RESEARCH ARTICLE

Expression of Tumor Necrosis Factor Receptor-associated Factor 6 in Lung Cancer Tissues

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Abstract

Background: Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) has been reported to be associated with the development of various cancers. However, the role of TRAF6 in lung cancer remains unclear. Objective: To explore the expression and clinicopathological significance of TRAF6 protein in lung cancer tissues. Materials and Methods: Three hundred and sixty-five lung cancer samples and thirty normal lung tissues were constructed into 3 microarrays. The expression of TRAF6 protein was determined using immunohistochemistry (IHC). Furthermore, correlations between the expression of TRAF6 and clinicopathological parameters were investigated. Results: The expression of TRAF6 in total lung cancer tissues (365 cases), as well as in small cell lung cancer (SCLC, 26 cases) and non-small cell lung cancer (NSCLC, 339 cases) was significantly higher compared with that in normal lung tissues. The ROC curve showed that the area under curve of TRAF6 was 0.663 (95%CI 0.570~0.756) for lung cancer. The diagnostic sensitivity and specificity of TRAF6 were 52.6% and 80%, respectively. In addition, the expression of TRAF6 was correlated with clinical TNM stage, tumor size and lymph node metastasis in all lung cancers. Consistent correlations were also observed for NSCLCs. Conclusions: TRAF6 might be an oncogene and the expression of TRAF6 protein is related to the progression of lung cancer. Thus, TRAF6 might become a target for diagnosis and gene therapy for lung cancer patients.

Keywords: TNF receptor-associated factor 6 - lung cancer - immunohistochemistry - TNM stage

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Introduction

Over the past two decades (from 1990 to 2010), there has been a steady decline in the lung cancer death rate as a result of advances in prevention, early detection and treatment. However, lung cancer burden was highest for men in more developed regions compared with that in lower developed regions (Kim et al., 2014). According to the statistics, 224, 210 new cases will be diagnosed as lung cancer and 159, 260 will die from it in the United States in 2014 (Siegel et al., 2014). Lung cancer is still ranked the first in all tumor mortality, which is the most common factor in cancer deaths in both males (aged 40 years and older) and females (aged 60 years and older) (Siegel et al., 2014). In China, lung cancer is the first leading cause of death from all cancers (Chen et al., 2014). Non-small cell lung cancer (NSCLC) comprises 75-85% of newly diagnosed lung cancers. Over 70% of NSCLC patients present with advanced disease, and the overall 5-year survival rate for NSCLC is only 16% (Chen et al., 2013c). Major efforts have been made to identify molecular markers that predict prognosis and response to additional therapy. However, the specific markers have not been achievable (Chen et al., 2012; Chen et al., 2013a; 2013b; 2013c; Gazala et al., 2013; Granger et al., 2013). Therefore, the research for effective molecular markers for diagnosis and treatment of lung cancer is an important issue at present.

Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) has a tumor necrosis factor receptor (TNFR)-associated factor (TRAF) domain in its carboxyl terminus and has a RING finger domain, a cluster of zinc fingers and a coiled-coil domain, which are also present in other TRAF family proteins (Ishida et al., 1996). TRAF6 was firstly identified by Ishida's (Ishida et al., 1996) and it binds to the amino-terminal region of the CD40 cytoplasmic tail, which is distinct from the binding domain for TRAF2, TRAF3, and TRAF5. Meanwhile, TRAF6 plays a critical role in immune (Kobayashi et al., 2003) and inflammation. Unlike other TRAFs, it is also involved in IL-1 signaling, leading to the activation of NF-kB (nuclear factor kappaB) (Ishida et al., 1996; Lee and Lee, 2002). TRAF6 and TRAF3 maintain the balance of apoptosis and anti-apoptosis in RAdncCD40L-infected

