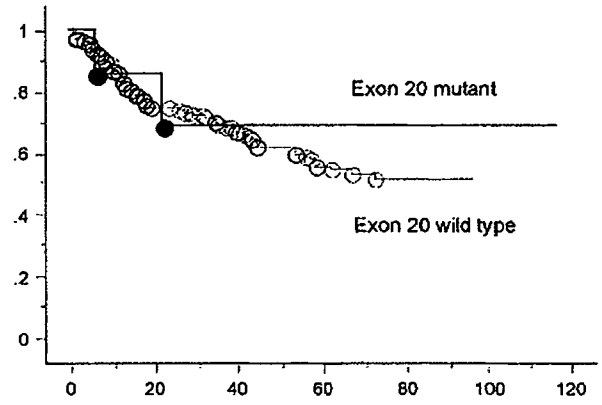

Fig. 2 The prognosis from patients with exon 20 insertion mutation ($n=7$, 2 were dead) and the patient without exon 20 insertion mutation *EGFR* ($n=315$, 92 were dead) was not significantly different (Log-rank test, $p=0.7186$, Breslow–Gehan–Wilcoxon test, $p=0.8593$).

3.3. Clinical course of two recurrent lung cancer patients treated with gefitinib

Case 1: 58-year-old adenocarcinoma woman with no history of smoking underwent surgery at Kinki-chuo Chest Medical Center. A molecular analysis revealed 772_773 insertion N (2312-14 insertion AAC) mutations at *EGFR* exon 20. Three years later, the recurrent lung cancer was treated with gemcitabine, vinorelbine and Uracil-Ftegafur in addition to radiotherapy. Because the treatment failed, gefitinib treatment was started at 2004. The patient died from progressive disease about 6 months after gefitinib administration. Case 2: 72-year-old adenocarcinoma man with no history of smoking underwent surgery at Nagoya City University Hospital. A molecular analysis revealed 772_773 insertion V (2312-14 insertion GGT) at *EGFR* exon 20 (Fig. 3), and wild type at *Kras* codon 12/13. Multiple lung metastasis were treated with Uracil-Ftegafur, however, the treatment failed. Gefitinib treatment was started at 2005. But the tumor size was increased (Fig. 3) and the treatment was quitted at 3 months.

4. Discussion

We obtained findings that exon 20 insertion type *EGFR* mutations tend to be higher in female gender and never smoker, as like as other *EGFR* mutation subtypes [8–14]. From the original three papers published by Lynch et al., Paez et al. and Pao et al., there was no *EGFR* exon 20 insertion subtypes. Shigematsu et al. reported that 12 of 617 (1.9%) had exon 20 insertion mutation, however, 356 of 617 patients were either from Japan or Taiwan [11]. Sonobe et al. reported that the 2 of 154 cases (1.3%) had *EGFR* exon 20 insertion mutations. These data suggested that *EGFR* mutations at exon 20 might be also higher in East Asian. More interestingly, patients with exon 20 mutation did not respond to gefitinib therapy.

Although many reports have identified more than 30 different mutations in the tyrosine kinase domains of *EGFR*, the vast majority of which can be grouped into three major types, including in-frame deletion at exon 19, single-nucleotide substitution at exon 18 or 21 and in-frame duplication at exon 20 [8–14]. To date, only the L858R missense mutation in exon 21 and deletions in exon 19 have been proven to be activating mutations [4,5,10,14]. On the

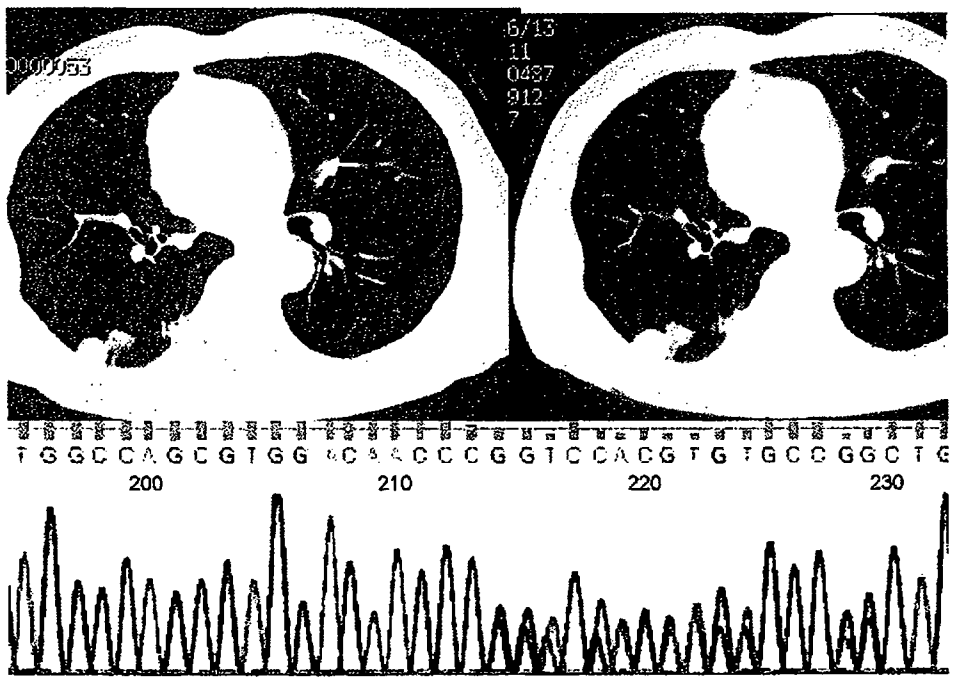

Fig. 3 CT examination before (left) and after (right) gefitinib therapy revealed increased tumor size. A molecular analysis revealed 772_773 insertion V (2312-14 insertion GGT) at *EGFR* exon 20 (below).

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other hands, Greulich et al. reported that transformation by the D770_N771 ins NPG (exon 20) *EGFR* insertion mutant was remarkably insensitive to gefitinib and erlotinib, as inhibition of colony growth in soft agar required exposure to 100-fold higher concentrations (>1 mM) of these agents than was required to inhibit colony formation by cells expressing the *EGFR* missense mutants or deletion mutant [14]. No significant inhibition of anchorage-independent growth of cells expressing D770_N771ins NPG *EGFR* was observed at 3 mM gefitinib or erlotinib [14]. Greulich et al. also reported that all three lung adenocarcinoma patients with known exon 20 insertion mutants of *EGFR* have failed to show a clinical response to treatment and have instead achieved only stable disease with erlotinib [14]. *In vitro* analysis, cells expressing the *EGFR* deletion and insertion mutants formed colonies in soft agar with a higher efficiency than that of cells expressing the missense mutants, comparable to the colony formation efficiency of cells expressing polyoma middle T antigen, suggested these mutants were oncogenic [14]. Interestingly, the irreversible *EGFR* inhibitor CL-387, 785 [20] is more effective than gefitinib or erlotinib for inhibition of colony formation by cells expressing the exon 20 insertion mutant [14]. CL-387, 785 had an even greater effect on colony formation by cells expressing L858R [14], and this compound was previously found to be active against *EGFR* containing the exon 20 point mutation T790M, associated with resistance to gefitinib and erlotinib [21]. Thus the distinct inhibitor sensitivity of various *EGFR* mutants argues that therapies may need to be targeted against specific mutant forms of a protein, whereas generalized inhibition of a particular oncogenic target may not be sufficient.

Conflict of interest

None declared.

