Table IV. (Continued)

Accession No.	Gene Name	Location	Function Summary	Fold Change*
NM_005766	FERM, RhoGEF (ARHGEF), and pleckstrin domain protein 1 (chondrocyte-derived) (FARP1)	13q32.2	The resulting protein contains a predicted ezrin-like domain, a Dbl homology domain, and a pleckstrin homology domain; it is believed to be a member of the band 4.1 superfamily whose members link the cytoskeleton to the cell membrane	1.46
NM_003938	δ-Adaptin, partial CDS	19p13.3	The AP3D1 subunit is implicated in intracellular biogenesis and trafficking of pigment granules and possibly platelet dense granules and neurotransmitter vesicles	1.46
NM_002354	Tumor-associated calcium signal transducer 1 (*TACSTD1*)	2p21	Member of the GA733 family	1.45
L48689	Voltage-dependent Na^+ channel β-1 subunit; β 1	Unknown	Unknown	1.44
NM_032233	Chromosome 14 open reading frame 154 (C14orf154)	14q32.3	Unknown	1.44
NM_000939	Proopiomelanocortin (adrenocorticotropin/ β-lipotropin/α-melanocyte stimulating hormone/ β-melanocyte stimulating hormone/ β-endorphin (POMC)	2p23.3	Unknown	1.44
NM_014989	Regulating synaptic membrane exocytosis 1 (*RIMS1*)	6q12-q13	The RIM family of active zone proteins likely functions as protein scaffolds that help regulate vesicle exocytosis during short-term plasticity	1.43
AF283787	Immunoglobulin heavy chain variable region	Unknown	Unknown	1.42
NM_002503	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, β (*NFKBIB*)	19q13.1	I κ B-β; may be T3-dependent transcriptional activator	1.42
NM_017771	PX domain containing serine/ threonine kinase (*PXK*)	3p21.2	May be a signaling protein that interacts with SH3 domains; contains a PX (Phox) domain and a Wiskott-Aldrich syndrome homology region	1.42

*The fold change is the difference in gene expression between women and men.

males and females be clarified. To this end, we demonstrated the feasibility of using expression profiles to differentiate sexually distinctive genes in stage I lung adenocarcinoma. We examined only stage I lung adenocarcinoma because these tumors had similar characteristics.

A previous study reported that the prognosis was better for female than for male lung cancer[1]; especially women with early-stage lung cancer had longer survival rates after surgical resection compared with males.[25–28] Clinically, male and female differences have not been distinguished therapeutically, and sex-specific differentiation has not been considered in World Health Organization tumor staging. We believe ours is the first report

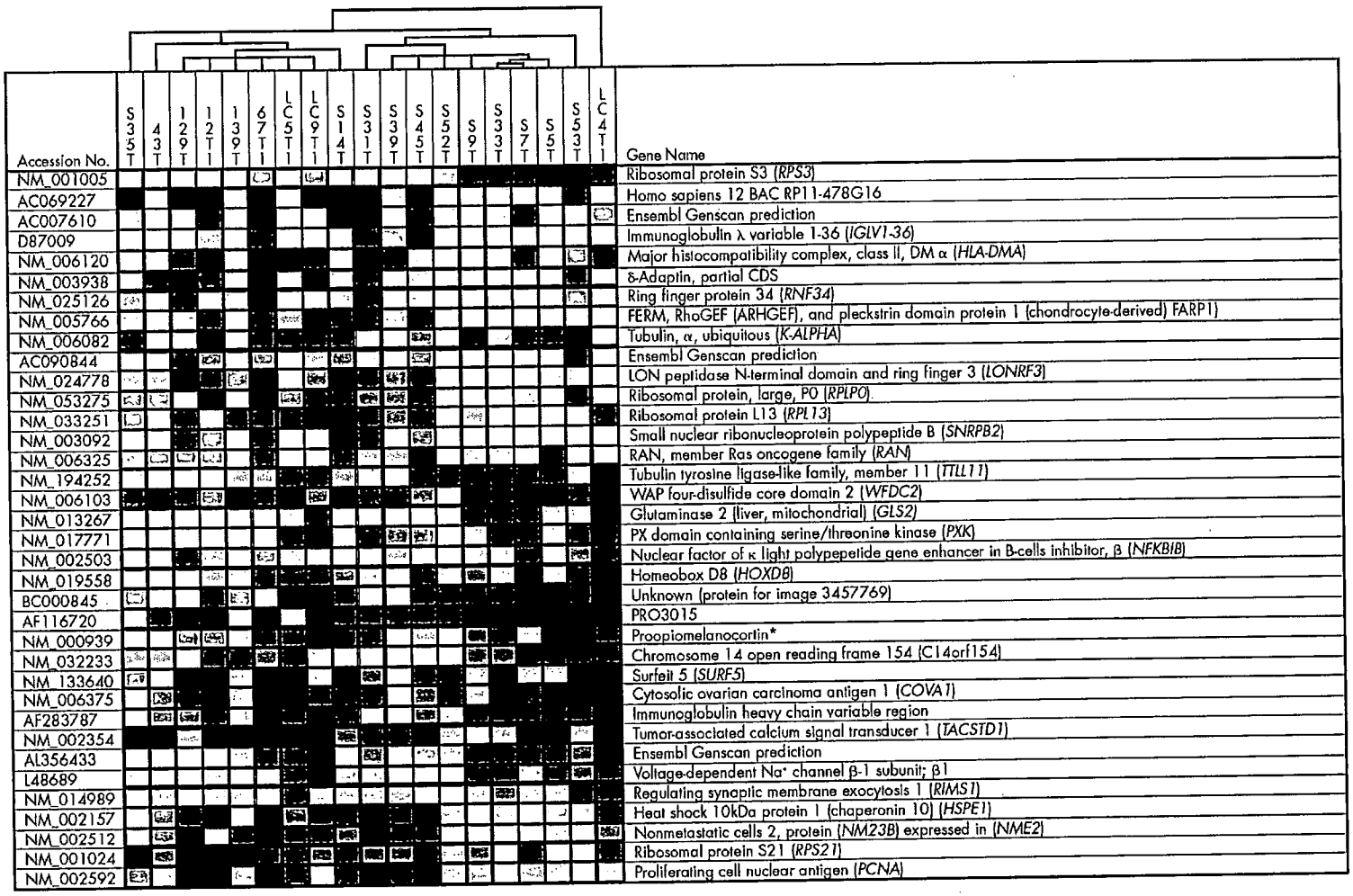

Figure 1. Expression portrait of 36 genes with significantly altered expression in female lung adenocarcinoma, according to microarray analysis. Patients were divided into 2 groups, men (n = 13) and women (n = 6). In the 19 patients examined, 24 genes were overexpressed and 12 were underexpressed. The signal indicator reflects the expression intensity of each gene in these samples. The darkest red indicates expression in patients that is 3 times as strong as that in normal lung tissue; the darkest green indicates a level of expression one third that of normal lung tissue. *Adrenocorticotropin/β-lipotropin/α-melanocyte stimulating hormone/β-melanocyte stimulating hormone/β-endorphin.

