

Epidermal Growth Factor Receptor Gene Mutations in Early Pulmonary Adenocarcinomas

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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

Introduction

Lung cancer is the leading cause of cancer death worldwide¹⁾; despite much effort to conquer this disease, the overall survival rate is approximately 10%. Recently, molecular therapy targeting the epidermal growth factor receptor (EGFR) has become the second- or third-line treatment for selected patients with non-small-cell lung cancer (NSCLC).^{2,3)} Tyrosine kinase inhibitors such as gefitinib^{4,5)} and erlotinib,⁶⁾ were developed to inhibit signal transduction pathways mediated by the EGFR, thus selectively suppressing proliferation of lung cancer cells that carry activating mutations of the region encoding the cleft within the EGFR protein that binds adenosine triphosphate (ATP).^{7,8)} The mutations detected were single-base

substitutions or small in-frame deletions occurring in the known “hot spot” for mutation, exons 18, 19 and 21. Importantly, these mutations were detected selectively in tumors responding to gefitinib or erlotinib,^{7,8)} which frequently were adenocarcinomas in women who never smoked. As these activating mutations are more frequent tumors in Asians than Westerners, the significantly higher response rates to gefitinib in Japanese reported in multi-institutional clinical trials²⁾ were attributed to high prevalence of *EGFR* gene mutations in this population.

While an association between gefitinib responsiveness and *EGFR* mutations has been demonstrated, when *EGFR* mutations occur during carcinogenesis in the lung still is unclear. Noguchi et al.⁹⁾ classified small peripheral adenocarcinomas into six types based on tumor growth patterns; types A and B represented in situ peripheral bronchioloalveolar carcinomas that did not involve lymph nodes¹⁰⁾ and required computed tomography (CT) for detection. Type C appears to be advanced slightly beyond types A and B, showing active fibroblastic proliferation. On the other hand, types D (poorly differentiated adenocarcinoma), E (tubular adenocarcinoma), and F (papillary adenocarcinoma with compressive and destructive

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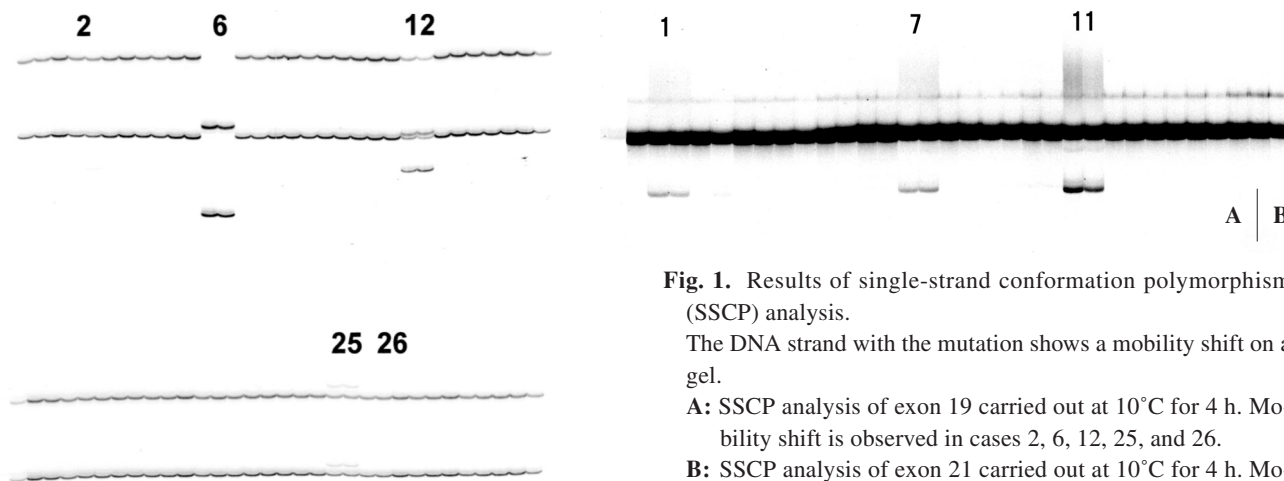


Fig. 1. Results of single-strand conformation polymorphism (SSCP) analysis.

The DNA strand with the mutation shows a mobility shift on a gel.

A: SSCP analysis of exon 19 carried out at 10°C for 4 h. Mobility shift is observed in cases 2, 6, 12, 25, and 26.

B: SSCP analysis of exon 21 carried out at 10°C for 4 h. Mobility shift is observed in cases 1, 7, and 11. All of these abnormal DNA strands were sequenced to identify the altered bases.

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 criteria¹¹⁾ 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'-AGGTGACCCTTGTC-TCTGTGTTCT-3' and 5'-CACCAGACCATGAGAGGCCCTGCG-3' were used to amplify 216 base pairs in exon 18 by two-step PCR at an annealing temperature of 68°C; 5'-GATCACTGGGCAGCATGTGGCACC-3' and 5'-TGGACCCCCACACAGCAAAGCAGA-3' to amplify 199 base pairs in exon 19 by two-step PCR at annealing temperature of 68°C; and 5'-TTCCC-ATGATGATCTGTC-3' and 5'-ATGCTGGCTGACCTAAA-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.

