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## Overexpression of T-box Transcription Factor 5 (TBX5) Inhibits Proliferation and Invasion in Non-Small Cell Lung Carcinoma Cells

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T-box transcription factor 5 (TBX5), a member of the conserved T-box transcription factor family that functions in organogenesis and embryogenesis, has recently been identified as a critical player in cancer development. The aim of this study was to determine the role of TBX5 in non-small cell lung carcinoma (NSCLC). Immunohistochemistry was used to detect the correlation between levels of TBX5 and clinicopathological features of NSCLC patients in tissue microarray. Expression of TBX5 in NSCLC tissues and cell lines was evaluated by quantitative PCR and Western blot. The role of TBX5 in regulating proliferation, colony formation, invasion, and apoptosis of NSCLC cells was evaluated in vitro. Finally, a tumorigenicity assay was performed to determine the effect of TBX5 on tumor growth in vivo. The levels of TBX5 in NSCLC tissues were significantly correlated with the TNM stage ( $p=0.016$ ), histopathologic type ( $p=0.029$ ), and lymph node status ( $p=0.035$ ) of NSCLC. TBX5 overexpression markedly suppressed in vitro NSCLC cell proliferation, colony formation, and invasion and induced apoptosis. In vivo tumor growth was significantly suppressed by TBX5. TBX5 has a tumor-suppressing effect in NSCLC and may serve as a therapeutic target for diagnoses and treatment of NSCLC.

**Key words: T-box transcription factor 5 (TBX5); Non-small cell lung carcinoma (NSCLC); Tumor growth; Proliferation; Invasion**

### INTRODUCTION

Lung cancer is the most common malignant tumor and the leading cause of cancer-associated mortality<sup>1</sup>. There were 1.8 million people diagnosed with lung cancer in 2012 worldwide, and there were 1.6 million associated deaths according to the Centers for Disease Control and Prevention (CDC). Based on histopathology features, lung cancer can be classified as non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC)<sup>2</sup>. NSCLC, which accounts for over 80% of lung cancer cases, is usually diagnosed at an advanced tumor node metastasis (TNM) stage, resulting in the loss of surgical therapeutic options and a poor 5-year survival rate (<15%)<sup>3</sup>. Several markers aimed at the diagnosis of lung cancer have been developed during the past few years<sup>4,5</sup>; however, their applications were limited because of a lack of sensitivity and specificity. The initiation and progression of lung cancer have been proposed to be a multistage process with dysfunction of genes, which participate in the regulation of cell cycle, proliferation, differentiation,

and apoptosis<sup>6</sup>. Identifying novel tumor suppressors and exploring their antitumor mechanisms during the pathogenesis of NSCLC will be of great value for the diagnosis and treatment of NSCLC.

T-box transcription factor 5 (TBX5) belongs to the large T-box transcription factor gene family, which were originally known to be regulators of organ development<sup>7</sup>. Members of this family have been found to be involved in processes including cell–cell signaling, proliferation, apoptosis, and migration. Several TBX genes were also suggested to play an inhibitory role in breast, melanoma, and pancreatic cancers<sup>8</sup>. The TBX5 gene is located on the human chromosome 12q24.1 and includes eight exons that encode for 518 amino acids. TBX5 protein functions as a transcription factor that binds to T-box elements on its target gene to regulate heart differentiation and development<sup>9,10</sup>. Recently, the novel tumor-regulating role of TBX5 has been discovered. High expression levels of TBX5 were found to be associated with an unfavorable survival rate in stages I and II of gastric adenocarcinoma<sup>11</sup>.

Polymorphisms near the *TBX5* gene were shown to be correlated with an increased risk for esophageal cancer.<sup>12</sup> Rosenbluh et al. reported that *TBX5* is critical in promoting colon cancer progression through forming a transcriptional regulatory complex with YAP1 and  $\beta$ -catenin to initiate antiapoptotic gene expression<sup>13</sup>. However, a recent study demonstrated that epigenetic inactivation of *TBX5* is associated with a poor survival rate among colon cancer patients. Furthermore, overexpression of *TBX5* significantly inhibits the proliferation, invasion, and colony formation and promotes apoptosis of colon cancer cells<sup>14</sup>, strongly suggesting *TBX5* as a tumor suppressor gene.

Although diverse levels and roles of *TBX5* in different types of cancers have been reported, the regulation and function of *TBX5* in NSCLC remain unclear. A genome-wide analysis has identified *TBX5* to be an important transcription regulator of genes that were differentially expressed between lung adenocarcinoma and normal patients<sup>15</sup>. A study on genome-wide methylation analysis suggested that *TBX5* is a methylation-sensitive gene that can potentially serve as an early diagnosis marker of NSCLC<sup>16</sup>. Taken together, these facts indicate that it is very important to investigate the expression pattern and role of *TBX5* in NSCLC, and the conclusions of this study may provide valuable information for the early detection and treatment of NSCLC.

## MATERIALS AND METHODS

### *Tissue Microarray*

A tissue microarray (#TC0219; Auragene, Changsha, Hunan Province, P.R. China) was established based on 120 specimens (110 NSCLC tissues, 6 adjacent nontumor tissues, and 4 normal tissues) from 110 patients who were diagnosed with NSCLC according to pathological examinations. Patient profile consisted of 38 women and 72 men ranging in age from 24 to 79 years (median age: 55 years), among whom 20 were diagnosed with squamous cell lung carcinoma (SCC), 53 with adenocarcinoma (AC), and 37 with large cell carcinoma (LCC).

