Adverse prognostic impact of TGFB1 T869C polymorphism in non-small-cell lung cancer

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Abstract: Previously, several polymorphisms in TGFB1 have been identified in non-small-cell lung cancer (NSCLC), and the variants, C-509T, T869C, and G915C, have been demonstrated to associate with higher circulating levels of TGF-β1. However, little is known about the prognostic value of TGFB1 polymorphisms in cancers. In this study, by genotyping the TGFB1 T869C polymorphism in a total of 261 patients with NSCLC using DNA from blood lymphocytes, we first found that NSCLC patients, especially those with allele C carriers, had significantly higher serum TGF-β1 levels than healthy individuals. By using chi-square (χ²) test and Fisher’s exact test, we noticed that TC/CC genotypes were positively correlated with smoking, clinical TNM stage, lymph node, and distant metastasis in NSCLC patients. Kaplan–Meier analysis showed that patients with TT genotype had a better overall survival than the allele C carriers (TC + CC). Finally, multivariate analysis confirmed histology, lymph node, and distant metastasis but not serum TGF-β1 T869C polymorphism as independent prognostic factors for NSCLC. Taken together, our data, as a proof of principle, suggest that T869C polymorphism in TGFB1 may act as a genetic modifier in NSCLC progression and a promising prognostic marker of survival in NSCLC patients.

Keywords: genotype and multivariate analysis, single-nucleotide polymorphisms, prognosis

Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide. Nearly 75%–85% of patients with primary lung cancer are pathologically diagnosed with non-small-cell lung cancer (NSCLC). Despite intensive progress in preclinical and clinical studies, the prognosis of NSCLC is still poor, with a 5-year survival rate of only 15%. Currently, accumulating studies are underway to reveal the specific prognostic biomarkers that can predict the prognosis in patients with NSCLC. This may largely help to guide medical care of NSCLC patients, with either conventional chemotherapy or targeted therapies.

Genetic factors are critically involved in modulating cellular microenvironment and affecting carcinogenesis in a complicated manner. Single-nucleotide polymorphisms (SNPs) are the most common form of human genetic variation. Emerging evidence has demonstrated that SNPs affect gene expression and further increase the potential risk of cancer. Meanwhile, the prognostic value of SNPs has been revealed in multiple human cancers, including NSCLC.

TGFB1 T869C is a multifunctional cytokine that regulates many aspects of cellular function, including cellular proliferation, differentiation, migration, apoptosis, and immune surveillance. In cancers, it has been reported that TGFB1 acts as a tumor suppressor at early stages by inhibiting cell proliferation and as a tumor promoter at enhanced stages by facilitating tumor progression and metastasis. In lung cancer, several polymorphisms in the TGFB1 gene have been reported previously, including rs1800469,
A total of 261 NSCLC patients were recruited from The First Affiliated Hospital of Harbin Medical University between January 2006 and December 2012. All patients were diagnosed and histopathologically confirmed with NSCLC and without prior history of other cancers. None of the patients included in this study received radiotherapy, chemotherapy, hormone therapy, or other related antitumor therapies prior to surgery. Blood samples were collected from these patients for genotyping and detection of serum TGF-β1 levels. Follow-ups were done every 3 months from the enrolled time until death or the last time of follow-up. The maximum follow-up time was 72 months, and the median follow-up time was 22.7 months. As a result, 213 patients with complete follow-ups were subjected to survival analysis. For certification of smoking, nonsmokers were defined as those who smoked <1 cigarette per day for <1 year; otherwise, they were considered as smokers. The control subjects were matched to the cancer cases on the basis of gender and age (±5 years). All the cases and the controls were ethnic Chinese, and they resided in Harbin City or in the surrounding regions. This study was approved by the ethics committees of The First Affiliated Hospital of Harbin Medical University, and written informed consent was obtained from each individual for this study.

**TGFB1 T869C genotyping**

Genomic DNA was extracted from blood samples using commercially available QIAamp DNA purification kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The SNP T869C of the TGFB1 gene was determined by polymerase chain reaction-restriction-fragment length polymorphism (PCR-RFLP) using primers 5'-TATGAGGATGTTGGCGTGT-3' (forward) and 5'-GGGGTGTTTTACGTTGATG-3' (reverse). The PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems, San Francisco, CA, USA) thermal cycler. Each PCR amplification reaction was conducted in a total volume of 25 μL. The reaction mixture included 50 ng genomic DNA, 2.5 μM MgCl₂, 200 μM dNTPs, 1 unit of Taq polymerase (Qiagen), and 200 μM primers. The PCR amplification consisted of a preliminary denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 61°C for 1 min. Genotyping results and statistical analyses were determined independently by two authors in a blinded manner.

