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Prognostic significance of TBX2 expression in non-small cell lung cancer

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Abstract T-box2 (TBX2) expression has been reported to be related to aggressive tumor features. However, the role of TBX2 in non-small-cell lung cancer (NSCLC) tumorigenesis has never been elucidated. So we aimed at investigating the potential role of TBX2 in NSCLC. TBX2 expression was evaluated by qRT-PCR and Western blotting in 50 paired fresh lung cancer tissues as well as immunohistochemistry on 212 paraffin-embedded sections. We showed that the expression level of TBX2 was significantly increased in NSCLC as compared with the adjacent noncancerous tissue. Positive expression level of TBX2 was associated with histological type, lymph node metastasis and distant metastasis. Kaplan–Meier survival curves showed that positive expression level of TBX2 was associated with poor overall survival (OS) and progression-free survival of NSCLC patients. Results showed that TBX2 positivity was an independent prognostic factor for OS (HR 1.87, 95 % CI 1.004–3.153, $p = 0.012$). On the basis of these results, we suggested that TBX2 protein expression may be an unfavorable independent prognostic parameter for NSCLC.

Introduction

Lung cancer has been the leading cause of cancer-related deaths in developed countries (Jemal et al. 2008). Although significant advances have been made with therapies, the 5-year survival rate of lung cancer is below 20 % (Tsim et al. 2010). The best chance of achieving long-term survival is in complete surgical resection; however, even in resected stage IA patients, 30 % succumb from their disease within 5 years (Tsuchiya et al. 2007). Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and usually grows and spreads more slowly than small cell lung cancer. Recent advances in molecular biology have provided important insights into molecular prognostic biomarkers. The identification of new molecular prognostic parameters will be helpful for planning the treatment of NSCLC patients after surgical resection (Filipits and Pirker 2011).

T-box2 (TBX2) is a member of the T-box family of transcription factors that are crucial in embryonic development (Abrahams et al. 2010). Recent studies suggest that T-box factors may also play a role in controlling cell cycle progression and in the genesis of cancer (Abrahams et al. 2010). TBX2 is involved in several cancers such as breast cancer, melanoma and pancreatic cancer (Barlund et al. 2000; Mahlamaki et al. 2002; Vance et al. 2005). But, so far, its expression pattern and significance in NSCLC remains unknown. This study was designed to investigate the expression of TBX2 in NSCLC and explore the correlations between TBX2 expression and clinicopathological features. We demonstrated that TBX2 was upregulated in NSCLC and

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may be a promising independent prognostic factor in NSCLC.

Materials and methods

Patient and tissue samples

Group I included 50 fresh NSCLC cancerous samples. The tumor and non-tumor tissues were cut in half and processed separately for both western blotting and qRT-PCR.

Group II included 212 NSCLC patients who underwent resection (lobectomy and mediastinal lymph node dissection with microscopic examination of margins showing no tumor cells) from January 2001 to January 2004 at the Shandong Provincial Hospital Affiliated to Shandong University were included in this retrospective study.

Patients who have previous malignant disease, second primary tumor, or those who received preoperative radiotherapy and/or chemotherapy were excluded. 4 μm-thick sections were stained with hematoxylin and eosin. Each case was reassigned for tumor, node, metastasis (TNM) classification. The following clinical and pathologic parameters were retrospectively reviewed and analyzed for each case: age at surgical resection, gender, smoking habits, histological type, pathological TNM stage, nodal status, metastasis, survival time after surgery. This study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University. Written informed consent was obtained from all patients.

Follow up

All patients had follow-up records and were observed at 3-month intervals during the first 3 years and at 6 month intervals thereafter. Overall survival (OS) was defined as the time from the tumor resection to the date of death; progression-free survival (PFS) was defined as the time from the tumor resection to the date of disease relapse/progression or the date of death.

Real-time quantitative PCR

Total RNA was isolated tissue using TRIZOL according to the manufacturer's protocol (Invitrogen). RNA was reverse transcribed using First Strand cDNA System (Invitrogen) according to the manufacturer's instructions. TBX2 forward primer was 5′-TTGGCTGCTCTAACCCTCTAG-3′, and the reverse primer was 5′-GCT GTTGCT GACACAAATTC-3′; GAPDH forward primer was 5′-GCAC CA CCAACTGCTTAG C-3′, and the reverse primer was 5′-GCATGGACTGTGGTCATGA-3′. The PCR amplification were performed for 35 cycles of 94 °C for 30 s,

