that the expression level of H-FABP was significantly different between the responder (PR and CR) and nonresponder (PD) groups ($P = 0.0031$, Mann-Whitney U test) and also between the patients with MR or SD and the nonresponder group ($P = 0.0047$, Mann-Whitney U test). These results indicate that up-regulation of H-FABP in tumor tissues can be monitored by routine clinical methods.

Discussion

We identified 87 protein spots of which the intensity was statistically significantly different between samples from the responder (CR and PR) and nonresponder (PD) groups in the training set. Application of a data-mining procedure allowed identification of a set of nine protein spots that accurately distinguished between responders and nonresponders. The different expression levels of these nine protein spots allowed classification of 13 of 14 of our test PR and PD cases in accordance with their clinical response to gefitinib. These protein spots classified cases showing a MR to gefitinib (MR) into the responder group. The intermediate cases, SD, were categorized into both responder and nonresponder groups. The usefulness of our findings will be validated in a larger clinical data set.

Table 3. List of proteins for the response to gefitinib

Spots no.*	Rank	Accession no.†	Identtified protein†	MW (DA)‡	pI‡	Ion charge state (+)	MZ (obs)§	Mass‖	δ¶	Miss**	Mascot ions score††	Peptide sequence
384	5	Q96RP9	Ig mu chain C region	49,557	6.35	2	810.3	1,617.7	0.91	0	74	QVGSGVTTDQVQAEAK
						2	640.1	1,277.5	0.63	0	47	YAATSQVLLPSK
671	1	P01876	Ig α-1 chain C region	37,655	6.08	2	919.2	1,836.0	0.32	0	68	QEPSQGTTTFAVTSILR
						2	771.8	1,540.7	0.91	0	54	DASGVTFTWTPSSGK
1090	7	Q9UNH7	SNX 6	46,649	5.81	2	636.5	1,270.5	0.55	0	73	NLVELAELELK
						2	577.0	1,152.2	−0.33	0	39	SLVDYENANK
1182	8	P50453	Cytoplasmic antiproteinase 3	42,404	5.61	2	816.4	1,629.8	0.95	0	82	IEELLPGSSIDAETR
						2	626.6	1,249.4	1.66	0	75	AFQSLLTEVNK
						2	591.0	1,179.5	0.47	0	63	LVLVNAIYFK
						2	757.5	1,513.6	−0.56	0	47	LQEDYDMESVLR +Oxidation (M)
1292	6	P40121	Macrophage capping protein	38,518	5.88	2	633.8	1,264.4	1.18	0	85	VSDATGQMNLTK
						2	676.8	1,351.4	0.05	0	79	YQEGGVESAFHK
						2	932.1	1,861.1	1.11	0	50	MQYAPNTQVEILPQGR +Oxidation (M)
						2	659.8	1,317.3	0.23	0	41	EGNPEEDLTADK
1711	3	Q8NBJ7	Sulfatase modifying factor 2	33,857	7.78	2	792.5	1,581.7	1.32	0	112	MGNTPDSASDNLGFR
						2	779.9	1,557.6	0.15	0	95	GASWIDTADGSANHR
						2	740.0	1,477.6	0.36	0	83	LPTEEEWEFAAR
						2	613.2	1,224.4	−0.02	0	66	FLMGTNSPDSR
						2	629.9	1,256.5	1.27	0	55	SVLWWLPVEK
						2	818.0	1,633.8	0.12	1	55	RLPTEEEWEFAAR
						2	837.7	1,672.9	0.48	0	47	LEHPVLHVSWNDAR
2091	9	P09211	Glutathione *S*-transferase P	23,225	5.44	2	647.5	1,292.5	0.44	0	36	MLLADQGQSWK +Oxidation (M)
2182	4	P02794	Ferritin heavy chain	21,094	5.30	2	823.4	1,643.8	1.04	0	91	MGAPESGLAEYLFDK +Oxidation (M)
						2	648.3	1,294.5	0.03	0	53	NVNQSLLELHK
2478	2	P05413	Fatty acid–binding protein, heart	14,727	6.34	2	735.2	1,467.5	0.81	0	103	LGVEFDETTADDR
						2	798.7	1,595.7	−0.32	1	73	LGVEFDETTADDRK
						2	603.3	1,204.3	0.26	0	70	WDGQETTLVR
						2	455.0	907.0	1.04	0	67	SLGVGFATR
						2	774.7	1,546.8	0.56	0	61	QVASMTKPTTIIEK
						2	438.0	873.0	0.88	0	54	NGDILTLK
						1	889.6	889.0	−0.41	0	45	SIVTLDGGK

Abbreviation: pI, isoelectric point.
*Spot numbers refer to those in Fig. 1B (Supplementary Fig. S3).
†Accession nos. of proteins were derived from Swiss-Prot and National Center for Biotechnology Information nonredundant databases.
‡Theoretical molecular weight and isoelectric point were obtained from Swiss-Prot and the ExPASy database (http://au.expasy.org).
§Experimental m/z value.
‖Relative molecular mass calculated from the peptide sequence.
¶Difference (error) between the experimental and calculated masses.
**Number of missed cleavage sites.
††Mascot ions score (http://www.matrixscience.com/search_form_select.html).

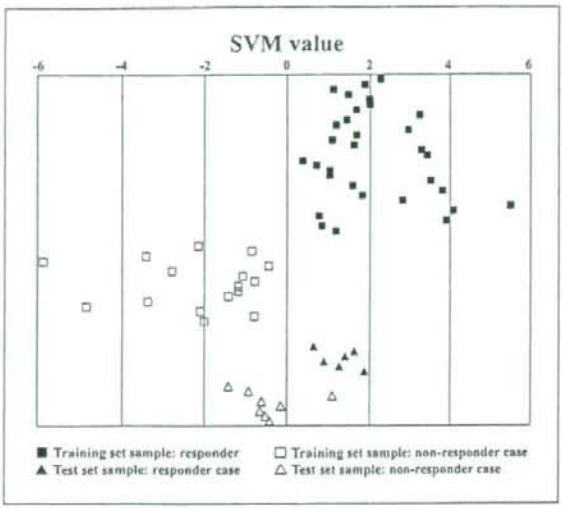

Fig. 2. Predictive performance of the nine spots was validated by examining the SVM value of each sample in the group.

We identified the proteins whose expression was correlated with response to gefitinib and found associations with the EGFR signal pathway and with the biology of lung cancer. Sorting nexin (SNX) 6 is a member of a SNX family that functions in the intracellular trafficking of plasma membrane receptors (33). SNXs form complexes with other SNXs and with plasma membrane receptors. In complexes with SNX1, SNX2, and SNX4, SNX6 interacts with the intercellular portion of the EGFR as well as with transforming growth factor-β receptor, insulin receptor, leptin receptor, and platelet-derived growth factor receptor (34). By binding to the kinase domain of the transforming growth factor-β receptor, SNX6 perturbs transforming growth factor-β signal transduction (34). The other SNX family, SNX1, decreases the expression of EGFR by activating the endosome-to-lysosome pathway with enterophilin-1 (35), although the functions of the complex of SNX6 and EGFR have not yet been reported. The functional association of SNX6 with oncogene product Pim-1, which has been implicated in the development of hematopoietic (36), gastric (37), and prostatic (38) malignancies, suggests the involvement of SNX6 in cancer biology. Kakiuchi et al. (21) reported that another SNX family member, SNX13, was correlated with the response to gefitinib in patients with NSCLC. These reports suggest that SNX6 might play an important role in signal transduction pathways that affect the phenotypes of lung cancer.