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carcinoma cells (Elmetwali et al., 2010; Jundi et al., 2012). The most abundant amounts of the TRAF1, TRAF2 and TRAF6 protein can be detected in thymus, testis and epidermis, while in other tissues the expression is low or even absent, which reveals a tissue-specific difference of TRAF protein expression in normal human tissues (Zapata et al., 2000). TRAF6 and TRAF2 exhibit high protein expression in 83 human cancer cell lines, such as leukemia/lymphoma, ovarian carcinoma, melanoma, lung cancer, colon carcinoma, prostate carcinoma, breast cancer and renal cell carcinoma cell lines (Zapata et al., 2000), which was associated with TNFa-induced cancer cell migration and invasion (Xiao et al., 2012; Chaudhry et al., 2013).

Recent studies have indicated that TRAF6 is involved in some cancers. However, the role and mechanism of TRAF6 in lung cancer have not been extensively investigated. The levels of TRAF6 expression, including its mRNA and protein, were validated in vitro of lung cancer (Starczynowski et al., 2011; Zhong et al., 2013). However, only mRNA of TRAF6 was detected in clinical patient samples (Starczynowski et al., 2011). Therefore, in the current study, we focused on the expression of TRAF6 protein and its association with clinicopathological parameters in lung cancer patients

Materials and Methods

Study design

This retrospective study included 365 cases of lung cancer and 30 cases of normal lung tissues. All tissues were made into 3 microarrays. All lung cancer cases were initial pneumonectomies without treatment and were randomly selected from operations performed in the First Affiliated Hospital of Guangxi Medical University, *P*. R. China between January 2010 and December 2012. The age of the lung cancer patients ranged from 11 to 84 years old, with a mean age of 57.67 years. The normal lung tissues were collected from autopsies without any lung diseases in the same Hospital between March 2009 and November 2012. The age of the normal lung cases ranged from 19 to 73 years old, with a mean age of 54.03 years. Histologic examination revealed the tumor specimens mainly consisted of adenocarcinomas (127 cases), squamous cell carcinomas (175 cases), adenosquamous carcinoma (28 cases), undifferentiated carcinomas (8 cases), large cell carcinoma (1 case) and small cell carcinoma (26 cases). The 127 adenocarcinomas were further split into acinar adenocarcinomas (83 cases), papillary adenocarcinomas (19 cases), broncholoalveolar cell carcinomas (18 cases) and mucinous carcinoma (7 cases). All clinicopathological information provided from medical records has been summarized in Table 1-4. The study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was obtained from the patients and clinicians for the usage of the samples for research. All samples were reviewed and diagnosed by two independent pathologists.

Evaluation of immunostaining

TRAF6 antibody was purchased from Santa Cruz Biotechnology, Inc., Heidelberg, Germany (sc-8409, 1: 300 dilution). All stained sections were evaluated and scored independently by two pathologists with no prior knowledge of the clinicopathological outcomes of the patients. The mean percentage of positive cells were scored as 0 (0 %), 1 (1-25 %), 2 (26-50 %), 3 (51-75 %), and 4 (76-100 %). The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), 3 (strong). Final pathological scores were obtained for each case by multiplying the percentage and the intensity score. Staining with the score over 2 was regarded as positive.