### *Clinical Specimens*

Human NSCLC and their adjacent noncancerous tissues were collected from patients with NSCLC at the Third Xiangya Hospital, Central South University from March to June of 2015. None of the patients received radiotherapy, chemotherapy, or other anticancer treatments before surgery. The histological features of all specimens were evaluated by pathologists according to the WHO criteria. Informed consent was collected from each participant. All the methods in this study were in accordance with approved guidelines, and this study was approved by the medical ethics committee of the Third Xiangya Hospital, Central South University.

### *Immunohistochemical Staining*

Immunohistochemical staining was performed with human tissue sections to determine *TBX5* levels. Briefly, samples were rehydrated with decreasing concentrations of alcohol (100%, 95%, 85%, and 75%) and finally with distilled water. Antigen retrieval was performed in boiled sodium citrate buffer (0.01 mol/L; #P019IH; Auragene) under high pressure. Sections were then treated with 3% hydrogen peroxide and blocked with 100  $\mu$ l of nonimmunized goat serum before incubation with *TBX5* antibody (#Ab49308; 1:200; Abcam, Cambridge, UK) overnight at 4°C. After washing with phosphate-buffered saline (PBS), sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (#SA009; Auragene). DAB staining (#P013IH; Auragene) was then performed to detect the activity of HRP. Immunohistochemical evaluation was performed by two pathologists who followed double-blinded principles to score the staining intensity with the following criteria: <10 staining was considered to be negative staining (-),  $\geq$ 10% staining was considered to be positive staining [among which 10%–25% was weak staining (+)], 25%–75% was moderate staining (++) and 75%–100% was intense staining (+++). For evaluation of *TBX5* expression in the tissue microarray, negative to weak staining (- ~ +) was considered to be low staining, and moderate to intense staining (++ ~ +++) was considered to be high staining.

### *Cell Culture*

Human bronchial epithelial (HBE) cell line and human cancer cell lines including NIC-H1650, NIC-H1299, A549, and NIC-H596 were obtained from the Auragene cell bank. Cells were cultured with Dulbecco's modified Eagle's medium (DMEM; #SH30022.01; Hyclone, Logan, UT, USA) or RPMI-1640 medium (#SH30809.01; Hyclone) supplied with 10% FBS (#900-108; Gemini, West Sacramento, CA, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and maintained in a humidified cell incubator (5% CO<sub>2</sub>, 37°C).

### *Lentivirus Infection*

Human *TBX5* cDNA was cloned using the following primers: *TBX5*-F-*Eco*RI (5'-CCGGAATTCGCCACCATGGCCGACGCAGACGAGG-3'), *TBX5*-R-*Mlu*I (5'-CCGGACGCGTTTAGCTATTGTCTGCTCCACTCTGGCAC-3'). cDNA was then cloned into the PGMLV-CMV-MCS-EF1-ZsGreen1-T2A-Puro vector and transfected into the 293T cell line together with plasmids that encode envelope and other viral proteins (Gag, Pol, and env). Transfection efficiency was confirmed by fluorescence ratio, and cell culture supernatant was collected after 48–72 h. Viruses were stored in a -80°C freezer, and titer was determined in 293T cells before future use.

For lentiviral infection in NSCLC cell lines, cells were plated into 96-well plates ( $5 \times 10^3$  cell/well) and cultured for 24 h until reaching 30%–50% confluence. Lv-TBX5 and Lv-NC were added into the cell culture medium at MOI (multiplicity of infection)=20 for the A549 cell line and MOI=40 for NCI-H596 along with 5  $\mu$ g/ml polybrene. Twelve hours after infection, culture medium was refreshed, and cells were cultured for another 24 h before becoming ready for further treatments.

#### *Quantitative Real-Time PCR*

The total RNA of lung cancer cell line samples was extracted using a TRIzol kit (#R1022; Dongsheng Bio., Guangzhou, Guangdong Province, P.R. China). Reverse transcription of mRNA was carried out with a reverse transcription kit (#K1622; Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBRGreen qPCR Mix (#P2092; Dongsheng Bio.) on an ABI 7300 real-time PCR system. Primers for qPCR are as follows: TBX5, 5'-AAGTAAAGAAATATCCCGTGGTC-3' (sense) and 5'-AGACTCGCTGCTGAAAGG-3' (antisense);  $\beta$ -actin, 5'-AGGGGCCGGACTCGTCATACT-3' (sense) and 5'-GGCGGACCACCATGTACCCT-3' (antisense). The  $\beta$ -actin gene was used as an endogenous control, and all qRT-PCRs were performed in triplicate.

