



# Experimental study on the suppressive effect of B3GNT3 on the apoptosis of lung adenocarcinoma cells and its application in early screening for lung adenocarcinoma

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**Background:** Lung adenocarcinoma, as a common histological type in lung cancer, the overall survival is very low, and the prognosis is poor because it is difficult to find and easily recurs. Therefore, this study aimed to explore the role of the secreted protein beta-1,3-N-acetylglucosaminyltransferase 3 (B3GNT3) in the development of lung adenocarcinoma and to evaluate its potential significance for early clinical biomarker screening.

**Methods:** The mRNA expression profiles of patients with lung adenocarcinoma and normal controls were analyzed via The Cancer Genome Atlas (TCGA) database. Serum samples of clinical lung cancer patients and healthy people were obtained, and the differences in B3GNT3 expression in different stages of lung adenocarcinoma and in healthy tissues were compared. Kaplan-Meier (K-M) curves were drawn to clarify the influence of high and low expression of B3GNT3 on the prognosis of patients. Peripheral blood samples from patients with lung adenocarcinoma and healthy people were obtained clinically, and receiver operating characteristic (ROC) curves were drawn to clarify the sensitivity and specificity of B3GNT3 expression for the diagnosis of lung adenocarcinoma. Lung adenocarcinoma cells were cultured *in vitro*, the expression of B3GNT3 was knocked down by lentivirus infection. The expression of the apoptosis-associated genes was detected by reverse transcription-polymerase chain reaction (RT-PCR).

**Results:** The secreted protein B3GNT3 is significantly differentially expressed in the serum of patients with lung adenocarcinoma versus normal controls. Subgroup analysis according to lung adenocarcinoma clinical stage showed that the higher the clinical stage of lung adenocarcinoma was, the higher the B3GNT3 expression. Enzyme-linked immunosorbent assay (ELISA) revealed that B3GNT3 expression was significantly increased in the serum of patients with lung adenocarcinoma and significantly decreased after surgery. By inhibiting programmed cell death-ligand 1 (PD-L1), the level of apoptosis was significantly increased and the proliferative capacity was significantly inhibited. In contrast, the level of apoptosis was significantly increased and the proliferation ability was significantly inhibited after simultaneous overexpression of B3GNT3 and inhibition of PD-L1.

**Conclusions:** High expression of the secreted protein B3GNT3 in lung adenocarcinoma is closely related to prognosis and can serve as a potential biological marker for the early screening of lung adenocarcinoma.

**Keywords:** Lung adenocarcinoma; beta-1,3-N-acetylglucosaminyltransferase 3 (B3GNT3); programmed cell death-ligand 1 (PD-L1); apoptosis; early clinical screening

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## Introduction

The incidence and mortality rates of lung cancer rank first among those of all malignant tumors, posing a serious public health problem. There are approximately 1.2 million patients with lung cancer worldwide, and those dying of lung cancer account for 25% of those dying of malignant tumors. In recent years, the proportion of lung adenocarcinoma has gradually increased in lung cancer (1). Non-small cell lung cancer (NSCLC) accounts for 80% of total lung cancer cases. As the most common histological type of NSCLC, lung adenocarcinoma is more common in elderly patients, with characteristics of early metastasis, rapid progression, high incidence rate and high mortality rate (2,3). Most patients with lung adenocarcinoma are diagnosed at an advanced stage (4). At present, the treatments for lung adenocarcinoma mainly include surgery, radiotherapy and chemotherapy. Although the above treatments can reduce the risk of death in patients with lung adenocarcinoma to a certain extent, the overall survival of patients with lung adenocarcinoma is still very low, and the prognosis is poor because it is difficult to find and easily recurs (5). Therefore, clarifying the molecular mechanisms of lung adenocarcinoma and identifying novel candidate biomarkers are prerequisites for the early diagnosis of lung adenocarcinoma, which is a key factor for effective treatment.

Recently, many scholars have tried to achieve early diagnosis in patients with lung adenocarcinoma by screening for effective biological markers (6). Tumor markers refer to a class of substances abnormally synthesized and released by malignant tumor cells or a class of substances that increase the host's response to tumor stimulation, existing in tumor

cells or hosts' tissues, body fluids or excrement in the forms of enzymes, hormones, antigen or oncogene products (7,8). Serum tumor markers have the advantages of corresponding high specificity, high sensitivity, convenience, ease of access, and minimal trauma, and research on tumor markers has also been ongoing for a long time (9). Serological tumor markers [alpha fetoprotein (AFP), carcinoembryonic antigen (CEA), etc.] have been widely promoted and applied in the clinic for liver cancer, colorectal cancer, mammary cancer and other diseases (10). However, effective serum tumor markers have not been screened for lung adenocarcinoma.

With profound research on genomics, numerous researchers have attempted to obtain specific tumor markers through bioinformatic analysis of databases such as The Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus (GEO) database and by screening of long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) in lung cancer (11-13). However, comprehensive specific serum tumor marker screening for lung adenocarcinoma via TCGA database analysis has not been performed.

On this basis, this study preliminarily screened specific serum tumor markers in patients with lung adenocarcinoma through TCGA database analysis. The sensitivity and specificity of the identified marker were defined through validation in patients with lung adenocarcinoma. Moreover, lung adenocarcinoma cell lines were cultured *in vitro*, and the specific molecular regulatory mechanism of this tumor marker for lung adenocarcinoma was finally defined by molecular biological techniques. On the basis of previous studies, this study further explored the significance of beta-1,3-N-acetylglucosaminyltransferase 3 (B3GNT3) in early screening of lung adenocarcinoma and its specific molecular regulation mechanism for the occurrence and development of lung adenocarcinoma. Finally, it is clear that B3GNT3 is the key upstream regulator of programmed cell death-ligand 1 (PD-L1). We present the following article in accordance with the MDAR reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-22-1175/rc>).

## Methods

### Data source and analysis

The TCGA database was searched for tumor samples of patients with lung adenocarcinoma and normal control samples, and differentially expressed genes were defined as those with  $|\log_2\text{fold change (FC)}| > 2$  and  $P_{\text{adj}} < 0.05$

### Highlight box

#### Key findings

- High expression of the secreted protein B3GNT3 in lung adenocarcinoma is closely related to prognosis.

#### What is known and what is new?

- Serological tumor markers have been widely promoted and applied in the clinic for liver cancer, colorectal cancer, mammary cancer and other diseases.
- B3GNT3 is the key upstream regulator of PD-L1.

