**Advances in Brief**

**p53 Mutations in Non-Small Cell Lung Cancer in Japan: Association between Mutations and Smoking**

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**Abstract**

The p53 gene has been implicated as a tumor suppressor gene involved in the pathogenesis of lung cancer. Our previous study revealed that the p53 gene is frequently mutated with a distinct nucleotide substitution pattern in small cell lung cancer specimens in Japanese patients. In this study, we examined 30 primary, resected non-small cell lung cancer samples in Japanese patients using complementary DNA-polymerase chain reaction and sequencing. Mutations changing the p53 coding sequence were found in 14 of 30 tumor samples (47%), while G:C to T:A transversions which are uncommon in other cancers such as colon cancer were the most frequently observed mutations, in agreement with an earlier report on non-small cell lung cancer in American patients. Furthermore, the present study shows for the first time that in univariate and multivariate analyses, the presence of p53 mutations is closely associated with lifetime cigarette consumption.

**Introduction**

The incidence of lung cancer is dramatically increasing in Japan and is expected to become the leading cause of cancer deaths in the near future (1). Unfortunately, as many as 90% of patients diagnosed with lung cancer still die of their disease, but recent progress in the molecular biology of lung cancer has reached a point where it can provide a basis for novel strategies in the prevention and treatment of this disease (2, 3). Accumulating evidence indicates that changes both in dominant oncogenes and in tumor suppressor genes are probably necessary for malignant transformation of normal bronchial epithelial cells. Among such genetic abnormalities, the p53 gene appears to be the most frequent target (4–8). Our previous study revealed that a different nucleotide substitution pattern exists in small cell lung cancer in Japan when compared to that in the United States, suggesting the involvement of a distinct mutagenic process in each of these two populations (7).

Thus, the current study was initiated (a) to determine the frequency, location, and nature of p53 mutations in NSCLC in Japan and to compare them with those of NSCLC in the United States and (b) to correlate p53 mutations with clinical data. Our findings showed that p53 mutations in NSCLC tumors in Japanese patients are similar to those in American patients, although p53 mutations at a prominent hot spot (codon 273) known in the United States were not observed in cases in Japan. We also found for the first time that the presence of p53 mutations is closely associated with lifetime cigarette consumption.

**Materials and Methods**

**Tumor Samples, Clinical Data, and Statistical Analyses.** Thirty tumor specimens from 30 patients with NSCLC were obtained during surgery at the Aichi Cancer Center in Japan. Tumors were quick frozen and stored at −135°C until use. DNA and RNA were prepared from tumor samples as described previously (9). For clinical and postsurgical pathological stages, the new international staging system for lung cancer was used (10). Univariate and multivariate analyses of factors potentially related to p53 mutations were performed by means of the logistic regression model to investigate the single and joint effects of these factors. All molecular genetic studies were done without knowledge of the clinical data.

**cDNA/PCR Amplification and Sequencing.** First-strand cDNA was synthesized using 25 µg of total cellular RNA with random hexanucleotide primers, and subsequent PCR was performed with one-fifth of the synthesized cDNA, as described previously (11). The primers used to amplify the entire open reading frame of p53 cDNA were 5′-AGTCAGCTTTGACCGTGACCTCCTGATT and antisense, 5′-AGTCGAATTCTCGTGGAACAGAAGTGGAAG. cDNA/PCR amplification was performed for 30 cycles at 94°C (1 min), 58°C (2 min), and 72°C (6 min) followed by 10 min of extention at 72°C. After digestion with HindIII and EcoRI, the PCR products were cloned into the HindIII-EcoRI site of pGEM7Zf(+) (Promega, Madison, WI). The plasmid DNA prepared from pooled clones was sequenced using p53 specific primers and a DSP-240 DNA processor (Seiko, Tokyo, Japan) by the dideoxy method, as described previously (7). Identified mutations were confirmed by separate cDNA/PCR amplification and subsequent sequencing.

**Genomic DNA/PCR Amplification and Sequencing.** For tumor samples which exhibited large deletions at the exon-exon boundaries in p53 cDNAs, we analyzed the putative abnormal splicing junctions. One µg of genomic DNA was amplified with appropriate primers (each set specific for the mutation involved). The genomic DNA/PCR amplification consisted of 30 cycles (94°C for 1 min, 58°C for 2 min, 72°C for 6 min) after an initial denaturation step (94°C for 5 min). Direct sequencing for genomic DNA/PCR products was performed using nested primers as described previously (11).

