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Original Article

Increased expression of TUSC3 in non-small cell lung cancer

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Abstract: Aims: To examine the expression of tumor suppressor candidate 3 (TUSC3) in non-small cell lung cancer (NSCLC) compared to adjacent normal tissue, and its correlation with patients' clinicopathological features. Methods: Forty NSCLC patients were recruited, including 30 patients with adenocarcinoma and 10 patients with squamous cell carcinoma. Expression of TUSC3 in tumor tissue and matched normal tissue was determined by real-time PCR and immunohistochemistry (IHC). The epidermal growth factor receptor (EGFR) mutation was detected by amplification refractory mutation system (ARMS)-Scorpions analysis. TUSC3 expression in several NSCLC cell lines was assayed by Western blotting. Results: Expression of TUSC3 is significantly higher in tumors than their adjacent normal tissues in 40 paired NSCLC specimens at both mRNA and protein level ($P<0.05$). More importantly, increased TUSC3 expression at the mRNA level correlated significantly with gender and age ($P<0.05$). However, no statistical significance was found between TUSC3 expression and smoking, T status, lymph node metastasis, differentiation and EGFR mutation status at neither mRNA nor protein level ($P>0.05$). Western analysis detected increased TUSC3 level in several NSCLC cell lines includes A549, H322, H460 and SPC-A1 compared to the control, human bronchial epithelial (HBE) cells. Conclusion: TUSC3 is upregulated in NSCLC tumors compared to adjacent normal tissue, as well as in several NSCLC cell lines, suggesting its potential oncogenic role in NSCLC.

Keywords: Non-small cell lung cancer, TUSC3, immunohistochemistry, EGFR

Introduction

Lung cancer is one of the most common cancers and the leading cause of cancer mortality worldwide [1]. Non-small cell lung cancer (NSCLC) is the most common subtype and accounts for 80-85% of all lung cancer [1]. Despite the advances in treatment options, such as surgical resection, chemotherapy, radiation therapy, and combination of chemotherapy and chemoradiation modality [2], little improvement has been achieved over the past few decades in term of patients' survival. Thus, understanding the molecular pathogenesis of NSCLC and identifying new targets and strategies for effective therapy is still of great importance.

Tumorigenesis is a multi-step process driven by genetic and/or epigenetic alterations, which cause the activation of oncogenes and inactivation of tumor suppressors. TUSC3 (tumor suppressor candidate 3), also named N33, was first cloned from a metastatic prostate carcinoma carrying a homozygous deletion [3]. It is located on chromosome band 8p22 [3]. Several studies have reported that TUSC3 was downregulated and thus was generally regarded as a putative tumor suppressor in prostate and ovary cancer [4, 5]. Deletions or mutations of TUSC3 are associated with mental retardation [6-8]. As a subunit of the oligosaccharyltransferase complex, TUSC3 is capable of modulating glycosylation pattern in ovarian cancer cells [9].

Although TUSC3 is known to have multiple functions and is regarded as a tumor suppressor in prostate and ovarian cancer, its expression level and role in NSCLC is still unclear. Thus, in

Table 1. Clinicopathological characteristics of patients with NSCLC

	Case No.	%
Total patients	40	
Gender		
Male	32	80.0
Female	8	20.0
Age (median)		
<60	28	70.0
≥60	12	30.0
Smoking History		
Ever	20	50.0
Never	20	50.0
Pathological Stage		
IA	15	37.5
IB	13	32.5
IIA	3	7.5
IIB	1	2.5
IIIA	8	20
T status		
T1a	15	37.5
T1b	3	7.5
T2a	18	45.0
T2b	1	2.5
T3	1	2.5
T4	2	5.0
Lymph node metastasis		
N0	30	75.0
N1	4	10.0
N2	6	15.0
Tumor Type		
Adenocarcinoma	30	75.0
Squamous cell carcinoma	10	25.0
Differentiation		
Well	12	30.0
Moderate	21	52.5
Poor	7	17.5
EGFR mutation		
Negative	26	65.0
Positive	14	35.0

this study, we explored the expression of TUSC3 at both mRNA and protein level in 40 NSCLC tumors and their paired adjacent normal tissues. The correlation between TUSC3 expression and patients' clinicopathological factors and EGFR mutation status was also analyzed. In addition, we also tested TUSC3 expression in several NSCLC cell lines.