#### What is the implication, and what should change now?

- B3GNT3 can serve as a potential biological marker for the early screening of lung adenocarcinoma.

according to the DESeq2 software package in R 4.0.3. A total of 3,612 detectable secreted proteins in peripheral blood were obtained through the MetaSecKB database. MetaSecKB is a secretome and subcellular proteome knowledgebase specifically designed for metazoan, i.e., human and animals. According to the P<sub>adj</sub> and |log<sub>2</sub>FC|, volcano plots were drawn to visualize the expression of secreted proteins. Finally, the secreted proteins closely related to the occurrence and development of lung cancer were screened out.

### *Clinical sample source*

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Qiqihar Medical University (No. 20200425). Informed consent was taken from all individual patients. Data for patients with lung adenocarcinoma treated surgically in our hospital from January 2018 to January 2019 were collected retrospectively according to the inclusion and exclusion criteria. The inclusion criteria were as follows: (I) patients first diagnosed with lung adenocarcinoma in our hospital and (II) patients who did not undergo radiotherapy, chemotherapy or immunotherapy before surgery. The exclusion criteria were as follows: (I) patients with multiple tumors; (II) patients who underwent radiotherapy, chemotherapy and immunotherapy before surgery; and (III) patients with a history of severe liver and renal dysfunction and mental illness. The same number of healthy people who had received physical examinations from hospitals were obtained by propensity ratio analysis and assigned to the normal control group. Peripheral blood samples were collected from the control and lung adenocarcinoma groups 1 week before surgery and from patients with lung adenocarcinoma 1 week after surgery.

The diagnostic criteria for lung adenocarcinoma was as follows: all patients were diagnosed with lung adenocarcinoma through chest computed tomography (CT), sputum exfoliative cytology examination, fiberoptic bronchoscope, lung puncture or surgical pathological examination, with clinical stages of Ia–IVb.

### *Cell culture*

The human normal lung epithelial cell line BEAS-2B and

four human lung adenocarcinoma cell lines (A549, H1975, Calu3 and H1395) were purchased from American Type Culture Collection (ATCC). According to the criteria provided by ATCC for cell culture, the normal lung epithelial cell line BEAS-2B was cultured with BEGM medium CC-3170 (Lonza/Clonetics, CC-3170). The four human lung adenocarcinoma cell lines were cultured with RPMI medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin and 100 mg/L streptomycin. The cells were placed in an incubator at 37 °C with 5% CO<sub>2</sub> for culture.

### *Quantitative polymerase chain reaction (qPCR)*

Total RNA in tissues and cells was extracted with TRIzol reagent, which was reverse transcribed into cDNA with the RNA reverse transcription kit, 5X All-In-One RT MasterMix (ABM, Cat. No. G492, China), taking complementary DNA (cDNA) as a template for qPCR with EvaGreen 2X qPCR MasterMix (ABM, Cat. MasterMix-S). The B3GNT3 forward primer sequence was CAGCACGTTTCAGAACTTCCTC, the reverse primer sequence was GCGCACATAGTTGCTAGGGG, and the results were analyzed by relative quantitative analysis.

### *Enzyme-linked immunosorbent assay (ELISA)*

Fresh blood from patients with tumors was placed on ice for half an hour and centrifuged at 15,000 rpm at 4 °C for 30 min. The upper serum level was taken and processed with a human B3GNT3 ELISA kit (Sai Berry, Cat. SEJ574Hu02, China) to determine B3GNT3 expression in serum.

### *Short hairpin RNA (shRNA) constructs and transfection*

shRNA plasmids (Hanheng Biotechnology, China) with the target sequence CCGCTTCCTACCTTATGAGAT were constructed for B3GNT3 gene expression interference and packaged as lentivirus (with a titer of 810 IU/mL). The cells were spread in 6-well plates. When the confluence of the cells reached 50–70%, 20 μL shRNA was added to each well, the transfection efficiency was observed by microscopy after 48 h, and the transfected cells were screened with 2 μg/mL puromycin. After 5 days of screening, the medium was replaced with normal medium and subamplified to a confluence of 70% for subsequent experiments. The

efficiency of B3GNT3 knockdown was determined by qRT-PCR and Western blotting (WB).

### WB

The cells of each group were collected with efficient RIPA lysis buffer (Solarbio, Cat. R0020, China), lysed at 4 °C, and then centrifuged at 15,000 rpm for 15 min for protein extraction. The protein was quantified with a bicinchoninic acid (BCA) protein quantification kit (Thermo Fisher, Cat. 23235, USA). After the quantification of the protein concentration, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was conducted for each tissue/cell. Subsequently, the proteins separated by electrophoresis were transferred to a polyvinylidene fluoride (PVDF) membrane with a transfer device; the membrane was then blocked with 5% skim milk for 1 h and rinsed in tris-buffered saline with Tween 20 (TBST). Primary antibodies against the target proteins were then added for incubation at 4 °C overnight, and then a secondary antibody was added for incubation for 1 hour. Finally, the PVDF membrane was exposed to test through a chemiluminescence imaging system.

In this study, rabbit anti-B3GNT3 antibody (Abcam, Cat. ab70156, UK) was adopted as the B3GNT3 antibody with a dilution ratio of 1:1,000; rabbit anti- $\beta$ -actin antibody (Abcam, Cat. 8227) was used as the internal reference antibody with a dilution ratio of 1:1,000; rabbit anti-Bax antibody (Abcam, ab32503) was used as the BAX antibody with a dilution ratio of 1:2,000; rabbit anti-Bcl2 antibody (Abcam, ab32124) was used as the Bcl2 antibody; and HRP anti-rabbit IgG antibody (Abcam, ab288151) was used as the secondary antibody.

### Cell Counting Kit-8 (CCK-8) proliferation assay

Lenti-shB3GNT3 cells and the control cells were inoculated in 96-well plates at 1,000 cells/well and cultured for 24, 48, 72 and 96 h. CCK-8 detection reagent was added at 1:10 (Vazyme Biotech, Cat. A311-02, China), and the optical density (OD) was detected at 450 nm.

### Apoptosis detected by ELISA

Apoptosis was detected by an ELISA apoptosis kit (meeck, cat. APOAC-1KT, USA). After A549 cells were treated under different grouping conditions, the cells were first collected by centrifugation and then resuspended in 200  $\mu$ L of cytolytic buffer, followed by quiescence for

30 min. After centrifugation at 1,000 r/min for 10 min, 20  $\mu$ L of the supernatant was removed and added to the ELISA plate. Then, 80  $\mu$ L of immunoreactive reagent was added to each well of the plates and incubated for 2 h at room temperature. The supernatant was removed, and the well was rinsed three times. Then, 100  $\mu$ L of buffer was added and incubated for 30 min. The OD was measured at a wavelength of 405 nm, with the substrate buffer as the blank control group.

