

Defective Expression and DNA Variants of TGFB_{R2} in Chinese Small Cell Lung Carcinoma

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1. Introduction

Lung cancer is one of the most commonly diagnosed malignant tumors, and has the highest death rate of all cancer types. Both the incidence rate and death rate of lung cancer have increased rapidly worldwide during the last 50 years. Lung cancer has now become the leading cause of cancer death in males, and the second most common cause of cancer death in females, after breast cancer. According to data provided by the International Agency for Research on Cancer, about 1.6 million new lung cancer patients were confirmed in 2008, accounting for 13% of the total cancer cases, while about 1.4 million patients died, amounting to 18% of the total deaths caused by cancer worldwide (Jemal et al., 2011).

Lung cancer can be divided according to histological subtype into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with the latter accounting for about 14% of new lung cancer cases in the USA and Europe in 2004 (Jemal et al., 2004). The clinical and histological features of SCLC were first recognized by Barnard in 1926 as being distinct from those of other types of lung cancer (Barnard, 1926). SCLC cells develop from lung Kulchitsky cells, and SCLC can be further subdivided into three different types: oat-cell type, intermediate-cell type and mixed-cell type (Travis, 1999). Smoking is the key risk factor for SCLC, and more than 95% of patients develop SCLC as a result of tobacco smoking. Smoking more cigarettes and prolonging the duration of smoking can both increase the risk of developing SCLC (Brownson et al., 1992), while stopping smoking reduces its risk, compared to persistent smokers (Khuder and Mutgi, 2001; Jackman and Johnson, 2005). SCLC is very aggressive and the median survival time without treatment is less than 4 months. Chemotherapy and radiotherapy represent the two major treatments for SCLC. According to the standards developed by the Veterans Administration Lung Cancer Study Group, SCLC can be divided into two stages: a limited stage and an extensive stage (Simon, 2003). Cancer cells in limited-stage SCLC are restricted to the ipsilateral hemithorax

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and can be treated by both chemotherapy and radiation therapy. About 20% of patients are cured after treatment, and the median survival time is about 18 months. Patients with extensive-stage SCLC have a high response rate to chemotherapy, which is the primary treatment for this disease, but the median survival time is only about 9 months because most patients relapse and the results of salvage therapy are poor (Janne et al., 2002; Demedts et al., 2010).

Transforming growth factor-beta (TGF- β) belongs to a large superfamily of cell cytokines, and is an important component of several cellular metabolic pathways. TGF- β signaling pathways regulate many aspects of cellular function, such as cellular proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis, immune surveillance and survival (Jakowlew, 2006). TGF- β plays a very complex dual role in cancer development, progress and metastasis (Akhurst & Derynck, 2001; Elliott & Blobe, 2005). TGF- β inhibits primary tumor development and growth by inducing cell cycle arrest and apoptosis as a tumor suppressor during the early phase of tumorigenesis (Arteaga, et al. 1993), but also promotes tumor invasion and metastasis by inducing the epithelial-mesenchymal transition in some epithelial cells, indicating that TGF- β can also act as a tumor promoter in the late stage of cancer (Miyazono, 2009).

There are two types of TGF- β signaling pathways; Smad-dependent and Smad-independent pathways. In Smad-dependent TGF- β signaling pathways, autocrine or exogenous TGF- β binds to the TGFBR2 and TGFBR1 membrane receptors. TGFBR2 then phosphorylates TGFBR1, which activates receptor-regulated Smads (also known as R-Smads). The R-Smads usually comprise Smad2 and Smad3. Activated Smad2 and Smad3 form complexes with Smad4, the common-partner Smad (co-Smad) in mammals. The subsequent R-Smad-co-Smad complexes shuttle between the nucleus and cytoplasm, and interact with various transcription factors and transcriptional co-activators such as AP-1, Sp1, p300, and SMIF to regulate the transcription of target genes (Derynck & Zhang, 2003). The phosphorylation of R-Smads can be blocked by inhibitory Smad, which starts the ubiquitination and degradation of the R-Smad-co-Smad complexes, thus inhibiting signal transduction (Itoh & ten Dijke, 2007). This TGF- β signal transduction pathway mainly regulates cell metabolism through this network involving cell cycle capture and apoptosis. In addition to Smad-mediated signaling pathways, TGF- β also activates other pathways, including Erk, JNK and p38 MAPK kinase pathways, via Smad-independent mechanisms (Moustakas & Heldin, 2007).