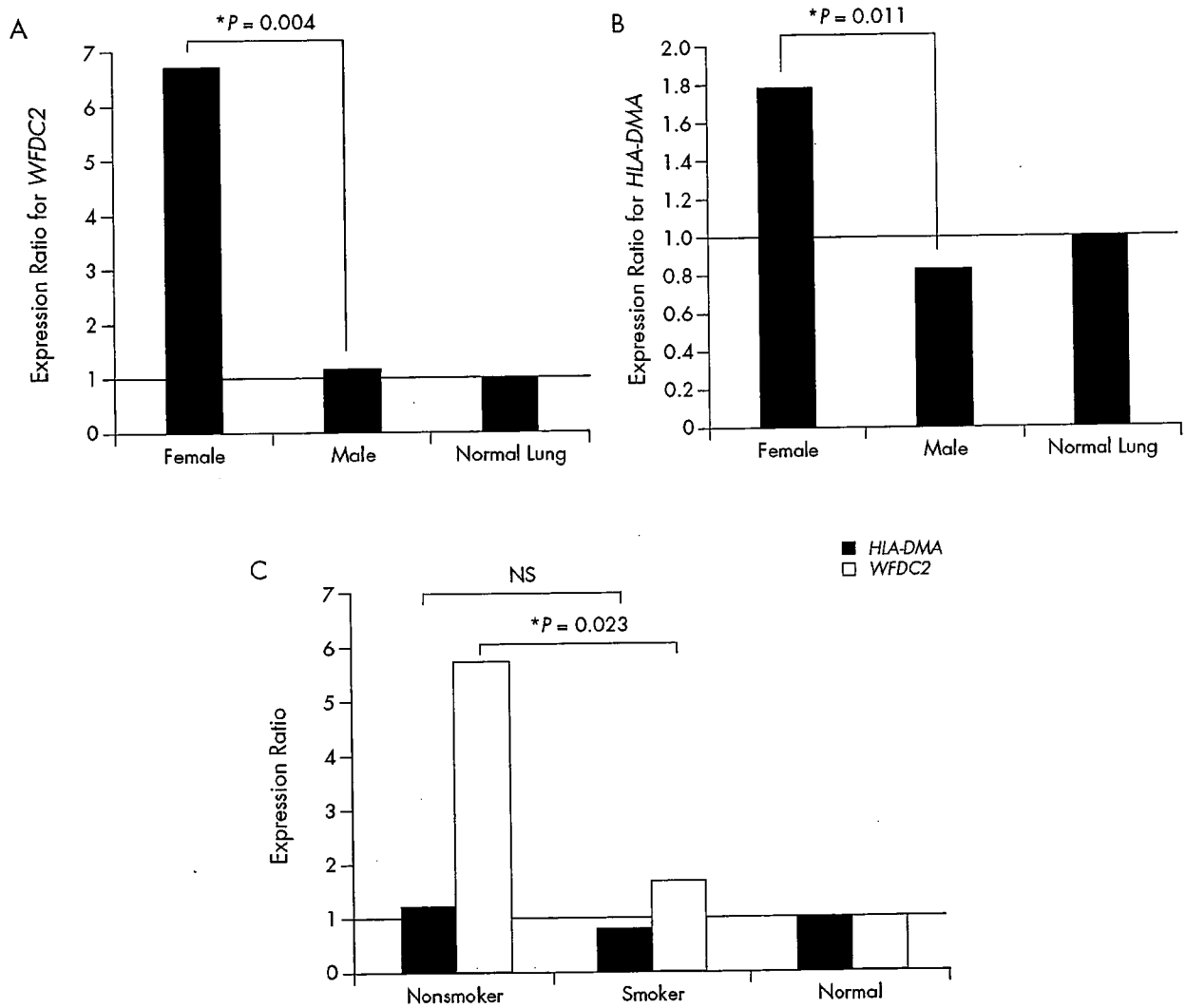

Figure 2. Real-time quantitative polymerase chain reaction analysis comparing women and men with lung adenocarcinoma. (A) WAP four-disulfide core domain 2 (*WFDC2*) expression in female adenocarcinoma was significantly higher than that in male adenocarcinoma ($P = 0.004$). (B) Major histocompatibility complex, class II, DM α (*HLA-DMA*) expression in female adenocarcinoma was significantly higher than that in male adenocarcinoma ($P = 0.011$). (C) Expression of *WFDC2* in nonsmokers was significantly higher than that in smokers ($P = 0.023$), but *HLA-DMA* expression was not significant in either group. NS = not significant. *Determined by *t* test.

of differences in gene expression in lung adenocarcinoma.

It has been demonstrated that in lung cancer, there is a loss of heterozygosity (LOH) in 1p, 2q, 3p, 5q, 8p, 9p, 9q, 11p, 13q, 17p, 18q, or 22q,[29] and that there might be oncogenes, where chromosomal amplifications were observed, in 1p, 2p, 8q, 12p, 17q, or 18q.[30] In this study, we selected 36 genes that altered expression by sex; many of these genes are located in chromosomal regions where genetic alterations were observed.

In underexpressed genes in females, heat shock 10 kDa protein 1 (*HSPE1*), whose function remains unclear, is located at 2q33.1,[31]

where LOH was reported for squamous cell carcinoma of the lung, although the frequency of LOH was 12.9% in the progression of squamous cell lung cancer.[32] In our study, we did not examine LOH status, but judging from our results, the genes that were underexpressed and located on the region reported to have LOH might have tumor suppressive functions, especially in female lung adenocarcinoma. However, overexpression of *HSPE1* was observed in carcinogenesis of the large bowel and uterine exocervix.[33] Expression level was relative in our study; in other words, underexpressed genes in females were overexpressed in males, whether the level reached statistical significance or not. Considering these phenomena, *HSPE1* might contribute differently to carcinogenesis in male and female lung adenocarcinoma.

WFDC2 is located at 20q12-q13.2.[34] It has been reported that the *WFDC2* gene is amplified in ovarian carcinomas and that *WFDC2* protein is a biomarker for ovarian carcinoma.[35] DNA amplification at chromosomal region 20q12-q13 was found to be common in breast cancer.[36] In ovarian tumors, 20q12-q13 amplification was associated with poor survival and more aggressive tumor pathology than those tumors with a normal 20q copy number.[36,37]

HLA-DMA is located at 6p21.3.[38,39] Chatterjee et al[40] believe there might be a suppressor gene on 6p21.3 in cervical carcinoma. LOH has been frequently found at 6P21.3 in lung cancer: 53% in non–small cell lung cancer, 36% in small cell lung cancer.[41]

In our study, *WFDC2* and *HLA-DMA* expression also was verified using Q-PCR. These genes were overexpressed in female patients compared with male patients, similar to our findings using microarray analysis. It has been suggested that *WFDC2* might be a particular oncogene for several female adenocarcinomas,[35,36] although there has already been speculation that a tumor suppressor gene might exist at 6p21.3.[40] Considering these factors, *HLA-DMA* might influence oncogenic systems in female lung adenocarcinomas.

Thus, we studied *WFDC2* and *HLA-DMA* expression in smokers and nonsmokers. Although there was significant overexpression of *WFDC2* in nonsmoking patients, *HLA-DMA* expression was not found to be significant in either group. This suggests that *WFDC2* was related to smokers developing adenocarcinoma and was not associated with sex-specific differences. However, we found the results of *HLA-DMA* analysis to be consistent with those from a previous study by Fu et al,[1] who reported *HLA-DMA* might be associated with sex-specific differences in the development of lung adenocarcinomas. It is necessary to carefully interpret the array results when discussing these types of studies.

PCNA is a 36-kDa molecular-weight protein acting as a subunit of DNA polymerase delta, and is therefore associated with DNA replication.[42] Increases in PCNA expression have been reported to lead to the disruption of growth control and may lead to malignant transformation,[43] and lung cancer with high expression of PCNA has been reported to result in worse outcomes.[42] Interestingly, PCNA was underexpressed in female adenocarcinoma compared with male cancer in this study. Our results suggest that these differences in "key point genes" might characterize male and female lung adenocarcinomas. Clustering analysis of significant genes also revealed 2 distinct molecular portraits between male and female cancer.

CONCLUSION

Our results suggest a difference in gene expression profiles in male and female lung adenocarcinoma. These findings may aid in the exploration of the epidemiologic differences between female and male cancer. Although our study was a small preliminary investigation, we hope it will provide useful information for the development of novel treatments of lung adenocarcinoma that consider sex-specific differences.