We tried to identify the proteins whose expression was associated with EGFR mutation. Because gefitinib is a specific inhibitor of EGFR and mutation of EGFR is considered to be a predictive marker for gefitinib sensitivity, we had expected some similarity between the set of proteins predicting sensitivity to gefitinib and the set of proteins reflecting EGFR mutation status. However, only sulfate modifying factor 2 was common to the two sets. Search of the PubMed database revealed no association of sulfate modifying factor 2 with the EGFR pathway and no evidence for its involvement in resistance to chemotherapy. Similarly, the other proteins correlated with EGFR mutation status had no obvious involvement in the EGFR pathway. Functional studies on these proteins will contribute to further understanding of EGF signaling in cells and to discovery of novel therapeutic targets in lung cancer.

2D-DIGE is a high-performance proteomic technology and a powerful tool to develop candidate biomarkers. However, 2D-DIGE requires expensive fluorescent dyes and well-trained operators to run the gels. Thus, routine clinical studies with multiple large-format two-dimensional gels and a 2D-DIGE protocol are unlikely to be practical. Application of our results requires a simple and cost-effective method that can be used routinely in the clinic. In addition, as we need to examine the expression of multiple proteins, a practical tool for simultaneously measuring the amount of the other proteins is required. With that in mind, we validated measurement of the differential expression of H-FABP by the use of a commercially available ELISA kit (MARLIT-M H-FABP) that is routinely used in hospitals for the early diagnosis of acute myocardial infarction using serum samples. The expression level of H-FABP in tumor tissues as monitored by the ELISA assay was highly correlated with that by 2D-DIGE, and a significant difference in H-FABP expression was observed between responders (CR + PR), minor responders (MR + SD), and nonresponders (PD). Thus, our results can provide a simple and direct method to predict the response to gefitinib.

H-FABP functions in intracellular lipid transport, storage, and metabolism. As H-FABP is highly expressed in heart and released into plasma after myocardial injury, it has been used as a plasma marker for early diagnosis of acute myocardial infarction and stroke. However, many lines of evidence also suggest an association of H-FABP with cancer biology. Higher expression of H-FABP was observed in a more tumorigenic small-cell lung cancer cell line (39) compared with its counterpart. Increased expression of H-FABP is associated with tumor aggressiveness, metastasis, and poor prognosis of gastric cancer (40). In contrast, H-FABP is known to have growth-inhibitory activity in breast cancer cells (41), and breast cancer does not express H-FABP because of gene silencing by hypermethylation (42). These observations suggest complexity in the way that H-FABP is involved in the progression of cancer. Recently, Loeffler-Ragg et al. (43) reported that another FABP family member, E-FABP, is up-regulated in gefitinib-resistant colon cancer cell lines compared with gefitinib-sensitive cell

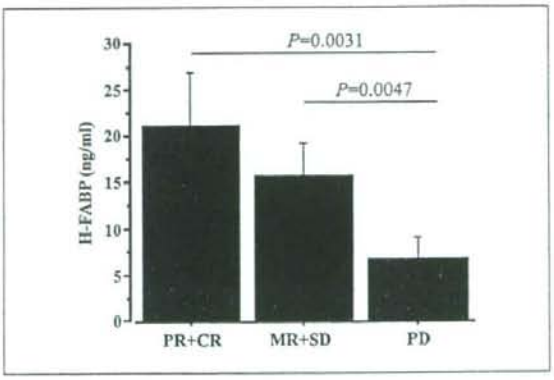

Fig. 3. ELISA assay for H-FABP. The differential expression level of H-FABP was validated by ELISA assay.

lines. Further study on the contribution of the FABP family to cancer phenotypes, including resistance to chemotherapy, will provide novel insights into cancer biology.

In conclusion, our proteomic study has identified proteins whose expression can predict the response to gefitinib in patients with recurrence of lung adenocarcinoma. Large-scale validation of the present results and functional analysis to elucidate the contribution and synergies of the identified proteins in the response to gefitinib will assist in developing novel therapeutic strategies for lung cancer.

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Gene expression profiling of epidermal growth factor receptor/KRAS pathway activation in lung adenocarcinoma

Tatsuhiro Shibata,[1,2,7] Satoko Hanada,[3,8] Akiko Kokubu,[1] Yoshihiro Matsuno,[4] Hisao Asamura,[5] Tsutomu Ohta,[3] Michiie Sakamoto[6] and Setsuo Hirohashi[1,2]

[1]Cancer Genomics Project, [2]Pathology Division, [3]Center for Medical Genomics, National Cancer Center Research Institute, Tokyo, 104-0045; [4]Clinical Laboratory Division, [5]Division of Thoracic Surgery, National Cancer Center Hospital, Tokyo, 104-0045; [6]Department of Pathology, Keio University, School of Medicine, Tokyo, 160-8582, Japan

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We examined the genome-wide expression profiles of 86 primary lung adenocarcinomas and compared them with the mutation status of the four key molecules (EGFR, ERBB2, KRAS and BRAF) in the EGFR/KRAS/BRAF pathway. Unsupervised classification revealed two subtypes (the bronchial type and the alveolar type) of lung adenocarcinoma. Mutually exclusive somatic mutations of the epidermal growth factor receptor (EGFR) gene (36/86, 41.8%), K-ras gene (11/86, 12.8%) and BRAF gene (1/86, 1.1%) were detected. KRAS mutations were observed significantly frequently in bronchial-type tumors, whereas the frequencies of EGFR mutations were similar in both the alveolar and bronchial types. Twenty-seven genes showed increased expression in EGFR-mutated tumors and these included molecules that function in the EGFR/KRAS/BRAF pathway (EGFR, AKT1 and BCR). In particular, expression of BCR, which is required for EGFR protein degradation, was induced by EGF stimulation, suggesting a negative feedback loop in lung cancer. A subgroup of the alveolar type tumors showed significantly better prognosis than other tumors. Integrated analysis of genetic and gene expression profiling aimed to delineate inherent oncogenic pathways in cancer will be valuable not only for the understanding of molecular pathogenesis, but also for discovering novel biomarkers and predicting clinical outcome. (*Cancer Sci* 2007; 98: 985–991)

Lung cancer is one of the leading causes of cancer death worldwide, and lung adenocarcinoma accounts for more than 50% of all lung cancers.[1] Lung adenocarcinoma comprises a heterogeneous group of tumors with broad ranges of histopathology, combinations of genetic alterations and gene expression patterns.[2,3]

Recently, molecular-targeted therapies, which modulate key molecules (such as kinases) of the essential signal pathways that cancer cells exploit for survival, have considerably increased the possibility of personalized cancer therapy.[4,5] It has been discovered that epidermal growth factor receptor (EGFR) gene mutations were closely associated with better responsiveness to EGFR tyrosine kinase inhibitors (EGFR-TKIs).[6–8] Activation of EGFR transfers signals through the ERBB2, RAS and RAF proteins in the downstream pathway, and importantly oncogenic mutations of the erbB2, K-ras or B-raf genes have also been reported in lung cancer.[9] The EGFR/ERBB2/KRAS/BRAF pathway is frequently (more than 50%) altered in lung adenocarcinoma and, consistent with their epistatic roles in the signal pathway, EGFR, ERBB2, KRAS and BRAF mutations exist in a mutually exclusive way.[9] Therefore this pathway is one of the most promising therapeutic targets for lung cancer, and many pharmacological compounds targeting the components of the pathway have been developed and clinically tested.[10]