**Results and Discussion**

**Identification of p53 Mutations in NSCLC Tumor Specimens.** We examined exons 4 through 9 of p53 in cDNA prepared from 30 NSCLC tumor specimens which were surgically removed as part of potentially curative resections from 30 patients at the Aichi Cancer Center. p53 mutations were found in 14 of these 30 NSCLC tumors (47%) (Table 1). Eleven of the 14 samples contained missense mutations, while 1 tumor sample...
**Table 1: p53 gene mutations in NSCLC**

<table>
<thead>
<tr>
<th>Tumor sample</th>
<th>Codon</th>
<th>Nucleotide substitution</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>L111</td>
<td>154</td>
<td>GGG to GTC</td>
<td>Gly to Val</td>
</tr>
<tr>
<td>L124</td>
<td>157</td>
<td>GTC to TTC</td>
<td>Val to Phe</td>
</tr>
<tr>
<td>L110</td>
<td>175</td>
<td>GCC to CTC</td>
<td>Arg to Leu</td>
</tr>
<tr>
<td>L116</td>
<td>179</td>
<td>CAT to CGT</td>
<td>His to Arg</td>
</tr>
<tr>
<td>L112</td>
<td>194</td>
<td>CTT to CGT</td>
<td>Leu to Arg</td>
</tr>
<tr>
<td>L213</td>
<td>245</td>
<td>GGC to TGC</td>
<td>Gly to Cys</td>
</tr>
<tr>
<td>L107</td>
<td>248</td>
<td>CGG to CAG</td>
<td>Arg to Gin</td>
</tr>
<tr>
<td>L206</td>
<td>249</td>
<td>AGG to ATG</td>
<td>Arg to Met</td>
</tr>
<tr>
<td>L215</td>
<td>250</td>
<td>CCC to TTC</td>
<td>Pro to Phe</td>
</tr>
<tr>
<td>L203</td>
<td>275</td>
<td>TGT to TTT</td>
<td>Cys to Phe</td>
</tr>
<tr>
<td>L168</td>
<td>282</td>
<td>CGG to TGG</td>
<td>Arg to Trp</td>
</tr>
<tr>
<td>L114</td>
<td><strong>216–218</strong></td>
<td>3-base pair deletion</td>
<td>Val to deletion</td>
</tr>
<tr>
<td>L126</td>
<td><strong>Intron 3</strong></td>
<td>AG to TG</td>
<td>Inframe deletion of codons 33–125</td>
</tr>
<tr>
<td>L108</td>
<td><strong>Intron 7</strong></td>
<td>5-base pair deletion</td>
<td>Inframe deletion of codons 262–269</td>
</tr>
</tbody>
</table>


* Both mutant and wild-type p53 mRNA were expressed in these tumor samples.

**Fig. 1:** p53 missense mutations in NSCLC tumors of the Japanese patients in this study are shown above the schematic diagram for p53 protein compared with findings in NSCLC tumors of American patients (6). The five conserved regions (C) and the two predicted large T-antigen binding regions (C) are indicated (14). Arrows, points of mutation.

In general, the type of mutations reflects the mutagens involved. For example, G:C to T:A transversions can be caused by benzo[a]pyrene which is contained in cigarette smoke (12). G:C to T:A transversions, which are uncommon in p53 mutations in other types of human cancers (13), were observed in NSCLC tumors at a frequency of 50%. Our results are in agreement with those previously reported for lung cancer, suggesting a common mutagen involved in lung cancer (5–7).

The mutations found in NSCLC were scattered throughout the low charge region in the central part of the p53 protein (Fig. 1) (14). We note that together with the data of our previous analysis of small cell lung cancer (7), no p53 mutations at codon 273 which is a known hot spot in American patients (6) have been found in Japan thus far (0 of 26). These observations may suggest a possible involvement of a distinct mutagenic process between these two populations as discussed below, although the number of cases studied is still too small to draw this conclusion definitely.