Table 1. Results of the EGFR mutation analysis

Case	Age	Gender	Smoking status (SI)	Pathologic type	Noguchi's classification	Largest dimension	Pathologic stage (mm)	v	ly	EGFR mutation analysis (exons 18, 19, and 21)	Changes in amino acids
1	71	F	Never smoked	W/D Ad	C	25	T1N0M0 IA	-	-	exon 21 T2573 G	L858 R
2	81	F	Never smoked	W/D Ad	F	25	T1N0M0 IA	-	-	exon 19 2235-2249 del (15 base)	E746-A750 del
3	77	M	Current smoker (1,000)	P/D Ad	D	31	T2M0N0 IB	2+	-	Normal	Normal
4	69	F	Current smoker (450)	W/D Ad	B	10	T1N0M0 IA	-	-	Normal	Normal
5	68	F	Never smoked	P/D Ad	D	25	T1N2M0 IIIA	1+	1+	Normal	Normal
6	71	M	Never smoked	P/D Ad	D	60	T2N1M0 IIB	2+	1+	exon 19 2239-2251 del (13 base), ins C	L747-T751 del, ins P
7	70	F	Never smoked	W/D Ad	C	15	T1N0M0 IA	-	1+	exon 21 T2573 G	L858 R
8	65	M	Never smoked	La	NA	70	T2M0N0 IB	2+	1+	Normal	Normal
9	65	M	Current smoker (1,140)	P/D Ad	D	15	T4N1M0 IIIB	2+	1+	Normal	Normal
10	55	M	Current smoker (2,960)	M/D Ad	F	25	T1N0M0 IA	2+	-	Normal	Normal
11	69	M	Current smoker (400)	W/D Ad	C	28	T1N0M0 IA	-	-	exon 21 T2573 G	L858 R
12	71	F	Never smoked	W/D Ad	F	20	T1N1M0 IIA	-	-	exon 19 2235-2249 del (15 base)	E746-A750 del
13	55	M	Current smoker (760)	P/D Ad	D	50	T2M0N0 IB	-	-	Normal	Normal
14	54	F	Never smoked	W/D Ad	A	10	T1N0M0 IA	-	-	Normal	Normal
15	72	F	Current smoker (1,060)	M/D Sq	NA	60	T2N1M0 IIB	-	-	Normal	Normal
16	76	M	Current smoker (340)	W/D Ad	E	60	T4N0M0 IIIB	-	-	Normal	Normal
17	74	M	Current smoker (1,650)	P/D Ad	D	20	T1N0M0 IA	1+	-	Normal	Normal
18	66	M	Current smoker (920)	M/D Sq	NA	37	T2M0N0 IB	-	-	Normal	Normal
19	71	F	Never smoked	La	NA	22	T4N0M0 IIIB	-	-	Normal	Normal
20	77	M	Current smoker (1,140)	Sm	NA	34	T2M0N0 IB	2+	-	Normal	Normal
21	64	M	Current smoker (150)	P/D Ad	D	15	T1N0M0 IA	-	-	Normal	Normal
22	43	M	Current smoker (500)	M/D Ad	E	35	T2N2M0 IIIA	1+	2+	Normal	Normal
23	57	M	Ex-smoker (150)	P/D Ad	D	45	T2N1M0 IIB	1+	1+	Normal	Normal
24	58	M	Current smoker (300)	P/D Ad	D	27	T1N0M0 IA	-	-	Normal	Normal
25	71	M	Current smoker (1,530)	P/D Ad	D	35	T2N2M0 IIIA	1+	2+	exon 19 G2203 A	G735 S
26	67	F	Never smoked	W/D Ad	C	28	T1N0M0 IA	-	-	exon 19 2239-2253 del (15 base)	L747-T751 del
27	78	M	Never smoked	W/D Ad	C	18	T1N0M0 IA	-	1+	Normal	Normal
28	76	M	Current smoker (560)	W/D Ad	C	15	T1N0M0 IA	-	1+	Normal	Normal
29	78	M	Ex-smoker (280)	W/D Ad	C	27	T1N0M0 IA	-	-	Normal	Normal
30	49	M	Current smoker (750)	W/D Ad	A	10	T1N0M0 IA	-	-	Normal	Normal

F, female; M, male; SI, smoking index (cigarettes/day \times years); v, microscopic vascular invasion; ly, microscopic lymph vessel invasion; W/D, well-differentiated; M/D moderately differentiated; P/D poorly differentiated; Ad, adenocarcinoma; La, large cell carcinoma; Sm, small cell carcinoma; Sq, squamous cell carcinoma; NA, not applicable; G, guanine; C, cytosine; T, thymine; A, adenine; L, leucine; R, arginine; E, glutamic acid; A, alanine; T, threonine; P, proline; G, glycine; S, serine.

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

Table 2. Association of EGFR mutations and clinicopathologic features

Factors	EGFR (exons 18, 19, 21)		<i>p</i> value*
	Mutation	No mutation	
Male	3	16	0.104
Female	5	6	
Smoker	2	17	0.028**
Non-smoker	6	5	
p-stage IA	5	10	0.682
p-stages IB–IIIB	3	12	

*, Fisher's exact probability test; **, significant difference.

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 sum-

marized 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 classification⁹ 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.

Table 3. Reported mutations in the EGFR gene in seven studies

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

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.

Our previous study¹⁷⁾ 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 *EGFR* gene locus, tumors carrying *EGFR* mutations could be detected by FISH more easily. This point should be further examined.

In conclusion, *EGFR* mutations were detected in early pulmonary adenocarcinomas. We believe that *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.

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