

Reduced transcription of the *Smad4* gene during pulmonary carcinogenesis in idiopathic pulmonary fibrosis

KIYOSHI TAKENAKA¹, AKIHIKO GEMMA¹, AKINOBU YOSHIMURA¹, YOKO HOSOYA¹, MICHIIYA NARA¹, YUKIO HOSOMI¹, TETSUYA OKANO¹, SHINOBU KUNUGI⁴, KIYOSHI KOIZUMI³, YUH FUKUDA⁴, KAZUTSUGU UEMATSU², KAZUO SHIMIZU³ and SHOJI KUDOH¹

Departments of ¹Internal Medicine, Divisions of Pulmonary Medicine, Infectious Diseases and Oncology, ²Pathology, and

³Surgery, Divisions of Endocrine, Cardiovascular and Thoracic Surgery, Nippon Medical School, Tokyo 113-8602;

⁴Division of Pulmonary Medicine, Saitama Medical Center, Kamoda, Kawagoe-shi, Saitama 350-8550, Japan

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Abstract. Patients with idiopathic pulmonary fibrosis (IPF) have an increased risk of developing lung cancer. To identify key molecules involved in malignant transformation in IPF, we analyzed the expression profiles of lung and lung tumor tissue from patients with lung cancer and IPF (lung cancer/IPF) using cDNA arrays and real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Reduced expression of the *Smad4* gene was identified in all eight tumor samples from the lung cancer/IPF patients using real-time RT-PCR. Expression levels of *Smad4* were significantly lower in tumors from lung cancer/IPF patients than in those from lung cancer patients without IPF or in lung cancer cell lines ($p < 0.01$). Mutational analysis of *TGF- β type II receptor* and *Smad4* was performed using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). The methylation status of the *Smad4* promoter was analyzed using methylation-specific PCR with subsequent sequence analysis. No mutations were detected in the eight tumor samples, but hypermethylated regions were detected in the *Smad4* promoter in two of the eight tumors with reduced *Smad4* expression. Promoter reporter assays showed that the activity of the *Smad4* promoter containing the sequence of the methylated region was significantly stronger than that of the *Smad4* promoter with a deleted methylated region ($p < 0.002$). Our findings indicate that the loss of the growth inhibitory response to TGF- β signaling may be crucial in pulmonary carcinogenesis or in the progression of lung cancer in IPF patients in whom

TGF- β is overexpressed; hypermethylation of the *Smad4* promoter region may be one mechanism by which this occurs. These findings are useful for the development of preventive measures or treatment for lung cancer patients with IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease of uncertain etiology. Patients with IPF have been shown to be 14.1 times more likely to develop lung cancer than the general population, and more than 10% of IPF patients die of lung cancer (1,2). Currently, few IPF patients with lung cancer can be treated by surgery, radiotherapy and/or chemotherapy due to pulmonary dysfunction or severe adverse effects. The development of new therapies is therefore essential. To clarify the mechanisms of pulmonary carcinogenesis in IPF, we previously analyzed microdissected tissues of the metaplasias and bronchiolar epithelia of IPF patients. Using polymerase chain reaction (PCR)-based microsatellite analysis, fluorescent *in situ* hybridization (FISH) and immunohistochemistry of the fragile histidine triad (FHIT) protein, we observed frequent allelic losses and reduced expression of the *FHIT* gene (3), and suggested that *FHIT* gene inactivation may be involved in tumorigenesis in patients with IPF.

To identify key molecules involved in malignant transformation in IPF patients, we analyzed the expression profiles of cancer-related genes in lung tumor and paired IPF tissue from patients with lung cancer and IPF (lung cancer/IPF) using cDNA array analysis and real-time quantitative RT-PCR. It is often difficult to obtain fresh samples for expression analysis from patients with lung cancer/IPF, as only a small percentage undergo surgical resection. When we examined 64 lung cancer patients with IPF in our hospital, 14 (21.9%) were undergoing surgery. Of the 14, frozen lung cancer and tissues affected by IPF were available for expression analysis from eight patients. Real-time RT-PCR was used to confirm results obtained by cDNA array analysis, and the mechanism of transcriptional change was analyzed by mutational analysis, methylation-specific PCR and promoter reporter assays with or without the sequence including the methylated region.