To achieve personalized therapy by choosing the most effective therapeutic agents and minimizing their undesirable effects, we need to gain a comprehensive view of signal transduction networks and their association with biological phenotypes in cancer. In relation to genetic activation of the EGFR/ERBB2/KRAS/BRAF pathway, many questions still remain, such as whether there exist any molecular or histopathological features of the activated EGFR/ERBB2/KRAS/BRAF pathway and how the pathway interacts with other signal pathways. Such information would be valuable for understanding the molecular pathogenesis of lung cancer as well as for identifying appropriate biomarkers for molecular diagnosis and discovering novel therapeutic targets for individualized therapy.

Materials and Methods

Surgical specimens. Surgical specimens from 86 Japanese patients with lung adenocarcinoma who were diagnosed and underwent surgery at the National Cancer Center Hospital, Tokyo, Japan, between June 1997 and May 2002 were examined. Clinical features of analyzed cases are shown in Table 1. The study protocol was approved by the institutional review board of the National Cancer Center.

RNA extraction and microarray analysis. Each frozen sample was homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted. Biotin-labeled cRNA was synthesized from 5 μg of total RNA using the Superscript Choice System (Invitrogen) and BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). Hybridization to the microarray HG-U95Av2 (Affymetrix, Santa Clara, CA, USA) and detection of the signals were carried out according to the manufacturer's instructions.

Immunohistochemical analysis. Five-micrometer-thick sections of the formalin-fixed paraffin-embedded tumors were deparaffinized. After heat-induced epitope retrieval, the sections were incubated with mouse monoclonal anticytokeratin 6 (KRT6) antibody (clone D5/16 B4, DakoCytomation, Glostrup, Denmark), mouse monoclonal antimucin 5AC (MUC5AC) antibody (clone 45M1, Zymed, South San Francisco, CA, USA), mouse monoclonal anti-Surfactant Apoprotein A (SP-A) (clone PE10, DakoCytomation) and rabbit polyclonal anti-Clara cell antigen (CC-10) antibody (Santa Cruz, Santa Cruz, CA, USA) at a dilution of 1:100. The sections were incubated with a biotinylated secondary antibody against mouse or rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:200 and then with the Vectastain ABC reagent (Vector Laboratories). Tumors containing more than 10% stained tumor cells in the largest representative section were considered positive.

[7]To whom correspondence should be addressed. E-mail: tashibat@ncc.go.jp
[8]Present address: Department of Pulmonary Medicine, Faculty of Medicine, Tokyo Medical and Dental University, Tokyo, Japan.

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Table 1. Clinicopathological features of analyzed cases

			Cluster A (n = 42)	Cluster B (n = 44)	P-value
Gender	Male	49	29	20	0.027
	Female	37	13	24	
Age (average)		35–83 (62.4)			
Smoking habit	Never	41	17	24	0.19
	Former	23	10	13	
	Current	22	15	7	
Stage	I	52	23	29	0.29
	II or III	34	19	15	
Differentiation	Well	37	11	26	0.002
	Moderate or Poor	49	31	18	
EGFR mutation	Exon 18	1	1	0	0.25
	Exon 19	18	9	9	
	Exon 20	0	0	0	
	Exon 21	17	5	12	
KRAS mutation	Exon 1	10	8	2	0.019
	Exon 2	1	1	0	
BRAF mutation	Exon 11	0	0	0	ND
	Exon 15	1†	0	1	

†V600E mutation. EGFR, epidermal growth factor receptor.

Laser-capture microdissection, DNA extraction and mutational analysis. Five-micrometer-thick sections of the methanol-fixed paraffin-embedded tumors were subjected to laser-capture microdissection using the LM200 system (Arcturus, Mountain View, CA, USA). Corresponding normal lung epithelial cells were obtained by scraping and were used as a control. DNA was extracted using a standard method. We amplified exons 18, 19, 20 and 21 of the EGFR gene, exons 19 and 20 of the erbB2 gene, exons 1 and 2 (covering codons 12, 13 and 61) of the K-ras gene, and exons 11 and 15 of the B-raf gene by polymerase chain reaction (PCR) using High Fidelity Taq polymerase (Roche, Mannheim, Germany) and appropriate primers (primer sequences available on request). All PCR products were purified and analyzed by sequencing.

Quantitative reverse transcriptase-PCR. Five micrograms of total RNA was isolated from a randomly selected 28 cases and was reverse-transcribed (High Capacity cDNA Archive kit, Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was carried out in triplicate and evaluated using TaqMan probes for each target gene (Applied Biosystems) and the ABI 7500 system (Applied Biosystems). The relative expression of each gene was determined by comparing its expression to that of beta-actin.

Cell culture. EGFR mutated lung adenocarcinoma cell lines (NCI-H1650 and NCI-H1975)[7] were maintained as recommended (American Tissue Cell Collection, Manassa, VA, USA). The cell lines were serum-starved and stimulated with 10 ng/mL epidermal growth factor (Sigma, St. Louis, MO, USA) for the indicated time. To inhibit EGFR activation, the specific EGFR inhibitor AG1478 (Sigma, 10 μM) was added.

Statistical analysis of microarray data. For unsupervised clustering of lung adenocarcinoma, we first selected 685 genes based on two criteria. First, the average difference (which is correlated with gene expression level) exceeded 1000 in more than 5% of the 86 tumors, and second, the average difference varied by at least twofold from the median in at least 25% of the samples. Data analysis was carried out using the Gene Spring (Silicon Genetics, Redwood City, CA, USA) software packages and visualized using the Tree and View software package (Stanford University). We used the significance analysis of microarrays software package (SAM; http://www-stat.stanford.edu/~tibs/SAM/index.html) to select genes differentially expressed in each of the tumor subtypes.[11] The output criteria selected for SAM included a difference of 1.5-fold or more between the two groups and a false discovery rate (FDR) of less than 5%.

Other statistics. The unpaired t-test and the χ^2 test were used for comparisons of proportions. The Kaplan-Meier method was used to estimate survival as a function of time, and log-rank analysis was used to compare the differences between subgroups. Cox proportional hazard modeling was carried out to determine independent prognostic factors.

Results

Gene expression profiling of lung adenocarcinoma revealed histologically and clinically heterogenous subtypes. We analyzed the expression of 12 625 probe targets (representing more than 10 000 unique genes) in 86 primary lung adenocarcinomas using an oligo-nucleotide-based microarray. We selected 685 genes whose expressions were relatively abundant and varied widely among the samples analyzed (Appendix I Table 1) and carried out two-dimensional hierarchical clustering analysis to classify the lung adenocarcinomas according to similarities in their patterns of expression (Fig. 1a).