Both Smad-dependent and -independent TGF- β signaling pathways start by binding TGF- β to its transmembrane receptor TGFBR2, which then activates the downstream signal transduction. However, TGFBR2 expression is often reduced or even blocked in tumor cells (Levy & Hill, 2006). In bladder cancer, deficient TGFBR2 expression leads to loss of the growth inhibition function of TGF- β , and loss of expression of TGFBR2 has been shown to correlate with tumor grade (Tokunaga, et al., 1999). Other studies also found that inactivation of TGFBR2 played a central role in the development and progression of human gastric cancer, and TGFBR2 expression has shown a strong association with the degree of malignancy in gastric cancer (Chang, et al., 1997). The expression of TGFBR2 was also reduced in breast cancer (Gobbi, et al. 2000). Although the reasons for defective TGFBR2 expression are still unknown, loss of or reduced expression of TGFBR2 may be caused by histone deacetylation in lung cancer cell lines (Osada et al., 2001).

TGFBR2 mutations have also been observed in tumor cells. A DNA variant with a frameshift mutation in the poly(A)₁₀ repeat, resulting in microsatellite instability (MSI), has been

detected in the coding region of the TGFBR2 gene in several types of tumors, including colon cancer, gastric cancer, and gliomas (Markowitz et al. 1995; Pinto et al., 1997; Izumoto et al. 1997). This frameshift could affect gene function and be related to cancer development. This MSI also been detected in both NSCLC and SCLC (Kim et al., 2000; Tani et al., 1997), though the mutation rate seems to be much lower than that of deficient TGFBR2 expression rate in lung cancer. A previous study identified a novel microdeletion (c.492_507del) in giant cell carcinoma (GCC) and large cell carcinoma (LCC) patients, compared to other NSCLC subtypes. This 16-bp microdeletion introduced a premature stop codon at positions 590–592 of the cDNA, resulting in a truncated TGFBR2 protein with a mutated transmembrane domain and loss of a kinase domain. Although the mutated TGFBR2 played an important role in the abrogation of TGF- β signal transduction in LCC cells (Wang et al., 2007), it was not correlated with the reduced TGFBR2 expression seen in NSCLC (Xu et al., 2007). However, TGFBR2 has rarely been studied in Chinese SCLC samples and its role in TGF- β insensitivity in this population thus remains unknown. The present study therefore examined the levels of TGFBR2 expression in 27 pairs of formalin-fixed, paraffin-embedded SCLC tumors and compared them with NSCLC samples. The entire cDNA region and promoter of the gene was then sequenced to identify the causal variants in the TGFBR2 gene that accounted for its defective expression.

2. Materials and methods

2.1 Specimens

Twenty-seven formalin-fixed, paraffin-embedded SCLC samples and their corresponding normal tissues were collected by the Laboratory of the Department of Thoracic Surgery, Changhai Hospital between 2000 and 2007. All the patients had undergone pulmonary resection for primary SCLC at Changhai Hospital and had provided informed consent, and none had received preoperative radiotherapy or chemotherapy. The demographic and clinical features of these SCLC cases are summarized in Table 1. This research was conducted with the official approval of the academic advisory board of the Institute of Genetics, Fudan University, Shanghai, P. R. China.

An additional 65 formalin-fixed, paraffin-embedded NSCLC samples and their corresponding normal tissues were collected between 2005 and 2007, as a control group to compare with SCLC (Table 2). These tissues were also provided by the Laboratory of the Department of Thoracic Surgery, Changhai Hospital after obtaining the patients' consent. None of these patients had received radiotherapy or chemotherapy prior to surgery.

2.2 Immunohistochemistry

Expression of TGFBR2 was detected by immunohistochemistry assay using a monoclonal antibody against the extracellular domain of TGFBR2 (R & D Systems, Minneapolis, MN) via two-step immunohistochemical staining using the EnVision system (DAKO Cytomation, Denmark), as described in our previous report (26). In brief, after the paraffin sections were deparaffinized and hydrated, serial 4- μ m thick sections were placed into 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase activity. For antigen retrieval, the sections were treated with boiling 0.01 mol/L citrate buffer (pH 6.0) for 25 min and then incubated with 10% fetal calf serum for 20 min at room temperature. After the blocking serum was removed, the sections were incubated with the primary antibody (1:50) at room temperature for 1 h, followed by rinsing three times with phosphate-buffered saline (PBS).