Correspondence to: Dr Akihiko Gemma, Department of Internal Medicine, Divisions of Pulmonary Medicine, Infectious Diseases and Oncology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan
E-mail: agemma@nms.ac.jp

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Materials and methods

Tissue samples, RNA and DNA extractions. Frozen lung cancer and IPF tissues from eight lung cancer/IPF patients were available for expression analysis. All patients provided written/oral informed consent for the use of the tissues. All tissues were frozen in liquid nitrogen after surgery and stored at -80°C until analysis. The lung cancers by histological type included four cases of squamous cell carcinomas, two of adenocarcinoma, one of small cell carcinoma and one of large cell carcinoma. All patients were male, ranging in age from 64 to 77 years. Using RT-PCR, we also analyzed *Smad4* gene expression in four lung cancer (adenocarcinoma) patients without IPF and eight lung cancer cell lines: Lu65, A549, PC7, PC9 and PC14 (adenocarcinoma cell lines) and NCI-N231, NCI-H69 and Lu135 (small cell carcinoma cell lines). The Lu65 and Lu135 cell lines were provided by Y. Shimosato and T. Terasaki (National Cancer Center Research Institute, Tokyo, Japan). NCI-N231, A549 and NCI-H69 were obtained from the American Type Culture Collection (Rockville, MD). PC7, PC9 and PC14 were obtained from IBL (Gunma, Japan). RNA and genomic DNA were isolated from each sample using standard previously described protocols (4,5).

cDNA array hybridization and analysis of hybridization signals. mRNA was isolated from total RNA by incubation with oligo-dT-magnetic beads (Toyobo Co., Osaka, Japan). The Gene Navigator cDNA Array System-Cancer Selected (Toyobo Co.) was used for cDNA analysis as previously reported (5). One hundred and seventy-seven species of human DNA fragments were spotted in duplicate on a filter. The genes on the filter included cancer-related genes, house-keeping genes and non-mammalian genes as negative controls. A list of the set of genes on the filter can be found at the site: (<http://www.toyobo.co.jp/seihin/xr/product/genenavigator.html>). To construct the probes, reverse transcription was performed using Reverse Transcriptase, ReverTra Ace (Toyobo Co.), a random 9mer (Toyobo Co.) as the primer and 5 μg of polyA RNA. The probes were labeled with biotin by incorporation of biotin-16-deoxyuracil triphosphate (dUTP) during the synthesis of cDNA. The filters were pre-incubated in 20 ml of PerfectHyb (Toyobo Co.) at 68°C for 30 min. The biotin-labeled probes were denatured and added to the pre-hybridization solution. The filters were incubated overnight at 68°C in the hybridization mixture. After washing, specific signals on the filters were detected by the Imaging High Chemifluorescence Detection kit (Toyobo Co.). Vistra ECF substrate (AttoPhos) (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as the chemifluorescence substrate. A chemifluorescence image of the filter was acquired by FluorImager (Amersham Pharmacia Biotech). Gene expression images were quantified by measuring the intensity of the signals using Imagene (BioDiscovery, Los Angeles, CA). Signal intensity among filters was compared using E-Gene Navigator Analysis (GeneticLab, Sapporo, Japan). The background threshold was set at a level 5-fold higher than the negative control. The signal intensities were normalized by comparing the expression of the housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). A significant difference in gene expression between two examined tissue

samples was defined as a ≥ 3 -fold difference in the signal intensity ratio.

Real-time quantitative RT-PCR. Quantitative PCR was performed using the ABI PRISM 7700 Sequence Detector (Perkin Elmer/Applied Biosystems, Foster City, CA). PCR primers and TaqMan fluorogenic probes were designed using the Primer Express software program (Perkin-Elmer/Applied Biosystems). Their sequences are shown in Table I. Using a random hexamer and ReverTra Ace- α , 1 μg of each total RNA was reverse transcribed. A portion of the cDNA was used for quantitative PCR in a 50- μl volume using the designed primers, TaqMan probes and Master Mix (PCR buffer, MgCl_2 , dATP, dCTP, dGTP, dUTP, AmpErase UNG and AmpliTaq Gold DNA polymerase) (Perkin-Elmer/Applied Biosystems). The initial thermal cycle conditions were 50°C for 2 min and 95°C for 10 min as recommended by the manufacturer. The cycle conditions were 95°C for 15 sec and 60°C for 1 min, and the number of cycles was 40. The RNA level of a gene was expressed as the ratio of the level of RNA of that gene to the level of *GAPDH* RNA in the sample.

