Background: Epidermal growth factor receptor (EGFR) gene mutations are frequently found in pulmonary adenocarcinomas.

Materials and Methods: Various lung cancers (n=30) including 8 small adenocarcinomas were examined for EGFR gene mutations in three exons.

Results: Mutations were detected in 32% of adenocarcinomas. Exon 19 mutations were detected in 5 tumors, often advanced stages: 1 in Noguchi’s pathologic type C, 2 in type D, and 2 in type F. Exon 21 mutations were detected in 3 tumors, all small adenocarcinomas in type C, at pathologic stage IA.

Conclusion: We suspect that exon 21 mutations are early events in small bronchioloalveolar carcinomas, while exon 19 mutations are later events occurring in adenocarcinomas of various types. (Ann Thorac Cardiovasc Surg 2007; 13: 87–92)

Key words: epidermal growth factor, lung cancer, adenocarcinoma, mutation, bronchioloalveolar carcinoma
growth) are regarded as more aggressive cancers. This classification is widely considered to accurately depict the diverse array of pulmonary adenocarcinomas. We therefore sought to clarify when EGFR mutation occurred during development of small pulmonary adenocarcinomas, using the Noguchi’s classification to estimate relative time points in tumor development.

Materials and Methods

Patient characteristics
Resected lung cancer tissues from 30 patients who underwent lobectomy and systematic lymph node dissection in Tokyo Medical University Hospital were studied with respect to EGFR gene mutations. Histologic types included 25 adenocarcinomas and 5 other carcinomas including 2 squamous cell carcinomas, 2 large cell carcinomas, and 1 small cell carcinoma. Noguchi’s pathologic classification was applied to all adenocarcinomas, including some tumors larger than 20 mm. Adenocarcinomas included 2 in type A, 1 in type B, 7 in type C, 10 in type D, 2 in type E, and 3 in type F. Pathologic (p-) stages of the 30 carcinomas according to international staging criteria were IA in 15, IB in 5, and IIA to IIIB in 10. The p-IA tumors included 8 small adenocarcinomas with a largest dimension below 20 mm. Written informed consent for genetic analysis of the resected tumor was obtained from all patients. In the operating room, immediately upon resection of a pulmonary lobe containing a primary lung cancer, about 500-mg sample was removed from the tumor, immersed in liquid nitrogen, and stored at −80°C until genetic study.

Detection of the EGFR gene mutation
Genomic DNA was extracted from the stored tumor using a REDExtract - N-Amp Tissue PCR kit (Sigma, St. Louis, MO). The three exons in the EGFR gene (exons 18, 19, and 21) reported to include frequent mutation sites were amplified by polymerase chain reaction (PCR). Primer sequences of 5′-AGGTGACCCTTTGTCTTGATCCCTTCTGTTCT-3′ and 5′-CACCAGACCATGA-GAGGCCCTGTG-3′ were used to amplify 216 base pairs in exon 18 by two-step PCR at an annealing temperature of 68°C; 5′-GATCACTGGGCAGCATGTCATTCC-3′ and 5′-TGGACCCCACACAGCAAAGCAGA-3′ to amplify 199 base pairs in exon 19 by two-step PCR at annealing temperature of 68°C; and 5′-TTCCCATGATGCCCTGCTG-3′ and 5′-ATGCCTGGCCTGA-CCTAAA-3′ to amplify 232 base pairs in exon 21 by three-step PCR at an annealing temperature of 55°C. Amplified sequences within each exon initially were screened for mutations by single-strand conformation polymorphism (SSCP) analysis using 14% polyacrylamide gels. Samples were electrophoresed at 72 V/cm under two different conditions, 10°C for 4 h and 20°C for 2 h. Isolated DNA strands showing a mobility shift on gels were cut from gels, and these isolated DNA strands were sequenced using cycle sequencing kit (BigDye Terminator version 3.1, Applied Biosystems, Foster City, CA) in a DNA analyzer (Applied Biosystems 3730x).

Statistical analysis
Differences in distribution of EGFR mutations between two groups were tested by Fisher’s exact probability test.
A p value less than 0.05 was considered to indicate significance.

Results

SSCP analysis detected shifts of amplified single-strand DNAs in electrophoretic gels in 8 samples (Fig. 1, A and B). DNA fragments showing abnormal mobility shifts on gels were cut and sequenced. Altered sequences were determined in all 8 samples. Patient characteristics and results of EGFR mutation screening are shown in Table 1. Mutations were detected only in adenocarcinomas. 

Mutations in exon 19 were detected in 5 tumors including 1 in type C, 2 in type D, and 2 in type F according to Noguchi’s classification. These include one point mutation resulting in replacement of G735 by S and four small deletions of 13 to 15 base pairs. The deletions caused omission of five amino acids (E746 to A750) in 2 tumors and omission of a slightly different sequence in 2 others (L747 to T751). One of the latter tumors also had insertion of cytosine at the deletion point, resulting in insertion of P where the others were omitted. P-stages included
stage IA in 2 tumors, stage IIA in 1, stage IIB in 1, and stage IIIA in 1.

Mutations in exon 21 were detected in three tumors, all in Noguchi type C and p-stage IA. All represented substitution of G for T at nucleotide 2573, resulting in an amino acid substitution (L858R). No mutations were detected in exon 18.

All EGFR mutations were detected only in adenocarcinomas, which showed a frequency of the EGFR mutations of 32% (8/25). Relationships between EGFR mutations and clinicopathologic features are shown in Table 2. Frequency of mutations did not differ between p-IA and the more advanced stages p-IB to IIIB (p = 0.682). EGFR gene mutations were more frequent in patients who never smoked than in current or previous smokers (p = 0.028). Although mutations were more frequent in women (50%) than in men (15%), this difference was not statistically significant (p = 0.104).