#### *Western Blot Analysis*

Cells were rinsed with cold PBS before being incubated with RIPA lysis buffer (#P002A; Auragene) containing proteinase inhibitors. Tissue samples were homogenized in RIPA lysis buffer with inhibitors. Protein mix was extracted after 20 min of incubation on ice and centrifuged at 13,000 rpm for 20 min. Protein concentration was determined by BCA assay, and samples were then prepared (2 mg/ml for cell lines, 4 mg/ml for tissues) with sodium dodecyl sulfate-loading buffer (#P003B; Auragene). For the immunoblot assay, equal amounts of protein from different cell lines (10  $\mu$ g/sample) and tissues (20  $\mu$ g/sample) were electrophoresed on a 12% sulfate-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were then blocked with 5% milk-TBST, incubated overnight at 4°C with a primary antibody in 3% BSA-TBST, washed with TBST, and incubated with a secondary antibody. Results were detected by AuraECL chemiluminescence kit (#P001WB-1; Auragene). Antibodies used included anti-TBX5 antibody (#Ab49308; 1:1,000; Abcam), anti-CDKN2A antibody (#AM2391; 1:800; Abzoom, Dallas, TX, USA), anti-SNCG antibody (#AM4078; 1:800; Abzoom), caspase 3 polyclonal antibody (19677-1-AP; 1:600; Proteintech, Rosemont, IL, USA), anti-caspase 8 antibody (#ab25901; 1:500; Abcam), anti-PARP antibody (#BM0432; 1:1,000; Abzoom), goat anti-rabbit IgG-HRP

(#SA009; 1:15,000; Auragene), and goat anti-mouse IgG-HRP (#SA001; 1:15,000; Auragene).

#### *Cell Proliferation Assay*

The effect of TBX5 levels on the proliferation of NSCLC cell lines was examined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells (infected with Lv-NC or Lv-TBX5) were plated into 96-well plates at a density of  $5 \times 10^3$  cell/well with a total volume of 100  $\mu$ l/well. MTT solution (10  $\mu$ l; #A600799-0005; Sangon Biotech, Shanghai, P.R. China) was added into each well at the given time points (every 24 h) followed by an additional 4 h of incubation (5% CO<sub>2</sub>, 37°C). Formazan that formed in each well was solubilized with 150  $\mu$ l of DMSO, and the optical density (OD) of the solution was measured at 570 nm (#MK3; Thermo Fisher) for further quantification of cell viability and proliferation.

#### *Clonogenic Assay*

A549 and NCI-H596 cells in the exponential phase were infected with Lv-NC or Lv-TBX5, and cells were then digested with 0.25% trypsin and made into single-cell suspensions in culture medium. Cell density was measured with a cell counter (#3516; Marienfeld, Cologne, Germany). Five hundred cells were plated into 10 ml of culture medium and maintained in an incubator with 5% CO<sub>2</sub> at 37°C for 2–3 weeks until cell colonies were visible under the naked eye. To quantify the colonies, they were fixed with 4% paraformaldehyde, stained with GIMSA (#G1010; Solarbio, Beijing, P.R. China), and counted under an ordinary optical microscope. Colonies that contained fewer than 10 cells were excluded; colony formation ratio=(colony count/cell seeded) $\times$ 100%.

#### *Cell Invasion Assay*

After infection with Lv-NC or Lv-TBX5, A549 and NCI-H596 cells were harvested and suspended in serum-free medium at a density of  $4 \times 10^5$  cells/ml. Matrigel Transwell chambers (#354480; Corning, Corning, NY, USA) were pretreated with serum-free medium, filled with 250  $\mu$ l of cell suspension (100,000 cells/chamber), and inserted into 24-well plates that contained 500  $\mu$ l/well cell growth medium (10% FBS). After a 24-h incubation (5% CO<sub>2</sub>, 37°C), cells and ECMatrix gel that remained in the upper chambers were carefully removed. Cells that migrated into the lower chambers were stained with crystal violet solution (#C0121; Beyotime Biotech, Haimen, Jiangsu Province, P.R. China) and visualized under a microscope. Stained cells were treated with glacial acetic acid for further measurement of OD value under a wavelength of 570 nm.



### Flow Cytometry Assay

Cells infected with Lv-NC or Lv-TBX5 were collected by trypsin without EDTA, washed with PBS before being incubated with annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions using Annexin-V-FITC-PI double staining kit (#KGA108; KeyGEN BioTECH, Nanjing, Jiangsu Province, P.R. China). Flow cytometric analysis (#FACSCanto II; BD Biosciences, Franklin Lakes, NJ, USA) was then performed to detect the staining.

### In Vivo Tumorigenicity Assay

BALB/c-nude mice (male, 4 weeks old, five mice/treatment group) were purchased from the Hunan SJA Laboratory Animal Co. (Changsha, Hunan Province, P.R. China) and maintained under free access to rodent chow diet and water. When mice reached 6 weeks old, A549 cells infected with Lv-NC or Lv-TBX5 were inoculated by subcutaneous injection ( $4 \times 10^6$  cells/injection) into the axilla area. Tumor size and bodyweight of the mice were monitored every 3 days. Tumor volume was calculated by the equation:  $[V (\text{volume, mm}^3) = L (\text{major axis, mm}) \times S^2 (\text{minor axis, mm}) / 2]$ . Mice were decapitated 30 days after injection, and tumors were isolated for further measurement.

mm)  $\times S^2$  (minor axis, mm)/2]. Mice were decapitated 30 days after injection, and tumors were isolated for further measurement.