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Original Article

Epidermal Growth Factor Receptor Gene Mutations in Early Pulmonary Adenocarcinomas

Haruhiko Nakamura, MD, PhD,[1] Norihito Kawasaki, MD,[1] Masahiko Taguchi, MD, PhD,[1] and Harubumi Kato, MD, PhD[2]

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 worldwide[1]; 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 trials[2] 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 nodes[10] 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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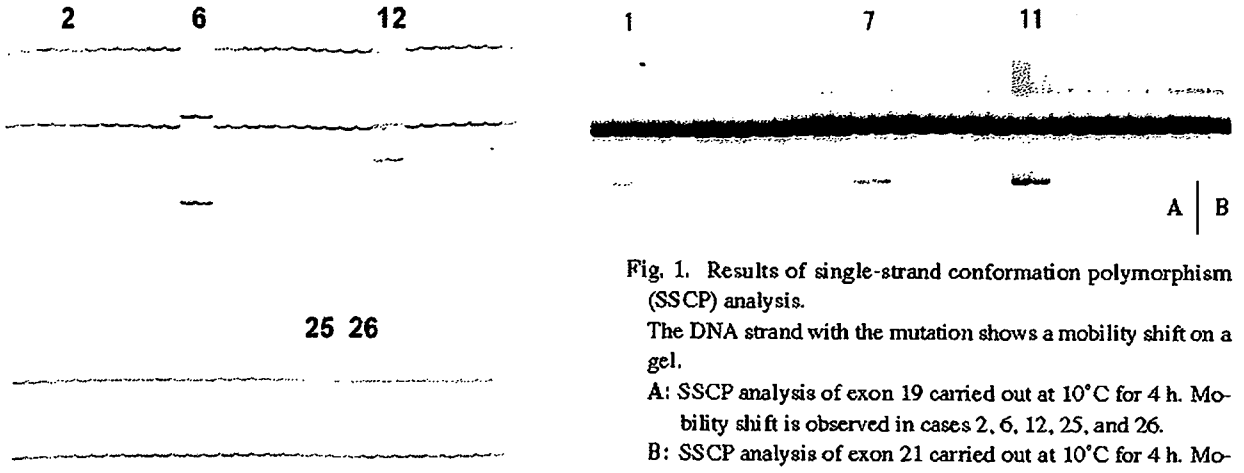

Fig. 1. Results of single-strand conformation polymorphism (SSCP) analysis.
The DNA strand with the mutation shows a mobility shift on a gel.
A: SSCP analysis of exon 19 carried out at 10°C for 4 h. Mobility shift is observed in cases 2, 6, 12, 25, and 26.
B: SSCP analysis of exon 21 carried out at 10°C 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 criteria[10] 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′-AGGTGACCCTTGTCTCTGTGTTCT-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′-TTCCCATGATGATCTGTC-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 × 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)		p 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 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 occurences 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 study[17] 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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Epidermal Growth Factor Receptor Mutation Detection Using High-Resolution Melting Analysis Predicts Outcomes in Patients with Advanced Non–Small Cell Lung Cancer Treated with Gefitinib

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Abstract

Purpose: Epidermal growth factor receptor (*EGFR*) mutations, especially deletional mutations in exon 19 (DEL) and L858R, predict gefitinib sensitivity in patients with non–small cell lung cancer (NSCLC). In this study, we validated *EGFR* mutation detection using high-resolution melting analysis (HRMA) and evaluated the associations between *EGFR* mutations and clinical outcomes in advanced NSCLC patients treated with gefitinib on a larger scale.

Experimental Design: The presence of DEL or L858R was evaluated using HRMA and paraffin-embedded tissues and/or cytologic slides from 212 patients. In 66 patients, the results were compared with direct sequencing data.

Results: HRMA using formalin-fixed tissues had a 92% sensitivity and a 100% specificity. The analysis was successfully completed in 207 patients, and DEL or L858R mutations were detected in 85 (41%) patients. The response rate (78% versus 8%), time-to-progression (median, 9.2 versus 1.6 months), and overall survival (median, 21.7 versus 8.7 months) were significantly better in patients with *EGFR* mutations ($P < 0.001$). Even among the 34 patients with stable diseases, the time-to-progression was significantly longer in patients with *EGFR* mutations. Patients with DEL ($n = 49$) tended to have better outcomes than those with L858R ($n = 36$); the response rates were 86% and 67%, respectively ($P = 0.037$), and the median time-to-progression was 10.5 and 7.4 months, respectively ($P = 0.11$).

Conclusions: HRMA is a precise method for detecting DEL and L858R mutations and is useful for predicting clinical outcomes in patients with advanced NSCLC treated with gefitinib.

Gefitinib (Iressa; AstraZeneca) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor. Phase II studies have shown gefitinib antitumor activity in patients with advanced non–small cell lung cancer (NSCLC; refs. 1, 2). Several studies have shown that the response rate to gefitinib is higher in women, patients with adenocarcinoma, never smokers, and Japanese or East Asians (1–3); subsequently, somatic mutations in the kinase domain of *EGFR* were suggested to be a determinant of gefitinib sensitivity (4, 5). Since then, many retrospective studies have consistently revealed that *EGFR* mutations, mainly in-frame deletions including amino acids at codons 747 to 749 in exon 19 (DEL) and a missense mutation at codon 858 (L858R) in exon 21, are associated with tumor response, time-to-progression, and overall survival in NSCLC patients treated with gefitinib (6–8).

In our previous study, which clearly showed a correlation between *EGFR* mutations and gefitinib sensitivity in patients with recurrent NSCLC after surgical resection of the primary tumor (6), we used methanol-fixed, paraffin-embedded surgical specimens and did laser capture microdissection and direct sequencing, which we considered to be the most precise methods available for identifying mutations at that time. However, these methods are not useful in clinical practice for the treatment of advanced NSCLC for two reasons. First, the diagnostic samples of advanced NSCLC tumors, unlike surgical specimens, contain a small amount of tumor cells and are highly contaminated with normal cells. Second, laser capture microdissection and direct sequencing require special

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Table 1. Patient characteristics (N = 212)

	n (%)
Age (y)	
Median (range)	62 (29-84)
Sex	
Women	92 (43)
Men	120 (57)
Smoking history*	
Never smokers	96 (45)
Former smokers	38 (18)
Current smokers	78 (37)
Histology	
Adenocarcinoma	193 (91)
Others	19 (9)
Performance status†	
0	59 (28)
1	123 (58)
2	22 (10)
3	8 (4)
Stage	
III	42 (20)
IV	75 (35)
Recurrence after surgery	95 (45)
Gefitinib therapy	
First line	89 (42)
Second line	66 (31)
Third or more line	57 (27)

*Never smokers were defined as patients who have never had a smoking habit and former smokers were defined as patients who had stopped smoking at least 1 y before diagnosis.
† At the beginning of gefitinib therapy.

instruments and cost time and money. Recently, high-resolution melting analysis (HRMA) using the dye LCGreen I (Idaho Technology) was introduced as an easy, quick, and precise method for mutation screening (9), and we established a method for detecting DEL and L858R mutations using HRMA. Our cell line study revealed that DEL and L858R mutations could be detected using HRMA in the presence of 10% and 0.1% mutant cells, respectively (10). We also showed that the two major mutations could be identified by HRMA using DNA extracted from archived Papanicolaou-stained cytologic slides with 88% sensitivity and 100% specificity (10).

In this study, we validated *EGFR* mutation detection by HRMA using DNA extracted from archived paraffin-embedded tissues. We also did the HRMA in advanced NSCLC patients treated with gefitinib on a larger scale using archived tissues and/or cytologic slides.

Patients and Methods

Patients. Among 364 consecutive patients with NSCLC who began receiving gefitinib monotherapy (250 mg/d) at the National Cancer Center Hospital between July 2002 and December 2004, 212 patients were retrospectively analyzed using HRMA. One hundred fifty-two patients were excluded from the analysis because tumor samples were not available (n = 126) or their informed consent to the genetic analysis was not obtained (n = 26).