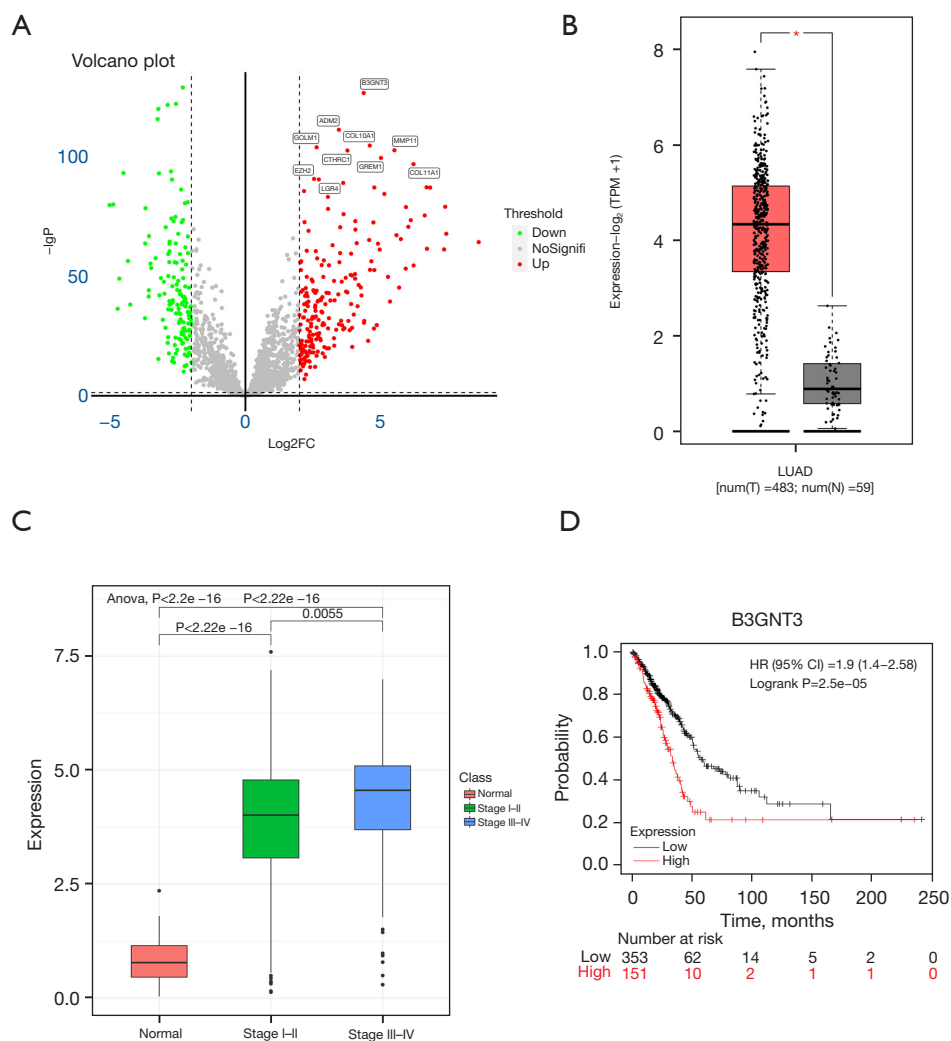
### Statistical analysis

Statistical analyses of the data used were performed in SPSS 24.0 (IBM, New York, USA) software. For the count variables, the chi-square test was used. For measurement data the independent samples *t*-test was used for those that obeyed normal distribution, and the results were expressed as mean  $\pm$  standard deviation; for those that did not obey normal distribution the Mann-Whitney U test was used, and the results were expressed as median [quartiles].  $P < 0.05$  indicates a statistical difference.

## Results

### *Bioinformatics analysis revealed that B3GNT3 is a key secreted protein of potential blood biomarkers for lung cancer*

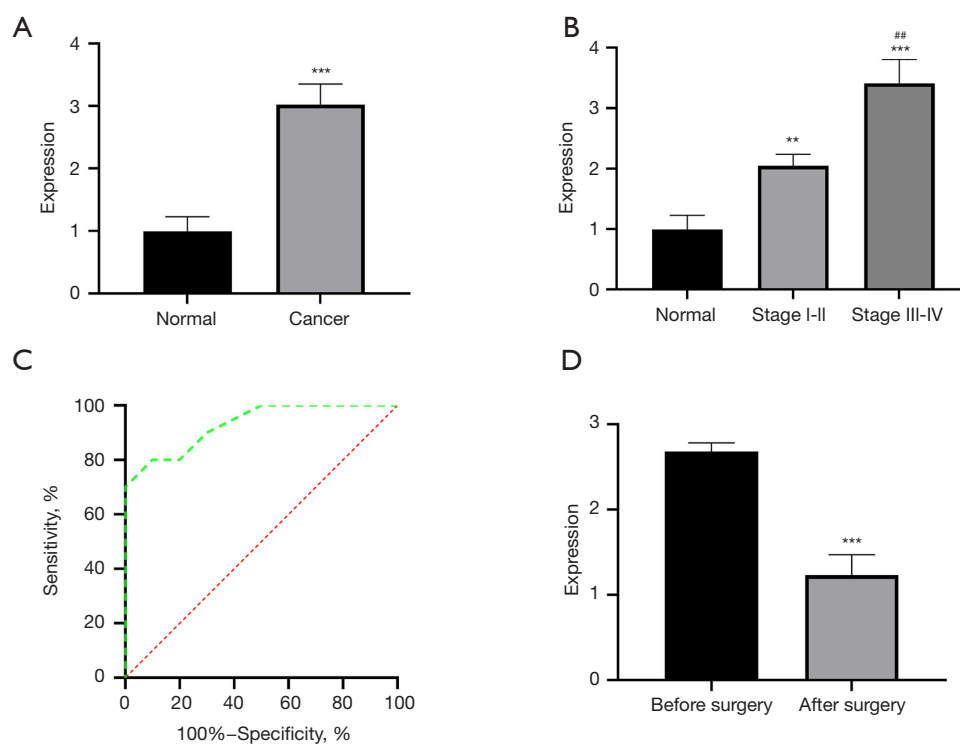
A total of 535 tumor samples and 59 normal control samples were obtained after searching the TCGA database. A total of 209 up-regulated genes and 140 down-regulated genes were set as differentially expressed genes from these samples. The differentially expressed genes acquired from the TCGA database were cross-compared with the MetaSecKB database, and 3,612 detectable secreted proteins in peripheral blood were obtained. Screening conditions were set for the differentially expressed secreted proteins ( $|\log_2FC| > 2$ ,  $P_{adj} < 0.05$ ), and the variability of each secreted protein is shown by volcano plots (*Figure 1A*). The results showed that B3GNT3 ( $\log_2FC = 4.381387$ ,  $P < 0.01$ ) was the secreted protein with the most significant differential expression. The analysis of 535 samples from patients with cancers and 59 samples from normal controls in TCGA showed significantly high B3GNT3 expression in samples from patients with lung adenocarcinoma (*Figure 1B*). Further detection of B3GNT3 expression in each sample from patients with lung adenocarcinoma according to the clinical stage of patients with tumors showed that the higher the clinical stage was, the