58 °C for 45 s, and 72 °C for 30 s, on a Applied Biosystems 7900HT (Applied Biosystems) with SYBR Green Real-time PCR Master Mix (Takara). The expression level was normalized against GAPDH. The relative quantitative value was expressed using the 2-$\Delta\Delta$Ct method. Each experiment was repeated three times.
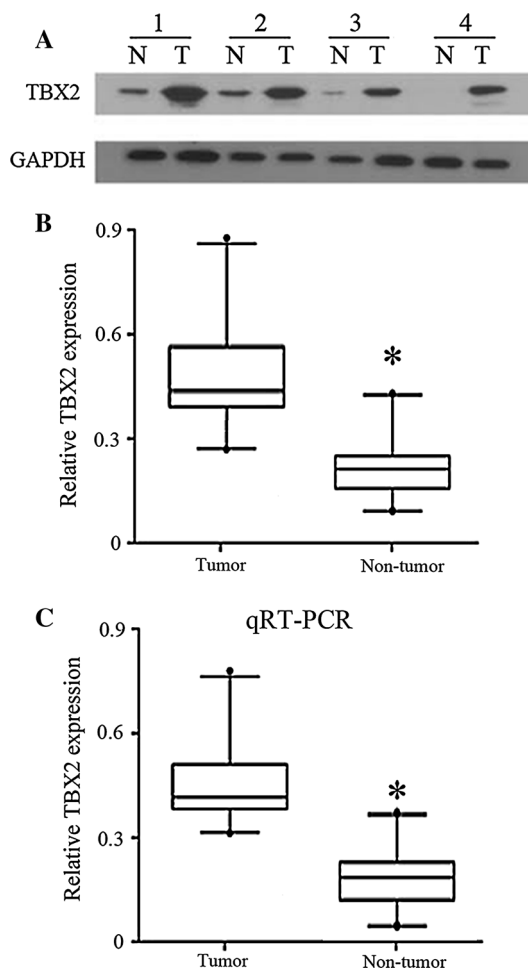
Western blot assay

Total proteins from tissues were lysed in lysis buffer containing protease inhibitor. Protein concentration was determined using a Bio-Rad protein assay system (Bio-Rad). Equivalent amounts of proteins were separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes (Millipore). After being blocked in Tris buffered saline (TBS) containing 5 % non-fat mTBX2, the membranes were incubated with anti-TBX2 (C-17) and GAPDH (Santa Cruz) at 4 °C for 12 h and then with horseradish peroxidase conjugated anti-Rabbit antibody for 2 h at room temperature. ECL detection reagent (Amersham LifeScience) was used to demonstrate the results.

Immunohistochemical Staining

After dewaxing in xylene and rehydrating stepwise in ethanol, sections were subjected to heat-induced antigen retrieval. Endogenous peroxidase activity and nonspecific binding were blocked with 3 % H_2O_2 and nonimmune sera, respectively. Sections were then incubated with primary antibodies overnight at 4 °C. The primary antibodies were used in this study: rabbit anti-BX2 antibody (C-17, 1:100, Santa Cruz). The following day, primary antibody was detected using the appropriate labeled Streptavidin–Biotin kit (Maixin Biotechnology, Fuzhou, China) according to the manufacturer's instructions. Immunolabeled sections were visualized with 3,3′-diaminobenzidine and counterstained with hematoxylin. Sections were stained in parallel without primary antibody to provide a negative control.

Evaluation of immunohistochemical staining

Two investigators separately evaluated all the specimens in a blinded manner. Variant cases were reviewed and discussed until a consensus was obtained. Five areas were selected at random and scored. TBX2 expression was evaluated using a semi-quantitative scoring system where both intensity and percentage of positive tumor cells were analyzed (Zhang et al. 2012; Chen et al. 2013). The intensity of staining was scored as 0, 1 for weak, 2 for moderate, and 3 for strong. The percentage of positive tumor cells was scored as 0 for 0 %, 1 for 1–10 %, 2 for 11–50 %, and 3 for 51–100 %. The sum of both scores was used to identify two categories of expression: negative

Fig. 1 TBX2 expression in non-small cell lung cancer (NSCLC) tissues and paired adjacent noncancerous tissues. **a** Representative Western blots of TBX2 in cancerous tissues and the matched adjacent noncancerous tissues from 4 NSCLC patients. **b** Relative expression of TBX2 protein in 50 non-small cell lung cancer (NSCLC) tissues and paired adjacent noncancerous tissues. **c** Relative TBX2 mRNA expression in all 50 non-small cell lung cancer (NSCLC) tissues and the matched adjacent noncancerous tissues. The *box plots* in **b** and **c** describe the relative expression of TBX2. The ends of the boxes define the 25th and 75th centiles, a *line* indicates the median, and *bars* define the 5th and 95th centiles. Individual outliers are also shown. *$p < 0.01$

expression (with a total score ≤ 2) and positive expression (with a total score ≥ 3).