Materials and methods

Patients

A total of 40 NSCLC patients from the Department of Thoracic Surgery & Pathology, Nanfang Hospital, Southern Medical University were enrolled. This study was approved by the ethic committee at Southern Medical University and a signed consent form was obtained from each patient. These patients underwent complete tumor resection between 2013 and 2014 and did not receive any neoadjuvant treatment.

All tumors and paired normal tissues were obtained from surgical specimens. The matched normal tissues were at least 3 cm away from the edge of the corresponding tumors.

Quantitative real-time RT-PCR

Total RNA was isolated using TRIzol™ method (Invitrogen, California, United States), and first-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Promega, Wisconsin, United States). Single-strand cDNA was used as template for subsequent real-time PCR (qPCR). Two microliters (μl) of cDNA from each tissue were used for qPCR assay. Templates were amplified using QuantiTect SYBR Green PCR kit (QIAGEN, California, USA). Primer sequences for TUSC3 used in RT-PCR were: 5'-CCT CAG CGG CAG TGT TCT G-3' and 5'-CCT CAT CAT AGT CCA CCA TAC TGA A-3'. qPCR was initiated at 95°C for 10 minutes, and then amplified for 40 cycles at 95°C for 15 s, 60°C for 60 s, and 72°C for 45 s. The amplified fluorescence signal in each specimen was measured at the late extension step of each cycle. To quantify each gene, serial 10-fold dilutions of human genomic DNA was used as control.

Immunohistochemistry and quantification

TUSC3 antibody (sc-390556) was purchased from Santa Cruz Biotechnology (Shanghai, China). The tissue sections were deparaffinized in xylene and rehydrated through a graded series of alcohol to distilled water. After optimization of immunohistochemistry conditions, antigen retrieval for TUSC3 was performed using pressure chamber with pH 6 Target Retrieval Solution (DAKO, Denmark) pretreat-

