μg/mL of syringin, 1.82 μg/mL of lobetyol, and 2.06 μg/mL of lobetylin for 48 h, which represented their concentrations in 20 μg/mL CLP. (f) The synergistic inhibition of lobetyol and lobetylin on the proliferation of A549 cells, which was calculated by using CompuSyn software. Data were expressed as means ± SD. The groups marked with different letters suggested significant differences, $P < 0.05$.

dependent manners 24–72 h after administration with 1.25–20 μg/mL of CLP, respectively. The IC_{50} values of CLP on A549 cell proliferation for 24, 48, and 72 h were 13.5, 10.5, and 8.6 μg/mL, respectively. Most notably, treatment with 20 μg/mL of CLP for 24–72 h did not affect the viabilities of human normal lung epithelial BEAS-2B cells (Figure 1(c)), suggesting its low cytotoxicity.

To determine the anticancer pharmacodynamic substances in CLP, the six compounds isolated from CLP were investigated *in vitro* in A549 cell model. As shown in Figure 1(d), lobetyol, lobetyolin, and atractylenolide III had significant inhibition on the cell proliferation of A549 cells, while other compounds showed little or no effects. Considering that the contents of those six compounds in CLP were clear, the contribution of each compound to the antiproliferative activities of CLP could be calculated by comparing their overall and individual inhibition. The inhibitory rates of CLP (20 μg/mL), syringin (9.38 μg/mL), lobetyol (1.86 μg/mL), lobetyolin (2.10 μg/mL), and atractylenolide III (0.22 μg/mL) on the proliferation of A549 cells were 65%, 8.1%, 21.8%, 24.4%, and 2.3%, respectively. Therefore, lobetyol and lobetyolin contributed approximately 71% to the inhibitory effects of CLP on lung cancer cell proliferation (Figure 1(e)). Although the content of syringin in CLP was approximately fivefold higher than those of lobetyol and lobetyolin, the antiproliferative activity of syringin was approximately fourfold less. Thus, lobetyol and lobetyolin could be the main anticancer compounds. Moreover, the combination index of lobetyol and lobetyolin, which were mixed at 5 different concentrations (Figure 1(f)), was around the additive baseline 1, indicating their additive effects.

3.3. Effects of CLP on A549 Cell Migration, Invasion, and Colony Formation. As shown in Figure 2, the width of wound scratch in the control group was significantly reduced 48 h after CLP treatment. However, compared with the untreated group, wound closures were significantly decreased in the CLP-treated groups ($P < 0.05$), indicating the inhibition of CLP on A549 cell migration. Moreover, the results of transwell assay showed that approximately 250 cells invaded the lower chamber in the control group after CLP treatment for 48 h. However, the number of cells in the lower chamber was significantly decreased in the CLP-treated groups compared with the control group ($P < 0.05$), indicating the inhibition of CLP on A549 cell invasion. Similarly, CLP dose dependently inhibited the colony formation of A549 cells. But colonies were hardly found in 10 μg/mL of the CLP-exposed group.

3.4. Effects of CLP on A549 Cell Apoptosis. As shown in Figure 3, the apoptosis rates of the normal cells were only 5% but it was significantly increased in the CLP-treated groups in a concentration-dependent manner ($P < 0.05$), indicating that CLP induced A549 cell apoptosis. Furthermore, the number of untreated A549 cells at the G1 phase was 67%, which was much lower than those of CLP-treated groups ($P < 0.05$). In other words, CLP obviously caused an accumulation of A549 cells at the G1 phase and decreased in the S phase in a concentration-dependent manner ($P < 0.05$).

3.5. Effects of CLP on the Expression of Ras/PI3K/AKT Signals. At 48 h after CLP treatment, the expression levels of Ras, PI3K, AKT, and pAKT were measured by Western blot analysis. In Figure 4, CLP significantly inhibited the expression levels of Ras, PI3K, AKT, and pAKT compared with the control group ($P < 0.05$). However, there were no significant differences of PTEN expression among the control and CLP-treated groups ($P > 0.05$). Therefore, the proapoptotic effect of CLP on lung cancer cells did not depend on the activation of PTEN. Furthermore, after CLP treatment, the expression of Bcl-2, caspase-9, and caspase-3 was significantly reduced but the levels of Bax, clv-caspase 9, and clv-caspase 3 were significantly increased with the increase of CLP concentration ($P < 0.05$).

Since the results of flow cytometry assay showed the cell cycle arrest at the G1 phase induced by CLP, the effect of CLP on the expression of cyclin D1 and CDK4, which were critical for the G1/S transition, was further examined. The results in Figure 4 displayed that the expression of cyclin D1 and CDK4 in the CLP-treated groups was significantly decreased compared with that in the control group ($P < 0.05$), supporting the G1/S arrest of cell cycle exposed by CLP. Notably, the expression of GSK-3β, which was related to stabilization of cyclin D1, was significantly upregulated after CLP treatment, indicating that CLP arrested A549 cells at the G1 phase via mediating the GSK-3β/cyclin D1/CDK4 pathway.

3.6. Antitumor Effects of CLP In Vivo. The growth of orthotopic tumor was monitored using the IVIS Lumina LT imaging system. Tumor volumes and weights were represented by radiance. As shown in Figure 5, the tumor growth of the model group was very fast, especially on the 17th day after challenge. As shown in Figure 5, the volumes of tumor in the model group were much higher than the lung. However, both tumor volumes and weights in the CLP-treated groups were much less than those in the model group ($P < 0.05$), indicating that CLP could effectively inhibit tumor growth after 15 days of treatment. Moreover, the tumor volumes and weights in the CLP-H group were significantly decreased compared with those in the DDP group ($P < 0.05$), indicating the stronger potential of CLP on the inhibition of tumor growth in vivo. Interestingly, no significant change was observed in the body weights of tumor-bearing mice between the model and DDP group, while the mice in the CLP-treated group gained more weights than those in the model group ($P < 0.05$). Therefore, CLP would be less toxic to the mice than DDP.

TUNEL staining was used to visualize the cell apoptosis in tumor tissue. As shown in Figure 6, the apoptotic cells (marked as green) hardly existed in the model group, while tumor tissue from the CLP-treated group exhibited a higher percentage of apoptotic cells compared with that from the model group. Therefore, CLP inhibited tumor growth by the augmentation of apoptotic tumor cells.

The tumor cells characterized with markedly large nuclei were aligned tightly and irregularly in the tissues of the model group. However, after CLP treatment, the adhesion of human lung adenocarcinoma cells disappeared and

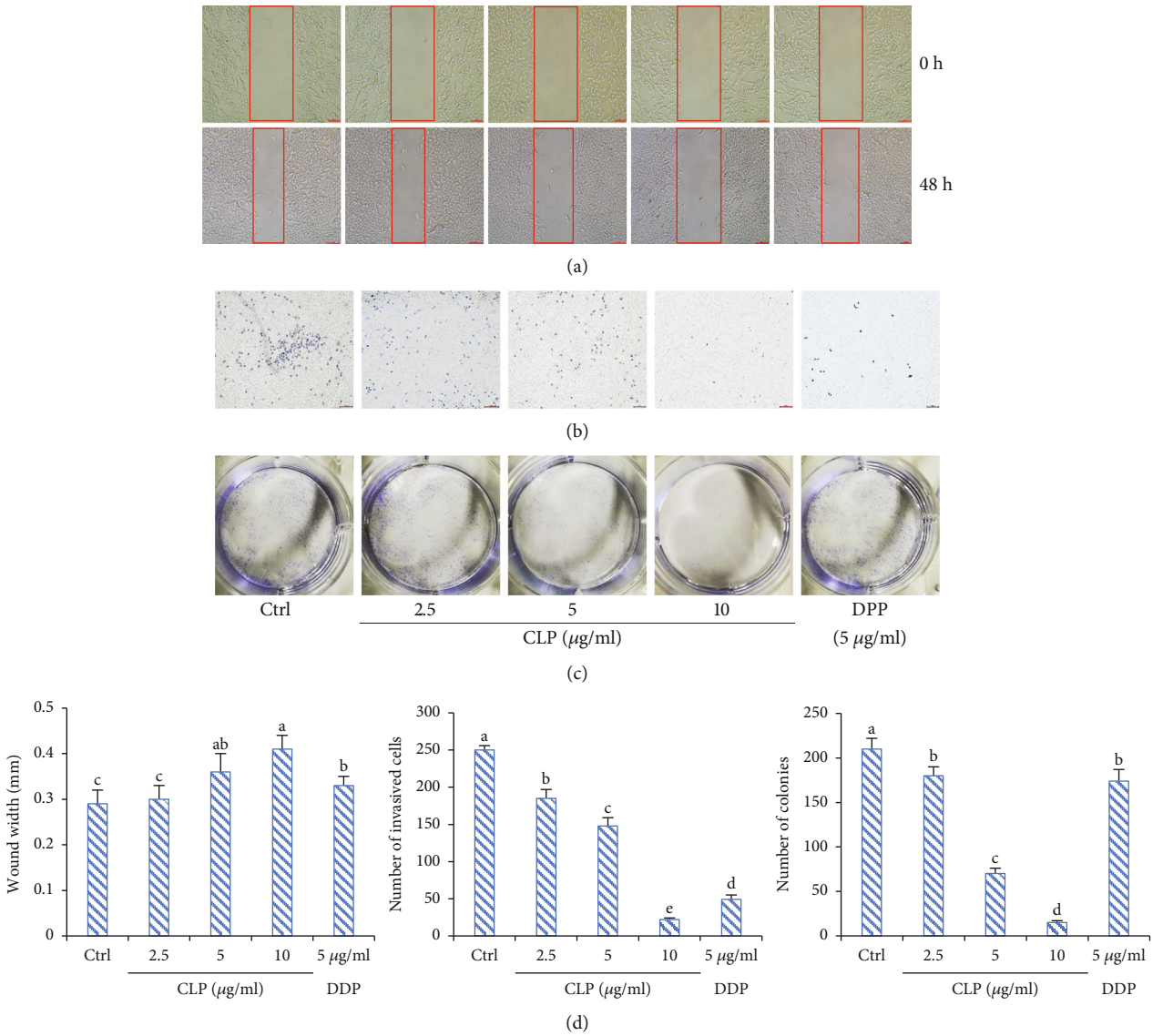


Figure 2: Effect of CLP on the migration, invasion, and colony formation of A549 cells. (a) Cell migration was determined by a wound healing assay. (b) Cell invasion was determined by a Transwell assay. A549 cells were treated with CLP (2.5, 5, and 10 μg/mL) or DDP (5 μg/mL) for 48 h, respectively. (c) The number of colonies of the A549 cells, which were treated with CLP (2.5, 5, and 10 μg/mL) or DDP (5 μg/mL) for 7 days, was counted under low-magnification light microscope (×100). (d) The quantitative results of migration, invasion, and colony formation assay. Data were expressed as means ± SD. The groups marked with different letters suggested significant differences, $P < 0.05$.

separated from the surrounding cells, the cell volume was reduced, and the nucleoplasm was condensed. In addition, the immunohistochemistry results displayed that the tumor tissues in the CLP-treated groups presented the low brown expression of Ki-67, an antigen indicating the proliferative state of active tumor cells, while those in the tumor tissues of the model group were comparatively high (Figure 7). Similarly, the expressing profiles of pAKT in the tumor tissues were consistent with those of Ki-67. All these results demonstrate that CLP effectively inhibited the growth of A549 cells *in vivo*.

3.7. Antibacterial Activity of CLP. In Figure 8, the α community richness among the model and CLP-treated groups was considered to assess the effects of CLP on lung dysbiosis in A549 tumor-bearing mice. The levels of 3 indexes (ACE, Chao1, and Shannon), which reflected the microbiota diversities, were significantly increased in the CLP-treated groups as the dose increased compared with those in the model group ($P < 0.05$). The levels of Simpson in CLP-treated groups were decreased compared with those in the model group, but the Simpson levels were significantly different among the CLP-treated groups. Furthermore, the relative abundances of microbes in *Veillonella*, *Streptococcus*, and *Megasphaera* families in the lung tissues of A549 tumor-bearing mice were much higher than those in the CLP-treated mice ($P < 0.05$), while the relative abundances of *Alloprevotella* and *Actinomyces* in the model mice were remarkably lower than those in the CLP-treated mice

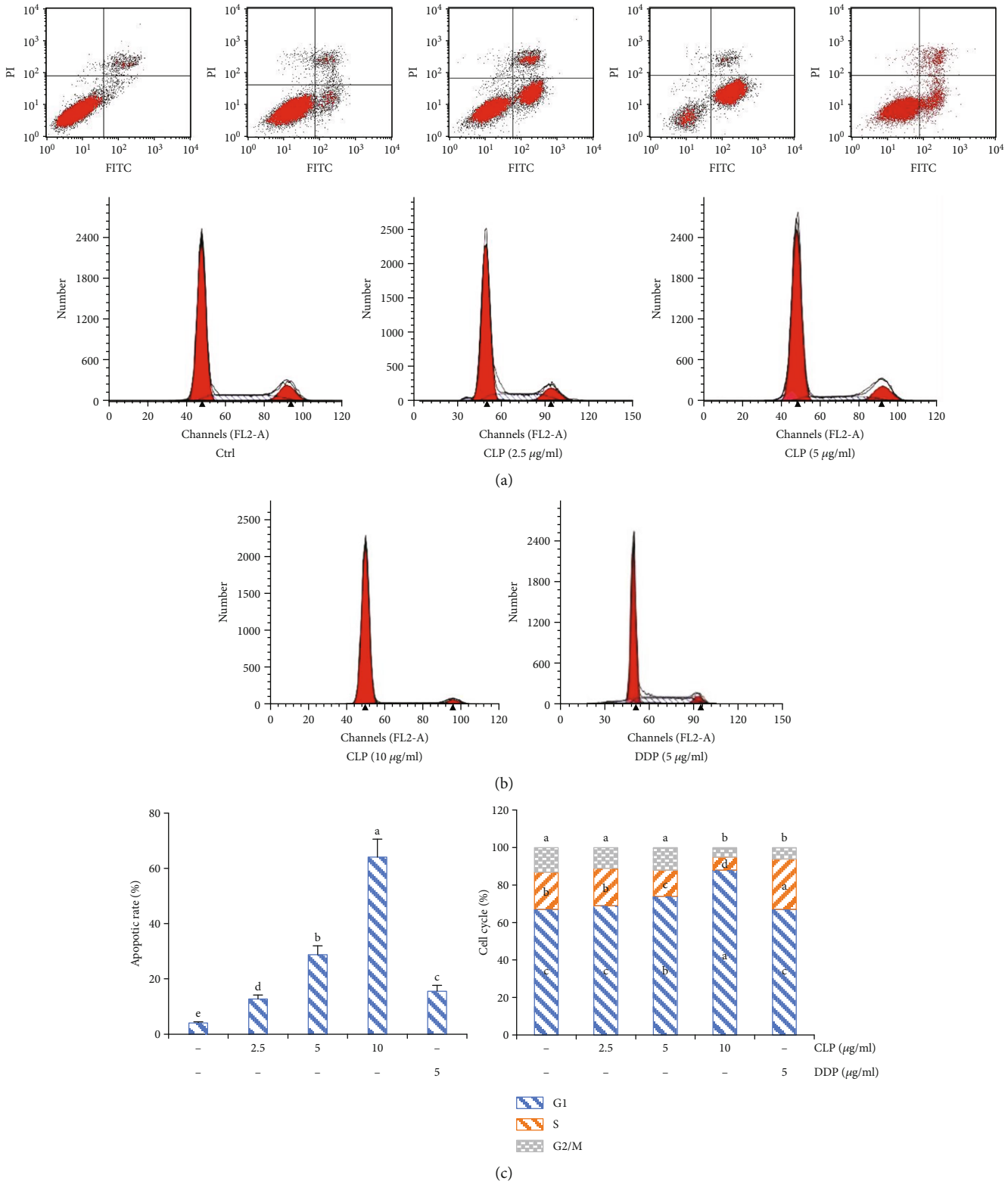


Figure 3: CLP induced the apoptosis of A549 cells. (a) The effects of CLP on A549 cell apoptosis. (b) Effect of CLP on cell cycles of A549 cells. Flow cytometry was used to detect apoptotic rates and cell cycles of A549 cells. Data were expressed as means ± SD. The groups marked with different letters suggested significant differences, $P < 0.05$.

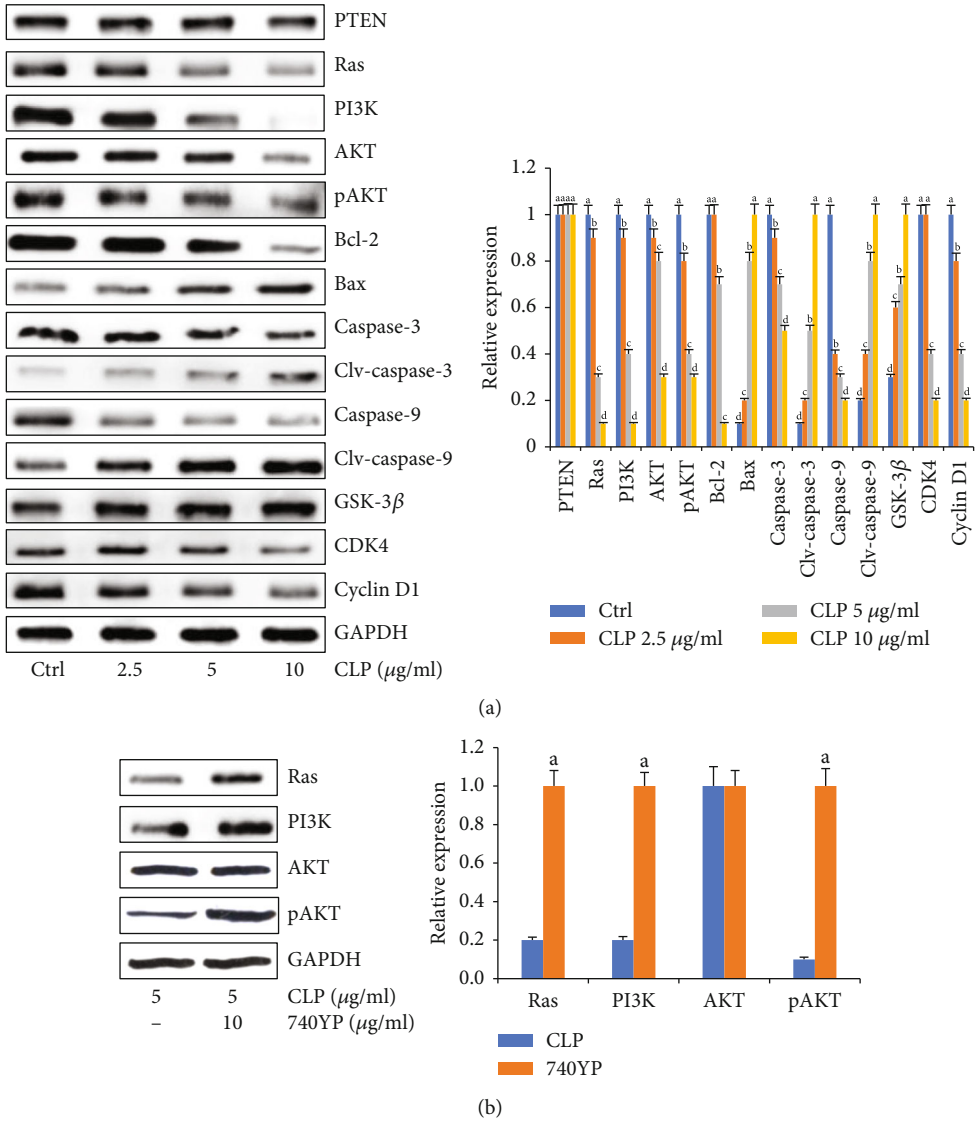


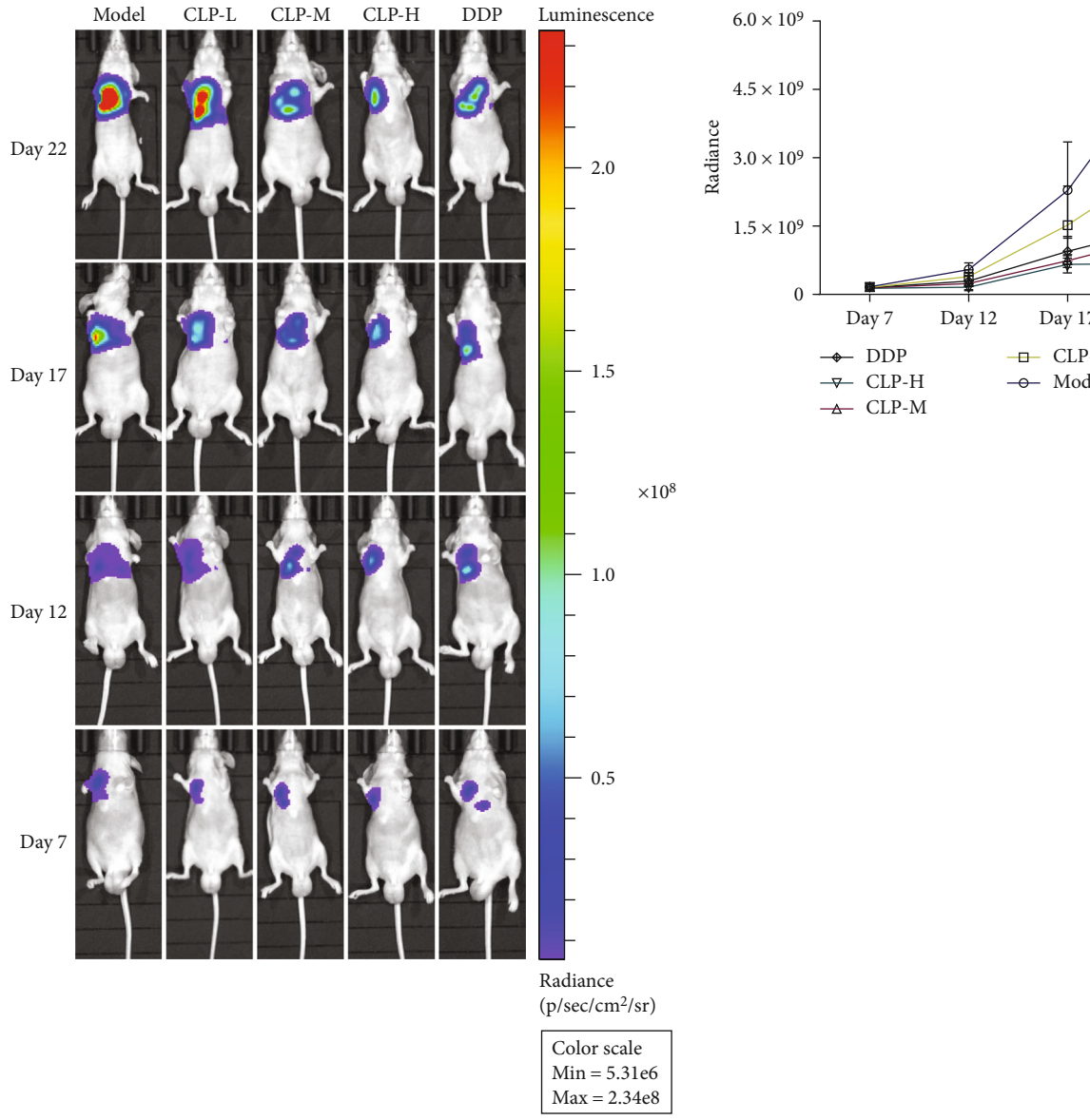
Figure 4: Effects of CLP on the protein expression of the Ras/PI3K/AKT pathway. (a) Representative bands of key protein expression of the Ras/PI3K/AKT pathway (left). The quantitative results of protein expression in each group (right). (b) PI3K activator 740YP reversed the inhibition of CLP on the protein expression of the Ras/PI3K/AKT pathway (left). The quantitative results of protein expression in each group (right). Data were expressed as means ± SD. The groups marked with different letters suggested significant differences, $P < 0.05$.

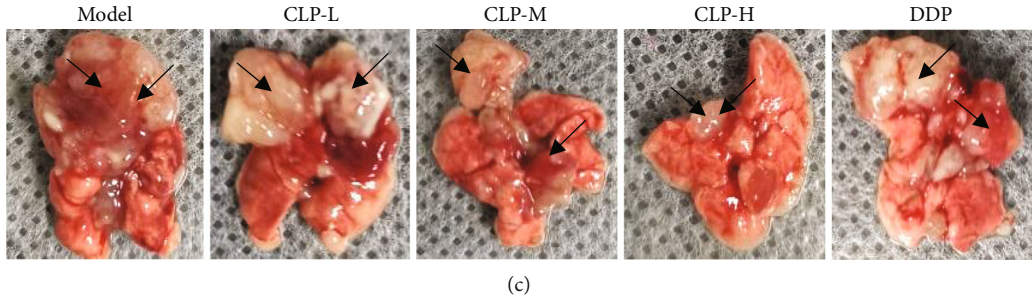
($P < 0.05$). Therefore, CLP improved the lung dysbiosis of the mice with lung cancer.

CLP significantly inhibited the growth of *S. pyogenes* and *S. aureus* with MIC values of 1.94 and 2.37 mg/mL and MBC of 1.94 and 4.74 mg/mL. Furthermore, the diameters of bacteriostatic zones of CLP were 12 and 7 mm. These results suggested the potential antibacterial activities of CLP, which would contribute to its regulation on lung dysbiosis induced by cancer cells.

4. Discussion

Lung cancer remains the most common malignancies globally. Its molecular mechanisms have been widely studied, and the need for novel therapeutic approaches is also growing. Although platinum- or tyrosine kinase inhibitor-based chemotherapy has been the standard protocol for treating lung cancer, the chemotherapy efficacy is greatly limited by the drug resistance and toxic effects [33–35]. Traditional Chinese medicine combined with chemotherapy has been frequently used to prevent the lung cancer, which could improve the prognosis and decrease the complications of the patients [8]. The efficacy and safety of these adjuvant therapies (such as kanglaite injection, kushen injection, Feiyanning, Javanica oil, and *Astragalus* extract) have been scientifically evaluated [36–38]. Therefore, a therapy should be developed to explore plant-derived compounds with high efficacy, low toxicity, and novelty mechanisms. The herb *C. lanceolata* root contains many bioactive components, including polyphenols, saponins, alkaloids, and polysaccharides [9]. Among these compounds, polyacetylenes were the characteristic and main effective constituents of





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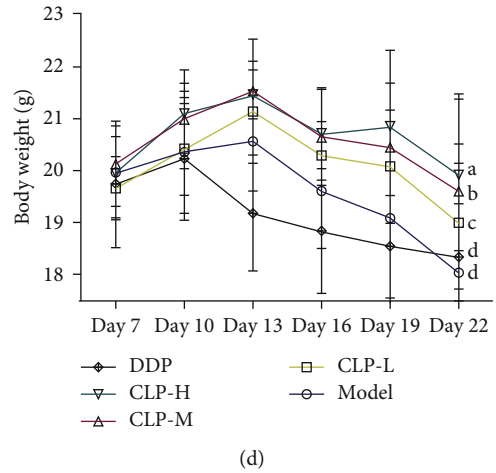

(d)

Figure 5: Antitumor effects of CLP on the A549 tumor-bearing mouse model. The nude mice were randomly divided into five subgroups as follows: model, treated with saline; CLP-L, treated with 10 mg/kg of CLP; CLP-M, treated with 20 mg/kg of CLP; CLP-H, treated with 40 mg/kg of CLP; and DDP, treated with 5 mg/kg of DPP. (a) Tumor growth was monitored every 5 days by using the IVIS Lumina LT imaging system. (b) Tumor growth in the mouse model. (c) Morphological observation of tumor tissue (black arrows). (d) Body weight changes. Data were expressed as means ± SD. The groups marked with different letters suggested significant differences, $P < 0.05$.

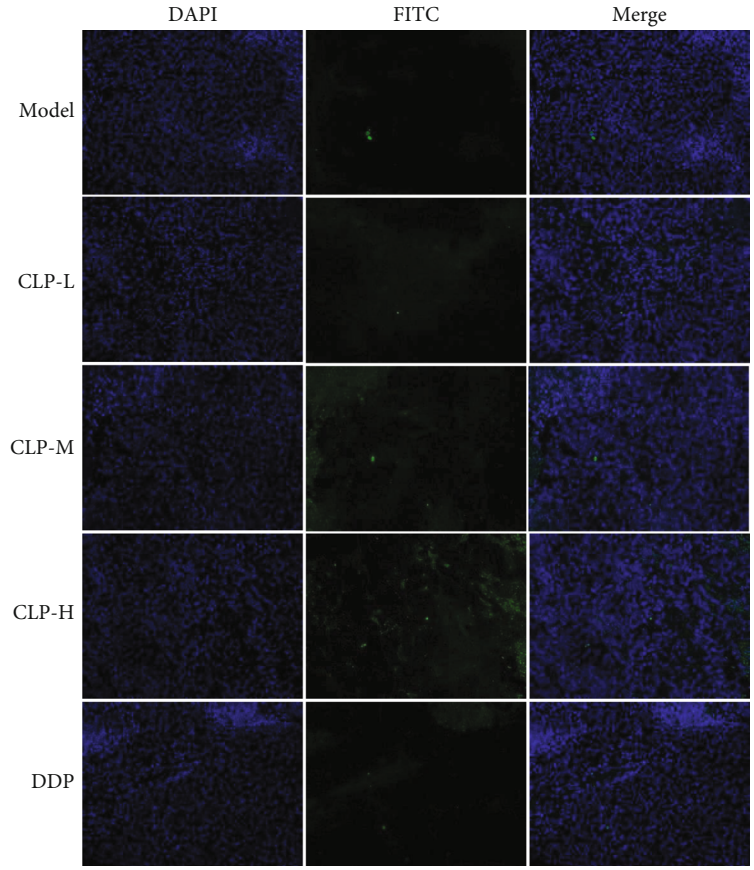

Figure 6: Effects of CLP on the cell apoptosis of tumor tissues in A549 tumor-bearing mice. TUNEL assay was used to detect apoptotic cells in tumor tissue sections. All images were taken at ×200 magnification.