Statistical analysis

Statistical analyses were performed using the SPSS version 17.0. The *t* test was used to analyze the data from qRT-PCR and Western blot in the tissues. Clinical and histopathologic information and the results from the immunohistochemical studies were entered into a database. The significance of TBX2 expression for tumor was analyzed by the Kaplan–Meier method, and the differences were evaluated by the log-rank test. Multivariable survival

analyses were performed with the Cox proportional hazards model. Differences were considered significant if the P-value from a two-tailed test was <0.05.

Results

TBX2 expression were upregulated in NSCLC tissues

First, we used Western blot to detect 50 fresh NSCLC cancerous samples and paired adjacent noncancerous tissues from the same patient. We found the TBX2 protein expression level was significantly higher in cancerous tissues than that in corresponding adjacent non-cancerous tissues (Fig. 1a, $p < 0.001$). In consistence, the qRT-PCR demonstrated the same trend (Fig. 1b, $p < 0.001$). These data suggest a potential role for TBX2 in NSCLC.

TBX2 expression in NSCLC and its association with clinicopathological features

Immunohistochemistry was employed to examine the protein expression of TBX2 in primary NSCLC specimens and normal adjacent tissues. Immunoreactivity of TBX2 was observed primarily in the nuclei (Fig. 2). TBX2 positivity is significantly higher than that in adjacent non-cancerous normal tissues [60.8 % (129/212) vs 9 % (19/212), $p < 0.001$].

The clinical features of patients, including age, gender, histology, tumor stage, metastasis and TBX2 expression were summarized in Table 1. Correlation analysis demonstrated that TBX2 expression was correlated with histological type ($p = 0.014$), lymph node metastasis ($p = 0.008$) and distant metastasis ($p = 0.021$).We did not find any correlation between TBX2 expression and other patient characteristics.

Positive TBX2 expression correlates with poor prognosis

Kaplan–Meier survival curves demonstrated that patients with positive TBX2 expression exhibited inferior OS ($p < 0.001$) and PFS ($p < 0.001$) compared to those with negative expression of TBX2 (Fig. 3). Furthermore, for the purpose of avoiding the influence caused by univariate analysis, the expression of TBX2 as well as other parameters was examined in multivariate Cox analysis. Results showed that TBX2 positivity was an independent prognostic factor for OS (HR 1.87, 95 % CI 1.004–3.153, $p = 0.012$, Table 2).
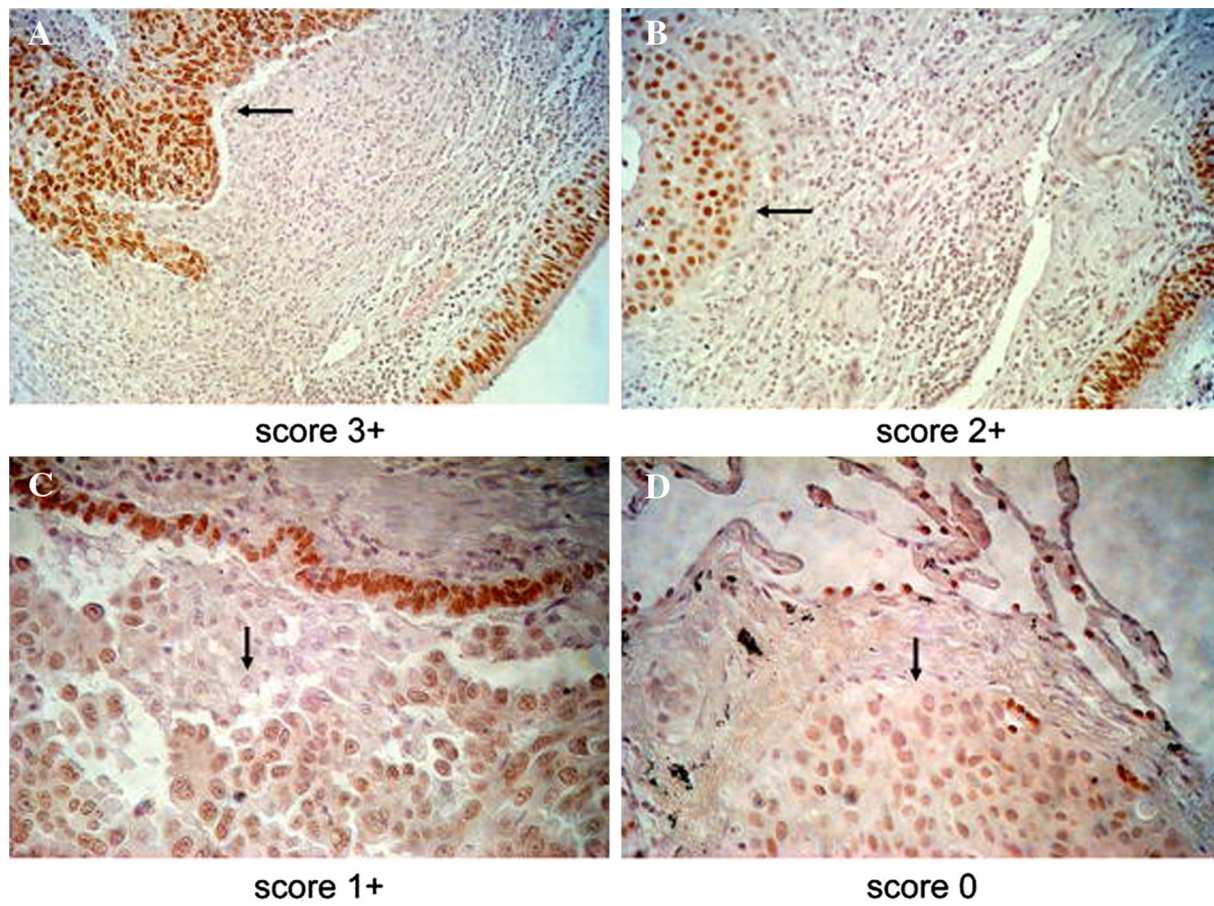
Fig. 2 Immunohistochemical staining of TBX2 expression in non-small cell lung cancer (NSCLC) tissues. **a** 3 + IHC score (intense nuclear staining) in a tumor sample; **b** 2 + IHC score (moderate nuclear staining) in a tumor sample; **c** 1 + IHC score (weak nuclear staining) in a tumor sample; D. 0 IHC score (lack of staining) in a tumor sample. *Black arrows* indicate tumor tissue, 200×