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REFERENCES

1. Fu JB, Kau TY, Severson RK, Kalemkerian GP. Lung cancer in women: Analysis of the national Surveillance, Epidemiology, and End Results database. *Chest.* 2005;127:768–777.
2. Sekine I, Nishiwaki Y, Yokose T, et al. Young lung cancer patients in Japan: Different characteristics between the sexes. *Ann Thorac Surg.* 1999;67: 1451–1455.
3. Santos-Martinez MJ, Curull V, Balanco ML, et al. Lung cancer at a university hospital: Epidemiological and histological characteristics of a recent and a historical series [in Spanish]. *Arch Bronconeumol.* 2005;41:307–312.
4. Patel JD, Bach PB, Kris MG. Lung cancer in US women: A contemporary epidemic. *JAMA.* 2004; 291:1763–1768.
5. Chen KY, Chang CH, Yu CJ, et al. Distribution according to histologic type and outcome by gender and age group in Taiwanese patients with lung carcinoma. *Cancer.* 2005;103:2566–2574.
6. Ranson M, Hammond LA, Ferry D, et al. ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: Results of a phase I trial. *J Clin Oncol.* 2002;20:2240–2250.
7. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [published correction appears in *J Clin Oncol.* 2004;22:4811]. *J Clin Oncol.* 2003;21:2237–2246.
8. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science.* 2004;304:1497–1500.
9. Travis WD, Colby TV, Corrin B, et al. *WHO International Histological Classification of Tumors: Histological Typing of Lung and Pleural Tumors.* 3rd ed. Berlin, Germany: Springer-Verlag; 1999.
10. Onda M, Emi M, Yoshida A, et al. Comprehensive gene expression profiling of anaplastic thyroid cancers with cDNA microarray of 25 344 genes. *Endocr Relat Cancer.* 2004;11:843–854.
11. Onda M, Emi M, Nagai H, et al. Gene expression patterns as marker for 5-year postoperative prognosis of primary breast cancers. *J Cancer Res Clin Oncol.* 2004;130:537–545.
12. Tsai CA, Chen YJ, Chen JJ. Testing for differentially expressed genes with microarray data. *Nucleic Acids Res.* 2003;31:e52.
13. Cleveland WS. Robust locally weighted regression and smoothing scatterplots. *J Amer Stat Assoc.* 1979; 74:829–836.
14. Quackenbush J. Microarray data normalization and transformation. *Nat Genet.* 2002;32(Suppl): 496–501.
15. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A.* 1998; 95:14863–14868.
16. Onda M, Akaishi J, Asaka S, et al. Decreased expression of hemoglobin beta (HBB) gene in anaplastic thyroid cancer and recovery of its expression inhibits cell growth. *Br J Cancer.* 2005; 92:2216–2224.
17. Onda M, Nagai H, Yoshida A, et al. Up-regulation of transcriptional factor E2F1 in papillary and anaplastic thyroid cancers. *J Hum Genet.* 2004;49: 312–318.
18. National Center for Biotechnology Information. GenBank. Available at: http://www.ncbi.nlm.nih.gov/entry.fcgi?db=Nucleotide&cmd=search&term=nucleotide.
19. Sasaki S, Nakamura T, Arakawa H, et al. Isolation and characterization of a novel gene, hRFI, preferentially expressed in esophageal cancer. *Oncogene.* 2002;21:5024–5030.
20. Budde LM, Wu C, Tilman C, et al. Regulation of IkappaBbeta expression in testis. *Mol Biol Cell.* 2002;13:4179–4194.
21. Morre DJ, Sedlak D, Tang X, et al. Surface NADH oxidase of HeLa cells lacks intrinsic membrane binding motifs. *Arch Biochem Biophys.* 2001;392: 251–256.
22. Harari PM, Huang SM. Searching for reliable epidermal growth factor receptor response predictors. *Cancer Res.* 2004;10:428–432.
23. Godfried MB, Veenstra M, van Sluis P, et al. The *N-myc* and *c-myc* downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. *Oncogene.* 2002;21:2097–2101.
24. Mitsudomi T, Kosaka T, Endoh H, et al. Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with post-

operative recurrence. *J Clin Oncol.* 2005;23:2513–2520.
25. Bach PB, Cramer LD, Warren JL, Begg CB. Racial differences in the treatment of early-stage lung cancer. *N Engl J Med.* 1999;341:1198–1205.
26. Alexiou C, Onyeaka CV, Beggs D, et al. Do women live longer following lung resection for carcinoma? *Eur J Cardiothorac Surg.* 2002;21:319–325.
27. Ferguson MK, Karrison T. Does pneumonectomy for lung cancer adversely influence long-term survival? *J Thorac Cardiovasc Surg.* 2000;119:440–448.
28. de Perrot M, Licker M, Bouchardy C, et al. Sex differences in presentation, management, and prognosis of patients with non-small cell lung carcinoma. *J Thorac Cardiovasc Surg.* 2000;119:21–26.
29. Girard L, Zochbauer-Muller S, Virmani AK, et al. Genome-wide allelotyping of lung cancer identifies new regions of allelic loss, differences between small cell lung cancer and non-small cell lung cancer, and loci clustering. *Cancer Res.* 2000; 60:4894–4906.
30. Shimizu S, Mitsudimi T. Molecular abnormalities of lung cancer. *Nippon Rinsho.* 2002;60(Suppl 5): 73–77.
31. Hansen JJ, Bross P, Westergaard M, et al. Genomic structure of the human mitochondrial chaperonin genes: HSP60 and HSP10 are localised head to head on chromosome 2 separated by a bidirectional promoter. *Hum Genet.* 2003;112:71–77.
32. Endo C, Sagawa M, Sato M, et al. Sequential loss of heterozygosity in the progression of squamous cell carcinoma of the lung. *Br J Cancer.* 1998;78: 612–615.
33. Cappello F, Bellafiore M, David S, et al. Ten kilodalton heat shock protein (HSP10) is overexpressed during carcinogenesis of large bowel and uterine exocervix. *Cancer Lett.* 2003;196:35–41.
34. Clauss A, Lilja H, Lundwall Å. A locus on human chromosome 20 contains several genes expressing protease inhibitor domains with homology to whey acidic protein. *Biochem J.* 2002;368:233–242.
35. Hellstrom I, Raycraft J, Hayden-Ledbetter M, et al. The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma. *Cancer Res.* 2003;63:3695–3700.
36. Tanner MM, Grenman S, Koul A, et al. Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. *Clin Cancer Res.* 2000;6:1833–1839.
37. Diebold J, Mosinger K, Peiro G, et al. 20q13 and cyclin D1 in ovarian carcinomas. Analysis by fluorescence in situ hybridization. *J Pathol.* 2000; 190:564–571.
38. Kersemaekers AM, van de Vijver MJ, Kenter GG, Fleuren GJ. Genetic alterations during the progression of squamous cell carcinomas of the uterine cervix. *Genes Chromosomes Cancer.* 1999;26:346–354.
39. Mazurenko N, Attaleb M, Gritsko T, et al. High resolution mapping of chromosome 6 deletions in cervical cancer. *Oncol Rep.* 1999;6:859–863.
40. Chatterjee A, Pulido HA, Koul S, et al. Mapping the sites of putative tumor suppressor genes at 6p25 and 6p21.3 in cervical carcinoma: Occurrence of allelic deletions in precancerous lesions. *Cancer Res.* 2001;61:2119–2123.
41. Virmani AK, Fong KM, Kodagoda D, et al. Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. *Genes Chromosomes Cancer.* 1998;21: 308–319.
42. Caputi M, Esposito V, Groger AM, et al. Prognostic role of proliferating cell nuclear antigen in lung cancer: An immunohistochemical analysis. *In Vivo.* 1998;12:85–88.
43. Fukami-Kobayashi J, Mitsui Y. Overexpression of proliferating cell nuclear antigen in mammalian cells negates growth arrest by serum starvation and cell contact. *Jpn J Cancer Res.* 1999;90:286–293.

