Reduced *ING1b* Gene Expression Plays an Important Role in Carcinogenesis of Non-Small Cell Lung Cancer Patients

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ABSTRACT

Purpose: **We performed a clinical study on *ING1b* gene expression and *p53* gene status in relation to *p53* target genes.**

Experimental Design: **Eighty-eight tumors from surgically treated non-small cell lung cancer (NSCLC) patients were studied. PCR-single-strand conformational polymorphism after sequencing was performed to investigate *ING1* and *p53* gene status. Quantitative reverse transcription-PCR was performed to evaluate the gene expression of *ING1b*, *p21*, and *bax*. The results of p21 and bax expression were confirmed with immunohistochemistry.**

Results: **Only two carcinomas (2.3%) had nonmissense mutations of *ING1b*. Thirty-seven carcinomas (42.0%) had reduced *ING1b* gene expression. Thirty-seven carcinomas (42.0%) had mutations of *p53*. In total, 63 carcinomas (71.6%) had either reduced *ING1b* expression or mutant *p53*. The *p21* gene expression ratio was significantly lower in the *ING1b*-reduced tumors than in the *ING1b*-positive tumors ($P = 0.0029$). Similarly, the *bax* gene expression ratio was significantly lower in the *ING1b*-reduced tumors than in the *ING1b*-positive tumors ($P < 0.0001$), and it was also significantly lower in tumors that had either reduced *ING1b* expression or mutant *p53* than in tumors that had both positive *ING1b* expression and wild-type *p53* ($P = 0.0331$).**

Conclusions: **Reduction of *ING1b* gene expression was associated with reduced *p21* and *bax* gene expression in NSCLCs. The present study is the first clinical report to confirm the positive role of *ING1b* in regulating *p21* and *bax* gene expression. *ING1b* might be one of the tumor suppressor genes that could play a role in carcinogenesis in NSCLC patients.**

INTRODUCTION

It is widely accepted that malignant tumors are caused by the accumulation of genetic alterations, including tumor suppressor genes and oncogenes (1, 2). NSCLCs[2] are also variably affected by the inactivation of tumor suppressor genes such as *p53* (3, 4) and *p16* (5) or the activation of oncogenes such as K-*ras* (4). These genetic alterations principally control the cell cycle, tumor growth, and other malignant behavior. Such heterogeneity in the genetic alterations might result in the variety of clinical behavior of NSCLCs. Therefore, it is important to classify NSCLCs according to their gene status. However, our previous studies on *p53*, *p16*, and K-*ras* in Japanese NSCLC patients showed that mutations of *p53* occurred in 35.4% of patients, reduced *p16* expression occurred in 36.3% of patients, and mutations of K-*ras* occurred in 8.3% of patients (4, 5). In total, 62.0% of NSCLCs had abnormalities in some of the three genes, and 38.0% of NSCLCs had no abnormality in any of these three genes. This result led us to investigate the existence of other new tumor suppressor genes or new oncogenes in NSCLCs.

Recent studies have suggested that the *ING1* gene encodes proteins associated with cell cycle regulation (6, 7), apoptosis (8), and neoplastic transformation (9). The human *ING1* gene is located in chromosome 13q33–34 (10), and it contains three exons (11). The human *ING1* gene produces four isoforms (11–14). Because all four ING1 protein isoforms have a PHD finger motif, they are considered to have transcriptional activity (11). Among these four isoforms, the *ING1b* transcript consists of exons 1a and 2, and its isoform is also known as p33. Recent experimental studies have demonstrated that ING1b proteins can interact with several molecules, such as p53 (7, 15), HAT, and histone deacetylase complexes (16, 17). We considered, therefore, that *ING1b* might play an important role in carcinogenesis. We performed a clinical study on *ING1b* gene expression in relation to *p53* gene status in NSCLC patients. In addition, because *p53* has biological functions through several of its target genes (18–20), evaluation of its target genes in relation to *p53* gene status is important to clarify their influence on carcinogenesis. Among several p53 target genes, *p21* and *bax* are associated with cell cycle regulation and apoptosis (18–20). In addition, they are reported to be prognostic factors in NSCLC

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[2] The abbreviations used are: NSCLC, non-small cell lung cancer; HAT, histone acetyltransferase; SSCP, single-strand conformational polymorphism; RT-PCR, reverse transcription-PCR; PHD, plant homeodomain; PIP, PCNA interacting protein.