High-resolution melting analysis. On a protocol approved by the Institutional Review Board of the National Cancer Center Hospital, we did the following genetic analyses. Formalin-fixed, paraffin-embedded tissues and/or Papanicolaou-stained cytologic slides containing sufficient tumor cells (at least 1% of nucleated cells) were selected after microscopic examination by a pathologist (K.T.). The detailed analysis method has been described previously (10). Briefly, DNA was extracted from the tissues and/or cytologic slides using a QIAamp DNA Micro kit (Qiagen). PCR was done using dye LCGreen I and primers designed to amplify a region containing E746-I759 of *EGFR* [DEL-specific primer, AAAATTCCCGTCGCTATC (forward) and AAGCAGAAACTCACATCG (reverse)] or L858 of *EGFR* [L858R-specific primer, AGATCACA-GATTTTGGGC (forward) and ATTCTTTCTCTTCCGCAC (reverse)] on a LightCycler (Roche Diagnostics). The PCR products were denatured at 95°C for 5 min and cooled to 40°C to form heteroduplexes. The LightCycler capillary was then transferred to an HR-1 (Idaho Technology), a HRMA instrument, and heated at a transition rate of 0.3°C per second. Data were acquired and analyzed using the accompanying software (Idaho Technology). After normalization and temperature adjustment steps, melting curve shapes from 78.5°C to 85.5°C were compared between samples and control samples. Human Genomic DNA (Roche Diagnostics) was used as a control sample with wild-type (WT) *EGFR*. Samples revealing skewed or left-shifted curves from those of control samples were judged to have mutations. All analyses were done in a blinded fashion.

Table 2. Clinical validation of HRMA and direct sequencing without laser capture microdissection

	HRMA without LCM			Direct sequencing without LCM (6)
	Formalin-fixed tissues	Methanol-fixed tissues	Cytologic slides (10)	
n	66	66	29	66
Successfully analyzed, n (%)	63 (95)	66 (100)	28 (97)	66 (100)
True positive	34	36	14	28
True negative	26	29	12	29
False positive	0	0	0	0
False negative	3	1	2	9
Sensitivity (%)	92	97	88	76
Specificity (%)	100	100	100	100
Positive predictive value (%)	100	100	100	100
Negative predictive value (%)	90	97	86	76

NOTE: The results of these analyses were compared with those of direct sequencing with LCM (used as the "gold standard" method). True positive is defined as the correct detection of deletional mutations in exon 19 or L858R.
Abbreviation: LCM, laser capture microdissection.

Table 3. *EGFR* mutations among patient subgroups

	n	*EGFR* mutations				*P*
		DEL	L858R	Total	%	
Total	207	49	36	85	41	
Sex						
Women	89	31	17	48	54	0.001
Men	118	18	19	37	31	
Smoking history						
Never smokers	93	30	19	49	53	0.002*
Former smokers	38	12	10	22	58	
Current smokers	76	7	7	14	18	
Histology						
Adenocarcinoma	189	48	35	83	44	0.007
Others	18	1†	1‡	2	11	

*Comparison between never smokers and others.
† Pleomorphic carcinoma.
‡ Adenosquamous carcinoma.

Clinical validation of HRMA. Direct sequencing with and without laser capture microdissection had been done in 66 patients with recurrent NSCLC after surgery in the previous study (6). In these patients, HRMA was done using both formalin-fixed and methanol-fixed surgical specimens without laser capture microdissection, and the results were compared with the results of direct sequencing with laser capture microdissection, which we considered to be the gold standard method.

Radiologic evaluation. One board-certified radiologist (U.T.) who was unaware of the patients' mutational statuses reviewed the baseline, the first follow-up, and confirmatory imaging studies and classified the tumor responses into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) using standard bidimensional measurements (11). In patients without measurable lesions, significant clinical benefit and disease progression were defined as clinical PR and clinical PD, respectively. Patients who died before the follow-up imaging studies were classified as PD. SD was subdivided into minor response (MR), long SD, and short SD. MR was defined as a ≥25% decrease in the sum of the products of the perpendicular diameters of all measurable lesions, and long SD meant that SD lasted for >6 months. Responders were defined as patients with CR, PR, or clinical PR.

Statistical analysis. The associations among *EGFR* mutations, patient characteristics, and tumor responses to gefitinib were assessed using a χ^2 test. The differences in time-to-progression and overall survival according to the patient subgroups were compared using Kaplan-Meier curves and log-rank tests. The starting point of the time-to-progression and overall survival was the first administration of gefitinib. Multivariate analyses using logistic regression models and Cox proportional hazard models were done to assess the association between the clinical outcomes and the following factors: age (<70 versus ≥70 years), sex, smoking history (never smokers versus others), histology (adenocarcinoma versus others), performance status (0/1 versus 2/3), stage (recurrence after surgery versus III/IV), prior chemotherapy (yes versus no), and the mutational status of *EGFR* (mutant versus WT). All analyses were done using the SPSS statistical package (SPSS version 11.0 for Windows; SPSS, Inc.).

Results

Patient characteristics. The patient characteristics are listed in Table 1. All the patients were East Asians: 210 Japanese, 1 Korean, and 1 Chinese. The median follow-up time for the survivors was 29.7 months (range, 10.7-49.8 months).

Clinical validation of HRMA. The clinical validation of the HRMA results using various samples is shown in Table 2. The sensitivity of HRMA using DNA extracted from formalin-fixed tissues was 92%, significantly higher than that of direct sequencing without laser capture microdissection but lower than that of HRMA using methanol-fixed tissues. The specificity and positive predictive values were 100% in all the analyses.

Mutational analysis. HRMA was completed in 207 patients. Five patients could not be successfully analyzed because of incomplete PCR. Of the 207 patients, 130 were analyzed using tissue samples (96 samples were obtained by thoracotomy, 17 by mediastinoscopic lymph node biopsy, 9 by thoracoscopic lung or pleural biopsy, 5 by resection or biopsy of distant metastases, and 3 by transbronchial lung biopsy), and 117 were analyzed using cytology samples (43 samples were obtained by bronchial brushing or washing, 40 from pleural effusion, 9 by transbronchial needle aspiration, 8 from pericardial effusion, 7 by needle aspiration of superficial lymph nodes, 6 by percutaneous needle aspiration of lung tumors, and 4 from sputum). In 40 patients who were analyzed using both tissue and cytology samples, 4 had inconsistent results; mutations were detected only in tissue samples and not in cytology samples (3 patients) or vice versa (1 patient). These four patients were judged to have mutations because false-negative results were more common than false-positive results in the validation of HRMA. Consequently, DEL and L858R mutations were detected in 49 (24%) and 36 (17%) patients, respectively, and these mutations were mutually exclusive. The other 122 (59%) patients were classified as having WT *EGFR* in this study, although some of them may have had minor mutations. As

Table 4. *EGFR* mutations and response to gefitinib

	Responders		SD			PD	Response rate (%)	*P*
	CR	PR	MR	Long SD	Short SD			
WT	0	10	2	4	17	89	10/122 (8)	$<10^{-23}$
Mutant	2	64*	6	4	1	8†	66/85 (78)	
DEL	0	42	2	2	1	2	42/49 (86)	0.037
L858R	2	22	4	2	0	6	24/36 (67)	
Total	2	74	8	8	18	97	76/207 (37)	

*Including four clinical responders without measurable lesions.
† Including a patient who had no measurable lesions at baseline.