Unsupervised classification demonstrated that lung adenocarcinomas were largely divisible into two clusters (clusters A and B, Fig. 1a). We compared gene expressions between the two clusters and selected genes that showed significant differences in expression (Appendix I Table 2). Tumors in cluster A preferentially expressed genes associated with the cell cycle/cell proliferation, the extracellular matrix, and those expressed specifically in bronchial epithelium (KRT6 and MUC5AC, Fig. 2a,b).[12,13] In contrast, genes expressed significantly in cluster B included growth factor inhibitors, enzymes involved in lipid metabolism, and alveolar pneumocyte markers (such as CC10 and surfactant pulmonary-associated protein C (SFTPC)).[14] In accordance with their similarities to normal pulmonary epithelium, we named these clusters the bronchial type (cluster A) and alveolar type (cluster B), respectively. To validate our microarray analyses, we carried out immunohistochemical analysis of the classifying molecules. We examined the expressions of KRT6, Mucin 5AC, Surfactant apoprotein A (SFTPA) and CC-10 (Fig. 2c–f). As shown in Figure 1(b) and Table 1, KRT6 (bronchial type 12/42 (28.5%) and alveolar type 4/44 (9%), $P = 0.02$), SFTPA (bronchial type 11/42 (26.2%) and alveolar-type 31/44 (70.5%), $P < 1 \times 10^{-4}$) and

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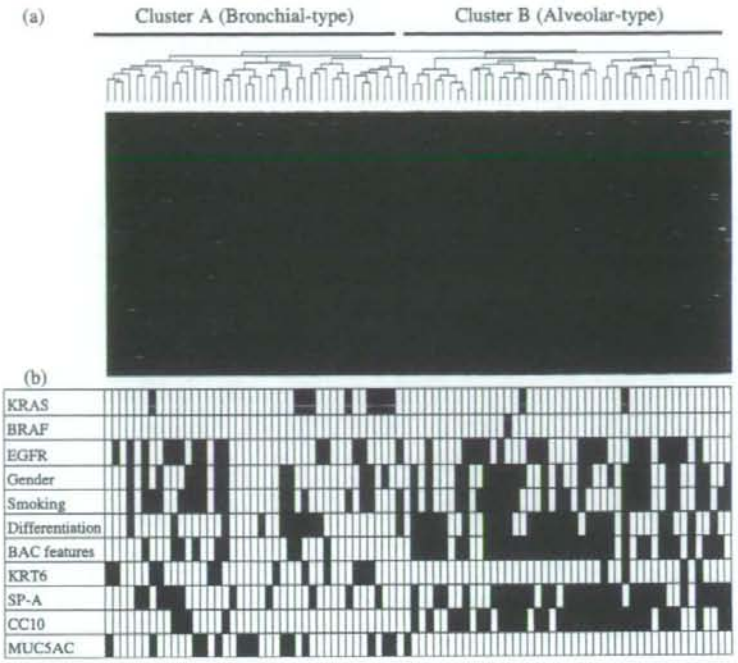

Fig. 1. (a) Unsupervised hierarchical clustering analysis of 86 lung adenocarcinomas. Hierarchical clustering of 86 primary lung adenocarcinomas was carried out based on the expression of 685 transcripts. The normalized expression index for each transcript is indicated by a colored bar. Unsupervised classification revealed two clusters (cluster A, bronchial type, and cluster B, alveolar type) that are shown above the dendrogram of the samples. (b) Clinical features, mutation status of epidermal growth factor receptor (EGFR), KRAS and BRAF, and immunohistochemical expression profile of KRT6, Mucin 5AC, Surfactant apoprotein A (SP-A) and CC10 in 86 lung adenocarcinomas. Clinicopathological features (gender, smoking status, differentiation and bronchioloalveolar carcinoma [BAC] features) of each case are shown. Solid boxes indicate cases in which the patient was female, a non-smoker and the tumor was well-differentiated and contained BAC features, while clear boxes indicate cases in which the patient was male, a smoker, and the tumor was moderately/poorly-differentiated. The presence of mutations in the EGFR, KRAS and BRAF genes is shown by a solid box. Immunohistological positivity for KRT6, SP-A, CC-10 and MUC5AC is shown by a solid box.

CC-10 (bronchial type 4/42 (9.5%) and alveolar type 32/42 (76.2%), $P < 1 \times 10^{-8}$) were preferentially expressed in bronchial-type and alveolar-type tumors, respectively. Mucin 5AC was detected only in bronchial-type tumors (15/42, 35.7%).

To determine whether this gene-expression-based classification had any association with clinical features, we compared clinicopathological factors between these two groups (Fig. 1b). Bronchial-type tumors had a stronger tendency to occur in males ($P = 0.02$) and to show histologically poorer differentiation ($P = 0.002$) than alveolar-type tumors. We also found that alveolar-type tumors frequently ($P < 0.0001$) showed lepidic growth that is characterized as BAC (bronchioloalveolar carcinoma) features, while bronchial-type tumors exhibited solid growth (Fig. 2g, h, Table 1). Therefore these two subtypes represent clinically and histologically distinct subgroups of lung adenocarcinoma.

Comparison of gene expression profiling and EGFR/ERBB2/KRAS/BRAF mutation status in lung adenocarcinoma. We examined oncogenic mutations of the four key molecules (EGFR, ERBB2, KRAS and BRAF) in this pathway simultaneously and attempted to analyze whether any of them were associated with the classification of lung adenocarcinoma described above. To this end, we obtained tumor cells from 86 lung adenocarcinomas by microdissection and determined the mutation status of the EGFR (exons 18–21), ERBB2 (exons 19 and 20), KRAS (exons 1 and 2) and BRAF (exons 11 and 15) genes. We detected somatic mutations of the EGFR gene (36/86, 41.8%), K-ras gene (11/86, 12.8%) and B-raf gene (1/86, 1.1%) in our cohort (Table 1). We were unable to find any mutation in exons 19 and 20 of the erbB2 gene. The occurrence of these mutations appeared to be mutually exclusive. As reported previously,[15] EGFR mutations were frequently observed in female ($P = 0.046$) and non-smoking patients ($P = 0.034$), whereas there was no significant association between the presence of KRAS mutation and any of the clinical background factors examined (age, gender, smoking habit, tumor differentiation and pathological stage).

We then examined whether these alterations had any association with the classification obtained by gene-expression profiling (Fig. 1b). EGFR mutations were more frequent in alveolar-type (21/44, 47.7%) than in bronchial-type (15/42, 35.7%) lung adenocarcinoma, although not to a significant degree ($P = 0.25$). On the other hand, we found that KRAS mutations were significantly more frequent in the bronchial type (9/42, 21.4%) than in the alveolar type (2/44, 4.5%) ($P = 0.019$).

Identification of mRNA expression features associated with the presence of EGFR mutation. Because our unsupervised classification of lung adenocarcinoma based on genome-wide gene-expression pattern failed to detect any specific subgroup of tumors that harbored frequent EGFR mutations and identification of candidate biomarkers to predict EGFR mutation should be clinically valuable, we next attempted to determine whether any expression signature was associated with the presence of EGFR mutation. Comparison of gene expression profiles between EGFR-mutated and EGFR-wild-type tumors detected 26 genes whose expression was significantly increased in EGFR-mutated tumors (Table 2). Interestingly, these included molecules (EGFR and AKT1) that are known to function in the EGFR/KRAS/BRAF pathway. They also included secreted/membrane-associated molecules such as PTK7. To validate these results, we carried out quantitative RT-PCR analysis of these genes in 28 lung tumor samples (14 EGFR-mutated and 14 EGFR-wild-type tumors, and there was no significant difference in clinocopathological features between the two groups). As shown in Figure 3(a), the expressions of these genes differed significantly between EGFR-mutated and EGFR-wild-type tumors.