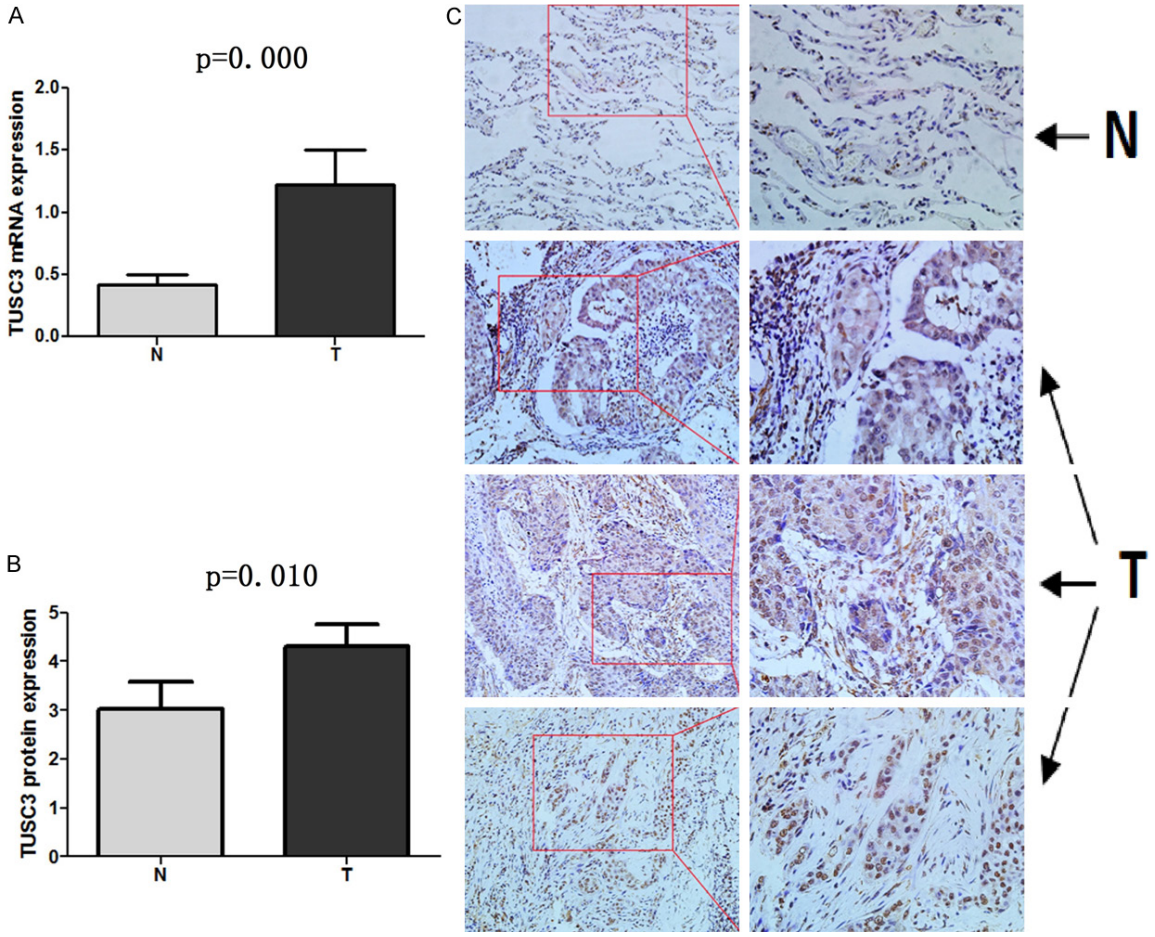

Figure 1. Increased expression of TUSC3 in the tumor samples of 40 NSCLC patients compared to their adjacent normal tissue. N: normal tissue, T: tumor tissue. A: Increased expression of TUSC3 at mRNA level measured by qRT-PCR. B: Increased expression of TUSC3 at protein level measured by immunohistochemistry (IHC). C: Representative of IHC staining, pictures on the right side are zoomed images of the area in the red box on the corresponding picture on the left.

ment. These slides were blocked with hydrogen peroxide/methanol. After rinsing, the slides were incubated with the primary antibody at 4°C over night. TUSC3 antibody was diluted 1:100 (sc-390556, Santa Cruz Biotechnology, USA). Target signals were detected with LSAB peroxidase Kit and DAB (DAKO, Denmark). The stained slide were then lightly counterstained with hematoxylin.

Quantification of the stained slides was conducted independently by two pathologist and their results were averaged. Specifically, five fields were randomly chosen for a given slide and the ratio of positive cells versus total number of cells in the field, intensity of the staining of positive cells compared to background were quantified. Score was assigned as below: based on positive cell ratio: no positive cell, score 0; 1-25%, score 1; 26-50%, score 2; 51-75%, score 3; 76-100%, score 4. Based on staining intensity: no above background staining: score 0; light yellow staining, score 1; dark yellow staining, score 2; brownish yellow staining, score 3; dark brown staining, score 4. The two score were then added and averaged. Five slides from each tumor and five slides from matched normal tissue were quantified and averaged. A final score >4 was defined as strong staining, and <4 was defined as weak staining.

Western blotting

The following human lung cancer cell lines were collected and assayed for TUSC3 expression: GLC-82, a lung carcinoma cell line; A549, H322 and SPC-A1, three NSCLC cell lines; H460, a

Table 2. The relationship between TUSC3 mRNA expression and clinicopathological parameters in NSCLC

	All cases	TUSC3 mRNA expression level		*P* value
		Low expression (%)	High expression (%)	
Gender				
Male	32	18 (56.25%)	14 (43.75%)	0.003
Female	8	2 (25.00%)	6 (75.00%)	
Age (median)				
<60	25	16 (64.00%)	9 (36.00%)	0.022
≥60	15	4 (26.67%)	11 (83.33%)	
Smoking History				
Ever	20	8 (40.00%)	12 (60.00%)	0.206
Never	20	12 (60.00%)	8 (40.00%)	
Stage				
I and II	32	16 (50.00%)	16 (50.00%)	0.317
III and IV	8	4 (50.00%)	4 (50.00%)	
T status				
T1	18	8 (44.44%)	10 (55.56%)	0.525
T2-4	22	12 (54.55%)	10 (45.45%)	
Lymph node metastasis				
Negative	30	14 (46.67%)	16 (53.33%)	0.465
Positive	10	6 (60.00%)	4 (40.00%)	
Tumor Type				
Adenocarcinoma	30	16 (53.33%)	14 (46.67%)	0.465
Squamous cell carcinoma	10	4 (40.00%)	6 (60.00%)	
Differentiation				
Well	12	6 (50.00%)	6 (50.00%)	1
Moderate and poor	28	14 (50.00%)	14 (50.00%)	
EGFR mutation				
Negative	27	16 (59.26%)	11 (40.74%)	0.091
Positive	13	4 (30.77%)	9 (59.23%)	