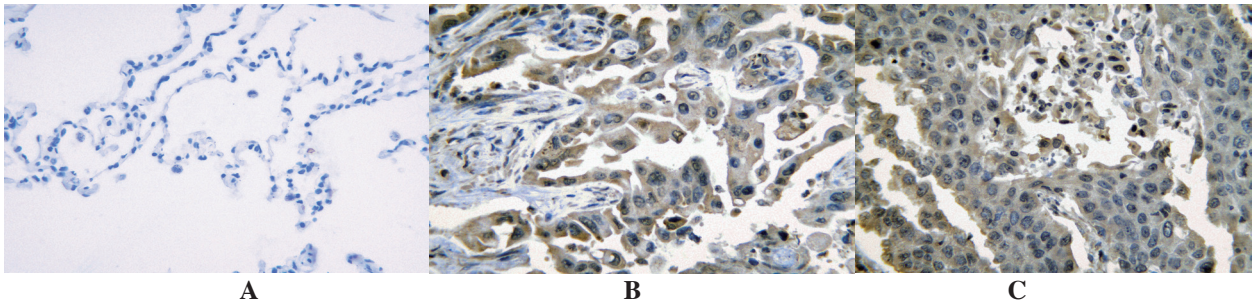
Statistical analysis

SPSS20.0 (Munich, Germany) was used for statistical

Table 1. Expression of TRAF6 Protein in Lung Cancer and Normal Lung Tissues

Tissue	n	TRAF6 negative (n, %)	TRAF6 positive (n, %)	P-value
Normal lung	30	24 (80)	6 (20)	
lung cancer	365	173 (47.4)	192 (52.6)	0.001
SCLC	26	9 (34.6)	17 (65.4)	0.001
NSCLC	339	164 (48.4)	175 (51.6)	0.001
AC	127	63 (49.6)	64 (50.4)	0.003
Acinar	83	43 (51.8)	40 (48.2)	0.007
Papillary	19	9 (47.4)	10 (52.6)	0.019
Broncholoalveolar cell	18	5 (27.8)	13 (72.2)	<0.001
Mucinous	7	6 (85.7)	1 (14.3)	0.732
SCC	175	86 (49.1)	89 (50.9)	0.002
ASC	28	12 (42.9)	16 (57.1)	0.004
UC	8	2 (25)	6 (75)	0.003
LCC	1	1 (100)	0	0.642

*P value was obtained as compared to normal lung; No difference was found between the expression of TRAF6 protein in SCLC and NSCLC (P=0.176); SCLC: small cell lung cancer, NSCLC: non-small cell lung cancer, AC: Adenocarcinoma; SCC: Squamous cell carcinoma, ASC: Adenosquamous carcinoma, UC: Undifferentiated carcinoma, LCC: Large cell carcinoma



A B C

Figure 1. Immunohistochemical Staining of Tumor Necrosis Factor (TNF) Eceptor-associated Factor 6 (TRAF6) in lung tissues. TRAF6 signaling was predominantly observed in the cytoplasm of lung cancer cells. Negative staining in normal lung tissue (**A**). Positive expression in lung adenocarcinoma (**B**) and squamous carcinoma (**C**), 400x.

analysis. Kruskal-Wallis H was used to assess the difference of TRAF6 status among lung cancer histological subtypes, pathological classifications and grading. Mann-Whitney U tests were selected for the comparison of TRAF6 expression for the parameters of tumor staging (TNM), age, gender, lymph node metastasis and distal metastasis. The associations between TRAF6 expression levels and the clinicopathological features were evaluated by using Spearman's correlation. Receiver operator characteristic curve (ROC) was employed to identify the diagnostic value of TRAF6 protein. Statistical significance was determined at a P <0.05 level.

Results

Differential expression of TRAF6 between lung cancer and normal lung tissues

Typical staining patterns of the TRAF6 expression in the cytoplasm were shown in Figure 1. Among the 365 lung cancer patients studied, 192 cases (52.6 %) were TRAF6 positive, while the positive TRAF6 expression ratio was 20% in normal lung tissues (6 in 30 cases), significantly lower than that in lung cancer tissues (Table 1). Higher level of the TRAF6 protein expression was also found in SCLC, as compared with the normal lung tissues (Table 1). Also, the difference could be noticed between NSCLC and normal lung tissues. Furthermore, ROC curve was performed to identify the diagnostic value of TRAF6 level in lung cancer. The area under

Table 2. Relationship between the Expression of TRAF6 Protein and Clinicopathological Parameters in Lung Cancer