Since BCR has recently been shown to regulate the degradation of EGFR protein after ligand stimulation[16] we hypothesized that induction of *BCR* expression in EGFR-mutated tumors might constitute a negative feedback loop. *BCR* overexpression was frequent in EGFR-mutated tumors (16/36, 44.4%) compared to in EGFR-wild-type ones (1/50, 2%). To determine whether *BCR* expression is regulated by EGFR activation in lung

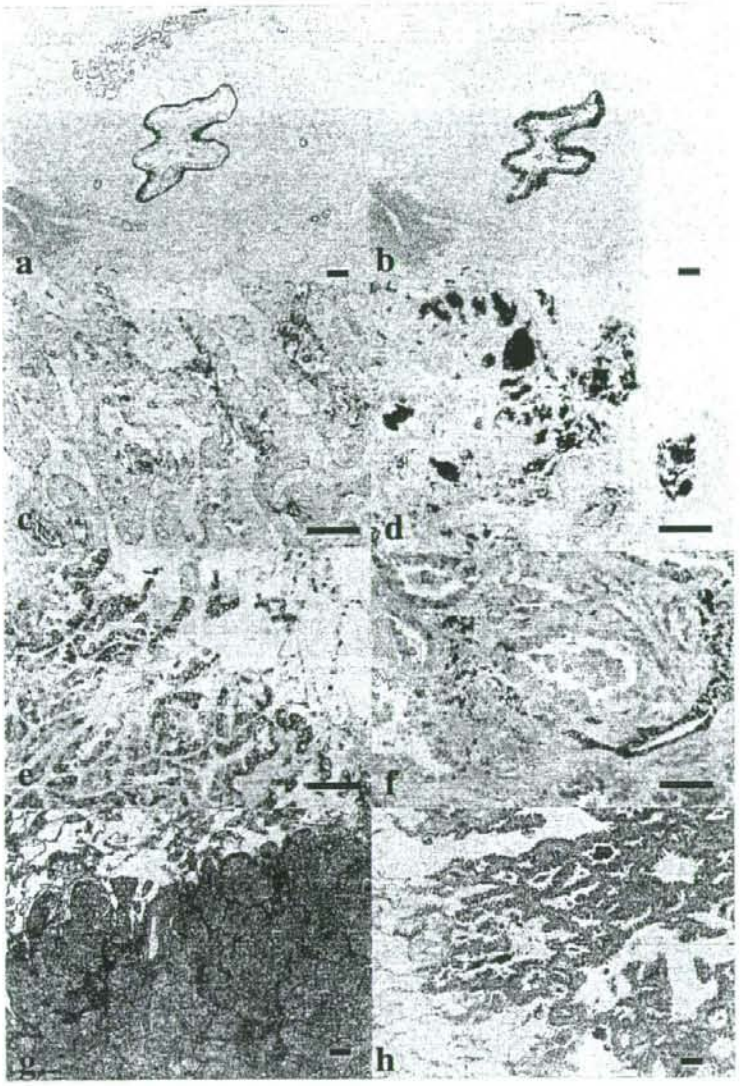

Fig. 2. Immunohistochemical analyses of classifying molecule expression in lung adenocarcinoma and normal lung. Bronchial epithelia but not alveolar pneumocytes were positive for both KRT6 (a, ×100) and MUC5AC (b, ×100). Representative immunohistochemical expression of KRT6 (c, ×200), MUC5AC (d, ×200), Surfactant apoprotein A (SP-A) (e, × 200) and CC-10 (f, ×200) in lung adenocarcinoma is shown. The representative histologies of bronchial-type (left) and alveolar-type (right) tumors (Hematoxylin-eosin stained, ×100). Bar, 200 μm.

cancer cells, we stimulated lung cancer cells with EGF and examined the resulting change in BCR mRNA expression *in vitro*. As shown in Figure 3(b), expression of BCR mRNA was up-regulated (2.2–3.0-fold) in response to EGF stimulation. On the other hand, when we inhibited EGFR activation pharmacologically in EGFR-mutated lung cancer cell lines, we observed a significant decrease (0.6-0.4-fold) of BCR mRNA expression (Fig. 3c).

Prognostic significance of molecular classification of lung adenocarcinoma. We examined whether our molecular classification of lung adenocarcinoma has any association with clinical outcome. The presence of genetic alterations in the EGFR/KRAS/BRAF pathway was not associated with prognosis (data not shown). There was no significant difference in prognosis between the alveolar and bronchial types ($P = 0.07$, Fig. 4b). However, when we divided the alveolar type into two subgroups, we found that a subgroup of the alveolar type showed remarkably better prognosis than the other tumors (Fig. 4c).

Discussion

In this study, we attempted to investigate the biological significance of oncogenic pathway activation in lung cancer. Previous gene expression analyses of lung cancer elucidated heterogenous subgroups with specific gene expression signatures in lung adenocarcinoma.[17–21] Our unsupervised hierarchical clustering based on gene expression profiling revealed two distinct subgroups (bronchial and alveolar types) of lung adenocarcinoma, which are associated with clinical backgrounds and largely consistent with these studies. Especially the TRU (terminal respiratory unit)-type of lung adenocarcinoma[20] shares similar histological and molecular features with the alveolar type. We concurrently analyzed somatic mutations of the components of the EGFR/KRAS/BRAF pathway (ERBB2, EGFR, KRAS and BRAF genes). As reported previously,[8,15] EGFR mutations were frequently observed in female, non-smoking patients, but there was no significant association between the presence of EGFR mutations

