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

Kotaro Kameyama, Cheng-long Huang,1 Dage Liu, Daiki Masuya, Takashi Nakashima, Shinichi Sumitomo, Yuji Takami, Moritoshi Kinoshita, and Hiroyasu Yokomise

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 nonsense 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). NSCLCs2 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.

Received 2/19/03; revised 6/9/03; accepted 6/16/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported in part by Grant 12470240 (to C-I. H.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

1 To whom requests for reprints should be addressed, at The Second Department of Surgery, Kagawa Medical University, I.-5701, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. Phone: 81-87-891-2191; Fax: 81-87-891-2192; E-mail: chuang@kms.ac.jp.

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.
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 ING1b, p21, and bax 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

<table>
<thead>
<tr>
<th>Table 1</th>
<th>PCR primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ING1b</strong></td>
<td>Sense primer for exon 1a</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for exon 1a</td>
</tr>
<tr>
<td></td>
<td>Sense primer for part 1 of exon 2</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for part 1 of exon 2</td>
</tr>
<tr>
<td></td>
<td>Sense primer for part 2 of exon 2</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for part 2 of exon 2</td>
</tr>
<tr>
<td></td>
<td>Sense primer for part 3 of exon 2</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for part 3 of exon 2</td>
</tr>
<tr>
<td></td>
<td>Sense primer for part 4 of exon 2</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for part 4 of exon 2</td>
</tr>
<tr>
<td></td>
<td>Sense primer for ING1b expression</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for ING1b expression</td>
</tr>
<tr>
<td><strong>p53</strong></td>
<td>Sense primer for exon 5</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for exon 5</td>
</tr>
<tr>
<td></td>
<td>Sense primer for exon 6</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for exon 6</td>
</tr>
<tr>
<td></td>
<td>Sense primer for exon 7</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for exon 7</td>
</tr>
<tr>
<td></td>
<td>Sense primer for exon 8</td>
</tr>
<tr>
<td></td>
<td>Antisense primer for exon 8</td>
</tr>
<tr>
<td><strong>p21</strong></td>
<td>Sense primer</td>
</tr>
<tr>
<td></td>
<td>Antisense primer</td>
</tr>
<tr>
<td></td>
<td>Sense primer</td>
</tr>
<tr>
<td></td>
<td>Antisense primer</td>
</tr>
<tr>
<td><strong>bax</strong></td>
<td>Sense primer</td>
</tr>
<tr>
<td></td>
<td>Antisense primer</td>
</tr>
</tbody>
</table>
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 mM citrate buffer solution at pH 6.0 and cooled to room temperature for 20 min. After quenching the endogenous peroxidase activity with 3% H2O2 (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 for 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.

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 χ2 test. All Ps 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-T substitution at the third nucleotide of codon 173 (Fig. 1A). The other carcinoma had a C-to-T transition at the third nucleotide of codon 145. These two tumors had nonmissense mutations in ING1b. One carcinoma had a G-to-A substitution at the third nucleotide of codon 173. B, a carcinoma with a missense mutation of p53 [Arg (CGG) to Trp (TGG) at codon 248].
no tumor with missense mutations of the coding regions of \textit{ING1b} among the 88 NSCLCs we studied.

\textbf{\textit{ING1b} Gene Expression in NSCLCs.} In our pilot study using normal lung tissues, the standardized \textit{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 \textit{ING1b} gene expression ratio varied greatly (0.768 ± 0.404; Table 2; Fig. 2A). With regard to tumor histology, the standardized \textit{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. \textit{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 \textit{ING1b}-positive group, and 37 carcinomas (42.0%) were classified into the \textit{ING1b}-reduced group (Table 2). There was no significance in \textit{ING1b} gene expression in relation to gender, smoking, tumor status, nodal status, or pathological stage.

\textbf{\textit{p53} Gene Status in NSCLCs.} Of the 88 NSCLCs studied, 41 carcinomas (46.6%) had mutations of \textit{p53} (Fig. 1B). All cases had point mutations, and four cases with missense 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 \textit{p53} (Table 2). With regard to tumor histology, 10 tumors (28.6%) had mutations in \textit{p53} among the 35 adenocarcinomas. Twenty-two tumors (45.8%) had mutations in \textit{p53} among the 48 squamous cell carcinomas. All large cell carcinomas had mutations in \textit{p53}.