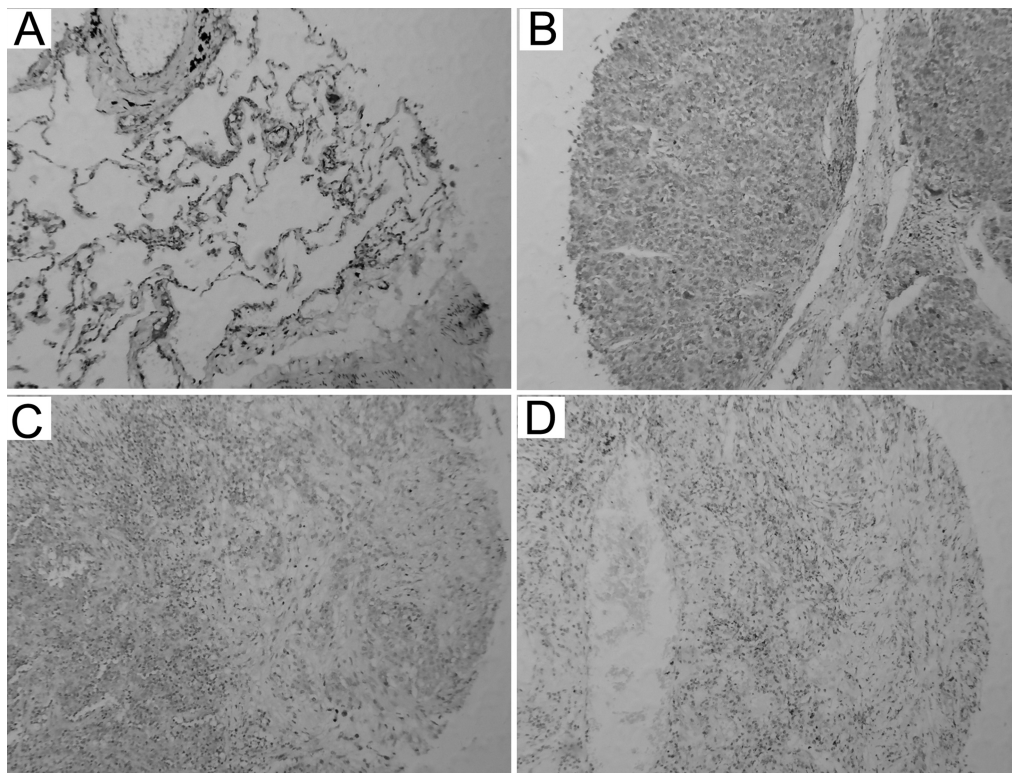
### Statistical Analysis

Comparison of the TBX5 levels between normal and NSCLC tissues, and correlation between TBX5 and clinicopathological features were computed by chi-square test. Comparisons of protein levels, mRNA levels, OD value, cell numbers, and tumor parameters were performed with Student's two-tailed *t*-test. Data were analyzed using SPSS 17.0 statistical software, represented as mean  $\pm$  standard error of the mean (SEM). A value of  $p < 0.05$  was considered to have statistical significance.

## RESULTS

### Correlation of TBX5 Levels With Clinicopathological Features of NSCLC

To evaluate the potential involvement of TBX5 in NSCLC, immunohistochemical staining of sections from the tissue microarray was performed. Results were categorized



**Figure 1.** Representative immunohistochemistry staining of T-box transcription factor 5 (TBX5) protein in non-small cell lung carcinoma (NSCLC) and adjacent nontumor tissues. (A) Nontumor adjacent tissue section showed intensive positive staining of TBX5. (B) Intensive (+++), (C) moderate (++) and (D) weak (+) positive staining of TBX5 in NSCLC tissue sections. Low TBX5 expression level was defined as negative and weak staining, and high TBX5 expression level was defined as moderate and intensive staining.



as low or high TBX5 levels (Fig. 1). Based on the staining result, we analyzed the relationship between TBX5 expression and clinical parameters of NSCLC. TBX5 expression showed a significant correlation with TNM stage ( $p=0.016$ ), histology ( $p=0.029$ ), and lymph node status ( $p=0.035$ ) of NSCLC (Table 1). However, there was no correlation between TBX5 expression and age, gender, or pathological differentiation (all  $p>0.05$ ).

*Expression of TBX5 in NSCLC Tissues and Cell Lines*

We further examined the TBX5 protein levels in human NSCLC tissue and matched normal adjacent tissue by Western blot (Fig. 2A). Results showed that TBX5 expression was significantly lower in NSCLC tissue compared to adjacent tissue ( $p<0.05$ ) (Fig. 2B), which strongly indicated that TBX5 is negatively associated with NSCLC. We also tested mRNA levels (Fig. 2C) and protein levels (Fig. 2D) of TBX5 from normal and lung cancer cell lines. Consistently, TBX5 was found to be expressed at significantly higher levels in the normal bronchial epithelial (HBE) cell line than in lung cancer cell lines, which included A549, NIC-H1650, NIC-H1299, and NIC-H596 ( $p<0.05$ ) (Fig. 2E).

*Effect of TBX5 Overexpression on NSCLC Cell Line Proliferation, Colony Formation, and Invasion*

Because TBX5 was found to be greatly downregulated in NSCLC tissues and cell lines, we performed overexpression of TBX5 in lung cancer cell lines A549 and NIC-H596 to test the effect of TBX5 on proliferation, clonogenicity, and invasion. The MTT assay showed significant inhibition of cell proliferation in A549 and NIC-H596 cells that were overexpressed with TBX5 (Fig. 3A and B). The clone formation assay (Fig. 3C) showed that TBX5 infection greatly reduced the number of colonies that were formed in both lung cancer cell lines when compared to the control groups (Fig. 3D). Moreover, the migration capacity of A549 and NIC-H596 cells was greatly blocked upon TBX5 overexpression (Fig. 3E and F). These results suggest that TBX5 plays an inhibitory role in the proliferation, colony formation, and invasion of NSCLC cells.