Codonopsis species. Although lobetyolin, a maker polyacetylene glycoside in *C. lanceolata*, reduces hepatic XO activity and inhibits the tumor growth of H22 hepatoma cell xenografts in mice [39, 40], the antitumor activities of *C. lanceolata* and its exact mechanisms have been largely unknown. In the study, for the first time, six compounds (syringin, codonopilodiynoside A, lobetyol, isolariciresinol, lobetyolin, and atractylenolide III) were isolated and identified in the

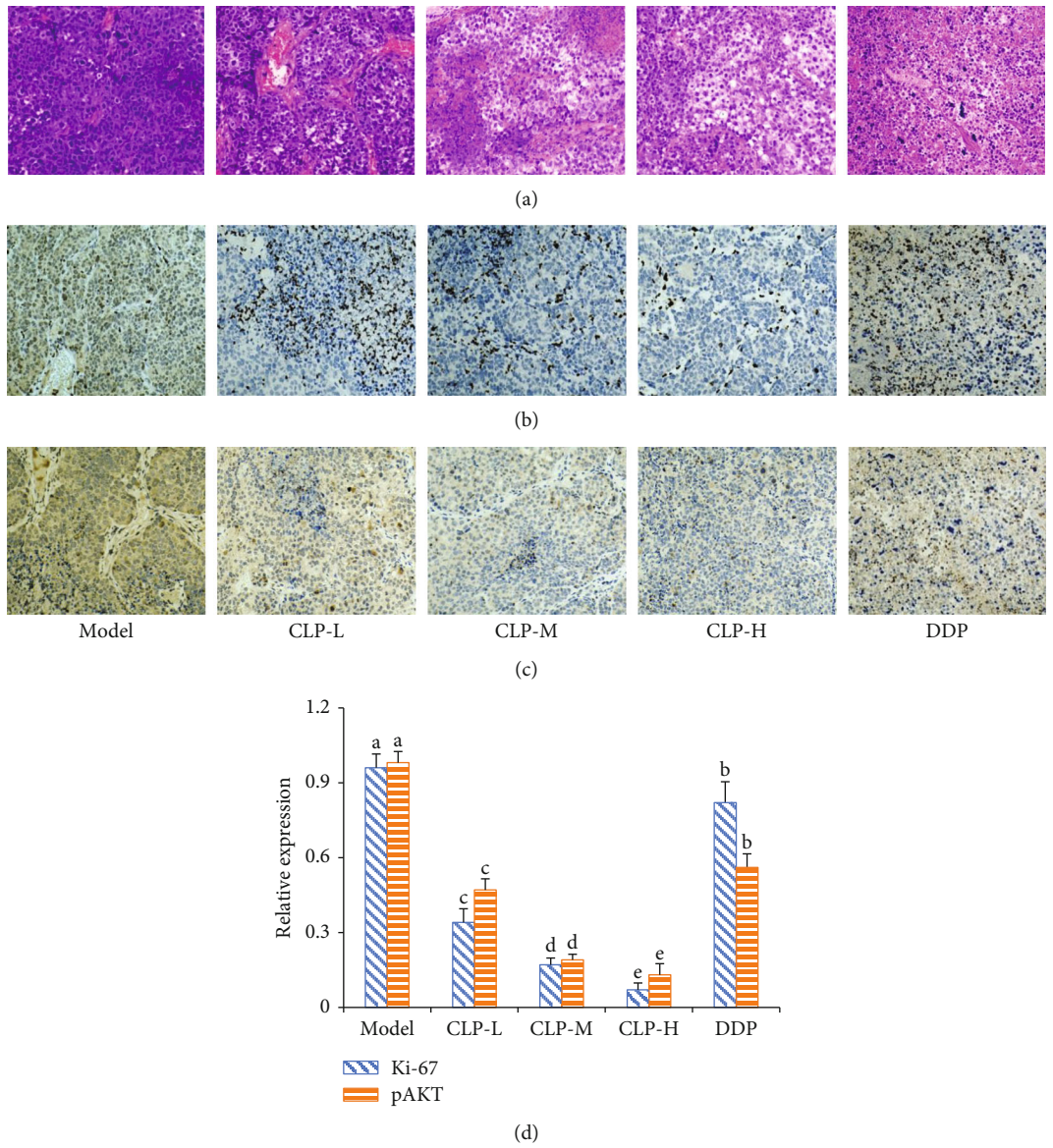

Figure 7: Effects of CLP on cell proliferation in the tumor tissues of A549 tumor-bearing mice. (a) Representative images of HE staining after treatment with CLP. (b) Immunohistochemistry assay was used to detect the proliferative levels of Ki-67 and pAKT in tumor tissue sections. All images were taken at ×200 magnification. (c) Relative expression of Ki-67 and pAKT in tumor tissue sections. Data were expressed as means ± SD. The groups marked with different letters suggested significant differences, $P < 0.05$.

effective fraction of *C. lanceolata* (i.e., CLP). After comparison among the contents and *in vitro* antitumor activities of each compound, lobetyol and lobetyolin contributed approximately 71% to the effects of CLP on lung cancer cell proliferation, indicating that they were the main anticancer compounds in *C. lanceolata*. Then, we investigated the therapeutic potentialities and mechanisms of CLP on A549 cells *in vitro* and *in vivo*. The results showed that CLP obviously suppressed the proliferation, migration, and invasion of A549 cells in the dose- and time-dependent manners. It also inhibited tumor growth in A549 nude mouse xenografts. However, it did not reduce the proliferation of human normal lung epithelial BEAS-2B cells, suggesting its low cytotoxicity.

The proteins involved in the PI3K/AKT pathway are abnormally expressed in human cancers, which participate in every process within cancer cells [41, 42]. PI3K could be activated by Ras and subsequently transduces intracellular signaling by directly binding with the pleckstrin homology domains of various proteins and participates in many physiological processes including cell cycle regulation, DNA repair, cell apoptosis, and glycometabolism [43–45]. AKT inhibits proapoptotic Bcl-2 family members Bax, phosphorylates GSK-3β, and negatively regulates caspase-9, which then cleave and activate caspase-3, thereby increasing the expression of antiapoptotic components and cell survival signals [46]. In the study, we found that CLP could not impact the levels of PTEN but could significantly downregulate the levels of Ras, PI3K, and pAKT in A549 cells, indicating that CLP acted as an inhibitor by inactivation of the Ras/PI3K/AKT pathway. Moreover, CLP significantly

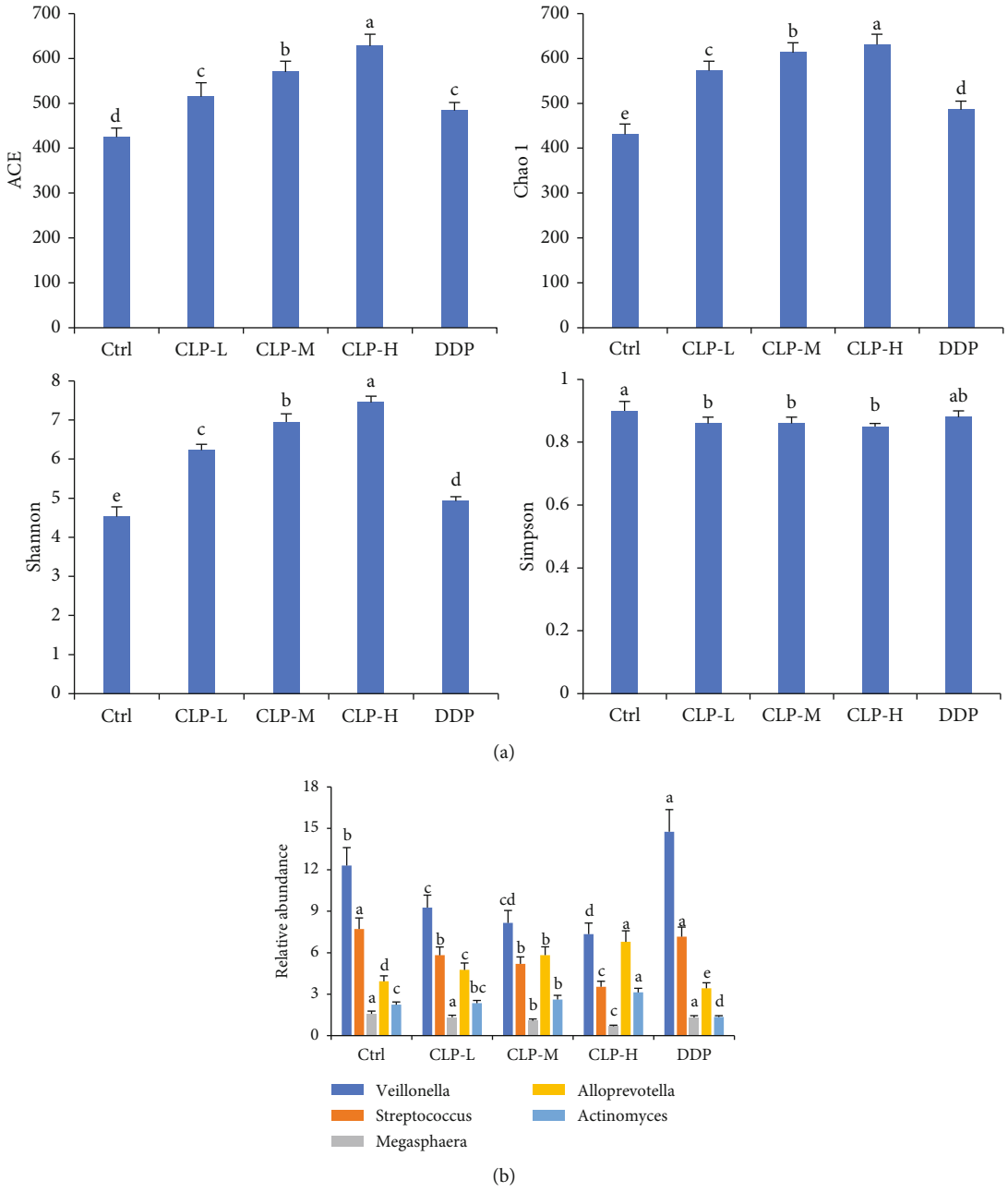

Figure 8: Effects of CLP on lung microbiota of A549 tumor-bearing mice. (a) Alpha diversities of lung microbiota. ACE, chao, Shannon, and Simpson indicated the diversities of lung microbiota. (b) Relative abundance of the differently expressed microbiota at the family levels. Data were expressed as means ± SD. The groups marked with different letters suggested significant differences, $P < 0.05$.

upregulated levels of Bax, clv-caspase-9, and clv-caspase-3 but downregulated the levels of Bcl-2, caspase-9, and caspase-3 in A549 cells. However, all those profiles could be reversed by PI3K activator 740YP. Therefore, CLP induced the apoptosis of A549 cells by modulating the Ras/PI3K/AKT pathway.

In addition, cell cycle is an essential component involved in the processes of cell proliferation. Uncontrolled cell proliferation is one of the salient features of cancer [47, 48]. Cyclin D1 is an abnormally expressed maker in cancers which promotes the G1 to S phase transition by binding to CDK4 [49]. Photophosphorylation of AKT inhibited the activation of GSK-3β, resulting in the stabilization of cyclin D1 [50]. In the study, cell cycle was significantly arrested at the G1 phase in a dose-dependent way after CLP treatment. CLP obviously increased the expression of GSK-3β but reduced the levels of cyclin D1 and CDK4 in A549 cells. In addition, in the A549 nude mouse xenograft model, Ki-67 staining results indicated that CLP markedly decreased the proliferative state of cells in tumor tissue sections. Therefore, the antiproliferative effects of CLP would be consistent with the arrest of the G1 phase.

Emerging evidence had showed that lung microbiota plays crucial roles in pathogenesis and progression of lung

cancer [51–53]. On one hand, the respiratory bacterial load as well as changes in the bacterial community contributes to tumor cell proliferation, survival, and tissue invasion. Specifically, ACE, Chao1, and Shannon are three common indices reflecting microbiota diversity and richness, while there was a negative correlation between the Simpson level and microbiota diversity. The results showed that CLP treatment could increase the levels of ACE, Chao1, and Shannon but could reduce the Simpson level, indicating that CLP improved lung dysbiosis through increasing microbiota diversity. On other hand, it has been known that the lower airways of patients with lung cancer were enriched with oral pathogenic bacteria *Veillonella*, *Prevotella*, and *Streptococcus* [54–57]. Interestingly, CLP not only decreased the abundance of those oral pathogenic commensals in the lung tissues of tumor-bearing mice but also directly inhibited the growth of *S. pyogenes* and *S aureus*, which are common lung pathogenic bacteria in patients with lung cancer [58, 59], indicating its antimicrobial potential on tumor-related dysbiosis. But its underlying antimicrobial mechanism would be further considered.

In summary, CLP inhibited proliferation and induced apoptosis of A549 cells, which were arrested at the G1/S phase, and suppressed growth of lung cancer in the nude mouse xenograft models. It also significantly upregulated the expression of Bax, GSK-3β, clv-caspase-9, and clv-caspase-3 and downregulated the expression of Ras, Pi3K, pAKT, cyclin D1, CDK4, Ras, Bcl-2, caspase-9, and caspase-3 in A549 cells, which all were reversed by the PI3K activator. But CLP hardly altered the expression of PTEN. Thus, it indicated that CLP induced apoptosis of A549 cells via regulating the Ras/PI3K/AKT pathway. Moreover, CLP exerted antibacterial activities in vitro and improved the lung dysbiosis of tumor-bearing mice. It could be a therapeutic candidate for the prevention and treatment of human lung cancer.

Data Availability

The data is available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Bibliometric Analysis of the Scientific Literature on Rheumatoid Arthritis-Associated Interstitial Lung Disease

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Background. In recent years, the number of studies on rheumatoid arthritis-related interstitial lung disease (RA-ILD) has been increasing, which has led to many publications on this topic. Our purpose is to identify research trends in RA-ILD and analyze the most-cited RA-ILD-related high-quality scientific publications. *Methods*. All publications on RA-ILD in the Core Collection database of Web of Science were searched. The publication year, country, institution, total citations, and journal were extracted and analyzed. We used VOSviewer software or an online bibliometric analysis platform for cooccurrence analysis of the keywords, institutions, and countries involved. The 100 most frequently cited RA-ILD publications were analyzed. *Results*. In total, 596 publications related to RA-ILD were obtained. Over time, the frequency of RA-ILD publications has increased. Globally, the United States provides the most publications on RA-ILD ($n = 195$). The institution with the highest publication output was the Mayo Clinic ($n = 43$). The journal "Annals of the Rheumatic Diseases" published most with 93 articles and received 338 citations. A clinical description was the most common research topic in RA-ILD-related publications. *Conclusions*. In recent years, there has been an increasing number of studies on RA-ILD, and related publications have increased rapidly. This study is the first bibliometric study of RA-ILD-related publications. It can be used as a guide for clinicians and can help researchers choose research directions of interest in this field.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by articular and extra-articular manifestations affecting approximately 1-2% of the general population [1]. Interstitial lung disease (ILD) is an extra-articular manifestation of RA, which occurs frequently in up to 80% of patients with RA. This may be the result of chronic immune activation and inflammation in RA, or the pulmonary toxicity caused by immunomodulatory drugs used to treat RA [2–6]. The prevalence of RA-ILD ranges from 1% to 58%, depending on the diagnostic means used and the severity in the RA population studied [6, 7]. Currently, ILD is the second leading cause of death in patients with RA after cardiovascular disease [8]. Research on RA-ILD has increased to include its natural history, pathogenesis, radiological evaluation, clinical manifestations, and treatment [9–12]. However, the trend of RA-ILD research is unclear, and the most influential research in this field has not been systematically determined. Therefore, our purpose was to provide a bibliometric study of publications on RA-ILD.

Bibliometric analysis is a convenient and reliable statistical method that can quantitatively and qualitatively evaluate research trends in the research field. This analysis has long been used in the field of medical research and has been widely accepted by scientific researchers [13–15]. To the best of our knowledge, no bibliometric studies on RA-ILD have been published to date. Therefore, in this study, we use

bibliometric statistical methods to identify the most influential publications and analyze the research status and trends in the RA-ILD research field.

2. Materials and Methods

2.1. Datasource. All the data of this study were obtained from articles retrieved from the core collection database of Web of Science on July 1, 2021.

2.2. Search Strategy. The retrieval steps and strategies were as follows: Title = rheumatoid arthritis AND Title = (interstitial lung disease OR interstitial pneumonia) AND Language = English AND Document type = (review OR article) AND Time span =1980 to 2021.

2.3. Statistical Tools. VOSviewer, an online bibliometric analysis platform (https://bibliometric.com), and Excel software were used to extract and analyze all data. VOSviewer is a software that is usually used to visually analyze the collaborative network between countries, institutions, and authors and cocitation of keyword clusters to analyze research trends and hotspots. The role of the online bibliometric analysis platform is similar to that of VOSviewer. Excel software was used to extract and analyze various details of the publication, including author, title, journal, year of publication, institution, country, journal impact factors, and number of total citations.

2.4. Data Extraction. According to the retrieval steps and strategies, the two authors independently fetched the article information and discussed the differences until they reached a consensus. Data were obtained from the core collection database of Web of Science, and the publication information was extracted and analyzed using Excel, online bibliometric analysis, and VOSviewer software.

3. Results

3.1. Publication Analysis. A total of 596 RA-ILD research articles were found in the core collection database of the Web of Science. The number of articles increased from 1981 to 2021 (Figure 1(a)). Quantitative analysis shows that in the past 10 years, global research on RA-ILD has increased rapidly, from four articles from 1981 to 1985 to 326 articles from 2016 to 2020. This result shows that RA-ILD has attracted increasing attention, and the research process of RA-ILD continues to accelerate.

3.2. Countries Analysis. These articles cover 46 countries and regions. Globally, the United States (US) published the most studies ($n = 195$), followed by Japan ($n = 105$), the United Kingdom ($n = 66$), Spain ($n = 58$), China ($n = 49$), Italy ($n = 40$), South Korea ($n = 32$), France ($n = 23$), Mexico ($n = 22$), and Canada ($n = 14$) (Figures 1(b) and 1(c)).

The online bibliometric analysis platform was used to analyze cooperative relations between countries. The visual analysis shows that the USA has always been the center of RA-ILD research in the world, and Japan, France, China, and South Korea have been found to be potential research powers. (Figure 1(d)).

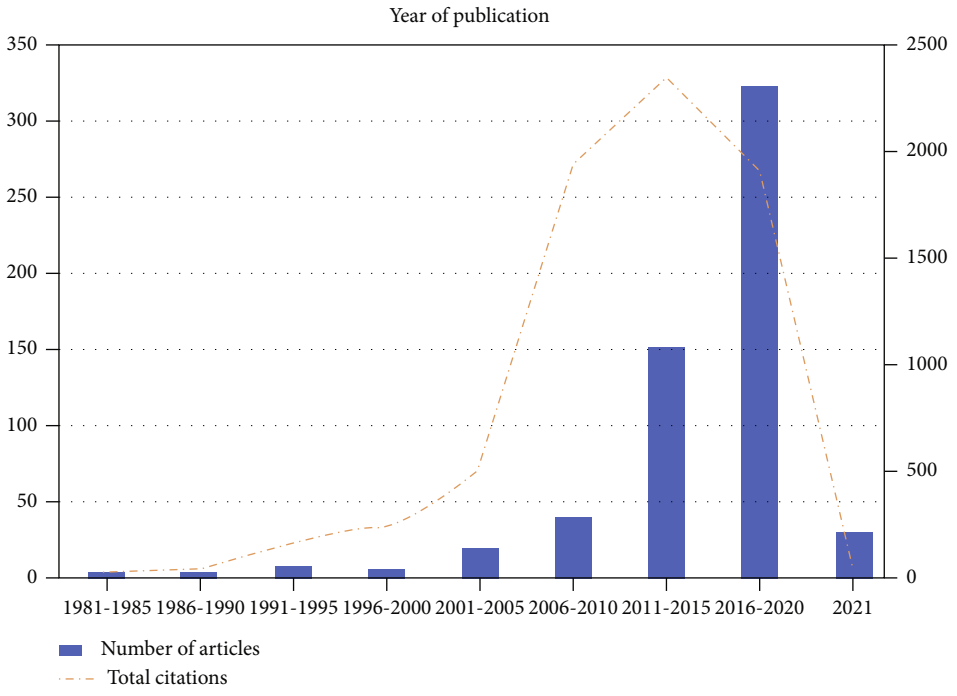
3.3. Institutions Analysis. All the publications involve 1000 institutions. The results show that many institutions in the United States actively participate in RA-ILD research. The 10 most productive institutions internationally were Mayo Clinic ($n = 43$), National Jewish Health ($n = 41$), Brigham and Women's Hospital ($n = 31$), Colorado State University ($n = 31$), University of California, San Francisco ($n = 19$), University of Ulsan ($n = 19$), University of Modena and Reggio Emilia ($n = 17$), Harvard Medical School ($n = 15$), Queen Elizabeth's Hospital ($n = 15$), and University of Miami ($n = 15$), respectively (Figure 2(a)). According to the citation report, Mayo Clinic's articles were cited the most, namely, 1408 times, followed by the National Jewish Health, which was cited 858 times, and the University of California, San Francisco, 851 (Figure 2(b)).

VOSviewer software was used to analyze the extent of cooperative relations between institutions. The institution with the most links, i.e., the highest link strength was recorded by the National Jewish Health Organization ($n = 131$), followed by the University of Colorado ($n = 123$), Mayo Clinic ($n = 119$), and Brigham and Women's Hospital ($n = 117$). In the VOSviewer software, the width of the line reflects the close relationship of interinstitution cooperation. The National Jewish Health had close collaborations with the University of Colorado and Mayo Clinic. Mayo Clinic had large collaborations with National Jewish Health, University of Colorado, Brigham and Women's Hospital, and Harvard Medical School (Figure 2(c)).

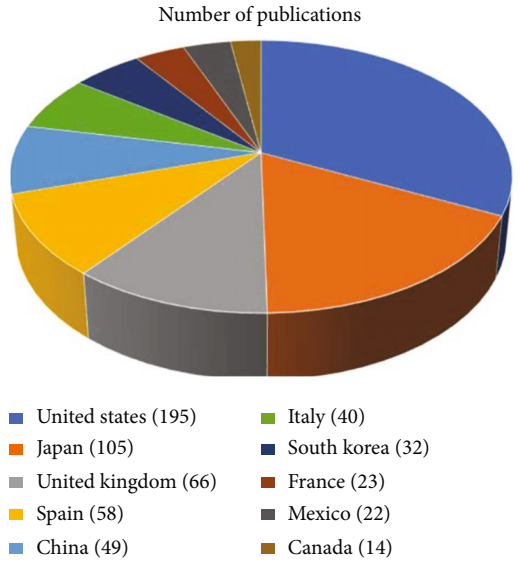
3.4. Journals Analysis. All 596 articles in this study were published in 123 journals. Among these, the journals which had published at least 20 articles on the topic accounted for 73.8% of the total (Table 1). The five journals with the most articles on the topic were Annals of the Rheumatic Diseases, Arthritis & Rheumatology, American Journal of Respiratory and Critical Care Medicine, Rheumatology, and Arthritis and Rheumatism. Moreover, articles in the Annals of the Rheumatic Diseases have been cited the most. More than 20 journals published on RA-ILD, and the average impact factor was 11.8, indicating a high level of reliability of the included studies.

3.5. Research Status and Analysis. VOSviewer software was used to analyze the cooccurrence analysis of keywords in the RA-ILD research articles. When the minimum number of keywords appearing in the publication was set to five, 72 keywords were selected and divided into four clusters:

"Clinical-Features," "Pathological-Features," "Treatment," and "Prevalence and mortality." In the "Clinical-Features" cluster, the most common keywords were "pneumonia," "idiopathic pulmonary-fibrosis," and "prognosis." In the "Pathological-Features" cluster, the most frequent keywords were "rheumatoid arthritis," "interstitial lung disease," and "fibrosis." In the "treatment" cluster, the most frequent keywords were "classification," "criteria," and "safety." In the "prevalence and mortality" cluster, the most



(a)



(b)

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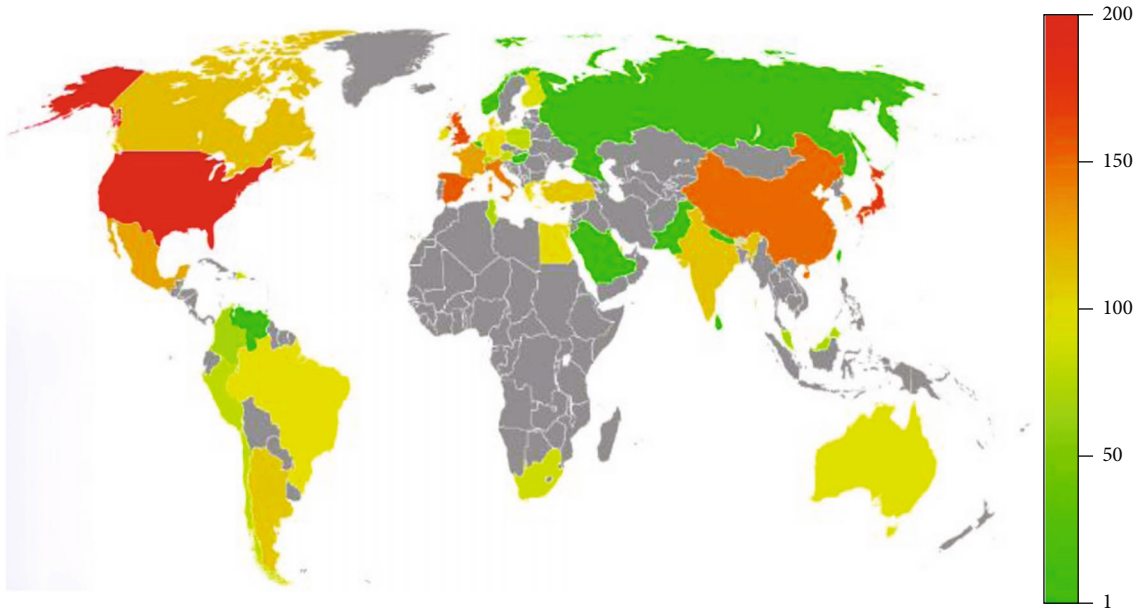

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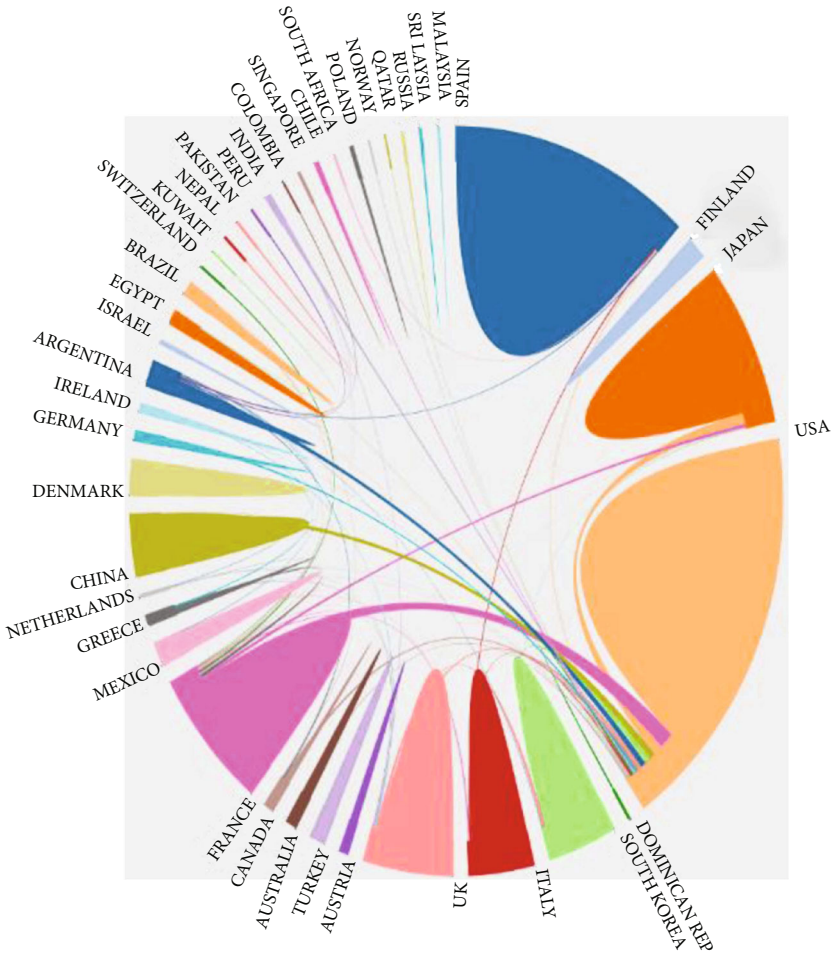


(d)

Figure 1: Overview of publications. (a) Number of publications and citations from 1981 to 2021. (b) Sources of publications. (c) Top 10 countries. (d) International collaborations.

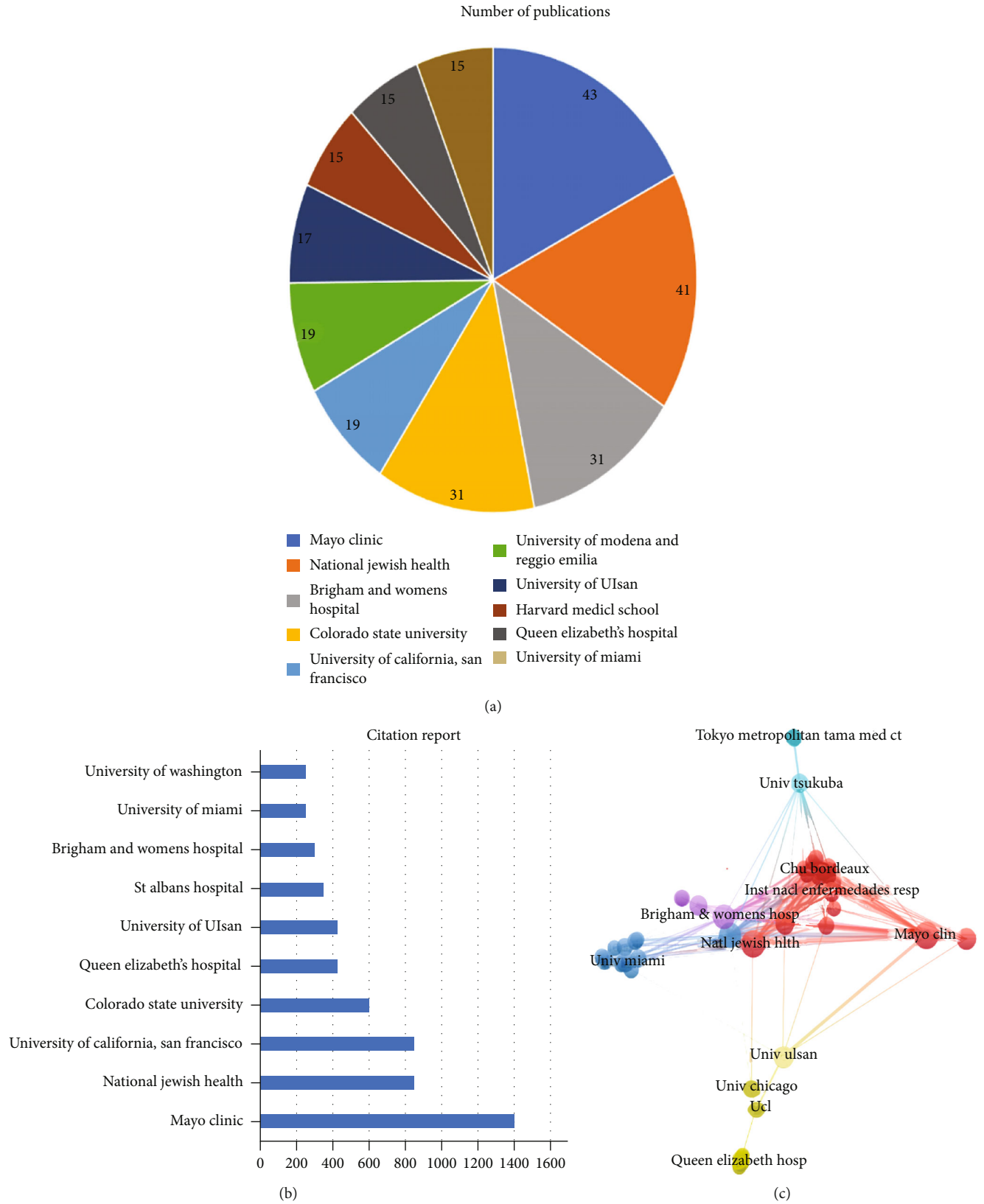


Figure 2: Highest impact institutions. (a) The top 10 institutions (publications). (b) The top 10 institutions (citations). (c) Institutional collaborations.