**Discussion**

In this study we initially screened for mutations using PCR-SSCP, which enabled us to detect small amounts of abnormal tumor-derived DNA fragments among largest amounts of normal DNA derived from interstitial tissue. We successfully detected mutations within coding regions of the EGFR gene in 32% of unselected Japanese patients with adenocarcinoma. All gene mutations resulted in changes of amino acids. Lynch et al.\(^7\) reported 10 tumors carrying five types of EGFR mutations causing amino acid alterations, 2 representing mutations that we also detected (E746–A750 del and L858R). Paez et al.\(^8\) reported 22 tumors carrying four types of mutations, 3 being types that we detected. EGFR mutations detected in seven studies including our present one\(^7,8,12–15\) are summarized in Table 3. In all studies exons 19 and 21 represented “hot spots” for mutations, which frequently were found in non smokers and in women.

Kosaka et al.\(^13\) detected EGFR mutations more frequently in moderately and well differentiated adenocarcinomas than in poorly differentiated adenocarcinomas. This is of considerable interest as gene mutations occurring in less invasive cancers have been reported as relatively rare. Moreover, EGFR mutations are frequent in tumors affecting nonsmokers, while most altered genes in lung cancers such as RAS, p53, and FHIT were found more frequently in heavy smokers than in nonsmokers.

According to the hypothesis of multistep carcinogenesis, gene mutations tend to accumulate in late-stage disease or highly malignant cancers, a generalization that seems not to apply to EGFR mutations.

Our present study disclosed EGFR mutations in early-stage adenocarcinomas. Noguchi’s pathologic classification\(^9\) represents an effort to depict the sequence of carcinogenesis for peripherally located adenocarcinomas. When chest CT is used to screen for lung cancer, most peripheral small shadows showing pure ground glass opacity prove to be atypical adenomatous hyperplasia or noninvasive bronchioloalveolar carcinoma, Noguchi types A and B. In our present study we found a point mutation in exon 21 in 3 Noguchi type C tumors, all representing p-IA disease. This suggests that exon 21 mutations in the EGFR gene may be relatively early occurrences in the development of bronchioloalveolar carcinoma. In contrast, mutations in exon 19 were found in more advanced tumors such as Noguchi types D, E, and F. These results suggest the possibility that malignant grades of pulmonary adenocarcinoma may be related to mutation at different sites within the EGFR gene. Although a relationship between exons affected and disease stage or adenocarcinoma subtype was not mentioned in previous studies, Tokumo et al.\(^14\) reported significantly higher prevalence of mutations in exon 19 in tumors from men than women. Minna et al.\(^16\) also suggested different biologic activities of different affected exons, given that point mutations in exon 21 are heterozygous, including one normal allele, while the normal allele is severely underrepresented in tumors with small exon 19 deletions. These differences may be related to disease stages, histopathologic grade, and lineage of adenocarcinomas. We suspect that exon 21 is likely to be altered in the noninvasive Noguchi type A to C sequence (well differentiated bronchioloalveolar carcinoma), while exon 19 might be altered in more aggressive types such as D, E, and F.

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**Table 2. Association of EGFR mutations and clinicopathologic features**

<table>
<thead>
<tr>
<th>Factors</th>
<th>EGFR (exons 18, 19, 21)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutation</td>
<td>No mutation</td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Smoker</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>Non-smoker</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>p-stage IA</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>p-stages IB–IIIB</td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>

* Fisher’s exact probability test; **, significant difference.
Our previous study\(^7\) revealed that lung cancer cells can be effectively detected in cytologic specimens using fluorescence in situ hybridization (FISH) techniques. If EGFR mutations might be closely associated with chromosomal aberrations around the \(\text{EGFR}\) gene locus, tumors carrying \(\text{EGFR}\) mutations could be detected by FISH more easily. This point should be further examined.

In conclusion, \(\text{EGFR}\) mutations were detected in early pulmonary adenocarcinomas. We believe that \(\text{EGFR}\) mutations in exon 21 are relatively early events during development of pulmonary adenocarcinomas, especially small bronchioloalveolar carcinomas (Noguchi type A to C). In contrast, mutations in exon 19 occur in various types of adenocarcinoma, often at later stages. These results of our small series should be examined further in larger numbers of patients.

### Acknowledgment

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### References


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**Table 3. Reported mutations in the \(\text{EGFR}\) gene in seven studies**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Type of mutation</th>
<th>Number</th>
<th>Amino acid changes</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>Point mutations</td>
<td>10 (4.0%)</td>
<td>G719S</td>
<td>5(^a) (2.0%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G719C</td>
<td>2(^a) (0.8%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Others</td>
<td>3 (1.2%)</td>
</tr>
<tr>
<td>19</td>
<td>Small deletions</td>
<td>118 (47.2%)</td>
<td>del E746–A750</td>
<td>65 (26.0%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Other deletions and/or insertions</td>
<td>53 (21.2%)</td>
</tr>
<tr>
<td></td>
<td>Insertions or duplications</td>
<td>5 (2.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Point mutations</td>
<td>1 (0.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Point mutations</td>
<td>2 (0.8%)</td>
<td>S768I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insertions or duplications</td>
<td>2 (0.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Point mutations</td>
<td>112 (44.8%)</td>
<td>L858R</td>
<td>110(^b) (44.0%)</td>
</tr>
<tr>
<td></td>
<td>Other point mutations</td>
<td>2 (0.8%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Studies summarized include our present results and those in references\(^7,8,12–15\). G, glycine; S, serine; C, cysteine; E, glutamic acid; A, alanine; I, isoleucine; L, leucine; R, arginine; \(^a\), A point mutation in another exon was also present in 1 tumor. \(^b\), A point mutation in another exon was also present in 8 tumors.


