Molecules and Cells

TGFBI Promoter Methylation is Associated with Poor Prognosis in Lung Adenocarcinoma Patients

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Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide and has high rates of metastasis. Transforming growth factor beta-inducible protein (TGFBI) is an extracellular matrix component involved in tumour growth and metastasis. However, the exact role of TGFBI in NSCLC remains controversial. Gene silencing via DNA methylation of the promoter region is common in lung tumorigenesis and could thus be used for the development of molecular biomarkers. We analysed the methylation status of the *TGFBI* promoter in 138 NSCLC specimens via methylation-specific PCR and evaluated the correlation between *TGFBI* methylation and patient survival. *TGFBI* promoter methylation was detected in 25 (18.1%) of the tumours and was demonstrated to be associated with gene silencing. We observed no statistical correlation between *TGFBI* methylation and clinicopathological characteristics. Univariate and multivariate analyses showed that *TGFBI* methylation is significantly associated with poor survival outcomes in adenocarcinoma cases (adjusted hazard ratio = 2.88, 95% confidence interval = 1.19-6.99, $P = 0.019$), but not in squamous cell cases. Our findings suggest that methylation in the *TGFBI* promoter may be associated with pathogenesis of NSCLC and can be used as a predictive marker for lung adenocarcinoma prognosis. Further large-scale studies are needed to confirm these findings.

Keywords: Hypermethylation, MSP, NSCLC, Prognosis, *TGFBI*

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality worldwide, and non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases (Siegel et al., 2014). Despite advances in early detection and standard treatment, more than 60% of NSCLC patients are diagnosed with distant metastasis, and NSCLC prognosis remains poor across all disease stages (Spiro & Silvestri, 2005). The molecular mechanisms underlying NSCLC are complex and heterogeneous, thereby presenting multiple and distinct clinical and histological features (Yano et al., 2011). A global variation also occurs in the epidemiology, biology, and treatment of NSCLC (Zhou et al., 2011). Ultimately, a better understanding of the molecular changes in carcinogenesis and metastasis in NSCLC could provide new insights for the identification of promising biomarkers for disease diagnosis, prognosis, and treatment (Sato et al., 2007). Alteration of DNA methylation patterns is recognized as a crucial factor in initiation and progression of NSCLC (Lu & Zhang, 2011). In a recent study, we demonstrated aberrant methylation of metastasis-associated genes in Korean NSCLC patients (Choi et al., 2008; Kim et al., 2007; 2017).

Transforming growth factor beta-induced protein (TGFBI, also known as keratoepithelin or βig-H3) is a secreted extracellular matrix (ECM) component that plays a crucial role in

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tumour growth and metastasis (Thapa et al., 2007). Interestingly, TGFBI can have dual function in cancer by acting as either a tumour suppressor or promoter (Ween et al., 2012). Loss of *TGFBI* expression caused by promoter hypermethylation has been reported in various cancers (Fang et al., 2014; Kang et al., 2007; Shah et al., 2008; Shao et al., 2006), whereas strong *TGFBI* expression was associated with cancer progression and metastasis in gastrointestinal tumours (Yokobori & Nishiyama, 2017). Unfortunately, the exact role of TGFBI in NSCLC remains controversial (Kim et al., 2003; Sasaki et al., 2002; Shin et al., 2012; Wen et al., 2011; Zhao et al., 2006). Therefore, to further elucidate the function and clinical significance of TGFBI in NSCLC, we investigated the methylation status of the *TGFBI* gene promoter region in 138 tumour tissues from NSCLC patients using methylation-specific polymerase chain reaction (MSP) and evaluated the correlation between *TGFBI* methylation pattern and patient survival.

MATERIALS AND METHODS

Patients and tissue samples

Tumour tissues and matched non-malignant lung tissue specimens (n = 138) were provided by the National Biobank of Korea - Kyungpook National University Hospital (KNUH), which is supported by the Ministry of Health, Welfare, and Family Affairs. This study was conducted with the approval of the Ethics Committee of KNUH (No. 2014-04-210, 08/08/2014) and written informed consent was obtained from all of the participants prior to obtaining the samples. A total of 88 patients were adenocarcinoma (ADC) and 50 patients were squamous cell carcinoma (SQC). The clinicopathological characteristics of the patients are summarized in Table 1.