Table 1: Active journals on rheumatoid arthritis-associated interstitial lung disease.

Journal	Article	Total citation	Mean citation	Impact factor
Annals of the Rheumatic Diseases	93	338	3.63	19.103
Arthritis & Rheumatology	75	219	2.92	10.995
American Journal of Respiratory and Critical Care Medicine	53	514	9.70	21.405
Rheumatology	38	710	18.68	20.543
Arthritis and Rheumatism	36	552	15.33	5.532
European Respiratory Journal	30	537	17.90	16.671
Clinical Rheumatology	18	248	13.78	4.098
Chest	12	485	40.42	2.878
Internal Medicine	11	156	14.18	2.048
Clinical and Experimental Rheumatology	10	98	9.80	4.473
Respiratory Medicine	8	258	32.25	3.772
Modern Rheumatology	8	108	13.50	3.023
Seminars in Arthritis and Rheumatism	6	208	34.67	5.532
Rheumatology International	6	154	25.67	2.631
Journal of Rheumatology	6	147	24.50	4.666
New England Journal of Medicine	6	133	22.17	91.245
PLos one	6	127	21.17	3.24
Scandinavian Journal of Rheumatology	6	104	17.33	3.641
Respirology	6	92	15.33	6.424
JCR-Journal of Clinical Rheumatology	6	26	4.33	3.517

frequent keywords were "prevalence," "mortality," and "risk" (Figure 3(a)).

To better understand the dynamic process of the RA-ILD research trends, we evaluated the evolution of the keywords (Figure 3(b)). We assigned colors based on the year the keyword appears in the article. For example, the yellow keyword appears later than the purple keyword. In the early stages, "idiopathic pulmonary fibrosis," "alveolitis," and "systemic sclerosis" were the main topics. Trends in recent years show that the terms "management," "predictors," "inflammation," and "progress" are becoming more and more popular.

3.6. The 100 Most-Cited Publications. The 100 most-cited publications on RA-ILD were published between 1984 and 2020 (Table 2). The analysis indicated that 2001-2005 was the period when most of these studies were published, with 41 publications, followed by 2016-2020, with 29 publications (Figure 4(a)).

The 100 most-cited articles were from 18 countries and regions. Thirty-four articles were published by authors from the USA, followed by Japan ($n = 20$), the United Kingdom ($n = 11$), China ($n = 7$), Italy ($n = 5$), Canada ($n = 3$), Austria ($n = 3$), Spain, France, and Germany ($n = 2$), and Mexico, Argentina, Australia, Bangladesh, Denmark, Finland, and Ireland ($n = 1$) (Figure 4(b)).

Of these 100 articles, the Mayo Clinic Medicine and National Jewish Health each generated seven publications, resulting in their being the most represented institutions on this topic, followed by the University of California in San Francisco ($n = 5$) and Queen Elizabeth Hospital ($n = 4$) (Figure 4(c)).

Overall, there were 52 different journals which published the 100 articles. "Rheumatology" was the most productive journal, with 8 articles and 632 citations, followed by "Arthritis and Rheumatism," with five articles and 529 citations (Table 3).

When considering the individual authors' academic contributions, Jay H Ryu, provided 11 publications, followed by Joyce C Lee and Eric L Matteson, each with 8 publications (Table 4).

The most common research topic on RA-ILD addressed the clinical description ($n = 44$), followed by clinical research ($n = 13$), diagnosis ($n = 8$), mortality ($n = 7$), and risk factors ($n = 6$) (Figure 4(d)).

4. Discussion

ILD is one of the most common complications of RA and poses a great challenge to clinicians and researchers [16]. The prevalence of RA-ILD ranged from 1% to 58% in the different studies, which was related to the diagnostic techniques used and the study population that was included [17–19]. According to the literature, there are many risk factors for RA-ILD, including male sex, smoking, older age, high disease activity of RA, characteristics of extra-articular diseases (subcutaneous nodules), and seropositive RA autoantibodies (rheumatoid factor and anticitrulline protein antibody) [2, 20–23]. The most common presenting symptoms include exertional dyspnea, tachypnea, and bibasilar inspiratory crackles. In the advanced stages of the disease, symptoms of cyanosis, edema, and pulmonary hypertension may occur, leading to a reduced quality of life [24].

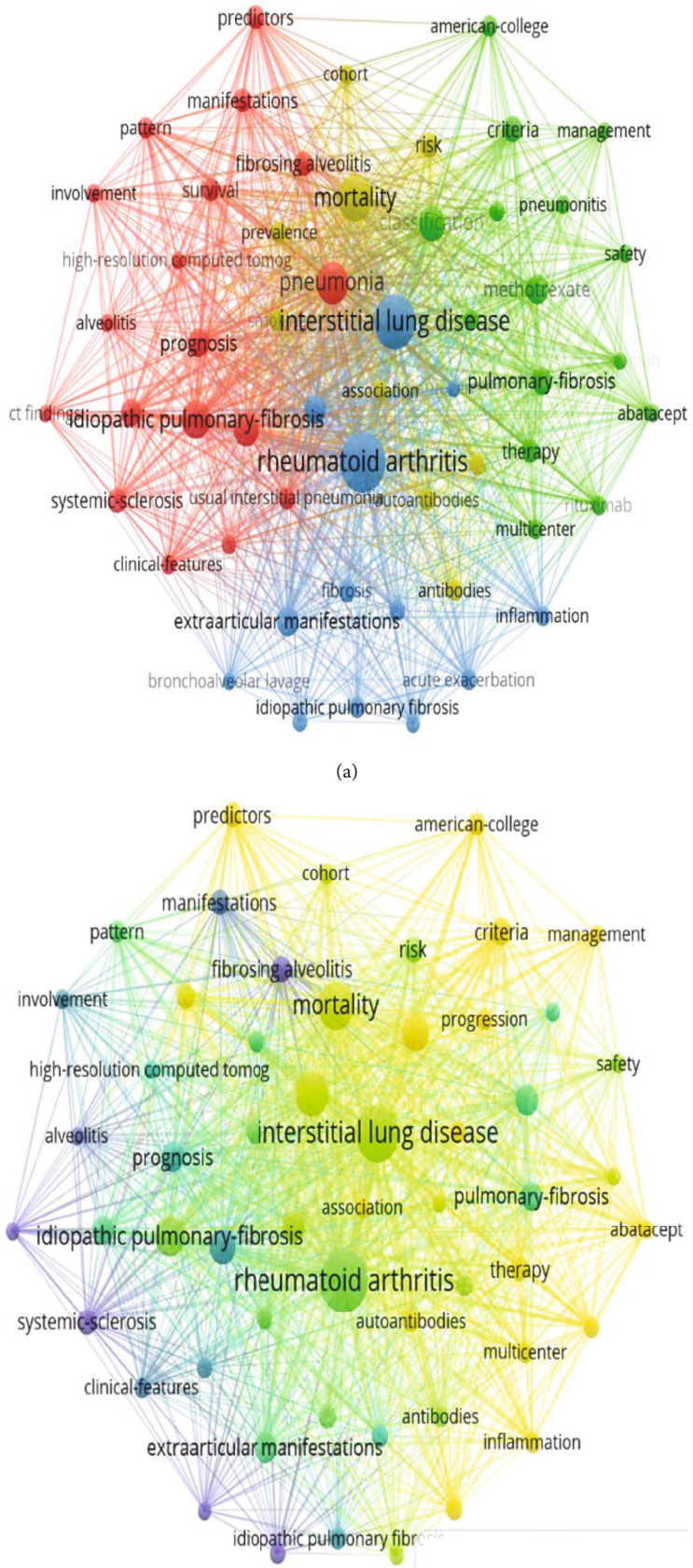


Figure 3: Keyword analysis. (a) Cluster analysis of keywords. (b) Evolution of keyword frequency.

Table 2: The top 100 cited publications on rheumatoid arthritis-associated interstitial lung disease.

Rank	Title	Author	Journal	Year	Total citation	Citation/year
1	Incidence and mortality of interstitial lung disease in rheumatoid arthritis: a population-based study	Bongartz, T.	Arthritis and Rheumatism	2010	324	27
2	Usual interstitial pneumonia in rheumatoid arthritis-associated interstitial lung disease	Kim, E. J.	European Respiratory Journal	2010	283	23.58
3	Histopathologic pattern and clinical features of rheumatoid arthritis associated interstitial lung disease	Lee, H. K	Chest	2005	245	14.41
4	Rheumatoid arthritis-interstitial lung disease-associated mortality	Olson, A. L.	American Journal of Respiratory and Critical Care Medicine	2011	222	20.18
5	Interstitial lung disease in recent onset rheumatoid arthritis	Gabbay, E.	American Journal of Respiratory and Critical Care Medicine	1997	220	8.8
6	Rheumatoid arthritis-related interstitial lung disease: associations, prognostic factors and physiological and radiological characteristics-a large multicentre UK study	Kelly, C. A.	Rheumatology	2014	205	25.63
7	Progressive preclinical interstitial lung disease in rheumatoid arthritis	Gochuico, B. R.	Archives of Internal Medicine	2008	198	14.14
8	Rheumatoid arthritis-associated interstitial lung disease the relevance of histopathologic and radiographic pattern	Kim, E. J.	Chest	2009	189	14.54
9	Interstitial lung disease has a poor prognosis in rheumatoid arthritis: results from an inception cohort	Koduri, G.	Rheumatology	2010	149	12.42
10	Interstitial lung diseases induced or exacerbated by DMARDS and biologic agents in rheumatoid arthritis: A systematic literature review	Roubille, C.	Seminars in Arthritis and Rheumatism	2014	126	15.75
11	Predictors of mortality in rheumatoid arthritis-associated interstitial lung disease	Solomon, J. J.	European Respiratory Journal	2016	124	20.67
12	MUC5B promoter variant and rheumatoid arthritis with interstitial lung disease	Juge, P. A.	New England Journal of Medicine	2018	108	27
13	Influence of anti-TNF therapy on mortality in patients with rheumatoid arthritis-associated interstitial lung disease: results from the British Society for Rheumatology Biologics Register	Dixon, W. G.	Annals of the Rheumatic Diseases	2010	108	9
14	High-resolution computed-tomography of the lungs in patients with rheumatoid-arthritis and interstitial lung-disease	Mcdonagh, J	British Journal of Rheumatology	1994	102	3.64
15	Leflunomide use and the risk of interstitial lung disease in rheumatoid arthritis	Suissa, S.	Arthritis and Rheumatism	2006	95	5.94
16	Different risk factors between interstitial lung disease and airway disease in rheumatoid arthritis	Mori, S.	Respiratory Medicine	2012	89	8.9
17	A population-based cohort study of rheumatoid arthritis-associated interstitial lung disease: comorbidity and mortality	Hyldgaard, C.	Annals of the Rheumatic Diseases	2017	83	16.6
18	Predictors of mortality in rheumatoid arthritis-related interstitial lung disease	Assayag, D.	Respirology	2014	83	10.38
19	Rheumatoid arthritis-associated interstitial lung disease: radiologic identification of usual interstitial pneumonia pattern	Assayag, D.	Radiology	2014	77	9.63
20	Correlation between HRCT findings, pulmonary function tests and bronchoalveolar lavage cytology in interstitial lung disease associated with rheumatoid arthritis	Biederer, J.	European Radiology	2004	74	4.11
21	Association of fine specificity and repertoire expansion of anticitrullinated peptide antibodies with rheumatoid arthritis associated interstitial lung disease	Giles, J. T.	Annals of the Rheumatic Diseases	2014	71	8.88

Table 2: Continued.

Rank	Title	Author	Journal	Year	Total citation	Citation/year
22	Fibrosing interstitial pneumonia predicts survival in patients with rheumatoid arthritis-associated interstitial lung disease (RA-ILD)	Solomon, J. J.	Respiratory Medicine	2013	71	7.89
23	Effect of rituximab on the progression of rheumatoid arthritis-related interstitial lung disease: 10 years' experience at a single centre	Yusof, M. M.	Rheumatology	2017	69	13.8
24	Shared genetic predisposition in rheumatoid arthritis-interstitial lung disease and familial pulmonary fibrosis	Juge, P. A.	European Respiratory Journal	2017	69	13.8
25	Rheumatoid arthritis (RA)-specific autoantibodies in patients with interstitial lung disease and absence of clinically apparent articular RA	Gizinski, A. M.	Clinical Rheumatology	2009	68	5.23
26	Detection of rheumatoid arthritis-interstitial lung disease is enhanced by serum biomarkers	Doyle, T. J.	American Journal of Respiratory and Critical Care Medicine	2015	67	9.57
27	Leflunomide-induced interstitial lung disease: prevalence and risk factors in Japanese patients with rheumatoid arthritis	Sawada, T.	Rheumatology	2009	66	5.08
28	Acute exacerbation in rheumatoid arthritis-associated interstitial lung disease: a retrospective case control study	Hozumi, H.	BMJ open	2013	63	7
29	The lung in rheumatoid arthritis focus on interstitial lung disease	Spagnolo, P.	Arthritis & Rheumatology	2018	62	15.5
30	Abatacept in patients with rheumatoid arthritis and interstitial lung disease: a national multicenter study of 63 patients	Fernandez-Diaz, C.	Seminars in Arthritis and Rheumatism	2018	62	15.5
31	Rheumatoid arthritis (RA) associated interstitial lung disease (ILD)	O'Dwyer, D. N.	European Journal of Internal Medicine	2013	62	6.89
32	Rheumatoid arthritis treatment and the risk of severe interstitial lung disease	Wolfe, F.	Scandinavian Journal of Rheumatology	2007	60	4
33	Rheumatoid arthritis complicated with acute interstitial pneumonia induced by leflunomide as an adverse reaction	Kamata, Y	Internal Medicine	2004	56	3.11
34	Morphologic and quantitative assessment of CD20+ B cell infiltrates in rheumatoid arthritis-associated nonspecific interstitial pneumonia and usual interstitial pneumonia	Atkins, S. R.	Arthritis and Rheumatism	2006	55	3.44
35	Biomarkers of rheumatoid arthritis-associated interstitial lung disease	Chen, J.	Arthritis & Rheumatology	2015	54	7.71
36	Acute exacerbation of preexisting interstitial lung disease after administration of etanercept for rheumatoid arthritis	Hagiwara, K.	Journal of Rheumatology	2007	52	3.47
37	Nonspecific interstitial pneumonia pattern as pulmonary involvement of rheumatoid arthritis	Yoshinouchi, T	Rheumatology International	2005	49	2.88
38	Progressive decline of lung function in rheumatoid arthritis-associated interstitial lung disease	Zamora-Legoff, J. A.	Arthritis & Rheumatology	2017	48	9.6
39	Retrospective study of the clinical characteristics and risk factors of rheumatoid arthritis-associated interstitial lung disease	Zhang, Y. F.	Clinical Rheumatology	2017	46	9.2
40	Rheumatoid arthritis-interstitial lung disease in the United States: prevalence, incidence, and healthcare costs and mortality	Raimundo, K.	Journal of Rheumatology	2019	45	15
41	The multifaceted aspects of interstitial lung disease in rheumatoid arthritis	Cavagna, L.	Biomed Research International	2013	45	5
42	Association of human leukocyte antigen with interstitial lung disease in rheumatoid arthritis: a protective role for shared epitope	Furukawa, H.	PLos one	2012	45	4.5

Table 2: Continued.

Rank	Title	Author	Journal	Year	Total citation	Citation/ year
43	Clinical and radiological features of acute-onset diffuse interstitial lung diseases in patients with rheumatoid arthritis receiving treatment with biological agents: importance of Pneu	Kameda, H.	Internal Medicine	2011	45	4.09
44	Is incident rheumatoid arthritis interstitial lung disease associated with methotrexate treatment? Results from a multivariate analysis in the ERAS and ERAN inception cohorts	Kiely, P.	BMJ open	2019	44	14.67
45	High resolution computed tomography pattern of usual interstitial pneumonia in rheumatoid arthritis-associated interstitial lung disease: relationship to survival	Yunt, Z. X.	Respiratory Medicine	2017	44	8.8
46	Interstitial lung disease in rheumatoid arthritis: recent advances	Kim, D. S.	Current Opinion in Pulmonary Medicine	2006	44	2.75
47	Treatment of rheumatoid arthritis-associated interstitial lung disease: a perspective review	Iqbal, K.	Therapeutic Advances in Musculoskeletal Disease	2015	43	6.14
48	Clinical course and outcome of rheumatoid arthritis-related usual interstitial pneumonia	Song, J. W.	Sarcoidosis Vasculitis and Diffuse Lung Diseases	2013	42	4.67
49	Increased levels of interleukin-33 associated with bone erosion and interstitial lung diseases in patients with rheumatoid arthritis	Zhu X. Y	Cytokine	2012	42	4.2
50	Incidence of and risk factors for interstitial pneumonia in patients with rheumatoid arthritis in a large Japanese observational cohort, IORRA	Shidara, K.	Modern Rheumatology	2010	42	3.5
51	Interstitial lung-disease in rheumatoid-arthritis - assessment with high-resolution computed-tomography	Fujii, M	Journal of Thoracic Imaging	1993	42	1.45
52	Patterns of interstitial lung disease and mortality in rheumatoid arthritis	Zamora-Legoff, J. A.	Rheumatology	2017	41	8.2
53	Standard and pocket-size lung ultrasound devices can detect interstitial lung disease in rheumatoid arthritis patients	Cogliati, C.	Rheumatology	2014	41	5.13
54	Potential risk of TNF inhibitors on the progression of interstitial lung disease in patients with rheumatoid arthritis	Nakashita, T.	BMJ open	2014	41	5.13
55	A fatal case of acute exacerbation of interstitial lung disease in a patient with rheumatoid arthritis during treatment with tocilizumab	Kawashiri, S.	Rheumatology International	2012	41	4.1
56	Interstitial lung disease in patients with rheumatoid arthritis: comparison with cryptogenic fibrosing alveolitis over 5 years	Rajasekaran, A.	Journal of Rheumatology	2006	41	2.56
57	Interstitial lung disease in patients with rheumatoid arthritis: a comparison with cryptogenic fibrosing alveolitis	Rajasekaran, B. A.	Rheumatology	2001	40	1.9
58	A roadmap to promote clinical and translational research in rheumatoid arthritis-associated interstitial lung disease a dance promote clinical and translational research	Doyle, T. J.	Chest	2014	39	4.88
59	Rheumatoid arthritis-related interstitial lung disease (RA-ILD): methotrexate and the severity of lung disease are associated to prognosis	Rojas-serrano, J.	Clinical Rheumatology	2017	38	7.6
60	Rheumatoid arthritis associated interstitial lung disease: a review	Assayag, D.	Medicina-Buenos Aires	2014	38	4.75
61	Sonographic assessment of interstitial lung disease in patients with rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus	Moazedi-Fuerst, F.	Clinical and Experimental Rheumatology	2015	37	5.29

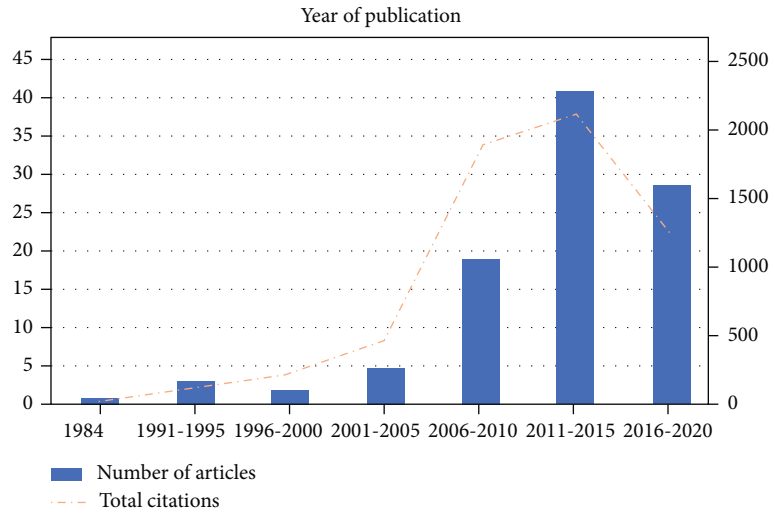
Table 2: Continued.

Rank	Title	Author	Journal	Year	Total citation	Citation/year
62	Association of cross-reactive antibodies targeting peptidyl-arginine deiminase 3 and 4 with rheumatoid arthritis-associated interstitial lung disease	Giles, J. T.	PLos one	2014	37	4.63
63	Clinical and laboratory factors associated with interstitial lung disease in rheumatoid arthritis	Restrepo, J. F.	Clinical Rheumatology	2015	35	5
64	Rheumatoid arthritis interstitial lung disease: mycophenolate mofetil as an antifibrotic and disease-modifying antirheumatic drug	Saketkoo, L. A.	Archives of Internal Medicine	2008	34	2.43
65	Survival and quality of life in rheumatoid arthritis-associated interstitial lung disease after lung transplantation	Yazdani, A.	Journal of Heart and Lung Transplantation	2014	33	4.13
66	Rheumatoid arthritis-associated autoantibodies and subclinical interstitial lung disease: the multi-ethnic study of atherosclerosis	Bernstein, E. J.	Thorax	2016	32	5.33
67	Nintedanib reduces pulmonary fibrosis in a model of rheumatoid arthritis-associated interstitial lung disease	Redente, E. F.	American Journal of Physiology-Lung Cellular and Molecular phy	2018	31	7.75
68	Profibrotic effect of IL-17A and elevated IL-17RA in idiopathic pulmonary fibrosis and rheumatoid arthritis-associated lung disease support a direct role for IL-17A/IL-17RA in human fib	Zhang, J.	American Journal of Physiology-Lung Cellular and Molecular phy	2019	30	10
69	Variable course of disease of rheumatoid arthritis-associated usual interstitial pneumonia compared to other subtypes	Nurmi, H. M.	BMC Pulmonary Medicine	2016	30	5
70	Risk of interstitial lung disease associated with leflunomide treatment in Korean patients with rheumatoid arthritis	Ju, J. H.	Arthritis and Rheumatism	2007	30	2
71	Anti-cyclic citrullinated peptide antibody is associated with interstitial lung disease in patients with rheumatoid arthritis	Yin, Y. F.	PLos one	2014	29	3.63
72	A novel model of rheumatoid arthritis-associated interstitial lung disease in SKG mice	Keith, R. C.	Experimental Lung Research	2012	29	2.9
73	Treatment strategies for a rheumatoid arthritis patient with interstitial lung disease	Kelly, C.	Expert Opinion on Pharmacotherapy	2008	28	2
74	Rheumatoid arthritis disease activity predicting incident clinically apparent rheumatoid arthritis-associated interstitial lung disease: a prospective cohort study	Sparks, J. A.	Arthritis & Rheumatology	2019	27	9
75	Changes in peripheral CD19(+) Foxp3(+) and CD19(+) TGF beta(+) regulatory B cell populations in rheumatoid arthritis patients with interstitial lung disease	Guo, Y. Y.	Journal of Thoracic Disease	2015	27	3.86
76	Interstitial lung disease in patients with rheumatoid arthritis: spontaneous and drug induced	Hallowell, R. W.	Drugs	2014	27	3.38
77	HLA-A * 31 : 01 and methotrexate-induced interstitial lung disease in Japanese rheumatoid arthritis patients: a multidrug hypersensitivity marker?	Furukawa, H.	Annals of the Rheumatic Diseases	2013	27	3
78	Asymptomatic preclinical rheumatoid arthritis-associated interstitial lung disease	Chen, J.	Clinical & Developmental Immunology	2013	27	3
79	Rheumatoid arthritis-associated interstitial lung disease and idiopathic pulmonary fibrosis: shared mechanistic and phenotypic traits suggest overlapping disease mechanisms	Paulin, F.	Revista de Investigacion Clinica-Clinical and Translational Investig	2015	26	3.71
80	Lymphoid interstitial pneumonia in juvenile rheumatoid-arthritis	Lovell, D.	Journal of Pediatrics	1984	26	0.68
81	Ultrasound screening for interstitial lung disease in rheumatoid arthritis	Moazedi-Fuerst, F. C.	Clinical and Experimental Rheumatology	2014	25	3.13

Table 2: Continued.

Rank	Title	Author	Journal	Year	Total citation	Citation/year
82	Interstitial pneumonia due to cytomegalovirus following low-dose methotrexate treatment for rheumatoid-arthritis	Aglas, F.	Arthritis and Rheumatism	1995	25	0.93
83	Therapeutic management of patients with rheumatoid arthritis and associated interstitial lung disease: case report and literature review	Diamanti, A. P.	Therapeutic Advances in Respiratory Disease	2017	24	4.8
84	Interstitial lung disease in rheumatoid arthritis: response to IL-6R blockade	Mohr, M.	Scandinavian Journal of Rheumatology	2011	24	2.18
85	Tocilizumab therapy in rheumatoid arthritis with interstitial lung disease: a multicentre retrospective study	Manfredi, A.	Internal Medicine Journal	2020	23	11.5
86	Recent advances in the pathogenesis, prediction, and management of rheumatoid arthritis-associated interstitial lung disease	Johnson, C.	Current Opinion in Rheumatology	2017	23	4.6
87	Abatacept therapy in rheumatoid arthritis with interstitial lung disease	Mera-Varela, A.	Journal of Clinical Rheumatology	2014	23	2.88
88	The clinical significance of HRCT in evaluation of patients with rheumatoid arthritis-associated interstitial lung disease: a report from China	Zou, Y. Q.	Rheumatology International	2012	23	2.3
89	A case of adalimumab-associated interstitial pneumonia with rheumatoid arthritis	Yamazaki, H.	Modern Rheumatology	2010	23	1.92
90	Prevalence and effects of emphysema in never-smokers with rheumatoid arthritis interstitial lung disease	Jacob, J.	Ebiomedicine	2018	22	5.5
91	Association of disease activity with acute exacerbation of interstitial lung disease during tocilizumab treatment in patients with rheumatoid arthritis: a retrospective, case-control study	Akiyama, M.	Rheumatology International	2016	22	3.67
92	Predicting outcomes in rheumatoid arthritis related interstitial lung disease	Jacobt, J.	European Respiratory Journal	2019	21	7
93	Plasma miRNA expression profiles in rheumatoid arthritis associated interstitial lung disease	Oka, S.	BMC Musculoskeletal Disorders	2017	21	4.2
94	Patients with limited rheumatoid arthritis-related interstitial lung disease have a better prognosis than those with extensive disease	Sathi, N.	Rheumatology	2011	21	1.91
95	Risk of serious infection in patients with rheumatoid arthritis-associated interstitial lung disease	Zamora-Legoff, J. A.	Clinical Rheumatology	2016	20	3.33
96	Possible effect of abatacept on the progression of interstitial lung disease in rheumatoid arthritis patients	Nakashita, T.	Respiratory Investigation	2016	20	3.33
97	Up-to-date information on rheumatoid arthritis-associated interstitial lung disease	Suda, T.	Clinical Medicine Insights-Circulatory Respiratory and Pulmonary	2015	20	2.86
98	Eternacept for the treatment of patients with rheumatoid arthritis and concurrent interstitial lung disease	Horai, Y.	Journal of Clinical Pharmacy and Therapeutics	2012	20	2
99	Myofibroblasts and S-100 protein positive cells in idiopathic pulmonary fibrosis and rheumatoid arthritis-associated interstitial pneumonia	Yoshinouchi, T.	European Respiratory Journal	1999	20	0.87
100	The performance of the GAP model in patients with rheumatoid arthritis associated interstitial lung disease	Morisset, J.	Respiratory Medicine	2017	19	3.8

In addition to its impact on the quality of life, RA-ILD places a huge burden on the medical system, with an average total medical cost of more than $170,000 per patient over five years [8]. Our statistical and quantitative analysis shows a gradual increase in RA-ILD research results from 2011 to 2020, with more researchers and physicians focusing on this area of research. Despite the wide range of RA-ILD research, an analysis of the current status and trends in RA-ILD research is not clear. In this study, we analyzed, discussed, and described the current status, priorities, and trends of RA-ILD research. At the same time, our study will help RA-ILD researchers gain a more comprehensive understanding of the current state of RA-ILD research and thus guide the direction of future research.



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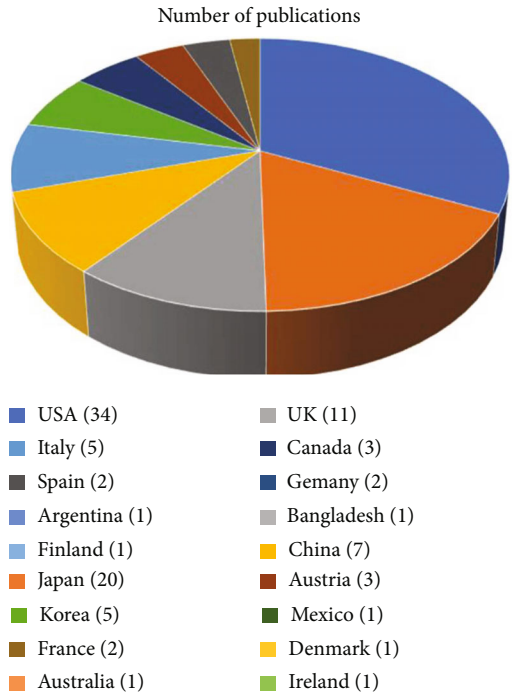

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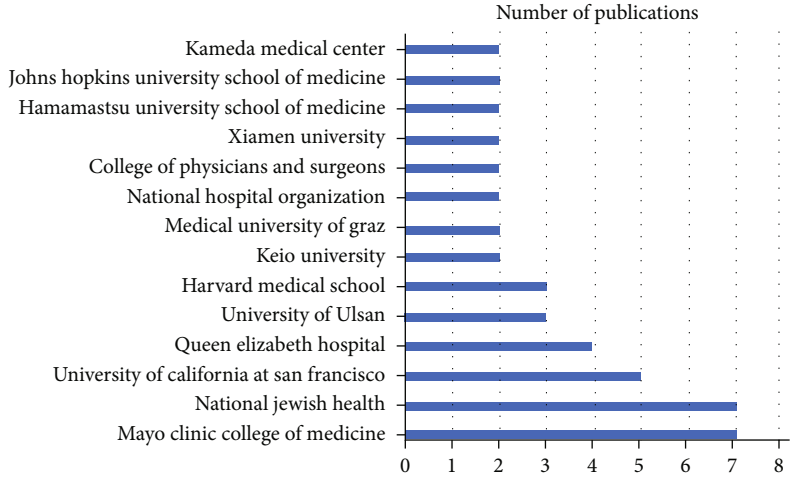

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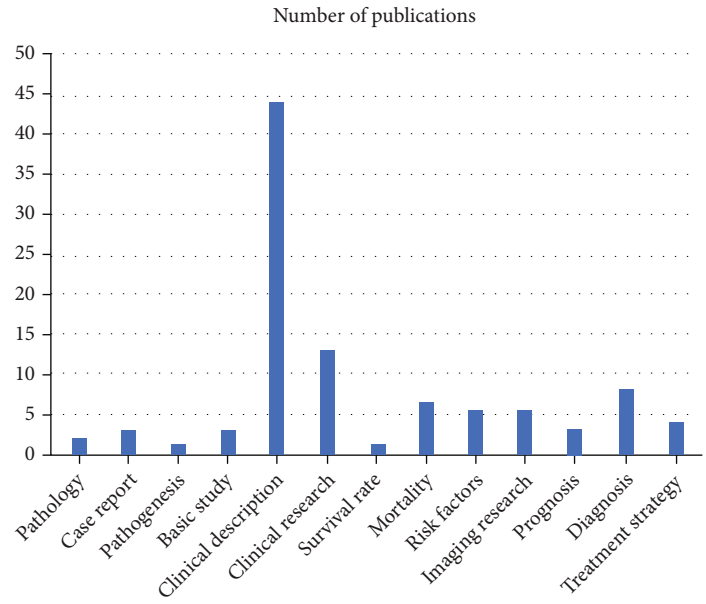

(d)

Figure 4: Top 100 most-cited publications on RA-ILD. (a) Year of publication. (b) Distribution of country. (c) Institution analysis. (e) Publication topics.

