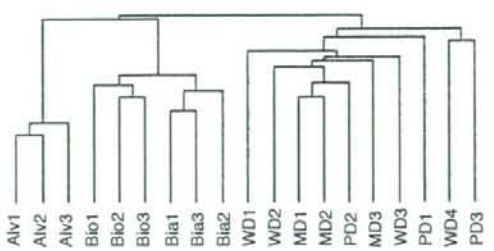

Figure 3 A dendrogram of hierarchical clustering for noncancerous lung epithelial cells and lung adenocarcinoma cells. Alv1-3 Alv3 are type II alveolar cells, Bio1 3 Bio3 are bronchiolar epithelial cells, Bia1 3 Bia3 are bronchial epithelial cells, and WD1 3 WD3, MD1 3 MD3 and PD1 3 PD3 are lung adenocarcinoma cells.

epithelial cells. Among 14837 probe sets that showed 'present calls' for at least one of the 19 samples, 2372 sets, which showed 'absent calls' for all nine epithelial cell samples, were considered as being not expressed in epithelial cells. Among the 2372 sets, a number of probe sets showed high signal intensities in AdC cells. To further define genes significantly and commonly overexpressed in AdC cells, probe sets that showed signal intensities of >3000 in at least three AdC samples were selected (Table 1). Nine genes of 10 probe sets, SGNE1, COL11A1, STK6, TFPI2, ATP10B, TM4SF4, GAGED2, MCM6 and TOP2A, were selected under this criterion. There was no gene that showed overexpression in all 10 AdC cases, instead, those genes showed overexpression in three to five of them.

To confirm the overexpression of the nine genes in lung AdC, we examined the expression of these genes in 45 cases of macrodissected primary lung AdC cells and 14 cases of macrodissected noncancerous lung cells by real-time RT–PCR analysis. To make a comparison of expression levels for each gene among the cases easier, mean levels of expression for each gene in 45 cases of AdCs were adjusted to 1000 and the relative values for expression were compared among the cases. Mean levels of expression in AdC cells were significantly higher (P 0 0.05) than those in noncancerous lung cells in four genes of SGNE1, COL11A1, GAGED2 and TOP2A (Table 2, Figure S1). In particular, the mean levels of COL11A1 and GAGED2 expression in noncancerous lung cells was more than 40 times lower than those in AdC cells. Thus, it was concluded that four genes of SGNE1, COL11A1, GAGED2 and TOP2A were significantly overexpressed in lung AdC cells.

Identification of differentiation markers for lung adenocarcinoma

We next attempted to identify genes that are differentially expressed among three different anatomic regions of lung epithelial cells, because those genes are strong candidates for differentiation markers of alveolar, bronchiolar and bronchial epithelial cells, respectively. Among 12465 probe sets that showed 'present calls' in at least one of the nine noncancerous epithelial cell

Table 1 List of genes highly expressed in lung adenocarcinoma cells and not expressed in noncancerous lung epithelial cells

Gene symbol	Gene title	WD1	WD2	WD3	WD4	MD1	MD2	MD3	PD1	PD2	PD3	Average of intensity	Number of tumor sample (3000)
SGNE1	Secretory granule, neuroendocrine protein 1 (7B2 protein)	A	509	A	4238	A	450	6481	5229	5185	5015	3872	5
COL11A1	Collagen, type XI, alpha 1	1372	1161	1180	810	7531	9339	17160	A	1291	3725	4841	4
STK6	Serine/threonine kinase 6	1577	1287	953	2923	930	4529	845	7352	8193	9631	3822	4
TFPI2	Tissue factor pathway inhibitor 2	A	21724	1909	A	A	1061	A	A	19507	3055	9451	3
ATP10B	ATPase, Class V, type 10B	3390	6536	A	A	1262	A	18870	A	A	A	7514	3
TM4SF4	Transmembrane 4 superfamily member 4	A	A	A	4956	A	A	10932	3412	A	A	6433	3
COL11A1	Collagen, type XI, alpha 1	1218	1478	876	A	11067	9580	17135	A	1210	2565	5641	3
GAGED2	G antigen, family D, 2	5118	6928	A	A	1954	A	A	A	4705	A	4676	3
MCM6	MCM6 minichromosome maintenance deficient 6	A	2623	1619	A	2703	7071	1315	3508	4326	2532	3212	3
TOP2A	Topoisomerase (DNA) II alpha 170kDa	A	1645	A	A	900	3953	796	843	6177	3557	2553	3

A: absent or marginal.

Table 2 Expression of nine candidate tumor markers in lung adenocarcinoma cells, noncancerous lung cells and peripheral blood cells

Gene symbol	AdC (n¼45) mean7s.d.	NL (n¼14) mean7s.d.	PB (n¼5) mean7s.d.
SGNE1	1000711104	146794*	977*
COL11A1	1000711693	22753*	0*
STK6	10007900	7907756	43067 3254
TFPI2	10007 2039	3867219	0.037 0.02*
ATP10B	10007 1235	471 71059	12 0007 8177*
TM4SF4	10007 3756	15712	0
GAGED2	10007 1908	23728*	0*
MCM6	10007 794	8907 285	171 7 263*
TOP2A	10007 1234	1877 99*	457 49*

*Significantly different (P 0 0.05) from AdC by student's t-test. Abbreviations: AdC, adenocarcinoma; NL, noncancerous lung cells; PB, peripheral blood cells.

samples, 3089 showed 'present calls' in all the nine samples, and thus were considered as being expressed in any region of epithelial cells analysed. In contrast, 356 probe sets showed cell type specific patterns of gene expression among alveolar, bronchiolar and bronchial epithelial cells (Table S2). The remaining 9020 probe sets did not show any correlation between their present/absent calls and cell type specificities.

Among the 356 probe sets, 121 probe sets showed 'present calls' only in alveolar cells, and 'absent/marginal calls' in bronchiolar/bronchial epithelial cells, while 40 probe sets showed 'present calls' in alveolar/bronchiolar epithelial cells, and 'absent/marginal calls' in bronchial epithelial cells, suggesting that genes for these 161 probe sets were expressed predominantly in alveolar/bronchiolar epithelial cells. On the other hand, 154 probe sets showed 'present calls' in bronchiolar/bronchial epithelial cells, but 'absent/marginal calls' in alveolar epithelial cells, and another 22 probe sets showed 'present calls' only in bronchial epithelial cells, and 'absent/marginal calls' in alveolar/bronchiolar epithelial cells, suggesting that genes for these 176 probe sets were expressed predominantly in bronchiolar/bronchial epithelial cells. The number of probe sets that were positive only in bronchiolar epithelial cells was 14, and the number of probe sets positive in both alveolar and bronchial epithelial cells and negative in bronchiolar epithelial cells was only five.

However, if signal intensities of >5000 were considered as being highly expressed, only 16 genes were judged as being expressed in a cell-type specific manner (Table 3). There were five genes that were expressed only in alveolar epithelial cells. Three other genes of four probe sets were expressed in alveolar/bronchiolar epithelial cells and not in bronchial epithelial cells. Thus, these eight genes were judged as being strong candidates for differentiation markers for alveolar/bronchiolar epithelial cells. As expected, SFTPA2, SFTPB and SFTPC were classified into this group (Otto, 2002). However, it was noted that five genes of CLDN5, RARRES2, RAFTLIN, CLST11240 and DF were shown to be expressed only in type II alveolar epithelial cells, indicating that these genes could be unique and noble markers for type II alveolar epithelial cells. None of the probe sets were selected as being present only in bronchiolar or bronchial epithelial cells under this criterion. Instead, there were eight genes that showed positive in bronchiolar/bronchial epithelial cells and negative in alveolar epithelial cells, suggesting that these eight genes were differentiation markers for bronchiolar/bronchial epithelial cells. As expected, SCGB1A1 was judged as being highly expressed in bronchiolar and bronchial lung epithelial cells (Broers et al., 1992). None of the probe sets were selected as being 'present' in alveolar/bronchial epithelial cells but absent/marginal in bronchiolar epithelial cells. Thus, in total, 16 genes of 17 probe sets were selected as being differentiation markers for lung epithelial cells.

Subclassification of lung adenocarcinomas according to the expression profiles of differentiation makers

Most lung AdCs are thought to originate from type II alveolar epithelial cells or bronchiolar epithelial cells and often retain their phenotypes, in particular in well-differentiated type AdCs. Thus, it was interesting to classify the 10 AdC samples based on the expression profiles of the 17 probes selected above. As shown in Figure 4, all the 10 AdC cases were classified into a group of type II alveolar epithelial cells. In particular, one of four well differentiated types (WD3) was classified in the same branch as three cases of type II alveolar epithelial cells, and two other cases of well differentiated types (WD1 and WD4) were also classified into the same sub-branch as type II alveolar epithelial cells. Thus, although pathological classification of differentiation stages did not always correlate with the classification based on the expression profiles of the 17 probes, well differentiated types of AdC often retained the expression profiles of type II alveolar epithelial cells.