**Figure 1** Bioinformatic analysis showed that B3GNT3 is a key secreted protein and a potential blood biomarker for lung cancer. (A) Volcano plot of the differential expression of secreted proteins in the serum of patients with lung adenocarcinoma and normal controls, where red dots represent significantly upregulated genes and green dots represent significantly downregulated genes. (B) Comparison of B3GNT3 expression levels in the serum of patients with lung adenocarcinoma and normal controls based on the TCGA database. (C) Changes in B3GNT3 expression in tumor tissues of patients with different clinical stages of lung adenocarcinoma. (D) OS survival plot of B3GNT3 gene expression in patients with lung adenocarcinoma. \*,  $P < 0.05$ . LUAD, lung adenocarcinoma; TPM, transcripts per million; B3GNT3, beta-1,3-N-acetylglucosaminyltransferase 3; HR, hazard ratio; TCGA, The Cancer Genome Atlas; OS, overall survival.

higher the B3GNT3 expression in patients with lung adenocarcinoma (Figure 1C,  $P < 0.05$ ), suggesting a gradual increase in B3GNT3 expression during disease progression in patients with lung adenocarcinoma. Patients with lung adenocarcinoma were divided into high and low expression groups according to the median B3GNT3 expression, and Kaplan-Meier (K-M) curves were drawn to assess the survival of both groups, which showed that the

survival cycle of the high B3GNT3 expression group was significantly worse than that of the low B3GNT3 expression group (Figure 1D). The results of the above bioinformatics analysis showed that B3GNT3 is closely associated with tumor grade in patients with lung adenocarcinoma, and the prognosis of patients with high B3GNT3 expression was significantly worse. Therefore, B3GNT3 may serve as a key secreted protein and potential blood biomarker in lung cancer.

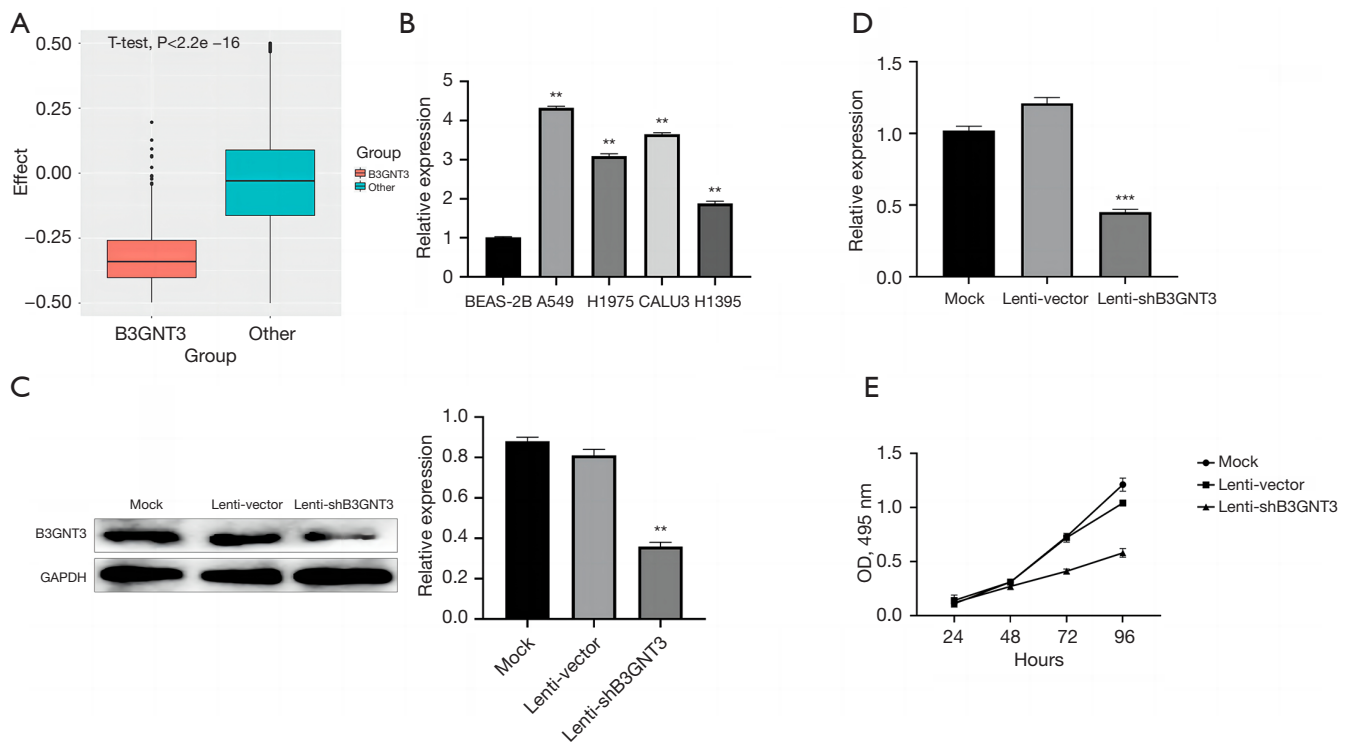


**Figure 2** Increased B3GNT3 expression in the serum of patients with lung adenocarcinoma. (A) B3GNT3 expression in the serum of patients with lung adenocarcinoma and normal controls. (B) B3GNT3 expression levels in different stages of lung adenocarcinoma. (C) ROC curve drawn based on B3GNT3 expression in the serum of normal controls and patients at different pathological stages. (D) Preoperative and postoperative B3GNT3 expression levels in the same patient with lung adenocarcinoma. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ##, indicates comparison with stage I–II,  $P < 0.01$ . B3GNT3, beta-1,3-N-acetylglucosaminyltransferase 3; ROC, receiver operating characteristic.