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

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

\textbf{Expression of \textit{p21} in NSCLCs.} With regard to \textit{p21} gene expression evaluated by RT-PCR, the standardized \textit{p21} gene expression ratio of NSCLCs varied greatly (0.352 ± 0.376; Fig. 2B). With regard to tumor histology, the standardized \textit{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.

\textbf{\textit{p21} protein expression was evaluated by immunohistochemistry, and \textit{p21} expression exhibited a nuclear staining pattern (Fig. 4A). In addition, the standardized \textit{p21} gene expression ratio evaluated by RT-PCR was highly associated with the percentage of \textit{p21}-positive tumor cells evaluated by immunohistochemistry staining (\(r = 0.790\); \(P < 0.0001\)). The immunohistochemical results agreed well with those from the RT-PCR assays, and 89.8% of the samples coincided exactly.

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

\begin{table}[h]
\centering
\caption{\textit{ING1b} gene expression and \textit{p53} status in 88 NSCLC patients according to clinicopathological characteristics}
\begin{tabular}{|l|c|c|c|c|c|}
\hline
& \textbf{No. of patients} & \textbf{Standardized gene expression ratio} & \textbf{Wild-type} & \textbf{Mutant} & \textbf{P} \\
\hline
\textbf{Gender} & & & & & \\
\textbf{Male} & 61 & 0.804 ± 0.431 & 37 & 24 & 0.4404 & 30 & 31 & 0.0122 \\
\textbf{Female} & 27 & 0.688 ± 0.327 & 14 & 13 & & 21 & 6 & \\
\hline
\textbf{Smoking} & & & & & \\
\textbf{Nonsmoker} & 22 & 0.699 ± 0.328 & 11 & 11 & 0.3823 & 17 & 5 & 0.0340 \\
\textbf{Smoker} & 66 & 0.792 ± 0.426 & 40 & 26 & & 34 & 32 & \\
\hline
\textbf{Tumor status} & & & & & \\
\textbf{T_1/T_2} & 54 & 0.790 ± 0.408 & 32 & 22 & 0.7547 & 36 & 18 & 0.0369 \\
\textbf{T_3/T_4} & 34 & 0.735 ± 0.402 & 19 & 15 & & 15 & 19 & \\
\hline
\textbf{Nodal status} & & & & & \\
\textbf{N_0} & 59 & 0.737 ± 0.429 & 32 & 27 & 0.3136 & 35 & 24 & 0.7109 \\
\textbf{N_1/N_2} & 29 & 0.832 ± 0.345 & 19 & 10 & & 16 & 13 & \\
\hline
\textbf{Pathological stage} & & & & & \\
\textbf{Stage I} & 34 & 0.769 ± 0.447 & 19 & 15 & 0.7547 & 23 & 11 & 0.1439 \\
\textbf{Stage II/III} & 54 & 0.768 ± 0.379 & 32 & 22 & & 28 & 26 & \\
\hline
\textbf{Histological type} & & & & & \\
\textbf{Adenocarcinoma} & 35 & 0.661 ± 0.291 & 17 & 18 & 0.1446 & 25 & 10 & 0.1106 \\
\textbf{Squamous cell carcinoma} & 48 & 0.830 ± 0.461 & 31 & 17 & & 26 & 22 & \\
\textbf{Large cell carcinoma} & 5 & 0.934 ± 0.394 & 3 & 2 & & 0 & 5 & \\
\hline
\textbf{Total} & 88 & 0.768 ± 0.404 & 51 & 37 & & 51 & 37 & \\
\hline
\end{tabular}
\end{table}
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. 4C). 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. 5A). 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. 5B). 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. 6A). 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. 6B). 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...
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. 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.
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 G1 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 chemotheraphy, radiotherapy, or p53 gene therapy (44) because ING1b and p53 work cooperatively. p53 status is a major predictor of response to chemotheraphy or radiotherapy in human cancers (45). Therefore, ING1b-reduced tumors may be resistant to chemotheraphy 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.

REFERENCES


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

Kotaro Kameyama, Cheng-long Huang, Dage Liu, et al.