Polymerase chain reaction-single strand conformation polymorphism analysis. Mutations in exons of the *Smad4* and *TGF- β receptor type II* (*TGF- β RII*) genes were analyzed by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP). In each genomic DNA sample from the lung cancers, 11 exons of the *Smad4* gene and two exons of the *TGF- β RII* gene were amplified separately with the PCR primers as previously reported (6-8) (Table II). Mutation of the *Smad4* promoter (9) was also analyzed using a primer pair as follows: forward 5'-TGCTCAGTGGCT TCTCG-3' and reverse 5'-TAGGGGAGAGCAGGAAG-3'. The PCR reaction tube mix contained 25 pmol of each primer labeled with fluorescein isothiocyanate (FITC), 2.5 mM dNTPs, 1.25 units of Ex Taq DNA polymerase and 1X Ex Taq buffer (Takara, Tokyo, Japan) in a final reaction volume of 20 μl . The 20- μl reaction mixture was placed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) when the heating block reached 94°C . PCR conditions for each set of primers were optimized. Standard denaturing and extension temperatures of 94 and 72°C , respectively, were used. Annealing temperatures were 56°C for *Smad4* analysis, and the number of cycles was 35 or 45, respectively (6-8). FITC-labeled PCR products were denatured, cooled on ice and loaded on neutral 6% polyacrylamide gels with or without 5% (vol/vol) glycerol as described previously (4). After electrophoresis, the gels were analyzed with the FluorImager.

Methylation analysis. The methylation status of the *Smad4* promoter was analyzed by methylation-specific PCR (MSP) (10) using the CpGenome DNA Modification Kit (Intergen Co., Purchase, NY). The fragment selected for this analysis was a CG-rich region, including 550 μl of freshly prepared non-coded sodium bisulfite mixture (Intergen Co.). These solutions were incubated at 50°C for 16 h. In bisulfite modification, all unmethylated cytosines are deaminated and converted to uracils, while the 5'-methylcytosines remain unaltered. The DNA samples were purified by ethanol precipitation and resuspended in 25 μl of TE (10 mM Tris/

Table I. List of primer and probe sequences used in the real-time reverse transcriptase-polymerase chain reaction of the indicated genes.

Gene	Sense primer sequence (5'-3')	Antisense primer sequence (5'-3')	Probe sequence (5'-3')
<i>Smad4</i>	aaaacggccatcttcagcac	aggccagtaatgtccggga	acccgcctatgccgccc
<i>GAPDH</i> ^a	gaaggtgaaggtcggagtc	gaagatggatgggatttc	caagctcccgttctcagcc

^aGlyceraldehyde-3-phosphate dehydrogenase.

Table II. List of primer sequences for amplification of each exon of each gene.

Gene	Sense primer sequence (5'-3')	Antisense primer sequence (5'-3')
<i>Smad4</i>		
Exon 1-1	tcagaaattggagacatatt	tctttttctccttcagcttc
Exon 1-2	agtgaacatttgcaaaagag	gttttaaatctgccaccatag
Exon 2	aaagtgtcttcataatgatgac	ttcttaggatgaaacaaactac
Exon 3	cttcattgtaatgattaatgtttc	aagagaaagtagtaagaaacag
Exon 4-1	gattttagggtgtattatattacttg	ctctgtcgtatgcacgattac
Exon 4-2	ttgtccactgaaggacattc	gctgactacatctgattctag
Exon 5	cataagatgacatctatgaatg	gcttttataaaggctgcctac
Exon 6	taaaagcaaattaacccatgtg	cccttacaacaaaacaagag
Exon 7	tgaaagtttagcattagacaac	cgtttcaatcaccactaaatc
Exon 8	tttctcatgggaggtgttc	caatttttaagtaacttactgac
Exon 9-1	tattaagcatgctatacaactctg	ctgtctaagtagtaactctg
Exon 9-2	caaagggtgtgcagttggaatg	ctccaccagatttcaattc
Exon 10	gaattttctttatgaactcatag	tttaaaaaagaatgaaaagcatac
Exon 11-1	ctgatgtcttccaaactctttctg	ggcatggatgaagtcattcgtc
Exon 11-2	ctgatgtcttccaaactctttctg	tgtattttgtagtccaccatc
<i>TGF-β RII</i> ^a		
Exon 3	tccaatgaatctcttcaactc	cccacacccttaagagaaga
Exon 7	ccaactcatggtgtccctttg	tctttggacatgccagcctg

^aTransforming growth factor-β receptor type II.