**Materials and methods**

**Study population**

Enzyme-linked immunosorbent assay (ELISA) analysis of serum TGF-β1 levels

After centrifuging blood samples, serum samples were obtained and stored frozen at −70°C until used. Quantitative determination of the serum levels of TGF-β1 was measured by a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA). Briefly, each serum sample or recombinant human TGF-β1 was diluted and added to the microtiter plates precoated with TGF-β1-soluble receptor type II and allowed to incubate overnight at 4°C. After the incubation, the plate was washed three times with 1× Phosphate Buffered Saline add Tween-20 (PBS-T) and incubated with biotinylated anti-human TGF-β1 for 2 hours at room temperature. Subsequently, horseradish peroxidase (HRP)-conjugated streptavidin was added to the plate and allowed to incubate for 30 minutes at room temperature. Color development was performed using a tetramethyl benzidine–H₂O₂ mixture and was terminated by 0.5 mol/L sulfuric acid. Finally, the absorbance of each well at 490 nm was determined using a spectrophotometer.

**Statistical analysis**

All statistical analyses were performed using SPSS19.0 software (SPSS Company, Chicago, IL, USA). Hardy–Weinberg equilibrium (HWE) of the T869C polymorphism was tested by standard χ² test. Survival time was calculated from the date of diagnosis to the date of death or to the last time of follow-up. Correlation of TGFB1 SNP with clinicopathological parameters in patients with NSCLC was evaluated by chi-square (χ²) test or Fisher’s exact test. The different survival times based on TGFB1 SNP were estimated using Kaplan–Meier method and compared by the log-rank test. Univariate or multivariate Cox regression analysis was done to determine the prognostic factors of NSCLC prognosis by estimating the crude hazard ratios (HRs). P-values <0.05 were considered statistically significant.

**Results**

TGFBI T869C polymorphism contributes to elevated serum TGF-β1 levels in NSCLC

The demographics of the cases and controls enrolled in the current study are shown in Table 1. No significant differences
were found between the cases and controls regarding the age or gender distribution and smoking history. A total of 261 cases with NSCLC were enrolled in our study. The general clinical characteristics of the enrolled patients are summarized in Table 2. The genotype distributions of TGFBI T869C polymorphism among the cases were in HWE. TGFBI T869C polymorphism results in a Leu10Pro substitution in the hydrophobic core of signal peptide (Figure 1A). Notably, this transition has been associated with higher circulating TGF-β1 levels, and this variant has been found to increase the risk of breast cancer. By ELISA analysis of the serum TGF-β1 levels in 261 NSCLC patients and 95 healthy controls, we noticed that the mean serum TGF-β1 value (17.1±4.9 ng/mL) was significantly higher than that of healthy controls (10.6±2.6 ng/mL; Figure 1B). Moreover, in both cases and controls, TGF-β1 level was certainly increased in individuals with smoking history (Figure 1C). Specially, patients with TC or CC genotype showed increased serum TGF-β1 levels compared with TT genotype carriers, indicating that T869C polymorphism also certainly contributes to increased serum TGF-β1 levels in NSCLC (Figure 1D).

**TGFBI T869C polymorphism predicts a poor prognosis in NSCLC patients**

To further determine the prognostic value of TGFBI T869C polymorphism in NSCLC, the χ² test was used to evaluate the correlation between T869C genotypes and corresponding patients’ clinicopathological features, including age, gender, smoking, TNM stage, histology, lymph node metastasis, and distant metastasis. As shown in Table 2, allele C was positively associated with smoking history (P=0.046), clinical stage (P=0.000), lymph node metastasis (P=0.000), and distant metastasis (P=0.000), whereas no significant correlations were found in age, gender, and histology.

Then, by Kaplan–Meier analysis and log-rank test, we observed a different survival rate between different genotypes of TGFBI T869C. The survival rate for NSCLC patients with TT or TC genotype was significantly lower than that for patients with CC genotype (Figure 2A and B, P=0.023). However, no remarkable difference in the survival rate was found between TT and TC genotype (Figure 2A).