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Abnormalities of Epidermal Growth Factor Receptor in Lung Squamous-Cell Carcinomas, Adenosquamous Carcinomas, and Large-Cell Carcinomas

Tyrosine Kinase Domain Mutations Are Not Rare in Tumors With an Adenocarcinoma Component

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BACKGROUND. Tyrosine kinase domain (TKD) gene mutations of the epidermal growth factor receptor gene (*EGFR*) have proven to be clinically significant in nonsmall-cell lung cancer (NSCLC), particularly in adenocarcinoma. However, TKD mutations together with deletion mutations in the extracellular domain of *EGFR* (EGFRvIII) have not been fully investigated in NSCLC except for adenocarcinoma. The present study sought to gain further insight into the significance of *EGFR* mutations in NSCLC by focusing on nonadenocarcinoma NSCLC.

METHODS. *EGFR* TKD mutations were investigated using direct sequencing and mutation-specific polymerase chain reaction (PCR), and EGFRvIII mutations were examined using reverse transcriptase-PCR in samples from 42 NSCLC patients and 6 NSCLC cell lines excluding adenocarcinoma.

RESULTS. *EGFR* TKD mutations were detected in 1 of 7 (14%) squamous-cell carcinomas with an adenocarcinoma component and 2 of 4 (50%) adenosquamous carcinomas. In contrast, *EGFR* TKD mutations were not identified in 24 pure squamous-cell carcinomas without any adenocarcinoma component, 7 large-cell carcinomas, or 6 cell lines. EGFRvIII was detected solely in 1 of 7 large-cell carcinomas (14%), but not in 31 squamous-cell carcinomas, 4 adenosquamous carcinomas, or 6 cell lines.

CONCLUSIONS. These results suggest that *EGFR* TKD mutations are found in NSCLCs with an adenocarcinoma element. Patients with such lesions are thus considered candidates for molecular therapies targeting EGFR. *Cancer* **2007;109:741–50.** © *2007 American Cancer Society.*

KEYWORDS: lung cancer, nonsmall-cell lung cancer (NSCLC), squamous-cell carcinoma, adenosquamous carcinoma, large-cell carcinoma, epidermal growth factor receptor (EGFR), mutation, EGFRvIII, overexpression.

Various mutations within the epidermal growth factor receptor gene (*EGFR*) have recently been found in nonsmall-cell lung cancer (NSCLC).[1–17] These mutations tend to cluster in the tyrosine kinase domain (TKD) of the gene. The potential impact of *EGFR* mutations in NSCLC has attracted substantial attention from researchers and clinicians because the presence of mutations may critically influence the effects of tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, against lung cancer. Numerous studies have suggested that gefitinib is more effective against tumors harboring *EGFR* mutations than against tumors without such

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TABLE 1
Frequency of *EGFR* TKD Mutations in NSCLC

AD		SQ		LA		ADSQ		Others		
MUT	N	MUT	N	MUT	N	MUT	N	MUT	N	Authors
15 (21%)	70	—	—	—	—	—	—	1 (2%)	49	Paez et al.
37 (17%)	213	—	—	—	—	—	—	9 (5%)	178	Bell et al.
114 (39%)	289	—	—	—	—	—	—	6 (3%)	230	Shigematsu et al.
36 (46%)	79	—	—	—	—	—	—	2 (3%)	17	Suzuki et al.
23 (26%)	88	0 (0%)	25	—	—	—	—	—	—	Pao et al.
39 (57%)	69	0 (0%)	24	—	—	—	—	1 (13%)	8	Huang et al.
29 (67%)	43	4 (40%)	10	—	—	—	—	0 (0%)	1	Chou et al.
14 (22%)	65	1 (5%)	21	—	—	—	—	2 (50%)	4	Han et al.
115 (54%)	215	0 (0%)	15	—	—	—	—	1 (9%)	11	Tam et al.
12 (21%)	58	1 (5%)	21	0 (0%)	1	—	—	2 (22%)	9	Cappuzzo et al.
60 (56%)	108	0 (0%)	31	0 (0%)	9	—	—	0 (0%)	6	Sonobe et al.
39 (10%)	375	0 (0%)	454	0 (0%)	31	—	—	—	—	Marchetti et al.
37 (45%)	82	0 (0%)	35	0 (0%)	2	1 (100%)	1	—	—	Tokumo et al.
29 (40%)	72	0 (0%)	45	0 (0%)	2	0 (0%)	1	—	—	Tomizawa et al.
110 (49%)	224	0 (0%)	35	0 (0%)	9	1 (20%)	5	0 (0%)	4	Kosaka et al.
136 (42%)	224	0 (0%)	102	1 (4%)	27	0 (0%)	1	1 (25%)	4	Sugio et al.

AD indicates adenocarcinoma; NSCLC, nonsmall-cell lung cancer; SQ, squamous cell carcinoma; LA, large cell carcinoma; ADSQ, adenosquamous carcinoma; MUT, number of cases with *EGFR* TKD mutations.

TABLE 2
Frequency of EGFRvIII in NSCLC

AD		SQ		LA		ADSQ		Others		
MUT	N	MUT	N	MUT	N	MUT	N	MUT	N	Authors
0 (0%)	10	2 (15%)	13	—	—	2 (100%)	2	1 (14%)	7	Garcia de Palazzo et al.
0 (0%)	26	0 (0%)	32	0 (0%)	7	—	—	—	—	Jungbluth et al.
19 (41%)	46	10 (42%)	24	1 (17%)	6	—	—	—	—	Okamoto et al.
0 (0%)	123	3 (5%)	56	—	—	—	—	—	—	Ji et al.

AD indicates adenocarcinoma; NSCLC, nonsmall-cell lung cancer; SQ, squamous cell carcinoma; LA, large cell carcinoma; ADSQ, adenosquamous carcinoma; MUT, number of cases with EGFRvIII.

mutations, and similar results have been reported for erlotinib, although these hypotheses have yet to be confirmed in randomized controlled studies.[1–3,6–9,11]

These types of mutations are widely recognized as occurring more frequently in adenocarcinomas than in tumors of other histology.[2–17] The frequency of *EGFR* TKD mutations in NSCLC except for adenocarcinoma is reported to be generally low (Table 1). These results lead clinicians to refrain from using gefitinib on patients with nonadenocarcinoma NSCLC. However, a subset of nonadenocarcinoma NSCLCs has been reported as harboring *EGFR* mutations in several studies.[2–5,7–11,14,16,17] These findings prompted us to investigate whether nonadenocarcinoma NSCLCs with *EGFR* mutations display any specific clinical features.

Deletion of exons 2–7 in *EGFR* gene (EGFRvIII), which is often found in glioblastoma,[18] has also been identified in a subset of NSCLC patients, although the frequency of this mutation appears to vary (Table 2).[19–22] EGFRvIII is also a possible target of TKIs, and revealing the presence of EGFRvIII mutation in NSCLC is thus considered clinically relevant.[22] However, EGFRvIII has not been investigated in NSCLC as intensively as *EGFR* TKD mutations. We previously reported that, similar to patients in Western countries, EGFRvIII is very rare in lung adenocarcinoma among Japanese.[23] The present study also investigated EGFRvIII in nonadenocarcinoma NSCLC to elucidate whether EGFRvIII has any contribution to tumorigenesis for this type of lung cancer.

Furthermore, overexpression of epidermal growth factor receptor (EGFR) has also been shown to be related to *EGFR* mutations[23] and susceptibility to TKIs in NSCLC.[11] We therefore investigated EGFR expression in these samples to explore whether this has any impact on nonadenocarcinoma NSCLC.

Overall, this study was intended to reveal whether nonadenocarcinoma NSCLCs contain EGFR abnormalities and can thus be possible targets of molecular therapy. We demonstrate herein that *EGFR* TKD mutations are not rare in NSCLCs with an adenocarcinoma component and EGFRvIII can be found in patients with large-cell carcinoma.