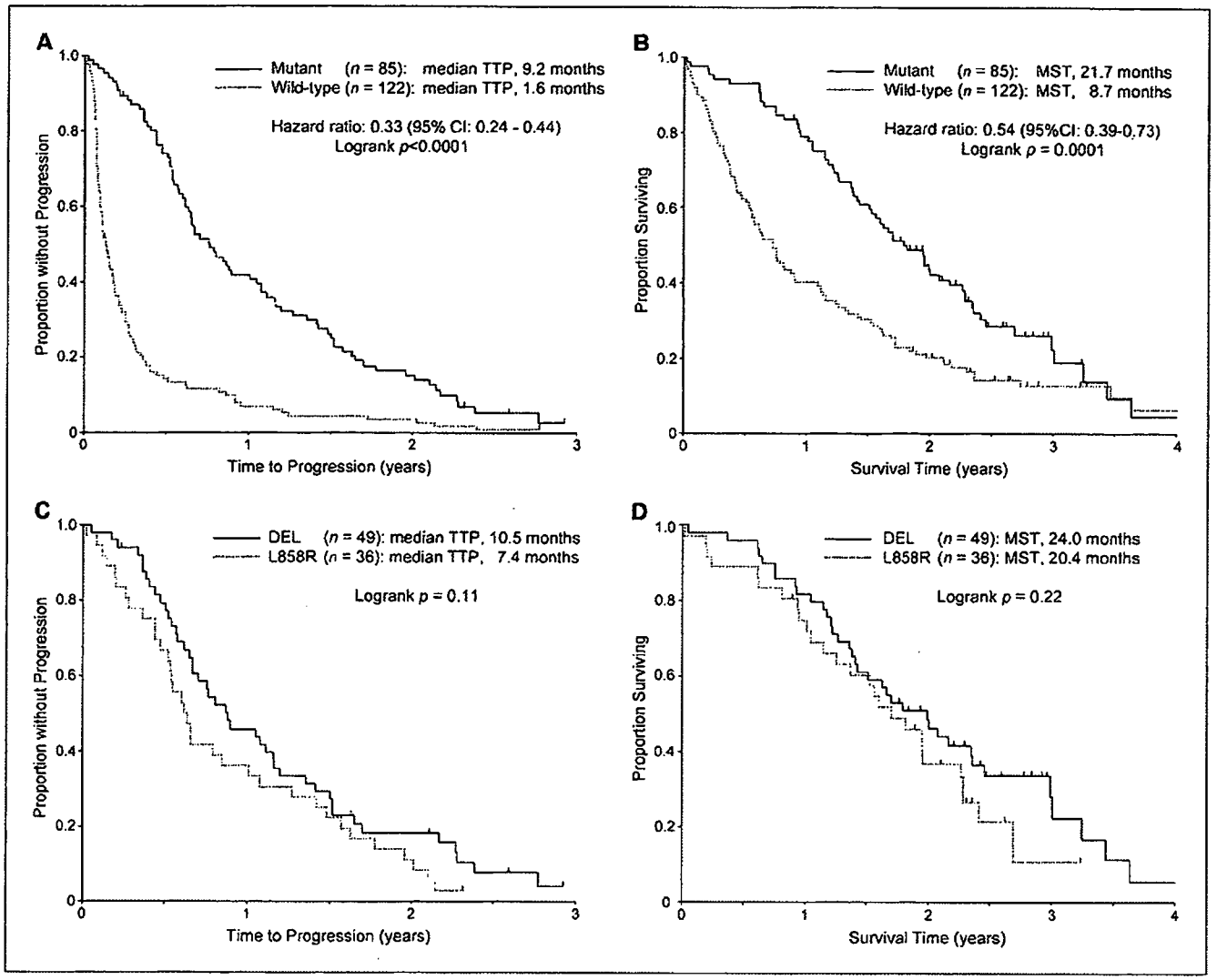

Fig. 1. Kaplan-Meier plot of time-to-progression (*A*) and overall survival (*B*) for patients with or without *EGFR* mutations. Kaplan-Meier plot of time-to-progression (*C*) and overall survival (*D*) for patients with DEL or L858R mutations. TTP, time-to-progression; MST, median survival time.

shown in Table 3, *EGFR* mutations were detected more frequently in women, never smokers, and patients with adenocarcinoma. Patient characteristics were not significantly different between patients with DEL mutations and those with an L858R mutation.

EGFR mutations and clinical outcomes. The association of the mutational status of *EGFR* and the response to gefitinib is shown in Table 4. The response rate was significantly higher in patients with *EGFR* mutations than in those with WT *EGFR* (78% versus 8%; $P < 10^{-23}$). Among patients with *EGFR* mutations, those with DEL mutations had a higher response rate than those with an L858R mutation (86% versus 67%; $P = 0.037$). Tumor responses were classified as SD in 11 patients with *EGFR* mutations and in 23 patients with WT *EGFR*. Among the patients with SD, a MR and/or a long SD (>6 months) were observed more frequently (91% versus 26%; $P = 0.0004$) and the time-to-progression was significantly longer (median, 6.9 versus 4.4 months; $P = 0.019$) in the patients with *EGFR* mutations than in the patients with WT *EGFR*.

As shown in Fig. 1, the time-to-progression (median, 9.2 versus 1.6 months; $P < 0.0001$) and overall survival (median, 21.7 versus 8.7 months; $P = 0.0001$) were significantly longer in patients with *EGFR* mutations than in those with WT *EGFR*. Patients with DEL mutations tended to have a longer time-to-progression (median, 10.5 versus 7.4 months; $P = 0.11$) and overall survival (median, 24.0 versus 20.4 months; $P = 0.22$) than those with an L858R mutation, although the difference did not reach statistical significance.

Clinical outcomes among subgroups of patients are shown in Table 5. In the univariate analysis, sex, smoking history, and histology were significant predictive factors for gefitinib sensitivity.

In the multivariate analyses, the mutational status of *EGFR* was an independent predictive factor of response [odds ratio, 38.9; 95% confidence interval (95% CI), 15.7-96.5; $P < 0.001$], time-to-progression (hazard ratio, 0.33; 95% CI, 0.24-0.45; $P < 0.001$), and overall survival (hazard ratio, 0.48; 95% CI, 0.34-0.67; $P < 0.001$). A poor performance status (2/3) was an

independent predictor of a shorter time-to-progression (hazard ratio, 1.80; 95% CI, 1.19-2.72; $P = 0.006$) and overall survival (hazard ratio, 3.97; 95% CI, 2.56-6.16; $P < 0.001$), and a history of prior chemotherapy was another independent predictor of a shorter overall survival (hazard ratio, 1.59; 95% CI, 1.14-2.23; $P = 0.006$). However, other clinical characteristics, including sex, smoking history, and histology, were not independent predictive factors for any clinical outcomes.

Discussion

In the current study, we showed the practicality of our new HRMA method for detecting two major *EGFR* mutations, DEL and L858R. The sensitivity and specificity of the analysis were 92% and 100%, respectively, when archived formalin-fixed, paraffin-embedded tissues were used without laser capture microdissection. Given the similar results that were obtained when Papanicolaou-stained cytologic slides were used (10), DEL and L858R mutations can likely be detected from such archived samples with about a 90% sensitivity and 100% specificity. Because the mutations were detected by HRMA even when only a small proportion (0.1% or 10%) of mutant cells existed (10), laser capture microdissection or other enrichment procedures are not needed in most cases. This is a major advantage of HRMA over direct sequencing because direct sequencing requires laser capture microdissection for accurate evaluation (6). However, there remained some risk of indeterminate or false-negative results because the DNA might have degenerated during sampling or the preservation of the archived samples. In fact, an analysis using methanol-fixed tissues, which are known to preserve DNA better than formalin-fixed tissues (12), was stable with no indeterminate and fewer false-negative results. Thus, an even higher sensitivity can be expected when fresh tumor samples are used. In any event, HRMA was successfully used to identify *EGFR* mutations and, more importantly, predict the clinical outcomes of gefitinib-treated patients with a high sensitivity and specificity.

Although the detection of *EGFR* mutations can provide patients with NSCLC and their physicians with critical information for optimal decision making, such tests are not common in clinical settings mainly because of the difficulty and impracticality of direct sequencing. Recently, highly sensitive nonsequencing methods to detect *EGFR* mutations in small tumor samples contaminated with normal cells have been reported (10, 13-21). Among them, HRMA has the advantages of being able to identify the mutations with less labor, time, and expense; PCR and the melting analysis can be done in the same capillary tube within a few hours, and the running cost is only about 1 U.S. dollar per sample. HRMA is expected to be one of the most practical methods for detecting *EGFR* mutations in clinical settings.