*Effect of TBX5 Overexpression on Apoptosis of the NSCLC Cell Line*

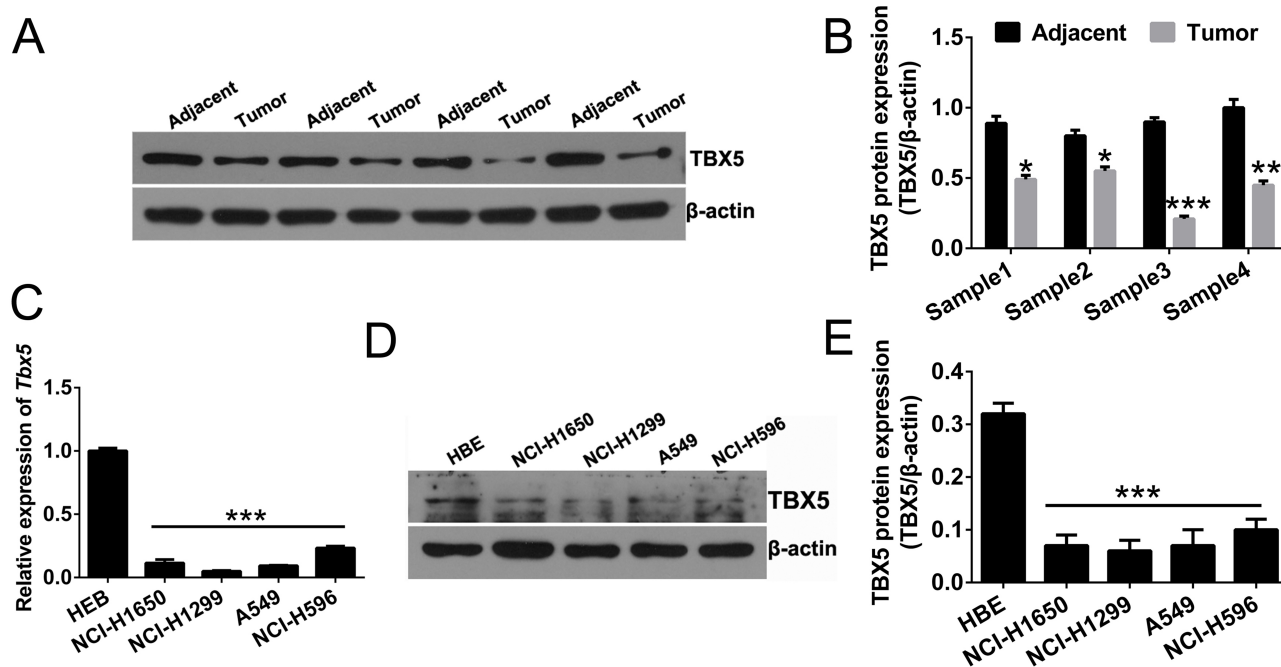
We then examined the effect of TBX5 on apoptosis by flow cytometry and Western blotting. Lung cancer cells

**Table 1.** Correlation of TBX5 Expression and Clinicopathological Features of NSCLC Patients

Clinicopathological Features	No. of Cases	TBX5 Expression		p Value
		High	Low	
Age(years)				0.566
≥55	61	25	36	
<55	49	23	26	
Gender				0.319
Female	38	14	24	
Male	72	34	38	
Histology				0.029*
Squamous cell carcinoma	20	14	6	
Adenocarcinoma	37	15	22	
Large cell carcinoma	53	19	34	
Clinical stage				0.016*
I	33	21	12	
II	16	7	9	
III+IV	61	20	41	
Lymph node status				0.035*
Metastasis	49	27	22	
No metastasis	61	21	40	
Pathological differentiation				0.932
Well	32	14	18	
Moderately	58	26	32	
Poorly	20	8	12	

TBX5, T-box transcription factor 5; NSCLC, non-small cell lung carcinoma; TNM, tumor node metastasis.

\* $p<0.05$ .



**Figure 2.** TBX5 levels in nontumor adjacent tissue, NSCLC tissue, and NSCLC cell lines. (A) TBX5 expression in four pairs of matched normal and cancer tissues.  $\beta$ -Actin protein was used as endogenous loading control. (B) Quantitative analysis of TBX5 levels (calibrated with  $\beta$ -actin levels of each sample) from (A). (C) qPCR detection of TBX5 mRNA levels from lung cancer cell lines. Data represent three independent experiments. (D) TBX5 protein levels in human bronchial epithelium cell line HBE, pleural effusion-derived cell line NCI-H1650, lymph node-derived cell line NCI-H1299, adenocarcinoma cell line A549, and lung adenosquamous cell line NCI-H596. (E) Quantitative analysis of TBX5 levels (calibrated with  $\beta$ -actin levels of each sample) from (D). Data represent mean  $\pm$  SEM with at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

overexpressed with TBX5 were found to contain a significantly higher population of cells that undergo apoptosis, indicated by increased staining of phosphatidylserine (PS) by annexin V-FITC and intracellular nucleic acid by PI (Fig. 4A and B). Further evaluation by Western blot showed that TBX5 overexpression significantly induced apoptotic markers including cleaved caspase 3, cleaved caspase 8, cyclin-dependent kinase inhibitor 2A (CDNK2), and nuclear enzyme poly-(ADP-ribose) polymerase (PARP) (Fig. 4C). In addition, TBX5 suppressed the tumor marker gene synuclein  $\gamma$  (SNCG) (Fig. 4D).