Table 3: Journal with more than three of the 100 most-cited publications on rheumatoid arthritis-associated interstitial lung disease.

Journal	Article	Total citation	Mean citation	Impact factor
Rheumatology	8	632	79	3.494
Arthritis and Rheumatism	5	529	105.8	5.532
Clinical Rheumatology	5	207	41.4	4.098
European Respiratory Journal	5	517	103.4	16.671
Annals of the Rheumatic Diseases	4	289	72.25	19.103
Arthritis & Rheumatology	4	191	47.75	10.995
Respiratory Medicine	4	223	55.75	3.772
Rheumatology International	4	135	33.75	2.631

4.1. Publication Trends in RA-ILD Research. The number of articles related to RA-ILD has increased rapidly over the last 10 years. Globally, the USA ranks first in terms of the number of publications and citations, indicating that the USA has led to research on RA-ILD in the past few years. In terms of institutional contributions, the institution with the highest publication output is the Mayo Clinic (USA) and ranked first in the total citations. This reflects the institution's leadership in the field of RA-ILD research. Analysis of cooperation between countries and institutions shows that regional clusters are usually geographically specific. As a leader in the world economy and science, the USA has the most frequent cooperation with Japan, France, China, and South Korea. Researchers working on RA-ILD should pay close attention to them and collaborate with these institutions and countries. Annals of the Rheumatic Diseases, Arthritis & Rheumatology, American Journal of Respiratory and Critical Care Medicine, Rheumatology, and Arthritis and Rheumatism are the five most prolific journals in RA-ILD.

4.2. Research Foci. Keyword analysis results showed that RA-ILD, rheumatoid arthritis, interstitial lung disease, and pneumonia were keyword cluster centers. In the early stages, "idiopathic pulmonary fibrosis," "alveolitis," and "systemic sclerosis" were the main topics. In recent years, more common keywords have included "management," "predictors," "inflammation," and "progression."

4.3. The Most-Cited Articles. The most-cited publication in RA-ILD was the 2010 article in Arthritis and Rheumatism by Bongartz et al. with 324 citations: "Incidence and mortality of interstitial lung disease in rheumatoid arthritis: a population-based study," which introduced incidence, risk factors, and mortality of RA-ILD [2]. The mean follow-up time of 582 RA patients and 603 non-RA patients was 16.4 years and 19.3 years, respectively. The lifetime risk of ILD was 7.7% in patients with rheumatoid arthritis and 0.9% in those without rheumatoid arthritis. Studies have shown that the prevalence of ILD is higher in older male patients and in

Table 4: Most frequent authors of the 100 most-cited publications on rheumatoid arthritis-associated interstitial lung disease.

Author	Article	First author	Last author	Co-author
Ryu, Jay H.	11	0	0	0
Lee, Joyce S.	8	0	2	6
Matteson, Eric L.	8	2	4	2
Brown, Kevin K.	6	0	2	4
Collard, Harold R.	6	0	1	5
Kelly, Clive A.	6	2	4	0
Kim, Dong Soon.	6	1	1	4
Rosas, Ivan O.	6	0	3	3
Solomon, Joshua J.	6	2	1	3
Doyle, Tracy J.	5	2	1	1
Ascherman, Dana P.	5	0	2	3
Fischer, Aryeh.	5	0	0	0
Swigris, Jeffrey J.	5	0	2	3

individuals with more severe RA parameters. RA patients diagnosed with ILD have poorer survival than RA patients without ILD, and ILD accounts for approximately 13% of the excess mortality in RA patients compared to the general population.

"Usual interstitial pneumonia in rheumatoid arthritis-associated interstitial lung disease" by Kim et al. in 2010 was the second most-cited article with 283 citations [16]. The authors determined that the pattern of common interstitial pneumonia (IP) found on high-resolution computed tomography (HRCT) is important for the prognosis of RA-ILD. Eighty-two patients with RA-ILD were identified retrospectively. "We determined the relationship between survival and the pattern of IP common on HRCT and compared it with patients diagnosed radiologically with idiopathic pulmonary fibrosis. Twenty (24%) of the 82 patients with RA-ILD had definite common IP. Survival in patients with RA-ILD was lower than that in patients without this pattern, similar to the survival of patients with idiopathic pulmonary fibrosis. In addition, a clear pattern of common IP on HRCT was associated with poor survival. Analysis of feature-specific HRCTs showed that traction bronchiectasis and cellular fibrosis were associated with poor survival. Women and a higher baseline carbon monoxide lung diffusing capacity were associated with better survival."

"Histopathological and clinical features of interstitial lung disease associated with rheumatoid arthritis" by Lee et al. was the third most-cited article with 245 citations [25]. The authors studied the histopathological patterns and clinical characteristics of patients with RA-ILD according to the American Thoracic Society/European Respiratory Society consensus classification of idiopathic IP. "Eighteen patients with RA who underwent surgical lung biopsy for suspected ILD were included in this study. This study revealed diverse histopathological findings. Ten patients had a common interstitial pneumonia (UIP) pattern, six patients had a nonspecific interstitial pneumonia (NSIP) pattern, and two patients had inflammatory airway disease with tissue-type pneumonia. Thus, the UIP pattern appears to be more common than the NSIP pattern in our study population."

4.4. Limitations. Our study had several limitations. First, we extracted information related to RA-ILD from the Core Collection database of the Web of Science. It is possible that some influential publications were not included in this database and were therefore excluded from our study. Second, the date of our retrieval and extraction of data was July 1, 2021. Part of the data correspond to dynamic changes, but the trend of changes will not be extensive. Third, we retained only English articles in our search strategy.

5. Conclusions

Quantitative analysis showed that in the past 10 years, global research on RA-ILD has increased rapidly. Of all the countries, the USA publishes most articles on RA-ILD.

The USA has contributed the most to the RA-ILD literature. Mayo Clinic, National Jewish Health, Brigham and Women's Hospital, Colorado State University, and University of California, San Francisco are the most prolific institutions associated with RA-ILD research. Annals of the Rheumatic Diseases, Arthritis & Rheumatology, American Journal of Respiratory and Critical Care Medicine, Rheumatology, and Arthritis and Rheumatism are the top five most popular journals on RA-ILD publications.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yuan Zhang, Tingxiao Zhao, and Tianjin Wu contributed equally to this study.

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Research Article

Prevalence and Clinical Significance of Occult Pulmonary Infection in Elderly Patients with Type 2 Diabetes Mellitus

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The occult pulmonary infection is the most common complications in elderly patients with type 2 diabetes mellitus (T2DM). Since its etiological characteristics has not been clarified, infection control remains a serious problem for public health. To investigate the prevalence and clinical significance of occult pulmonary infection in elderly T2DM patients, in this study, 573 elderly patients cochallenged with T2DM and community-acquired pulmonary infection from January 2018 to December 2020 were selected in the hospitals and divided into occult pneumonia group (OP, $n = 249$) and nonoccult pneumonia group (NOP, $n = 324$) according to the nature of infection. Clinical medical records were analyzed retrospectively to summarize the infection characteristics of elderly diabetics with occult pneumonia. The prevalence of the cases (278/324, 85.8%) in NOP group was not higher than that in OP group (206/249, 82.7%; $P > 0.05$). Also, there was not significant difference in the distribution of isolated pathogens among the positive patients. The length of hospitalization and mortality of OP patients were significantly higher than those NOP patients. Multivariate logistic regression showed that advanced age, comorbidities, hypothyroidism, senile dementia, and prolonged bed rest were independent risk factors for occult pneumonia in elderly diabetic patients. Therefore, the results demonstrated that the pulmonary infection in elderly patients with diabetes mellitus is often occult. Gram-negative bacteria are the predominant pathogens and cause poor prognosis. Advanced age, comorbidities (senile dementia, hypothyroidism), and prolonged bed rest are the independent risk factors for occult pneumonia.

1. Introduction

Diabetes mellitus is an endocrine and metabolic disease with high incidence and genetic tendency, which seriously affects the quality of life of patients and their offspring [1]. In the 1980s, the incidence of diabetes mellitus in China was only 1%. Nowadays, diabetics in China account for about 1/5 of the world's total. More than 90% of the diabetic patients in China suffer from type 2 diabetes mellitus (T2DM), and the incidence is increasing year by year [2]. Early onset of T2DM is hidden and hard to cure after onset, which brings heavy financial burden to families and society [3–5]. At present, the specific pathogenesis of T2DM is not clear. It is generally believed to be caused by the interaction of various factors such as obesity, abnormal glucose and lipid metabolism, and inflammatory reactions [6–9].

Clinical trials showed that the incidence of pulmonary infection in patients with T2DM was significantly higher than that in nondiabetic patients, and the older the patient is, the greater the vascular fragility, with poor blood glucose control, deterioration of immune function, and a higher risk of pulmonary infections [10–12]. Occult pulmonary infection, which is more common in elderly patients, is a kind of pulmonary infection with no obvious respiratory symptoms [13–15]. It is difficult to determine the location of lesion and make initial diagnosis. At present, scholars do not have a precise definition and diagnostic criteria for occult pulmonary infection, but most elderly patients have multiple underlying diseases. Pulmonary infection with atypical clinical symptoms may lead to missed diagnosis, misdiagnosis, neglect of the disease, and missing the optimal opportunity for treatment, which may result in malignant

progress of the disease and even endanger lives of patients [16]. Thus, it should be paid more attention. At present, the research on occult pulmonary infection is still in its infancy at home and abroad. Understanding the clinical status and risk factors of occult pulmonary infection in elderly patients with T2DM is of great clinical significance for controlling infection and improving the prognosis of patients. In this study, the case data of elderly patients with T2DM complicated with community-acquired pulmonary infection were retrospectively analyzed; the clinical etiological characteristics, treatment, and prognosis of such patients were summarized; and their risk factors were preliminarily analyzed to provide reference for clinical intervention.

2. Method

2.1. Study Population and Definitions. Totally, 573 elderly patients with T2DM complicated with community-acquired pulmonary infection treated in the second affiliated hospital of Zhejiang Chinese Medical University from January 2018 to December 2020 were selected. Inclusion criteria were employed as follows: (1) meeting diagnostic criteria of T2DM [17]; (2) meeting diagnostic criteria for community-acquired pulmonary infection [18]; (3) age ≥ 65 years; (4) hospital stay ≥3 days; and (5) clinical data fully available. Exclusion criteria: (1) complicated with infection of other sites; (2) changes in leukocyte level caused by other noninfectious diseases; (3) presence of immune system disorders; (4) antimicrobial drugs taken before the first collection of pathogenic specimens; (5) complicated with other chronic pulmonary diseases. The patients were divided into occult pneumonia group and nonoccult pneumonia group according to whether the pulmonary infection is occult or not. Diagnostic criteria for occult pneumonia were employed as follows: (1) no typical respiratory symptoms, including cough, sputum, and chest pain; (2) diagnosis confirmed by computed tomography (CT); (3) patients with no obvious abnormalities in CT and unable to complete CT examination, positive sputum culture or blood culture, and elevated inflammatory indicators are effective after empirical anti-infective therapy. Also, the indexes of blood routine, urine routine, and hepatic and renal function were examined to exclude vasculitis, and the throat swabs were detected by RT-PCR analysis to exclude various acute infection including COVID-19. The flow chart of the study protocol and diagnostic work up was presented in Figure 1. This study has been approved by the ethics committee of the second affiliated hospital of Zhejiang Chinese Medical University.

2.2. Clinical Information. Clinical data of patients were collected, including age, gender, living habits (smoking history, drinking history), underlying diseases (hypertension, coronary disease, senile dementia, etc.), hypothyroidism, invasive operations (invasive airway operation and indwelling catheter), nasal feeding, long-term bed rest, glucocorticoid use, results of etiology and drug sensitivity analysis, length of hospital stay, and mortality. The biochemical indicators, such as white blood cell (WBC), procalcitonin (PCT), and albumin (ALB), were detected by automatic biochemical analyzer (HITACHI 7180, Japan) in the serum. Sputum samples were collected from the patients at the second affiliated hospital of Zhejiang Chinese Medical University and cultured in blood agar media for the microbiological and genomic analysis. Positive cultures with colonies suspected of lethal pathogens and resistant bacteria were sent to the laboratory of microbiology of hospital for further DNA extraction.

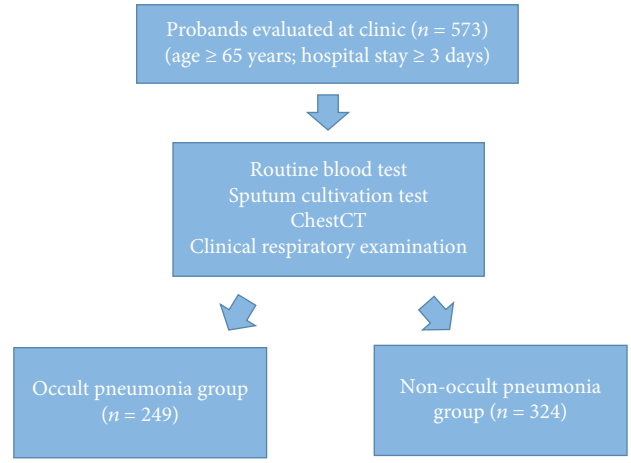

Figure 1: The patients underwent a comprehensive evaluation, including routine blood test, sputum cultivation test, chest CT, and clinical respiratory examination.

2.3. Statistical Analysis. GraphPad Prism 9 (GraphPad Software, San Diego, USA) was used for data processing and analysis. Measurement data were presented as mean ± standard deviation. Comparisons were by T-test. Enumeration data were presented in rate (%). Comparisons were made by χ^2-test. The risk factors of occult pneumonia in elderly diabetic patients were analyzed by logistic regression. $P < 0.05$ was taken as statistically significant.

3. Result

3.1. Clinical Characteristics. After a detailed assessment according to the inclusion and exclusion criteria, a total of 573 subjects were considered in the analysis. Their mean age was 72.7 ± 8.8 years, and male patients were 53.6%. A history of smoking was present in 33.0% and drinking in 14.5%. All the patients had basic diseases, in which 16.8% had two more basic diseases.

3.2. Clinical Characteristics of Pulmonary Infection in T2DM Patients. The distribution of pathogenic bacteria detected in the two groups was shown in Table 1. Totally, 206 induced sputum samples were positive to the pathogenic bacteria culture in 249 patients of the occult group (OP), whose positive rate was 82.7%, and 157 kinds of pathogenic bacteria were detected among 249 OP samples. In other hand, 278 samples were positive in 324 patients of the nonoccult group (NOP), whose positive rate was 85.8%, and 183 kinds of pathogenic bacteria were detected. However, there was no significantly difference in the positive rate of pathogen culture between

Table 1: The distribution of pathogenic bacteria detected from the patients in OP and NOP groups.

Pathogens	OP ($n = 249$)	NOP ($n = 324$)
Gram-negative bacteria	**134 (53.8%)**	**198 (61.1%)**
Klebsiella pneumoniae	34 (13.7%)	69 (21.3%)
Pseudomonas aeruginosa	39 (15.7%)	55 (17.0%)
Acinetobacter baumannii	36 (14.5%)	28 (8.6%)
Escherichia coli	9 (3.6%)	21 (6.5%)
Enterobacter cloacae	0 (0.0%)	18 (5.6%)
Pseudomonas maltophilia	16 (6.4%)	7 (2.2%)
Gram-positive bacteria	**79 (31.7%)**	**80 (24.7%)**
Streptococcus pneumoniae	30 (12.0%)	23 (7.1%)
Staphylococcus aureus	22 (8.8%)	30 (9.3%)
Staphylococcus epidermidis	18 (7.2%)	16 (4.9%)
Hemolytic Staphylococcus	9 (3.6%)	0 (0.0%)
Enterococcus	0 (0.0%)	11 (3.4%)
Fungi	**36 (14.5%)**	**46 (14.2%)**
Candida albicans	19 (7.6%)	33 (10.2%)
Candida tropicalis	12 (4.8%)	11 (3.4%)
Others	5 (2.0%)	2 (0.6%)

the two groups ($\chi^2 = 1.012$, $P > 0.05$). Although the components of pathogens detected in positive patients were not identical, there was no significantly difference in the distribution of pathogens detected in positive patients ($\chi^2 = 2.519$, $P > 0.05$).

3.3. Treatment and Prognosis of Pulmonary Infection in T2DM Patients. All patients received conventional treatments, such as blood glucose reduction, anti-infection, and nutritional replenishment. The empirical anti-infection treatment was employed by mainly using antimicrobial drugs such as carbapenems and β lactam/enzyme inhibitors. When the condition did not improve, sensitive drugs were selected according to the results of drug sensitivity. Imipenem and vancomycin were used cautiously in patients with resistance changes. In the contrast of the prognosis of the two groups as shown in Table 2, patients in the occult group had a higher length of hospital stay and case fatality rate than those in the nonoccult group ($P < 0.05$).

3.4. Univariate Analysis of Occult Pneumonia in T2DM Patients. As shown in Table 3, the results by using univariate analysis presented that there were significantly differences in age, senile dementia, hypothyroidism, WBC, PCT, C-reactive protein (CRP), and prolonged bed rest between the two groups ($P < 0.05$). It indicated that those indexes could contribute to the development and outcome of the occult pneumonia in T2DM patients.

3.5. Multivariate Analysis of Occult Pneumonia in T2DM Patients. As showed in Table 4, the results obtained from multivariate logistic regression analysis presented that advanced age, senile dementia, hypothyroidism, and prolonged bed rest were independent risk factors for occult pneumonia in elderly diabetic patients ($P < 0.05$), whose OR values were all more than 1, and P values were less than 0.05, indicating the statistical significance.

Table 2: The prognostic indicators between the patients in OP and NOP groups.

Prognostic indicators	OP ($n = 249$)	NOP ($n = 324$)	P value
Length of hospitalization (d)	18.5 ± 4.8	11.6 ± 3.3	$P < 0.01$
Mortality (%)	20 (8.0%)	9 (2.78%)	P <0.01

4. Discussion

Numerous studies have confirmed that diabetes is an important risk factor for pulmonary infection. Several reasons have been found: (1) high blood glucose levels provide good growth conditions for pathogenic bacteria, which facilitates their mass multiplication; (2) hyperglycemia increases plasma osmotic pressure and attenuates the chemotaxis, phagocytosis, and bactericidal ability of neutrophils, resulting in decreased clearance of pulmonary pathogens; (3) hyperglycemia can reduce cellular immunity and the body's anti-infective ability, increasing the risk of infection; (4) diabetes mellitus is often associated with uremia, ketoacidosis, and other complications. The patients have metabolic disorders, negative nitrogen balance, and a higher probability of infection. And in patients with vasculopathy, reduced tissue blood flow also reduces the absorption of antibiotics [19–22]. Occult pneumonia refers to insidious clinical symptoms and/or insidious lesion sites, which are not easily diagnosed at the initial diagnosis, often without typical symptoms of respiratory tract infection such as cough, sputum, and chest pain, and are mostly seen in the elderly population. At present, there are few clinical studies on occult pneumonia, and there are no accepted definitions and diagnostic criteria. According to the previous reports, elderly patients were the high-risk population of occult pulmonary infection. Elderly patients have a substantially increased risk of pulmonary infection since all physiological functions of the body were in an attenuated state, and their typical respiratory symptoms were very easily masked by underlying diseases. When the pulmonary infection overlaps with the sagittal position of the heart or spine, chest X-ray (CXR) often fails to confirm the diagnosis, resulting in misdiagnosis or delay in anti-infective treatment and even multiorgan dysfunction, thus endangering patient's life [11, 23–25]. Therefore, understanding the clinical manifestations and risk factors of occult pulmonary infections in elderly diabetic patients is important for controlling infections and improving prognosis.

In this study, the predominant pathogens of elderly patients with occult pulmonary infection and nonoccult pulmonary infection were all Gram-negative bacteria, marked by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. The results were basically consistent with previous reports, and the inconsistency in specific proportions might be related to different geographical environments [12, 26]. Moreover, patients in both groups were

Table 3: Univariate analysis of the occult pneumonia in the patients with T2DM.

Clinicopathologic characteristics		OP ($n = 249$)	NOP ($n = 324$)	t value	P value
Age (years)	65-70	119	164	21.02	<0.01
	71-80	76	132		
	>80	54	28		
Gender	Male	132	175	0.06	0.81
	Female	117	149		
History of smoking	Smoked	89	100	1.52	0.22
	Never smoked	160	224		
History of drinking	Drank	44	39	3.61	0.06
	Never drank	205	285		
Underlying diseases	Hypertension	95	126	0.03	0.86
	Coronary heart disease	59	74	0.06	0.81
	Senile dementia	83	51	24.32	<0.01
	Hypothyroidism	72	61	8.04	<0.01
Biochemical indexes	WBC ($\times 10^9$/mL)	8.65 ± 1.62	10.87 ± 2.67	11.58	<0.01
	PCT (μg/L)	0.18 ± 0.02	0.21 ± 0.02	17.80	<0.01
	CRP (mg/L)	34.6 ± 8.6	32.4 ± 5.6	3.70	<0.01
	ALB (g/L)	33.7 ± 5.9	34.5 ± 6.4	1.53	0.13
Invasive operation	Have	54	75	0.17	0.68
	Not	195	249		
Prolonged bed rest	Prolonged	45	66	0.48	0.49
	Never prolonged	204	258		

Table 4: Multivariate analysis of the occult pneumonia in the patients with T2DM.

Characteristics	β	SE	Wald value	OR value	95% CI	P value
Age	0.69	0.32	4.67	1.98	1.07-3.69	0.03
Senile dementia	0.39	0.17	5.25	1.47	1.06-2.04	0.02
Hypothyroidism	0.41	0.20	4.53	1.51	1.03-2.21	0.03
Prolonged bed rest	0.24	0.09	6.64	1.27	1.06-1.52	0.01

SE: standard error; OR: odds ratio; CI: confidence interval.

treated with comprehensive treatment, and the treatment was basically similar. After the diagnosis of pulmonary infection in elderly patients, it is necessary to carefully assess the severity of the disease with reference to the physiological characteristics of the elderly population and select sensitive antibiotics to control the infection. Prompt drug replacement is necessary in case of resistance changes in the causative organism. For patients who are receiving many antibiotics and invasive procedures, the possibility of drug-resistant strains should be considered. Patients with long-term medication and malnutrition should be cautioned against the occurrence of superinfection. In addition, patients in the occult group had longer hospital stays and a higher case fatality rate, suggesting that the prognosis of elderly patients with diabetes mellitus complicated with occult pulmonary infection was worse.

It has been known that the patients with T2DM are prone to pneumonia for a variety of reasons, including impairment of immune function, impairment of pulmonary function, and ischemia-hypoxia due to hyperglycemia-induced collage synthesis decrease and secondary vascular endothelial changes [11]. In elderly patients with severe pneumonia, fasting glucose values ≥11 mmol/L and glycosylated hemoglobin >7% were significantly and positively associated with increased risk of death. High glycosylated hemoglobin was an independent risk factor for increased mortality risk [12, 27]. However, oral antidiabetic drugs have been demonstrated to be associated with community-acquired pneumonia [28]. Notably, any combination with thiazolidinediones and other antidiabetic drugs had been considered to increase higher risk of community-acquired pneumonia, while the use of DPP-4 inhibitors or metformin dose not display this danger [29–32].

We also found that the risk factors of occult pulmonary infection in elderly patients with T2DM were analyzed. The results showed that age, senile dementia, hypothyroidism,

WBC, PCT, ALB, and prolonged bed rest were related to occult pulmonary infection, and that advanced age, senile dementia, hypothyroidism, and prolonged bed rest were independent risk factors for occult pneumonia. Patients of advanced age have a low capacity to respond to stress. This was because mass neutrophils remain in the viscera and the adhesion of neutrophils was enhanced after infection. Their peripheral blood levels were not increased but decreased. The body temperature might also decrease or remain normal. More cough reflex inhibition made the cough symptoms mild or without cough. The onset was insidious due to lack of fever, cough, and other typical clinical symptoms [33]. For patients with senile dementia, the brainstem had poor regulation of respiratory function, and the lungs had a greatly diminished ability to clear pathogens, which, combined with the fact that they often present with language impairment and had difficulty expressing subjective sensation, thereby delaying the treatment [34]. Studies have shown that multiple neuroendocrine hormone abnormalities are present in patients with diabetes mellitus, manifested by glucocorticoid hypersecretion and dysfunction of hypothalamo-hypophyseal-thyroidal axis (HHTA) [35–37]. Moreover, with increasing age, HHTA also becomes progressively aged, leading to hypothyroidism manifested by reduced respiration, hypothermia, and hyporeflexia, which can mask infectious symptoms and lead to occult pulmonary infections [38–40]. In addition, long-term bed ridden patients have relatively elevated diaphragmatic position, and the scope of their imaging ghosting is enlarged, making it difficult to obtain good and comprehensive evidence from chest X-ray imaging. Therefore, the infection could be easily concealed.

In summary, the pulmonary infections in elderly patients with diabetes mellitus are often occult. The predominant pathogens are Gram-negative bacteria. The prognosis is poor. Advanced age, comorbidities (senile dementia, hypothyroidism), and prolonged bed rest are the independent risk factors for occult pneumonia. Clinical attention should be paid to the following points for such elderly patients: (1) improve lung CT examination and laboratory inflammatory indicator test as soon as possible to raise the detection rate of occult pulmonary infection; (2) aggressively give anti-infective treatment after the diagnosis of occult pulmonary infection; select sensitive antibiotics and adjust medication regimen in a timely manner according to treatment effect; (3) aggressively treat the underlying diseases of elderly patients, especially those with senile dementia and hypothyroidism, to avoid occult pulmonary infections. However, this study is a retrospective study with a low level of evidence. Future efforts will be made to carry out prospective, multicenter, and large-sample studies to further analyze the status of occult pulmonary infections in elderly patients with endocrine diseases.

Data Availability

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

Conflicts of Interest

The authors declare no conflict of interests.

Acknowledgments

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Research Article

A Biological Insight into the Susceptibility to Influenza Infection in Junior Rats by Comprehensive Analysis of lncRNA Profiles

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Long noncoding RNAs (lncRNAs) have been reported to participate in regulating many biological processes, including immune response to influenza A virus (IAV). However, the association between lncRNA expression profiles and influenza infection susceptibility has not been well elucidated. Here, we analyzed the expression profiles of lncRNAs, miRNAs, and mRNAs among IAV-infected adult rat (IAR), normal adult rat (AR), IAV-infected junior rat (IJR), and normal junior rat (JR) by RNA sequencing. Compared with differently expressed lncRNAs (DElncRNAs) between AR and IAR, 24 specific DElncRNAs were found between IJR and JR. Then, based on the fold changes and *P* value, the top 5 DElncRNAs, including 3 upregulated and 2 downregulated lncRNAs, were chosen to establish a ceRNA network for further disclosing their regulatory mechanisms. To visualize the differentially expressed genes in the ceRNA network, GO and KEGG pathway analysis was performed to further explore their roles in influenza infection of junior rats. The results showed that the downregulated DElncRNA-target genes were mostly enriched in the IL-17 signaling pathway. It indicated that the downregulated lncRNAs conferred the susceptibility of junior rats to IAV via mediating the IL-17 signaling pathway.

1. Introduction

Influenza is an infectious respiratory disease mainly caused by influenza viruses [1]. Against the background of the continuous COVID-19 coronavirus pandemic, influenza virus is still a serious threat to public health with not only morbidity from 25% to 90% but also the mortality rate that is fluctuated from 1% to 3% and more than 500,000 people all over the world die from influenza every year [2, 3]. Notably, approximately 870,000 children in preschool are annually hospitalized worldwide due to influenza [4]. Though infectious respiratory disease can occur in all ages, children are more susceptible to influenza infection and have more severe symptoms because of the imperfect immune system [5]. Compared with other age groups, rates of influenza infection are usually the highest in children, especially in children with underlying chronic medical conditions [6]. Moreover, it is reported that host genetic factors play an important role on child susceptibility to influenza infection [7]. However, the molecular mechanisms underlying the influenza-associated genes and progression of influenza remain largely ambiguous.

Long noncoding RNAs (lncRNAs) are defined as a series of transcript RNAs which are more than 200 nucleotides with limited or no protein-coding capacity [8–10]. Accumulating evidences indicate that they perform various functions as regulatory RNAs in diverse biological processes [11–14]. Recently, lncRNAs have been reported to act as prominent regulators of virus-host interactions [15–19]. Although host lncRNAs have been proved to involve in regulating innate immunity against viral infection and replication, the relationship between lncRNA profiles and child susceptibility has been unknown [20].

In this study, we performed RNA sequencing (RNA-seq) in the rat samples to investigate the host immune response to influenza A virus (IAV). Based on RNA-seq data, the specific differentially expressed lncRNAs (DElncRNAs) were identified to construct a ceRNA regulatory network. Furthermore, the molecular mechanisms of the differentially expressed genes were investigated by bioinformatic analysis. Therefore, this study explored the DElncRNA profiles as the potential targets against IAV infection and uncovered a novel regulatory mechanism, which would provide a new insight into the susceptibility factors for IAV infection in children.

2. Materials and Methods

2.1. IAV-Infected Rat Model. Male SD rats (aged 3 and 6 weeks old) were purchased from Zhejiang experimental animal center (Zhejiang, China) and bred under specific pathogen-free conditions. The influenza A virus (H1N1 subtype) was obtained from the Zhejiang Provincial center for disease control and prevention (Zhejiang, China). The infection was induced under sevoflurane by nasal inoculation of IAV, which had been challenged for 5 times in mice with tissue culture infective dose ($TCID_{50}$) of $10^{3.5}$ [21]. After intranasally infected with IAV, the rats were randomly divided into four groups (namely, IAV-infected adult rat (IAR), normal adult rat (AR), IAV-infected junior rat (IJR), and normal junior rat (JR)). Each group had 5 rats, and the rats were sacrificed at day 3 postinfection. The experiments were performed following protocols approved by the animal ethics committee, Zhejiang experimental animal center, and implemented in accordance with the local guide for the care and use of laboratory animals.