We analyzed consecutive gefitinib-treated patients in a single institution on a larger scale than any other previous report. The mutational analysis by HRMA was successful in 207 patients and confirmed strong and independent associations between the two major *EGFR* mutations and clinical outcomes. Clinical predictors, such as sex, smoking history, and histology, added little predictive information to that provided by the mutational analysis. We believe that the mutational status of *EGFR* is the most important predictor of clinical outcomes in gefitinib-treated patients.

Among the patients without the two major mutations, 8% were responders. This result may be due to false-negative HRMA results, other *EGFR* mutations, or other determinants of gefitinib sensitivity. As for other *EGFR* mutations, the direct sequencing of exons 18 to 24 was done in four responders without DEL or L858R mutations, and one of them had G719C and S768I mutations. Although missense mutations at codon 719 of *EGFR* (G719C, G719S, or G719A) may be associated with gefitinib sensitivity, the predictive significance of these mutations is unclear because the number of reported patients is small (6). At present, we consider the accurate detection of the two major *EGFR* mutations to be sufficient for optimal decision making.

Recently, the *EGFR* copy number was reported to be another predictor of gefitinib sensitivity (6, 22, 23), and Cappuzzo et al. (22) suggested that this factor was a stronger predictor of overall survival than *EGFR* mutations. Our previous study also showed that the *EGFR* copy number evaluated by quantitative

Table 5. Clinical outcomes among subgroups of patients

	n	Response rate (%)	*P*	Median TTP (mo)	*P*	MST (mo)	*P*
Total	207	37		3.7		14.5	
Sex							
Women	89	51	<0.001	5.6	0.17	18.3	0.15
Men	118	26		2.3		9.6	
Smoking history							
Never smokers	93	51	<0.001*	6.2	0.073*	16.9	0.22*
Former smokers	38	47		5.2		14.5	
Current smokers	76	14		2.2		9.1	
Histology							
Adenocarcinoma	189	40	0.004	4.3	0.060	15.1	0.10
Others	18	6		1.6		4.9	
EGFR mutations							
DEL/L858R	85	78	<0.001	9.2	<0.001	21.7	<0.001
WT	122	8		1.6		8.7	

Abbreviations: TTP, time-to-progression; MST, median survival time.
*Comparison between never smokers and others.

PCR was associated with response; however, an increased *EGFR* copy number was concentrated in patients with *EGFR* mutations and was not an independent predictor of response and overall survival (6). In the current study, we showed that *EGFR* mutations were associated with better outcomes even among patients with SD. The interpretation of this result is difficult because a long SD might be caused by intrinsic characteristics independent of treatment; however, this result suggested that *EGFR* mutations predicted not only "super responders" but also "non-super responders" who gained a clinical benefit. Contrary to these findings, Cappuzzo et al. (22) showed that *EGFR* mutations predicted only responders and were not associated with overall survival, whereas *EGFR* copy number was associated with both response and SD and was an independent predictor of overall survival. Although the reason of these discrepancies is unclear, we consider that if *EGFR* mutations are accurately identified, *EGFR* copy number adds little information for patient selection, at least in Japanese patients.

About the outcomes of patients with DEL or L858R mutations, our larger scale study produced results similar to those of some previous studies, which indicated that DEL mutations were associated with better outcomes after EGFR tyrosine kinase inhibitor treatment than an L858R mutation (24–27). Further investigations are needed to clarify the difference in the biological characteristics of the two mutations. However, in the current study, the difference was small and even patients with an L858R mutation had favorable outcomes: the response rate was 67%, the median time-to-progression was 7.4 months, and the median survival time was 20.4 months. We now think that both DEL and L858R mutations should be treated equally in clinical decision-making.

In conclusion, the detection of DEL and L858R mutations using HRMA is accurate and practical. Using HRMA, we confirmed a strong association between the two major *EGFR* mutations and clinical outcomes in patients with advanced NSCLC treated with gefitinib.

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Epidermal growth factor receptor mutation status and clinicopathological features of combined small cell carcinoma with adenocarcinoma of the lung

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In lung cancer, somatic mutations of epidermal growth factor receptor (*EGFR*) are concentrated in exons 18–21, especially in adenocarcinoma (Ad), but these mutations have rarely been reported in small cell lung carcinoma (SCLC). Combined SCLC is rare, and the *EGFR* mutation status and its relationship to the clinicopathological features of this tumor type have not yet been elucidated. We retrospectively studied six patients with combined SCLC with Ad components among 64 consecutive patients who underwent resection of SCLC. The clinicopathological features of each patient were reviewed, especially for the distribution pattern of the Ad component and lymph node metastases. *EGFR* mutations were screened by high-resolution melting analysis in each case, and were confirmed by sequencing of each mutation in the microdissected SCLC or Ad components. Regarding *EGFR*, no specific mutation was detected in five of the six patients, whereas one female patient who had never smoked had a missense mutation. In this case, both the SCLC and Ad components shared the same mutation in exon 21 (L858R). We identified a patient with combined SCLC with Ad sharing an identical *EGFR* mutation in both the SCLC and Ad components. In addition to the clinicopathological characteristics of this rare histological type of lung cancer, these findings provide useful information for better understanding the biology, natural history and clinical management of SCLC. (*Cancer Sci* 2007; 98: 1714–1719)

Small cell lung carcinoma (SCLC) accounts for 15–20% of all lung cancers worldwide.[1] SCLC is known to be more sensitive than non-SCLC to chemotherapy, but shows a more aggressive clinical course. The median survival time without treatment is 2–4 months.[2,3] Approximately 20% of patients with limited SCLC achieve a cure, but most patients with SCLC will relapse, and relapsed or refractory SCLC has a uniformly poor prognosis with a 5-year survival rate of less than 5%.[4]

According to the 2004 World Health Organization (WHO)/International Association for the Study of Lung Cancer (IASLC) classification of lung and pleural tumors,[5] 'combined SCLC' is defined as SCLC combined with an additional component that consists of any of the histological types of non-SCLC, usually adenocarcinoma (Ad), squamous cell carcinoma (Sq) or large cell carcinoma. Combined SCLC is rare, and has been reported to account for less than 1–3.2% of all SCLC.[6,7] However, a high proportion (12–26%) of SCLC patients who undergo surgical resection show combination with non-SCLC.[8–12]

In a clinical setting, the distinction of SCLC from non-SCLC is critical because of major differences in patient management and prognosis. Recently, molecular targeted therapy has been developed using agents such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which exerts antitumor activity in patients with advanced non-SCLC (especially Ad) with *EGFR* mutations. High expression of EGFR has been reported in various epithelial malignant tumors, including lung cancer,[13,14] and somatic mutations in the kinase domain of *EGFR* are suggested to be strongly correlated with sensitivity to EGFR tyrosine kinase inhibitor.[15,16] These mutations are concentrated in exons 18–21 of *EGFR*, and approximately 90% of *EGFR*-mutant patients with lung Ad have mutations in two hot spots: in-frame deletion at codons 747–749 (DEL) in exon 19, and a missense mutation at codon 858 (L858R) in exon 21.[17,18] Although these mutations have rarely been reported in SCLC, two recent studies have demonstrated *EGFR* mutation in SCLC.[19,20]

In the present study, we retrospectively investigated six resected cases of combined SCLC with an Ad component to elucidate the clinicopathological features of this rare tumor, especially the ratio of each tumor component, the distribution patterns of the Ad component, and the status of lymph node metastasis. The *EGFR* mutation status in surgically resected specimens was also analyzed for each histological type in the same tumor.

Materials and Methods

Patients and histological diagnosis. A search of our surgical pathology files covering the period January 1982 to December 2004 yielded 64 consecutive patients with SCLC who had undergone surgical resection at the National Cancer Center Hospital, Tokyo, Japan. For the purposes of the present study, we identified six patients with combined SCLC with an Ad component. The research protocol was approved by the Institutional Review Board.