#### Impact of TBX5 on Tumorigenicity of NSCLC Cell Lines In Vivo

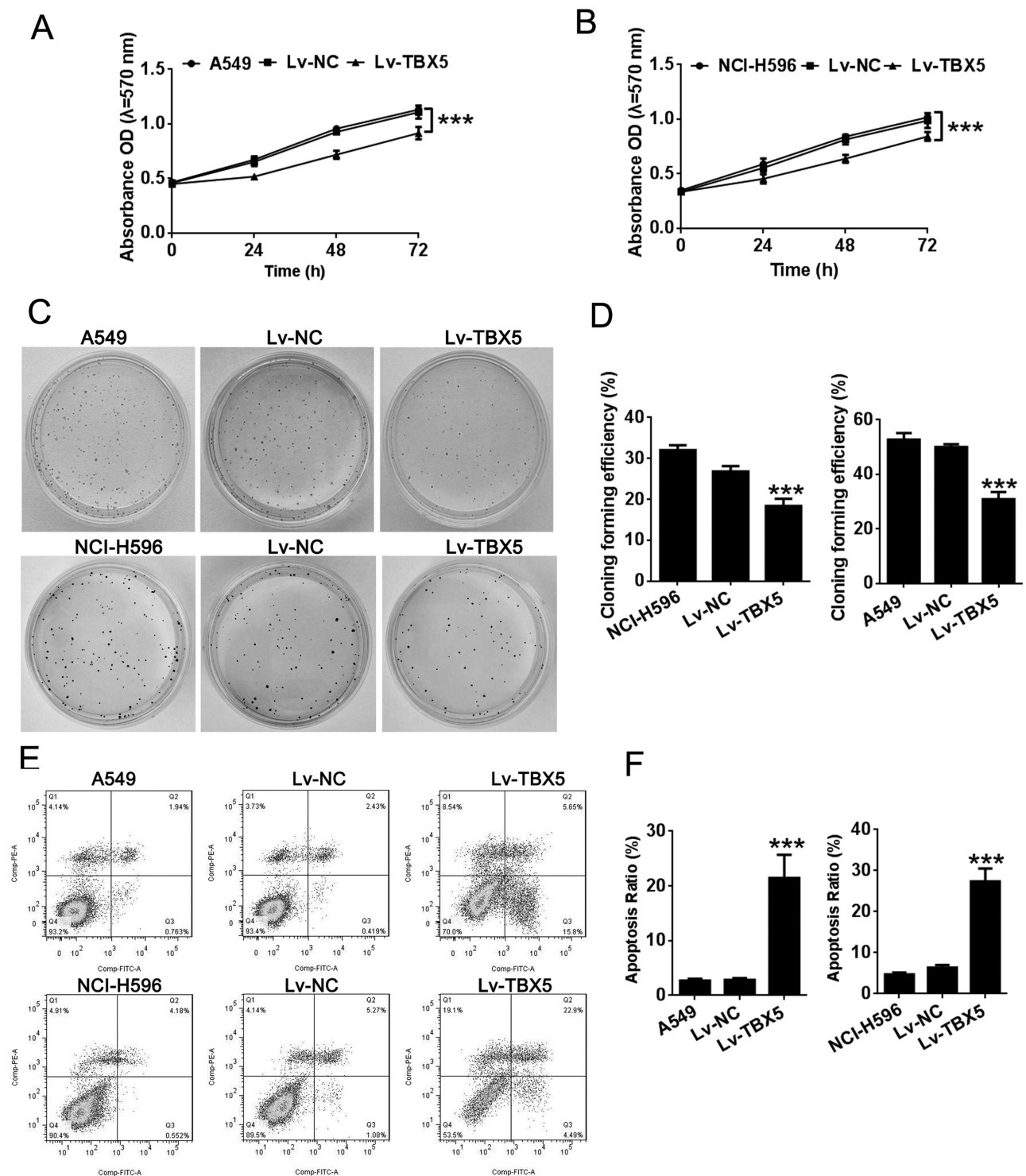
Finally, A549 cells infected with control or the TBX5 virus were tested for tumorigenesis capacity in BALB/c-nude mice. Increased TBX5 levels dramatically blunted the tumorigenic capacity of A549 cells (Fig. 5A). Tumor volume and weight were significantly decreased with TBX5 overexpression (Fig. 5B and C). These results strongly suggest that TBX5 exerts a tumor suppression effect in vivo.