Expression of differentiation markers in lung adenocarcinoma cells

We next examined the expression of the 16 genes in the same set of macrodissected lung AdC cells and noncancerous lung cells by real-time RT–PCR analysis (Table 4). Although we failed to design primer sets for the amplification of mRNAs specific to two genes (Affy. ID numbers 205382 and 222271 in Table 3), 14 other genes were successfully analysed by this method. To make a comparison of the differences in relative expression levels for each gene among samples analysed,

Table 3 Differentiation markers for alveolar, bronchiolar and bronchial epithelial cells

	Affy ID	Gene symbol	Gene title	Alv1	Alv2	Alv3	Bio1	Bio2	Bio3	Bia1	Bia2	Bia3
Alv>5000												
1	204482_at	CLDN5	Claudin 5	15701	8974	7236	A	A	A	A	A	A
2	209496_at	RARRES2	Retinoic acid receptor responder 2	11239	8937	7875	A	A	A	A	A	A
3	212646_at	RAFTLIN	Raft-linking protein	5678	9562	10146	A	A	A	A	A	A
4	219719_at	CLST11240	CLST11240 protein	5909	9539	6623	A	A	A	A	A	A
5	205382_s_at	DF	D component of complement (adipsin)	8647	6841	5026	A	A	A	A	A	A
Alv, Bio>5000												
1	38691_s_at	SFTPC	Surfactant, pulmonary-associated protein C	178779	192474	116806	56026	31575	9874	A	A	A
2	218835_at	SFTPA2	Surfactant, pulmonary-associated protein A2	158701	128093	128257	73531	13013	19787	A	A	A
3	209810_at	SFTPB	Surfactant, pulmonary-associated protein B	72022	90827	56839	42108	88852	14317	A	A	A
4	37004_at	SFTPB	Surfactant, pulmonary-associated protein B	79606	72026	45462	43899	69857	9413	A	A	A
Bio, Bia>5000												
1	205725_at	SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)	A	A	A	188781	226145	219147	153690	184343	193646
2	222271_at	—	Homo sapiens transcribed sequences	A	A	A	35583	27525	54945	56002	61019	43471
3	217109_at	MUC4	Mucin 4, tracheobronchial	A	A	A	8064	6719	31106	39510	16442	18559
4	222068_s_at	LOC123872	Similar to RIKEN cDNA 4930457P18	A	A	A	7617	10603	38771	22222	18860	19909
5	221185_s_at	DKFZp434B227	Hypothetical protein DKFZp434B227	A	A	A	15513	7275	16015	19882	15887	16400
6	220636_at	DNAI2	Dynein, axonemal, intermediate polypeptide 2	A	A	A	7932	8834	21730	17325	16602	7066
7	208140_s_at	DKFZp586M1120	Hypothetical protein DKFZp586M1120	A	A	A	7713	5961	11610	12516	8652	7415
8	210445_at	FABP6	Fatty acid binding protein 6, ileal (gastrotropin)	A	A	A	5223	11248	5769	13570	7823	7829

A: absent or marginal. There are two Affy IDs, 209810 and 37004, for the SFTPB gene.

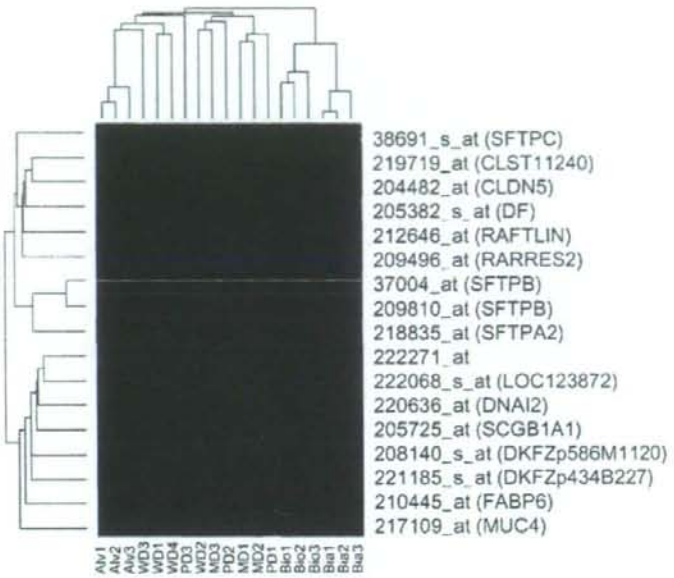

Figure 4 Two-dimensional hierarchical clustering for noncancerous lung epithelial cells and lung adenocarcinoma cells with 17 probe sets corresponding to 16 differentiation marker genes. Alv1 – Alv3 are type II alveolar cells, Bio1 – Bio3 are bronchiolar epithelial cells, Bia1 – Bia3 are bronchial epithelial cells, and WD1 – WD3, MD1 – MD3 and PD1 – PD3 are lung adenocarcinoma cells.

Table 4 Expression of 14 candidate differentiation markers in lung adenocarcinoma cells, noncancerous lung cells and peripheral blood cells

Gene symbol	AdC (n¼45) mean7s.d.	NL (n¼14) mean7s.d.	PB (n¼5) mean7s.d.
CLDN5	1000 7 1000	15185 7 7407*	20741 7 16296
RARRES2	1000 7 1318	4955 7 1727*	0*
RAFTLIN	1000 7 1259	2810 7 1345*	414 7 172*
CLST11240	1000 7 1083	26500 7 35000*	0*
SFTPC	1000 7 6487	10216 7 26355	0.01 7 0.01
SFTPA2	1000 7 3712	12354 7 20058*	94 7 65
SFTPB	1000 7 3986	5322 7 11846	0.001 7 0.001
SCGB1A1	1000 7 1926	14444 7 15556*	1385 7 111
MUC4	1000 7 1819	428 7 469	0*
LOC123872	1000 7 2000	6000 7 4750*	0*
DKFZp434B227	1000 7 1079	2382 7 1719*	7584 7 5831
DNAI2	1000 7 1250	491 7 7 3583*	4083 7 2583
DKFZp586M1120	1000 7 1299	5480 7 3107*	0*
FABP6	1000 7 1448	1500 7 1362	9035 7 5460*

*Significantly different ($P < 0.05$) from AdC by student's t-test. Abbreviations: AdC, adenocarcinoma; NL, noncancerous lung cells; PB, peripheral blood cells.

and of those between lung AdCs and noncancerous lung cells for differentiation marker genes with those for tumor marker genes easier, mean levels of expression for each gene in 45 cases of AdCs were also adjusted to 1000 and the relative values for expression were compared among samples (Table 4). Interestingly, the mean value of MUC4 in lung AdCs was higher than that in noncancerous lung cells. The result indicated that expression of the MUC4 gene was retained in a large fraction of lung AdC cases. Alternatively, it is also possible that the MUC4 gene was aberrantly overexpressed in a subset of lung AdCs. In either case, the result suggested that the MUC4 gene could be useful as a differentiation marker whose expression is retained in lung AdCs. Expression of three other genes, SFTPC, SFTPB and FABP6, was retained relatively frequently in lung AdCs, since the mean values were not significantly different between lung AdCs and noncancerous lung cells. However, in the remaining 10 genes, mean values in lung AdCs were significantly lower than those in noncancerous lung cells, suggesting that those genes were downregulated in a large fraction of lung AdCs.

To further evaluate whether expression of these genes in AdC cells correlates with the histological differentiation of AdC cells, levels of mRNA expression for each

gene were compared among 45 AdC cases (Table 5). In this table, to compare between the expression levels of each gene and histological differentiation of the AdC cases, cases with expression over the mean levels of 14 cases of noncancerous lung cells are indicated by plus marks. For CLDN5, CLST11240, SCGB1A1 and DKFZp586M1120, none of the 45 AdC cases showed higher expression levels than noncancerous lung cells, thus, these genes were deleted from this table. The other 10 genes showed high expression levels in various fractions of AdC cases. SFTPA2, SFTPB and SFTPC showed high expression levels only in the well differentiated type, whereas RAFTLIN, LOC123872 and DFKZp434B227 showed high expression levels in the well and moderately differentiated types. Interestingly, MUC4 and FABP6 showed high levels not only in well and moderately differentiated types but also in the poorly differentiated type, and DNAI2 showed a high level only in a case of the moderately differentiated type and RARRES2 showed high levels in one case of moderately and poorly differentiated types, respectively. Thus, these genes were expressed to various extents and irrespective of differentiation status in lung AdCs.

Expression of lung adenocarcinoma markers in peripheral blood cells

We next examined the expression of nine tumor marker genes in five cases of PB cells. Expression of three genes, COL11A1, TM4SF4 and GAGED2, was not detected

Table 5 Retention of differentiation marker gene expression in lung adenocarcinoma

Case No.		Differentiation markers									
		RARRES2	RAFTLIN	SFTPC	SFTPA2	SFTPB	MUC4	LOC123872	DKFZp434B227	DNAI2	FABP6
Well	1										
	2		+						+		+
	3						+				
	4				+	+	+				
	5		+					+	+		
	6										
	7								+		+
	8			+							+
	9						+		+		+
	10										
	11										
	12										
	13		+				+				
	14						+				
	15										
	16					+					
Moderately	1										
	2										
	3										
	4										
	5	+					+		+		+
	6										
	7						+				
	8										
	9										
	10						+	+		+	
	11						+				
	12										
	13										
	14										
	15										
	16		+								+
	17						+				
	18										
Poorly	1						+				
	2	+					+				
	3										
	4						+				
	5										
	6										+
	7										
	8										
	9										
	10										+
	11										

and expression of two other genes, SGNE1 and TFPI2, was extremely low in PB cells (Table 2). On the other hand, the remaining four genes, STK6, ATP10B, MCM6 and TOP2A, were expressed at considerable levels in PB cells. We next examined the expression of 14 differentiation marker genes in five cases of PB cells. Seven of the 14 genes, CLDN5, RAFTLIN, SFTPA2, SCGB1A1, DKFZp434B227, DNAI2 and FABP6, were expressed at adequate levels in PB cells. However, five other genes, RARRES2, CLST11240, MUC4, LOC123872 and DKFZp586M1120, were not expressed, and the remaining two genes, SFTPB and SFTPC, were expressed at extremely low levels in PB cells (Table 4).