Patient No.	Age	Gender	Type	Subtype	TNM	Stage	Smoking Status		Family History	TGFBR2 expression		
							Pack*Year	Status		Normal	Tumor	
S1	32	Male	SCLC	Limited	T2N1M0	IIa	No	No	No	9	9	Pr
S2	65	Male	SCLC	Limited	T2N1M0	IIa	720	No	No	9	9	Pr
S3	46	Female	SCLC	Limited	T2N2M0	IIIa	No	No	No	9	9	Re
S4	52	Female	SCLC	Limited	T2N1M0	IIa	No	No	No	9	9	Pr
S5	64	Male	SCLC	Limited	T2N1M0	IIa	Little	No	No	9	9	Re
S6	45	Male	SCLC	Extensive	T4N3M0	IIIb	600	No	No	9	9	Re
S7	46	Male	SCLC	Limited	T2N2M0	IIIa	200	No	No	9	9	Re
S8	63	Male	SCLC	Limited	T2N1M0	IIa	600	No	No	9	9	Re
S9	47	Male	SCLC	Extensive	T4N2M0	IIIb	600	No	No	9	9	Pr
S10	69	Male	SCLC	Limited	T1N0M0	Ia	600	No	No	9	9	Re
S11	57	Female	SCLC	Limited	T2N1M0	IIa	No	No	No	9	9	Pr
S12	48	Male	SCLC	Limited	T3N3M0	IIIb	300	No	No	9	9	Re
S13	57	Male	SCLC	Limited	T3N2M0	IIIa	No	No	No	9	9	Re
S14	62	Male	SCLC	Limited	T2N1M0	IIa	500	No	No	9	9	Re
S15	46	Male	SCLC	Limited	T2N2M0	IIIa	520	No	No	9	9	Re
S16	57	Female	SCLC	Limited	T1N2M0	IIIa	600	No	No	9	9	Re
S17	63	Male	SCLC	Limited	T2N1M0	IIa	600	No	No	9	9	Pr
S18	37	Female	SCLC	Limited	T2N2M0	IIIa	No	No	No	9	9	Pr
S19	52	Male	SCLC	Extensive	T4N0M1	IV	No	No	No	9	9	Pr
S20	77	Male	SCLC	Limited	T2N1M0	IIa	1200	No	No	9	9	Re
S21	60	Female	SCLC	Limited	T2N1M0	IIa	Unknown	No	No	9	9	Pr
S22	50	Male	SCLC	Limited	T2N2M0	IIIa	300	No	No	9	9	Re
S23	55	Male	SCLC	Extensive	T4N0M0	IIIa	600	Yes	Yes	9	9	Re
S24	71	Male	SCLC	Limited	T1N0M0	Ia	1600	No	No	9	9	Pr
S25	43	Male	SCLC	Limited	T3N0M0	IIb	400	No	No	9	9	Re
S26	63	Male	SCLC	Limited	T1N2M0	IIIa	No	No	No	9	9	Re
S27	71	Male	SCLC	Limited	T2N2M0	IIIa	Unknown	No	No	9	9	Re

Re: reduced TGFBR2 expression in tumor tissues, Loss: loss of TGFBR2 expression, Pr: preserved TGFBR2 expression.

The staining score of each tissue is the product of the proportion of positive staining cells and intensity scores.

Table 1. Clinical features and TGFBR2 expression of the 27 SCLC patients

Type	Total	TGFBR2 expression	
		Pr	Re
AdC	33	21	12
SqC	27	18	9
Ad-SqC	3	2	1
Atypical Carcinoid	1	1	
Low Differentiation Sarcoma	1		1
Grand Total	65	42	23

Re: reduced TGFBR2 expression in tumor tissues, Pr: preserved TGFBR2 expression.

Table 2. Clinical features and TGFBR2 expression in NSCLC samples

The sections were then incubated with a working solution of horseradish peroxidase-labeled goat anti-mouse immunoglobulin, as provided in the EnVision kit, for 30 min. Finally, the peroxidase activity was developed with 3,3-diaminobenzidine tetrahydrochloride and hydrogen peroxide. Because NSCLC develops from bronchial epithelium precursors, human normal bronchial epithelium was used as a positive control. A negative control for each specimen was provided by treating the sections with PBS instead of the primary antibody.