MATERIALS AND METHODS

Patients

A total of 96 consecutive Japanese patients with NSCLC underwent surgery in the Department of Thoracic Surgery at Kyorin University Hospital between May 2001 and March 2003. Postoperative diagnoses were made by trained pathologists. Patients diagnosed with squamous-cell carcinoma, adenosquamous carcinoma, or large-cell carcinoma were enrolled for further analysis. Pathological diagnosis was based on the criteria of the World Health Organization classification system.[24] Briefly, squamous-cell carcinoma comprises tumors with <10% adenocarcinoma component. In particular, squamous-cell carcinoma without a detectable adenocarcinoma component was termed 'pure squamous-cell carcinoma' in this study. Adenosquamous carcinoma comprises tumors with a 10% to 90% adenocarcinoma component. Adenocarcinoma comprises tumors with a >90% adenocarcinoma component, and patients with this diagnosis were excluded from the present study. After surgery, some patients underwent chemotherapy and/or radiotherapy with various regimens, including gefitinib therapy in 5 patients with recurrence. Written informed consent to analyze tissue DNA, RNA, and protein was obtained from each patient before operation. Results of molecular analyses on these patients have not previously been reported.

Patient Data

Clinical data were obtained from in- and outpatient medical records. The following criteria were used to classify smoking status: never smoker, patients who had smoked <100 cigarettes in their lifetime; former smoker, patients who had stopped smoking ≥12 months before diagnosis; and current smoker.

Cell Lines and Clinical Samples

Lung squamous-cell carcinoma cell lines EBC-1, LK2, and Sq-1 and large-cell carcinoma cell lines 86-2 and Lu99 were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Squamous-cell carcinoma cell line RERF-LC-AI was purchased from Riken Bioresource Center (Tsukuba, Japan). Lung adenocarcinoma cell lines NCI-H1650 and H1975, which have been reported to show delE746-A750 and L858R, respectively, were purchased from the American Type Culture Collection (Manassas, VA) and used as positive controls for *EGFR* TKD mutations.[25] The glioblastoma cell line U87MGΔEGFRSH, which has been reported to show EGFRvIII, was kindly donated by Professor Webster K. Cavenee (Ludwig Institute for Cancer Research, San Diego) and was used as a positive control for EGFRvIII mutation.[26] Tumor samples and visually normal lung tissues distant from the tumor were immediately frozen after resection and preserved at −80°C. Visually normal lung tissues were confirmed as containing no tumor component on pathological examination. DNA, RNA, and protein were extracted from these samples and cell lines according to methods previously described.[23]

Direct Sequencing of *EGFR* TKD

Mutations of *EGFR* in lung cancer cluster within exons 18–21, a main portion of TKD.[1,2] Direct sequencing analysis of *EGFR* from exons 18–21 using genomic DNA was therefore performed, as previously reported,[23] for all NSCLC samples, including the 8 NSCLC cell lines.

EGFR TKD Mutation-Specific PCR

Lung cancer samples are frequently intermingled with large amounts of normal tissue. In addition, several NSCLC cell lines tested herein reportedly harbor an L858R mutation in only a minor population of cells (1%–10%). The sensitivity of sequencing analysis, which usually needs >30% mutant DNA, was thus insufficient to detect mutations in such contaminated tumor samples. A mutation-specific polymerase chain reaction (PCR) method that we previously developed was thus used to detect the major *EGFR* TKD mutations with high sensitivity. This method can simultaneously detect delE746-A750 and L858R, which together account for approximately 70% of *EGFR* TKD mutations, with a low percentage of mutant DNA (2.5% for delE746-A750 and 0.25% for L858R) being detectable. The precise method has been described elsewhere.[27] In brief, PCR was performed with 100 ng of extracted DNA and primers specific for delE746-A750 and L858R were added together with the PCR mix. PCR conditions were as follows: 95°C for 5 minutes; then 45 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds; followed by a final 10 minutes at 72°C. Consequently, a 153-bp band and a 104-bp band are detected in samples containing delE746-A750 and L858R, respectively. For the detection of delT751-K758, we set up PCR amplifying the area of DNA including this deletion. This PCR can

clearly discriminate a short form of DNA with this deletion (241 bp) from germline DNA (265 bp).

Analysis of EGFRvIII

EGFRvIII is generated by total deletion of exons 2–7 (801 bp) in the extracellular domain of the *EGFR* gene. Reverse transcriptase PCR (RT-PCR) is reportedly useful in detecting this deletion in glioblastoma,[18] and was thus used in this study. For detecting the 801-bp deletion, tumor cDNA and primers were subjected to 40 cycles of PCR amplification. PCR primers used were as follows: sense, 5′-GTA TTG ATC GGG AGA GCC G-3′; antisense, 5′-GTG GAG ATC GCC ACT GAT G-3′.[23] In addition, direct sequencing was used to confirm the presence of EGFRvIII in samples showing positive results according to RT-PCR. The sense primer for RT-PCR was also used as a primer for sequencing analysis.

Western Blotting

Western blotting was used to analyze expression of EGFR protein rather than immunohistochemistry, as the results of the latter method are considered less quantitative and reproducible than the former.[28] For EGFR Western blotting analysis, 100 μg of tumor or normal lung protein was used. Protein samples were subjected to Western blotting analyses using anti-EGFR polyclonal antibody (Santa Cruz Biologicals, Santa Cruz, CA) according to the manufacturer's instructions. The level of expression in each tumor was determined as follows: (0), very weak or no EGFR band (170 kD), similar to normal lung; (1), easily visible band; and (2), very strong band similar to levels of β-actin. An EGFR expression level of 1 or 2 was defined as indicating EGFR overexpression.[23]

Microdissection

To elucidate which component includes *EGFR* TKD mutations in tumors containing both adenocarcinoma and squamous-cell carcinoma components, separate mutational analysis of each component was performed using microdissection techniques. For adenosquamous carcinomas and squamous-cell carcinomas with an adenocarcinoma component, a trained expert pathologist performed laser capture microdissection using LM200 (Arcturus, Mountain View, CA) and manual microdissection of both squamous-cell carcinoma and adenocarcinoma component on 8 μm-thick hematoxylin and eosin (H&E)-stained, formalin-fixed, paraffin-embedded histology sections. DNA was extracted from each microdissected tissue by incubation with proteinase K (200 μg/mL) for 24 hours at 37°C, and *EGFR* TKD mutation-specific PCR was performed using DNA extracted from both squamous-cell carcinoma and adenocarcinoma components.

Statistical Analysis

The significance of differences in categorical data was tested using the χ^2 test or Fisher exact test.

RESULTS

Patient Characteristics

Postoperative pathological examinations revealed 42 nonadenocarcinoma NSCLCs (31 squamous-cell carcinomas, 7 large-cell carcinomas, and 4 adenosquamous carcinomas) among 96 NSCLC tumors. The 31 squamous-cell carcinomas comprised 24 pure squamous-cell carcinomas (77%) and 7 squamous-cell carcinomas with an adenocarcinoma component (23%). Patient characteristics in the present study are summarized in Table 3. Patients comprised 39 men (93%) and 3 women (7%) with a median age of 73 years (range, 40–84 years), and 30 current smokers (71%), 8 former smokers (19%), and 4 never smokers (10%).

EGFR Mutations in Cell Lines

Sequencing analysis revealed delE746-A750 mutation in H1650 and L858R mutation in H1975, as previously reported.[23] In contrast, no squamous-cell carcinoma cell lines or large-cell carcinoma cell lines displayed *EGFR* TKD mutations even according to mutation-specific PCR (data not shown). The absence of L858R mutations in squamous-cell carcinomas tested in the present study (LK2, EBC-1, Sq-1, and RERF-LC-AI) was inconsistent with the previous study demonstrating that these lines harbor the L858R mutation in various proportions of cells (LK2, 1%; EBC-1 and Sq-1, 10%; RERF-LC-AI, 100%).[29] RT-PCR and direct sequencing revealed the EGFRvIII mutation only in the U87MGΔEGFRSH glioblastoma cell line, but not in any lung cancer cell lines (Fig. 1).