Moreover, to identify the risk factors that correlated with the patients’ outcome, we carried out univariate and multivariate analyses. The univariate Cox regression analyses showed that the genotype of TGFBI T869C, histology, TNM stage, lymph node metastasis, and distant metastasis might be associated with the prognosis of NSCLC (Figure 3A). The multivariate Cox regression analysis revealed histology (risk ratio [RR]=1.008, 95% confidence interval [CI]: 1.002–1.014, P=0.009), lymph node metastasis (RR=2.558, 95% CI: 1.305–5.014, P=0.006), and distant metastasis (RR=1.817, 95% CI: 1.817–2.751, P=0.004) as independent prognostic factors of the overall survival in patients with NSCLC (Figure 3B).

**Discussion**

In the present study, we demonstrated the prognostic value of TGFBI T869C polymorphisms in patients with NSCLCs. The results suggest that testing for the existence of these three genotypes in TGFBI T869C may help to identify patient...
subgroups at high risk of poor prognosis, thereby helping to make therapeutic decisions in the management of NSCLC. To the best of our knowledge, this is the first study to determine the prognostic effect of TGFB1 polymorphism on the clinical outcomes of patients with NSCLC.

Previously, a large body of evidence has demonstrated that TGFB1 polymorphisms contribute to lung cancer susceptibility. Several studies have also been conducted to determine the function of TGFB1 polymorphisms in radiation pneumonia susceptibility in lung cancer patients. These findings suggest that several polymorphisms of TGFB1 gene could be used as genetic biomarkers for predicting the susceptibility of lung cancer. Notably, several studies have shown that C-509T, T869C, and G915C polymorphisms of TGFB1 gene are associated with increased serum TGF-β1 levels. Consistent with this notion, in this study, compared to healthy controls, patients with NSCLC had a higher serum TGF-β1 level. Moreover, patients with a TC or CC genotype in the T869C polymorphism of TGFB1 gene had a higher TGF-β1 level than those with a TT genotype.

Mutations in TGFB1 and its receptors, or associated downstream kinases of TGF-β1 signaling, play key roles in the development of cancer. Most NSCLC patients exhibit resistance to the tumor-suppressive role of TGF-β1
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Figure 3 Prognostic factors in Cox proportional hazards model.
Notes: Univariate (A) and multivariable (B) analyses were performed to identify prognostic factors for NSCLC. All the bars correspond to 95% CI. Bold numbers represent P-values with significant differences.
Abbreviations: NSCLC, non-small-cell lung cancer; CI, confidence interval; HR, hazard ratio.

at advanced stage as a result of several alterations, such as mutations in SMAD4 and aberrant serum TGF-β1 levels.\textsuperscript{12,23,24} TGF-β1 acts as a tumor promoter through interactions with tumor microenvironment. It is widely reported that TGF-β1 can suppress a repertoire of immune response, including cytotoxic T lymphocytes, macrophages, and natural killer cells.\textsuperscript{25} Therefore, we hypothesized that dysregulation of TGF-β1 signaling induced by T869C polymorphism of TGFB1 gene might contribute to a poor prognosis in patients with NSCLC. In this study, we noticed that the genotypic distribution of T869C polymorphism in TGFB1 gene showed significant correlation with smoking history, clinical stage, lymph node metastasis, and distant metastasis. In addition, allele C of TGFB1 (T869C) was associated with increased risk of tumor-related death, suggesting that SNP T869C of TGFB1 gene might be a potential prognostic marker for patients with NSCLC. However, the clear mechanisms underlying the unconfirmed roles of TGF-β1 in NSCLC warrant further investigation.

Besides, several limitations in the current study must be specified. First, the healthy controls enrolled in our study were selected on the basis of gender and age, which might contribute to a bias. Second, replication in a validation cohort was not carried out. Finally, other SNPs (C-509T and G915C) of TGFB1 gene that contribute to increased TGF-β1 levels should also be evaluated to test the common roles of those SNPs in NSCLC.

Conclusion
Our findings suggest that the T869C polymorphism of TGFB1 gene was correlated with clinical progression of NSCLC patients and might be a genetic predictor for NSCLC prognosis. Furthermore, validation in a larger cohort and related functional characterizations are needed to confirm these observations.
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Disclosure
The authors report no conflicts of interest in this work.

References