2.2. Histopathology. The whole lungs of each group were stripped away and fixed with 10% neutral buffered formalin after perfused with 5 ml of PBS. The fixed tissues were embedded in paraffin, sectioned into 5 μm sections, deparaffinized, rehydrated, and then stained with hematoxylin and eosin. Infections in the lungs were assessed by the level of the formation of lymphoid aggregates and leucocyte infiltration of the airway space, combined with the degree of perivascular lymphocytic aggregation within the sections [22, 23].

2.3. RNA Sample Collection and Library Preparation. Three micrograms of total RNA is from each sample of IAR, AR, IJR, and JR as the initial amount to establish four lncRNA libraries. Firstly, an Ribo-zero™ GoldKits (RiBOBiTech, Guangzhou, China) was used to remove ribosomal RNA (rRNA). Secondly, different index tags were chosen to establish lncRNA libraries according to the instructions of the NEB Next Ultra Directional RNA LibraryPrep Kit for Illumina (NEB, Ispawich, USA). Finally, the established libraries were used for Illumina sequencing.

2.4. RNA-seq Data Acquisition and Quality Control. After RNA-seq, a good deal of sequencing raw data was obtained from 4 independent samples. To ensure the quality of informatic analysis data, we filtered the raw reads to acquire high-quality clean reads by avoiding adapter-polluted reads, low-quality reads, Ns reads, and rRNA mapping reads and then performed further bioinformatic analysis. Bioinformatic analysis was based on the clean reads.

2.5. Differential Expression Analysis of lncRNAs. The expression values of lncRNAs in each sample were normalized using the Fragments per Kilobase per Million Mapped Fragments (FPKM). FPKM was defined per million sequence number as the number of lncRNA expression, of which the total number of aligned reads were used by normalized expression values. The data of the lncRNAs from 4 samples was separately recombined, to make a comparison using DEGseq software. The DElncRNAs were filtrated with $|\log 2\text{Ratio}| \geq 1.5$ and $q \leq 0.05$ as the screening conditions to obtain the up- and downregulated genes.

2.6. Construction of ceRNA Regulatory Network. The interactions between DElncRNAs and miRNA were predicted using miRanda, PITA, and TargetScan. The lncRNA-miRNA interaction pairs were recognized as targeted relationship when successfully predicted in two websites at least. In addition, miRNA-mRNA interaction pairs predicted by the above method were also selected. Next, the lncRNA-miRNA-mRNA ceRNA regulatory network was established by Cytoscape software (Version 3.8.2) [24].

2.7. Functional Enrichment Analysis of Differentially Expressed lncRNAs. To illustrate gene ontology or molecular pathway enrichment, the WEB-based Gene SeT AnaLysis Toolkit (WebGestalt) was used to perform GO functional annotation, including biological process (BP), cellular component (CC), and molecular function (MF), and to analyze KEGG pathway enrichment for the significant DElncRNA target genes [25].

2.8. Statistical Analysis. Normalization FPKM analysis was used to control the quality of the sequence data of lncRNAs, miRNAs, and mRNAs in our study. Statistical comparisons of the data were analyzed by SPSS software (SPSS 26, Chicago, IL, USA). Multiple comparisons among all groups were performed by one-way analysis of variance (ANOVA). Meanwhile, comparisons between two groups were performed by Student's test. $P < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. Lung Histopathology. The results of hematoxylin and eosin staining clearly showed that compared with IAV-uninfected (AR and JR) groups, the lung tissue sections of IAV-infected (IAR and IJR) groups had higher levels of leucocyte infiltration and platelet aggregation in the airway and perivascular spaces (Figure 1). However, those features were hardly found in uninfected groups. It suggested the serious lung injury and inflammation induced by IAV.

3.2. Sequencing Data Filtering and Alignment Analysis. After RNA-seq, plentiful raw data were got from 4 samples (AR, IAR, JR, and IJR). According to the manufacturer protocols, the raw data were filtered to obtain high-quality reads and sequences for further bioinformatic analysis. The total

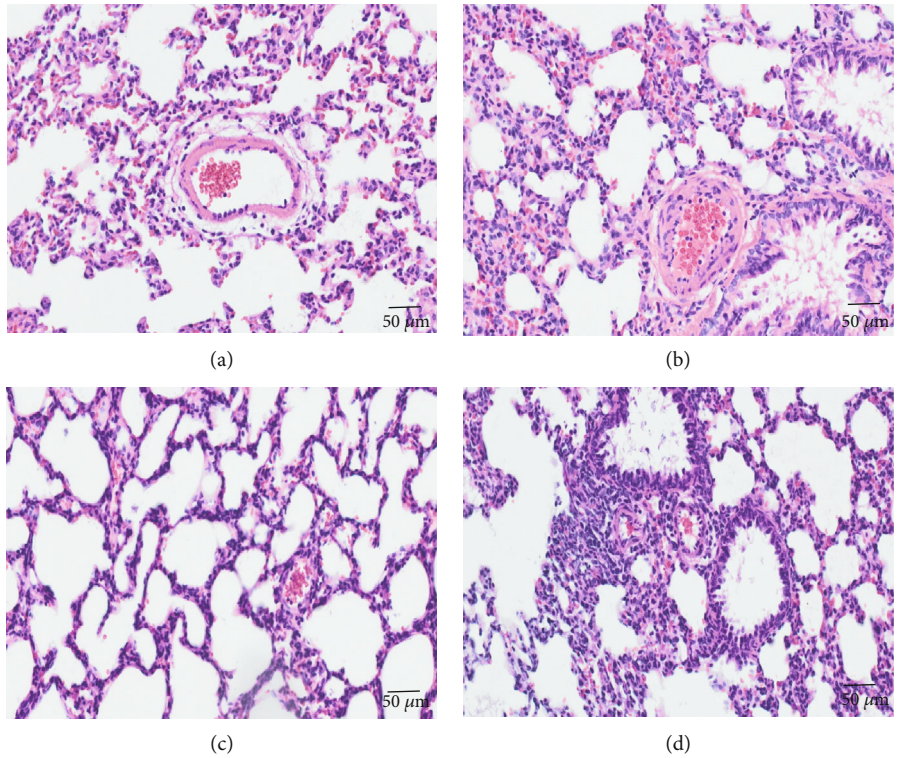

Figure 1: Histopathological analysis for lung tissues at day 3 postinfection. Representative HE-stained sections of lung tissues from (a) normal adult rat (AR), (b) influenza-infected adult rat (IAR), (c) normal junior rat (JR), and (d) influenza-infected junior rat (IJR). Scale bar = 50 μm for all images.

Table 1: Statistical result of the RNA-seq data quality test and alignment analysis.

Sample	Sequencing data				Alignment data		
	Raw reads	Clean reads	Raw bases	Clean bases	Total reads	Mapped reads	Multimap reads
AR	100132938	96394520 (96.27%)	15019940700	14459178000 (96.27%)	96394520	92470381 (95.93%)	4981812 (5.17%)
IAR	105647358	101644822 (96.21%)	15847103700	15246723300 (96.21%)	101644822	97262759 (95.69%)	5317435 (5.23%)
JR	100492130	96947954 (96.47%)	15073819500	14542193100 (96.47%)	99474110	95373552 (95.88%)	6231592 (6.26%)
IJR	102616336	99474110 (96.94%)	15392450400	14921116500 (96.94%)	96947954	92944082 (95.87%)	5626577 (5.80%)

numbers of raw reads, clean reads, and clean bases of all samples are shown in Table 1. To identify lncRNA sequences, we aligned the filtered data using HiSAT2 and the mapping rates were more than 95% of total reads, which indicated not only a high utilization of the sequencing reads but also reliable results in the subsequent analysis.

3.3. Identification of Differentially Expressed lncRNAs. Based on the filtered data, compared with AR, 763 DelncRNAs were found in IAR, and similarly, 763 in IJR when compared with JR. In addition, $|\log 2\text{Ratio}| \geq 1.5$ and $q < 0.05$ as the screening conditions were performed to further identify the DElncRNAs by using DEGseq. To visualize DElncRNAs between IAV-infected groups and normal groups in both adult and junior rats, the volcano plots and heatmaps were illustrated (Figure 2). Compared the expression profiles in IAR and AR samples, a total of 66 known DElncRNAs were selected for further analysis. Moreover, it was totally 64 known DElncRNAs, compared IJR with JR. To identify the unique known DElncRNAs between IJR and JR, we selected top 5 DElncRNAs filtered by fold changes and P value. The 5 specific DElncRNAs compared IJR with JR were selected as candidates for further bioinformatic analysis.

3.4. Targeted miRNA Analysis of Differentially Expressed lncRNAs. To determine the function of the 5 specific DElncRNAs, we predicted the targeted miRNAs of DElncRNAs by using three online websites: miRanda, PITA, and TargetScan. The lncRNA-miRNA interaction was recognized as targeted relationship when successfully predicted in two websites at least. Then, the targeted miRNAs were selected which were differentially expressed and negatively associated with the expressions of the lncRNAs. Finally, 25 lncRNA-miRNA pairs were identified and associated with the severity of IAV infection in junior rats after online prediction.

3.5. Identification of Potential ceRNA (lncRNA-miRNA-mRNA) Regulatory Network. The miRNA-mRNA interaction pairs were also predicted by the same method according to

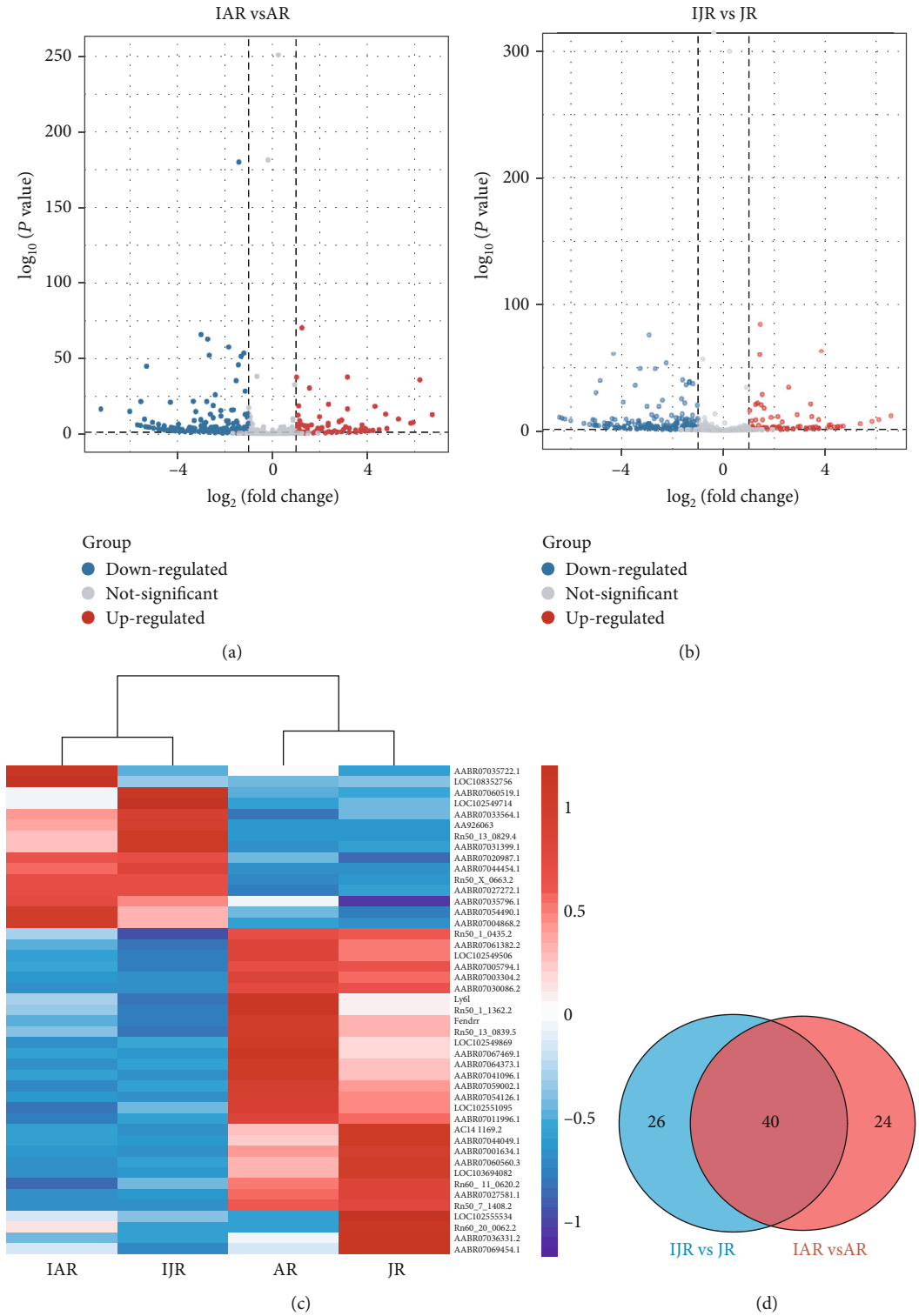

Figure 2: Identification of differentially expressed lncRNAs. The volcano plot showing the differentially expressed lncRNAs compared IAR with AR (a) and compared IJR with JR (b). (c) All the expression level of the differentially expressed lncRNAs in each sample was analyzed by the hierarchical cluster. (d) Venn diagram showing the overlap of DElncRNAs between the IAR vs. AR group and the IJR vs. JR group.

the binding free energies and the binding mode. The targeted mRNAs were also negatively associated with the expression of miRNAs. As the translation of mRNAs might be regulated by lncRNAs via sponging miRNAs, a lncRNA-mRNA competing interaction pair would be considered if the mRNA and the lncRNA significantly shared common miRNAs. Thus, top 10 miRNA-mRNA interaction pairs of each DElncRNA-target miRNAs filtered by fold changes were selected in this study. Based on lncRNA-miRNA and miRNA-mRNA regulatory relationships, a competitive endogenous RNA (ceRNA)

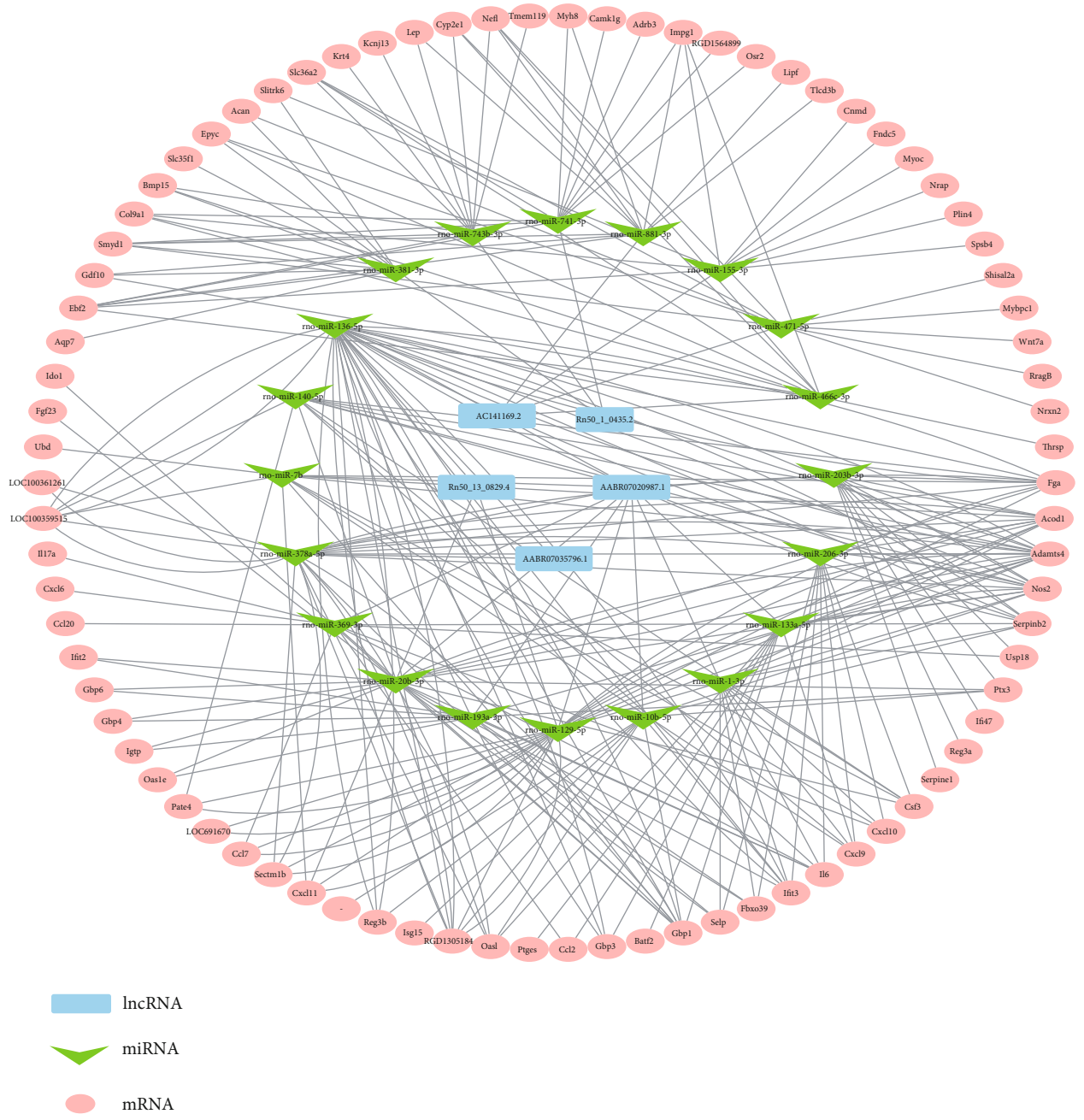

Figure 3: Identification of potential ceRNA (lncRNA-miRNA-mRNA) regulatory network. The lncRNA-miRNA-mRNA network of 3 upregulated DElncRNAs and 2 downregulated DElncRNAs was visualized in Cytoscape.

network of lncRNA-miRNA-mRNA was established to further explore the regulatory mechanisms of lncRNAs, including up- and downregulated lncRNAs (Figure 3). Then, we analyzed the ceRNA networks by using CytoHubba analysis and the hub miRNAs competitively bound by ceRNAs were presented, which included rno-miR-20-3p, rno-miR-136-5p, and rno-miR-378a-5p. Therefore, those results indicated that rno-miR-20-3p had been the potential acted as a novel prognostic indicator for IAV.

3.6. Functional Enrichment Analysis. To further investigate the roles of these DElncRNA-associated mRNA genes in ceRNA regulatory networks, GO and KEGG pathway analyses were performed by using WebGestalt database. The results showed that the obviously enriched BP included biological regulation, response to stimulus, and metabolic process. The CC contained membrane, extracellular space, and endomembrane system, whereas MF covered protein binding, ion binding, and nucleotide binding. On the other hand, these DElncRNA-associated mRNAs were annotated by KEGG pathway analysis. As shown in Figure 4, the function of these mRNAs was mainly involved in cytokine-cytokine receptor interaction, IL-17 signaling pathway, chemokine signaling pathway, TNF signaling pathway, and Toll-like

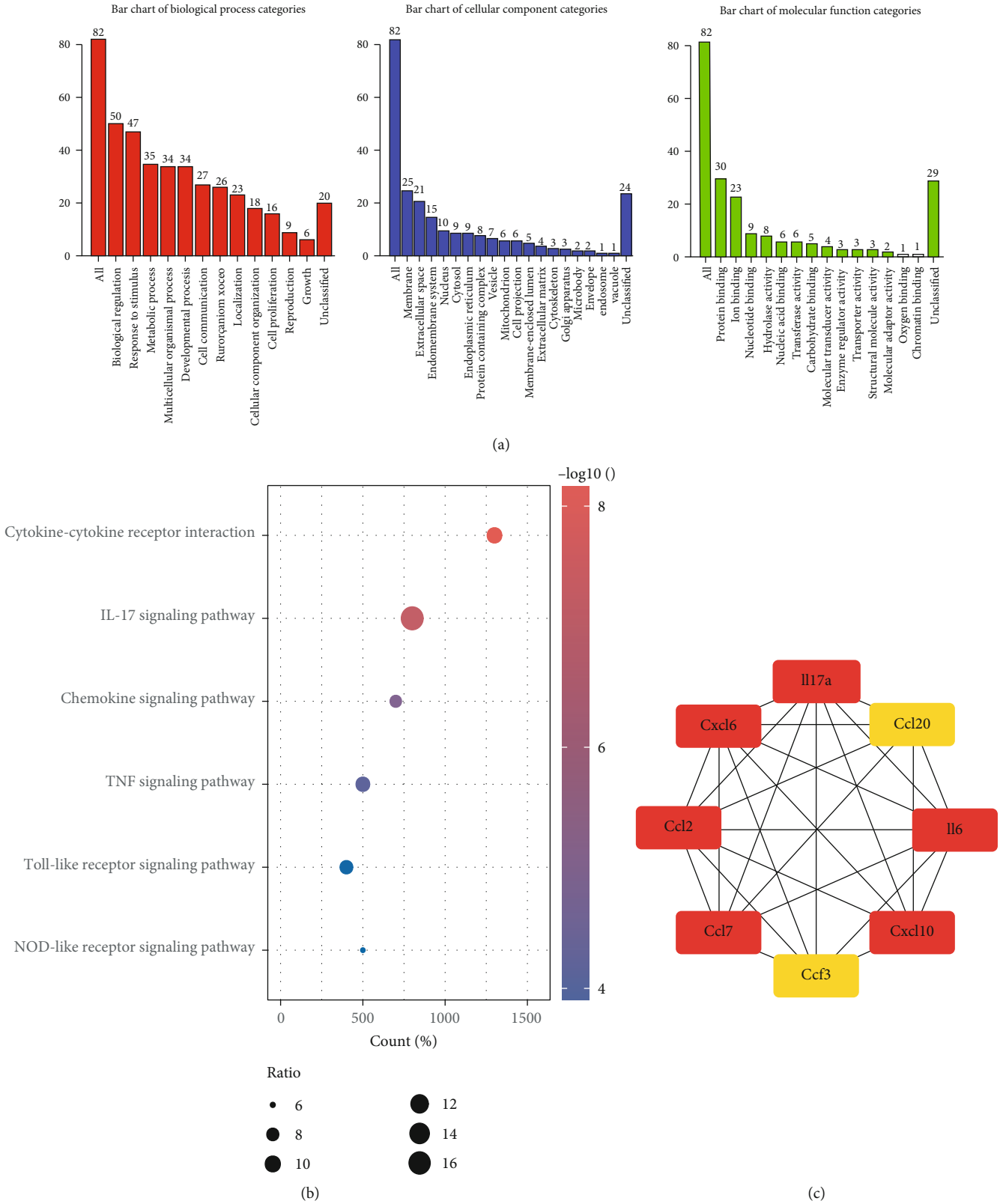

Figure 4: Functional enrichment analysis. (a) GO analysis for DEmRNAs in the ceRNA regulatory network, including the significant biological processes, molecular functions, and cellular components of DEmRNAs. (b) Bubble plots showing the crucial pathways for DEmRNAs by KEGG pathway analysis. (c) The IL-17 signaling pathway-related genes were evaluated by CytoHubba, with a high score shown in red and a low score shown in yellow.

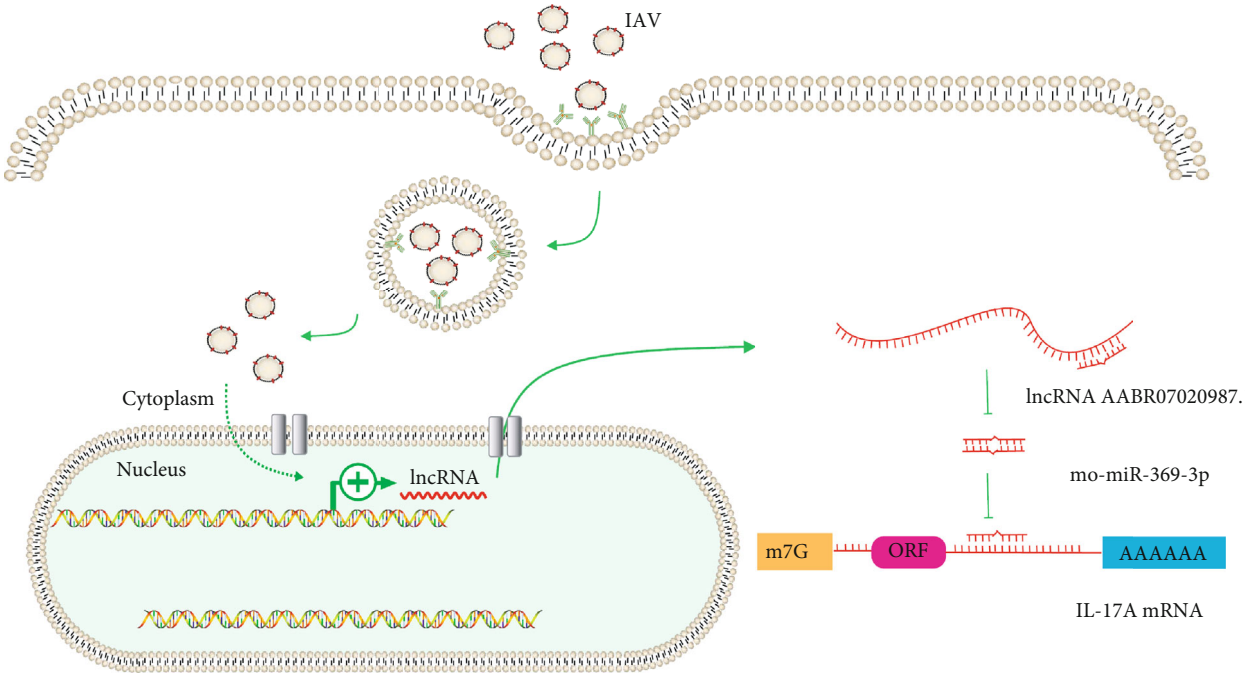

Figure 5: The schematic diagram of the mechanisms of the ceRNA regulatory network after influenza infection in junior rats. When the juniors were infected with influenza virus, the expression of lncRNA AABR07020987.1 expression was indirectly promoted, and then, the sponge adsorption of rno-miRNA-369-3p was enhanced, resulting in the weakened inhibition of rno-miRNA-369-3p on IL-17A mRNA.

receptor signaling pathway. Among these pathways, the IL-17 signaling pathway presented the largest gene hits, including IL-17A, CXCL16, CCL7, IL-6, CCL2, CXCL10, CCL20, and CSF3. Compared with CCL20 and CSF3, other proinflammatory cytokines and chemokines marked with red color in Figure 4(c) exerted high scores, indicating that they would play crucial roles in the susceptibility of junior rats to IAV.

4. Discussion

Influenza is an acute respiratory infection caused by influenza virus, which leads to respiratory symptoms, fever, and even a series of systemic symptoms in children [26]. Compared with adults, children as immunocompromised individuals are more susceptible to be infected with IAV [27, 28]. Therefore, it is vital to make a treatment plan to elucidate the susceptibility to children for clinical prevention and treatment of IAV infection. Numbers of evidences have illustrated that host lncRNAs act as either positive or negative regulators of the innate antiviral response, facilitating influenza virus replication [29–31]. In the study, we detected the gene expression profiles with the focus on lncRNAs in IAR, AR, IJR, and JR by using RNA-seq, having found plenty of differentially expressed lncRNAs. The results revealed that lncRNA AABR07020987.1, AABR07035796.1, and Rn50_13_0829.4 were significantly downregulated, while Rn50_1_0435.2 and AC141169.2 were significantly upregulated in IAV-infected junior rats, indicating that these DElncRNAs had potential to be novel biomarkers for evaluating the prognosis and diagnosis of IAV challenge. In addition, a ceRNA regulatory network was constructed to investigate the lncRNA-miRNA-mRNA regulatory relationship. Moreover, we explored the most remarkably enriched molecular function, cellular component, and biological processes of these DElncRNA-associated mRNA by GO and KEGG analysis. The results showed that cytokine-cytokine receptor interaction, IL-17 signaling pathway, chemokine signaling pathway, TNF signaling pathway, and Toll-like receptor (TLR) signaling pathway were enriched in the IJR group vs. the JR group. Growing evidence had revealed that the first three are involved in the development of severe lung immunopathology via recruiting B lymphocytes to the sites of pulmonary influenza virus infection and subsequently increased the susceptibility and severity in children, while TLRs as the well-known innate immune recognition receptors played the essential roles in host defense and inflammation during viral infections [32–37].

Among the pathways, the IL-17 signaling pathway presented the largest gene hits. IL-17 signaling pathway-related genes such as IL-17A, CXCL16, CCL7, IL-6, and CCL2 that regulated inflammatory responses in IJR were significantly upregulated. In addition, we found that the IL-17A gene was one of the most upregulated IL-17 signaling pathway-related genes. Omidian et al. [38] demonstrated that IL-17A was a potent proinflammatory cytokine which prevented host from pathogenic microorganism infections like IAV infection. Moreover, IL-17A was also involved in the immunopathogenesis of IAV-induced acute lung injury, which was relevant to disease severity and dysregulation of IL-17A could lead to susceptibility to infectious diseases [39]. Besides, overexpressed IL-17A could induce neutrophil activation as well as chemotaxis. When exposed to viruses, higher airway neutrophil activity increased susceptibility to viral infection [40]. Since TLR4 antagonists reduced influenza-induced mortality in rats,

neutrophils could enhance susceptibility by deriving oxidized phospholipids (TLR4 agonists) and neutrophil proteases can also degrade antiviral peptides, which eliminated viral load and directly averted susceptibility to viral infection [41, 42]. Therefore, these results demonstrated that targeting lncRNAs in mediating the IL-17-dependant pathway could be a novel strategy for repressing viral infection.

According to bioinformatic analysis based on RNA-seq data, we speculated that lncRNA AABR07020987.1 positively affected the expression of IL-17A by acting as a ceRNA to compete with IL-17A mRNA for binding sites of rno-miR-369-3p (Figure 5). Targeting lncRNA AABR07020987.1 could be conducive to improve susceptibility to IAV in immature individuals. The lncRNA AABR07020987.1-rno-miR-369-3p-IL-17A axis could be a therapeutic potential to avert susceptibility to IAV. Therefore, this ceRNA network should be further verified by using qPCR and luciferase assays to investigate the role of IL-17A on the susceptibility to IAV in children.

5. Conclusion

In conclusion, 5 novel DElncRNAs (3 upregulated lncRNA (i.e., AABR07020987.1, AABR07035796.1, and Rn50_13_0829.4) and 2 downregulated (i.e., Rn50_1_0435.2 and AC141169.2)) were identified in the lung tissues of IAV-infected junior rats when compared with those in the normal junior rats and infected adult rats. Further bioinformatic analysis indicated that these DElncRNA-mediated ceRNA networks are mainly involved in the IL-17 signaling pathway and chemokine signaling pathway. Therefore, these results provided a new therapeutic strategy to improve susceptibility of influenza in children via targeting the DElncRNA-mediated ceRNA network.