Lung cancer		n	TRAF6 negative (n, %)	TRAF6 positive (n, %)	P-value
Gender	Male	299	152 (50.8)	147 (49.2)	0.5
	Female	96	45 (46.9)	51 (53.1)	
Age (years)	<60	218	109 (50)	109 (50)	0.956
	≥60	177	88 (49.7)	89 (50.3)	
TNM	I-II	299	157 (52.5)	142 (47.5)	<0.001
	III-IV	63	14 (22.2)	49 (77.8)	
Lymph node metastasis	Yes	128	41 (32)	87 (68)	<0.001
	No	234	130 (55.6)	104 (44.4)	
Tumor diameter (cm)	≤7	314	165 (52.5)	149 (47.5)	<0.001
	>7	48	6 (12.5)	42 (87.5)	
Distal metastasis	Absent	346	167 (48.3)	179 (51.7)	0.069
	Present	16	4 (25)	12 (75)	

Table 3. Relationship between the Expression of TRAF6 Protein and Clinicopathological Features in SCLC

SCLC		n	TRAF6 negative (n, %)	TRAF6 positive (n, %)	P-value
Gender	Male	21	9 (42.9)	12 (57.1)	0.076
	Female	5	0	5 (100)	
Age (years)	<60	15	5 (33.3)	10 (66.7)	0.875
	≥60	11	4 (36.4)	7 (63.6)	
TNM	I-II	13	5 (38.5)	8 (61.5)	0.351
	III-IV	10	2 (20)	8 (80)	
Lymph node metastasis	Yes	13	3 (23.1)	10 (76.9)	0.392
	No	10	4 (40)	6 (60)	
Tumor diameter (cm)	≤7	19	7 (36.8)	12 (63.2)	0.1551
	>7	4	0	4 (100)	

SCLC: small cell lung cancer

Table 4. Relationship between the Expression of TRAF6 Protein and Clinicopathological Features in NSCLC

NSCLC		n	TRAF6 negative (n, %)	TRAF6 positive (n, %)	P-value
Gender	Male	254	124 (48.8)	130 (51.2)	0.779
	Female	85	40 (47.1)	45 (52.9)	
Age (years)	<60	181	88 (48.6)	93 (51.4)	0.924
	≥60	158	76 (48.1)	82 (51.9)	
TNM	I-II	286	152 (53.1)	134 (46.9)	<0.001
	III-IV	53	12 (22.6)	41 (77.4)	
Lymph node metas	Yes	115	38 (33)	77 (67)	<0.001
	No	224	126 (56.2)	98 (43.8)	
Tumor diameter (cm)	≤7	295	158 (53.6)	137 (46.4)	<0.001
	>7	44	6 (13.6)	38 (86.4)	
Distal metastasis	Absent	16	4 (25)	12 (75)	0.056
	Present	323	160 (49.5)	163 (50.5)	
Pathological grading	I	39	23 (59)	16 (41)	0.254 a*
	II	92	49 (53.3)	43 (46.7)	
	III	130	59 (45.4)	71 (54.6)	
Histology patterns	Adenocarcinoma	127	63 (49.6)	64 (50.4)	0.535 a
	Squamous cell carcinoma	175	86 (49.1)	89 (50.9)	
	Adenosquamous carcinoma	28	12 (42.9)	16 (57.1)	
	Undifferentiated carcinoma	8	2 (25)	6 (75)	
	Large cell carcinoma	1	1 (100)	0	
Adenocarcinoma classification	acinar adenocarcinoma	83	43 (51.8)	40 (48.2)	0.065 a
	Papillary adenocarcinoma	19	9 (47.4)	10 (52.6)	
	Broncholoalveolar cell carcinoma	18	5 (27.8)	13 (72.2)	
	Mucinous carcinoma	7	6 (85.7)	1 (14.3)	

a Kruskal-Wallis H test; No significant difference was found between each histology pattern or adenocarcinoma classification; *Pathological grading I *vs* II : p=0.549, I *vs* III : p=0.138, II *vs* III: p=0.248; NSCLC: non-small cell lung cancer

curve (AUC) of TRAF6 was 0.663 (95%CI 0.570~0.756, *P*=0.003). The sensitivity and specificity were 52.6% and 80%, respectively.