Table 3. TUSC3 expression in the 40 non-small cell lung cancer tissues and paired normal tissues by immunohistochemistry staining

	TUSC3 expression level			*P* value
	Negative	Weak	Strong	
Tumor tissues	8	9	23	0.003
Normal tissues	22	1	17	

NSCLC cell line derived from the pleural fluid of a NSCLC patient. HBE (human bronchial epithelial) cell line was used a normal control. Western blotting was conducted as described (9, 10) with β-actin (1:2000 dilution, Santa Cruz Biotechnology, USA) as an internal control. TUSC3 antibody was the same as mentioned above with 1:1000 dilution.

EGFR mutation detection

DNA samples were extracted from the resected tumor tissues using a QIAamp DNA mini Kit (Qiagen, Germany) and were serially diluted to 50 ng/μl for use as DNA templates. Mutations in EGFR exon 19 and 21 were detected by ARMS analyze using ADx EGFR Mutations Detection Kit (Amoy Diagnostics, Xiamen, China), which has been approved by the State Food and Drug Administration (SFDA) for clinical usage in mainland China.

Statistical analysis

All statistical analyses were performed using SPSS for Windows software, version 13.0 (SPSS Inc, Chicago, Illinois, United States). We used a paired t-test to compare the TUSC3 level between tumors and matched normal tissues. The χ^2 test was applied to find the correlation between the TUSC3 level and clinicopathologic parameters.

Results

Patient characteristics

Demographic, clinical and histopathological data of the patients are shown in **Table 1**. The median age of patients was 56 years (range 38 to 71 years), and the majority were male (80%). Half of the patients (50%) were current or previous smokers. The 40 NSCLC tumors consist of 10 squamous cell carcinomas (SCC) and 30 adenocarcinomas (AC).

TUSC3 mRNA expression

TUSC3 mRNA expression was detected by RT-PCR. Of the 40 paired normal tissue and

Table 4. The relationship between TUSC3 protein expression and clinicopathological parameters in NSCLC

	All cases	TUSC3 protein expression		*P* value
		Low expression	High expression	
Gender				
Male	32	14 (43.75%)	18 (56.25%)	0.161
Female	8	2 (25.00%)	6 (75.00%)	
Age (median)				
<60	25	10 (40.00%)	15 (60.00%)	1
≥60	15	6 (40.00%)	9 (60.00%)	
Smoking History				
Ever	20	10 (50.00%)	10 (50.00%)	0.196
Never	20	6 (30.00%)	14 (70.00%)	
Stage				
I and II	32	14 (43.75%)	18 (56.25%)	0.162
III and IV	8	2 (25.00%)	6 (75.00%)	
T status				
T1	18	8 (44.44%)	10 (55.56%)	0.604
T2-4	22	8 (30.00%)	14 (70.00%)	
Lymph node metastasis				
Negative	30	13 (43.33)	17 (56.67)	0.317
Positive	10	3 (30.00%)	7 (70.00%)	
Tumor Type				
Adenocarcinoma	30	12 (40.00%)	18 (60.00%)	0.317
Squamous cell carcinoma	10	4 (40.00%)	6 (60.00%)	
Differentiation				
Well	12	6 (50.00%)	6 (50.00%)	0.162
Moderate and poor	28	10 (35.71%)	18 (64.29%)	
EGFR mutation				
Negative	27	10 (37.04%)	17 (62.96%)	0.581
Positive	13	6 (46.15%)	7 (53.85%)	