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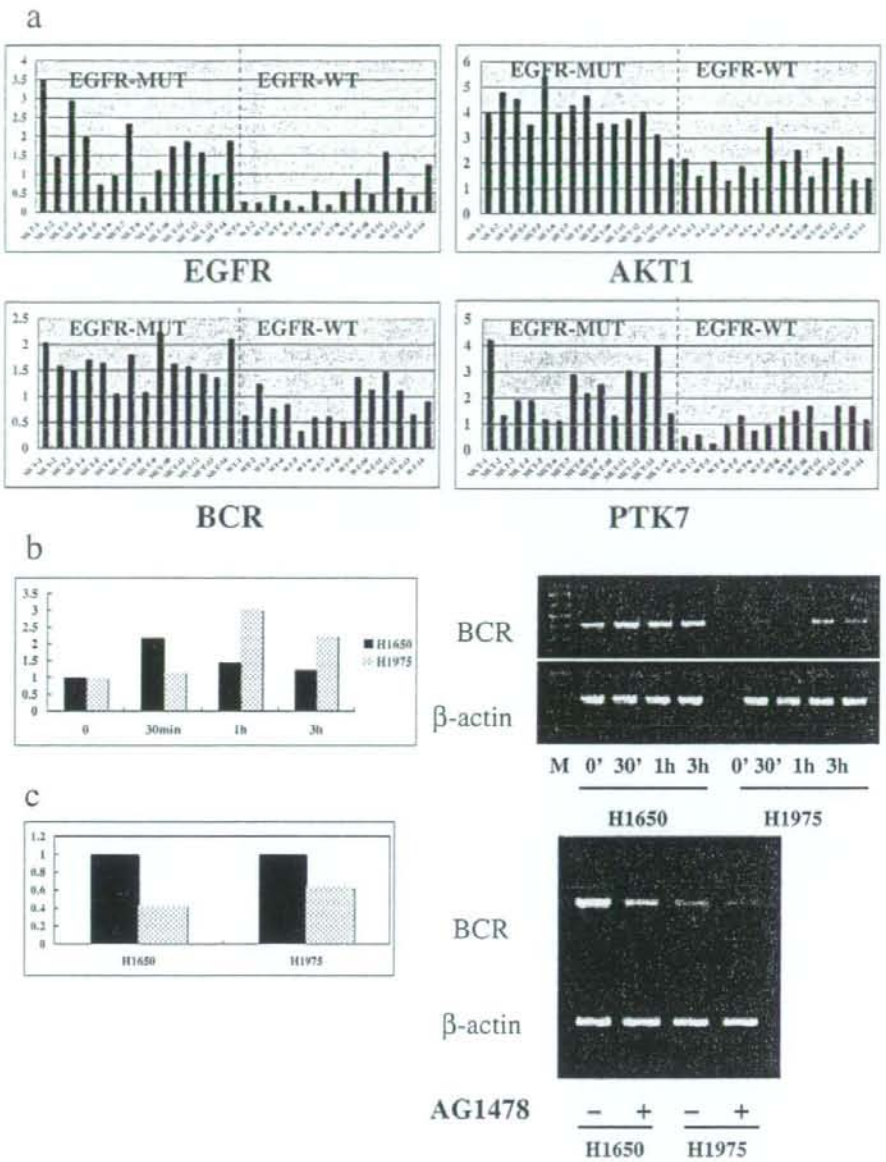

Fig. 3. Validation of distinctive expression between epidermal growth factor receptor (EGFR)-mutated and EGFR-wild-type tumors by quantitative reverse transcription-polymerase chain reaction (RT-PCR). (a) mRNA expression of the EGFR, AKT1, BCR and PTK7 genes was quantified in 14 EGFR-mutated (EGFR-MUT-1–14) and 14 EGFR-wild-type (EGFR-WT-1–14) lung adenocarcinomas. The normalized expressions (relative to beta-actin expression) of the four genes in 28 tumors are shown. (b) BCR mRNA was induced by EGF stimulation in lung cancer cell lines. Relative BCR mRNA expression (compared to the expression at time 0) was subsequently quantified after EGF stimulation (30 min, 1 h and 3 h) in lung cancer cell lines (NCI-H1650: closed bar, NCI-H1975: hatched bar). Gel electrophoresis of PCR products (BCR, upper panel; β-actin, lower panel) at each time point was shown. (c) BCR mRNA expression was reduced by treatment with EGFR kinase inhibitor (AG1478). Relative BCR expression in AG1478 treated lung cancer cell lines was shown (control, closed bar; EGFR inhibitor, hatched bar). Gel electrophoresis of PCR products (BCR, upper panel; β-actin, lower panel) of control and AG1478-treated cells is shown.

and the classification based on the genome-wide gene expression pattern. This suggests that genetic activation of the EGFR gene may confer a growth advantage in both subgroups and that environmental or intrinsic factors might affect the frequency of EGFR mutations. Correlation analysis between gene expression patterns and the presence of EGFR mutations revealed a small group of tumors (the left branch in the bronchial subgroups) that shows frequent EGFR mutation and mixed expression of alveolar and bronchial signatures. This group might represent a transitional subgroup between the two types although further analysis will be required. The frequency of KRAS mutation detected in our study was comparable with that reported previously.[22] In

Table 2. Genes preferentially expressed in epidermal growth factor receptor (EGFR)-mutated lung adenocarcinoma

Ref Seq accession number	Gene symbol	Fold difference	*P*-value
NM_016335	PRODH	3.18	0.0005
NM_001669	ARSD	2.5	0.00078
NM_005228	EGFR	2.31	0.00392
NM_000095	COMP	2.11	0.00515
NM_001012329	CTNNBIP1	2.09	0.000181
NM_004327	BCR	1.89	0.0000272
NM_002885	RAP1GA1	1.83	0.0053
NM_003917	AP1G2	1.82	0.00148
NM_002821	PTK7	1.79	0.000147
NM_025179	PLXNA2	1.73	0.000158
NM_080920	GGTLA4†	1.73	0.000277
NM_004484	GPC3	1.73	0.00295
NM_001015881	TSC22D3	1.72	0.00219
NM_003944	SELENBP1	1.65	0.00252
NM_003317	TITF1†	1.59	0.0000193
NM_014698	TMEM63A	1.54	0.000214
NM_000548	TSC2	1.54	0.00141
NM_004689	MTA1	1.54	0.00362
NM_015316	PPP1R13B	1.53	0.00172
NM_001306	CLDN3	1.53	0.00704
NM_002933	RNASE1	1.53	0.00738
NM_001014431	AKT1	1.52	0.000155
NM_002134	HMOX2	1.51	0.000136
NM_015234	GPR116	1.5	0.0021
NM_004390	CTSH	1.5	0.00593
NM_000802	FOLR1	1.5	0.00761

†Genes previously reported to be preferentially expressed in EGFR-mutated lung cancer.

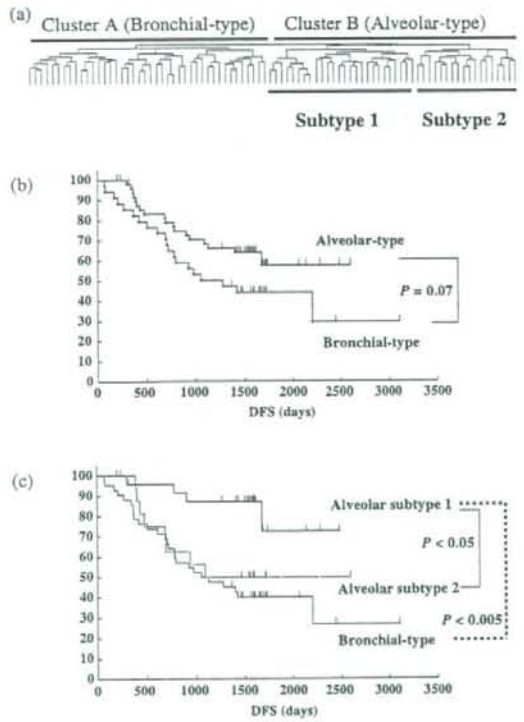

Fig. 4. Gene expression profiling of lung adenocarcinoma was significantly associated with better disease-free survival (DFS) after surgery. (a) Subclassification of the alveolar type tumors. (b, c) Kaplan-Meier plots of DFS (days) of lung adenocarcinoma stratified according to the gene expression profiling.

contrast to EGFR mutations, KRAS mutations were significantly frequent in a specific subgroup of lung adenocarcinoma (the bronchial type) but KRAS mutation status was not associated with any clinical features. Since KRAS mutations have been reported to occur frequently in mucinous-type or goblet cell-type lung adenocarcinoma[23,24] it is possible that bronchial-type adenocarcinoma may contain such histological subtypes.