0.1 mM EDTA, pH 7.5). PCR amplification was performed using methylation-specific primers that had been designed to distinguish methylated from unmethylated DNA. One primer set (forward 5'-AGTGAGAGAGGT TTTTGTGTAGG-3' and reverse 5'-CCACCACCACCA TCATC-3') was designed to anneal to unmethylated DNA that had undergone a chemical modification. A second primer set (forward 5'-GTTGAGGAG GGGCGGTTTGG-3' and reverse 5'-CCCTCCCAAAAATT CC-3') was designed to anneal to methylated DNA that had undergone a chemical modification. A third primer set (forward 5'-TGCTCAGTGGCTTCTCG-3' and reverse 5'-TAGGGG AGAGCAGGAAG-3') was designed to anneal to unmodified DNA. The PCR reaction mixture consisted of 10X PCR buffer, 2.5 mM dNTPs, 10 pmol of each primer, 1.25 U of AmpliTaq Gold and 2 ng of template DNA in a final volume of 25 μl. PCR was performed as follows: denaturation at 95°C for 12 min, denaturation at 95°C for 30 sec, annealing at 60°C for

45 sec and extension at 72°C for 45 sec for 35 cycles. A total of 10 μl of each allele-specific PCR sample was mixed with 2 μl of loading dye and loaded on a 1.2% agarose gel. The gel was stained with ethidium bromide.

Sequencing analysis. When bands of methylated DNA were detected in the methylation analysis, they were excised from the gels, purified with GeneClean II Kit (Bio 101, Vista, CA) and ligated into the pPCR Script Amp SK(+) cloning vector (Stratagene, La Jolla, CA). Subsequently, 8-10 independent clones were sequenced by fluorescent automated sequencing (Perkin Elmer/Applied Biosystems) (4).

Cloning and reporter assays of the promoter region of the *Smad4* gene. *Smad4* promoter fragments from normal human lung DNA were generated with Advantage GC 2 PCR (Clontech, Palo Alto, CA). The primer sequences were

Table III. Differential expression of genes in pulmonary fibrosis and lung carcinoma tissues in cDNA array analysis.

Gene name	Classification	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
<i>Smad4</i>	Regulatory transcription factors	IP > Ca ^a	IP > Ca ^a	IP = Ca ^b	IP > Ca ^a	IP = Ca ^b
<i>p21</i>	Cell cycle protein	IP > Ca ^a	IP > Ca ^a	IP = Ca ^b	IP = Ca ^b	IP = Ca ^b
<i>Metallothionein</i>	Metabolic enzyme	IP = Ca ^b	IP > Ca ^a	IP = Ca ^b	IP = Ca ^b	IP > Ca ^a
<i>MMP-7</i>	Signaling intermediates	IP = Ca ^b	IP > Ca ^a	IP = Ca ^b	IP = Ca ^b	IP > Ca ^a
<i>TIMP-1</i>	Signaling intermediates	IP = Ca ^b	IP > Ca ^a	IP = Ca ^b	IP > Ca ^a	IP = Ca ^b

^aThe signal intensity ratio of the gene was ≥ 3 -fold in pulmonary fibrosis tissue compared with lung carcinoma tissue. ^bThe difference in the signal intensity ratio of the gene between pulmonary fibrosis tissue and lung carcinoma tissue was < 3 -fold. *MMP-7*, matrix metalloproteinase-7; *TIMP-1*, tissue inhibitor of matrix metalloproteinase.

Table IV. Relative expression levels of *Smad4* using real-time reverse transcriptase-polymerase chain reaction.

Sample	Relative expression levels of <i>Smad4</i> ^a
Normal tracheal cell	1
Normal human lung	1.25
Tumors from lung cancer/IPF patients ^b	0.08 \pm 0.07
Tumors from lung cancer patients without IPF ^c	2.45 \pm 2.20
Cell lines ^d	0.58 \pm 0.41

^a*Smad4* expression intensity in sample/normal tracheal cells. ^bTumor samples from eight lung cancer patients with idiopathic pulmonary fibrosis (IPF). ^cTumor samples from four lung cancer patients without IPF. ^dLu65, A549, PC7, PC9, PC14, NCI-N231, NCI-H69 and Lu 135. Data are expressed as the mean \pm SD. The relative expression level of *Smad4* was significantly lower in tumors from lung cancer/IPF patients than in lung cancer cell lines ($p < 0.01$, Mann-Whitney U test).