2.3 Interpretation of the staining and data evaluation

All sections were examined by standard light microscopy and scored semi-quantitatively on the basis of the percentage of immunoreactive cells and on the intensity of the staining reaction. The samples were initially classified into one of four grades, according to staining intensity: 0 (negative staining, equivalent to the negative control), 1 (weak staining), 2 (medium staining) and 3 (strong staining). The percentages of positively-stained cells were assigned as 0 for 0-25%, 1 for 26-50%, 2 for 51-75% and 3 for 76-100%, respectively. The final score was determined as the product of the proportion and intensity scores, and ranged from 0-9. Samples were considered to be negatively stained if the final score was 0, and positively stained if the final score was 1-9. Moreover, cancer samples were classified as preserved- or reduced-type in terms of TGFBR2 expression, depending on whether the final score was the same as or less than that of its corresponding normal lung tissue.

2.4 DNA Extraction and mutation analysis

Target cells from formalin-fixed, paraffin-embedded tissue sections were microdissected and scraped into microtubes. After deparaffinization with xylene and washing in ethanol, DNA was extracted by standard proteinase K digestion and phenol-chloroform extraction (Sambrook & Maniatis, 1989).

The presence of the 16-bp microdeletion in exon 4, which was previously detected in LCC and GCC, was examined in all SCLC tissues using the following forward and reverse primers to amplify the fragments of 117/101 bp, representing the wild/mutant alleles: 5'-caccagcaatcctgacttggtg-3' and 5'-cggtaacgcggtagcagtag-3'. The MSI in exon 3 was detected by the STR method using an ABI 3100 Sequencer and the following forward and reverse primers were used to amplify the exon 3 fragment (normally 242 bp) of the TGFBR2 gene: 5'-tccaatgaatctcttactc-3' and 5'-ccacacccttaagagaaga-3'. c.1167 C>T in exon 4 of TGFBR2 was detected by direct sequencing using an ABI 3100 Sequencer and the following forward and reverse primers to amplify the exon 4 fragment (242 bp) of the TGFBR2 gene: 5'-

ccaagatgccatcgtg-3' and 5'-tcccaggctcaaggtaaagg-3'. The other primers used for promoter and exon sequencing are listed in Table 3.

Fragments	Region	Direction	Sequence (5' - 3')
TGFBR2 promoter	Promoter part1	Forward	aactacaaaacatgtacaccagg
TGFBR2 promoter		Reverse	ttcttaggtcgaagtctagagg
TGFBR2 promoter	Promoter part2	Forward	atgcagaatctctgcctgcctc
TGFBR2 promoter		Reverse	cgagagctttggccgacttt
TGFBR2 promoter	Promoter part3	Forward	gtaaatacttggagcgaggaactc
TGFBR2 promoter		Reverse	ttctgaacgtgcggtgggat
TGFBR2 exon	exon 1	Forward	tcggtctatgacgagcag
TGFBR2 exon		Reverse	gggaccccaggaagacc
TGFBR2 exon	exon 2	Forward	gggctggatcaagttcatttg
TGFBR2 exon		Reverse	ggagacagagatacactgactgtg
TGFBR2 exon	exon 3	Forward	tccaatgaatcttctcactc
TGFBR2 exon		Reverse	cccacaccttaagagaaga
TGFBR2 exon	exon 4-1	Forward	ccaactcttctctccttgtttg
TGFBR2 exon		Reverse	tccaagaggcactactctcatagg
TGFBR2 exon	exon 4-2	Forward	gtcgtttgctgaggctataagg
TGFBR2 exon		Reverse	ccaggctcaaggtaaagggatctagca
TGFBR2 exon	exon 5	Forward	ggcagctggaatataatgatgggc
TGFBR2 exon		Reverse	tgctcgaagcaacacatg
TGFBR2 exon	exon 6	Forward	ttctcttgggctgcacatg
TGFBR2 exon		Reverse	cctaagaggcaacttggttgaatc
TGFBR2 exon	exon 7	Forward	ccaactcatgtgtccctttg
TGFBR2 exon		Reverse	tctttggacatgccagcctg
TGFBR2 MSI	Exon 3	Forward	Fam-tccaatgaatctctcactc
TGFBR2 MSI		Reverse	cccacaccttaagagaaga
TGFBR2 LOH	Exon 4	Forward	ccaagatgccatcgtg
TGFBR2 LOH		Reverse	tcccaggctcaaggtaaagg