Table 1 Correlation between TBX2 expression with clinicopathologic features in 212 non-small-cell lung cancer (NSCLC) patients

Parameters	Group	Total	TBX2 expression		P
			Negative (%)	Positive (%)	
Gender	Male	132	53 (40.2)	79 (59.8)	0.441
	Female	80	30 (37.5)	50 (62.5)	
Age (years)	<60	110	52 (47.3)	58 (52.7)	0.425
	≥60	102	31 (30.4)	71 (69.6)	
Histological type	adenocarcinoma	78	45 (57.7)	33 (42.3)	0.014
	non-adenocarcinoma	134	38 (28.4)	96 (71.6)	
Tumor size (cm)	<3.5 cm	124	51 (41.1)	73 (58.9)	0.510
	≥3.5 cm	88	32 (36.4)	56 (63.6)	
TNM stage	I + II	130	60 (46.2)	70 (53.8)	0.319
	III	82	23 (28)	59 (72)	
Lymph nodes metastasis	No	180	79 (43.9)	101 (56.1)	0.008
	Yes	32	4 (12.5)	28 (87.5)	
Distant metastasis	No	188	81 (43.1)	107 (56.9)	0.021
	Yes	24	2 (8.3)	22 (91.7)	
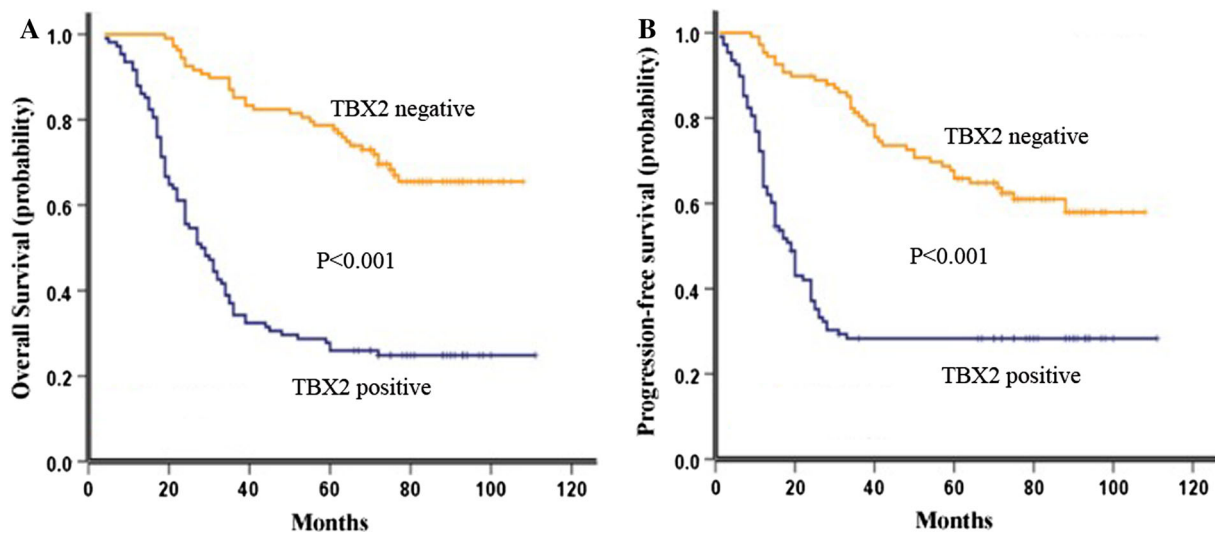
Fig. 3 Kaplan-Meier analysis of non-small cell lung cancer (NSCLC) patients according to TBX2 expression level. Positive TBX2 expression was positively correlated with poor OS (**a**) and PFS (**b**)