Abbreviations

LncRNAs:	Long noncoding RNAs
IAV:	Influenza A virus
IAR:	IAV-infected adult rat
AR:	Adult rat
IJR:	IAV-infected junior rat
JR:	Normal junior rat
RNA-seq:	RNA sequencing
DElncRNAs:	Differently expressed lncRNAs
TCID50:	Tissue culture infective dose
rRNA:	Ribosomal RNA
FPKM:	Fragments per Kilobase per Million Mapped Fragments
BP:	Biological process
CC:	Cellular component
MF:	Molecular function
ANOVA:	One-way analysis of variance
ceRNA:	Competitive endogenous RNA
TLR:	Toll-like receptor.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Review Article

The Role of Respiratory Flora in the Pathogenesis of Chronic Respiratory Diseases

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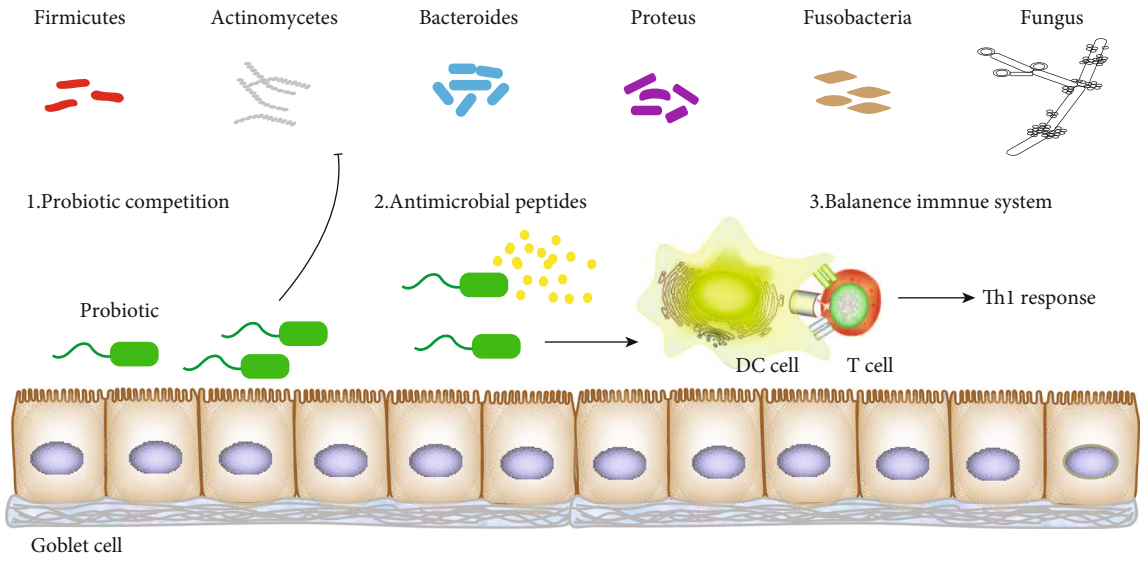
Large quantities of bacteria, including *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*, colonize the surface of the respiratory mucosa of healthy people. They interact and coexist with the local mucosal immune system of the human airway, maintaining the immune stability and balance of the respiratory system. While suffering from chronic respiratory diseases, the microbial population in the airway changes and the proportion of *Proteobacteria* is increased in patients with asthma. The abundance of the microbial population in patients with chronic obstructive pulmonary disease (COPD) is decreased, and conversely, the proportion of *Firmicutes* and *Proteobacteria* increased. The diversity of airway microorganisms in cystic fibrosis (CF) patients is decreased, while pathogenic bacteria and conditional pathogenic bacteria are proliferated in large numbers. The proportion of *Firmicutes* and *Proteobacteria* is increased in patients with upper airway cough syndrome (UACS), which replaces the dominance of *Streptococcus* and *Neisseria* in the pharynx of a normal population. Therefore, a clear understanding of the immune process of the airway flora and the immune dysfunction of the flora on the pathogenesis of chronic respiratory diseases can provide new ideas for the prevention and treatment of human respiratory diseases.

1. Introduction

The human body carries a huge group of microbial populations, which constitutes a complex and delicate ecosystem. The complex interaction between microbes and the human immune system determines the health of the human body. The airway is an open cavity connecting the human body with the outside world. It is always invaded by external microbes, so it has strong local immunity. For example, the airway contains not only motile cilia, strong secretory goblet cells, and secretory IgA on the mucosal surface but also abundant mucosal-associated lymphoid tissue under the mucosa, which is sufficient to cope with the invasion of external pathogens [1]. Once the local immunity of the airway is disturbed, acute and chronic respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and upper airway cough syndrome (UACS) are likely to be triggered (Figure 1). For a long time, the airway, especially the lower airway, has been considered amicrobic [2, 3]. The airway is a dynamic ecosystem full of microbial populations, which is closely related to the immunity and inflammatory response of the host [4]. As is in the intestine, there are certain types and quantities of microbial populations in the airway of healthy people, that is, normal flora. Under the influence of external factors, these normal florae continuously evolve to maintain the dynamic balance of respiratory microecology and resist and avoid the invasion and colonization of pathogens to the airway [5, 6]. However, under the influence of external factors, such as environmental pollution and antibiotic abuse, changes in respiratory flora may result in pathogenic infection. Even some strains in the normal flora may turn into conditional pathogenic bacteria, causing several respiratory diseases. The stability of the airway flora is greatly affected by the external environment, which affects the local airway immune balance and even leads to the immune dysfunction of the respiratory system, thereby adding lots of difficulties to the clinical treatment of respiratory diseases. From the microecological perspective, it is of great significance to pay attention to the local immunity of the airway



	Firmicutes	Actinomycetes	Bacteroides	Proteus	Fusobacteria	Fungus
Healthy upper airway	+ +	+ +	+ + +	+ +	+ + +	+ +
Asthma	+ + +	+ +	+ + +	+ + + +	+ + +	+ +
UACS	+ + + +	+ +	+ + +	+ + + +	+ + +	+ +
Healthy lower airway	+	+	+ +	+	+ +	+
COPD	+ +	+ +	+	+ +	+	+
CF	+	+ + + +	+ +	+	+ +	+

Figure 1: The profiles of respiratory flora in the occurrence and development of chronic respiratory diseases.

in the prevention and treatment of respiratory diseases [7, 8]. In recent years, with the development and application of molecular biology techniques, the research on the relationship between respiratory microflora with respiratory system immunity and diseases has developed rapidly. Growing studies have interpreted the role of complex respiratory microflora in the process of immune dysfunction in various respiratory diseases, which provides new ideas for exploring the pathogenesis and treatment of respiratory diseases [9, 10].

2. Flora Distribution and Local Immunity of the Airway in Healthy People

Large quantities of bacteria colonize the surface of the respiratory mucosa of healthy people, of which the main groups are *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, and *Fusobacteria* [11]. Bacteria regulate the species and quantity through the quorum-sensing system and locally produced antimicrobial peptides to maintain dynamic balance and achieve peaceful coexistence with the human body. The floras in the upper and lower airways are highly homologous, and the quantity of upper airway flora is greater than that of the lower airway, which means that there are no specific microbes in the upper and lower airways [12]. This is different from the normal intestinal flora. The human airway is mainly defended by the local mucosal immune system. Mucosal epithelial cells act as a physical barrier separating the internal and external environments. They are the main structure of the local innate immune system and the main carrier of the airway flora. There are cilia on the surface of respiratory mucosal epithelial cells, which can transport bacteria of the lower airway upwards, while the mucus secreted by goblet cells and secretory IgA antibodies produced by submucosal lymphohistiocytes can further maintain the local chemical immune barrier function of the mucosa and prevent the invasion and colonization of pathogens [13, 14]. However, the local immune system of the airway neither equips with the strong acid and alkali on the surface of alimentary canal mucosa and the powerful germicidal mechanism of excellent enzyme system nor the extremely anaerobic environment, which determines that there is no significant difference in the species but only in the number of bacteria in the upper and lower airway. This is the evolutionary result of the interaction and symbiosis between the airway local immunity and the flora [15].

3. Immunity of Respiratory Flora and Asthma

Asthma is a kind of disease that is well-recognized as immune dysfunction of the respiratory system, which is defined as a clinical syndrome of intermittent respiratory symptoms caused by viral upper respiratory infections, environmental allergens, or other stimuli, characterized by nonspecific bronchial hyperresponsiveness and airway inflammation [16, 17].

Due to the low positive rate of colony culture in sputum in the past and the insignificant effect of antibiotic treatment in the early stage of asthma, it was believed that there was no relationship between bacteria and asthma attacks. An epidemiological study had disclosed that bronchial infection could be the basis of asthma attacks in the adult during the outbreaks of bronchitis and pneumonia. This was also in line with the previously reported efficacy of macrolide antibiotics in the treatment of patients with chronic infectious asthma [18]. With the application of serological testing and PCR-based research methods, it had found that *Streptococcus pneumoniae* and *Chlamydia pneumoniae* infections were common during acute asthma attacks [19]. Analysis of the microbiota in bronchoalveolar lavage (BAL), tracheal brushings, and sputum microorganisms revealed that the respiratory microbiota of asthmatic patients who had a significant increase in the abundance of *Proteobacteria* in the flora was significantly different from that of healthy people [20]. Through the cultivation of respiratory specimens (Figure 2), it was found that the proportions of asthma in children with bacteria, such as *Moraxella catarrhalis*, *Haemophilus influenzae*, or *Streptococcus pneumoniae*, were increased significantly, which were related to the severity and acute exacerbation of asthma [21, 22]. The results from 16S rRNA sequencing showed that the proportion of *Proteobacteria*, including *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and other respiratory pathogens, in respiratory specimens of asthma patients was higher than that of the normal population. The increase in these pathogens was also found in patients with irregular inhaled hormone therapy, suggesting that this feature of respiratory flora was a characteristic of asthma itself and not simply a result of immunosuppression caused by inhaled hormones. In contrast, the proportion of *Prevotella* of *Bacteroidetes* in the airways of asthma patients was lower than that of the normal population [23–25].

The distribution of different bacterial communities is also related to the features of asthma. Airway microbes and asthma patients also have certain effects on the responsiveness of glucocorticoids. In vitro studies have shown that monocytes and macrophages cocultured with *Haemophilus influenzae* and *Haemophilus parainfluenza* were impaired in response to dexamethasone, which was found in some patients with hormone-resistant asthma [26]. Studies have shown that dexamethasone can inhibit tumor necrosis factor (TNF) production by macrophages and promote the phagocytosis of microparticles by human monocytes, thereby played an important role in immune-mediated tissue damage or tissue repair after infection [27, 28]. The possible mechanism of the effect of flora changes on the sensitivity to corticosteroids is the release of superantigens to produce oxidative stress, the release of cytokines, or the activation of host p38 mitogen-activated protein kinase (MAPK) [29].

These studies indicated that changes in respiratory flora would increase types of pathogenic bacteria but reduce types of normal flora and biomass in asthma patients, suggesting that the immune dysregulation of the respiratory flora may be one of the causes of asthma attacks.

4. Immunity of Respiratory Flora and COPD

COPD is a highly heterogeneous disease that seriously affects human health. It is characterized by persistent airflow obstruction and increasing airway chronic inflammatory response to harmful particles or gases. Exposure to noxious smoke and particles is a major driver for the progression of COPD. At the same time, other factors also play a part, such as genetic predisposition, nutritional status, and respiratory infection [30]. The role of microbial infection in the onset and development of COPD is a hot topic in the research of the respiratory microbiome. COPD is associated with the colonization of early pathogenic pathogens in the bronchus. The possible effect of changes in respiratory microbes on the progression of COPD can be explained by the circular vicious hypothesis [31]. Once the innate self-protective mechanism of the lung affected by smoke exposure and the airway microbial homeostasis is broken, pathogenic microbes can induce an inflammatory response of COPD, leading to progressive airway obstruction as well as pulmonary parenchyma injury by disrupting the balance of proteases and antiproteases in the lung. Direct supporting evidence for this hypothesis came from conventional biological research. A large increase in local immune and inflammatory components such as inflammatory corpuscles, cytokines, chemotactic factors, and proteases in the airways of patients with COPD could be detected by bronchoscopy or sputum examination. These pathological changes were consistent with chronic infection. Meanwhile, COPD patients with bronchiectasis harbored more airway bacteria, showing worse clinical outcomes [32]. Microbiological research can help us better understand the impact of microbes on the pathogenesis and progression of COPD. But whether differences in airway microbes have an impact on COPD clinical symptoms (such as the presence or absence of bronchitis) deserves further exploration.

Unlike the early changes in the microbiota of patients with asthma, there is no significant difference in respiratory flora between mild and moderate COPD patients and normal people [33]. Changes in flora are present only in patients with severe COPD (Forced expiratory volume in one second (FEV1) is less than 40% to 50% of the expected value). Microbiological analysis of sputum samples showed that the abundance of bacteria in the airway of patients with COPD was decreased, the proportion of *Proteobacteria* such as *Pseudomonadaceae*, *Burkholderiaceae*, and *Enterobacteriaceae* was increased, and the relative abundance of *Firmicutes* was decreased [34]. However, some scholars believe that *Firmicutes* and *Actinobacteria* were the dominant flora in the lungs of COPD patients. By sampling and sequencing the BALF of patients with moderate and severe COPD, Pragman et al. found that the structure of the flora in the lungs of these two groups of patients was changed and that *Firmicutes*, *Proteobacteria*, and *Actinobacteria* were abundant in the lower airways of these patients [35]. Microbiological analysis of bronchial biopsy showed that this change was related to the increase in local heterogeneity of respiratory microbes. This change in respiratory microbes associated with disease severity had also been reported in patients with CF [36].

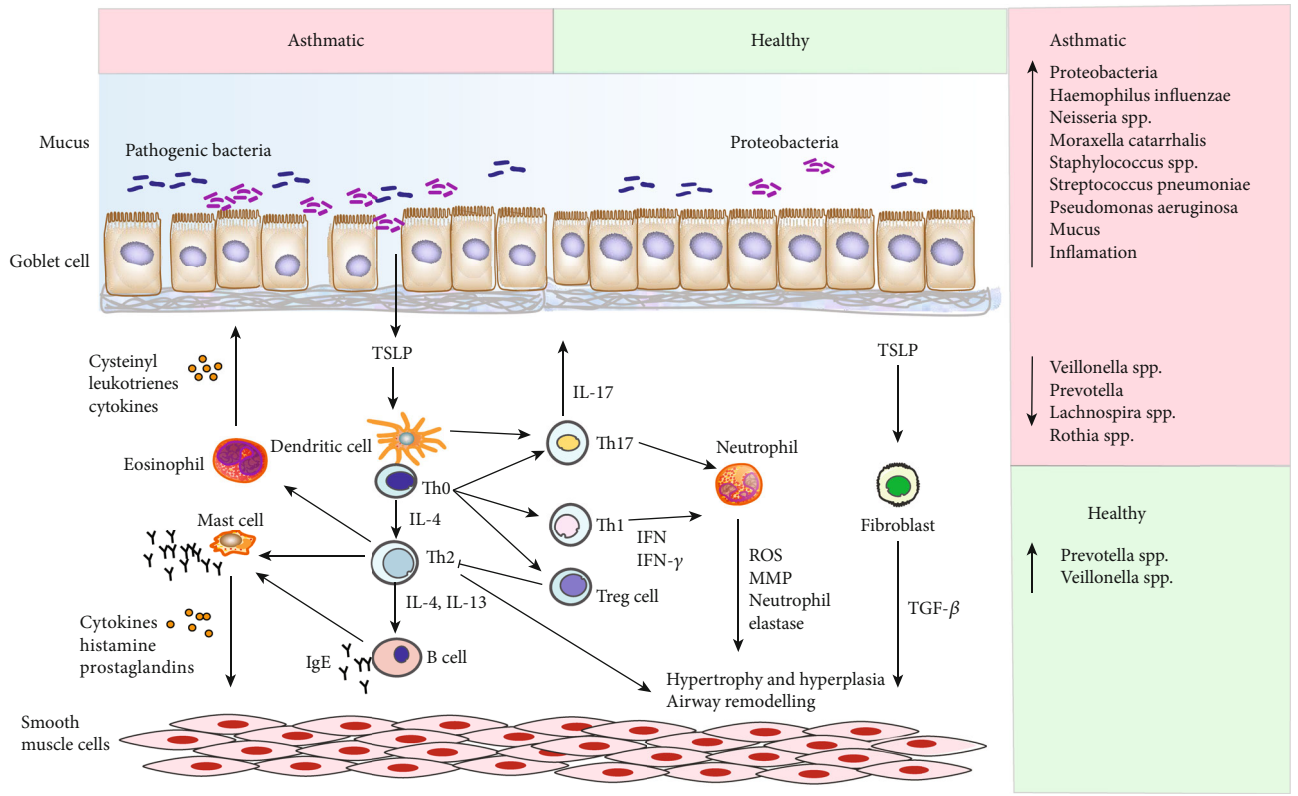


Figure 2: The roles of pathogens in the occurrence and development of asthma.

Microbiological studies showed that, at the acute exacerbation stage of COPD, there was no significant difference in respiratory microbes compared with the stationary phase [37]. The respiratory symptoms in the acute exacerbation stage of COPD are related to the colonization of new strains in the airway. Microbiological analysis showed a clear increase in the relative abundance of bacteria in one genus but without significant changes in other colonies. In the process of infection, the microbial pattern was completely different [38]. The latter had an obvious increase in the proportion of *Firmicutes* before the colonization of the dominant bacteria. This finding also supported the clinical features of acute exacerbation in both types. Taking into account the different microbial modes, different therapeutic measures are required. Although the composition of respiratory flora in patients with COPD has been clear through microbiological analysis, the role of respiratory flora in the pathogenesis of the disease remains to be further explored.

5. Immunity of Respiratory Flora and CF

CF is a familial autosomal recessive inherited congenital disease, which is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), resulting in the structural and functional alterations of the transmembrane protein in pulmonary CF, thereby causing chronic pulmonary infection and pulmonary function deterioration in patients, leading to early deaths [39]. CF is characterized by defective mucociliary clearance and chronic infection of complex microbial flora [40]. Infection, persistent inflammation, and acute periodic attacks lead to an irreversible decline in lung function. Although little is known about the factors contributing to acute exacerbation, antibiotic treatment can temporarily resolve lung symptoms and partially restore lung function. Acute exacerbations may be associated with changes in microbial density and the acquisition of new microbial strains.

Fodor et al. used massive pyrosequencing technology to identify changes in the airway microbial community structure of 23 CF patients during CF exacerbations and stabilities and obtained more than 350,000 sequences, representing nearly 170 distinct microbial floras [41]. Approximately 60% of the sequences obtained were from the known CF pathogenic bacteria *Pseudomonas aeruginosa* and *Burkholderiaceae*. Although antibiotic treatment and species richness declined slightly, the microbial community structure hardly changed. Moreover, the microbial composition in acute exacerbation was highly similar to that in the stationary phase, suggesting that exacerbations may be due to the spread of infection in the lungs, rather than changes in the composition of the microbial community.

The research through in vitro bacterial culture of the patients' sputa revealed that the predominant pathogenic bacteria were *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Staphylococcus aureus*. Meanwhile, other strains such as *Burkholderia*, *Stenotrophomonas maltophilia*, and *Achromobacteria xyloxydans* were also present in elderly patients [42, 43]. The diversity of microbial communities in the lungs of CF patients was decreased, and the degree of pulmonary inflammation was related to the decrease in

microbial diversity. Moreover, *Bacteroidetes* and *Fusobacteria* were the dominant strains in healthy individuals while *Actinobacteria* accounted for a larger proportion in CF patients. The structure of pulmonary florae was destroyed, pathogenic bacteria and conditional pathogens were multiplied, and florae were out of balance, which may be one of the causes of CF.

6. Immunity of Respiratory Flora and UACS

UACS, formerly known as postnasal drip syndrome (PNDS), is a syndrome of chronic cough caused by the reflux of allergic or nonallergic inflammatory secretions from the nasal cavity and nasopharynx into the pharynx [44]. It can be accompanied by a series of symptoms such as pharyngeal foreign body sensation, itchy pharynx, blocking sensation, and sputum adhesion in the pharynx. It is considered to be one of the most common causes of chronic cough. The pathogenesis of UACS remains unclear. Physicians in different countries have different definitions as well as treatments for UACS. The pathogenetic theories of UACS include the earliest theory of postnasal drip irrigation, the subsequent theory of chronic airway inflammation, and the more recent theory of sensory nerve hypersensitivity. Furthermore, some researchers believed that UACS was the clinical phenotype of irritable cough syndrome [45].

There is a slight difference in the types of pharyngeal flora between healthy people and patients with UACS. *Firmicutes* and *Proteobacteria* are the dominant flora in UACS that are different from the normal flora. Among these, *Lactobacillus* of *Firmicutes* and *Pseudomonas aeruginosa* of *Proteobacteria* have significantly increased and replaced the dominant position of original *Streptococcus* and *Neisseria* in the pharynx of normal people. Moreover, TRPV1 and TGF-β2 were increased significantly in UACS patients [46]. TRPV1 is a nonselective cation channel associated with cough sensitivity and widely distributed in sensory nerves from the upper to lower airways [47]. TRPV1 is regulated by multiple physicochemical factors, e.g., increased lactate secretion and the stimulation of inflammatory factors will lead to the increase of TRPV1. And it is highly expressed in pathological conditions such as chronic cough and high airway responsiveness. Another study showed significant elevation of both TGF-β1 and β2 in mammary secretions of dairy cows infected with *Pseudomonas aeruginosa* compared to those uninfected, demonstrating the correlation between *Pseudomonas aeruginosa* and TGF-β1 and β2. Meanwhile, *Pseudomonas aeruginosa* could also stimulate airway inflammation through a variety of known inflammatory pathways, leading to an increase in the prevalence of UACS [48].

7. Immunity of Respiratory Flora and Infection of Respiratory Fungal

Fungal colonization in the human body refers to the presence of a large number of fungi growing in the form of spores at a site where the human body communicates with the outside world, such as the alimentary canal, the upper airway, and urogenital tract, but without local tissues damage or symptoms [49]. Colonization is generally the final step in a long-lasting symbiotic or innocuous relationship between the fungus and the host and also is the first step in the conversion to fungal infection and the development of related diseases [50].

Contamination and colonization with *Candida* and *Aspergillus* in the upper airway are very common [51]. The results of ICU investigation in domestic general hospitals showed that *Candida* was the most common fungus colonizing in the lower airway, among which the infection rate of *Candida albicans* was the highest (37.91%), followed by *Aspergillus* (16.99%) [52]. Systemic or local immunosuppression caused by granulocytopenia in leukemia chemotherapy is an important cause of airway fungal infection. Pathogenic fungi can be colonized in patients, with infection and colonization by *Aspergillus fumigatus* and *Candida albicans* being the most common [53].

8. Inhaled Corticosteroids (ICSs) and Airway Flora Immunity

Glucocorticoid has a potent anti-inflammatory and immunosuppression function, which eliminates most pathogenic bacteria and improves the airway microenvironment by effectively reducing the levels of proinflammatory cytokines and airway inflammation. Inhaled corticosteroids, without obvious systemic side effects, can easily form an effective concentration in the airway and work directly, which is quickly destroyed by enzymes in alveoli and inactivated by the liver after entering the blood circulation.

Glucocorticoids can enter into the nucleus by binding with glucocorticoid receptor in the cytoplasm through the cell membrane and form hormone-hormone receptor complex, which combines with special DNA sequence to produce biological effect through the following three ways: (1) inhibiting the release of inflammatory factors such as TNF-α and leukotrienes by promoting the production of enzyme-linked protein I, resulting in the decrease of phospholipase A2-α; (2) inhibiting the production of inflammatory protein and its phospholipase A2-α by inactivating MAPK; (3) inhibiting the expression of cytokines, chemokines, cell adhesion factors, and their receptors by activating NF-κB pathway [54]. Glucocorticoids not only can directly inhibit inflammatory cells but also reduce the exudation of capillary and the formation of sputum. Studies have found that dexamethasone reduced lung inflammation by promoting the secretion of IL-12 and inhibiting the expression of IL-13 in rats [55]. However, there are still side effects in the long-term repeated use of inhaled corticosteroids to control respiratory diseases, such as dryness, hoarseness, and *Candida* infection in the oral and pharyngeal [56]. Furthermore, long-term use of glucocorticoids may also cause immunosuppression and aggravate the development of the disease by destroying the local mucosal barrier and microenvironment of the airway, resulting in the unbalance of the microbial flora. These will lead to changes in the microbiome and increase the risk of some systemic reactions such as airway infections (*pneumonia* and *mycobacterial* disease). Thus, rationally controlling the time of using hormones may reduce the incidence of adverse reactions.

Table 1: The protective effects of traditional Chinese medicine on asthma.

Mechanism	Traditional Chinese medicine	Reference
Regulating Th1/Th2 balance	Bulleyaconitine A	[71]
	Ligustrazine	[72]
	Selaginella uncinata flavonoids	[67]
	Turmeric (Curcuma longa)	[73]
Regulating Th17/Treg balance	Bufei Yishen formula	[74, 75]
	Baicalin	[76]
	Gu-Ben-Fang-Xiao-Tang	[77]
	Louqin Zhisou decoction	[78]
Regulating antigen presenting cells	Atractylodin	[79]
	Oligomeric proanthocyanidins	[80]
	Tilianin	[81]
	Wuhu decoction	[82]
Inhibiting oxidative stress	Citrus flavonoids	[83]
	Platycodi Radix	[84]
	Quercetin	[85]
	Saikosaponin A	[86]
	Wedelolactone	[87]
Inhibiting NLRP3 inflammasome	Abscisic acid	[88]
	Andrographolide	[89]
	EGCG	[90]

In addition, previous studies have proved that the diversity of oral microflora was decreased, while the proportion of *firmicutes* and *Bacteroidetes* was increased in normal obese children [57]. Because the diversity of flora is affected by many factors, maintaining the balance of flora is the main measure to reduce the occurrence of adverse reactions when infants are given Budesonide atomization therapy. Therefore, it is necessary to strengthen the detection of flora and maintain the balance of respiratory flora in clinical treatment to reduce the occurrence of adverse reactions [58].

Although ICSs have been widely used in the clinical treatment of respiratory diseases because of the strong anti-inflammatory activities, they could not effectively improve the inflammatory response of airway remodeling and proliferation. Most clinical reports have shown that excessive inhalation of ICSs may cause drug dependence or resistance and other adverse reactions, especially in children with asthma in the early stage, which will affect children's immune system and result in hormone-dependent. Therefore, exploring new anti-inflammatory drugs to combine or even replace hormones is the leading direction of drug development.

9. Probiotic Preparation and Improvement of Respiratory Flora Immunity

Probiotic refers to live biotherapeutic products (LBP) containing enough viable organisms with well-defined composition. It can change the composition of the flora at a certain site of the host through colonization, thereby improving the microecological balance of the host and playing a beneficial role [59]. On the one hand, probiotics can regulate the adaptive immune response of the host by promoting the release of cytokines from dendritic cells (DCs), altering the balance of Th1/Th2, shifting the immune response in the direction of Th1 cells, and inhibiting the response of Th2 cells. On the other hand, it can also play a role in the biological barrier by competing with pathogens and secreting antimicrobial peptides and other metabolites [60]. At present, probiotics have been used to treat diseases associated with immune dysregulation of the intestinal flora, such as diarrhea, obesity, type 2 diabetes, inflammatory bowel disease, and other diseases [61, 62]. In recent years, a probiotic composition has been invented, including *Lactobacillus rhamnosus* and *Lactobacillus plantarum*. This supplement inhibits the expression of inflammatory factors in the lung by activating TLR3 and RIG-1 signaling pathways in alveolar macrophages to promote IFN-β and also regulates lung flora and intestinal flora to alleviate respiratory syncytial virus (RSV) infection. Over-the-counter probiotics have been used in clinical trials to investigate their potential effects in various disease conditions, but it is required more stringent quality control to ensure the purity and efficacy of products. The challenge of detecting unwanted microbial contaminants is that the sensitivity of detection may be reduced in the presence of the required probiotic microorganisms. One of the strategies under study was to reduce or eliminate the growth of bacteria in the product for improving the sensitivity of detecting the contaminating microbes. FDA scientists developed and used recombinant phage lysine as a reagent to improve LBP purity detection through "mock" purity assays ("test-tube" studies)

where *Lactobacillus jensenii* represented the probiotic's product strain. However, the type of strains, dosage, and course of probiotic can affect the outcome of clinical application, and even the same flora will have different effects at different ages. Therefore, the prevention and treatment of respiratory infection with probiotics deserves further research.

10. Protective Effects of Natural Products against Various Stimuli-Induced Lung Dysbiosis

Natural products or traditional Chinese medicine have been reported to improve airway immunity, ameliorate the function of the blood-air barrier, inhibit inflammation, and reduce growth of pathogenic bacteria due to its advantages of multicomponent, multichannel, and multitarget (Table 1), which had the potential for the prevention and treatment of several respiratory diseases including COPD, asthma, and UACS [63]. Baicalin, a kind of flavonoid derived from Scutellaria baicalensis (Huangqin in Chinese), with broad-spectrum antibacterial activity and obvious anti-inflammatory activity, which can effectively improve the respiratory system inflammation and treat respiratory disease by inhibiting a variety of bacteria and fungi [64, 65]. Baicalin has an inhibitory effect on *Candida albicans* and *Staphylococcus aureus*, which was correlated with the concentration of baicalin, that is, the DNA synthesis of *Candida albicans* was decreased with the increase of drug concentration [66, 67]. Cryptotanshinone has antiasthmatic effect, the mechanism of which may be related to downregulating Th2 cytokines and reducing inflammatory cell infiltration [68]. The Yinqiao powder is equipped with the effect of clearing away heat and toxin by inhibiting *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [69]. Xiaochaihu decoction, a famous herbal medicine exerting antiallergic and antitussive effects, has been widely used for the treatment of asthma in clinics [70]. It can effectively reduce the frequency of asthma attacks and airway infection in children with asthma and alleviate airway inflammation by enhancing the immune function and antiallergic ability of airway mucosa. However, the current research is still insufficient. Most studies focus on in vitro antibacterial (*Streptococcus pneumoniae*, *Staphylococcus aureus*, etc.) activities of the agents, but few considered the antibacterial effect in vivo. And due to the complexity of pharmacodynamics, it is difficult to draw the conclusion whether antibacterial effects of drugs or anti-inflammatory effects play the decisive role in airway inflammation.

There is still a lack of robust research on pharmacological mechanisms. Therefore, further efforts are needed to elucidate the underlying mechanism in germ-free mice.