Discussion

Genes highly and specifically expressed in cancer cells are useful for molecular detection of cancer cells. Genes that are expressed in a tissue specific manner in precursor cells and whose expression is retained in cancer cells are useful for differential diagnosis to predict the origin of cancer cells. To identify these genes, we isolated lung AdC cells and noncancerous lung epithelial cells of three different anatomic regions by the LCM method. These cells were then used for oligo nucleotide microarray analysis of 14500 genes. Hierarchical clustering analysis revealed that lung AdC cells and noncancerous lung epithelial cells were significantly different in their gene expression profiles, suggesting the presence of genes up or downregulated specifically and commonly in lung AdC cells in comparison with noncancerous lung epithelial cells. Subsequently, nine genes were identified as being upregulated in AdC cells in vivo in comparison with noncancerous lung epithelial cells, and thus, as possible lung AdC specific tumor markers. Moreover real-time RT–PCR analysis against 45 AdCs and 14 noncancerous lung tissues revealed that expression of four of the nine genes, SGNE1, COL11A1, GAGED2 and TOP2A, was significantly high in lung AdCs in comparison with noncancerous lung tissues. The SGNE1 gene encodes a neuroendocrine protein called 7B2, and its serum levels are elevated in several types of neuroendocrine tumors, including small cell lung cancer (Vieau et al., 1991; Mbikay et al., 2001). The COL11A1 gene encodes one of the two alpha chains of type XI collagen, and it was previously reported that COL11A1 is expressed in colorectal cancers but not expressed in normal colon epithelia (Fischer et al., 2001). GAGED2 encodes a protein of a family of CT antigens and is known to be expressed in a variety of cancers (Egland et al., 2002). TOP2 is also known to be highly expressed in various types of cancers. Since these four genes are expressed in a variety of cancers, they are not likely to be expressed specifically in lung AdC cells. Therefore, molecular diagnosis with a set of these markers, rather than with any of a single marker, will be useful for the detection and characterization of lung AdC.

Hierarchical clustering analysis also revealed that there were considerable numbers of genes differentially expressed among three different types of noncancerous lung epithelial cells. Subsequently, we identified 16 genes that were expressed specifically in either alveolar/bronchiolar or bronchiolar/bronchial epithelial cells. As expected, SFTPA2, SFTPB and SFTPC were identified as genes that were expressed in alveolar/bronchiolar epithelial cells but not in bronchial epithelial cells (Otto, 2002). Five genes were identified as being expressed only in alveolar epithelial cells but not in bronchiolar/bronchial epithelial cells. In addition to CC10 (SCGB1A1), seven genes were identified as being expressed in bronchiolar/ bronchial epithelial cells, but not in alveolar epithelial cells (Broers et al., 1992). Thus, this is the first report showing the difference in gene expression profiles among three different regions of lung epithelial cells, and identifying various differentiation marker genes for respective epithelial cells. Moreover, hierarchical clustering analysis with these 16 genes indicated that the gene expression profile of lung AdC is more similar to that of type II alveolar epithelial cells than that of bronchiolar/bronchial epithelial cells. This finding supports the previous reports showing that lung AdCs are derived from type II alveolar epithelial cells and not from Clara cells (Ten Have-Opbroek et al., 1997; Otto, 2002). In particular, expression of 10 genes, SFTPA2, SFTPB, SFTPC, RARRES2, RAFTLIN, MUC4, LOC123872, DFKZp434B227, DNAI2 and FABP6, was retained in a considerable fraction of lung AdC cases. SFTPA2, SFTPB and SFTPC are typical examples of differentiation markers isolated in this study. However, MUC4 is known to be expressed in various normal epithelial cells as well as several types of carcinomas, including lung carcinoma (Hanaoka et al., 2001; Moniaux et al., 2004). At present, tissue specificities for the expression of the remaining six genes are unknown. Thus, further studies should focus on the characterization of these genes as lung epithelial cell-specific markers.

Finally, we found that five tumor markers, COL11A1, GAGED2, TM4SF4, SGNE1 and TFPI2, and seven differentiation markers, SFTPB, SFTPC, RARRES2, CLST11240, MUC4, LOC123872 and DKFZp586M1120 were not expressed or expressed at extremely low levels in PB cells. Up to the present, several genes have been used for detection of lung AdC cells in PB, such as CK19 and CEA (Matsunaga et al., 2002; Mitas et al., 2003). However, both the sensitivity and specificity of these genes for detection of lung AdC cells were not high enough to use as a single molecular marker in clinics. Thus, it will be important to further evaluate the specificity and sensitivity for the combinational use of the 12 markers identified in this study with several known markers for lung AdC cell detection in PB.

Materials and methods

Preparation of tissue samples

Ten primary lung AdCs and nine normal lung tissues used for oligonucleotide microarray analysis were obtained from 15 patients with lung AdC at surgery at the National Cancer

Center Hospital, Tokyo, Japan (Table S1). AdCs were histologically classified according to the Histological Typing of Lung and Pleural Tumors (Travis et al., 1999) and staged according to the TNM Classification of Malignant Tumors (Sobin, 2002). These tissues were embedded in OCT medium and frozen at −80°C as described previously (Kobayashi et al., 2004).

Another 45 lung AdC samples and 14 noncancerous lung tissues were obtained from 46 patients at surgery and kept at −80°C until RNA extraction. Total RNA was extracted from these macrodissected samples using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Peripheral blood cells were obtained from five individuals with no past histories of cancer, and total RNA from these samples was extracted using a QIAamp RNA Blood Mini Kit (QIAGEN).

Laser capture microdissection and total RNA extraction

The OCT-embedded tissues were cryostat sectioned at 8 mm thickness, mounted on glass slides, and then cells were microdissected using the PixCell II LCM (Arcturus Engineering, Mountain View, CA, USA) as described previously (Kobayashi et al.,, 2004). Approximately 100 type II alveolar epithelial cells, 200 bronchiolar epithelial cells, 200 bronchial epithelial cells and 500 AdC cells were captured onto a single transfer film from a single section. Total RNA was extracted from 2000 microdissected cells on the film using the Micro RNA Isolation Kit (Stratagene, San Diego, CA, USA) as described previously (Kobayashi et al., 2004).

RT–PCR analysis

RT–PCR analysis for SFTPB, SCGB1A1 and GAPDH were performed using RNA from approximately 1000 cells as described previously (Kobayashi et al., 2004).

cRNA preparation and GeneChip hybridization

Total RNA (10 ml) solutions, containing RNA from 1000 cells, were subjected to PCR-mediated global mRNA amplification using the TALPAT (T7 RNA polymerase promoter-attached, adaptor ligation-mediated, and PCR amplification followed by in vitro T7-transcription) method (Aoyagi et al., 2003). Biotin-labeled cRNA was prepared as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix Santa Clara, CA, USA) and hybridized to the human U133 GeneChip.

Microarray data analysis

Array chip slides were scanned with a GMS418 Array Scanner (Genetic Microsystems, Woburn, MA, USA), and image analysis of the array was performed according to the manufacturer's protocol. Raw data were analysed with an Affymetrix Microarray Analysis Suite (MAS) v4.0 and were globally normalized and scaled to a target intensity of 1000 to facilitate inter-array comparison. The MAS software generated absolute calls (present, marginal or absent), and the calls were used to determine whether a gene was expressed or not. After being filtered through 'absent call' for all samples, lists of genes expressed in AdC cells or noncancerous lung epithelial cells were ranked based on absolute call. Hierarchical clustering analysis was performed using the GeneSpring 6.1 (Silicon Genetics, Redwood City, CA, USA) software with unsupervised and supervised clustering of normalized data.

Real-time RT–PCR analysis

Real-time RT–PCR analysis was performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Lincoln Centre Drive Foster City, CA, USA). The expression level of each gene was normalized to RNA content for each sample by using GAPDH as an internal control. A primer pair and a TaqMan probe for each gene are listed in Table S3. PCR for each target gene and the reference gene GAPDH was performed in a single tube in duplicate, and the results were expressed as the average of the two independent tests.