### ***B3GNT3 expression was significantly increased in the serum of patients with lung adenocarcinoma***

According to the inclusion and exclusion criteria, this study ultimately included serum samples from 30 clinical patients with lung adenocarcinoma and 30 healthy volunteers subjected to propensity matching. Detection of serum B3GNT3 expression by ELISA showed significantly higher B3GNT3 expression levels in patients with lung adenocarcinoma than in normal controls (*Figure 2A*,  $P < 0.05$ ). The B3GNT3 expression levels of patients with lung adenocarcinoma were compared according to the clinical stage of lung adenocarcinoma, which showed that the expression level of B3GNT3 in tumor tissues of patients with stage I and IIa lung adenocarcinoma was significantly higher than that of normal controls, and the difference was significant. In addition, the comparison of patients with different clinical stages showed that the higher the clinical stage was, the higher the expression level of B3GNT3,

and the difference was statistically significant (*Figure 2B*,  $P < 0.05$ ). The K-M survival curve was drawn according to the expression level of B3GNT3 in lung adenocarcinoma tissue and showed that the prognosis of patients with high B3GNT3 expression was significantly worse than that of patients with low B3GNT3 expression ( $P < 0.05$ ). The areas under the receiver operating characteristic (ROC) curves (AUC) were 0.9373 and 0.9938, which indicated that B3GNT3 had high sensitivity and specificity for the diagnosis of lung adenocarcinoma (*Figure 2C*). Peripheral serum samples were collected from the patients 1 week before and after the surgery, and B3GNT3 expression in the serum was determined by ELISA. The results showed that B3GNT3 expression significantly decreased in the peripheral serum of patients after surgical resection of tumor tissues compared with that before surgery (*Figure 2D*,  $P < 0.05$ ). All the above results indicate that the expression level of B3GNT3 is significantly upregulated in the early stage of lung adenocarcinoma. With the progression



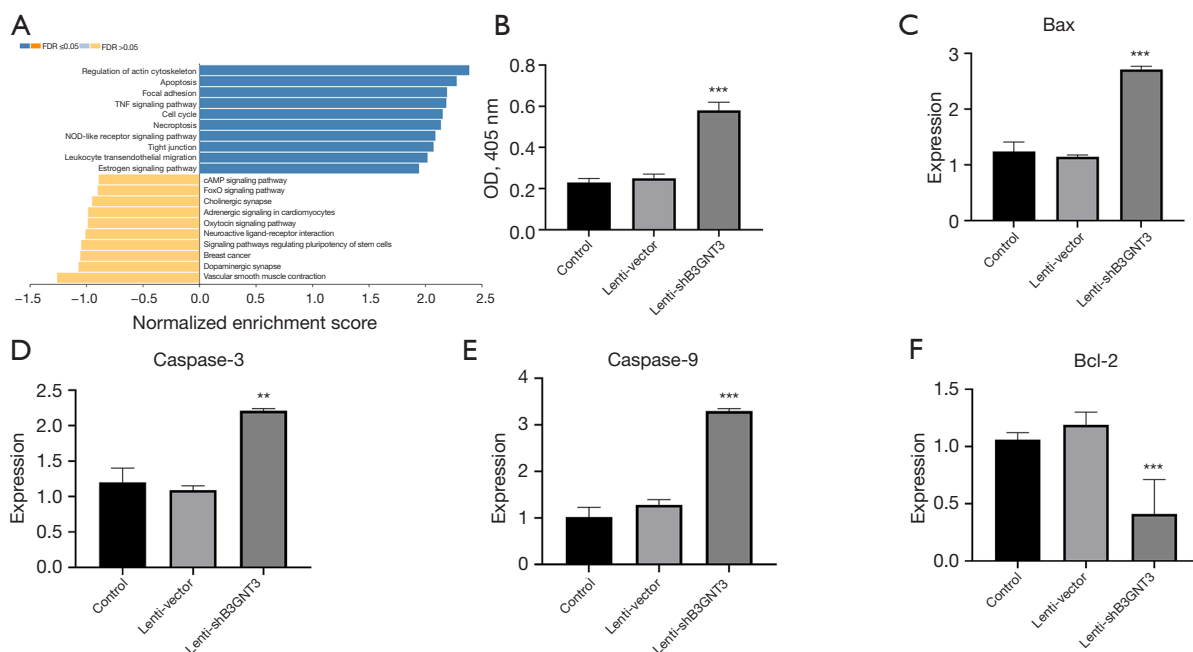
**Figure 3** Gene silencing of B3GNT3 genes suppressed the proliferation of lung adenocarcinoma cells. (A) A total of 2,000 genes were randomly selected from the DEPMap database and compared with B3GNT3 knockout cells for their effects on lung adenocarcinoma cells. (B) B3GNT3 expression levels in various lung adenocarcinoma cell lines were measured by qRT-PCR. (C) Efficiency of B3GNT3 knockdown was measured by qRT-PCR and WB. (D) Expression of B3GNT3 secreted in the culture medium was determined by the B3GNT3 ELISA kit. (E) Proliferative capacity of lenti-shB3GNT3 and lenti-vector in control cells was determined by the CCK-8 assay. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . B3GNT3, beta-1,3-N-acetylglucosaminyltransferase 3; OD, optical density; DEPMap, Cancer Dependency Map; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; WB, western blotting; ELISA, enzyme-linked immunosorbent assay; CCK-8, Cell Counting Kit-8.

of clinical stage, the expression of B3GNT3 is further increased, and the expression level of B3GNT3 is closely related to the prognosis of patients. Therefore, B3GNT3 can serve as a potential hematologic marker for lung adenocarcinoma.

### ***B3GNT3 gene silencing suppressed the proliferation of lung adenocarcinoma cells***

Based on the Cancer Dependency Map (DEPMap) database, the effects of B3GNT3 and 2,000 random genes on lung adenocarcinoma cells after CRISPR knockdown were compared. The results suggested that B3GNT3 knockout affects lung adenocarcinoma cells more significantly than 2,000 random genes (Figure 3A,  $P < 0.05$ ). Further detection of B3GNT3 expression in

different lung adenocarcinoma cells by qPCR showed that B3GNT3 expression in lung adenocarcinoma cells was significantly increased compared with that in normal lung epithelial cells (BEAS-2B cells) (Figure 3B,  $P < 0.05$ ). To explore the effect of B3GNT3 on the proliferative capacity of lung adenocarcinoma cells, the representative cell line A549 was selected from lung adenocarcinoma cells to construct the A549 cell line by constructing adenovirus transfection, and the efficiency of B3GNT3 knockdown was verified by qPCR and WB (Figure 3C,  $P < 0.05$ ). The medium was collected from the culture of knockdown cells and control cells, and the secreted B3GNT3 protein in the medium was detected with ELISA, showing that compared with the controls, B3GNT3 knockout cells secreted significantly less B3GNT3 (Figure 3D,  $P < 0.05$ ). Cell proliferation was detected by CCK-8 assay with



**Figure 4** B3GNT3 regulates the apoptosis of lung adenocarcinoma cells. (A) KEGG enrichment analysis revealed the signaling pathways enriched by differentially expressed genes between the high B3GNT3 and low B3GNT3 expression groups. (B) ELISA test for the apoptosis level of A549 cells after knocking down B3GNT3. (C-E) The expression of apoptosis-associated genes, including Bax, caspase-3 and caspase-9, in A549 cells after knocking down B3GNT3 was determined by RT-PCR. (F) The expression of Bcl-2, an apoptosis suppressor gene in A549 cells, was determined by RT-PCR after knocking down B3GNT3. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . FDR, false discovery rate; OD, optical density; B3GNT3, beta-1,3-N-acetylglucosaminyltransferase 3; KEGG, Kyoto Encyclopedia of Genes and Genomes; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction.

the B3GNT3 knockdown cell line, which showed that compared with that of the control cells, the proliferation capacity of B3GNT3 knockout cells was significantly reduced (Figure 3E,  $P < 0.05$ ). These results suggest that suppression of B3GNT3 expression can significantly suppress the proliferation of lung adenocarcinoma cells.