11. Conclusion

Changes in the immunity of respiratory flora play an important role in the development of multiple chronic respiratory diseases. In recent years, with the application of various new techniques in the detection of microbes in respiratory specimens, growing evidence showed that respiratory microbial population and its related local mucosal immunity were associated with the clinical manifestations, acute exacerbation, and prognosis of chronic respiratory immune disorders, such as asthma and COPD. Through studying the microbiological basis of the progression of human respiratory diseases, we can make a better understanding of the local immunological mechanism of the progression of these diseases, facilitate the judgment of disease types, predict the responsiveness to treatment, and evaluate the therapeutic effect. It provided a new idea for the clinical diagnosis, treatment, prediction, and prognosis of respiratory diseases.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

A New Lectin from *Auricularia auricula* Inhibited the Proliferation of Lung Cancer Cells and Improved Pulmonary Flora

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Lectins are widely distributed in the natural world and are usually involved in antitumor activities. *Auricularia auricula* (*A. auricula*) is a medicinal and edible homologous fungus. *A. auricula* contains many active ingredients, such as polysaccharides, melanin, flavonoids, adenosine, sterols, alkaloids, and terpenes. In this study, we expected to isolate and purify lectin from *A. auricula*, determine the glycoside bond type and sugar-specific protein of *A. auricula* lectin (AAL), and finally, determine its antitumor activities. We used ammonium sulfate fractionation, ion exchange chromatography, and affinity chromatography to separate and purify lectin from *A. auricula*. The result was a 25 kDa AAL with a relative molecular mass of 18913.22. Protein identification results suggested that this lectin contained four peptide chains by comparing with the UniProt database. The FT-IR and β-elimination reaction demonstrated that the connection between the oligosaccharide and polypeptide of AAL was an N-glucoside bond. Analyses of its physical and chemical properties showed that AAL was a temperature-sensitive and acidic/alkaline-dependent glycoprotein. Additionally, the anticancer experiment manifested that AAL inhibited the proliferation of A549, and the IC_{50} value was $28.19 \pm 1.92\,\mu$g/mL. RNA sequencing dataset analyses detected that AAL may regulate the expression of *JUN*, *TLR4*, and *MYD88* to suppress tumor proliferation. Through the pulmonary flora analysis, the bacterial structure of each phylum in the lectin treatment group was more reasonable, and the colonization ability of the normal microflora was improved, indicating that lectin treatment could significantly improve the bacterial diversity characteristics.

1. Introduction

Lectins are proteins or glycoproteins that have at least one carbohydrate or derivative binding site and are different from immunoglobulin in nature and do not have the function of catalytic enzymes. They can specifically recognize and bind to sugars or sugar chains without changing the covalent structure [1]. Lectins are widely distributed in nature, ranging from microorganisms to animals and plants. It was named lectin because it can agglutinate blood cells and make the blood cells show reticular sedimentation [2]. Fungi lectins are the most studied in the past decades and show different structures, functions, and carbohydrate-binding specificities [3]. Lectins have usually been used as a tool to distinguish between cell types and have been involved in several biological activities such as mitogenic [4], anti-insect [5], anti-inflammatory [6], antimicrobial [7], and antitumor [8] activities. Undoubtedly, lectins can serve as a therapeutic goldmine in the near future. In the past decade, a flux of interest in the study of lectins from natural sources has been observed [9]. Fungi have not only turned into a rich hotspot for new lectins with extraordinary sugar specificities but have also turned into potential candidates for biomedical applications [10]. Many microfungal strains from *Fusarium* sp. [11–13] and *Penicillium* sp. [14–16] have been investigated for lectin activity. Various fungal lectins exhibit interesting

physiological impacts such as mitogenic incitement of lymphocytes/splenocytes [17], suppression of cancer cell proliferation [18], and as immunomodulators [19]. In the study on the inhibition of tumor activity of lectin, Li et al. found that feeding mice *Pleurotus citrinopileatus* lectin at a dose of 5 mg/kg per day for 20 days could effectively inhibit the growth of 80% mouse sarcoma [20]. Li et al. found that purified *Hericium erinaceus* lectin with a molecular weight of 51 kDa could inhibit the proliferation of human liver cancer HepG2 and human breast cancer McF-7 cells [21]. Gondim et al. found that lectin in the seeds of a Brazilian fruit could inhibit the proliferation of human ovarian cancer A2780, lung cancer A549, breast cancer McF-7, and prostate cancer PC3 cells. In addition, lectin could block the retention of ovarian cancer cells in the G2/M phase, activate the expression of caspase 9, and delay cell apoptosis after 24 h of lectin action [22]. Chakkere et al. found that lectin in the fruit of Indian schistosomes could effectively inhibit the growth of human chronic myeloid leukemia K562, human colon cancer HT29, human cervical cancer HeLA, and human breast cancer McF-7 cells [23]. Lacerda et al. found that when the concentration of lectin was 100 mg/mL, it could inhibit 83% of mouse melanoma cells; lectin also had a protective effect on the stomach [24]. Liao et al. found that mussel lectin could bind to Gb3 on tumor cells and promote apoptosis of breast cancer cells [25].

Auricularia auricula (*A. auricula*) is a medicinal and edible homologous fungus [26]. In the biological classification system, *A. auricula* belongs to the fungus world basidiomycete. *A. auricula* contains many active ingredients, such as polysaccharides, melanin, flavonoids, adenosine, sterols, alkaloids, and terpenes, which can play a crucial role in the molecular recognition mechanism of cell-cell and cell-matrix interactions [27]. These macromolecules can reversibly bind to specific sugars and precipitate polysaccharides, glycoproteins, and glycolipids, so they can be used as cell recognition factors to participate in the targeting of tumor cells and have high medicinal value and antitumor activity. At an early stage, lectins in plants were studied deeply by scholars, most of which were about the seeds of leguminous plants. While for the fungus, Zhao et al. isolated two lectins with the same single subunit of 15.8 kDa from the fruiting body of *Agrocybe cylindracea*, and these lectins can bind to HeLa cells as a special signaling molecule and promote their apoptosis for the first time [28].

Lung cancer (LC) is one of the most dangerous malignancies with the fastest increase in morbidity and mortality. In the past 50 years, a significant increase in the incidence and mortality of lung cancer has been reported in many countries [29]. In recent years, LC is still one of the most common malignant tumors in human beings, and the incidence and mortality of LC are increasing year by year [30, 31]. There are two main types of lung cancer, namely, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) [32, 33]. Although researchers are beginning to develop therapeutic targets for LC, the prognosis of patients after treatment is poor, and the recurrence rate of patients after surgery is as high as 35-50% [34]. Therefore, it is essential to seek a novel therapy to improve the survival rate.

Therefore, this study was expected to isolate and purify lectin from *A. auricula*, determine the glycoside bond type and sugar-specific protein of *A. auricula* lectin (AAL), and finally determine its antitumor activities. By studying the biological activity of AAL and analyzing its ability to inhibit tumor proliferation, we can discover its important role in the research and development of clinical tumor drugs.

2. Materials and Methods

2.1. A. auricula and Lung Cancer Cell Lines. A. auricula was picked at the Nyingchi Prefecture, Millin County (Tibet, China), screening for the fruiting body that is large and full and with bright color for the test operation strain. A549 lung cancer cells were purchased from the Cell Bank of Shanghai Academy of Sciences.

2.2. Lectin Extract and Purification

2.2.1. Lectin Extract. To isolate AAL, a fresh *A. auricula* was taken, and its surface moisture was sucked dry with filter paper. After being dried by a freeze dryer, 5 grams of dried *A. auricula* was accurately weighed, 400 mL of 10 mM phosphate buffer was added, and the mixture was stirred into a homogenate by a blender. The supernatant was taken and centrifuged for 20 minutes at 2795 × g for a blood agglutination activity test. After the homogenate of *A. auricula* was precipitated by 20%-80% ammonium sulfate for 12 h, it was placed in a refrigerator at 4°C for overnight magnetic stirring for dialysis; then, the solution in the dialysis bag was transferred in batches to a freeze-drying machine for lyophilization and labeling. Lyophilized powder was used for a hemagglutination activity test and protein quantitative analysis to determine the optimal saturation of lectin extraction. The dry weight, protein concentration, and agglutination activity of the product were analyzed.

2.2.2. Lectin Purification. An AKTA Purifier100 protein purification system was selected for the purification of *A. auricula* lectin. 10 mM phosphate buffer solution and 20% ethanol solution were prepared in advance. The bubbles were removed by an ultrasonic cleaning machine for 10 min, then the AKTA system was connected. After the freeze-dried powder precipitated by ammonium sulfate was fully dissolved by phosphate buffer, the HiTrap DEAE Anion Exchange Column was prebalanced with phosphate buffer. When the samples attached to the DEAE column, phosphate buffer containing 0.1 M NaCl was used for elution at an elution rate of 0.5 mL/min and a system pressure of 0.3 MP. With the increase of the concentration of the eluent, the elution peak began to appear. According to the peak appearing time for collecting eluent, all the peak areas of the eluent were collected, and the active components of coagulation were determined by a hemagglutination test and then lyophilized in the freeze dryer after dialysis.

The dialysis freeze-dried powder was dissolved and combined with activated PSM-Sepharose in an ice bath for 2 h, the samples were placed in a refrigerated centrifuge and spun at1000 × g for 10 minutes, the supernatant was sucked out at a low temperature for preservation, PSM-Sepharose was

cleaned three times by phosphate buffer and combined with 0.1 M of glycine at different pH levels (pH 1.5, pH 3.0, and pH 4.5) in an ice bath for 10 min, the samples were placed in a refrigerated centrifuge for 10 minutes and spun at1000 × g, and the supernatant was taken and the pH was adjusted to neutral; the Tris-HCl with a pH of 8.5 was used. Four ultrafiltration tubes were placed in a refrigerated centrifuge for 1 h, 3 h, and 5 h, respectively, and the solution after ultrafiltration was lyophilized to obtain lectin, which was precisely weighed and the extraction rate of lectin was calculated. The initial supernatant was detected by a hemagglutination test. If there was activity, the above process should be repeated until the lectin was fully bound to PSM-Sepharose. According to the Laemmli method [35], the purity of lectin was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.3. Structural Identification. The molecular weight of lectin was determined by SDS-PAGE, and the bands were obtained by ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography. The relative molecular mass of lectin can be accurately determined by using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/TOF 5800 System) [36]. The 4000 Series Explorer V3.5 was used for data and graph processing.

The lectin was determined by a mass spectrometer (Applied Biosystems, MALDI-TOF/TOF 5800) for protein identification by primary and secondary mass spectrometry. Through UniProt database comparison, it was concluded that the lectin might contain peptides. The Edman degradation method is mainly used in the analysis of N-terminal amino acids. The lectins were first subjected to SDS-polyacrylamide gel electrophoresis, and the proteins in the gel were transferred to a PVDF membrane through an electric transfer slot. After setting by PPSQ-30 Analysis Software, N-terminal sequencing was performed on a PPSQ-30 automatic protein polypeptide sequencing instrument (Shimadzu). PPSQ-30 Data Processing Software was used for exporting data and graphs. The presence and the amount of lectin in the collected fractions were confirmed with the BCA Protein Quantification Kit (Pierce Biotechnology, Rockford, IL, USA). The sugar content of lectin was determined by the phenol-sulfuric acid method [37].

2.3. Analysis of Physicochemical Properties

2.3.1. Effects of Temperature and pH on Lectin Activity. A solution of 1 mg lectin was prepared and incubated in a water bath at 20°C-100°C for 40 min, respectively, to observe the coagulation activity of the lectin. The ability of agglutinating rabbit blood was detected by dissolving lectin in PBS with different pH levels.

2.3.2. Identifying the Sugar Specificity of Lectin. Lectins bind to sugars or proteins through sugar-binding sites (CRD), so lectins usually have specific binding sugars or proteins. After various sugars (derivatives) and proteins are added to the lectin solution, the inhibition of lectin coagulation activity is verified. Monosaccharides such as glucose, galactose, mannose, xylose, rhamnose, sialic acid, N-acetylgalactosamine, and pig gastric mucosa proteins were taken to prepare a sugar solution and a protein solution, respectively. The sugar and the protein solutions were diluted one by one, and then 30 μL was drawn from each of them and added to the 96-well plates and the same volume of 2% rabbit blood was added. They were left at room temperature for 1 hour. We observed whether the sugar solution and the protein solution have any influence on the rabbit blood agglutination and can eliminate the influence of themselves on the blood coagulation activity. We take 0.1 g AAL, make up a 100 μg/mL of lectin solution by using the PBS mixture, then draw 30 μL in turn and add it to each hole of the 96-well plate. We then add 30 μL of the concentration of the sugar solution and the protein solution, and we finally add 2% rabbit blood of the same volume, keeping the solutions at room temperature for 1 hour and then observing the clotting results.

2.4. Antitumor Activity Potential Evaluation of Lectin in A549 Cells

2.4.1. A549 Cell Culture. A549 lung cancer cells were cultured in RPMI 1640 medium (90% 1640 medium + 10% FBS + 1% double antibody, i.e., a mixture of penicillin and streptomycin), and the resuscitation lung cancer cells (A549) were cultured for 2-3 generations. The antitumor experiment was carried out when the cancer cells were in the logarithmic growth stage.

2.4.2. Cell Inhibition Analysis. The Cell Counting Kit-8 was used to detect the inhibition of lectin on the proliferation of tumor cells. Double distilled H_2O was added to the sides of the 96-well plates to prevent the medium from evaporating. The suitable holes in the 96-well plates were selected as sample holes and control holes, and three groups of parallel holes were set, respectively. The cells in the logarithmic growth stage were digested with trypsin, and culture medium was added to homogenate the cells into a cell suspension with a density of 2.5×10^4 cells/mL. The 200 μL cell suspension was inoculated in each well. Then, the 96-well plates were placed in a wet incubator at 37°C with 5% CO_2 for 24 hours. The next day, the 96-well plates were taken out, the original medium was sucked out, and an equal volume of PBS solution was added. Shaking gently, the mixture was sucked out and the whole process was repeated three times. The lectin medium with different concentrations of 100 μL were added, and the medium containing 1‰ DMSO was added into the control well; then, the culture was continued for 24, 48, or 72 hours. We added 10 μL CCK-8 solution to each well, then we incubated it in the incubator for 1 hour, and we detected OD_{450} with the enzyme marker. The inhibition rate of AAL on tumor cells was calculated according to the following formula (%).

$$\text{Cell inhibition rate}\,(\%) = \left[1 - \frac{\text{OD}_{\text{samples}}}{\text{OD}_{\text{control}}}\right] \times 100\%. \quad (1)$$

SPSS statistical analysis software was used to calculate half of the inhibitory concentration (IC_{50}) of lectin on tumor cells.

2.5. Study on the Antitumor Molecular Mechanism of AAL

2.5.1. RNA Sequencing. In order to further understand the molecular mechanism of AAL inhibiting the growth of tumor cells, we chose the A549 cells cultured with the initial concentration of 100 μg/mL lectin for 72 hours as the experimental group, and we chose the A549 cells cultured without lectin for 72 hours as the blank control group. There were 3 replicates in the experimental group and the control group for RNA sequencing.

2.5.2. mRNA Library Construction and Sequencing. Total RNA was extracted using the TRIzol Reagent (Invitrogen, CA, USA) following the manufacturer's procedure. The total RNA quantity and purity were analyzed using the 2100 Bioanalyzer and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number > 7.0. Approximately 10 μg of total RNA representing a specific adipose type was subjected to isolate poly(A) mRNA with poly-T oligoattached magnetic beads (Invitrogen). Following purification, the mRNA is fragmented into small pieces using divalent cations under elevated temperature. Then, the cleaved RNA fragments were reverse transcribed to create the final cDNA library in accordance with the protocol for the mRNA-Seq sample preparation kit (Illumina, San Diego, USA); the average insert size for the paired-end libraries was 300 bp (±50 bp). And then we performed the paired-end sequencing on an Illumina Hiseq 4000 at LC Sciences, USA, following the vendor's recommended protocol.

2.5.3. Bioinformatics Analysis. In this paper, we quantified gene expression abundance by calculating the FPKM value of the gene. We performed transcript abundance estimation and differentially expressed testing as follows: The mapped reads of each sample were assembled using StringTie. Then, all transcriptomes from Samples were merged to reconstruct a comprehensive transcriptome using perl scripts. After the final transcriptome was generated, StringTie and edgeR were used to estimate the expression levels of all transcripts. StringTie was used to estimate the expression level for mRNAs by calculating FPKM. The differentially expressed mRNAs and genes were selected with $\log 2(\text{fold change}) > 1$ or $\log 2(\text{fold change}) < -1$ and with statistical significance (P value < 0.05) by R package. The differentially expressed genes were screened when the inhibition rate of the A549 cells reached the maximum. The GO database and KEGG database were used for functional annotation and signal pathway enrichment analysis of the differentially expressed genes, and $P < 0.05$ was regarded as the significance threshold. According to the results of GO and KEGG functional annotation, tumor-related immune and apoptosis genes were screened out, and PPI analysis was performed to screen out key tumor factors. Relevant signal pathways were analyzed for the signal pathway gene network, so as to further search for tumor key genes.

2.6. The Regulatory Effect of Lectin on the Pulmonary Flora

2.6.1. Sample Collection. We took A549 cells at their logarithmic growth phase, stained them with 0.4% trypan blue staining solution, counted viable cells under a microscope to ensure that the cell viability is greater than 95%, and adjusted the concentration to 2.5×10^6 cells/mL with physiological saline. The mice were injected with 5×10^5 A549 cells per mouse through the tail vein to establish a tumor-bearing mouse model. All the mice were male mice, and they were divided into 2 groups by a random number table: the treatment group and the control group, each with 8 mice. After 2 weeks, the gavage operation of the corresponding experimental group was completed. We gavaged mice with 50 mg/kg lectin or 0.2 mL normal saline daily for 14 days. Lung tissues of mice were weighed, and genomic DNA was extracted from the left lung.

2.6.2. 16S Ribosomal RNA Gene Sequencing and Data Analysis. The V3-V4 region of the bacteria's 16S ribosomal RNA (rRNA) gene was amplified by PCR with barcode-indexed primers. Amplicons were then purified by gel extraction and were quantified using QuantiFluor-ST. After normalization, PCR amplicons were sequenced on an Illumina MiSeq platform (PE250). Alpha diversity was evaluated based on the following metrics: observed species and Shannon diversity index. A nonparametric two-sample t-test was used to compare the alpha diversity metrics between the control group and the treatment group.

3. Results and Discussion

3.1. Extraction of Lectin from A. auriculate. After a preliminary analysis, we found that ammonium sulfate with 80% saturation was the best extraction method for the crude extract of AAL (Figure 1(a)), and the clotting activity of the crude extract separated by 20% and 80% saturation of ammonium sulfate was 2^3, far higher than other extracts (Figures 1(c) and 1(d)). We also discovered that the crude extract protein concentration decreases firstly and then increased when the ammonium sulfate saturation increased, peaking at 80% saturation (Figures 1(b) and 1(d)). Considering the nature of the lectins, we determined that 80% saturation of ammonium sulfate is the best extracting method for crude extracting from *A. auriculate*.

3.2. Purification of Lectin from A. auriculate. A HiTrap DEAE column was subjected to ion exchange chromatography, and four elution peaks P1, P2, P3, and P4 appeared at 280 nm at last. The elution components corresponding to each peak were collected, and the results showed that component P2 had the largest absorption peak (Figure 2(a)), and we finally identified its coagulation activity as 2^5. Collected for freeze-drying, the proteins with specific binding capacity were bound to PSM-Sepharose by changing the pH of the system. There was a single elution peak of lectin isolated by affinity chromatography, which indicated that we got a single purity lectin through the extraction and purification steps (Figure 2(b)). In the process of affinity chromatography, elution conditions and ultrafiltration time were optimized, and hemagglutination activity was used as the evaluation criterion. When the coagulation activity of the eluent reached a maximum of 2^8 at pH 3.0 and ultrafiltration time of 3 h

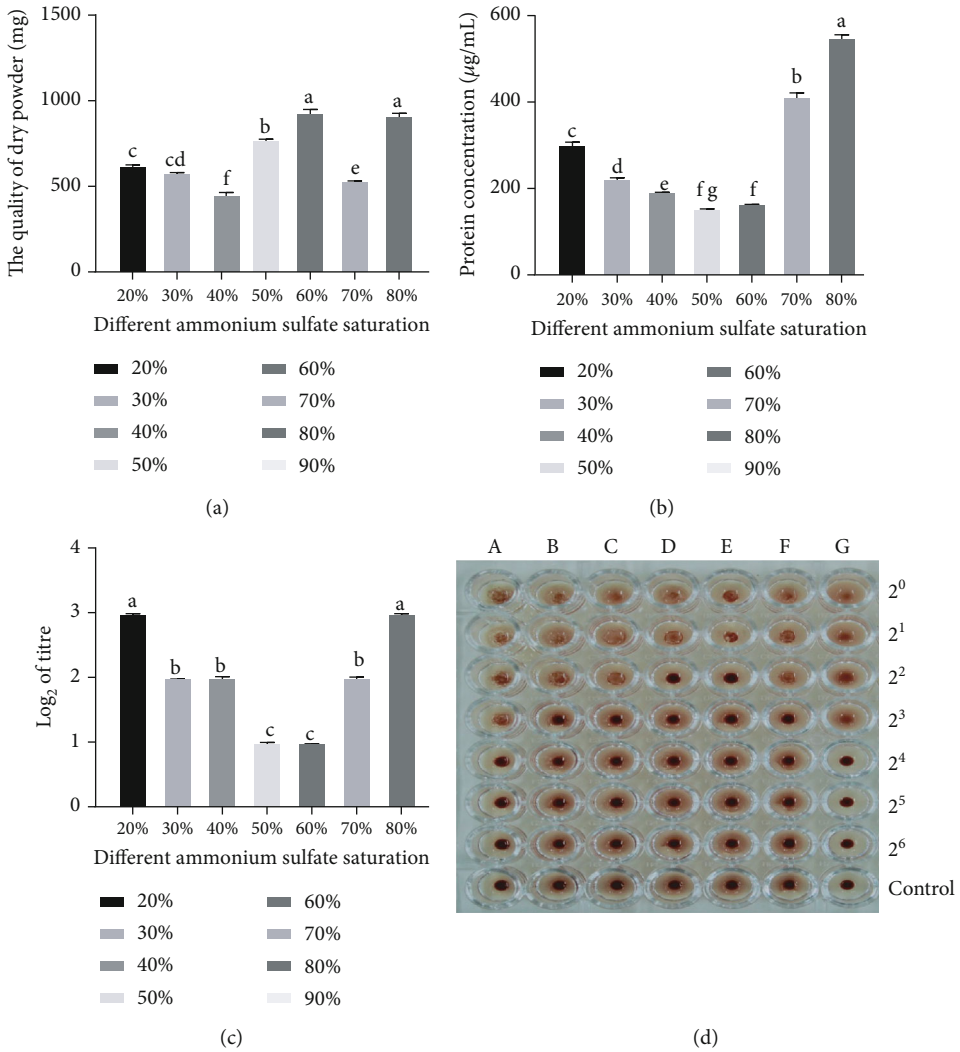


Figure 1: Effect of ammonium sulfate fractional precipitation on the extraction rate of lectin. Different ammonium sulfate saturations affect the quality of dry powder (a), protein concentration (b), and protein concentration (c). Hemagglutination activity of ammonium sulfate fractional precipitation (d) (A-G: crude extract precipitated by ammonium sulfate with a saturation of 20%-80%; 2^0-2^6: concentrations of lectin crude extract, respectively, in turn 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.13 mg/mL, 0.06 mg/mL, 0.03 mg/mL, and 0.015 mg/mL. PBS was used as control).

(Figures 2(c) and 2(d)), the optimal hemagglutination activity of this result was shown (Figure 2(e)). The possible reason was that the pH of the eluent was too small, which affected the activity of lectin, while if the pH was too large, the lectin cannot be eluted completely.

Through the separation and purification and the optimization of the conditions, a pure AAL was obtained, and the purification multiple of AAL was 30.53 (Table 1). According to the formula, the extraction rate of AAL was 0.068%.

In this experiment, ammonium sulfate precipitation, HiTrap DEAE anion exchange chromatography, and PSM-Sepharose 4B affinity chromatography were used to separate and purify AAL with a molecular weight of 25 kDa from *A. auricula* for the first time. By SDS-PAGE detection, the lectin was found to be a single-subunit protein. The extraction conditions were optimized, with 80% ammonium sulfate precipitation and extraction, as well as eluent pH 3.0 and ultrafiltration time 3 h during affinity chromatography, were selected as the optimal extraction scheme of lectin, and the extraction rate was 0.068%. After three steps of separation and purification, a single lectin was obtained from *A. auricula*, and the purification factor was 30.53. In recent years, many researchers have discovered lectin in edible fungi. Tateno et al. isolated a 35 kDa tetramer lectin from sulfur bacteria by Sepharose 4B one-step affinity chromatography. Jiang used ammonium sulfate precipitation and GlcNAc-sepharose 6B affinity chromatography to purify AAL-2 with a molecular weight of 43.175 kDa from *Agrocybe cylindracea* [38].

3.3. Determination of Relative Molecular Weight of AAL. The molecular weight of lectin was detected by SDS-PAGE. After the purification process of ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography, a band with a molecular weight of 25 kDa was obtained (Figure 3(a)). After the AAL treatment by β-mercaptoethanol, the SDS-PAGE results were still a band of 25 kDa

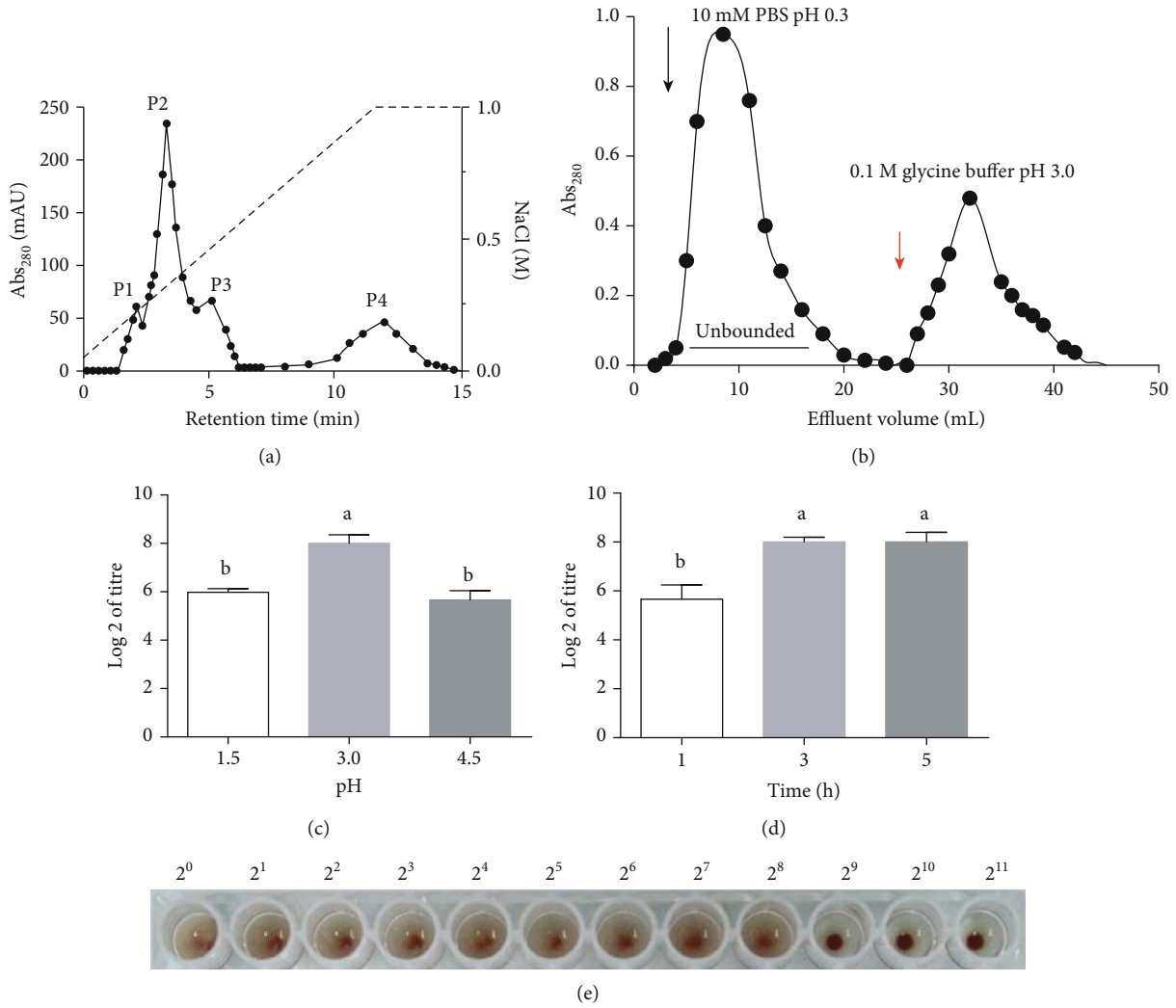


Figure 2: Processes and conditions for the purification of AAL. Ion exchange chromatograph of the crude extract of AAL (a). Affinity chromatograph of AAL (b). Effects of eluent conditions (c) and ultrafiltration time (d) on hemagglutination activity of AAL. Hemagglutination activity of AAL at different titres (e).

Table 1: Statistics of AAL extraction by different extraction methods.

Separation method	Total protein (mg)	Total coagulation activity (HU)	Specific activity (HU·mg-1)	Purification fold	Recovery rate (%)
Crude	620.00	3200	5.16	1	100
$(NH_4)_2SO_4$ precipitation	279.00	2840	10.18	1.79	88
HiTrap DEAE	36.20	1280	35.36	6.85	40
PSM-Sepharose 4B	6.50	1024	157.54	30.53	32

(Figure 3(b)). This suggested that AAL was a single subunit of glycoprotein.

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/TOF 5800) was used to analyze the relative molecular weight of AAL, and it was finally confirmed as 18913.22. The lectin protein was identified by a mass spectrometer (Applied Biosystems, MALDI-TOF/TOF 5800). Through UniProt database comparison, it was concluded that the lectin might contain four peptides, and the amino acid sequences were QIDAERK, TNHSVVTWNDK, RLNFTAGNPFPR, and VRELEQQVDSMTK, respectively. These sequences were not found in the existing *A. auricula* protein mass spectrometry library, so it was inferred to be a new protein in *A. auricula* and also determined to be a new lectin. The protein polypeptide sequencing machine was used to determine the amino acid sequence. Finally, the 13 amino acid sequences of the N-terminal were determined, and they are as follows: ITAPTTTSSAATE.