The surgically resected specimens were fixed in 10% formalin. All sections containing both tumor tissues and surrounding lung tissues were embedded in paraffin. Additional consecutive 5 μm-thick sections were cut from the tissue block and stained with hematoxylin and eosin. All histological diagnoses were reviewed by certificated pathologists (K. T., A. M. M. and Y. M.) based on the most recent WHO/IASLC classification of lung and pleural tumors.[5] Both clinical and pathological staging data for each patient have been reported according to the International Staging System for Lung Cancer.[21] Patient survival was calculated as the time between operation and death.

Immunohistochemistry and evaluation. For phenotypic analysis, paraffin section immunohistochemistry was carried out using the primary antibodies listed in Table 1, followed by subsequent labeling with the Envision+ horseradish peroxidase (HRP) system (DAKO, Carpinteria, CA, USA). For heat-induced epitope retrieval, sections stained for p63 were treated with 1.0 mmol/L

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Table 1. Results of immunohistochemistry

Patient no.	SCLC component (%)	Immunoreaction					Non-SCLC component (%)	Immunoreaction					No. tumor embolism cells per slice[†] (%)		
		CgA	SYN	NCAM	TTF-1	p63		CgA	SYN	NCAM	TTF-1	p63	SCLC	Ad	Sq
1	95	2+	3+	3+	3+	0	Ad, 5	1+	1+	1+	3+	0	30 (97)	1 (3)	–
2	80	3+	3+	3+	3+	0	Ad, 10	0	1+	1+	2+	1+	21 (84)	3 (12)	1 (4)
							Sq, 10	0	0	0	0	3+			
3	70	1+	3+	3+	3+	0	Ad, 30	0	1+	0	3+	0	38 (93)	3 (7)	–
4	55	2+	3+	3+	3+	0	Ad, 45	0	0	1+	1+	0	24 (92)	2 (8)	–
5	35	3+	3+	3+	3+	0	Ad, 60	1+	1+	1+	3+	1+	17 (100)	0 (0)	0 (0)
							Sq, 5	0	1+	0	0	2+			
6	5	Not done					Ad, 95	Not done					Not done		

CgA, chromogranin-A; NCAM, neural cell adhesion molecule; SCLC, small cell lung carcinoma; SYN, synaptophysin; TTF-1, thyroid transcription factor-1. Semiquantitative assessments of the percentage of positive tumor cells (0 = none, 1+ = 1–33%, 2+ = 34–66%, 3+ = 67–100%) were made. [†]We counted the number of lymph vessels with tumor embolisms confirmed by staining for D2-40 for a representative slide.

Table 2. Clinical characteristic of the patients with combined small cell lung carcinoma (SCLC) with adenocarcinoma (Ad)

Patient no.	Age/Sex	ECOG PS	Smoking status	Smoking index	Tumor location	Size (mm)	Stage (cTNM)	Preoperative diagnosis	Surgical procedure
1	74/Male	0	Current	2160	Peripheral	31	IIb (210)	Unknown	RLL[†]
2	66/Male	0	Ever	900	Peripheral	38	IIb (210)	Unknown	RM/LL[‡]
3	62/Female	0	Never	0	Peripheral	31	Ib (200)	SCLC	LUL
4	77/Male	1	Current	570	Peripheral	15	Ia (100)	Unknown	Left pneumonectomy
5	75/Male	0	Ever	1000	Peripheral	30	Ia (100)	Non-SCLC	RUL
6	76/Male	0	Current	1120	Peripheral	28	Ia (100)	Ad	RUL

Smoking index: (number of cigarettes smoked per day) × years. Adjuvant chemotherapy: [†]cyclosphosphamide + doxorubicin + vincristine × 1 cycle. [‡]Cisplatin + etoposide × 1 cycle followed by cisplatin + irinotecan × 3 cycles. LUL, left upper lobectomy; RLL, right lower lobectomy; RM/LL, right middle and lower lobectomy; RUL, right upper lobectomy.

ethylenediaminetetraacetic acid buffer (pH 8.0). Sections stained for chromogranin A (1:500, polyclonal; DAKO), synaptophysin (1:100, polyclonal; DAKO), neural cell adhesion molecule (NCAM) (1:200, Lu243; Nihon Kayaku, Tokyo, Japan), thyroid transcription factor (TTF)-1 (1:100, 8G7G3/1; DAKO), p63 (1:100, 4A4; DAKO) and D2-40 (1:50, D2-40; DAKO) were treated with 0.02 mol/L citrate buffer (pH 6.0). The slides were incubated overnight with each primary antibody. Diaminobenzidine was used as the chromogen, and hematoxylin as the counterstain.

Positive staining was defined as distinct linear membrane staining for neural cell adhesion molecule, cytoplasmic staining for chromogranin A and synaptophysin, and nuclear staining for TTF-1 and p63. Immunostaining of each of the SCLC and non-SCLC components was graded on a scale of 0–3+ according to the percentage of positive tumor cells (0 = none; 1+ = 1–33%; 2+ = 34–66%; 3+ = 67–100%). We then carried out immunohistochemical identification of lymph vessels with or without tumor embolisms for a representative slide.[22] After independent evaluation by two of us (T. F. and K. T.), judgment consensus was obtained by joint viewing of the slides using a multiheaded microscope.

Analysis of *EGFR* mutational status. In our previous study, we established a practical and precise non-sequencing method for detecting *EGFR* mutations involving high-resolution melting analysis (HRMA) using LCGreen I dye (Idaho Technology, Salt Lake City, UT, USA).[23] First we screened for the *EGFR* mutations, DEL and L858R, using the HRMA method in formalin-fixed paraffin sections obtained from surgically resected combined SCLC with Ad. Human genomic DNA (Roche Diagnostics, Basel, Switzerland) was used as a control sample with wild-type *EGFR*. Second, we used 10% formalin-fixed, paraffin-embedded surgical specimens of primary combined SCLC from patients demonstrating DEL or L858R by HRMA, and the DNA was extracted from each of the SCLC and Ad components, respectively, the areas of which were clearly determined morphologically after laser capture microdissection (Arcturus Engineering, Mountain View, CA, USA) of the tumor tissue.[24] Nested polymerase chain reaction (PCR) was carried out to amplify exons 19 and 21 of *EGFR* using previously described primers.[17] The PCR products were electrophoresed on 2% agarose gels and subcloned into the TA vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA), then the sequences were determined with M13 primers using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Results

Clinical characteristics. The clinical characteristics of the six patients are shown in Table 2. All patients were Japanese, aged between 62 and 77 years (mean 71.7 years). Five patients were male and one was female. Five patients were smokers whereas the remaining patient had never smoked. The median survival time of the six patients was 16.8 months (range 0.4–27.4 months); one patient died of heart failure 13 days after left pneumonectomy.

All six tumors were located in the peripheral portion of the lung. On clinical evaluation, three patients were staged as Ia (T1N0M0), one as Ib (T2N0M0) and two as IIb (T2N1M0). Preoperative pathological diagnoses were obtained in three patients and comprised one case each of SCLC, non-SCLC and Ad.

Pathological findings. Among six patients with combined SCLC with Ad, histological examination demonstrated that four had

Table 3. Histological findings of primary tumor and lymph node metastases, and epidermal growth factor receptor (*EGFR*) mutation

Patient no.	Stage (pTNM)	Ratio of each component (%)			Histological type of lymph node metastasis		BAC-like extension	*EGFR* mutation
		SCLC	Ad	Sq	Mediastinal	Hilar		
1	IIa (110)	95	5	0	Non†	SCLC	Absent	Wild type
2	IIIa (220)	80	10	10	SCLC	SCLC	Present	Wild type
3	IIIb (410)	70	30	0	Non†	Ad	Present	L858R
4	IIIb (420)	55	45	0	Ad	SCLC or Ad‡	Present	Wild type
5	IIIa (220)	35	60	5	Ad	SCLC or Ad‡	Present	Wild type
6	Ib (200)	5	95	0	Non†	Non†	Present	Wild type

†The patient had no mediastinal or hilar lymph node metastasis. ‡The patient had lymph node metastasis only from the SCLC component, and another lymph node showing metastasis only from the Ad component. Ad, adenocarcinoma; BAC, bronchioloalveolar carcinoma; hilar, hilar lymph node; L858R, mutation at codon 858 of *EGFR*; medical, mediastinum lymph node; pTNM, pathological TNM; SCLC, small cell lung carcinoma; Sq, squamous cell carcinoma.