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Detection of *EGFR* Mutations in Archived Cytologic Specimens of Non-Small Cell Lung Cancer Using High-Resolution Melting Analysis

Kiyoaki Nomoto, CT,[1] Koji Tsuta, MD,[1] Toshimi Takano, MD,[2] Tomoya Fukui, MD,[2] Karin Yokozawa,[3] Hiromi Sakamoto, MD,[4] Teruhiko Yoshida, MD,[4] Akiko Miyagi Maeshima, MD,[5] Tatsuhiro Shibata, MD,[5] Koh Furuta, MD,[3] Yuichiro Ohe, MD,[2] and Yoshihiro Matsuno, MD[1]

Key Words: Epidermal growth factor receptor; DNA mutation analysis; High-resolution melting analysis; Non-small cell lung cancer

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Abstract

*Mutations of the epidermal growth factor receptor (*EGFR*), particularly deletional mutations (DEL) in exon 19 and L858R in exon 21, are reportedly correlated with clinical outcome in patients with non–small cell lung cancer (NSCLC) receiving the EGFR tyrosine kinase inhibitors gefitinib and erlotinib, suggesting that detection of* EGFR *mutations would have an important role in clinical decision making. We established and validated an easy, inexpensive, and rapid method for detecting DEL and L858R from cytologic material by high-resolution melting analysis (HRMA). Dilution for sensitivity studies revealed that DEL and L858R were detectable in the presence of at least 10% and 0.1%* EGFR*-mutant cells, respectively. We analyzed 37 archived cytological slides of specimens from 29 patients with advanced NSCLC and compared the results with direct sequencing data obtained previously. Of 37 samples, 34 (92%) yielded consistent results with direct sequencing, 2 were false negative, and 1 was indeterminate. The sensitivity of this analysis was 90% (19/21) and specificity, 100% (15/15). These results suggest that HRMA of archived cytologic specimens of advanced NSCLC is useful for detecting* EGFR *mutations in clinical practice.*

Increased expression of epidermal growth factor receptor (EGFR) has been reported in carcinomas of various organs, including of the lung, and has been shown to have a crucial role in tumor progression.[1,2] Gefitinib (Iressa, AstraZeneca, Osaka, Japan) is an orally active, selective EGFR tyrosine kinase inhibitor that binds to the adenosine triphosphate binding pocket of the kinase domain and blocks downstream signaling pathways. Clinical phase 2 studies have demonstrated gefitinib antitumor activity in patients with advanced non–small cell lung cancer (NSCLC).[3,4] Although some of these studies have shown that the rate of response to gefitinib is higher in women, patients with adenocarcinoma, patients who have never smoked, and Japanese and East Asians,[3-5] no predictive molecular marker had been discovered until April 2004, when somatic mutations in the kinase domain of *EGFR* were suggested to be correlated with gefitinib sensitivity.[6,7] Thereafter, several studies revealed a strong association between *EGFR* mutations and clinical outcome in parameters such as response rate, time to progression, and overall survival in consecutive NSCLC patients treated with gefitinib.[8-10]

Many types of *EGFR* mutation have been identified.[6-16] They are concentrated in exons 18 to 21 of *EGFR*, close to the region encoding the adenosine triphosphate binding pocket, and about 90% of patients with *EGFR* mutations have mutations in 2 hotspots: in-frame deletions including amino acids at codons 747 to 749 (DEL) in exon 19 and a missense mutation at codon 858 (L858R) in exon 21.

The mutational status of *EGFR*, especially DEL and L858R, is a strong predictor of gefitinib sensitivity, and detection of such mutations would provide patients and physicians with important information for optimal choice of therapy. Therefore, analysis of a sufficient number of tumor samples in

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good condition and direct sequencing after laser capture microdissection (LCM) is considered the "gold standard" for detecting *EGFR* mutations. However, this approach is not necessarily practical for clinical use for a number of reasons. First, tumor samples with a large volume and in good condition are difficult to obtain in most cases of advanced NSCLC. Second, LCM and direct sequencing require special instruments and are time-consuming and costly. Therefore, it is necessary to establish practical and precise methods for detecting *EGFR* mutations from easily obtainable diagnostic samples, which usually contain a small number of tumor cells and a large number of normal cells.

The real-time reverse transcription–polymerase chain reaction (PCR) assay has been reported for detection of *EGFR* mutations.[17] In this method, many samples can be genotyped within a few hours without the need for post-PCR sample manipulation, although expensive fluorescence-labeled probes and restriction enzymes are needed. A new inexpensive dye, SYBR Green I, has been developed,[18] but this limits the melting resolution because of dye redistribution during melting.

Recently, studies have validated the usefulness of high-resolution melting analysis (HRMA) using LCGreen I dye for mutational analysis,[19-22] and another study has validated analysis using cytologic samples for c-kit.[23] The advantages of this approach are that labeling of either primer with dye is not needed and PCR amplification and melting analysis can be performed in the same capillary tube, minimizing sample handling and reducing the possibility of error and sample contamination. HRMA is easy, rapid, and inexpensive to perform and has considerable potential for mutation detection in clinical practice.

We report a new method for detecting DEL and L858R from archival Papanicolaou-stained cytologic slides by HRMA. We validated the method by comparing the results with direct sequencing data from specimens surgically resected from the same patients. We also performed a titration assay to evaluate the lower limit of the proportion of tumor cells for detection of *EGFR* mutations by using a mixture of wild-type (WT) and *EGFR*-mutant lung cancer cell lines.

Materials and Methods

Cell Lines and Titration Assay

We performed dilution for sensitivity studies using 3 lung adenocarcinoma cell lines, NCI-H1650, NCI-H1975, and NCI-A549, obtained from the American Tissue Cell Collection (Manassas, VA). The H1650 cell line contains a DEL mutation (delE746-A750), the H1975 cell line contains the L858R mutation,[24] and the A549 cell line contains WT *EGFR*.[25] *EGFR* copy numbers in the H1650, H1975, and A549 cells are reported to be 2, 3, and 2.48 per cell, respectively.[25,26] Dilutions of the *EGFR*-mutant cells (H1650 or H1975) with A549 cells were prepared using proportions of *EGFR*-mutant cells of 100% (no A549 cells), 10%, 1%, 0.1%, and 0% (no mutant cells). DNA extracted from each dilution was subjected to subsequent PCR assay.

DNA Extraction From Archived Cytologic Slides

With approval of the National Cancer Center Institutional Review Board, Tokyo, Japan, we performed *EGFR* gene analysis. Among the 66 cases analyzed in a previous study, diagnostic Papanicolaou-stained cytologic samples were available for 29. Of the patients, 5 had multiple (2 to 4) metachronous samples, and the total number of available cytologic samples was 37. Two clinical cytologists (K.N. and K.T.), who were unaware of the patients' characteristics and mutational status, examined these 37 samples. Cytologic parameters described for each slide included sampling procedure, approximate number of nucleated cells on each slide (<100, 100-499, 500-999, ≥1,000), and proportion of tumor cells among total nucleated cells (<10%, 10%-49%, 50%-89%, ≥90%). After this assessment, DNA was extracted from the cells on the slides using a QIAamp DNA Micro Kit (catalog No. 56304, QIAGEN, Valencia, CA) as follows: Coverslips were removed by immersion in xylene for 72 hours, and the slides were rinsed in 95% ethanol 3 times. Cells on the slides were removed by using sterilized disposable knives and suspended in ATL buffer containing Proteinase K in 1.5-mL tubes. Further procedures were performed according to the manufacturer's protocol.

In 2 samples with a small proportion of tumor cells, tumor cell–rich parts on the slides were marked with a diamond pen and selectively retrieved manually with a knife to enrich the proportion of tumor cells.

Polymerase Chain Reaction

Primer A was designed to amplify a region containing nucleotides 2235 to 2277 (amino acids E746 to I759) of *EGFR*, in which almost all reported deletional mutations in exon 19 occur.[6-16] The sequences of primer A were AAAATTCCCGTCGCTATC (forward) and AAGCAGAAACTCACATCG (reverse). Primer B was designed to amplify a region containing nucleotides 2573 and 2582, at which point mutations L858R and L861Q in exon 21 occur, respectively. L858R and L861Q account for about 96% and 2%, respectively, of all reported point mutations in exon 21.[6-16] The sequences of primer B were AGATCACAGATTTTGGGC (forward) and ATTCTTTCTCTTCCGCAC (reverse).

PCR was performed using these primers, Fast Start *Taq* Polymerase (Roche Diagnostics, Indianapolis, IN), and LCGreen I Gene Scanning Reagents (Idaho Technology, Salt Lake City, UT) on a LightCycler (Roche Diagnostics). The samples were denatured at 95°C for 10 minutes and then subjected

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to 37 cycles of denaturing for 10 seconds at 95°C, annealing for 10 seconds at 60°C, and extension for 5 seconds at 72°C with primer A and 45 cycles of denaturing for 10 seconds at 95°C, annealing for 5 seconds at 56°C, and extension for 5 seconds at 72°C with primer B.

High-Resolution Melting Analysis

The PCR products were denatured at 95°C for 5 minutes and cooled to 40°C in the LightCycler to form heteroduplexes. The LightCycler capillary was transferred to an HR-1 (Idaho Technology), an 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 *EGFR*.

Direct Sequencing

In a previous study, we performed direct sequencing of *EGFR* in 66 cases of NSCLC that relapsed after primary surgery. Methanol-fixed, paraffin-embedded surgical specimens of primary NSCLC were obtained, and DNA was extracted from laser capture microdissected tumor tissue. Nested PCR was performed to amplify exons 18 to 24 of *EGFR* using primers described previously.[8] Direct sequencing of the PCR products was performed using the ABI PRISM 3700 and 3100 DNA sequencers (Applied Biosystems, Foster City, CA).

Results

In the melting analysis using primer A (exon 19), 100% H1650 cells (EGFR DEL) gave a skewed curve from 100% A549 cells (EGFR WT). Mixtures of both cells gave gradual curves, and DEL could be detected in the presence of 10% but not 1% H1650 cells **Figure 1A**. In the analysis using primer B (exon 21), 100% H1975 cells (EGFR L858R) gave a left-shifted curve from 100% A549 cells. Mixtures of both cells gave gradual curves, and L858R could be detected in the presence of 0.1% H1975 cells **Figure 1B**.