Table 1 PCR primers

ING1b	
Sense primer for exon 1a	5′-TGCAGTGCTATTTTTTGAGGGG-3′
Antisense primer for exon 1a	5′-CGCCCCCGCCCATCCATCA-3′
Sense primer for part 1 of exon 2	5′-ACGCCTGTCCTTCTTGCCCC-3′
Antisense primer for part 1 of exon 2	5′-CTTGCCGCTGTTGCCCGCTG-3′
Sense primer for part 2 of exon 2	5′-TTCGAGGCGCAGCAGGAGCT-3′
Antisense primer for part 2 of exon 2	5′-CTTGGCCTTCTTCTCCTTGGG-3′
Sense primer for part 3 of exon 2	5′-CAGCAACCACGACCACGACG-3′
Antisense primer for part 3 of exon 2	5′-TGAGCCCCACGCACGAGAAG-3′
Sense primer for part 4 of exon 2	5′-CCTCCCCATCGACCCCAACG-3′
Antisense primer for part 4 of exon 2	5′-ACATTTTACACTCCTTGCACCTCA-3′
Sense primer for ING1b expression	5′-ATGTTGAGTCCTGCCAACGG-3′
Antisense primer for ING1b expression	5′-CGATCTGGATCTTCTCGTCG-3′
p53	
Sense primer for exon 5	5′-TCTGTCTCCTTCCTCTTCCT-3′
Antisense primer for exon 5	5′-TCTCCAGCCCCAGCTGCT-3′
Sense primer for exon 6	5′-TGATTCCTCACTGATTGCTCT-3′
Antisense primer for exon 6	5′-GAGACCCCAGTTGCAAACC-3′
Sense primer for exon 7	5′-TCTTGGGCCTGTGTTATCTC-3′
Antisense primer for exon 7	5′-AGGGTGGCAAGTGGCTCC-3′
Sense primer for exon 8	5′-GCTTCTCTTTTCCTATCCTGA-3′
Antisense primer for exon 8	5′-CGCTTCTTGTCCTGCTTGC-3′
p21	
Sense primer	5′-AGGATCCATGTCAGAACCGGCTGG-3′
Antisense primer	5′-CAGGATCCTGTGGGCGGATTAGGGCT-3′
bax	
Sense primer	5′-GGATGCGTCCACCAAGAAGC-3′
Antisense primer	5′-GCACTCCCGCCACAAAGATG-3′
β-*Actin*	
Sense primer	5′-GATATCGCCGCGCTCGTCGTCGAC-3′
Antisense primer	5′-CAGGAAGGAAGGCTGGAAGAGTGC-3′

patients (21, 22). Therefore, we performed an additional study to evaluate *p21* and *bax* gene expression in relation to *ING1b* and *p53* status.

MATERIALS AND METHODS

Patient Characteristics. From April 1999 to November 2000, 88 NSCLC patients, who underwent surgery at the Second Department of Surgery of Kagawa Medical University or the Department of Thoracic Surgery of the Japanese Red Cross Society Wakayama Medical Center, were studied. They included 35 patients with adenocarcinoma, 48 patients with squamous cell carcinoma, and 5 patients with large cell carcinomas. Tumor-node-metastasis (TNM) staging designations were made according to the postsurgical pathological international staging system (23). The patients' clinical records and histopathological diagnoses were fully documented.

PCR-SSCP and Sequencing for *ING1* and *p53* Gene Mutation. To investigate the mutations of *ING1* and *p53*, we performed PCR-SSCP and direct sequencing as described in previous reports (4, 13). The genomic DNA of tumors was extracted from frozen specimens by proteinase K digestion and phenol/chloroform extraction. For the coding region of exons 1a and 2 of the *ING1* gene, the primers shown in Table 1 were used (13). Exon 2 of *ING1* was amplified as four overlapping fragments with four primer sets, and then 30 cycles of PCR amplification of exons 1a and 2 of the *ING1* gene were performed. To detect the mutations of *p53*, 40 cycles of PCR amplification of exons 5–8 of the *p53* gene were performed using the primers shown in Table 1. Electrophoresis for SSCP was done to detect the mutant bands. Seven μl of PCR products were diluted with 10 μl of buffer consisting of 20 mM EDTA, 96% deionized formamide, and 5 mg/ml Dextran Blue 2000. Heating denaturation was performed at 95°C for 5 min, after which the samples were placed on ice for 5 min. Sixteen μl of this solution were then applied to each lane of a 7.5% neutral polyacrylamide gel. Electrophoresis was performed at 15 mA in buffer at a temperature of 18°C. The gel was stained with 2 μg/ml ethidium bromide and visualized under UV light. Finally, to ascertain the base changes and exclude the nonmissense mutations detected by PCR-SSCP, sequencing was performed using ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kits (Perkin-Elmer) according to the manufacturer's protocol.

Quantitative RT-PCR for *ING1b*, *p21*, and *bax* Gene Expression. Total cellular RNA was extracted from frozen tissue specimens by the acid guanidinium thiocyanate procedure. First-strand cDNA synthesis was performed with 5 μg of total RNA using a cDNA synthesis kit (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. For PCR amplification, we used a 1-μl aliquot of the reaction mixture. To obtain a reproducible quantitative performance of the RT-PCR assay for *ING1b*, *p21*, and *bax* gene expression, we used the primers shown in Table 1. The primers for *ING1b* were designed to span from exon 1a to exon 2 of the *ING1* gene on the basis of the nucleotide sequence. The primers for *p21* and *bax* were based on previously published reports (24, 25). β-*actin* DNA amplification was used as the internal PCR control (26). We titrated the amount of starting cDNA and the number of amplification cycles. All subsequent assays were carried out by using

the parameters that yielded amplification of every PCR product within a linear range. For *ING1b* gene expression, the reaction mixture was subjected to 31 PCR amplification cycles of 60 s at 94°C, 60 s at 55°C, and 90 s at 72°C. For *p21*, *bax*, and β-*actin* gene expression, the reaction mixture was subjected to 31 PCR amplification cycles of 60 s at 94°C, 60 s at 60°C, and 90 s at 72°C. Tubes containing all of the ingredients except the templates were included in all runs and served as negative reaction controls. Preparations of human adenocarcinoma cell line A-549 were used as positive controls for *ING1b*, *p21*, and *bax* gene expression.