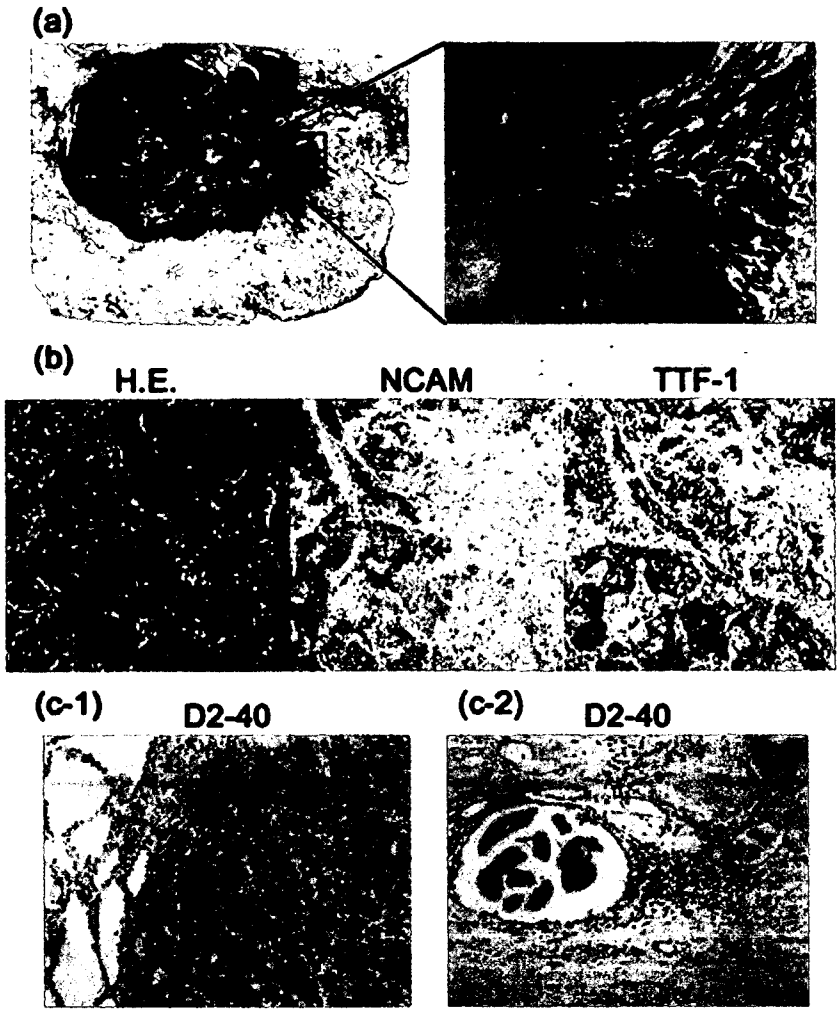

Fig. 1. Combined small cell lung carcinoma (SCLC) with adenocarcinoma (Ad). (a) The periphery of this tumor consisted of a non-mucinous bronchioloalveolar carcinoma-like extension (patient no. 3). (b) The transitional zone between the SCLC and Ad components had poorly differentiated cells, shown by the immunohistochemical studies (patient no. 1). (c) D2-40 with a membranous staining pattern of the lymph vessels. Tumor embolism of lymph vessels was confirmed by D2-40 staining (patient no. 3). (c-1) SCLC cell embolisms increased in number around the primary lesion. (c-2) Ad cell embolisms invaded the lymph vessels.

SCLC combined only with an Ad component (ratio of Ad in the tumor: 5, 30, 45 and 95%), whereas two had both Ad and Sq components (ratio of Ad/Sq: 10%/10% and 60%/5%, respectively). On pathological staging, one patient was staged as Ib (T2N0M0), one as IIa (T1N1M0), two as IIIa (T2N2M0) and two as IIIb (T4N1M0 and T4N2M0). In five of the six patients, the Ad components were observed in the peripheral part of the tumor showing a lepidic extension pattern, simulating bronchioloalveolar carcinoma. In the remaining one patient, Ad formed a minor component comprising approximately 5% of the tumor (Table 3). The Ad components in two patients showed a micropapillary growth pattern, whereas mucin production was not detected in any patient (Fig. 1a). The boundary between the SCLC and Ad components was not clear, and showed an

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indeterminate component that suggested gradual morphological transition from one to the other (Fig. 1b). In the two patients who also had combined Sq, the Sq component showed keratinization and was distinct from the SCLC component, but the border between the Ad and Sq components was unclear.

The results of immunohistochemical studies carried out in five cases are shown in Table 1. The specimen from patient no. 6 was not available. All of the SCLC components showed positive staining for at least one neuroendocrine marker. In addition, the Ad components in all five patients examined showed positive staining for at least one neuroendocrine marker, although semiquantitative assessments of the percentage of positive Ad cells were lower than those for SCLC cells in the same tumor. Also, the Ad components showed positive staining for TTF-1 in all five patients. TTF-1 staining of the SCLC component tended to be similar to that of the Ad component in terms of the percentage of positive cells. p63 immunostaining served as a good marker of Sq differentiation.

Status of lymph node metastasis. Five patients had pathologically confirmed hilar lymph node metastases, and three of them also had histologically proven mediastinal lymph node metastases, which had not been evident at the time of preoperative clinical evaluation (Table 3). Among these five patients with hilar lymph node metastases, two showed only SCLC in the metastatic lesion, one showed Ad only, and two showed SCLC or an Ad component that had developed separately in each lymph node. Among the three patients with mediastinal lymph node metastases, one had only SCLC in the nodes, and two had an Ad component only. Metastatic Ad components were found only in patients with a primary tumor in which Ad accounted for more than 30% of the total volume.

In the six patients, we identified tumor embolism of the lymph vessels immunohistochemically with D2-40 staining. There were approximately 800–1000 lymph vessels in each of these tumors per representative slide. The major component invading the lymph vessels around the tumors was SCLC cells. Even in the two patients who had mediastinal lymph node metastases with an Ad component, the SCLC cells tended to spread to the lymph vessels rather than the Ad cells (Table 1).

***EGFR* mutational status.** First, we analyzed 10 surgically resected samples from six patients with combined SCLC and Ad by HRMA. Analysis of exon 19 demonstrated curves identical to those of the control (wild type) in all samples, as shown in Fig. 2a. In the analysis of exon 21, thorough melting curves were obtained for two samples from patient no. 3, showing a different curve from the control, whereas the other eight samples demonstrated curves identical to the control (wild type), as shown in Fig. 2b. The normal lung tissue from patient no. 3, who was a female non-smoker, showed a wild-type curve, and therefore we judged that this patient had L858R in exon 21 of *EGFR*.

Next we confirmed this mutation in the SCLC and Ad components in patient no. 3. DNA was extracted from each SCLC and Ad component separately using laser capture microdissection or by manual microdissection, which was carried out for each clearly determined component on paraffin-embedded sections. Sequence analysis of subcloned PCR products obtained from the separate components was carried out. Examination of both SCLC and Ad components showed an identical mutation (L858R) in exon 21 (Fig. 3), confirming the results obtained by HRMA.