Our results revealed genes that were specifically expressed in tumors with genetic activation of the EGFR pathway (Table 2). They included genes (TITF1 and GGTLA4) whose expression has been previously reported to be associated with the presence of EGFR mutations.[20,25] Our analysis may also help to identify promising candidate biomarkers for prediction of EGFR activation and sensitivity to EGFR-TKIs in lung cancer. In particular, some of identified molecules (such as COMP, PTK7, PLXNA2, GPC3 and GRP116) are secreted or membrane-associated proteins that could be applicable for proteomic diagnosis using serum or sputum samples.

BCR is a multifunctional protein that forms a chimera protein with ABL in chronic myeloid leukemia.[26] Recently BCR was found to be a component of the endosomal sorting complex and positively regulates lysosomal degradation of the EGFR protein.[16] Ligand-induced down-regulation of growth factor receptors through endocytosis is the major regulatory mechanism that controls the duration and intensity of signal activation.[27] Our analysis revealed that expression of BCR was increased in EGFR-mutated tumors and that EGF stimulation rapidly induced BCR expression in lung cancer cells, supporting the hypothesis that

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BCR is a component of a negative feedback loop in EGFR signaling. Notably it has been reported that some types of EGFR mutation detected in lung cancer confer resistance to endosome-mediated degradation.[28] It is possible that these EGFR mutations may allow constitutive signal activation by overcoming this negative feedback loop and that destabilization of such oncogenic EGFR protein might be an alternative therapeutic strategy.

In our study cohort, we found that molecular classification of lung adenocarcinoma by gene expression profiling was significantly associated with patient prognosis. We found that a subgroup of the alveolar type was significantly better prognosis than other groups. There was no significant difference in clinicopathological features (gender, smoking habit, clinical stage, the presence of BAC features and EGFR mutation frequency) between the two subtypes (Appendix I Table 3). But this subgroup had more well-differentiated tumors ($P = 0.02$, Appendix I Table 3) and shows characteristic gene expression signatures including several transcriptional factors and secreted molecules (Appendix I Table 4). These gene products might also be useful for molecular diagnosis of lung adenocarcinoma. On the basis of our results, we propose that integrated analysis of genetic and gene expression profiling aimed to delineate inherent oncogenic pathways in cancer will be valuable not only for understanding molecular pathogenesis and identifying therapeutic targets, but also for clinical outcome in patients.

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Supplementary Material

The following supplementary material is available for this article:

Table S1.

Table S2.

Table S3.

Table S4.

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

Tomoya Fukui,[1,7] Koji Tsuta,[1] Koh Furuta,[2] Shun-ichi Watanabe,[3] Hisao Asamura,[3] Yuichiro Ohe,[4] Akiko Miyagi Maeshima,[5] Tatsuhiro Shibata,[6] Noriyuki Masuda[7] and Yoshihiro Matsuno[1,8]

[1]Clinical Laboratory Division, [2]Clinical Support Laboratory, [3]Thoracic Surgery Division, [4]Department of Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [5]Pathology Division, [6]Cancer Genomics Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [7]Department of Respiratory Disease, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Sagamihara-shi, Kanagawa 228-8555, Japan

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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

[8]To whom correspondence should be addressed.
E-mail: ymatsuno@med.hokudai.ac.jp

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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.

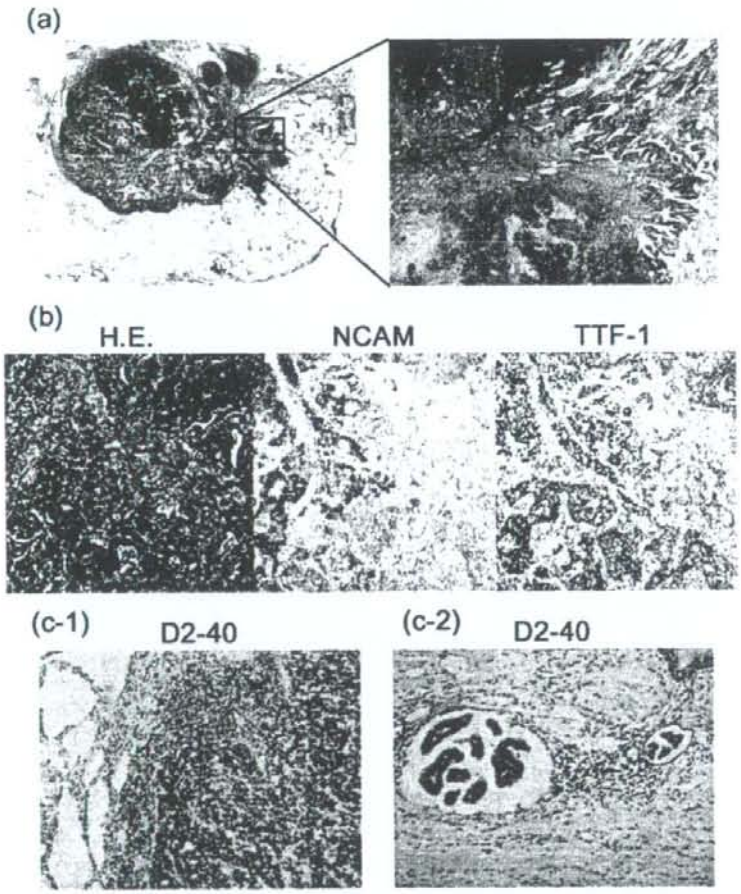

Fig. 1. Combined small cell lung carcinoma (SCLC) with adenocarcinoma (Ad). (a) The periphery of this tumor consisted of a non-mucinous bronchioloalveolar carcinoma-like extension (patient no. 3). (b) The transitional zone between the SCLC and Ad components had poorly differentiated cells, shown by the 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.

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

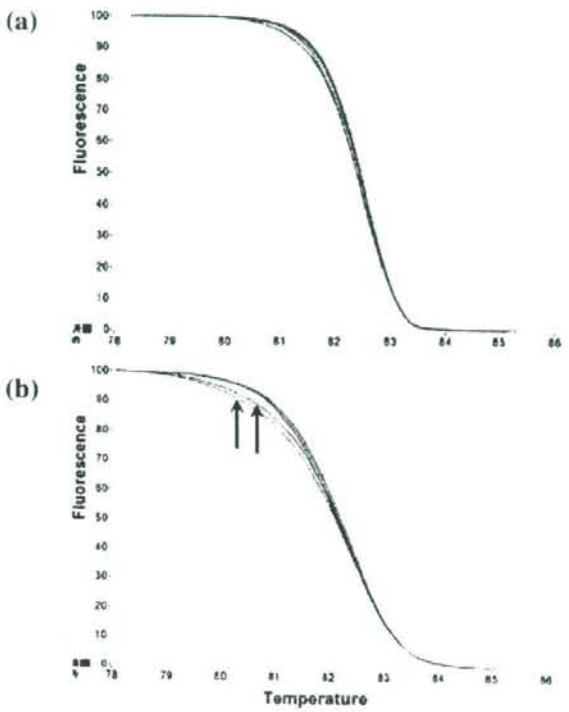

Fig. 2. Results of high-resolution melting analysis (HRMA). Adjusted melting curves obtained by HRMA of combined small cell lung carcinoma (SCLC) with primers designed to detect mutations in (a) exon 19 or (b) exon 21 of epidermal growth factor receptor (*EGFR*). Two samples from patient no. 3 were identified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