#### **The apoptosis level increased in A549 cells after knocking down B3GNT3**

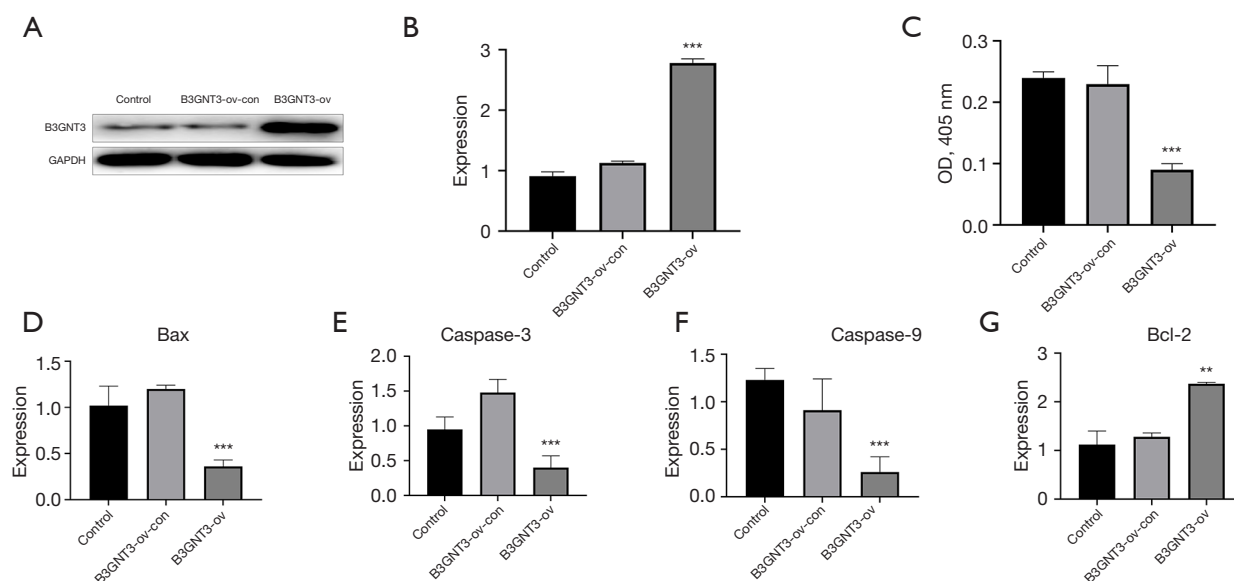
Lung adenocarcinoma samples were divided into high and low B3GNT3 expression groups according to the median B3GNT3 expression, and the differentially expressed genes between the high and low B3GNT3 expression groups were obtained. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analysis was conducted according to the acquired differentially expressed genes, which showed that the differentially expressed genes were mainly enriched in apoptosis-associated pathways (Figure 4A). The A549 cell line was cultured *in vitro*, and B3GNT3

expression in A549 cells was knocked down by adenovirus. The results of the apoptosis measured by ELISA showed that the expression of B3GNT3 was significantly increased after overexpression of B3GNT3; the expression of B3GNT3 was also significantly increased after overexpression of B3GNT3 and inhibition of PD-L1 (Figure 4B,  $P < 0.05$ ). RT-PCR showed that the expression of apoptosis-associated genes, including Bax (Figure 4C), caspase 3 (Figure 4D) and caspase 9 (Figure 4E), significantly increased ( $P < 0.05$ ), and the expression of the antiapoptotic gene Bcl-2 significantly decreased (Figure 4F,  $P < 0.05$ ) in A546 cells. The results of the above experiments suggest that B3GNT3 can suppress the apoptosis of lung adenocarcinoma cells.

#### **The apoptosis level of A549 cells was decreased after overexpressing B3GNT3**

To further clarify the effect of B3GNT3 on the apoptosis of A549 cells, a B3GNT3 mimic (overexpression) lentivirus was constructed to infect the A549 cell line. B3GNT3





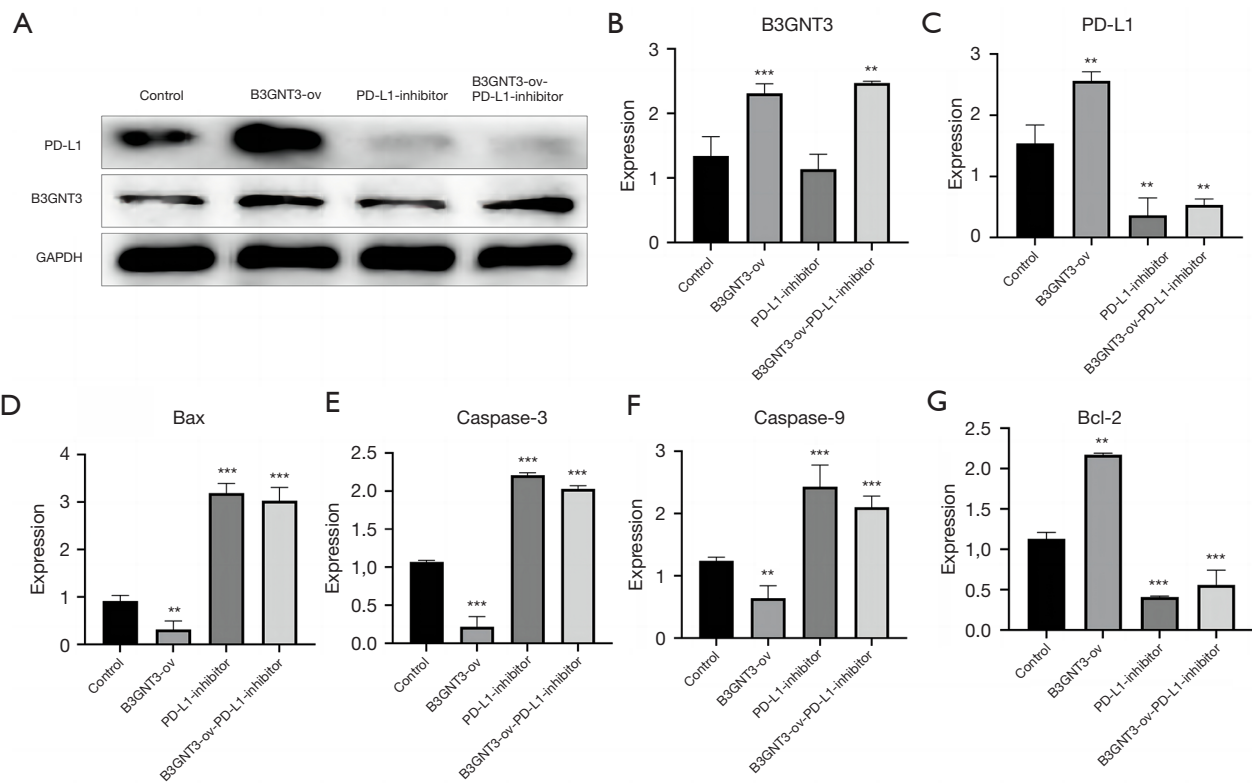
**Figure 5** The apoptosis level of A549 cells was suppressed after overexpressing B3GNT3. (A) B3GNT3 expression in A549 cells was determined by WB after overexpressing B3GNT3. (B) Semiquantitative analysis was conducted for the WB results. (C) Apoptosis of A549 cells was determined by ELISA. (D-F) The expression of apoptosis-associated genes, including Bax, caspase-3 and caspase-9, in A549 cells after overexpressing B3GNT3 was determined by RT-PCR. (G) Expression of the apoptosis suppressor gene Bcl-2 in A549 cells after overexpressing B3GNT3 was determined by B3GNT3. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ . B3GNT3, beta-1,3-N-acetylglucosaminyltransferase 3; OD, optical density; WB, western blotting; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction.