EGFR TKD Mutations in Patient Samples

EGFR mutations in TKD found in patient samples in this study are summarized in Table 3. Mutations within exons 18–21 were found in 1 of 31 squamous-cell carcinomas (3%; including 7 squamous-cell carcinomas with an adenocarcinoma component), and in 2 of 4 adenosquamous carcinomas by direct sequencing. No cryptic mutations were detected by mutation-specific PCR in tumor samples negative for mutations by direct sequencing. Case 33 had both delT751-K758 and I759N mutations, although the biological significance of the latter mutation is

TABLE 3
Characteristics and EGFR Abnormalities of Patients

Case	Age	Sex	Smoking	Histology	Differentiation	Adenocarcinoma component	*EGFR* mutation	EGFR expression	Postoperative recurrence	Gefitinib response	Prognosis
1	59	M	C60	SQ	mod			2			A
2	70	M	C76.5	SQ	well			1			A
3	78	M	F69	SQ	mod			2	yes		U
4	80	M	C120	SQ	mod			1	yes		U
5	55	M	C30	SQ	well			0	yes		D
6	61	F	C30	SQ	mod			0	yes		D
7	61	M	C50	SQ	mod			1			A
8	52	M	C100	SQ	mod			2			U
9	75	M	C100	SQ				2	yes		D
10	74	M	C40	SQ	poor			2	yes	PD	D
11	79	M	C90	SQ				2			A
12	69	M	N	SQ	well			0			D
13	69	M	C80	SQ				1			D
14	68	M	F15	SQ	mod			1			A
15	74	M	C55	SQ	mod			2			A
16	70	M	C75	SQ	mod			1	yes	PD	D
17	75	M	C50	SQ	mod			2			A
18	74	M	C50	SQ	mod			2			U
19	77	M	F159	SQ	poor			2	yes		D
20	69	M	C40	SQ	mod			2	yes		D
21	72	M	F30	SQ	poor			1	yes		A
22	74	M	C90	SQ	poor			2			A
23	63	M	C37.8	SQ	poor			1			A
24	81	M	C25.2	SQ	mod			0			A
25	67	M	F108	SQ	poor	yes	L858R	2	yes	NE	D
26	73	M	C50	SQ	poor	yes		1	yes		U
27	54	M	C30	SQ	mod	yes		2			A
28	66	M	C62.5	SQ	mod	yes		2			A
29	74	M	F77.5	SQ	well	yes		1	yes		D
30	73	M	N	SQ		yes		0	yes		A
31	84	M	C47.3	SQ	poor	yes		0	yes		D
32	79	M	F50	ADSQ	well	yes	del 1	1	yes		U
33	74	F	N	ADSQ	well	yes	del 6 and I759N	0			A
34	77	M	C	ADSQ	poor	yes		2			U
35	73	M	C100	ADSQ		yes		2	yes		D
36	44	M	C43.5	LA			EGFRvIII	2			D
37	83	F	F20.5	LA				2	yes		D
38	82	F	N	LA				2			A
39	40	M	C22	LA				1	yes	NE	U
40	54	M	C52.5	LA				1	yes	PD	A
41	47	M	C50.5	LA				2			U
42	61	M	C30	LA				2	yes		D

M indicates male; F, female; C, current smoker; F, former smoker; N, never smoker; Number, pack-year smoking; SQ, squamous cell carcinoma; ADSQ, adenosquamous carcinoma; LA, large cell carcinoma; well, well differentiated; mod, moderately differentiated; poor, poorly differentiated; L858R, nt. 2819T>G; del 1, del E746-A750, del nt. 2481–2495; del 6, del T751-K758,del nt. 2496–2519; I759N, nt. 2522A>T; EGFRvIII, deletion of exons 2–7; PD, progressive disease; NE, not evaluable; A, alive; D, dead; U, unknown.

considered unclear. Interestingly, the sole case of squamous-cell carcinoma with TKD mutation displayed an adenocarcinoma component. These findings indicate that all mutated cases were tumors with an adenocarcinoma component, and none of the 24 'pure squamous-cell carcinoma,' cases showed TKD mutation (Table 3). When the squamous-cell carcinoma with an adenocarcinoma component and adenosquamous carcinoma were combined, 3 of 11 such cases (27%) were shown to have TKD mutations. The difference in frequency of TKD mutations between tumors with or without an adenocarcinoma component was significant ($P = .007$). No large-cell carcinomas contained an adenocarcinoma component or displayed TKD mutations. In addition, no significant correlations between

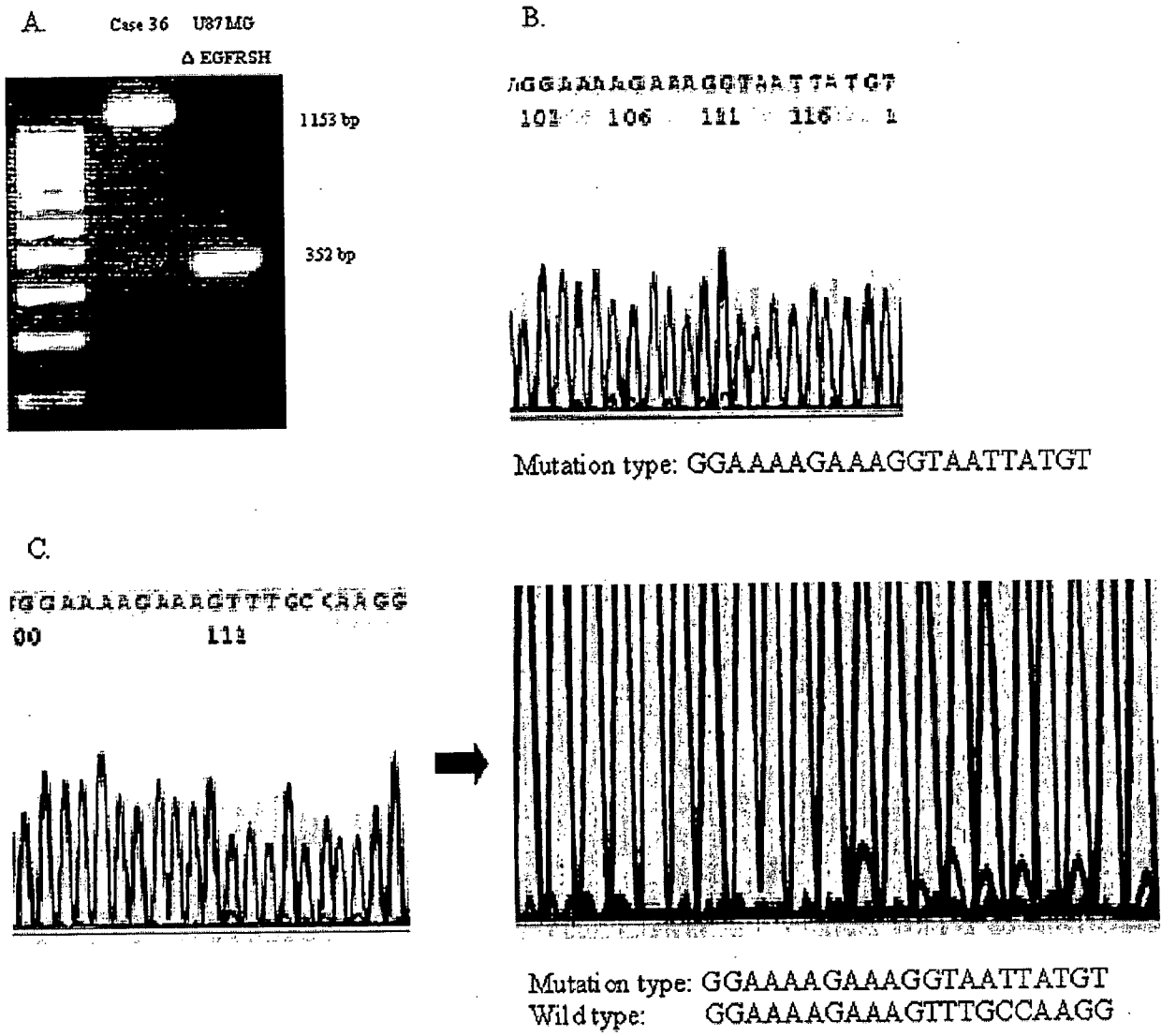

FIGURE 1. EGFRvIII mutation. (A) Results of reverse-transcriptase polymerase chain reaction (RT-PCR) for detecting EGFRvIII. The U87MGΔEGFRSH glioblastoma cell line showed a short 352-bp band, indicating the presence of EGFRvIII. One large cell carcinoma sample (Case 36) showed a weak 352-bp band together with wildtype allele. (B) Direct sequencing (sense direction) of EGFRvIII in U87MGΔEGFRSH. Mutant allele is dominant. (C) Direct sequencing (sense direction) of EGFRvIII in Case 36. Low signals for mutant DNA are visible.