designed on the basis of methylation analysis with the *MluI* or *BglIII* sites. Forward primer (5'-ACTACGCGTATGCTCA GTGGCTTCTCG-3') and reverse primers (1 5'-GTAAGA TCTTCGCCCTCCCAGAGGATTC-3' and 2 5'-GTAAGA TCTACATGGCGCGGTTACCTG-3') were designed to amplify the promoter region of *Smad4* with (reverse 1) or without (reverse 2) the sequence including the methylated region from the methylation analysis. First, PCR products including the promoter region of *Smad4* were subcloned into pPCR-Script Amp SK(+) cloning vector. Second, luciferase plasmids containing the *Smad4* promoter region were generated by cloning PCR products into the *MluI* and *BglIII* sites of pGL3-Basic Vector (Promega, Madison, WI). The luciferase plasmids with or without the *Smad4* promoter regions and pRL-TK vector (Promega) were co-transfected into A549 cells using the CalPhos™ Mammalian Transfection Kit (Clontech). Protein extracts were prepared, and luciferase activity was measured by the Dual-Luciferase Reporter Assay Systems (Promega).

Results

cDNA array analysis. We used cDNA array screening to identify genes differentially expressed in lung cancer and IPF tissues from lung cancer/IPF patients. A sufficient amount of total RNA for the cDNA array analysis could be extracted from only five of the eight patients. Comparison of chemi-fluorescent intensities produced by the lung cancer and IPF samples showed varying expression patterns, but *Smad4* was down-regulated in three of the five patients. *Metallothionein*, *p21*, *MMP-7* (matrix metalloproteinase-7) and *TIMP-1* (Tissue inhibitor of matrix metalloproteinase) were down-regulated in two of five patients (Table III). *TGF- β* , *TGF- β R-II* and other *Smads* were equally expressed between the pairs of lung cancer and IPF sample series.

Real-time quantitative reverse transcriptase-polymerase chain reaction. To confirm the differential expression of *Smad4*, we performed real-time quantitative RT-PCR (Table IV). Adequate amounts of total RNA for use in real-time RT-PCR from both lung cancer and IPF samples were available from six of eight lung cancer/IPF patients. In the remaining two patients, total RNA could only be extracted from the lung cancer tissue. We compared *Smad4* expression in patient lung cancers, normal tracheal cells and normal human lung tissue (BD Biosciences, Clontech, CA). All eight lung cancer/IPF patients showed down-regulation of *Smad4* expression in lung cancer compared to normal tracheal cells and normal human lung. There was no significant difference in *Smad4* expression in normal tracheal cells and normal human lung. Moreover, we compared *Smad4* expression in lung cancer/IPF and lung cancer without IPF patients. The relative expression level of *Smad4* in tumors from lung cancer/IPF patients (0.08 \pm 0.07) was significantly lower than in tumors from lung cancer without IPF patients (2.45 \pm 2.20) ($p = 0.0065$, Mann-Whitney U test). The relative expression level of *Smad4* was also significantly lower in tumors from lung cancer/IPF patients than in the lung cancer cell lines (0.58 \pm 0.41) ($p = 0.0053$, Mann-Whitney U test). Four of the six lung cancer/IPF patients showed down-regulation of *Smad4* expression in lung cancer tissue compared to IPF tissue; the other two patients showed almost equal levels of *Smad4* expression between the tissues.

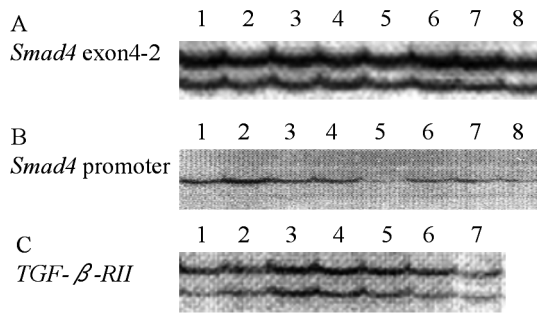


Figure 1. Representative results of PCR-SSCP analysis. (A) *Smad4* exon 4-2. (B) *Smad4* promoter and (C) *TGF-β RII* (transforming growth factor- β receptor type II). No abnormal mobility shift is present in any panels. Numbers on the panels indicate patient number.