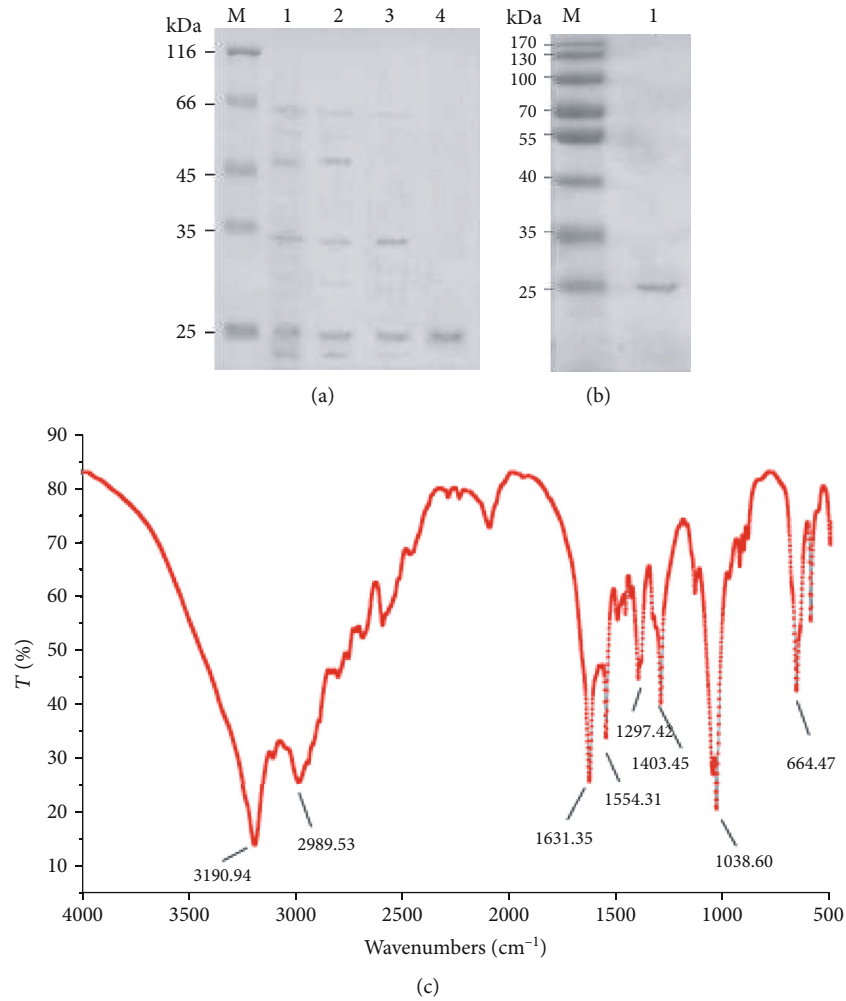


Figure 3: SDS-PAGE for the different periods of AAL and FT-IR detection. (a) Molecular weight detection of AAL by different extraction steps (M: standard molecular weight protein; 1: crude extraction components; 2: 80% ammonium sulfate precipitation component; 3: components of ion exchange chromatography; 4: affinity chromatography components). (b) Molecular weight of AAL was detected after β-mercaptoethanol treatment (M: standard molecular weight protein; 1: AAL after β-mercaptoethanol treatment). (c) FT-IR detection of AAL.

FT-IR was used to identify the binding mode of the oligosaccharide chain and the peptide chain of lectin. The infrared spectrum scanning range of lectin was from 400 cm^{-1} to 4000 cm^{-1}, and the absorption peak near 3190.94 cm^{-1} was the stretching vibration peak of the polysaccharide O-H bond. A wide absorption peak near 2989.53 cm^{-1} was the characteristic absorption peak of the C-H bond bending and vibration. The strong absorption peaks around 1631.35 cm^{-1} and 1554.31 cm^{-1} are the C=O bond stretching vibration peak and the N-H bond bending vibration peak on the amide. The absorption peak near 1403.45 cm^{-1} was characteristic of polysaccharide. The strong absorption peak near 1297.41 cm^{-1} was the stretching vibration absorption peak of the C-N bond on the amine. The strong absorption peak near 1038.60 $cm^{-1}$was either the C-O bond, the C-C bond stretching vibration, or the C-OH bond bending vibration of the polysaccharide chain. The strong absorption peak near 664.67 cm^{-1} is the bending vibration outside the C-H bond plane on the benzene ring (Figure 3(c)).

3.4. Analysis of Physicochemical Properties of Lectin from *A. auricula*

3.4.1. Effects of Temperature and pH on Hemagglutination Activity. A solution of 1 mg lectin was prepared and incubated in a water bath at 20°C-100°C for 40 min. The hemagglutination activity of the lectin was observed to remain unchanged within 50°C but gradually declined when higher than 50°C. At 90°C, the hemagglutination activity completely disappeared, indicating that the lectin was a temperature-sensitive glycoprotein. The lectin solution was incubated in a constant-temperature water bath at 50°C for 10-100 min, and the maximum hemagglutination activity was maintained within 40 min. With the increase of time, the hemagglutination activity decreased, and the hemagglutination activity remained unchanged after 80 min. The lectin was dissolved in PBS at different pH levels, and its ability to agglutinate rabbit blood was tested. At pH 2, there was no hemagglutination activity. With the increase of pH, the hemagglutination

activity of AAL increased gradually. At pH 7-8, the hemagglutination activity remained stable. While at pH 9, the hemagglutination activity decreased significantly. When the pH reached 10, the hemagglutination activity was completely lost, indicating that the lectin was an acid–base-dependent protein.

3.4.2. Sugar Specificity of AAL. When joining all kinds of sugars (derivative) and proteins in the AAL solution, respectively, to verify the inhibitory activity of blood coagulation, the results showed that glucose, galactose, mannose, rhamnose, and xylose monosaccharide sugar do not suppress AAL activity of blood agglutination. When N-acetyl galactosamine and sialic acid derivatives such as sugar were added to AAL, there was no activity of blood agglutination inhibition. The hemagglutination activity of AAL was inhibited only when porcine mucosal protein was added, and the minimum inhibitory concentration of lectin was 5 μg/mL, which indicated that the AAL did not have a specific recognition effect on monosaccharides and sugar derivatives, but had a specific recognition effect on mucoprotein (Table 2).

Table 2: The carbohydrate specificity of AAL.

Sugars (derivatives) and proteins	Minimum inhibition concentration (mM/μg·mL$^{-1)}$
Glc	N.i.[1]
Gal	N.i.
Man	N.i.
Rha	N.i.
Xyl	N.i.
GlcNAc	N.i.
PSM	5
SA	N.i.

[1]No inhibition.

3.4.3. Protein Quantification and Sugar Content of AAL. Using the BCA protein quantitative kits, we detected the protein concentrations of lectins using the linear regression equation $Y = 0.7968 + 0.1164X$, with the correlation coefficient $R^2 > 0.99$; the results were believable. The enzyme standard instrument had a 562 nm absorbance value and a sample hole measuring absorbance value of 0.283, according to the standard curve obtained by AAL protein concentration which was 209 μg/mL. According to the phenol-sulfuric acid method for determining the sugar content of AAL, the linear regression equation was $Y = 0.1803 + 0.0216X$, and the correlation coefficient was $R^2 > 0.99$, which indicate that the results were credible. The enzyme standard instrument had a 490 nm absorbance value and a sample hole measuring absorbance value of 0.296, according to the standard curve to calculate AAL sugar concentration which was 1.52 mg/mL; the polysaccharide content was 10.3%.

Previous studies have found that most lectins extracted from edible fungi are single-subunit proteins, and all of them show agglutination activity against erythrocyte. For example, Lin et al. found that lectin from edible fungi could agglutinate not only human erythrocytes but also rabbit erythrocytes, with stronger hemagglutination activity against rabbit erythrocytes [39]. Guo et al. studied the clotting activity of various edible fungi on chicken blood erythrocytes, and the results showed that most of them had blood clotting function [40]. In this experiment, lectin had agglutinating activity on rabbit blood, and the maximum titer was 2^8. Carbohydrate recognition domain (CRD) is used for the binding of lectin and for recognizing the position of the sugar chain. The sugar or protein specifically binding lectin from different sources is different. Osterne et al. obtained the specific lectin of α-cymene-D-mannoside from honeysuckle seeds which showed anti-inflammatory and cytotoxic activities [41]. Li et al. isolated and purified N-acetyl galactosamine/galactose-specific agglutinin from marine invertebrate mussels [42]. Wu et al. isolated trehalose-specific lectin from oyster sperm and demonstrated its structural model [43]. The results of the sugar inhibition of lectin showed that monosaccharides and sugar derivatives had no specific binding activity, while pig gastric mucosa protein (PSM) had an inhibitory effect on its coagulation activity. The minimum inhibition concentration was 5 μg/mL, indicating that lectin had specific binding to pig gastric mucosa protein. In addition, the coagulation activity of lectin decreased or was even lost under high temperatures and acidic and alkaline conditions, indicating that AAL is a kind of temperature-sensitive, acid–base-dependent glycoprotein. The protein quantitative results showed that the protein concentration of lectin was 209 μg/mL, and the sugar content of lectin detected by the phenol-sulfuric acid method was 10.3%. This indicates that lectin was a kind of complex glycoprotein with more sugar chains. Fourier transform infrared spectroscopy (FT-IR) and β-elimination reaction of the *A. auricula* lectin structure were analyzed, and the results show that the linkage between the lectin glycosyl chain and the polypeptide is an N-glucoside bond. A similar research also verified the lectin of the sugar chain with the connecting way of peptides. Yao et al. extracted lectins, and the structure of the lectins was analyzed by FT-IR, β-elimination reaction, and spectroscopy. The results show that the lectins are connected to the sugar and protein in the form of an O-glycosidic bond, and the secondary structure was mainly an α-helix and a random coil [44]. Cui et al. obtained a glycoprotein from the purification of *Grifola frondosa mycelia*, and they identified the glycoprotein by FT-IR, NMR, and β-elimination reactions as being attached to the sugar and protein by O-glucoside bonds. The roundabout spectrum results showed that the glycoprotein was mainly stable in the secondary structure of β-folding [45].

3.5. Study on the Antitumor Activity of AAL

3.5.1. Inhibition of the Proliferation of Lung Cancer Cell A549. This experiment set different concentrations of AAL for an early screening of A549 cells. The results showed that compared with the negative control group, when the concentration of lectins was 250 μg/mL, A549 cells showed a trend of apoptosis and the growth of A549 cells was significantly suppressed. When the concentration of the lectin was 100-200 μg/mL, the number of cells decreased and cell bubbles

appeared. When the concentration of the lectin was 50 μg/mL, the cell morphology of A549 cells did not change significantly. It was only observed that the cells began to gather and the cell volume increased, so it was judged that the cells began to appear together with the bubbles. Therefore, 100 μg/mL was chosen as the initial concentration of lectin to inhibit the proliferation of A549 cells.

In a compound sieve experiment, we chose 100 μg/mL of AAL as the initial concentration, and the concentration gradient was diluted (100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, and 6.25 μg/mL). A549 cells were treated for 24 h, 48 h, and 72 h under different lectin concentrations, then we used the CCK-8 Kits to test the lectin effect on the growth inhibition of A549 cells. The results showed that the growth of A549 cell inhibition was concentration and time dependent, the degree of A549 cell proliferation inhibition gradually strengthens with the increase of the concentration of lectins. And at the same concentration but processed at different times, the degrees of inhibition of A549 cells were different. When the concentration of AAL was 100 μg/mL and AAL was cultured for 72 hours, the inhibition rate of A549 cells reached a maximum of 74% (Figure 4).

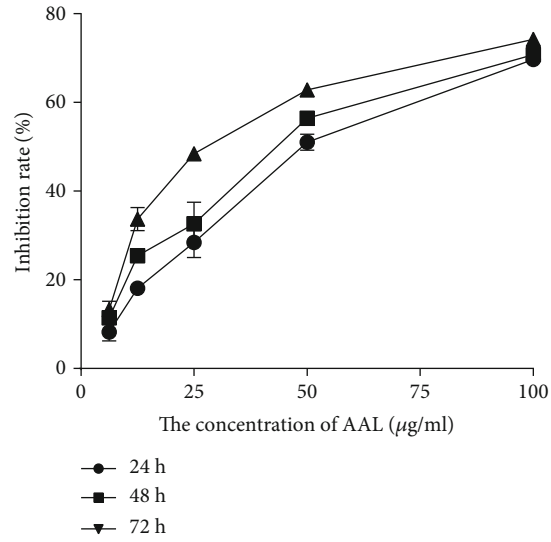


Figure 4: Inhibitory curve of AAL on A549.

3.5.2. Half-Inhibitory Concentration (IC_{50}) of AAL. The half-inhibitory concentration (IC_{50}) was the concentration at which the drug restricts the growth of half of the tumor cells. It is the standard to measure the effectiveness of the drug on the tumor cells, and it is used by new drugs to evaluate the targeting of cancer cells. Here, we studied the effect of *A. auricula* lectin on lung A549 cancer cells at 24 h, 48 h, and 72 h. Tumor cell proliferation inhibition rate was concentration and time dependent on AAL, and through the CCK-8 kit, we detected the proliferation of A549 cells. The IC_{50} was 50.17 ± 2.69 μg/mL for 24 h, 41.29 ± 2.22 μg/mL for 48 h, and 28.19 ± 1.92 μg/mL for 72 h. The results show that AAL had a good inhibition effect on the proliferation of A549 cells.

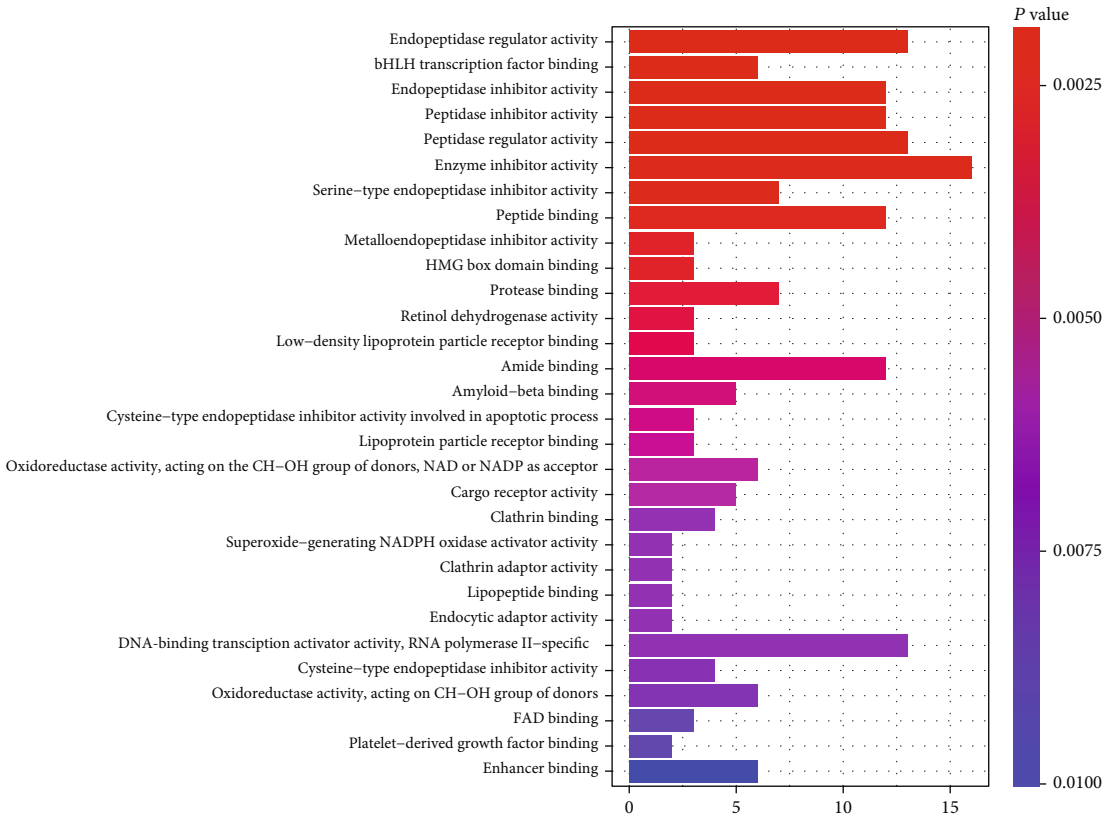
Cancer is a serious threat to human health. Previous studies found that there were 18.1 million new cancer cases and 9.6 million cancer deaths worldwide in 2018, and it is estimated that nearly half of all new cancer cases and more than half of all cancer deaths occurred in Asia; thus, the incidence of cancer is very serious. The incidence of cancer in China accounts for about 22% of the world's total, and the number of cancer cases is the highest in the world [29]. In recent years, lung cancer (LC) is still one of the most common malignant tumors in human beings, and the incidence and mortality of LC are increasing year by year [46, 47]. Currently, the drugs used to treat tumors are time-consuming, damaging, and expensive. Therefore, the pursuit of green, safe, and targeted edible antitumor drugs has become the top priority. Lectins found in terrestrial plants and edible fungi have antitumor activities, such as mulberry leaf lectins' sensitization to McF-7 by activating the P38 MAPK protein kinase to inhibit the signal transduction pathway mediated by fibrin [48, 49]. Tea mushroom lectin is easily absorbed by the lungs of mice, preventing 4T1 breast cancer cells from transferring to the lungs and preventing mice from suffering from secondary infection [50]. Ruthenate lectin acts as a molecular switch to control the apoptosis and autophagy of A549 cells. At the same time, lectin in the lymphatic fluid of marine mollusk shellfish also has a good inhibitory effect on tumor proliferation [51]. For example, the marine mussel lectin has the ability to recognize the sugar on the Gb3 receptor of tumor cells and thus inhibit the proliferation of McF-7 in breast cancer cells.

3.6. Study on the Antitumor Molecular Mechanism of Lectin. In order to study the antitumor molecular mechanism of AAL, we conducted RNA sequencing. The differential genes were screened when the inhibition rate of A549 cells reached the maximum. By RNA sequencing, different expression genes in A549 treated with lectin and without lectin were counted. A total of 350 differentially expressed genes were found, among which 194 were upregulated and 156 were downregulated.

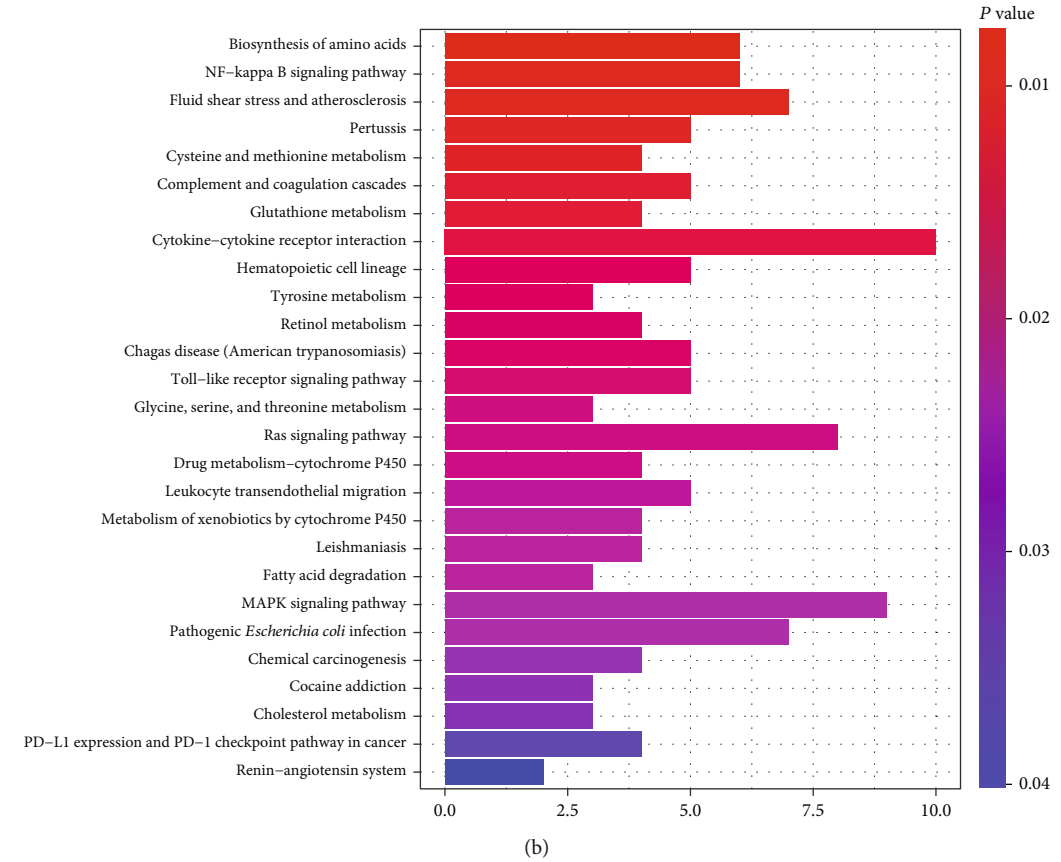
Through the GO and KEGG functional annotation and signal pathway enrichment analysis of the differentially expressed genes, many tumor-related genes and signaling pathways have been found, such as the NF-kappa B (NF-κB) signaling pathway, the Toll-like receptor signaling pathway, the PD-L1 expression and PD-1 checkpoint pathway in cancer, and the MAPK signaling pathway (Figures 5(a) and 5(b)).

3.7. Screening the Key Factors of Lectin Action on the A549 Cells. According to the results of GO and KEGG functional annotation, tumor-related immune and apoptosis genes were screened out, and PPI analysis was performed to screen out key tumor factors (Figure 5(c)). Relevant signaling pathways were analyzed for the signaling pathway gene network, so as to further search for tumor key genes (Figure 5(d)). The results showed that *JUN*, *TLR4*, and *MYD88* were the key regulatory factors.

By functional annotation of differentially expressed genes, it was found that GO mainly concentrated in endopeptidase regulator activity, endopeptidase inhibitor activity,



(a)



(b)

Figure 5: Continued.

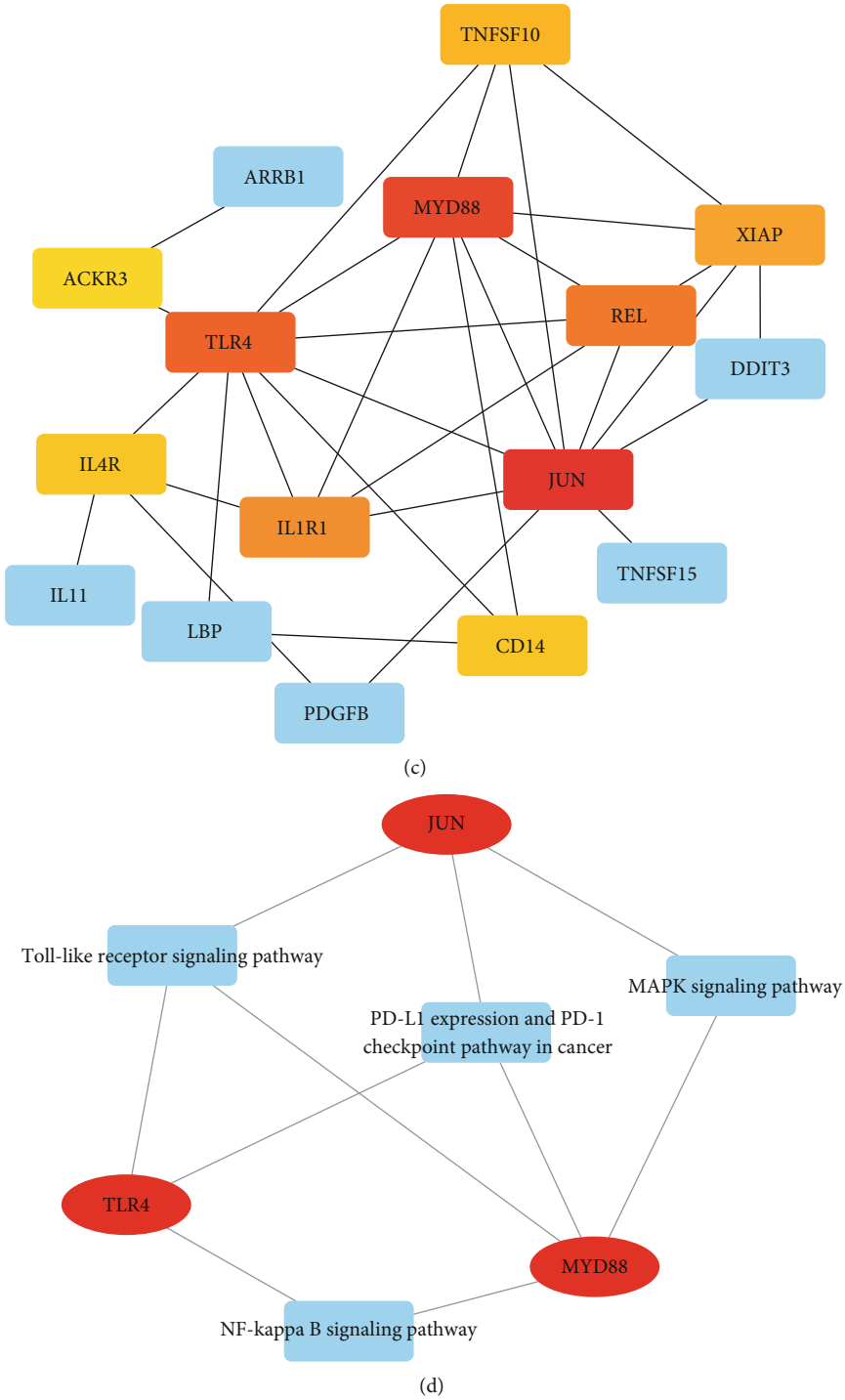


Figure 5: Antitumor molecular mechanism of lectin. GO annotation of the different expression genes (a). Signal pathway enrichment of the different expression genes (b). PPI plot of genes related to immunity and apoptosis (c). Network analysis of key genes and related signaling pathways (d).

enzyme inhibitor activity, and peptide binding and peptidase inhibitor activity. The KEGG pathway was mainly enriched in the NF-κB signaling pathway, the Toll-like receptor signaling pathway, the PD-L1 expression and PD-1 checkpoint pathway in cancer, and the MAPK signaling pathway. As we all know, NF-κB signaling plays an important role in inflammatory pathways and apoptosis [52]. Activation of the NF-κB signaling pathway is an important tumor-inducing modulator in many cancers such as cervical cancer, enabling tumor cells to evade apoptotic cell cycle checkpoints [53]. In addition, it is said that NF-κB plays a critical role in the inflammatory pathways of malignancies, such as activating NF-κB that encourages cell proliferation, survival, and angiogenesis [54]. Bacteria may disrupt the cell cycle by toxin

Table 3: The abundance distribution of bacteria.

Classification	Lectin treatment group	Control group
Firmicutes	3697	1172
Bacteroidetes	2321	108
Actinobacteria	1846	54
Proteobacteria	1211	475
Cyanobacteria	469	16
Chloroflexi	357	16
Acidobacteria	216	44
Fusobacteria	16	37
Others	315	43

production, resulting in cell growth with alterations in protein expression that control DNA repair, cell division, and apoptosis [55]. Furthermore, bacteria may alter the host immune response against malignant cells, and an association between microbiota composition and clinical immunotherapy response has recently been shown. Studies in animal models indicate that microbiota modulate the sensitivity of solid cancers to immune checkpoint inhibitors (ICIs), mainly cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and PD-1/PD-L1 [56]. As we all know, lectin has an effect on lung microflora [57]. So, lectin may affect the occurrence and development of lung cancer by influencing the microflora. The TLR signaling pathway consists of two subpathways: the *MYD88*-dependent pathway and the *MYD88*-independent pathway. There is increasing evidence that TLRs are an important regulator of tumor biology. The regulation of the TLR signaling pathway plays an important role in the occurrence or progression of tumors [58]. Grimmig et al. reported that the TLR signaling pathway promoted proliferation of pancreatic cancer cells [59]. Besides, *TLR3* has been found to stimulate cancer cell survival, proliferation, and progression in breasts [60], pharynx [61], and head and neck [62]. For lung cancer, the TLR pathway may play a key role in tumorigenesis and progression, especially in non-small-cell lung cancer (NSCLC) [63–65]. Therefore, some genetic variants in genes of this pathway may have a predictive value as a clinically potential biomarker for outcomes of NSCLC. Moreover, through the correlation analysis between PPI and genes, we found that *JUN*, *TLR4*, and *MYD88*, the three main regulatory genes of lectin, were all highly expressed in the tumors, and the expression of these three genes were inhibited after treatment by AAL.

3.8. Lectin Could Regulate the Pulmonary Flora. Compared with the control group (0.355 ± 0.041 g), the weight of the lung tissue in the lectin treatment group (0.242 ± 0.026 g) was significantly reduced, which proved that lectin could inhibit the growth of lung cancer cells in vivo. In sequencing analysis of respiratory flora of tumor-bearing mice after lectin treatment, 259 bacterial colonization species were detected in the control group and 538 bacterial colonization species were significantly increased in the lectin treatment group. Among them, 62 species of bacteria were common strains. The bacterial analysis results showed that compared with the control group, the lung microflora structure of the lectin treatment group was significantly different ($P < 0.05$), the bacterial structure of each phylum in the lectin treatment group was more reasonable, and the colonization ability of the normal microflora was improved, indicating that lectin treatment could significantly improve the bacterial diversity characteristics of the tumor-bearing mouse model (Table 3).

In recent years, with the in-depth development of molecular biology research technology, it has become clear that lung tissue is not always in a balanced state. Studies have shown that inhaled upper respiratory tract secretions can bring part of the oropharyngeal microbial community into the lung tissue; at the same time, the host activates the defense mechanism, which can effectively eliminate microbes and achieve the purpose of blocking infection. The manifestations of changes in the respiratory microecosystem are complex and diverse, and may eventually lead to local or systemic bacterial infections. However, in the course of lung cancer, the changes in the respiratory tract microflora have not been clearly understood.

In this study, by detecting changes in the respiratory tract flora of non-small-cell lung cancer-bearing mice, the effect of lectin treatment on the respiratory tract flora of A549 tumor-bearing mice was explored. The results of the study showed that *Firmicutes*, *Actinomycetes*, *Bacteroides*, *Proteobacteria*, and *Fusobacteria* in the respiratory tract have certain colonization in the respiratory tract of tumor-bearing mice. At the same time, compared with the control group, there are obvious differences in the structure of the lung flora in the lectin treatment group. The bacterial structure of each phyla in the lectin treatment group is more reasonable, indicating that lectin treatment can significantly increase the diversity of the lung flora of A549 tumor-bearing mice, promote the balance of respiratory flora, and enhance the body's biobarrier effect, reducing the colonization of pathogenic bacteria.

4. Conclusion

In this study, lectin was isolated from *A. auricula* by affinity chromatography and named AAL. Through 80% $(NH_4)_2SO_4$ precipitation, HiTrap DEAE anion exchange chromatography, and PSM-Sepharose 4B affinity chromatography (eluent pH 3.0, ultrafiltration time 3 h), the purification process of AAL was obtained, and the optimal extraction rate was 0.068%. AAL is a monosubunit protein with a molecular weight of 25 kDa. The relative molecular weight of lectin AAL as determined by MALDI-TOF/TOF is 18913.22. The N-terminal sequence was detected as ITAPTTTSSAATE. After protein identification and comparison with the UniProt database, it was concluded that the lectin contained 4 peptides, whose amino acid sequences were QIDAERK, TNHSVVTWNDK, RLNFTAGNPFPR, and VRELEQQVDSMTK. The connection between the oligosaccharide and polypeptide of AAL was the N-glucoside bond. Physical and chemical property analyses showed that AAL was a temperature-sensitive and acidic/alkaline-dependent glycoprotein. Additionally, the anticancer experiment manifested that AAL inhibited the proliferation of A549 and the IC_{50} value was $28.19 \pm 1.92\,\mu$g/mL. RNA sequencing and

TCGA dataset analyses detected that AAL may regulate the expression of *JUN*, *TLR4*, and *MYD88* to suppress tumor proliferation. We finally found a lectin that could regulate the pulmonary flora.

Data Availability

The data underlying the findings of this article will be shared by the corresponding authors upon reasonable request.

Additional Points

Sample Availability. Samples of the compounds are available from the authors.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

ZDL collected and identified the fruiting body of *Auricularia auricula*. ZDL, LL, and BX prepared the extract and isolated and purified the lectin. DDZ was involved in project administration. YLZ was involved in research design. XFY performed the cell-based testing. ZDL designed the research and performed the action mechanism of lectin and statistical analysis. All authors were involved in data analysis and manuscript writing.

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