Table 3. Primers used in the study

2.5 Statistical analysis

Data were analyzed using χ^2 tests, corrected χ^2 tests, or Fisher's exact tests. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1 TGFBR2 expression was more often reduced in SCLC than in NSCLC

TGFBR2 expression was assessed using immunohistochemistry. Normal human lung tissues and normal human bronchial epithelium were used as positive controls. Over 75% of cells in these tissues exhibited consistently strong staining, both showing staining scores of $3 \times 3 = 9$, indicating normal TGFBR2 expression (Figure 1). Immunostaining of TGFBR2 was performed in 27 SCLC tumor tissue samples and their corresponding normal tissues. All the normal tissues showed strong staining in over 75% cells with staining scores of 9. One SCLC

sample showed negative TGFBR2 expression (score of 0), while the remaining 26 were TGFBR2-positive. Furthermore, 16 of the total 27 SCLC tumor samples showed reduced TGFBR2 expression (score of 1–6) and 10 showed preserved expression (Table 1). None of the 65 NSCLC samples showed negative TGFBR2 expression (staining score of 0). In addition, only 35.4% (23/65) of all NSCLC tumor tissues showed reduced (score of 1–6) TGFBR2 expression and 64.6% (42/65) of tumors had preserved expression (score of 9) (Table 2). When adenocarcinoma and squamous cell carcinoma tissues were analyzed separately, the frequencies of preserved type were also higher (63.6% (21/33) and 66.7% (18/27) respectively) than those of reduced type (36.4% (12/33) and 33.4% (9/27) respectively). In contrast, the frequency of preserved type in SCLCs (47%, 10/27) was much lower than that of reduced type (63%, 17/27), indicating that reduced TGFBR2 expression was more frequent in SCLC cells (Table 1 & 2).

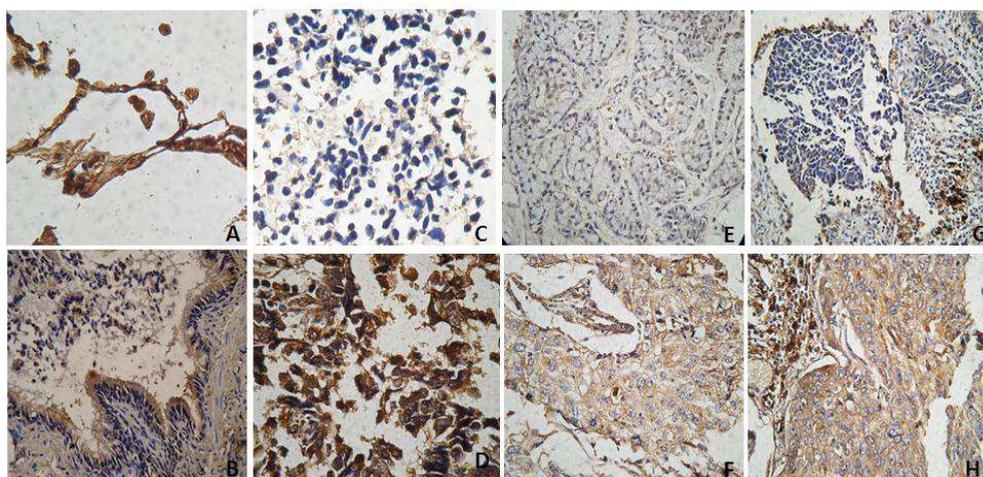


Fig. 1. Expression of TGFBR2 in lung cancer by immunohistochemical analysis ($\times 400$).

- A. The expression of normal lung epithelium;
- B. The expression of normal bronchus epithelium;
- C. Reduced expression of SCLC;
- D. Preserved expression of SCLC;
- E. Reduced expression of AdC;
- F. Preserved expression of AdC;
- G. Reduced expression of SCC;
- H. Preserved expression of SCC.

3.2 No significant relationship was found between TGFBR2 expression and clinical features in SCLC patients

The associations between TGFBR2 expression and other clinical features were analyzed. No significant associations were found between TGFBR2 expression and gender ($P = 1.00$), age ($P = 0.14$), tumor size ($P = 1.00$), nodal involvement ($P = 1.00$), metastasis ($P = 1.00$) or stage ($P = 0.12$) (Table 4).