Table 1. Correlation between *TGFBI* methylation and clinicopathological features of NSCLC patients

Variables	Methylation, n (%)	*P*
All subjects (n = 138)	25 (18.1)	
Age (years)		
≤ 65 (n = 70)	14 (20.0)	0.56
> 65 (n = 68)	11 (16.2)	
Gender		
Men (n= 94)	19 (20.2)	0.35
Women (n = 44)	6 (13.6)	
Smoking status		
Ever (n = 98)	19 (19.4)	0.54
Never (n = 40)	6 (15.0)	
Histologic types		
SQC (n = 50)	7 (14.0)	0.34
ADC (n = 88)	18 (20.5)	
TP53 mutations		
Negative (n = 80)	15 (18.8)	0.82
Positive (n = 58)	10 (17.2)	
Tumour status		
T1 (n=25)	4 (16.0)	0.34
T2 (n=87)	14 (16.1)	
T3 (n=22)	5 (22.7)	
T4 (n=4)	2 (50.0)	
Lymph node metastasis		
N0 (n=103)	15 (14.6)	0.06
N1 and N2 (n=35)	10 (28.6)	
Pathological stage		
Stage I (n = 92)	16 (17.4)	0.75
Stage II-IIIA (n = 46)	9 (19.6)	
Death		
Alive (n = 100)	15 (15.0)	0.12
Death (n = 38)	10 (26.3)	

SQC, Squamous cell carcinoma: ADC, adenocarcinoma

Cell culture, total RNA isolation and RT-PCR

Three human lung cancer cell lines (A549, H522, and H810) were obtained from the American Type Culture Collection (Manassas, USA). All cells were cultured following the instructions provided by the supplier. H522 cells were treated with the demethylating agent 5-aza-2′-deoxycytidine (5-AzadC) for 3 days. Culture media were replaced daily. Total RNA was extracted from cultured cells and primary tumour tissues using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA quality was confirmed via electrophoresis on a 1.2% agarose-formaldehyde gel. After removing residual DNA, first-strand cDNA synthesis was performed using SuperScript preamplification kit (Invitrogen). The resulting cDNA was amplified using the sense primer 5′-CGTGGTCCATGTCATCACCAAT-3′ and antisense primer 5′-GCATTCCTCCTGTAGTGCTT-3′ as previously described (Sasaki et al., 2002). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene for normalization of the *TGFBI* expression levels.

Genomic DNA isolation and methylation analysis

Genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, USA). Genomic DNA was treated with sodium bisulfite, and the methylation status of the promoter region of the *TGFBI* gene was analysed using MSP with primers specific to either unmethylated or methylated alleles. Primers used for the methylated reaction were 5′-GCCGCCCGCTTG CCCGTCGGTC-3′ (sense) and 5′-AAACCCGCCAAAATCGC GACG-3′ (antisense) primers. Primers used for the unmethylated reaction were 5′-GCTGCCTGCTTGCCTGTTGGTT-3′ (sense) and 5′-AAACCCACCAAAATCACAACA-3′ (antisense). All polymerase chain reaction (PCR) were carried out using reagents supplied in the GeneAmp DNA Amplification Kit using AmpliTaq Gold as the polymerase (PE Applied Biosystems, USA) on a PTC-100 instrument (MJ Research, Watertown, MA, USA). CpGenome™ Universal methylated and unmethylated DNA (Chemicon, USA) was used as the positive controls for methylated and unmethylated genes, respectively. Negative control samples without added DNA were included for each PCR run. PCR products were analysed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. Each MSP run was repeated at least once to confirm the results.