The amplified DNA samples were run on a 1% agarose gel, and the bands were visualized with ethidium bromide and photographed with a charge-coupled device camera recording system. Densitometric analysis of the photographic negatives was used for band quantification. The densitometric value obtained for the band of each PCR product in a given tumor sample was divided by the value of the β-*actin*, and the resultant ratio was referred to as the gene expression ratio. The expression ratio for a given tumor sample was then divided by the expression ratio of the human adenocarcinoma cell line A-549 to obtain the standardized *ING1b*, *p21*, and *bax* gene expression ratio. All values of gene expression ratio were expressed as mean ± SD.

Immunohistochemistry for p21 and bax Protein Expression. To confirm the results of *p21* and *bax* gene expression on RT-PCR, immunohistochemical studies were performed as described previously (21, 22). A mouse monoclonal antibody for p21 (clone EA10; Oncogene Science, Cambridge, MA) diluted 1:50 and a rabbit polyclonal antibody for bax (N-20; Santa Cruz Biotechnology Inc., Santa Cruz Biotechnology, CA) diluted 1:100 were used. Formalin-fixed paraffin-embedded tissue was cut in 4-μm sections and mounted on poly-L-lysine-coated slides. Sections were deparaffinized and rehydrated. The slides were then heated in a microwave for 10 min in a 10 μM citrate buffer solution at pH 6.0 and cooled to room temperature for 20 min. After quenching the endogenous peroxidase activity with 0.3% H_2O_2 (in absolute methanol) for 30 min, the sections were blocked for 2 h at room temperature with 5% BSA. Subsequently, duplicate sections were incubated overnight with the primary specific antibodies detecting p21 and bax, respectively. Slides were then incubated for 1 h with biotinylated antimouse IgG (Vector Laboratories Inc., Burlingame, CA) for p21 or biotinylated antirabbit IgG (Vector Laboratories Inc.) for bax. The sections were incubated with the avidin-biotin-peroxidase complex (Vector Laboratories) for 1 h, and antibody binding was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Finally, the sections were lightly counterstained with Mayer's hematoxylin. Sections of resected lung tumors known to express p21 or bax were used as positive controls. Sections incubated with normal rabbit IgG served as a negative reaction control for staining of p21 and bax.

All of the immunostained sections were reviewed by two pathologists who had no knowledge of the patients' clinical status. In cases of multiple areas of low intensity, five areas selected at random were scored, and in sections where all of the staining appeared intense, one random field was selected. The proportion of high and low staining tumor cells in each selected field was determined by counting individual tumor cells at high magnification. At least 200 tumor cells were scored per high-powered field. Finally, all sections were evaluated by the percentage of stained tumor cells, nuclear staining of p21, and cytoplasmic staining of bax, respectively.

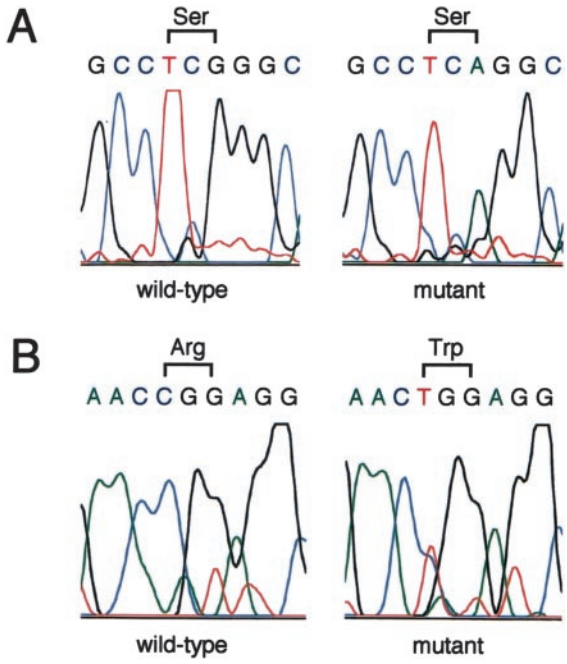

Fig. 1 Direct DNA sequencing for *ING1* and *p53* point mutation. *A*, a carcinoma with a nonmissense mutation of *ING1* [Ser (TCG) to Ser (TCA) at codon 173]. *B*, a carcinoma with a missense mutation of *p53* [Arg (CGG) to Trp (TGG) at codon 248].

Statistical Analysis. Because the distributions of the standardized gene expression ratio of *ING1b*, *p21*, and *bax* were normal distributions (Kolmogorov-Smirnov analysis, *ING1b*, $P > 0.9999$; *p21*, $P = 0.0749$; *bax*, $P > 0.9999$, respectively), the statistical significances of these gene expressions in relation to several clinical and pathological parameters were assessed by *t* test or ANOVA with the Bonferroni/Dunn test. In addition, because the *ING1b* expression cutoff line of 0.7 demonstrated the most significance in the relationships between *ING1b* expression and *p21*, *bax* expression, the sample was classified as an *ING1b*-reduced group when the standardized *ING1b* gene expression ratio was <0.7. If the standardized *ING1b* gene expression ratio was ≥ 0.7, the sample was classified as an *ING1b*-positive group. The statistical differences of *p53* gene status in relation to several clinical and pathological parameters were assessed by the χ^2 test. All *P*s were based on two-tailed statistical analysis, and a *P* of <0.05 was considered to indicate statistical significance.