EGFR TKD mutations and clinical features such as patient age ($P = .256$), gender ($P = .145$), and smoking status ($P = .145$) were identified.

Microdissection

Microdissection analysis was performed on 3 samples (2 adenosquamous carcinomas and 1 squamous-cell carcinoma with an adenocarcinoma component) with *EGFR* TKD mutations. By performing mutation-specific PCR separately for both squamous and adenocarcinoma components, mutations were detected in both components in all 3 cases (Fig. 2).

EGFRvIII in Patient Samples

RT-PCR of the *EGFR* extracellular domain in 42 samples revealed that only 1 case of large-cell carcinoma displayed a short 352-bp band indicating EGFRvIII mutation (Table 3). Direct sequencing revealed that this case included a minor population of EGFRvIII

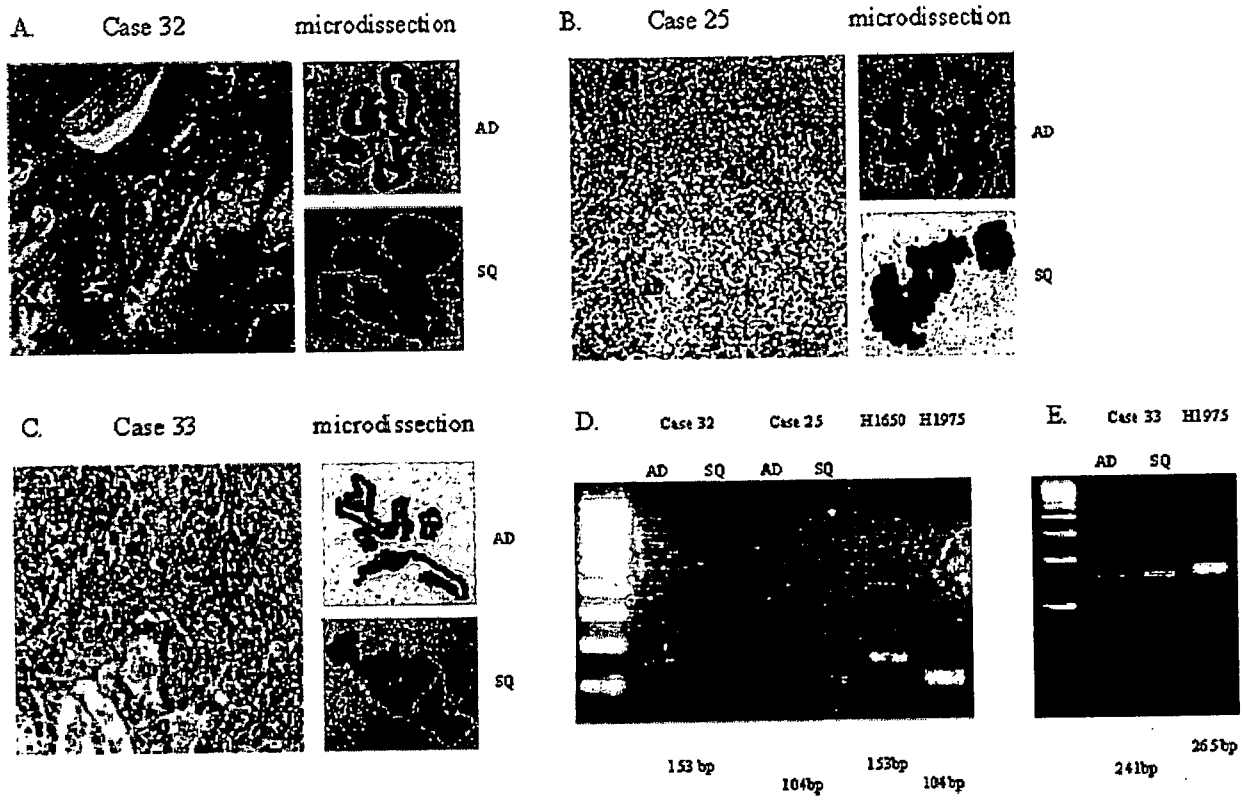

FIGURE 2. Results of microdissection and mutation-specific polymerase chain reaction (PCR). (A-C) Histological presentation of microdissection in 3 cases with *EGFR* mutations. Left: Pictures representing both squamous-cell carcinoma and adenocarcinoma components. SQ: squamous-cell carcinoma component; AD: adenocarcinoma component. Right: Upper and lower pictures show microdissected adenocarcinoma and squamous-cell carcinoma components, respectively. (D) Mutation-specific PCR for delE746-A750 and L858R in microdissected tissues. A 153-bp band indicating delE746-A750 is present in both squamous-cell carcinoma and adenocarcinoma components in Case 32. A 104-bp band indicating L858R is present in both components in Case 25. H1650: Positive control for delE746-A750; H1975: Positive control for L858R. (E) PCR detection of delT751-K758 in Case 33. A 241-bp band indicating delT751-K758 is present in both squamous and adenocarcinoma components in this case. H1975: Negative control for delT751-K758 showing a wildtype 265-bp band.

(Fig. 1B,C). This case also showed a normal 1153-bp band in addition to a 352-bp band.

EGFR Expression in Patient Samples

EGFR overexpression was present in 25 of the 31 squamous-cell carcinomas (81%), 3 of 4 adenosquamous carcinomas (75%), and 7 of 7 large-cell carcinomas (100%) (Fig. 3, Table 3). *EGFR* TKD mutations were identified in 3 of the 35 samples with EGFR overexpression (9%) and 1 of 7 samples without overexpression (14%). No significant correlation was noted between EGFR overexpression and mutation ($P = .421$).

DISCUSSION

This study analyzed *EGFR* gene mutations in detail in nonadenocarcinoma NSCLCs. Most strikingly, *EGFR* TKD mutations were found exclusively in tumors with an adenocarcinoma component, including adenosquamous carcinomas. Although many studies have reported that *EGFR* TKD mutations are exceptional in squamous-cell carcinoma, our results suggest that these mutations are not very rare in squamous-cell carcinoma with an adenocarcinoma component and adenosquamous carcinoma. Whereas no reliable data appear to be available regarding the frequency of squamous-cell carcinomas with an adenocarcinoma component, adenosquamous-cell carcinoma reportedly comprises 3% of all NSCLCs.[30] Our results suggest that a substantial portion of nonadenocarcinoma NSCLCs may harbor an *EGFR* mutation, and thorough histologic investigation to identify an adenocarcinoma component or genetic analyses to identify *EGFR* mutations are warranted for squamous-cell carcinomas.