Polymerase chain reaction-single strand conformation polymorphism analysis. One possible mechanism of the down-regulation of *Smad4* expression is the inactivation of *Smad4* by intragenic mutation. We performed PCR-SSCP analysis using 15 primer sets with intronic sequences designed to amplify *Smad4* exons 1-11 and one primer set designed to amplify the *Smad4* promoter. Exons 1-11 of the *Smad4* gene and the *Smad4* promoter were successfully amplified by PCR in eight of the lung cancer samples obtained from lung cancer/IPF patients. No SSCP variants were detected (Fig. 1A and B). Additionally, PCR-SSCP analysis was performed on the mutational hot spots of the *TGF-β RII* gene in the same specimens, but SSCP variants were not detected (Fig. 1C).

Methylation analysis. An alternative mechanism of transcriptional repression is the hypermethylation of CpG islands in the *Smad4* promoter. To investigate the methylation status of the *Smad4* promoter, MSP was performed on tumor DNA obtained from eight lung cancer/IPF patients. Among the eight patients, the methylated band of the *Smad4* gene was observed in samples obtained from the two patients whose *Smad4* expression was down-regulated in real-time quantitative RT-PCR analysis. The DNA sequences of the methylated and unmethylated bands were confirmed, and the representative results are shown in Fig. 2. In two cases, similar methylated regions in the *Smad4* promoter were observed.

***Smad4* promoter reporter assays.** To verify the biological significance of the hypermethylation of the *Smad4* promoter observed in methylation analysis, two *Smad4* promoter fragments from normal human lung DNA were generated with Advantage GC 2 PCR. The PCR primer pairs were designed to amplify the *Smad4* promoter with (promoter fragment 1) or without (promoter fragment 2) a 125 base pair including the methylated region of the *Smad4* promoter observed in the methylation analysis (Fig. 3). After subcloning, luciferase plasmids containing the *Smad4* promoter regions were generated by cloning PCR products into *Mlu*I and *Bgl*III sites of pGL3-Basic Vector. Luciferase assays were performed using the luciferase vector with or without *Smad4* promoters. The promoter activity/TK promoter activity ratio was significantly higher in cells transfected with the vector containing promoter fragment 1 (0.057 ± 0.034) (11 clones) than in cells transfected

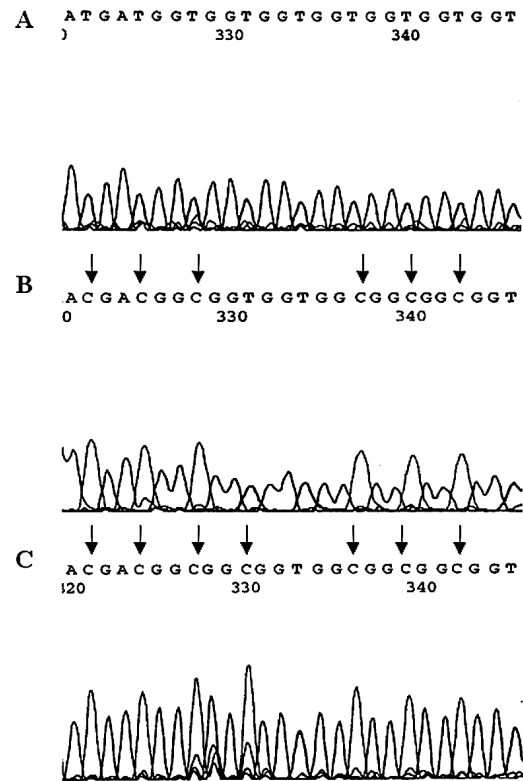


Figure 2. DNA sequence analysis of the MSP bands in lung cancers obtained from lung cancer/IPF patients. (A) Patient no. 3, unmethylated sequence. (B) patient no. 4, methylated sequence. (C) patient no. 8, methylated sequence. Arrows indicate methylated cytosines.

with the vector containing promoter fragment 2 (0.016 ± 0.005) (9 clones) ($p=0.002$, Mann-Whitney U test) (Fig. 4). These data indicate the presence of enhanced promoter activity within the region including the methylated sequence observed in the methylation analysis.

Discussion

The incidence of lung cancer is much higher in patients with IPF than in the general population (1,2). Predisposing factors in the development of lung cancer in IPF include squamous metaplasia, atypical epithelial cell proliferation with nuclear and cytoplasmic changes, and cuboidal and columnar epithelium in the terminal bronchioles (11). We previously reported that allelic loss of the *FHIT* gene was found in these epithelial regions and may be involved in carcinogenesis in the peripheral lung of patients with IPF (3).