RESULTS

***ING1* Gene Status in NSCLCs.** Of the 88 NSCLCs studied, only 2 carcinomas (2.3%) had point mutations of the coding regions of *ING1b*. One carcinoma had a G-to-A substitution at the third nucleotide of codon 173 (Fig. 1*A*). The other carcinoma had a C-to-T transition at the third nucleotide of codon 145. These two tumors had nonmissense mutations in nuclear localization sequences of *ING1b*. However, there was

Table 2 *ING1b* gene expression and *p53* status in 88 NSCLC patients according to clinicopathological characteristics

		ING1b				*p53*		
	No. of patients	Standardized gene expression ratio	Positive	Reduced	*P*	Wild-type	Mutant	*P*
Gender								
Male	61	0.804 ± 0.431	37	24	0.4404	30	31	0.0122
Female	27	0.688 ± 0.327	14	13		21	6	
Smoking								
Nonsmoker	22	0.699 ± 0.328	11	11	0.3823	17	5	0.0340
Smoker	66	0.792 ± 0.426	40	26		34	32	
Tumor status								
T_1/T_2	54	0.790 ± 0.408	32	22	0.7547	36	18	0.0369
T_3/T_4	34	0.735 ± 0.402	19	15		15	19	
Nodal status								
N_0	59	0.737 ± 0.429	32	27	0.3136	35	24	0.7109
N_1/N_2	29	0.832 ± 0.345	19	10		16	13	
Pathological stage								
Stage I	34	0.769 ± 0.447	19	15	0.7547	23	11	0.1439
Stage II/III	54	0.768 ± 0.379	32	22		28	26	
Histological type								
Adenocarcinoma	35	0.661 ± 0.291	17	18	0.1446	25	10	0.1106
Squamous cell carcinoma	48	0.830 ± 0.461	31	17		26	22	
Large cell carcinoma	5	0.934 ± 0.394	3	2		0	5	
Total	88	0.768 ± 0.404	51	37		51	37	

no tumor with missense mutations of the coding regions of *ING1b* among the 88 NSCLCs we studied.

***ING1b* Gene Expression in NSCLCs.** In our pilot study using normal lung tissues, the standardized *ING1b* gene expression ratio of normal lung tissues was 0.991 ± 0.142, the same as that of human adenocarcinoma cell line A-549 (data not shown). Of the 88 NSCLCs studied, the standardized *ING1b* gene expression ratio varied greatly (0.768 ± 0.404; Table 2; Fig. 2*A*). With regard to tumor histology, the standardized *ING1b* gene expression ratio was 0.661 ± 0.291 in adenocarcinomas, 0.830 ± 0.461 in squamous cell carcinomas, and 0.934 ± 0.394 in large cell carcinomas. *ING1b* gene expression had a tendency to be higher in squamous cell carcinomas than in adenocarcinomas ($P = 0.0601$ by Bonferroni/Dunn test). Furthermore, 51 carcinomas (58.0%) were classified into the *ING1b*-positive group, and 37 carcinomas (42.0%) were classified into the *ING1b*-reduced group (Table 2). There was no significance in *ING1b* gene expression in relation to gender, smoking, tumor status, nodal status, or pathological stage.

***p53* Gene Status in NSCLCs.** Of the 88 NSCLCs studied, 41 carcinomas (46.6%) had mutations of *p53* (Fig. 1*B*). All cases had point mutations, and four cases with nonmissense mutations were excluded from the mutant group and classified into the wild-type group in the clinical analysis. Finally, 37 carcinomas (42.0%) had mutations in *p53* (Table 2). With regard to tumor histology, 10 tumors (28.6%) had mutations in *p53* among the 35 adenocarcinomas. Twenty-two tumors (45.8%) had mutations in *p53* among the 48 squamous cell carcinomas. All large cell carcinomas had mutations in *p53*.

With regard to *p53* status in relation to clinicopathological parameters, the *p53* mutation rate of males was significantly higher than that of females (50.8% *versus* 22.2%, $P = 0.0122$; Table 2). The *p53* mutation rate of smoker patients was significantly higher than that of nonsmoker patients (48.5% *versus* 22.7%, $P = 0.0340$). With regard to tumor status, the *p53* mutation rate of T_3 to T_4 tumors was significantly higher than that of T_1 to T_2 tumors (55.9% *versus* 33.3%, $P = 0.0369$). However, there were no significant relationships between *p53* gene mutation and nodal status or pathological stage.

***ING1b* Gene Expression and *p53* Gene Status in NSCLCs.** Of the 88 NSCLCs studied, 25 carcinomas (28.4%) had both positive *ING1b* expression and wild-type *p53* (Fig. 3). Twenty-six carcinomas (29.5%) had reduced *ING1b* expression and wild-type *p53*. Twenty-six carcinomas (29.5%) had positive *ING1b* expression and mutant *p53*. Eleven carcinomas (12.5%) had both reduced *ING1b* expression and mutant *p53*. In total, 63 carcinomas (71.6%) had either reduced *ING1b* expression or mutant *p53*. In addition, there was no significant relationship between *ING1b* gene expression and *p53* gene status.