Concerning different histologic types of NSCLC, most of the histologic types of NSCLC including adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma and undifferentiated carcinoma showed significantly higher expression of TRAF6 than normal lung tissues. When adenocarcinoma was further divided into different patterns, remarkably higher expression of TRAF6 was found in acinar adenocarcinoma, papillary adenocarcinoma and broncholoalveolar cell carcinoma as compared to normal lung tissues (Table1).

Relationship of TRAF6 protein and other clinicopathological features in lung cancer

Correlations of TRAF6 protein and other clinicopathological features including TNM stage, lymph node metastasis and tumor size were found in lung cancer. The rate of the TRAF6 protein expression was remarkably higher in advanced stages (III and IV) than that in early stages (I and II). Higher levels of TRAF6 were found in lung cancer patients with lymph node metastasis and larger tumor size, compared with patients of corresponding traits in Table 2. Further analysis by spearman correlation indicated that the protein level of TRAF6 expression was significantly associated with TNM stages (r=0.230, *P*<0.001), tumor size (r=0.272, *P*<0.001) and lymph node metastasis (r=0.225, *P*<0.001). And we detected a marginal correlation between the expression of TRAF6 protein and distal metastasis (r=0.096, *P*=0.069). However, there was no correlation between the TRAF6 protein expression and other clinicopathological features, for instance, gender, age, histological differentiation grades, and so on (data not shown).

With regard to SCLC, there was no statistically difference between TRAF6 protein and all clinical pathological parameters in SCLC (Table3). No significant correlation was observed between TRAF6 expression and the parameters with spearman correlation analysis.

The difference between TRAF6 expression and the clinicopathological parameters of NSCLC were shown in Table 4. We discovered that the level of TRAF6 protein was higher in advanced stages, larger tumor, with lymph node metastasis than that in early stages, smaller tumor, without lymph node metastasis, respectively. Spearman test showed the consistent correlation between expression of TRAF6 and the following clinicopathological parameters: clinical TNM stages (r=0.222, *P*<0.001), lymph node metastasis (r=0.220, *P*<0.001) and tumor size (r=0.268, *P*<0.001) in 339 NSCLC patients. However, there was no correlation between the expression of TRAF6 protein and other clinicopathological features (data not shown).

In the subgroups of NSCLC, spearman correlation analysis also showed that TRAF6 protein was positively correlated with TNM stages (r=0.230, *P*<0.001), tumor size (r=0.329, *P*<0.001) and lymph node metastasis (r=0.307, *P*<0.001) in adenocarcinoma. Meanwhile in squamous carcinoma, we also found similar correlations between TRAF6 expression and TNM stages, tumor size and lymph node metastasis (data not shown).

Discussion

Lung cancer is one of the most lethal and aggressive neoplasms. With the development of genomic and basic research, increasing numbers of cell molecular biomarkers have been considered as specific diagnosing and targeted therapeutic agents for lung cancer (Uribe and Gonzalez, 2011; Hirose et al., 2012; Brothers et al., 2013; Ramshankar and Krishnamurthy, 2013; Wang et al., 2013). However, clear molecular biomarkers for the clinical diagnosis and treatment of lung cancer have not been identified. In addition, lung carcinogenesis is a multistep process and the mechanism of lung cancer is not fully understood. Therefore, we made great efforts to search for molecular biomarkers of lung cancer and investigate the potential role of the TRAF6 protein expression in the diagnosis and prediction of prognosis of lung cancer patients.