**Table 2: Correlation between clinical features and p53 mutations in NSCLC**

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Mutant</th>
<th>Normal</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>62 ± 11.7</td>
<td>62 ± 11.5</td>
<td>0.999</td>
<td>0.986</td>
</tr>
<tr>
<td>Sex</td>
<td>12</td>
<td>9</td>
<td>0.214</td>
<td>0.092</td>
</tr>
<tr>
<td>Clinicopathological status</td>
<td>3.14</td>
<td>3.12</td>
<td>0.911</td>
<td>0.937</td>
</tr>
<tr>
<td>Stage</td>
<td>T1</td>
<td>T1</td>
<td>1.055</td>
<td>0.908</td>
</tr>
<tr>
<td>Histology</td>
<td>Squamous cell</td>
<td>8</td>
<td>4</td>
<td>0.247</td>
</tr>
<tr>
<td>Smoking (cigarettes/day)</td>
<td>1026 ± 504</td>
<td>424 ± 425</td>
<td>1.003</td>
<td>0.009</td>
</tr>
</tbody>
</table>

* Data on 30 patients tabulated.
* Mean ± SD.
* For an increase of 1 year in age.
* NI, not included.
* Histology was dichotomized for analysis (squamous cell carcinoma versus other histological types).
* For an increase of 1 unit in lifetime cigarette consumption (1 cigarette/day for 1 year).
Correlation of \( p53 \) Mutations with Clinical Data. This study was unblinded after completing analysis of \( p53 \) mutations. Statistical analysis for the \( p53 \) mutations was then performed to correlate with the clinical data (Table 2). Mutations in the \( p53 \) gene were found even in small tumors (T1) without any nodal metastases (N0). There were no significant differences in the frequency of mutations with respect to tumor size (T), nodal involvement (N), or clinical stage. The frequency varied among the different histological subtypes of NSCLC (67% in squamous cell carcinoma and 35% in adenocarcinoma), a finding which generally agrees with a previous report in which American patients with \( p53 \) abnormalities were shown at 65% in squamous cell carcinoma and 33% in adenocarcinoma (6).

We could not find any statistically significant association between the presence of \( p53 \) mutations and age, sex, clinical stage, or histology. In univariate analyses, however, the amount of lifetime cigarette consumption (cigarettes/day \( \times \) years) was closely associated with \( p53 \) status (\( P < 0.01; \) Table 2). Using the logistic regression model, multivariate analysis was performed to investigate the independence of “lifetime cigarette consumption” as a predictive factor for mutation. This “lifetime cigarette consumption” was the only factor shown to be closely related to \( p53 \) mutations, after adjusting for other covariates (\( P < 0.05; \) Table 2). For instance, the odds ratio for \( p53 \) mutations to develop in smokers who consumed 20 cigarettes/day for 30 years can be estimated at a 5.3-fold increase over nonsmokers based on the computation (15).

\[ \text{Odds ratio} = \exp(\ln(1.003) \times 20 \text{ cigarettes/day} \times 30 \text{ yr}) \]

It is now clear that the \( p53 \) gene is the most frequent target among the known genetic alterations in lung cancer (Refs. 4–8; this study). As various epidemiological studies have also shown that major fractions of lung cancer can be attributed to smoking (16, 17), identifying the association between smoking and \( p53 \) mutations in this study is of particular interest and further supports the current public health efforts against smoking.

Our findings contradict the report by the NCI-Navy group which stated that there was no significant association between smoking and the \( p53 \) mutations in NSCLC tumor samples of American patients (6). We do not know the exact reasons for such a discrepancy, but one possible explanation may be differences in the genetic features between the two populations as related to carcinogen metabolism or DNA repair activity. In this regard, it should be noted that the incidence of lung cancer among Japanese Americans is 2 to 3 times lower than that of white Americans, a figure comparable to that among Japanese (18).

The results of this study, together with previous reports by us and by other researchers, indicate that the \( p53 \) gene is a good target for molecular epidemiological studies of various human cancers (7, 8, 13, 19, 20). Future studies carefully designed to search for the risk factors such as carcinogen exposure or higher sensitivity to certain carcinogens should provide a basis for the ultimate strategies in cancer prevention.

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References


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