We analyzed 37 archival cytologic samples from 29 patients by HRMA, and the results are summarized in **Table 1**, in comparison with the results obtained by direct sequencing from surgically resected specimens of each patient. Eleven samples were obtained by bronchial brushing or washing, 4 by transbronchial fine-needle aspiration (FNA), 4 by percutaneous FNA of lung tumors, 2 by FNA of superficial lymph nodes, 14 from pleural effusion, and 2 from pericardial effusion. The median time between sampling and analysis was 3 years (range, 1-8 years).

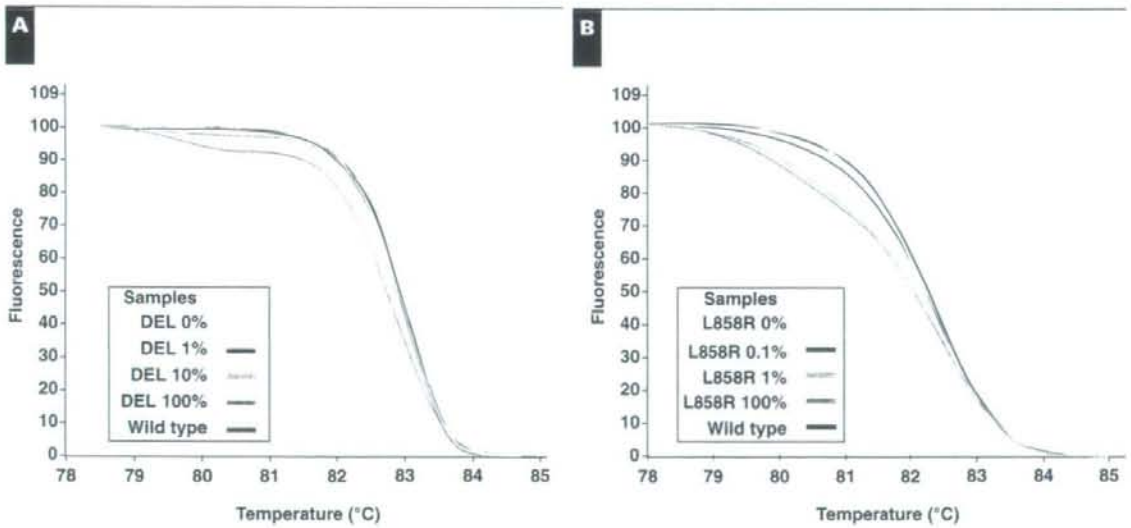

Figure 1 Adjusted melting curves obtained by high-resolution melting analysis of lung adenocarcinoma cells with primers designed to detect mutations in epidermal growth factor receptor (*EGFR*) exon 19 (**A**) or exon 21 (**B**). **A**, Mixtures of H1650 cells ($EGFR^{DEL}$) and A549 cells ($EGFR^{WT}$) revealed gradual curves; 100% and 10% H1650 cells were identified as containing a DEL mutation, and 1% H1650 cells were identified as wild type. **B**, Mixtures of H1975 cells ($EGFR^{L858R}$) and A549 cells ($EGFR^{WT}$) revealed gradual curves, and 100% to 0.1% H1975 cells were identified as containing the L858R mutation. DEL, deletional.

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In the analysis of exon 19, thorough melting curves were obtained in 35 samples, whereas the other 2 samples (5 and 21) could not be analyzed because PCR was not complete in these cases. Among the 35 samples, 12 gave curves that were different from a WT obtained for cell line A549, as shown in ▮Figure 2A▮, and 23 samples revealed almost the same curves with a WT ▮Figure 2C▮ (Figure 2A). Because the skewed curves for the 12 samples were analogous to the curve for H1650 cells, we judged that they had DEL. In the analysis of exon 21, 7 and 2 samples gave left- and right-shifted curves from a WT, respectively, and 28 samples gave almost identical curves with a WT ▮Figure 2B▮ and ▮Figure 2D▮. Because the left-shifted curves of the 7 samples were analogous to the curve for H1975, we judged that they had L858R.

As mentioned previously, 2 samples (21 and 25) showing right-shifted curves were considered inadequate for evaluation because of incomplete PCR (Figures 2C and 2D). Taken together, DEL was detected in 12 samples (8 patients) and L858R was detected in 7 samples (6 patients) among 37 samples (29 patients). Samples 5 and 25 were insufficient for judging genotypes of 1 hotspot but judged as containing mutations in the other hotspot (L858R and DEL, respectively). Therefore, the genotype was indeterminate in only 1 sample (case 21) and determined as WT in 17 samples (14 patients). Analysis of the 5 cases with multiple (2 to 4) metachronous samples revealed no differences of genotype in each case.

The results of HRMA were consistent with those of direct sequencing in all samples except samples 19 and 20 (Table 1), which revealed WT curves by HRMA, although

▮Table 1▮
HRMA Results for 37 Archival Cytologic Samples From 29 Patients

Sample No.	Histologic Type	Sampling Method	No. of Nucleated Cells	Proportion of Cancer Cells/Nucleated Cells (%)	Mutational Analysis by HRMA		Mutational Analysis by Direct Sequencing
					DEL	L858R	
1	Ad	TBAC	≥1,000	≥90	WT	L858R	L858R
2	Ad	PAC	500-999	≥90	WT	L858R	L858R
3	Ad	BC	500-999	≥90	WT	L858R	L858R
4	Ad	BC	500-999	10-49	DEL	WT	delL747-E749
5	Ad	BC	500-999	50-89	NE	L858R	L858R
6	Ad	BC	≥1,000	<10	WT	WT	WT
7	Ad	BC	100-499	≥90	WT	WT	WT
8	Ad	LN	≥1,000	50-89	WT	WT	WT
9a	Ad	BC	500-999	50-89	WT	WT	WT
9b		BC	500-999	≥90	WT	WT	WT
9c		BC	100-499	<10	WT	WT	WT
10	Ad	PL	≥1,000	10-49	DEL	WT	delE746-A750
11a	Ad	PL	≥1,000	≥90	WT	L858R	L858R
11b		PL	500-999	50-89	WT	L858R	L858R
12	Ad	PL	≥1,000	<10	WT	WT	WT
13	SCC	PAC	500-999	50-89	WT	WT	WT
14a	Ad	PC	≥1,000	50-89	DEL	WT	delE746-A750
14b		PL	500-999	50-89	DEL	WT	delE746-A750
14c		PL	≥1,000	50-89	DEL	WT	delE746-A750
14d		PL	500-999	10-49	DEL	WT	delE746-A750
15a	Pleo	BC	100-499	50-89	DEL	WT	delE746-A750
15b		BC	500-999	50-89	DEL	WT	delE746-A750
16	Ad	PAC	100-499	10-49	WT	WT	WT
17	Ad	PL	500-999	50-89	WT	L858R	L858R/E709K
18	Ad	TBAC	500-999	≥90	WT	WT	WT
19	Ad	PL	≥1,000	10-49	WT	WT*	L858R/S768I
20	Ad	PL	≥1,000	<10	WT	WT†	L858R
21	Ad	LN	100-499	≥90	NE	NE	delE746-A750
22	Ad	PL	≥1,000	<10	WT	WT	WT
23	Ad	TBAC	500-999	≥90	DEL	WT	delE746-A750
24	Ad	PL	≥1,000	50-89	WT	WT	WT
25	Ad	PL	100-499	<10	DEL	NE	delE746-A750
26a	Ad	TBAC	500-999	50-89	WT	WT	WT
26b		PL	≥1,000	<10	WT	WT	WT
27	Ad	PAC	≥1,000	50-89	WT	WT	WT
28	Ad	PC	500-999	50-89	DEL	WT	delE746-A750
29	Ad	BC	100-499	50-89	DEL	WT	delE746-A750

Ad, adenocarcinoma; BC, bronchial brushing or washing cytology; DEL, deletional mutation; HRMA, high-resolution melting analysis; LN, fine-needle aspiration cytology of superficial lymph nodes; NE, not evaluable; PAC, percutaneous fine-needle aspiration cytology; PC, pericardial effusion; PL, pleural effusion; Pleo, pleomorphic carcinoma; SCC, squamous cell carcinoma; TBAC, transbronchial fine-needle aspiration cytology; WT, wild type.

* WT after tumor cell–enrichment procedure.

† L858R after tumor cell–enrichment procedure.