Expression of *p21* in NSCLCs. With regard to *p21* gene expression evaluated by RT-PCR, the standardized *p21* gene expression ratio of NSCLCs varied greatly (0.352 ± 0.376; Fig. 2*B*). With regard to tumor histology, the standardized *p21* gene expression ratio was 0.247 ± 0.273 in adenocarcinomas, 0.394 ± 0.381 in squamous cell carcinomas, and 0.683 ± 0.692 in large cell carcinomas.

p21 protein expression was evaluated by immunohistochemistry, and p21 expression exhibited a nuclear staining pattern (Fig. 4*A*). In addition, the standardized *p21* gene expression ratio evaluated by RT-PCR was highly associated with the percentage of p21-positive tumor cells evaluated by immunohistochemical staining ($r = 0.709$; $P < 0.0001$). The immunohistochemical results agreed well with those from the RT-PCR assays, and 89.8% of the samples coincided exactly.

Expression of *bax* in NSCLCs. With regard to *bax* gene expression evaluated by RT-PCR, the standardized *bax* gene expression ratio of NSCLCs also varied greatly (0.816 ± 0.388; Fig. 2*C*). With regard to tumor histology, the standardized *bax* gene expression ratio was 0.717 ± 0.273 in adenocarcinomas,

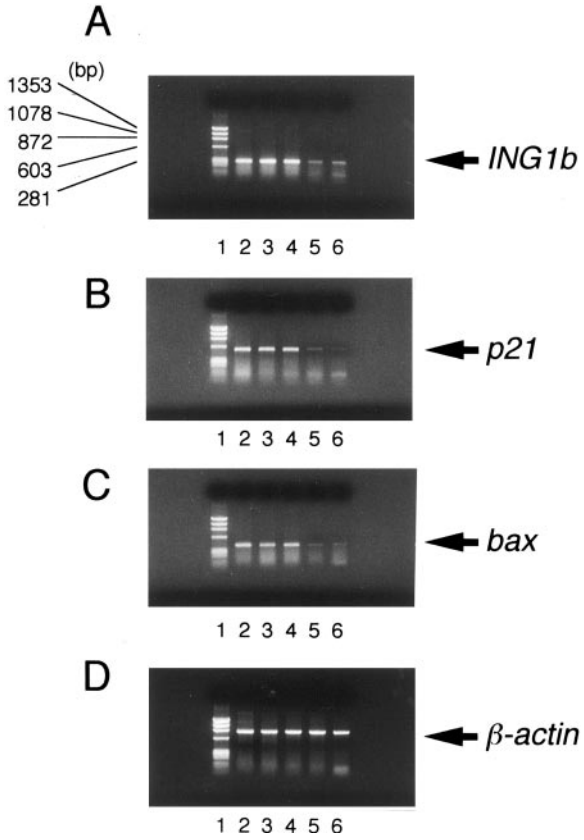

Fig. 2 Agarose gel electrophoresis of RT-PCR-amplified cDNA of *ING1b* (*A*), *p21* (*B*), *bax* (*C*), and β-*actin* (*D*). *Lanes 1*, size marker; *Lanes 2*, human adenocarcinoma cell line A-549; *Lanes 3* and *4*, adenocarcinoma and squamous cell carcinoma with positive gene expression of *ING1b*, *p21*, and *bax*; *Lanes 5* and *6*, adenocarcinoma and squamous cell carcinoma with reduced gene expression of *ING1b*, *p21*, and *bax*.

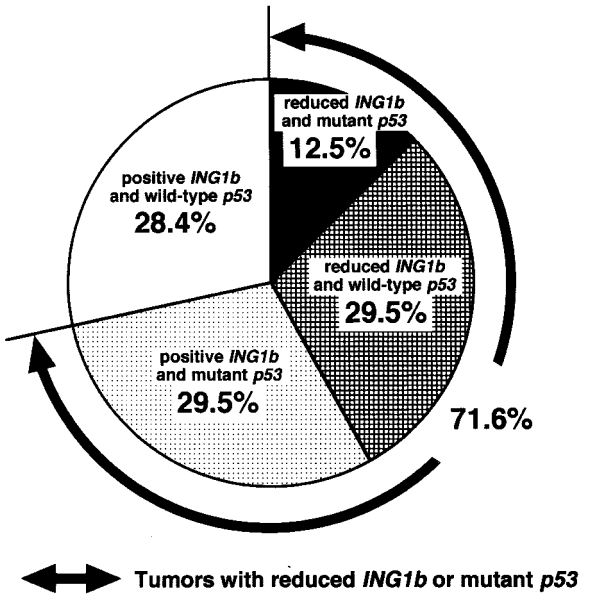

Fig. 3 Distributions of *ING1b* gene expression and *p53* gene status in 88 NSCLCs.