Table 2 Univariate and multivariate analysis of OS in 212 non-small-cell lung cancer (NSCLC) patients

Parameters	Univariate analysis			Multivariate analysis		
	HR	95 % CI	p	HR	95 % CI	p
Gender						
Male versus female	0.976	0.492–1.041	0.562			
Age (years)						
<60 versus ≥60	0.762	0.316–1.013	0.704			
Smoking status						
Never smoker versus smoker	2.497	1.213–4.015	<0.001	2.05	0.913–3.112	0.0011
TNM						
I–II versus III	0.855	0.607–1.193	0.506			
TBX2 expression						
Positive and negative	1.973	1.124–3.312	0.0013	1.87	1.004–3.153	0.012

Discussion

In the present study, we evaluated the expression of TBX2 by qRT-PCR and Western blotting in 50 paired fresh lung cancer tissues as well as immunohistochemistry on 212 paraffin-embedded tumor samples derived from a series of lung cancer patients. Our results showed that TBX2 was significantly upregulated in NSCLC tissues in comparison with adjacent noncancerous tissues. By immunohisto-chemistry, TBX2 was highly expressed in 60.8 % of tumors, and the TBX2 expression was clearly associated with histological type ($p = 0.014$), lymph node metastasis ($p = 0.008$) and distant metastasis ($p = 0.021$). Further-more, we investigated the correlations between TBX2 expression in human NSCLC tissues and clinical outcome of NSCLC. Our data suggest that the expression of TBX2 was an independent prognostic indicator for OS of NSCLC

patients by multivariate analysis. These results suggest a role of TBX2 expression as a novel biomarker in NSCLC.

TBX2 has also been reported to be upregulated in breast cancer cells (Barlund et al. 2000). Meanwhile, data have also revealed TBX2 potently downregulates the p19ARF tumor suppressor, thereby causing efficient immortaliza-tion of primary fibroblasts (Jacobs et al. 2000). Small interfering RNA-mediated down-regulation of Tbx2 expression results in a robust activation of p21 expression, implicating Tbx2 as a novel direct regulator of p21 expression and have implications for our understanding of the role of T-box factors in the regulation of senescence and oncogenesis, as well as in development (Prince et al. 2004). p21 was strongly induced in the Tbx2-deficient lung mesenchyme and deletion of p21 rescued, to a large degree, the growth deficits of Tbx2-deficient lungs (Lüdtke et al. 2013). Moreover, TBX2 is overexpressed in melanoma

cells (Vance et al. 2005) and pancreatic cancer cells (Mahlamaki et al. 2002), maintaining the tumor cell proliferation. In addition, methylation of the T-box 2 (TBX2) gene was associated with progression to muscle-invasive disease in pTa bladder cancer (Kandimalla et al. 2012). Also, targeting TBX2 in combination with chemotherapeutic drugs such as cisplatin could improve the efficacy of current anticancer treatments (Wansleben et al. 2013). However, we know little about whether TBX2 was implicated in NSCLC.

In summary, we present the clinical role for TBX2 in NSCLC in this study. Our study showed that overexpression level of TBX2 was significantly associated with aggressive features of NSCLC. TBX2 was an independent prognostic marker for NSCLC. TBX2 might act as a valuable marker for evaluating the clinical outcome of NSCLC.

Conflict of interest None.

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