	Cases	Gender		TGFBR2 expression		Age	P-value
		M	F	Re	Pr		
Age							
≤60	16 (59.26%)	11	5	10	6	32-57	P=1.0000
>60	11 (40.74%)	10	1	7	4	61-77	
Gender							
Male	21 (77.78%)	21	0	15	6		P=0.1358
Female	6 (22.22%)	0	6	2	4		
Tumor Size							
T1	4	3	1	3	1		P=1.0000
T2	16	11	5	14	9	T≥2	
T3	3	3	0				
T4	4	4	0				
Nodal involvement							
N0	5	5	0	3	2		P=1.0000
N1	10	8	2	14	8	N≥1	
N2	10	8	2				
N3	2	2	0				
Metastasis							
M0	26	20	6	16	10		P=1.0000
M1	1	1	0	1	0		
Stage							
I	2	2	0	1	1		P=0.1164
II	11	8	3	5	6		
III	13	10	3	11	2		
IV	1	1	0	0	1		

Table 4. Association between TGFBR2 expression and clinical features of 27 SCLC patients

3.3 TGFBR2 expression is related to tumor types

The relationship between TGFBR2 expression and histological type was analyzed. Samples were categorized as SCLC or NSCLC subtypes because they developed from different lung cells. As shown in Table 5, a significant association between TGFBR2 expression and histological type was identified ($P = 0.0151$), indicating the existence of a significant difference in TGFBR2 expression levels between SCLC and NSCLC subtypes (Table 5).

For further statistical analysis, NSCLC cases were divided into AdC, SqC, Ad-SqC and other subtypes. Because of the sample sizes, comparisons were only made between SCLC and AdC, and between SCLC and SqC. The results demonstrated significant differences in TGFBR2 expression between SCLC and AdC, and between SCLC and SqC ($P = 0.0402$ and 0.0293 , respectively) (Table 5).

3.4 Mutations in exon 4 of TGFBR2

In a previous study, we identified a microdeletion (c.492_507del) in patients with GCC and LCC. We therefore investigated the occurrence of this microdeletion in SCLC in the present study. Genomic DNA was extracted from 21 pairs of formalin-fixed, paraffin-embedded SCLC tissues and their corresponding normal tissues. The coding and promoter regions of

Item	n	Reduced	Preserved	P-value
(1)				
SCLC	27	17 (63.0%)	10 (37.0%)	0.0151
NSCLC	65	23 (35.4%)	42 (64.6%)	
(2)				
SCLC	27	17 (63.0%)	10 (37.0%)	0.0402
AdC	33	12 (36.4%)	21 (63.6%)	
(3)				
SCLC	27	17 (63.0%)	10 (37.0%)	0.0293
SqC	27	9 (33.3%)	18 (66.7%)	

Table 5. TGFBR2 expression in different subtypes of tumor

TGFBR2 were sequenced. The DNA from the other six pairs of tissues was degraded and was unsuitable for amplification. No microdeletion was observed in any of the tested SCLC samples.

However, another novel variant in exon 4 of TGFBR2 was identified in 11 of 21 SCLC tumor samples. This variant at c.1167 in the TGFBR2 coding region was T/T homozygous in eight out of 11 cases, and C/T heterozygous in the other three cases, compared with C/C homozygous in normal individuals. The corresponding normal samples for these were C/T heterozygous. In the other 10 pairs of samples, however, the site was C/C homozygous. These results suggest that loss of heterozygosity (LOH) occurred in the eight tumors whose alleles became T/T homozygous from C/T heterozygous (Figure 2 and Table 6).

Interestingly, this change was a synonymous mutation that did not alter the amino acid sequence. We investigated its effect on the expression of TGFBR2, and found that TGFBR2 expression was reduced in nearly all T carriers (81.8%), compared with that in normal tissues, while only 60% of CC carriers had reduced TGFBR2 expression (Table 6).

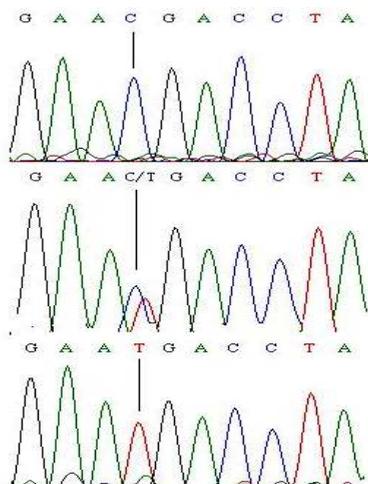


Fig. 2. LOH in SCLC.