0.860 ± 0.432 in squamous cell carcinomas, and 1.090 ± 0.485 in large cell carcinomas.

bax protein expression was evaluated by immunohistochemistry, and a cytoplasmic staining pattern was found for bax (Fig. 4*C*). The standardized *bax* gene expression ratio evaluated by RT-PCR was also associated with the percentage of bax-positive tumor cells evaluated by immunohistochemical staining ($r = 0.707$; $P < 0.0001$). The immunohistochemical results also agreed well with those from the RT-PCR assays, and 88.6% of the samples coincided exactly.

***p21* Gene Expression in Relation to ING1b Gene Expression and *p53* Gene Status.** With regard to *ING1b* gene expression, the standardized *p21* gene expression ratio was 0.452 ± 0.447 in 51 *ING1b*-positive tumors, whereas it was 0.214 ± 0.175 in 37 *ING1b*-reduced tumors (Fig. 5*A*). The standardized *p21* gene expression ratio was significantly lower in the *ING1b*-reduced tumors than in the *ING1b*-positive tumors ($P = 0.0029$ by *t* test).

With regard to *ING1b* gene expression and *p53* gene status, the standardized *p21* gene expression ratio was 0.386 ± 0.320 in 25 tumors that had both positive *ING1b* expression and wild-type *p53* and 0.339 ± 0.397 in 63 tumors that had either reduced *ING1b* expression or mutant *p53* (Fig. 5*B*). There was no significant difference in *p21* gene expression between these two groups.

***bax* Gene Expression in Relation to *ING1b* Gene Expression and *p53* Gene Status.** With regard to *ING1b* gene expression, the standardized *bax* gene expression ratio was 0.984 ± 0.360 in 51 *ING1b*-positive tumors, whereas it was 0.585 ± 0.298 in 37 *ING1b*-reduced tumors (Fig. 6*A*). The standardized *bax* gene expression ratio was significantly lower in the *ING1b*-reduced tumors than in the *ING1b*-positive tumors ($P < 0.0001$ by *t* test).

With regard to *ING1b* gene expression and *p53* gene status, the standardized *bax* gene expression ratio was 0.955 ± 0.322 in 25 tumors that had both positive *ING1b* expression and wild-type *p53* and 0.761 ± 0.400 in 63 tumors that had either reduced *ING1b* expression or mutant *p53* (Fig. 6*B*). The standardized *bax* gene expression ratio was significantly lower in tumors that had either reduced *ING1b* expression or mutant *p53* than in tumors that had both positive *ING1b* expression and wild-type *p53* ($P = 0.0331$ by *t* test).

DISCUSSION

The *ING1* gene is considered to have a role in cell cycle regulation (6, 7), DNA repair (15), apoptosis (8), and neoplastic transformation (9). It is widely preserved in many species, such as human beings (11), mice (27), and yeast (16, 28, 29). The human *ING1* gene produces four isoforms from three different promoter regions (11–14). Among these four isoforms, several facts from recent experimental studies demonstrated that *ING1b* has important biological functions. Cheung *et al.* (15) showed that overexpression of *ING1b* enhances the repair of UV-damaged DNA and that *p53* is required for this *ING1b*-mediated