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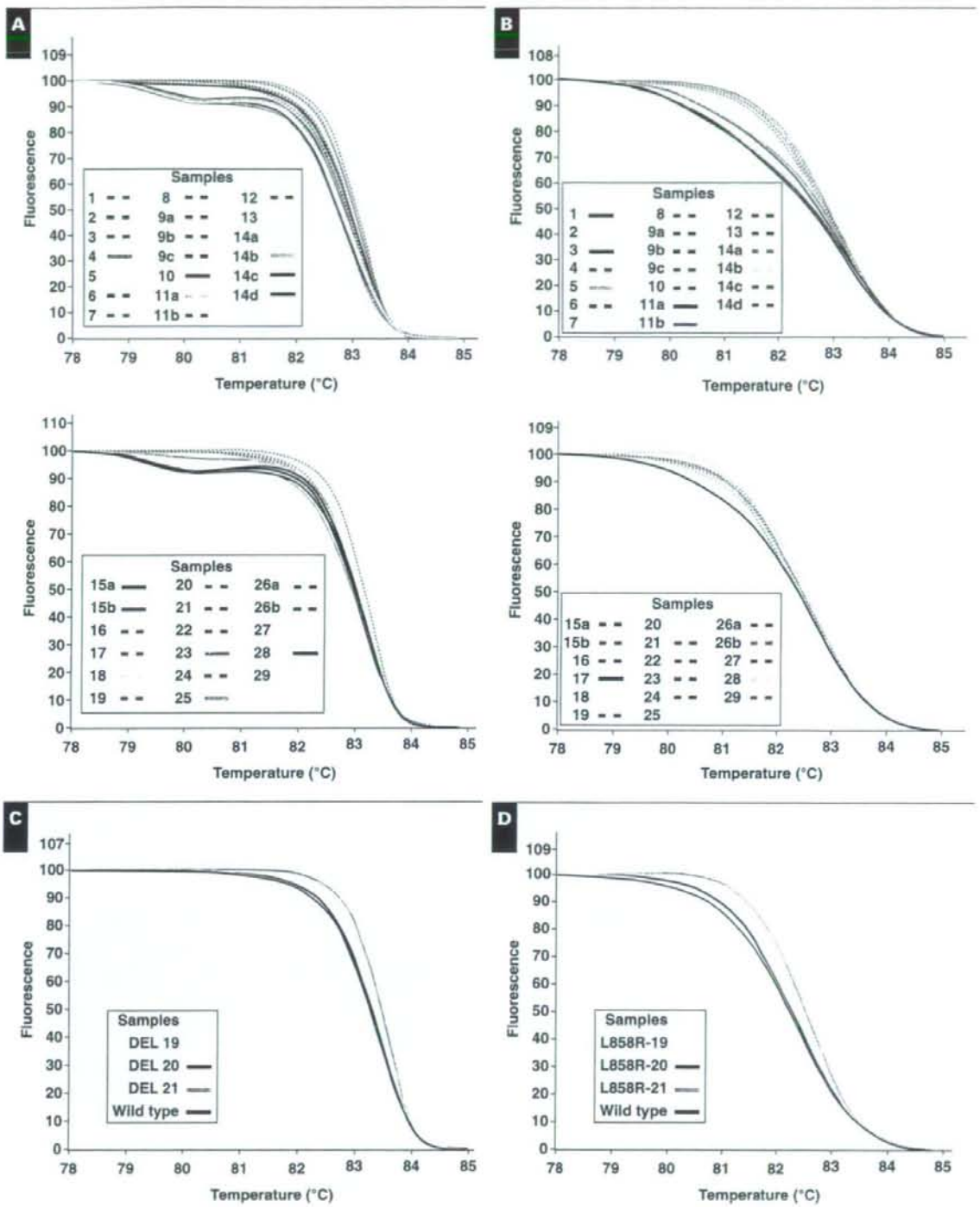

Figure 2 Adjusted melting curves of DNA extracted from archived cytologic slides in the analysis of epidermal growth factor receptor (*EGFR*) exon 19 (**A**) and exon 21 (**B**). Samples 4, 10, 14a-d, 15a-b, 23, 25, 28, and 29 were identified as containing deletional (DEL) mutations, and samples 1, 2, 3, 5, 11a-b, and 17 were identified as containing the L858R mutations. **C** and **D**, The curves of 3 samples (19-21) are shown in **C** (DEL) and **D** (L858R), but the curves were not obtained in 2 samples (5, DEL; 25, L858R) because of incomplete polymerase chain reaction.

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surgical specimens from the same patients showed the L858R mutation by direct sequencing. Thus, the results for these samples were considered false-negative. The cytologic appearances of these samples are shown in **Image 1**. Sample 20 contained only a small proportion (<10%) of cancer cells in a background of numerous benign nucleated cells, possibly explaining the false-negative result. In fact, we were able to detect the L858R mutation after tumor cell enrichment by manual dissection in sample 20. However, this was not the case for sample 19, which contained a moderately small proportion (10%-50%) of cancer cells, and the result remained negative even after tumor cell enrichment.

In summary, we identified DEL or L858R in 19 samples (14 patients) and WT *EGFR* in 15 samples (12 patients) accurately by HRMA, but 2 samples (2 patients) gave false-negative results and 1 sample (1 patient) was indeterminate. Accuracy was 92% (34/37) based on the number of samples and 90% (26/29) based on the number of patients. Among the 36 samples in which the genotype was determined, sensitivity was 90% (19/21) and specificity was 100% (15/15), or 88% (14/16) and 100% (12/12), respectively, based on the number of patients. These data indicate that this new method is useful for clinical decision making, especially when a patient is given a positive result.

Discussion

In the present study, we established and validated a new method for detecting 2 major *EGFR* mutations (DEL and L858R) using HRMA for cytologic samples. In a study using a cell line, the sensitivity of HRMA indicated that if at least 10% of cells in a sample were cancer cells, then both DEL and L858R were detectable. L858R was detectable even in 0.1% of L858R cells, whereas DEL could not be detected in 1% of DEL cells. Although the reason for this difference is unclear, the sensitivity is still sufficiently high for application to clinical practice.

We performed the HRMA using archival cytologic samples from 29 patients with NSCLC, and the results were quite consistent with the data obtained for the corresponding 26 cases by LCM plus direct sequencing, which were performed in a previous study.[8] The HRMA was completed in 8 of 9 tumor samples with known DEL mutations, and all 8 samples were identified as having DEL. DEL was detected accurately even in sample 25, in which tumor cells accounted for fewer than 10% of the cells on the slides. In the analysis of archived cytologic samples, no marked difference in detection accuracy was observed between DEL and L858R.

Two samples that contained a relatively small proportion of tumor cells gave false-negative results for L858R; one of these (sample 20) gave a positive result after tumor cell enrichment, but the other (sample 19) did not. The sensitivity of this analysis was 88% (14/16) based on the number of patients, and it increased to 94% (15/16) if tumor cells were enriched in the samples with a small proportion of tumor cells. These results indicate that cytologists will be required not only to diagnose whether samples are benign or malignant but also to evaluate whether they are suitable for molecular analysis.

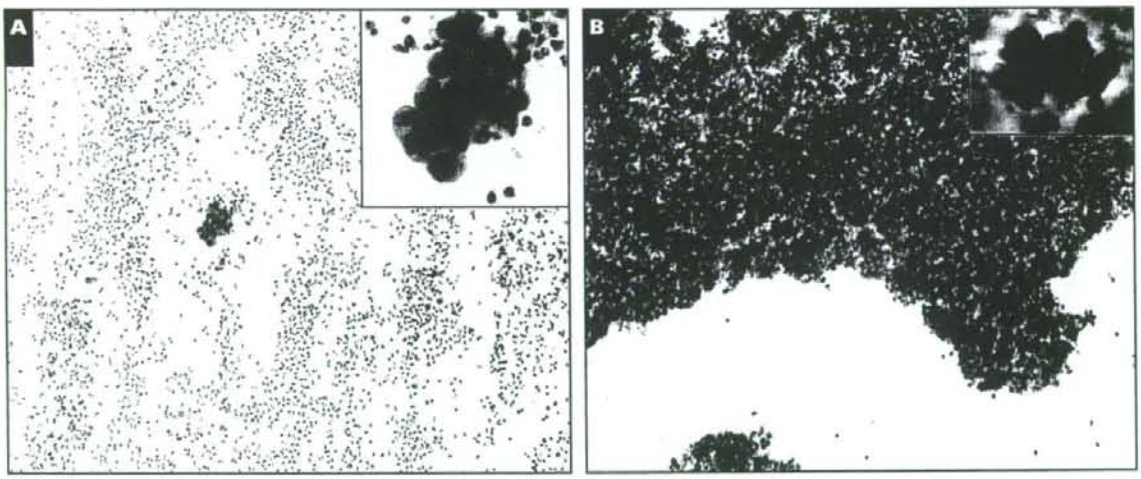

Image 1 Samples 19 (**A**) and 20 (**B**) show a few cancer cells in a background of many normal nucleated cells. L858R was not detected in these samples by high-resolution melting analysis (HRMA), although direct sequencing showed that DNA extracted from surgical specimens from the same patients had L858R. After retrieving tumor-rich parts selectively, L858R was detected by HRMA from a cytologic slide obtained simultaneously with sample 20, but this was not the case with sample 19 (×10). Insets, The tumor showed 3-dimensional clusters with nuclear atypia compatible with adenocarcinoma (×40).

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The mutational status of *EGFR* is a strong predictor of gefitinib sensitivity, and detection of such mutations would provide patients and physicians with important information for optimal choice of therapy. However, mutation detection has not become a common procedure in clinical practice because it often is difficult and impractical. Direct sequencing, which is a standard method for detecting mutations, requires high-quality DNA extracted from an adequate amount of pure tumor cells to obtain precise data and is costly and time-consuming.

Many researchers have tried to establish new methods for detecting *EGFR* mutations using small tumor samples contaminated with normal cells. To date, a number of nonsequencing methods for detecting mutations have been suggested, such as single-strand conformation polymorphism,[15,27] restriction fragment length polymorphism,[28,29] PCR amplification of specific alleles (also known as amplification refractory mutation system and allele specific amplification),[30-32] peptide nucleic acid–mediated PCR clamping,[33,34] peptide nucleic acid–locked nucleic acid PCR clamping,[35] denaturing gradient gel electrophoresis,[36] temperature gradient capillary electrophoresis,[37,38] denaturing high-performance liquid chromatography,[39,40] and high-density oligonucleotide arrays.[41] Some of these methods have been reported to give good results for detection of *EGFR* mutations[15,29,35,40]; however, they often require intensive labor or sophisticated instruments and, therefore, have not been adopted in clinical practice.