In the present study, we examined the expression of TRAF6 in lung cancer tissues by tissue microarray and immunohistochemistry. We found that TRAF6 expression in lung cancer was significantly higher than that in the normal lung tissues. Both of SCLC or NSCLC had the accordant upregulation of TRAF6 expression. We also assessed the diagnostic significance of TRAF6 protein by a ROC curve and found that TRAF6 protein has a moderate diagnostic value in lung cancer (AUC=0.663). In line with our results, Starczynowsk's (Starczynowski et al., 2011) reported that TRAF6 mRNA and protein expression were both highly expressed in 85 lung cancer cell lines. Additionally, TRAF6 protein was positively correlated with enhanced colony formation in NIH3T3 Cells. Starczynowski's (Starczynowski et al., 2011) also observed that TRAF6 overexpression resulted in malignant transformation of fibroblasts and tumor formation, whereas knockdown of TRAF6 suppressed human lung cancer growth and RAS-mediated tumor formation. Together with the report of Starczynowski's (Starczynowski et al., 2011), our current results support that TRAF6 might be an oncogene for lung cancer, playing a similar role as in other malignancies, such as breast cancer, leukemia and esophageal adenocarcinoma (Beroukhim et al., 2010), colon cancer (Sun et al., 2014) and osteosarcoma (Meng et al., 2012).

Then we went further to investigate the relationship between the expression of TRAF6 protein and diverse clinicopathological parameters in the current study relatively large series of lung cancer patients (n=365). TRAF6 expression was correlated with clinical TNM stages of lung cancer. In advanced stages (III and IV), the expression of TRAF6 was remarkably higher than that in early stages (I and II), which implied that TRAF6 could regulate tumor deterioration and progression. In the subgroup of NSCLC, the same correlation between TRAF6 expression and clinical TNM stages was observed. On the contrary to our findings, Liu's (Liu et al., 2012) found no correlation between TRAF6 expression and clinical stage. The cause of the contradiction may be due to the different cases included. Only patients at stage III

and IV were involved in the study of Liu's (Liu et al., 2012), while all of the stage I, II, III and IV were applied in the current study. Furthermore, the significantly higher TRAF6 expression was found in the groups of larger tumor size and groups with lymph node metastasis both in the whole lung cancer samples and in the NSCLC subgroup in the current study, which was in consistent with Liu's (Liu et al., 2012). Moreover, correlation analysis also showed the positive relationships between the TRAF6 protein level and tumor size, lymph node metastasis. Since the clinicopathological parameters of clinical TNM stage, tumor size and lymph node metastasis represent partially the deterioration and progress of lung cancer, TRAF6 might be a factor related to lung cancer development. Zhong et al reported that TRAF6 might be involved in the potentiation of growth, proliferation, and invasion of a human lung adenocarcinoma cell line A549 (Zhong et al., 2013). Together with the aforementioned reports, our current results indicate that TRAF6 might be valuable as a biomarker to predict the prognosis of lung cancer, especially NSCLC patients with greater certainty.

However, we have found no association between the expression of TRAF6 and any of the clinicopathological parameters in SCLC. Further larger scale studies are needed to investigate the role of TRAF6 in the progression of SCLC.

Our group previously found that TRAF6 functions as a bridge between miR-146a and the NF-kB pathway in NSCLC (Chen et al., 2013c). Besides miR-146a, TRAF6 has been proved to be the target genes for miR-125a (Guo et al., 2014) and miR-125b (Wang et al., 2014). However, the molecular mechanism of TRAF6 in lung cancer remains to be investigated. Further in vitro and in vivo experiments will be performed in the future.

In conclusion, upregulated expression levels of TRAF6 detected by immunohistochemistry were found in lung cancer, including SCLC and NSCLC. The expression of TRAF6 protein was positively related with clinical TNM stages, tumor size and lymph node metastasis. Together with previous reports, the current observations strongly suggest that TRAF6 might play a vital role in the tumorigenesis and deterioration of lung cancer. Further investigations are expected to evaluate the potential role and molecular mechanism of TRAF6 in lung cancer.

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