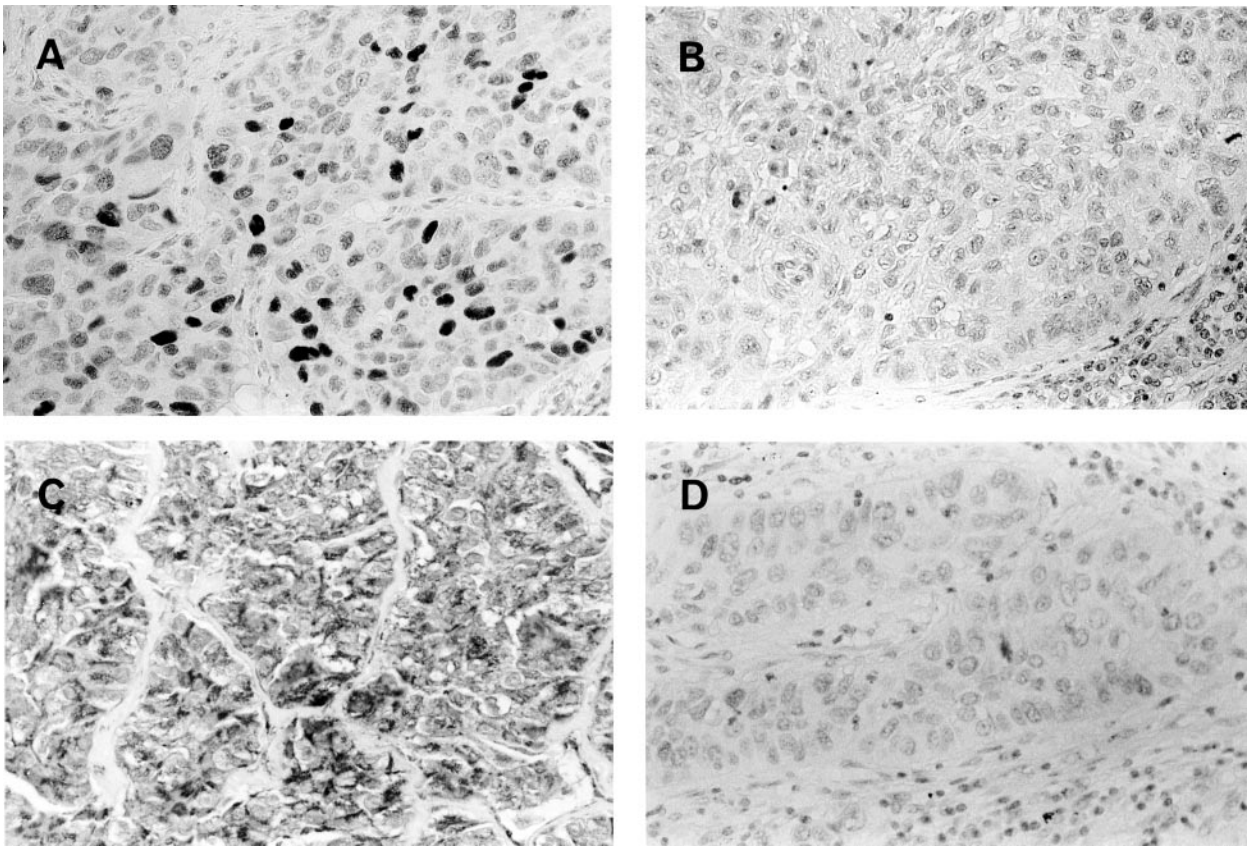

Fig. 4 Immunohistochemical staining of human NSCLC tissues using the avidin-biotin-peroxidase complex procedure. Original magnification, ×100. *A,* squamous cell carcinoma with positive p21 expression. *B,* squamous cell carcinoma with negative p21 expression. *C,* squamous cell carcinoma with positive bax expression. *D,* squamous cell carcinoma with negative bax expression.

DNA repair. It was also reported that the PIP domain, which is located in exon 1a of *ING1b* and binds to proliferating cell nuclear antigen, plays an important role in DNA repair and apoptosis (30). In addition, *p37*, a mouse homologue of *ING1*, is reported to be equivalent to human *ING1b* (27). It has been shown that the p37 binds to and interferes with the accumulation of p53 protein and that p37 forms a complex with p53 by immunoprecipitation (27). Furthermore, recent studies provide evidence that human ING1 proteins are involved in chromatin remodeling functions through stable physical association with protein complexes that have HAT and histone deacetylase activity (11, 16, 17). In particular, ING1b proteins affect the degree of physical association between proliferating cell nuclear antigen and p300 (17). ING1b proteins are therefore considered to be associated with DNA repair, apoptosis, and chromatin remodeling via the multiple protein complexes.

Considering these findings from experimental studies, we considered *ING1b* to be the most important of the four human *ING1* isoforms, and therefore, we performed this clinical study on *ING1b* expression in relation to *p53* in NSCLC patients. Our present study demonstrated that 42.0% of NSCLCs had reduced *ING1b* expression, whereas 46.6% of NSCLCs had mutations of the *p53* gene. In total, 71.6% of NSCLCs had either reduced *ING1b* expression or mutant *p53* gene. These tumors were considered to have some abnormalities in *ING1b*-associated *p53*-dependent pathways, which could cause the disruption of DNA repair and apoptosis and might result in accumulation of further genetic alteration and carcinogenesis (7, 15). Reduced *ING1b* expression, in particular, could play an important role in carcinogenesis in tumors with wild-type *p53*. In addition, our study in NSCLCs showed that *ING1b* expression is independent of *p53* gene status, which is in agreement with a previous report (31).

With regard to the dysfunction of the ING1 protein, previous clinical studies (32–34) revealed that mutation of the *ING1* gene is a rare event in human cancers, as seen in our present study. In contrast, reduced *ING1* gene expression has been reported to be frequent in human cancers (34–37). Although the true mechanism of reduced *ING1* expression is still not clear, it was suggested that the *ING1* gene and flanking regions are highly GC rich and that methylation of the promoter region may cause a loss of gene product (13).

Furthermore, combined study of the *p53* target gene in relation to the *p53* and *ING1b* status is also considered to be important to clarify the biological mechanism in the *p53*-dependent pathway. We therefore studied *p21* and *bax* gene expression in relation to *ING1b* and *p53* status. Also, we have demonstrated that reduced *ING1b* expression in NSCLCs is

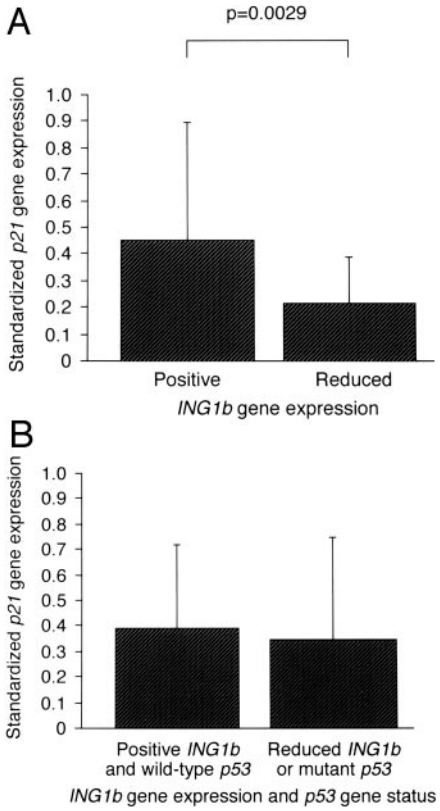

Fig. 5 *A*, standardized *p21* gene expression ratio of NSCLCs in relation to *ING1b* gene expression. *B*, standardized *p21* gene expression ratio of NSCLCs in relation to *ING1b* gene expression and *p53* gene status.