Statistical analysis

The correlation between the methylation status and the clinicopathological characteristics was analysed using a chi-square test for categorical variables. $P < 0.05$ was considered statistically significant. A logistic regression test was conducted to estimate the relationship between methylation and the covariates, namely, age, gender, smoking status, histology, and pathological stage. The overall survival (OS) of NSCLC patients according to methylation status of the *TGFBI* gene were compared using the Kaplan-Meier method and the log-rank test. Hazard ratio (HR) and 95% confidence intervals (CIs) were estimated using Cox proportional hazard model. All analyses were performed using the Statistical Analysis System for Windows, version 9.4 (SAS Institute, USA).

RESULTS AND DISCUSSION

Methylation status and expression of TGFBI gene in NSCLC samples

We analysed the methylation status of the human *TGFBI* gene in 138 primary NSCLCs and corresponding non-malignant lung tissues using MSP. Considering that promoter hypermethylation is usually associated with the functional silencing of tumour associated genes in cancer cells, we designed the MSP primers to span the 5′-untranslated region of the first exon (-54 to +75 relative to the translation start site). The target-specific primer set yielded a 128-bp single band of the expected size. Representative results of the MSP analyses are shown in Fig. 1A. Unmethylated bands were detected in all non-malignant and malignant tissues (200N, 200T, 205N, 205T, 246N, 246T, 256N, and 256T), thereby confirming the integrity of the DNA. Sequencing of the representative PCR products confirmed their methylation status and verified that all cytosines at non-CpG sites were converted to thymine (data not shown), thereby eliminating the possibility of incomplete bisulfite conversion. *TGFBI* promoter methylation was detected in 25 (18.1%) tumour tissues (205T and 246T) and eight (5.8%) matched non-tumour samples (205N) ($P = 0.003$), consistent with a previous observation that 16% (8/50) of lung cancer samples were methylated at the P5/P6 loci of *TGFBI* (Shah et al., 2008). Methylation of non-malignant lung tissues mostly concurred in the matching cancer tissues with methylated bands (data not shown), which could be caused by either premalignant changes in peritumoral normal tissue or contamination of non-malignant tissues with methylated cancer cells. Thus, the above results suggest that hypermethylation of the *TGFBI* promoter may be associated with pathogenesis of NSCLC.