To investigate the genetic events involved in the transformation of the epithelial region in patients with IPF, we performed cDNA array analysis to screen genes that were differentially expressed in lung cancer compared with IPF (5). Of the five patients examined, three showed down-regulation of the *Smad4* gene in lung cancer tissue compared with IPF tissue. Problems associated with global analysis include cross hybridization, selection of the control and the definition of significant differences. In this study, all eight lung cancer/IPF patients showed down-regulation of *Smad4* expression in lung cancer tissue compared to normal tracheal cells and normal

forward →

GCCAAACCTGAAATTACCCGGATGTGGTCCCCGCGCGCGCATGCTCAGTGGCTTCTCGACAA

GTTGGCAGCAACAACACGGCCCTGGTCGTCGTCGCCGCTGCGGTAAACGGAGCGGTTTGGGTG

GCGGAGCCTGCGTTTCGCGCCTTCCCGCTCTCCTCGGGAGGCCCTTCTGCTCTCCCTAGGCT

CCGCGGCCGCCCAGGGGGTGGGAGCGGGTGAGGGGAGCCAGGCGCCCAGCGAGAGAGGCC

CCCCGCCGCAGGGCGGCCCGGGAGCTCGAGGCGGTCCGGCCCCGCGCGGGCAGCGGCGCGGC

GCTGAGGAGGGGCGGCCTGGCCGGGACGCCTCGGGGCGGGGGCCGAGGAGCTCTCCGGGCC

GCCGGGGAAAGCTACGGGCCCCGTGCGTCCGCGGACCAGCAGCGCGGGAGAGCGGACTCCC

← reverse2

CTCGCCACCGCCCCGAGCCCAGGTAACCGCGCCATGTCCCTCCCTTCCCCGGCCGGGCCG

CGACCCCGCCTGTGGCTTCCCCGCCCCGGGGCGGGCTCCCGA_CGAC_GGG_CGG_CGG_CGG_C

← reverse1

GG_CGGCTGGGAGCGCGCGGAATCCTCTGGGAGGGCGA_CCGCGCGGCCTGACGAGCCGGGCC

GGGCGGGCCGGCTGAATGCCGGGCGGCGGTGCCTCGCGTCCCTCGGGCCCCAGCTCCGCTTG

CAGCTCGTGGGAGAATCAA

Figure 3. The *Smad4* promoter region was studied for methylation analysis and luciferase assay. Primers for luciferase assays were designed to amplify the promoter regions of *Smad4* with (promoter fragment 1) or without (promoter fragment 2) the methylated region on DNA sequence analysis of the MSP bands (Fig. 2). Primers for promoter fragment 1, forward and reverse 1; primers for promoter fragment 2, forward and reverse 2. Underlined C shows methylated cytosine on DNA sequence analysis of the MSP bands.

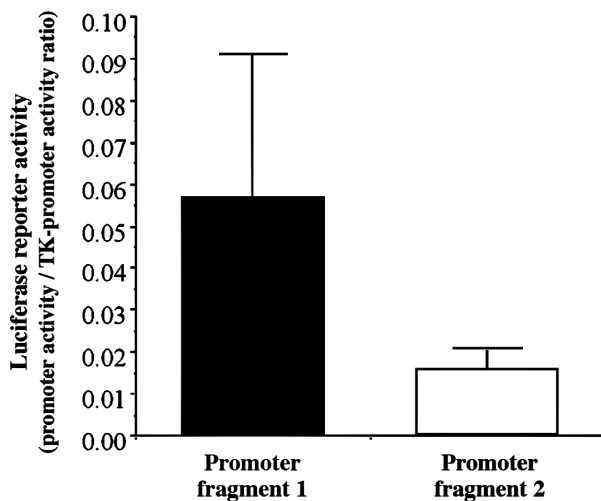


Figure 4. Luciferase assay with *Smad4* promoter reporters using normal human lung DNA. The promoter activity/TK promoter activity ratio was significantly higher in promoter fragment 1 (0.057 ± 0.034) than in promoter fragment 2 (0.016 ± 0.005) ($p=0.002$, Mann Whitney U test).

human lung tissue. Relative expression of the *Smad4* gene was significantly lower in tumors from lung cancer/IPF patients than in tumors from lung cancer without IPF patients or in lung cancer cell lines. These data imply that down-regulation of the *Smad4* gene may be involved in the development of lung cancer in pulmonary fibrosis.