## DISCUSSION

Despite the progress made in fighting cancer development during the past few decades, early detection and

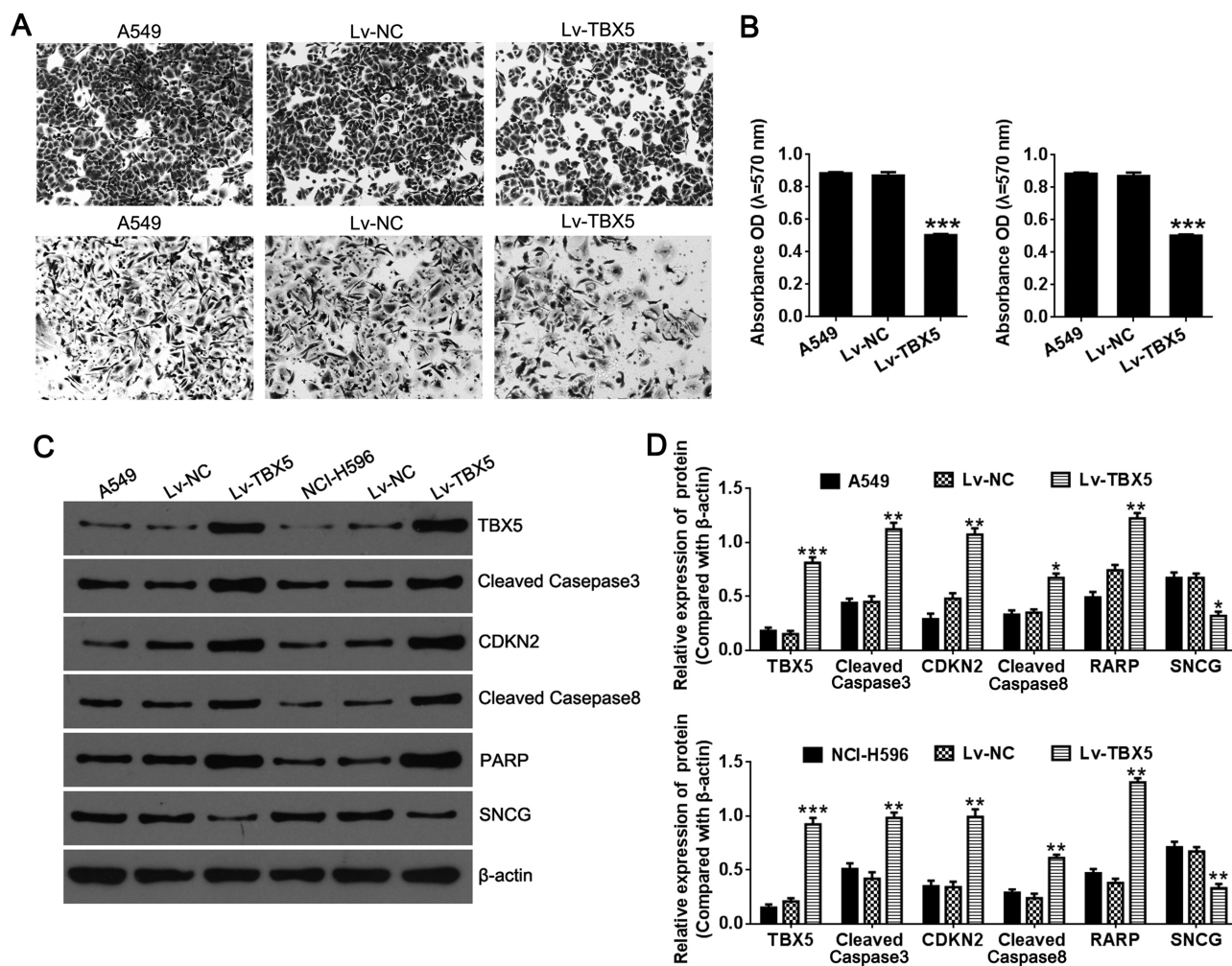
treatment of NSCLC remain elusive<sup>17</sup>. Understanding the biological mechanisms during NSCLC progression is critical for the identification of novel therapeutic options. Cumulative genetic alterations are considered to be the major causes for initiating the stepwise malignant transformation of normal respiratory epithelium, which ultimately results in lung cancer<sup>18</sup>. Therefore, genes that coordinate to regulate cancer progression have emerged as attractive candidates for anticancer research. In this study, we have identified TBX5, which regulates tumor cell proliferation, migration, and apoptosis, as a novel tumor suppressor in NSCLC.

Multiple studies have suggested that maintaining normal levels and function of TBX5 is critical during heart development<sup>19,20</sup>. More importantly, mutation and altered expression of TBX5 are known to cause several congenital diseases<sup>21,22</sup> and have recently been implicated in cancer<sup>14,23</sup>. Here we showed, for the first time, that TBX5 is highly expressed in normal adjacent tissues of NSCLC and cell lines, while its levels were markedly downregulated in NSCLC tissues and cells. These findings suggest a negative correlation of TBX5 levels with lung cancer progression, which is also consistent with the regulation of TBX5 in colon cancer<sup>14</sup>. Identifying disease-specific biomarkers of cancer is important for improving the