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

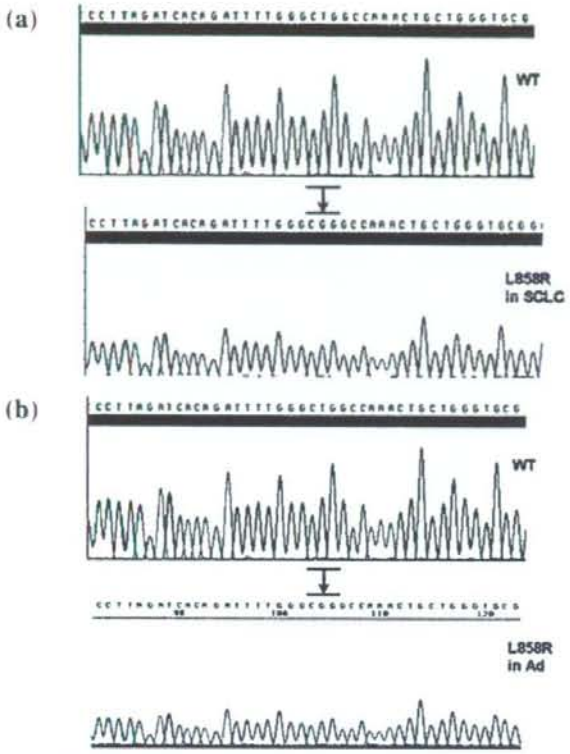

Fig. 3. Results of DNA sequencing from patient no. 3. The tumor of patient no. 3 was microdissected into the small cell lung carcinoma (SCLC) and adenocarcinoma (Ad) components. (a) Sequence analysis of the subcloned polymerase chain reaction (PCR) products from the microdissected SCLC component. (b)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

Acknowledgments

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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

Toshimi Takano,[1,6] Yuichiro Ohe,[1] Koji Tsuta,[2] Tomoya Fukui,[1] Hiromi Sakamoto,[5] Teruhiko Yoshida,[5] Ukihide Tateishi,[3] Hiroshi Nokihara,[1] Noboru Yamamoto,[1] Ikuo Sekine,[1] Hideo Kunitoh,[1] Yoshihiro Matsuno,[2] Koh Furuta,[4] and Tomohide Tamura[1]

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

Authors' Affiliations: [1]Division of Internal Medicine, [2]Clinical Laboratory Division, [3]Division of Diagnostic Radiology, and [4]Clinical Support Laboratory, National Cancer Center Hospital; [5]Genetics Division, National Cancer Center Research Institute; and [6]Division of Medical Oncology, Tokyo Kyosai Hospital, Tokyo, Japan

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Requests for reprints: Yuichiro Ohe, Division of Internal Medicine, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Phone: 81-0-3-3542-2511; Fax: 81-0-3-3542-6220; E-mail: yohe@ncc.go.jp.

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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[c]	
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.
[c]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[c]	1[b]	2	11	

*Comparison between never smokers and others.
[c]Pleomorphic carcinoma.
[b]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 m^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[c]	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.
[c]Including a patient who had no measurable lesions at baseline.

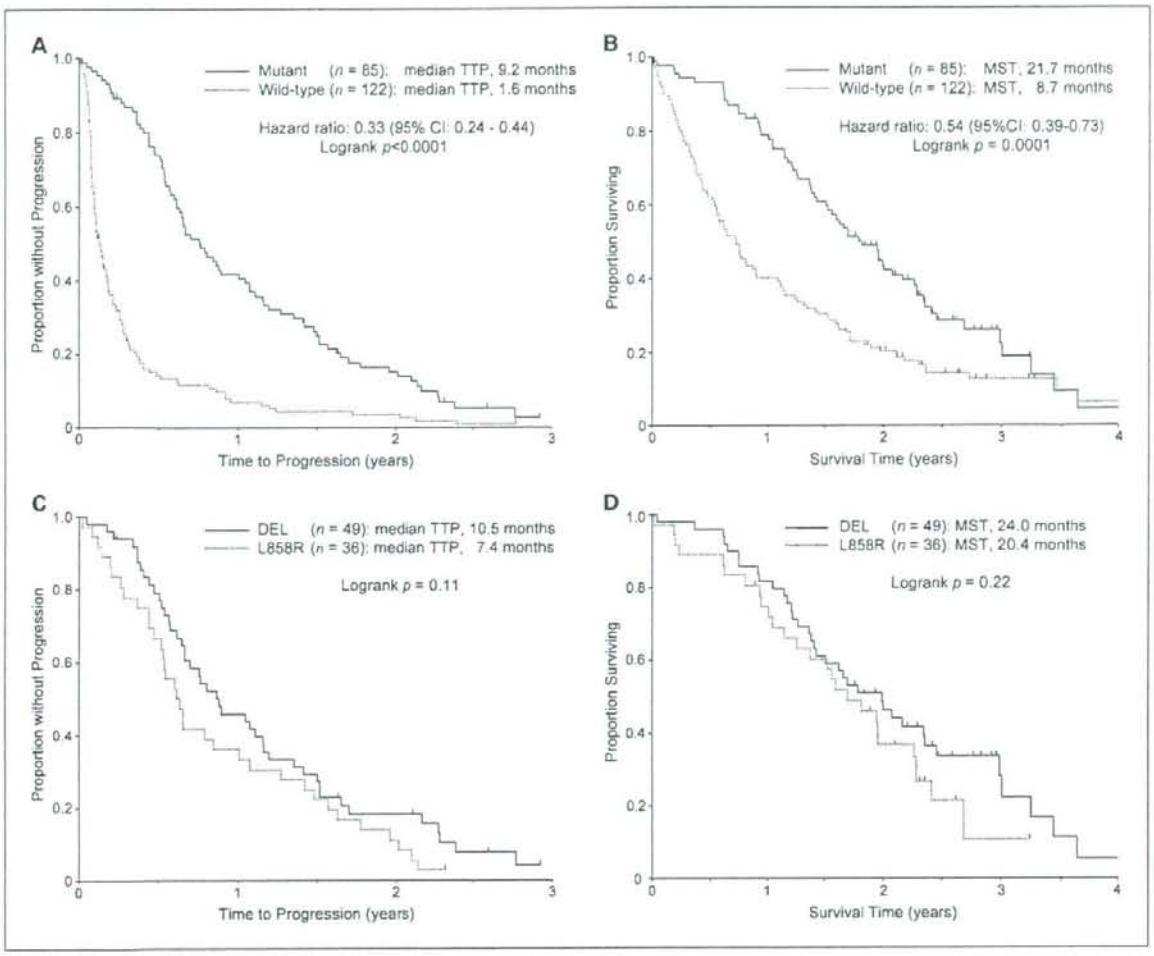

Fig. 1. Kaplan-Meier plot of time-to-progression (A) and overall survival (B) for patients with or without EGFR mutations. Kaplan-Meier plot of time-to-progression (C) and overall survival (D) for patients with DEL or L858R mutations. TTP, time-to-progression; MST, median survival time.

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