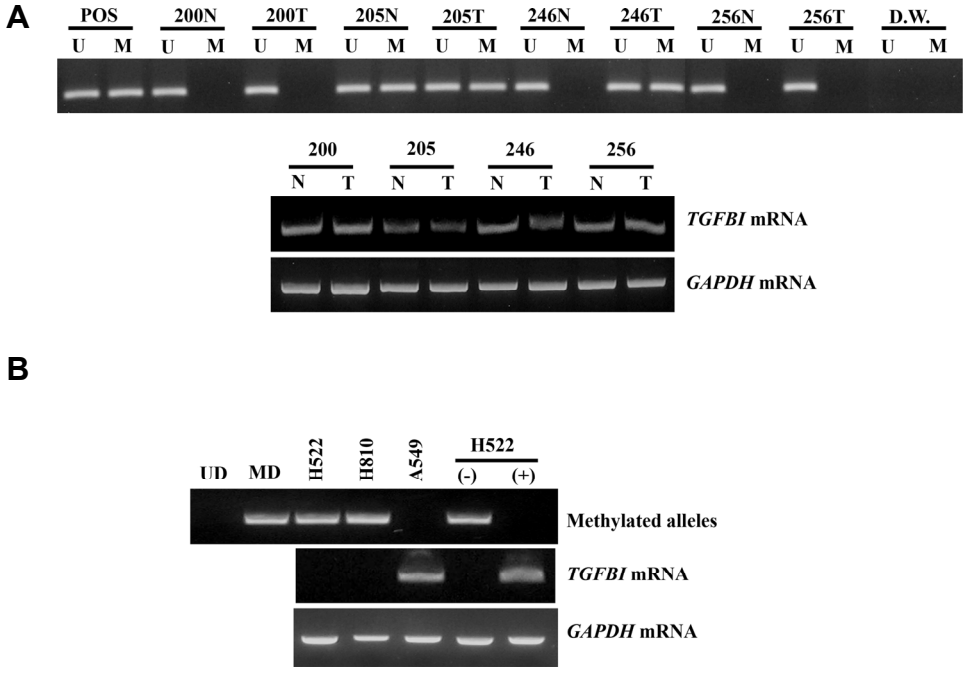

Fig. 1. Representative results of MSP and RT-PCR analyses of NSCLC tissue samples (A) and cell lines (B). Methylation status and expression of the *TGFBI* gene were analysed via MSP and RT-PCR analyses, respectively. (A) Tumour tissues and matched non-malignant lung tissue specimens showed methylated (205N, 205T, and 246T) or unmethylated alleles (200N, 200T, 205N, 205T, 246N, 246T, 256N, and 256T). CpGenome™ Universal methylated and unmethylated DNA was used as positive control for the methylated and unmethylated products, respectively. Distilled water was used as negative control. N, non-malignant tissue; T, tumor tissues; U and M, amplified products using primers targeting unmethylated or methylated sequences. *TGFBI* mRNAs were markedly decreased in 205N, 205T, and 246T tissues with methylated promoter. GAPDH was used as an internal loading control. (B) (-) indicated vehicle alone treatment and (+) indicated 20 μM 5-aza-2′-deoxycytidine (5-AzadC) treatment for 3 days.

We investigated whether methylation of the *TGFBI* promoter is correlated with gene silencing in both clinical tumour samples and tumour cell lines. Compared to unmethylated tissues, *TGFBI* expression levels were markedly downregulated in tumour and normal lung tissues (205N, 205T, and 246T) that are methylated in the *TGFBI* promoter (Fig. 1A). Similar results were confirmed by 5-AzadC experiments, exhibiting that methylated alleles disappeared in H522 cells and *TGFBI* mRNA was induced after treatment with the demethylating agent 5-AzadC for 3 days (Fig. 1B). Our results agree with those of recent studies demonstrating the correlation between promoter hypermethylation and significant downregulation of *TGFBI* expression in human cancer cells (Fang et al., 2014). Therefore, the above results indicated that CGI methylation is a key mechanism responsible for *TGFBI* downregulation.

Association of TGFBI methylation with clinicopathological parameters and clinical outcomes

Our results showed that *TGFBI* methylation was more frequent in patients with lymph node metastasis than in those without metastasis with a borderline significance (28.6% vs 14.6%, $P = 0.06$) (Table 1). However, no significant association was found in other clinicopathological factors, such as age, sex, smoking status, histology, *TP53* mutation, tumour status, pathological stage, and death (Table 1). Especially, TGFBI methylation was significantly correlated to death in ADC patients (Supplementary Table S1).

The median follow-up duration of the 138 NSCLC patients was 30.3 months (range, 3.2 to 97.8 months). Thirty eight patients (27.5%) died from cancer progression during the follow-up period and among them, twenty seven patients were ADC. Kaplan-Meier analysis showed that TGFBI methylation was significantly associated with worse OS in ADC patients but not in total patients (log-rank P [$P_{L\text{-}R}$] = 0.024 and, 0.080 respectively) (Fig. 2). Moreover, the above results were confirmed by Cox proportional hazard regression model (HR = 2.88, 95% CI = 1.19-6.99, $P = 0.019$) (Table 2). Therefore, *TGFBI* methylation in tumour samples could serve as an independent prognostic predictor of ADC patients.

Accumulating evidence has indicated that TGF-β signalling plays a dual role as a tumour suppressor in premalignant states and as a tumour promoter in advanced cancers (Massague, 2012). In addition, TGFBI, a downstream component of the TGF-β signalling pathway, has been shown to promote and/or inhibit cancer (Ween et al., 2012; Yokobori & Nishiyama, 2017). Moreover, the exact role of TGFBI and its expression patterns in NSCLC remain to be determined (Kim et al., 2003; Sasaki et al., 2002; Shin et al., 2012; Wen et al., 2011; Zhao et al., 2006). *TGFBI* is a secreted ECM protein