It has also been demonstrated that TGF- β is expressed in increased quantities in the lung tissue of patients with IPF (12,13). TGF- β is a multifunctional cytokine that inhibits

epithelial cell proliferation, and there is a strong correlation between malignant progression and loss of sensitivity to the antiproliferative effects of TGF- β . Tumor cells often escape the antiproliferative effects of TGF- β by the mutational inactivation or dysregulated expression of components in the TGF- β signal pathway (14). Loss of the growth inhibitory response to TGF- β may therefore be crucial in promoting tumor development in the lungs of IPF patients, where TGF- β is highly expressed.

Biological signals for TGF- β are transduced through heteromeric complexes of two transmembrane serine/threonine kinase receptors (TGF- β RI and TGF- β RII), then propagated to a family of Smads. Smads can be subdivided into three classes based on their functional properties: receptor-regulated Smads (Smads 1, 2, 3, 5 and 8), common Smads (Smad4) and antagonistic Smads (Smads 6 and 7) (15,16). *Smad4* was first identified as a candidate tumor suppressor gene in pancreatic carcinomas, in which nearly 40% of patients had lost or had an inactivated version of *Smad4*, and was initially known as *Deleted in pancreatic carcinoma locus 4 (DPC4)* (17). *Smad4* acts as a convergent node in the Smad pathways, downstream of TGF- β superfamily receptors. *Smad4* alterations are largely restricted to tumors of the pancreas and gastrointestinal tract (17,18), with a much lower frequency in those of the lung (19,20). To determine the mechanism of down-regulation of *Smad4* expression in lung carcinoma tissues in patients with IPF, we performed PCR-SSCP analysis. No SSCP variants were found in any exons of *Smad4* or the *Smad4* promoter in tumor samples from eight lung cancer/IPF patients. Therefore, we performed MSP to analyze the methylation status of the promoter regions of the *Smad4* gene. Aberrant methylation of

normally unmethylated CpG islands has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers (21,22). Methylation of gene promoters results in chromatin condensation, limiting accessibility of transcription factors to the DNA (23). In the present study, hypermethylation of the *Smad4* promoter was identified in two of eight (25.0%) patients who showed reduced *Smad4* expression in lung cancer.

To verify the biological significance of the hypermethylation of the *Smad4* promoter observed in methylation analysis, we studied the difference in *Smad4* promoter activity with or without the methylated region using reporter constructs. The decreased promoter activity observed when a 125 base pair including the methylated region was deleted demonstrated the significance of the region and indicated the potential involvement of DNA methylation in reduced expression of the *Smad4* gene in lung cancer/IPF patients.

TGF- β , *TGF- β RII* and other *Smads* were equally expressed between the pairs of lung cancer and IPF samples. It was reported that a mutation in exon 3 of the *TGF- β RII* gene was observed, although at a low incidence, in hyperplastic lesions of alveolar lining epithelial cells in IPF (24). This is a predisposing factor in the development of lung cancer in IPF patients. Mutations of the *TGF- β RII* gene have also been found in various tumors, but incidences of code-altering mutations are rare (25,26). If mutation of the *TGF- β RII* gene occurs in the pre-cancerous lesions of IPF, it would be identified in lung cancers developed from IPF fibrotic lesions. However, a mutation in exon 3 of the *TGF- β RII* gene was not detected in any of the eight patients examined in our study.

Our data suggests that loss of the growth inhibitory response to TGF- β signaling may be crucial in promoting tumor development in patients with IPF, and that hypermethylation of the *Smad4* promoter region may be one mechanism by which this occurs. Other possible mechanisms of *Smad4* down-regulation include alterations in chromatin structure due to histone deacetylation (27). Sporadic lung cancer was also reported to show frequent loss of sensitivity to the antiproliferative effects of TGF- β (28). The mechanism of resistance was reported to be reduced expression of the *TGF- β RII* gene through hypermethylation of the *TGF- β RII* gene promoter region and histone deacetylation (27). Inactivation of *Smad4*, which acts as a convergent node in the Smad pathways downstream of TGF- β superfamily receptors, may play a specific role in the process of pulmonary carcinogenesis in IPF.

In summary, our study demonstrated that down-regulation of the *Smad4* gene may be involved in lung carcinogenesis in patients with IPF. Further investigation in this area may aid in the development of novel preventative measures or modes of treatment for patients with lung cancer and IPF through the regulation of *Smad4* expression. Aberrant DNA methylation of the *Smad4* promoter region may be one possible therapeutic target because of differences between lung cancer and IPF lung tissue.

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