cancer sample, 26 of them (65.0%) had at least a 2.0-fold or greater expression of TUSC3 mRNA in the cancer tissues than in the paired normal tissues, and 34 of them (85.0%) had at least a 1.5-fold or greater expression of TUSC3 mRNA in the cancer tissues than in the paired normal tissue. Thus, TUSC3 mRNA levels were significantly increased in cancer tissues (**Figure 1A**). Furthermore, TUSC3 mRNA level correlated significantly with gender (female) and age (>60 yrs) ($P<0.05$) (**Table 2**). Increased mRNA level of TUSC3 was found in samples with EGFR positive mutation, although the relationship was not statistically significant in our data ($P=0.091$) (**Table 2**). No significant difference was observed between TUSC3 expression and tumor differentiation, tumor stage, lymph node metastasis or smoke history ($P>0.05$) (**Table 2**).

TUSC3 protein expression

TUSC3 protein level was measured by immunohistochemistry in cancer samples and paired normal tissues (**Figure 1B** and **1C**; **Table 3**). TUSC3 was expressed mainly in the nuclear or nuclear plus cytoplasm in tumor cells. The scoring was based on nuclear and cytoplasm staining. Immunostaining of TUSC3 was detected in epithelial cells in 45% (18/40) normal tissues and 80% (32/40) cancer tissues. Significant differences in positive staining rates were observed between tumor samples and normal tissue ($P=0.003$). TUSC3 protein levels were significantly increased in cancer tissues when compared with normal tissues (**Table 3**). However, no significant differences were observed between TUSC3 protein expression and gender, age, tumor stage, differentiation, lymph node metastasis, or EGFR mutation status (**Table 4**).

TUSC3 level in lung cancer cell lines

To test whether TUSC3 expression was also increased in NSCLC cell lines, we assayed its expression in several lung cancer cell lines by Western blot (**Figure 2**). Our result showed that TUSC3 level was elevated in NSCLC cell lines A549, H322, H460 and SPC-A1 compared with HBE, a human bronchial epithelial cell line as the normal control. No significant increase in TUSC3 expression was observed in GLC-82, a lung carcinoma cell line compared with HBE. This result supported that TUSC3 expression is elevated in NSCLC.

EGFR mutation status

A greater proportion of patients with tumors that were positive for EGFR mutation were female, non-smokers, with higher stage and lymph node metastasis when compared with patients with tumors that were negative with

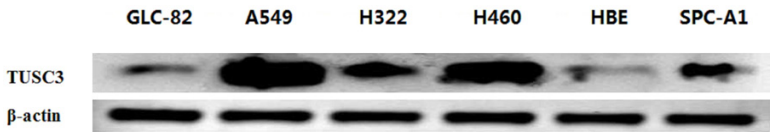

Figure 2. Increased expression of TUSC3 in several NSCLC cell lines. TUSC3 and control protein β-actin were measured by Western blot. HBE, a Human Bronchial Epithelial cell line was used as the normal control.

Table 5. The relationship between EGFR mutation and clinicopathological parameters in NSCLC

	All cases	EGFR mutation		*P* value
		Negative	Positive	
Gender				
Male	32	24 (75.00%)	8 (25.00%)	<0.001
Female	8	3 (37.50%)	5 (62.50%)	
Age (median)				
<60	25	17 (68.00%)	8 (32.00%)	0.453
≥60	15	10 (66.67%)	5 (33.33%)	
Smoking History				
Ever	20	17 (85.00%)	3 (15.00%)	0.018
Never	20	10 (50.00%)	10 (50.00%)	
Stage				
I and II	32	24 (75.00%)	8 (25.00%)	<0.001
III and IV	8	3 (37.50%)	5 (62.50%)	
T status				
T1	18	11 (61.11%)	7 (38.89%)	0.435
T2-4	22	16 (72.73%)	6 (27.27))	
Lymph node metastasis				
Negative	30	22 (73.33%)	8 (26.67%)	0.012
Positive	10	5 (50.00%)	5 (50.00%)	
Tumor Type				
Adenocarcinoma	30	19 (63.33%)	11 (36.67%)	0.134
Squamous cell carcinoma	10	8 (80.00%)	2 (20.00%)	
Differentiation				
Well	12	9 (75.00%)	3 (25.00%)	0.424
Moderate and poor	28	18 (64.29%)	10 (35.71%)	

EGFR mutation ($P<0.05$) (**Table 5**). Genetic mutations of EGFR in exon 19 were detected in 2 squamous cell carcinoma and 11 adenocarcinoma patients.