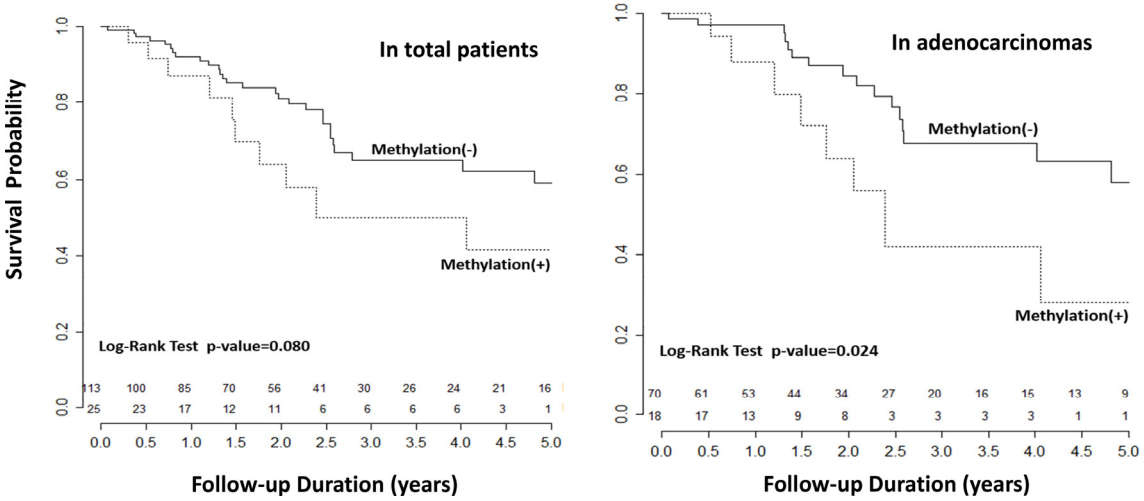

Fig. 2. Association of *TGFBI* methylation with poor prognosis of NSCLC patients. Kaplan-Meier survival curve of total and adenocarcinoma patients according to *TGFBI* methylation status. *P*-values from log-rank test.

Table 2. Association between *TGFBI* methylation and overall survival in NSCLC patients

Variables	$P_{L\text{-}R}$	Crude HR (95% CI)	*P*	Adjusted HR (95% CI)	*P*
Overall subjects	0.080	1.89 (0.92-3.90)	0.085	1.86 (0.89-3.86) [a]	0.097
ADC patients	0.024	2.60 (1.10-6.12)	0.029	2.88 (1.19-6.99) [b]	0.019

$P_{L\text{-}R}$, P-values from log-rank test.
Hazard rates (HR), Confidence interval (CI).
[a]Adjusted variables include age, smoking status, histology, *TP53*, and disease stage.
[b]Adjusted variables include age, smoking status, *TP53*, and disease stage.

that can function not only as a barrier for preventing the spread of tumour cells, but also as a reservoir for cell-binding proteins and growth factors that influence tumour cell behaviour (Thapa et al., 2007). Results of the current study showed that *TGFBI* methylation tended to occur in patient with lymph node metastasis and was significantly associated with poor survival outcomes in ADC patients, thereby suggesting that TGFBI acts as a tumour suppressor gene rather than an oncogene in NSCLC by regulating tumour metastasis. Recently, Wen et al. have demonstrated that TGFBI could function as an anti-metastatic protein in lung tumour cells. In addition, ectopic expression of TGFBI was found to stimulate cell adhesion and cytoskeleton formation by activating adhesion-associated signalling, inhibiting anchorage-independent growth activation, and inhibiting the activities of matrix metalloproteinases. Similarly, TGFBI-mediated apoptosis induction was found to be associated with improved response to chemotherapy in NSCLC (Irigoyen et al., 2010; Pajares et al., 2014). Taken together, our findings highlighted the anti-tumour role of the *TGFBI* gene in NSCLC and provided new diagnostic and therapeutic modalities for the clinical management of lung cancer.

Limitations of the present study include its retrospective design and relatively small sample size. Thus, the results were potentially influenced by selection bias. A major finding of this study is that epigenetic inactivation of TGFBI could serve as a useful predictor of a poor prognosis in ADC patients. However, further large-scale studies and longer follow-up periods are needed to verify the clinical significance of TGFBI.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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