- A. CC sequence in normal lung tissue from SCLC patients;
- B. TC sequence in normal lung tissue from SCLC patients;
- C. TT sequence in tumor tissue from SCLC patients.

Patient No.	Tissue	LOH	TGFBR2 expression
S1	+	TT	Pr
	-	CT	
S3	+	CC	Re
	-	CC	
S5	+	CC	Re
	-	CC	
S6	+	TT	Re
	-	CT	
S7	+	CC	Re
	-	CC	
S8	+	TT	Re
	-	CT	
S9	+	CC	Pr
	-	CC	
S11	+	CC	Pr
	-	CC	
S12	+	TT	Re
	-	CT	
S13	+	TT	Re
	-	CT	
S15	+	CT	Re
	-	CT	
S16	+	CC	Re
	-	CC	
S18	+	CC	Pr
	-	CC	
S19	+	TT	Pr
	-	CT	
S20	+	CT	Re
	-	CT	
S21	+	CC	Pr
	-	CC	
S22	+	CT	Re
	-	CT	
S23	+	TT	Re
	-	CT	
S25	+	CC	Re
	-	CC	
S26	+	TT	Re
	-	CT	
S27	+	CC	Re
	-	CC	

+: tumor tissues; -: normal lung tissue of patients;

Re: reduced TGFBR2 expression in tumor tissues, Pr: preserved TGFBR2 expression.

Table 6. Relationship between LOH in exon 4 and TGFBR2 expression

3.5 MSI in TGFBR2 in SCLC

Poly(A)₁₀/(A)₉ heterozygosity in exon 3 of TGFBR2, representing MSI, was detected in 60% of SCLC samples (9 out of 15), as shown in Table 7. However, no association between MSI and TGFBR2 expression was found (P = 0.264).

Patient No.	Tissue	MSI	TGF-BRII expression
S1	+	poly (A) ₁₀	Preserved
	-	poly (A) ₁₀	
S2	+	poly (A) ₁₀	Preserved
	-	poly (A) ₁₀	
S3	+	poly (A) ₁₀ / (A) ₉	Reduced
	-	poly (A) ₁₀ / (A) ₉	
S5	+	poly (A) ₁₀ / (A) ₉	Reduced
	-	poly (A) ₁₀ / (A) ₉	
S6	+	poly (A) ₁₀ / (A) ₉	Reduced
	-	poly (A) ₁₀ / (A) ₉	
S9	+	poly (A) ₁₀ / (A) ₉	Preserved
	-	poly (A) ₁₀	
S13	+	poly (A) ₁₀ / (A) ₉	Reduced
	-	poly (A) ₁₀	
S16	+	poly (A) ₁₀ / (A) ₉	Reduced
	-	poly (A) ₁₀ / (A) ₉	
S19	+	poly (A) ₁₀ / (A) ₉	Preserved
	-	poly (A) ₁₀	
S21	+	poly (A) ₁₀	Preserved
	-	poly (A) ₁₀	
S22	+	poly (A) ₁₀	Reduced
	-	poly (A) ₁₀	
S23	+	poly (A) ₁₀ / (A) ₉	Reduced
	-	poly (A) ₁₀ / (A) ₉	
S25	+	poly (A) ₁₀	Reduced
	-	poly (A) ₁₀	
S26	+	poly (A) ₁₀ / (A) ₉	Reduced
	-	poly (A) ₁₀ / (A) ₉	
S28	+	poly (A) ₁₀	Reduced
	-	poly (A) ₁₀	
S29	+	poly (A) ₁₀	Reduced
	-	poly (A) ₁₀	
S30	+	poly (A) ₁₀	Reduced
	-	poly (A) ₁₀	

+: tumor tissues; -: normal lung tissue of patients;

Re: reduced TGFBR2 expression in tumor tissues, Pr: preserved TGFBR2 expression.