HRMA is one of these new methods and has the advantage of being able to distinguish specific mutations from the WT sequence with less labor, time, and cost; PCR and the melting analysis can be performed in the same capillary tube within a few hours, and the running cost is only about $1 (US) per sample.

Detection of DEL and L858R using HRMA is accurate even when archived cytologic samples are used. Because HRMA involves little labor, time, and cost, it is expected to become one of the most practical and useful methods for detecting major *EGFR* mutations in cytologic materials from patients with NSCLC.

From the [1]Clinical Laboratory Division, [2]Division of Internal Medicine, and [3]Clinical Support Laboratory, National Cancer Center Hospital; and the [4]Genetics Division and [5]Pathology Division, National Cancer Center Research Institute, Tokyo, Japan.

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Address reprint requests to Dr Matsuno: Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.

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SHORT REPORT

Frequent *EGFR* mutations in brain metastases of lung adenocarcinoma

Shingo Matsumoto[1,2], Kenji Takahashi[1], Reika Iwakawa[1], Yoshihiro Matsuno[3], Yukihiro Nakanishi[4], Takashi Kohno[1], Eiji Shimizu[2] and Jun Yokota[1]*

[1]*Biology Division, National Cancer Center Research Institute, Tokyo, Japan*
[2]*Division of Medical Oncology and Molecular Respirology, Faculty of Medicine, Tottori University, Tottori, Japan*
[3]*Diagnostic Pathology Division, National Cancer Center Hospital, Tokyo, Japan*
[4]*Pathology Division, National Cancer Center Research Institute, Tokyo, Japan*

Lung adenocarcinomas often metastasize to the brain, and the prognosis of patients with brain metastases is still very poor. The *epidermal growth factor receptor* (*EGFR*) gene is mutated in a considerable fraction of primary lung adenocarcinomas, in particular those with drastic response to EGFR tyrosine kinase inhibitors. The present study was designed to elucidate the prevalence of *EGFR* mutations in brain metastases and the timing of their occurrence during cancer progression. *EGFR* mutations were detected in 12 of 19 metastatic lung adenocarcinomas to the brain (63%). This frequency was higher than those in previous studies for *EGFR* mutations at various stages of lung adenocarcinoma in East Asia, including Japan (*i.e.*, 20–55%). In 6 cases with *EGFR* mutations, the corresponding primary lung tumors were also examined for the mutations, and in all of them, the same types of *EGFR* mutations were detected also in the primary tumors. In 2 of them, second metastatic brain tumors in addition to the first ones were also available for analysis, and the same types of *EGFR* mutations were detected in both the first and second ones in both cases. These results indicate that *EGFR* mutations are present frequently in brain metastases and occur preceding brain metastasis. These findings will be highly informative for treatment of metastatic lung adenocarcinoma to the brain.
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Key words: *EGFR* mutation; metastatic brain tumor; lung adenocarcinoma

Epidermal growth factor receptor (EGFR) is a member of a family comprised of 4 homologous receptors, EGFR (ERBB1), HER-2/neu (ERBB2), HER-3 (ERBB3) and HER-4 (ERBB4). Ligand binding to EGFR leads to receptor tyrosine kinase (TK) activation and a series of downstream signaling activation that mediates proliferation, migration, invasion and suppression of apoptosis.[1] Recently, it was revealed that most lung adenocarcinoma patients who were responsive to gefitinib, an EGFR TK inhibitor, had somatic mutations in the kinase domain of the *EGFR* gene in their tumor cells.[2,3] Subsequently, it was reported that *EGFR* mutations are present in a considerable fraction of lung adenocarcinoma and occur more frequently in East Asian patients, including Japanese, than in Caucasian patients.[4–10] Furthermore, the incidence of *EGFR* mutations was significantly high in female patients and patients without smoking histories. In our previous study, *EGFR* mutations were detected frequently in noninvasive bronchioloalveolar carcinomas, suggesting that *EGFR* mutations occur early in the development of adenocarcinoma, and those with the mutations further progress to invasive and metastatic carcinomas.[11] However, to our knowledge, there is no report showing the prevalence of *EGFR* mutations in metastatic lung adenocarcinomas, and thus, it remains unclear whether *EGFR* mutations are indeed retained in metastatic lung adenocarcinoma or not. Elucidation of this issue will be implicative for treatment with EGFR TK inhibitors against advanced lung adenocarcinomas, which often metastasize systemically to diverse sites, such as brain, bone, adrenal glands and liver.[12] Therefore, we examined metastatic lung adenocarcinomas to the brain for *EGFR* mutations and compared the mutation status in the metastatic brain tumors with the corresponding primary tumors, if they were available for the analysis. We also examined these tumors for *KRAS* mutations, which have been reported as being mutually exclusive for *EGFR* mutations.[4,7]

A total of 21 metastatic brain tumor tissues were obtained from 19 patients who were treated during the period from 1986 to 2001 at the National Cancer Center Hospital, Tokyo, Japan. These tumor tissues were obtained at surgery or at autopsy. In 2 of the 19 cases, the second brain surgery was performed against the second recurrence in the brain 15 months and 24 months after the first brain surgery, respectively, and thus brain tumor tissues were obtained twice during their clinical courses. In 8 of the 19 cases, the corresponding primary lung tumors were obtained at lung surgery preceded by brain surgery. None of the patients were treated with gefitinib through all clinical courses. In 16 of the 19 cases, primary and metastatic tumors were macrodissected and were subjected to genomic DNA extraction by the method described previously.[13] In the remaining 3 cases, from which 3 primary tumors and 5 metastatic tumors were obtained, cancer cells were microdissected using the Pixcell Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA). Their genomic DNAs were isolated by SDS/proteinase K digestion and phenol/chloroform extraction as described previously.[14] Exons 18–21 of the *EGFR* gene and exons 1 and 2 of the *KRAS* gene were examined for mutations by genomic PCR amplification and direct sequencing of PCR products. PCR primer sequences and PCR conditions are described previously.[11]

Table 1 shows the result of all cases examined. In 12 of the 19 cases (63%), *EGFR* mutations were detected in their metastatic brain tumors. In 2 cases (cases 2 and 3), for which both the first and second metastatic brain tumors were available for the analysis, *EGFR* mutations were detected in both the metastatic tumors, and the type of mutation in the second metastatic tumor was the same as that in the first one in both cases. In 8 cases, for which primary lung tumors were also available for the analysis, 6 cases had *EGFR* mutations in their primary tumors. All metastatic tumors from the 6 cases with *EGFR* mutations in the primary tumors had the same types of mutations as those in the primary tumors, respectively. Figure 1 shows representative sequence chromatograms of case 1, for which cancer cells of primary and metastatic tumors were collected by microdissection. There was no case that *EGFR* mutations were not detected in primary tumors and were detected in the corresponding metastatic brain tumors. Additionally, there was also no case that *EGFR* mutations were detected only in primary tumors and were not detected in the corresponding metastatic brain tumors. Thus, it was shown that *EGFR* mutations in primary adenocarcinomas are retained in their metastatic brain tumors.

The first two authors contributed equally to this work.

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***Correspondence to:** Biology Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Fax: +81-3-3542-0807. E-mail: jyokota@gan2.ncc.go.jp

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TABLE I – *EGFR* AND *KRAS* MUTATIONS IN LUNG ADENOCARCINOMA WITH BRAIN METASTASIS

Case		Clinical characteristics					Mutation		
No.	Tumor	Age	Gender	Smoking	Stage[1]	Interval time (months)[2]	Gene	Exon	Amino acid change
1	P, M	48	F	–	IIIA	26	*EGFR*	19	E746-A750 del
2	P, M1, M2	43	F	–	IIA	M1, 18; M2, 33	*EGFR*	19	E746-E749 del, AT750-751VA
3	P, M1, M2	59	M	+	IIB	M1, 40; M2, 64	*EGFR*	19	L747-T751 del
4	P, M	49	F	–	IIIB	0.5	*EGFR*	19	E746-A750 del
5	P, M	54	M	+	IIIB	6	*EGFR*	19	E746-A750 del
6	P, M	70	M	+	IIIA	14	*EGFR*	21	L858R
7	P, M	64	M	+	IB	16			
8	P, M	58	M	+	IIIA	22			
9	M	56	M	+			*EGFR*	19	E746-A750 del
10	M	51	F	–			*EGFR*	19	E746-A750 del
11	M	56	F	–			*EGFR*	19	E746-A750 del
12	M	49	M	+			*EGFR*	19	E746-A750 del, T751A
13	M	59	M	+			*EGFR*	19	E746-T751del, SP752-753IS
14	M	48	F	–			*EGFR*	21	L858R
15	M	53	M	+			*KRAS*	1	G12C
16	M	59	M	+			*KRAS*	1	G12C
17	M	62	M	+					
18	M	74	M	+					
19	M	67	M	+					

P, primary tumor; M, metastatic brain tumor; M1, first metastatic brain tumor; M2, second metastatic brain tumor.
[1]Pathological stage according to the TNM classification at the time of lung surgery for the primary tumor.–[2]Interval time from lung surgery to brain surgery.