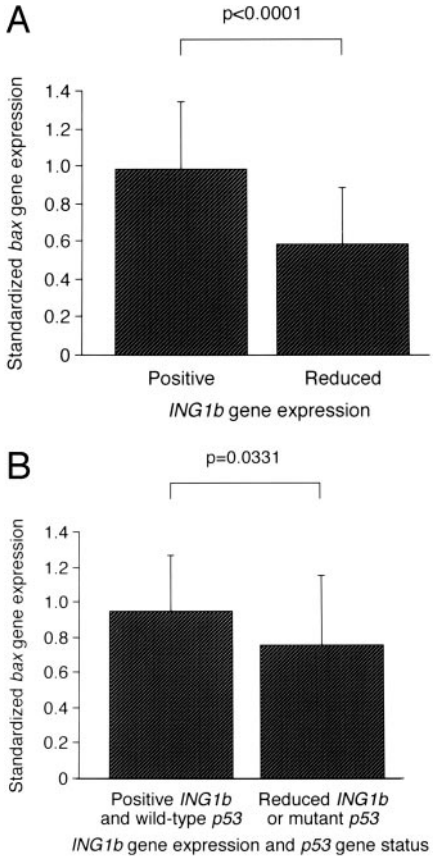

Fig. 6 *A*, standardized *bax* gene expression ratio of NSCLCs in relation to *ING1b* gene expression. *B*, standardized *bax* gene expression ratio of NSCLCs in relation to *ING1b* gene expression and *p53* gene status.

associated with reduced expression of the *p21* and *bax* genes. To our knowledge, the present study is the first clinical report to confirm the positive role of *ING1b* in regulating *p21* and *bax* gene expression.

Initially, p21, a potent inhibitor of cyclin-dependent kinases and a product of WAF1, was considered to be a downstream effector in the p53-dependent pathway of growth control (18). p21 is required for the p53-mediated G_1 arrest and apoptosis in response to DNA damage (19). Previous clinical studies in lung cancer patients showed that *p21* expression is related to tumor differentiation (38) and that reduced *p21* expression is associated with poor prognosis (21). Our study in NSCLCs has demonstrated that reduced *ING1b* expression is associated with reduced *p21* expression, regardless of whether the *p53* gene is wild type or mutant. This fact implies that reduced *ING1b* expression would affect the down-regulation of *p21* gene expression and that *ING1b* could act as one of the tumor suppressor genes in human cancers. There was no significant relationship between *p53* gene status and *p21* expression, however, in the NSCLCs we studied. Several clinical reports have also revealed that p53-independent *p21* expression is a common feature of human lung cancers (38, 39). This might be partly because of the p53-independent regulation of *p21* expression (40, 41). Although p53 is one of the regulators of *p21* gene expression, there are many p53-independent regulations of *p21* gene expression through several binding sites within the *p21* promoter (40). In particular, Sp1 binding sites are considered to play a major role in the regulation of *p21* transcription. In addition, a recent experimental study has demonstrated that ING1b proteins interact with proteins associated with HAT activity, such as p300 and CREB-binding protein (17). The p300/CREB-binding protein is also reported to cooperate with Sp1 to induce *p21* gene expression from Sp1 binding sites of the *p21* promoter (40). From these results, hypothetically, ING1b might regulate *p21* gene expression through the p53-independent pathway.

Similarly, bax is a member of the bcl-2 family and a regulator of apoptosis (42) and cell proliferation (43). The *bax* gene promoter region contains four motifs with homology to consensus p53-binding sites, and p53 is a direct activator of the *bax* gene (20). Our previous study (22) in NSCLC patients has shown that *bax* expression is associated with *p53* gene status and that *bax* expression is a prognostic factor in NSCLC patients. Our present study has demonstrated that reduced *ING1b* expression is associated with reduced *bax* expression and that tumors with either reduced *ING1b* or mutant *p53* have a significantly low expression of the *bax* gene. These results suggest that reduced *ING1b* expression would affect the down-regulation of *bax* gene expression through the p53-dependent pathway.

In summary, our study in NSCLCs demonstrated that the *ING1b* gene may act as a tumor suppressor gene and that reduced *ING1b* expression is associated with the down-regulation of *p21* and *bax* gene expression. These results raise clinical problems associated with chemotherapy, radiotherapy, or *p53* gene therapy (44) because ING1b and p53 work cooperatively. *p53* status is a major predictor of response to chemotherapy or radiotherapy in human cancers (45). Therefore, *ING1b*-reduced tumors may be resistant to chemotherapy and radiotherapy because of the dysfunction of the p53-dependent pathway. In addition, the effect of adenovirus-mediated *p53* gene therapy might be reduced in patients with *ING1b*-reduced tumors. The coinfection approach using adenovirus-mediated transfer of *p53* and *ING1b* may be indicated in patients with *ING1b*-reduced tumors (46). Therefore, it is important for the treatment of cancer patients to evaluate not only *p53* gene status but also *ING1b* gene expression.

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