Discussion

TUSC3, originally called N33, locates on chromosome band 8p22. High frequency of homozygous deletion of this chromosomal region is observed in prostate cancer, ovarian cancer and pancreatic cancer [3, 4, 11, 12]. It is reported that TUSC3 gene shares a high sequence homology with yeast Ost3p, a subunit of the oligosaccharyltransferase complex, suggesting an important role of protein N-glycosylation in cancer pathogenesis [11]. Downregulation of TUSC3 gene has been found in prostate, ovarian and pancreatic cancer [4, 5, 11, 12]. DNA hypermethylation of TUSC3 promoter is the mechanism underlying low expression of TUSC3 in ovarian cancer [13]. Knockdown of TUSC3 leads to increased proliferation and invasion of prostate cancer cells and ovarian cancer cells in vitro and increased tumor formation in vivo [4, 11]. Therefore, most studies suggest that TUSC3 is a tumor suppressor gene.

In this study, we evaluated TUSC3 mRNA and protein level in NSCLC tissues and matched normal tissues using qPCR and immunohistochemisty respectively. Opposite to previous studies, we found a higher expression of TUSC3 at both mRNA and protein level in NSCLC tumors than in normal tissues. In addition, we observed increased TUSC3 expression in four NSCLC cell lines. These results suggest that upregulation of TUSC3 may be involved in the initiation and progression of NSCLC. Our result that TUSC3 plays an oncogenic role in NSCLC is opposite to others showing that it functions as a tumor suppressor in other cancer types. This discrepancy might be due to different cancer types in study (prostate, ovarian versus lung cancer). TUSC3 might play distinct roles in the pathogenesis of different cancers. TUSC3 might even play different roles in different stages of cancer development, for example, Pils showed that N33 expression is lower only in

ovarian tumors of advanced stage (13), indicating that it is involved in later events such as metastasis rather than early tumorigenesis. Harak suggested that "TUSC3 mediated N-glycosylation might exert several different functions in tumorigenesis depending on cancer type and genetic background" (4).

In our present study, we also observed that there is a correlation between overexpression of TUSC3 at the mRNA level with gender and age. It is reported that more than 50% of NSCLC patients are older than 65 years while 30% are at least 70 years old at diagnosis [14]. Even after pulmonary resections, the morbidity and mortality rates still increase with increasing age [15]. Thus, based on our results, both age and TUSC3 overexpression are risk factors for NSCLC. We also observed stronger TUSC3 staining in the invasive front of the cancer nest than the cells surrounding the lumen or nest in 3 of the 40 samples. These three samples had an increased frequency of metastasis to lymph node, an increased depth of infiltration and stronger staining of TUSC3 than other samples (**Figure 1C**). Whether increased TUSC3 is associated with tumor invasiveness, tumor infiltration potential or metastasis needs further study.

Recently, EGFR mutation was found in most of non-smoker Asian adenocarcinoma (AC) of lung cancer patients [16]. These patients respond remarkably to the EGFR tyrosine kinase inhibitors (TKIs). Conversely, in patients with lung squamous cell carcinoma (SCC), mutation in EGFR are rare. In our study, the results is in agreement with the previous reports [17, 18]. EGFR mutation occurred more frequently in female, non-smokers and AC patients. However, we identified 2 somatic mutations in EGFR in Chinese SCC patients, despite the reportedly rare frequency of such mutations in European and North American SCC patients. Our results suggested that EGFR inhibitors may be effective for a subset of SCC patients, at least in the Chinese population.

Taken together, we detected increased TUSC3 expression at both mRNA and protein level in tumors of 40 NSCLC patients compared to their matched normal tissue. Increased TUSC3 level was also observed in several NSCLC cancer cell lines. Our data suggest that overexpression of TUSC3 may be involved in the initiation and progression of NSCLC. Further study on the exact role of TUSC3 in NSCLC is needed to further understand the role of TUSC3 in NSCLC, and why TUSC3 seems to play distinct roles in different cancer types.

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Disclosure of conflict of interest

None.

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