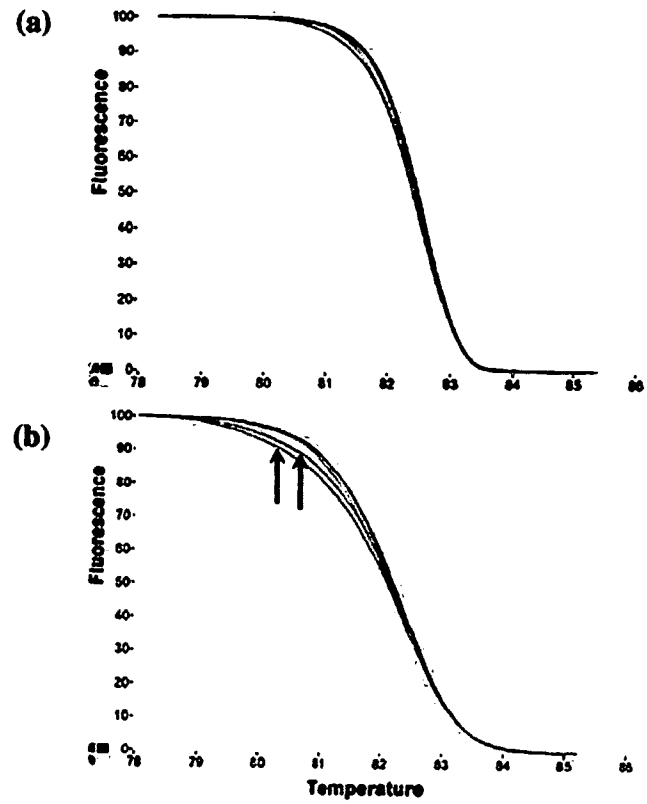

Fig. 2. Results of high-resolution melting analysis (HRMA). Adjusted melting curves obtained by HRMA of combined small cell lung carcinoma (SCLC) with primers designed to detect mutations in (a) exon 19 or (b) exon 21 of epidermal growth factor receptor (*EGFR*). Two samples from patient no. 3 were identified as containing the L858R mutations (↑). The DNA extracted from normal lung tissue of patient no. 3 was identified as wild type (not shown).

Discussion

The present study using microdissected tumor tissue is the first to report a patient with combined SCLC with Ad showing the *EGFR* mutation in both the SCLC and Ad components. *EGFR* mutations, especially DEL and L858R, have been reported in Ad of the lung. These somatic mutations in the kinase domain of *EGFR* have been shown to be predictive molecular markers for sensitivity to kinase inhibitors such as gefitinib (Iressa; AstraZeneca, Osaka, Japan). However, these mutations have rarely been demonstrated in SCLC. To our knowledge, there have been two reported cases of metastatic SCLC harboring DEL in exon 19 of *EGFR* showing responsiveness to EGFR tyrosine kinase inhibitors.[19,20,25] Considering that the diagnosis of SCLC is often based on small biopsy specimens that may not be sufficiently representative of the total tumor, there is a possibility that any combined component may be overlooked.

In a clinical setting, the distinction of SCLC from non-SCLC is critical because of major differences in management and prognosis between the two cancers. SCLC is well known to be more common in men and smokers, but so far SCLC with *EGFR* mutations has been detected only in female patients who have never smoked,[19,20] as was the case in our present female patient. Thus it seems reasonable to suggest that in clinically unusual SCLC patients, for example those who are non-smokers and female, showing peripheral nodular lesions and histological combination with Ad, *EGFR* mutation status should be analyzed because previous studies have shown that EGFR tyrosine kinase inhibitors are effective in patients with metastatic SCLC with *EGFR* mutations.

The present study is considerably informative with regard to the origin and histogenesis of SCLC. *EGFR* mutation is detected in patients with pre-invasive adenocarcinomatous lesions such as atypical adenomatous hyperplasia and bronchioloalveolar

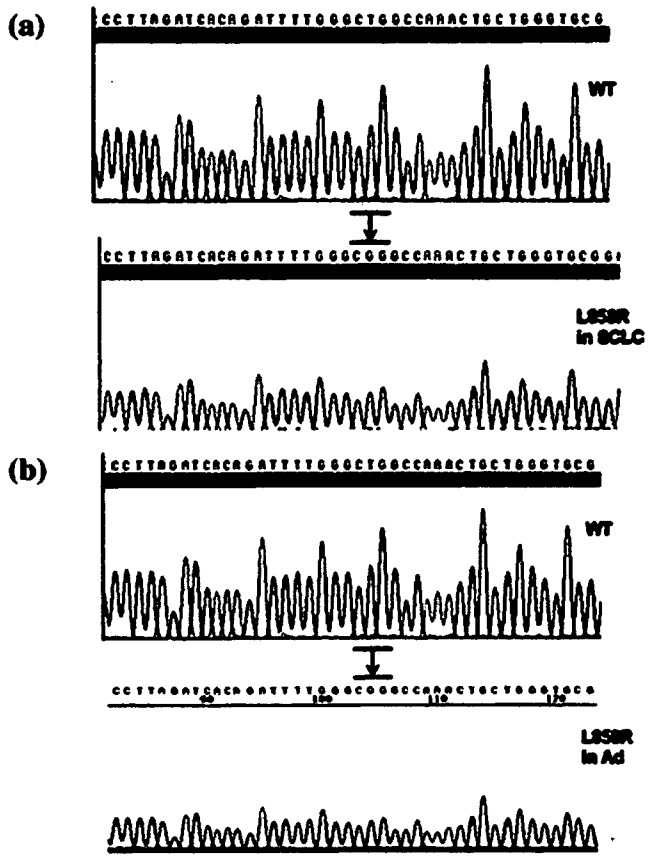

Fig. 3. Results of DNA sequencing from patient no. 3. The tumor of patient no. 3 was microdissected into the small cell lung carcinoma (SCLC) and adenocarcinoma (Ad) components. (a) Sequence analysis of the subcloned polymerase chain reaction (PCR) products from the microdissected SCLC component. (b) Sequence analysis of the subcloned PCR products from the microdissected Ad component. The patient had a tumor with L858R of EGFR, which was in both the SCLC and Ad components.

carcinoma, which eventually progress to invasive lung Ad.[26] In addition, *EGFR* mutations are also linked to Ad with a bronchioloalveolar carcinoma component.[27] Thus it is suggested that *EGFR* mutation occurs and plays a critical role in the early developmental stage of lung Ad. The mutation is detected more frequently in Ad in female non-smokers than in male smokers. In the present study, the only patient with SCLC harboring an *EGFR* mutation was female and a non-smoker, and the combined Ad component also harbored the same mutation. Moreover, as mentioned above, the two SCLC patients with *EGFR* mutation reported previously were also female and non-smokers. These findings imply that the mutations are an early genetic event in carcinogenesis of the lung and at least a certain proportion of SCLC may originate as a result of progression or transformation of Ad harboring *EGFR* mutation.

This phenomenon can also be linked to pathological features. The histological patterns of lymph node involvement showed that Ad components spread to mediastinal lymph nodes in the patients with hilar lymph node involved by SCLC or Ad component. Considering the status of tumor embolism of the lymph vessels observed using D2-40 staining, SCLC cell embolisms, but not Ad, increase in number around primary lesion in these tumors. It is suggested that a common uncommitted stem cell might differentiate into each component after involvement in a lymph node. Furthermore, positive staining for TTF-1, which is a highly specific immunohistochemical marker identifying carcinomas of pulmonary origin (especially non-mucinous Ad and SCLC),[28] was shown in the SCLC and Ad components, but not Sq. Previous studies have demonstrated TTF-1 expression in 83–100% of SCLC, but low expression in Sq.[29,30] These findings could be interpreted as being compatible with the hypothesis that SCLC and Ad originate from a common uncommitted stem (or precursor) cell originally expressing TTF-1.[31] It is possible to postulate that a fraction of SCLC possessing stem (or precursor) cell properties might have the potential to form an Ad component. In fact, in the present cases, there were some areas comprising morphologically indeterminate tumor cell components at the border of the SCLC and Ad components.

The rarity of patients with combined SCLC makes it difficult to determine the optimal management and biological characteristics of this tumor. However, the present findings suggest that the classical classification of lung cancer might provide insufficient management for a specified subpopulation in molecular targeted therapy. Although this retrospective study examined only a very limited number of lung carcinoma cases, we consider that the findings provide useful information for understanding the biology of this lung cancer and devising more effective forms of clinical management.

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