expression in cells was measured by WB (Figure 5A), and the results showed a significant increase in B3GNT3 expression in cells after overexpressing B3GNT3 (Figure 5B,  $P < 0.05$ ). The apoptosis of cells in each group detected by ELISA showed a significant decrease in the apoptosis rate after overexpressing B3GNT3 (Figure 5C,  $P < 0.05$ ). RT-PCR showed that the expression levels of apoptosis-associated genes, including Bax (Figure 5D), caspase 3 (Figure 5E) and caspase 9 (Figure 5F), decreased significantly ( $P < 0.05$ ), while the expression of the proliferation-associated gene Bcl-2 increased significantly (Figure 5G,  $P < 0.05$ ). After overexpressing B3GNT3 in A549 cells, the proliferation capacity of the cells increased significantly, while the apoptosis level was significantly decreased.

### **B3GNT3 regulates the apoptosis of lung adenocarcinoma cells via PD-L1**

To further clarify the specific molecular mechanism by which B3GNT3 regulates the apoptosis of lung adenocarcinoma cells, a B3GNT3 mimic lentivirus was

constructed to overexpress B3GNT3 in A549 cells. PD-L1 expression in cells was suppressed by adding a PD-L1 inhibitor, and the expression of B3GNT3 and PD-L1 in each group was detected by WB at the same time (Figure 6A). The results showed a significant increase in B3GNT3 expression after PD-L1 inhibition in cells with overexpression of B3GNT3 (Figure 6B,  $P < 0.05$ ). PD-L1 expression in cells significantly increased after overexpressing B3GNT3 and was significantly suppressed after inhibiting PD-L1 or both overexpressing B3GNT3 and inhibiting PD-L1 (Figure 6C,  $P < 0.05$ ). RT-PCR showed that the expression levels of the apoptosis-associated genes, including Bax (Figure 6D), caspase 3 (Figure 6E) and caspase 9 (Figure 6F), significantly decreased after overexpressing B3GNT3 ( $P < 0.05$ ), and Bcl-2 expression significantly increased (Figure 6G,  $P < 0.05$ ). The expression levels of apoptosis-associated genes, including Bax (Figure 6D), caspase 3 (Figure 6E) and caspase 9 (Figure 6F), increased significantly after inhibiting PD-L1 ( $P < 0.05$ ). Bcl-2 expression significantly decreased (Figure 6G,  $P < 0.05$ ). The expression of apoptosis-associated genes, including Bax



**Figure 6** B3GNT3 regulates apoptosis of lung adenocarcinoma cells via PD-L1. (A) Expression levels of B3GNT3 and PD-L1 in cells of each group determined by WB. (B,C) Semiquantitative analysis of the expression levels of B3GNT3 and PD-L1 in cells of each group determined by WB. (D-F) The expression levels of apoptosis-associated genes, including Bax, caspase-3 and caspase-9, in A549 cells overexpressing B3GNT3 detected by RT-PCR. (G) The expression of the proliferation-associated gene Bcl-2 in A549 cells overexpressing B3GNT3 detected by RT-PCR. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . PD-L1, programmed cell death-ligand 1; B3GNT3, beta-1,3-N-acetylglucosaminyltransferase 3; WB, western blotting; RT-PCR, reverse transcription-polymerase chain reaction.

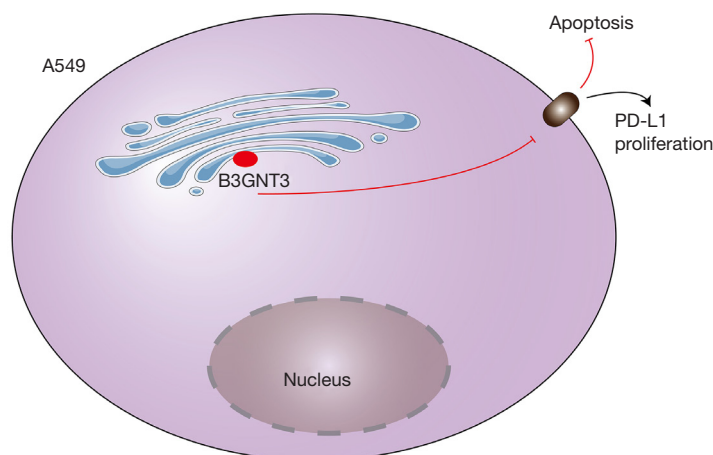
(Figure 6D), caspase 3 (Figure 6E) and caspase 9 (Figure 6F), in cells increased significantly after overexpression of B3GNT3 combined with inhibition of PD-L1 ( $P < 0.05$ ). Bcl-2 expression significantly decreased (Figure 6G,  $P < 0.05$ ). The above results indicate that B3GNT3 regulates the apoptosis of lung adenocarcinoma cells via PD-L1.