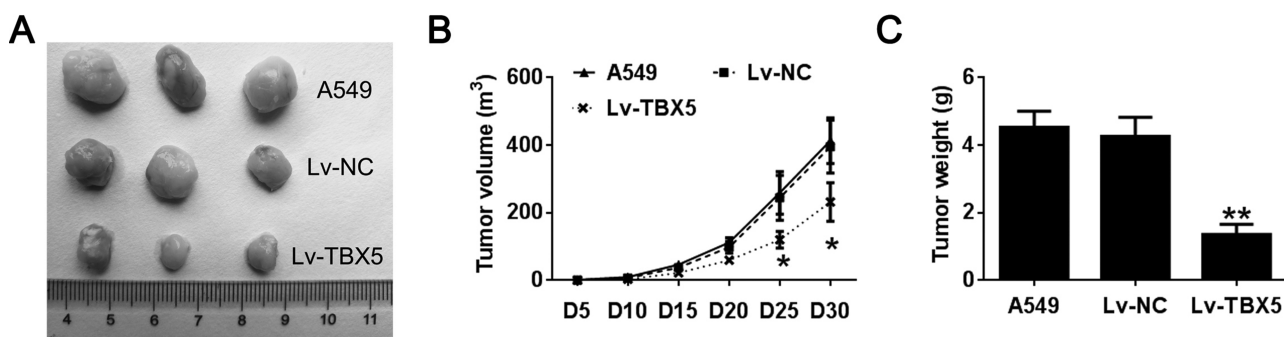


**Figure 3.** Effect of TBX5 overexpression on proliferation, clonogenicity, and apoptosis of NSCLC cell lines. TBX5 was overexpressed in A549 and NCI-H596 cell lines, (A, B) MTT assay was performed to test proliferation ability of cells. (C) Colony formation assay was carried out to evaluate the clone formation capacity of cell lines. (E) Apoptosis was evaluated by flow cytometry. The x-axis stands for propidium iodide (PI) staining to represent late apoptotic and necrotic cells. Q1 means necrotic cells, Q2 means late apoptotic cells, Q3 means early apoptotic cells, Q4 means live cells. (D, F) Quantitative analysis of (C) and (E). Data represent mean  $\pm$  SEM from three independent experiments with sample size=3 for each group. \*\*\* $p$ <0.001.





**Figure 4.** Effects of TBX5 overexpression on invasion and apoptotic signaling pathways of NSCLC cell lines. TBX5 was overexpressed in A549 and NCI-H596 cell lines. (A) Transwell assay was carried out to evaluate the invasive capacity of cell lines. (C) Protein levels of apoptotic markers were detected by Western blot. (B, D) Quantitative analysis of (A) and (C). Data represent mean  $\pm$  SEM from three independent experiments with sample size = 3 for each group. CDKN2, cyclin-dependent kinase inhibitor 2A; PARP, nuclear enzyme poly-(ADP-ribose) polymerase; SNCG, synuclein  $\gamma$ . \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 5.** Effect of TBX5 overexpression on the tumorigenesis ability of A549 NSCLC cell line in vivo. A549 cells overexpressed with control or TBX5 were inoculated into nude mice. (A) Representative appearance of tumors that were collected at the end of the study. (B) Tumor growth was monitored during the study. (C) Tumor weights were compared at the end of the study. Data represent mean  $\pm$  SEM with five independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .

accuracy of diagnosis and prognosis and for providing guidance for treatment. TBX5 levels were found to be significantly correlated with TNM stage, histopathologic type, and lymph node status of NSCLC, suggesting that TBX5 may be used as an independent biomarker for NSCLC.

We further demonstrated the tumor suppressive role of TBX5 in human NSCLC cell lines. By overexpressing TBX5 in A549 and NCI-H596 cells, we observed that TBX5 inhibits cancer cell proliferation and invasion and induces apoptosis. Consistently, previous data from other studies have shown that TBX5 inhibits cardiomyocyte proliferation<sup>24</sup> and inhibits osteosarcoma cell migration in a proliferation-independent manner<sup>9</sup>. It is also reported that TBX5 promotes the apoptosis of colon cancer cells<sup>14</sup>. Moreover, our results suggest that TBX5 induces early and late stage NSCLC cell line apoptosis, potentially through stimulating key genes in the apoptotic pathway, including cleaved caspase 3, cleaved caspase 8, CDKN2, and PARP. As a transcription factor, TBX5 functions through differential recruitment of other transcription factors and cofactors to regulate target gene expression. Its interaction partners include chromatin-modifying enzymes, chromatin remodeling complexes<sup>10</sup>, and transcription factors that are known to regulate cancer pathogenesis. GATA4, a transcription factor that cooperates with TBX5 in target gene transcription regulation<sup>25</sup>, is associated with colorectal and lung cancers<sup>26</sup>. NKx2-5, another important TBX5 cofactor, was identified as a tumor marker in prostate, colon, and breast cancers<sup>27</sup>. This knowledge provides the molecular basis for future studies on the mechanisms of TBX5 in regulating proliferation, invasion, and apoptosis.

To ascertain the tumor suppressive effect of TBX5 in vivo, we examined the tumorigenesis capacity of the NSCLC cell line under TBX5 overexpression conditions. An elevated TBX5 level substantially blocked tumor growth in nude mice, suggesting that TBX5 is an effective tumor suppressor during NSCLC development. However, gene expression and signaling pathways in tumors where TBX5 is overexpressed are to be further determined. Our in vivo data strongly imply that TBX5 is a promising candidate for future development of anti-NSCLC therapies.

In conclusion, our study showed that the TBX5 transcription factor is an important tumor suppressor gene in NSCLC. TBX5 may be used as a diagnostic marker and a drug target in the treatment of NSCLC. Additional efforts are necessary to explore the mechanisms of TBX5's antitumor effect. Further studies in a large clinical cohort of patients are required to establish the diagnostic and therapeutic applications of TBX5 in the area of NSCLC.

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