Table 7. MSI detection and relation with TGFBR2 expression

4. Discussion

Tumor cells are often able to escape from TGF- β -signaling-induced cell cycle capture and apoptosis. TGF- β has a dual function in tumor development (Akhurst & Derynck, 2001; Elliott & Blobe, 2005); it acts as a tumor suppressor during the initial stages of tumor development (Arteaga, et al. 1993), but promotes tumor progression during the later stages (Miyazono, 2009). High levels of TGF- β expression in tumor cells can induce tumor evolution by stimulating angiogenesis and through other potential immunosuppressive effects, as well as by directly affecting tumor cell invasion and metastasis (Pardali & Moustakas, 2007). These direct effects can be achieved via Smad-dependent pathways, or may be mediated by interference with these pathways (Derynck & Zhang, 2003). Changes in

the TGF- β signaling pathway may lead to abnormal signal transduction and cause dysregulated cell growth and differentiation. The first step in any mechanism involves binding of autocrine or paracrine TGF- β to the TGFBR2 receptor on the cell membrane, before activation of various downstream receptors can occur. TGFBR2 thus plays a key role in TGF- β signaling pathways, and its expression is reduced or blocked in many tumors (Chang et al., 1997; Tokunaga et al., 1999; Gobbi et al. 2000; Levy & Hill, 2006), resulting in partial or complete disruption of the TGF- β pathway.

Previous studies demonstrated that TGFBR2 expression in NSCLC differed between LCC and AdC, SqC or non-LCC cases, but the role of defective TGFBR2 expression in the initiation and/or development of SCLC (Xu et al., 2007), and its expression status in SCLC remain largely unknown. Furthermore, SCLC is phenotypically distinct from and much more malignant than NSCLC. We therefore compared TGFBR2 expression between SCLC and NSCLC. Immunohistochemical staining with TGFBR2 antibody revealed significant differences in the incidence of reduced expression in SCLC (63.0% of cases) versus AdC (36.4% of cases, $P = 0.0402$) and SqC (33.3% of cases, $P = 0.0293$), or SCLC versus NSCLC (35% of cases, $P = 0.0151$). These differences in expression levels between SCLC and NSCLC were consistent with the histopathologic classification of these tumors, suggesting that defective TGFBR2 expression might contribute to the initiation and/or development of SCLC.

To determine the reason for the reduced expression of TGFBR2, we examined the mutation status of c.492 507del in exon 4, but found no changes in this sequence in SCLC tumor samples. We subsequently determined the MSI status in exon 3, and identified a DNA variant with a frameshift mutation in the TGFBR2 poly(A)₁₀ repeat (which causes MSI) in the coding region of the TGFBR2 gene. A total of 60.0% of SCLC were poly(A)₁₀/(A)₉ heterozygous, but no association was found between the MSI and TGFBR2 expression. However, no MSI was identified in our previous study of NSCLC, suggesting that the MSI in SCLC is at least partly associated with its carcinogenesis. We also sequenced all seven exons and the promoter region of the TGFBR2 gene and identified a novel LOH at c.1167 in 38.1% (8/21) of SCLC tissues. Further analysis showed that most of the mutant T-allele carriers (81.8%) had reduced TGFBR2 expression in tumor tissues, compared with only 60% of C-allele carriers. These results suggest that the change from wild type to mutant type might contribute, at least in part, to the defective expression of TGFBR2 in SCLC patients, though further studies are needed to clarify the mechanisms responsible.

5. Conclusion

The present study identified reduced TGFBR2 gene expression levels in formalin-fixed, paraffin-embedded sections from most SCLC tumors examined, suggesting that this might contribute to the initiation and/or development of SCLC. Sequencing analysis also indicated that change of the wild-type C-allele to the mutant T-allele at c.1167 might contribute to the defective expression of TGFBR2 in SCLC patients. Another DNA variant with a frameshift mutation in the TGFBR2 poly(A)₁₀ repeat, leading to MSI, was found in the coding region of the TGFBR2 gene, but this was not associated with TGFBR2 expression.

These results suggest that defective expression of TGFBR2 might inactivate TGF- β signal transduction, leading to the loss of growth inhibition and acceleration of tumor formation, and that a C>T substitution at c.1167 might be partially responsible for this reduced expression of TGFBR2 in SCLC.

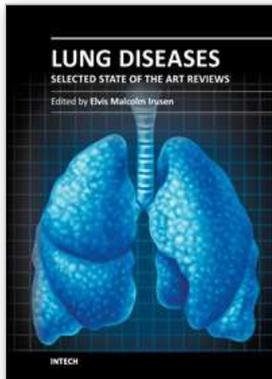
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