## Discussion

In this study, tumor samples from patients with lung adenocarcinoma and normal control samples were obtained from the TCGA database, and the key secreted protein, B3GNT3, which is closely related to the occurrence and development of lung adenocarcinoma, was examined. The close correlation between B3GNT3 and the prognosis of patients with lung adenocarcinoma was further clarified by human serological data detection

and *in vitro* cell experiments. Further mechanistic studies showed that B3GNT3 can regulate the apoptosis of lung adenocarcinoma cells and then regulate the progression of lung adenocarcinoma. The specific molecular regulation mechanism is detailed in Figure 7.

Lung cancer is one of the most common malignant tumors in the world; it also has a higher incidence in China than in other countries, and its mortality rate is the highest among all malignant tumors in China. Its early symptoms are not obvious in patients, and when the symptoms appear, generally, the disease has entered the advanced stage or metastasized, at which point the survival rate is low and the prognosis is poor. Chemotherapy and targeted treatment can significantly improve the prognosis in patients with lung cancer, but patients still inevitably develop drug resistance and relapse (14,15). Therefore, it is important to achieve early diagnosis of lung adenocarcinoma patients



**Figure 7** Research hypothesis map. B3GNT3 can regulate the apoptosis of lung adenocarcinoma cells by regulating PD-L1. B3GNT3, beta-1,3-N-acetylglucosaminyltransferase 3; PD-L1, programmed cell death-ligand 1.

and achieve effective prediction of patient prognosis. Many previous studies have tried to screen serum biomarkers of liver cancer, esophageal cancer and gastric cancer from the TCGA database, and the experimental results show that the selected markers have a good predictive ability for tumors (16-18). On this basis, in this study, 535 tumor samples and 59 normal control samples were obtained after searching the TCGA database, and differentially expressed genes were identified after analyzing mRNA data from the two sample groups. Differentially expressed genes were cross-compared with the secreted protein data obtained from the MetaSecKB database, and ultimately, B3GNT3 was identified as the most significantly differentially expressed secreted protein.

B3GNT3 is a type II transmembrane protein located in the Golgi apparatus. It is involved in the biosynthesis of the polyacetamide chain, generation of principal components of dimeric sialic acid Lewis A, regulation of I-selectin ligand function, lymphocyte trafficking and attribution of T cells (19-22). The *B3GNT3* gene is located on chromosome 19q13.1 and belongs to the  $\beta$ 3GlcNAcT gene family, which contains at least 8 different  $\beta$ 3GlcNAcT genes. Previous studies suggest that  $\beta$ 3GlcNAcT family members are closely related to the occurrence and development of malignant tumors (23-25). B3GNT3 was found to be closely related to the occurrence and development of breast cancer (26), but there was no report on its relationship with lung adenocarcinoma. In this study, various information of lung adenocarcinoma patients was obtained from the TCGA database at first, and then the patients were divided into

groups with high B3GNT3 expression and low B3GNT3 expression. K-M survival curve analysis revealed that the prognosis of patients with high B3GNT3 expression was significantly worse than that of patients with low B3GNT3 expression ( $P < 0.05$ ). The above results indicate that B3GNT3 expression has significant value in predicting the prognosis of patients. The research team further detected the expression of B3GNT3 in serum samples of lung adenocarcinoma patients and healthy volunteers by ELISA. The results showed that the expression level of B3GNT3 in the peripheral blood of lung adenocarcinoma patients was significantly higher than that in normal controls. The ROC curve was drawn, and the AUC values were calculated to be 0.9373 and 0.9938, which proved that the expression of B3GNT3 in serum could be used as a biomarker for the diagnosis of lung adenocarcinoma. Subsequently, a subgroup analysis of lung adenocarcinoma patients was performed according to their clinical stage. The results showed that the serum B3GNT3 level in patients with stage I and IIa lung adenocarcinoma was significantly higher than that in the control group, and with the increase in clinical stage, the expression level of B3GNT3 also increased. Therefore, B3GNT3 can be used as an important serum biomarker for the early screening of patients with lung adenocarcinoma. B3GNT3 can also be used as a serum biomarker for prediction of the prognosis of patients with lung adenocarcinoma.

To further clarify the specific molecular mechanism by which B3GNT3 regulates tumor progression in patients with lung adenocarcinoma, this study divided patients

with lung adenocarcinoma into high and low B3GNT3 expression groups according to mRNA information obtained from the TCGA database. KEGG analysis of differentially expressed genes in the two groups showed that the differentially expressed genes were mainly enriched in apoptosis-associated signaling pathways. To further clarify the regulatory effect of B3GNT3 on the apoptosis of lung adenocarcinoma cells, this study attempted to detect changes in proliferation and apoptosis by cultivating the cells *in vitro*. Moreover, B3GNT3 knockdown and overexpression lentiviruses were constructed and used to transduce lung adenocarcinoma cells, and changes in the proliferation and apoptosis of cells were detected. The results showed that the apoptosis rate of cells increased significantly after knocking down B3GNT3 and decreased significantly after overexpressing B3GNT3. Through bioinformatics and *in vitro* cell assays, this study clarified that B3GNT3 can regulate the disease progression of lung adenocarcinoma by suppressing the apoptosis of lung adenocarcinoma cells.

To further clarify the specific molecular mechanism of B3GNT3 in lung adenocarcinoma cells, this study attempted to use a PD-L1 inhibitor to overexpress B3GNT3. As one of the PD-1 ligands, PD-L1 can be expressed constitutively in different tissues under physiological conditions (27), mainly in activated T lymphocytes, B lymphocytes, dendritic cells (DC), monocytes, mesenchymal stem cells (MSCs), bone marrow (BM)-derived mast cells and various immune privileged organs (28,29). However, in the tumor immune microenvironment, the PD-1/PD-L1 axis is hijacked by cancer cells to evade immune surveillance. PD-L1 is expressed on different types of tumor cells, including glioblastoma, ovarian cancer, renal cell carcinoma, oral squamous cell carcinoma, colon carcinoma, and NSCLC cells (30-32). With the deepening of PD-L1 research, researchers have found that PD-L1 can also effectively suppress the apoptosis of tumor cells, thus promoting disease progression in tumor patients (33). To further clarify the specific molecular mechanism of PD-L1 and B3GNT3 in the apoptosis of lung adenocarcinoma cells, in this study, a PD-L1 inhibitor was added to B3GNT3 overexpression to detect the changes in apoptosis by cell assays. The results showed that apoptosis decreased after overexpressing B3GNT3, increased after inhibiting PD-L1, and increased after overexpressing PD-L1. The above findings suggest that B3GNT3 can regulate the apoptosis of lung adenocarcinoma cells via PD-L1.

## Conclusions

B3GNT3 is an effective serum biologic marker for the evaluation of lung adenocarcinoma. Moreover, B3GNT3 regulates the apoptosis of lung adenocarcinoma cells via PD-L1.

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## Footnote

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Qiqihar Medical University (No.20200425). Informed consent was taken from all individual patients.

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