Types of *EGFR* mutations detected in the present study were 10 in-frame deletions (83%) in exon 19 and 2 point mutations (17%) in exon 21. The most frequent mutation was a simple deletion of 5 amino acid residues from codon 746 to 750 (6/12, 50%). Both of the 2 point mutations were the leucine to arginine mutation at codon 858 (L858R). These 2 types of mutation are known to be the most common ones in lung cancer, especially in lung adenocarcioma. The remaining types of *EGFR* mutations detected were a simple deletion of 5 amino acid residues from codon 747 to 751 and 3 deletions coupled with 1 or 2 amino acid substitutions. No mutation was detected in exons 18 and 20 in the present study, although several point mutations and in-frame insertions have been identified in those exons in primary lung adenocarcinomas in previous studies.[4,7]

KRAS mutations were detected in 2 of 7 metastatic brain tumors without *EGFR* mutations. Both of the 2 mutations were the glycine to cysteine mutation at codon 12 (G12C). We also analyzed the association of *EGFR* mutations with clinicopathological characteristics, such as age, gender and smoking history (Table I). All female patients, who were never-smokers, had *EGFR* mutations in their tumors and the mutations were significantly more frequent in female patients (6/6, 100%) than in male patients (6/13, 46%) (Fisher's exact test, $p = 0.0436$). Therefore, the mutual exclusiveness of *EGFR* and *KRAS* mutations as well as frequent mutations in female nonsmokers was consistent with previous findings.[4,6–8,10]

We demonstrated here that *EGFR* mutations were frequently present in metastatic brain tumors of lung adenocarcinoma. In previous studies for *EGFR* mutations in various stages of lung adenocarcinomas in East Asia, the frequency of the mutations were 20–55%.[4,5,8,15] The higher incidence of *EGFR* mutations in our study raises a possibility that the mutations may be associated with metastasis of lung adenocarcinoma. In recent studies, it was suggested that *EGFR* mutations occur early in the development of lung adenocarcinoma.[11,16–18] Yoshida *et al.*[17] showed that *EGFR* mutations were present in 3% of atypical adenomatous hyperplasia (AAH), which is considered to be a precursor lesion of lung adenocarcinoma, and the presence of the mutations was increasingly frequent during sequential progression from AAH to invasive adenocarcinoma through bronchioloalveolar carcinoma (BAC). Our previous study also demonstrated that a majority of BACs had *EGFR* mutations.[11] Moreover, Tang *et al.*[18] reported that *EGFR* mutations identical to those in tumors were present in the histologically normal respiratory epithelium in 9 of 21 patients with lung adenocarcinoma carrying *EGFR* mutations in the tumors. In the present study, we also showed that all 6 cases with *EGFR* mutations in their metastatic brain tumors had the identical mutations in the corresponding primary tumors. Thus, *EGFR* mutations are likely to be an early genetic alteration in multistage carcinogenic processes of lung adenocarcinoma, and additional genetic alterations responsible for brain metastasis may occur in cancer cells with *EGFR* mutations. Indeed, our previous study, using the same samples as those from 16 of the 19 cases, showed the sequential accumulation of allelic losses during tumor progression. In particular, in case 6, which has *EGFR* mutations in both the primary and metastatic tumors, loss of heterozygosity (LOH) on chromosomes 2q, 13q and 18q was shown to accumulate during tumor progression.[19] In the present study, we showed that *EGFR* mutations, which had been present in the primary tumors at stages IIB-IIIB, were all retained in their brain metastases. Although *EGFR* mutations may be associated with the genesis and/or early progression of lung adenocarcinoma, our results showing the retention and frequent presence of the mutations in metastatic brain tumors indicate that lung adenocarcinomas with *EGFR* mutations may also have a higher potential of metastasizing to the brain. For this reason, it should be noted that *EGFR* gene alterations occur frequently in gliomas, a common brain tumor.[20,21] However, since there is no information on the prevalence of *EGFR* mutations in metastatic lung adenocarcinomas to sites other than the brain at present, further studies will be needed to clarify the association of *EGFR* mutations with metastatic sites of lung adenocarcinoma.

The brain is one of the most frequent metastatic sites of lung adenocarcinoma. Since traditional chemotherapy is not so effective against metastatic brain tumors of lung adenocarcinoma, radiotherapy is, to date, the main treatment for patients with them. Nevertheless, the prognosis of those patients is poor and median survival is only 3–6 months.[22] Interestingly, recent reports demonstrated that metastatic brain tumors of lung adenocarcinoma frequently and drastically responded to gefitinib, an EGFR TK inhibitor,[23–25] although *EGFR* mutations were not examined in these cases. Those reports could have an impact on treatment for metastatic brain tumors of lung adenocarcinoma; however, the role of gefitinib in therapeutic strategies against metastatic lung adenocarcinoma has not yet been established. This is the first report, to our knowledge, to analyze a considerable number of metastatic brain tumors of lung adenocarcinoma for *EGFR* mutations and to demonstrate the frequent presence of them. These results will provide us with a rationale for the use of this drug for treatment against metastatic brain tumors of lung adenocarcinoma.

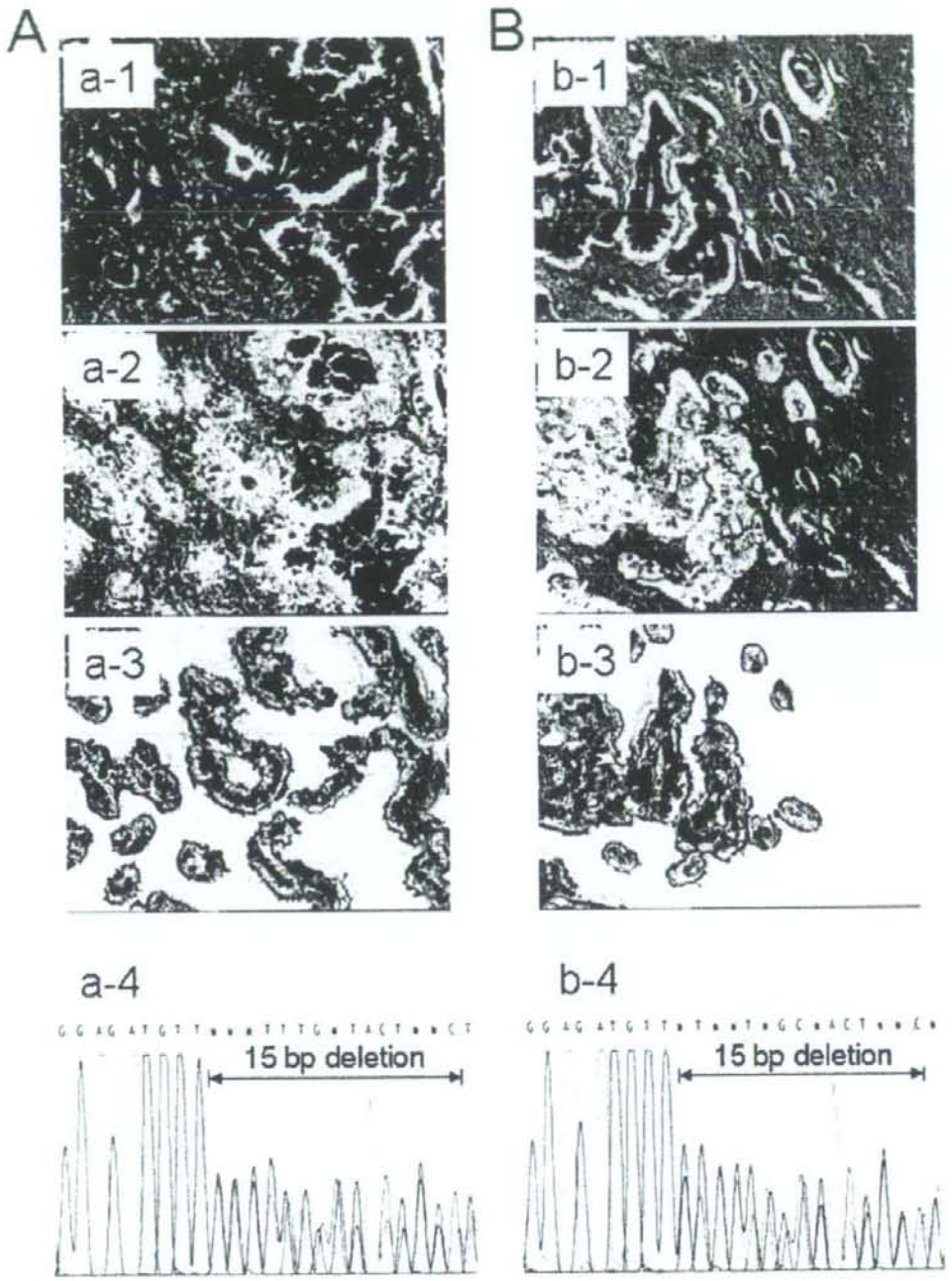

FIGURE 1 – Representative photographs of laser capture microdissection and sequence data obtained from a primary lung adenocarcinoma (*a*) and a corresponding metastatic brain tumor (*b*) in the same patient (case 1 in Table I; magnification of photographs, ×100). (*a-1*) and (*b-1*) shows the tumors in hematoxylin-stained tissue sections before microdissection. (*a-2*) and (*b-2*) shows the same sections after microdissection. (*a-3*) and (*b-3*) shows the cells captured on the transfer films. The sequence chromatogram from the primary tumor is shown in (*a-4*), and that from the metastatic brain tumor is shown in (*b-4*). Both show sequence chromatograms in exon 19 using antisense sequencing primer. A heterozygous in-frame 15 bp deletion was detected from both the samples, demonstrating a deletion of 5 amino acid residues from codon 746 to 750.

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