Previous studies investigating *EGFR* mutations have not precisely described whether the squa-

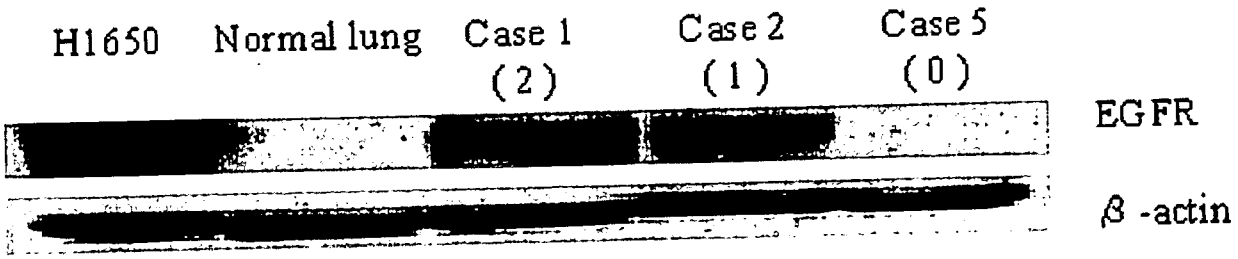

FIGURE 3. Analysis of epidermal growth factor receptor (EGFR) protein expression by Western blotting. EGFR protein expression was classified into 3 levels: (2), (1), and (0). Three representative samples (each corresponding to expression level (2), (1), and (0), respectively) together with a normal lung sample representing expression level (0) and the cell line H1650 representing level (2) are shown on the same blot. Expression of β-actin of the corresponding samples is shown below.

mous-cell carcinomas analyzed included an adenocarcinoma component, and many have even failed to separately analyze adenosquamous carcinomas. Actually, concordant with our results, studies discriminating adenosquamous carcinomas from squamous-cell carcinomas have uniformly shown that only the former type of tumors harbor mutations.[14,16] In the present study, microdissection analysis was performed to explore whether these mutations are restricted to an adenocarcinoma component. Consistent with the previous report,[31] *EGFR* TKD mutations were found in both adenocarcinoma and squamous-cell carcinoma components. These findings may suggest that squamous-cell carcinomas with an adenocarcinoma component are intrinsically different from pure squamous-cell carcinomas, and that an *EGFR* TKD mutation might have occurred in common progenitor cells destined to become both cell types in these tumors. However, the possibility remains that these findings are simply the result of artifacts due to contamination between the 2 cell types during analyses. Further studies are required to investigate biological differences between pure squamous-cell carcinomas and squamous-cell carcinomas with an adenocarcinoma component.

In addition to implications in tumorigenic mechanisms of *EGFR* TKD mutations, these results are also clinically important because patients with *EGFR* TKD mutations may be susceptible to TKI treatment. To date, no studies have precisely analyzed gefitinib sensitivity for squamous-cell carcinoma with an adenocarcinoma component. Similar to adenocarcinomas with mutations, the squamous-cell carcinoma component of these types of tumors with mutations may be susceptible to gefitinib therapy regardless of histology. Because of the small number of patients treated using gefitinib in this cohort, the efficacy of gefitinib could not be analyzed in our patients. Future studies with large subject populations will clarify this issue.

RT-PCR analysis of the extracellular domain of *EGFR* revealed that, similar to adenocarcinomas analyzed previously,[23] EGFRvIII is very rare in nonadenocarcinoma NSCLCs. In contrast, several reports have demonstrated that as much as 32% of NSCLCs, irrespective of histology, show this type of mutation.[19,21] This discrepancy may be attributable to the methods applied to detect EGFRvIII. The quality of antibody might influence rates of this mutation in previous studies using immunohistochemistry with EGFRvIII antibody. Most recently, a study investigating EGFRvIII in a large number of patients using RT-PCR has shown that, consistent with our results, this mutation is very rare in NSCLCs and found only in nonadenocarcinomas. These findings indicate that EGFRvIII contributes to tumor development in only a minor subset of NSCLCs.

In addition to *EGFR* mutations, we also analyzed EGFR protein expression in nonadenocarcinoma NSCLCs using Western blotting. EGFR protein overexpression was found in 35 of 42 nonadenocarcinoma NSCLCs (83%). Patients in our cohort thus showed a higher frequency of EGFR protein overexpression than those in a previous meta-analysis,[28] which may be because of differences in ethnicity. Whereas we have previously reported that EGFR overexpression is strongly correlated with TKD mutations in adenocarcinomas,[23] no such correlation was demonstrated in the present study for nonadenocarcinoma NSCLCs. This may be explained by differences in the histology of the tumors, although a definitive conclusion cannot be reached because of the small number of patients with TKD mutations in the present study. One recent report suggests that EGFR protein overexpression contributes more strongly to TKI susceptibility than *EGFR* TKD mutations.[11] Although gefitinib has failed to show clinical

efficacy in nonadenocarcinoma NSCLC patients,[32] other TKIs such as erlotinib or anti-EGFR antibody drugs may prove beneficial for tumors with EGFR overexpression.

In conclusion, we clarified that a subset of nonadenocarcinoma NSCLCs contains *EGFR* mutations. In particular, *EGFR* TKD mutations are not rare events in NSCLCs with an adenocarcinoma component. These molecular abnormalities will not only provide insights into the tumorigenesis and oncologic properties of these tumors, but will also render them possible targets for molecular therapies.

REFERENCES

1. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med.* 2004;350:2129–2139.
2. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science.* 2004;304:1497–1500.
3. Bell DW, Lynch TJ, Haserlat SM, et al. Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. *J Clin Oncol.* 2005;23:8081–8092.
4. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst.* 2005;97:339–346.
5. Suzuki M, Shigematsu H, Iizasa T, et al. Exclusive mutation in epidermal growth factor receptor gene, HER-2, and KRAS, and synchronous methylation of nonsmall cell lung cancer. *Cancer.* 2006;106:2200–2207.
6. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A.* 2004;101:13306–13311.
7. Huang SF, Liu HP, Li LH, et al. High frequency of epidermal growth factor receptor mutations with complex patterns in non-small cell lung cancers related to gefitinib responsiveness in Taiwan. *Clin Cancer Res.* 2004;10:8195–8203.
8. Chou TY, Chiz CH, Li CY, et al. Mutations in the tyrosine kinase domain of epidermal growth factor receptor is a predictive and prognostic factor for gefitinib treatment in patients with non-small-cell lung cancer. *Clin Cancer Res.* 2005;11:3750–3757.
9. Han SW, Kim TY, Hwang PG, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol.* 2005;23:2493–2501.
10. Tam YSI, Chung LP, Suen WS, et al. Distinct epidermal growth factor receptor and KRAS mutation pattern in non-small cell lung cancer patients with different tobacco exposure and clinicopathologic features. *Clin Cancer Res.* 2006;12:1647–1653.
11. Cappuzzo F, Hirsh FR, Rossi E, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst.* 2005;97:643–655.
12. Sonobe M, Manabe T, Wada H, et al. Mutations in the epidermal growth factor receptor gene are linked to smoking-independent, lung adenocarcinoma. *Br J Cancer.* 2005;93:355–363.
13. Marchetti A, Martela C, Felicioni L, et al. EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol.* 2005;23:857–865.
14. Tokumo M, Toyooka S, Kiura K, et al. The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. *Clin Cancer Res.* 2005;11:1167–1173.
15. Tomizawa Y, Iijima H, Sunaga N, et al. Clinicopathological significance of mutations of the epidermal growth factor receptor gene in patients with non-small cell lung cancer. *Clin Cancer Res.* 2005;11:6816–6822.
16. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res.* 2004;64:8919–8923.
17. Sugio K, Uramoto H, Oyama T, et al. Mutations within the tyrosine kinase domain of EGFR gene specifically occur in lung adenocarcinoma patients with a low exposure of tobacco smoking. *Br J Cancer.* 2006;94:896–903.
18. Worm K, Dabbagh P, Schwechheimer K. Reverse transcriptase polymerase chain reaction as a reliable method to detect epidermal growth factor receptor exon 2–7 gene deletion in human glioblastomas. *Hum Pathol.* 1999;30:222–226.
19. Garcia de Palazzo IE, Adams GP, Sundareshan P, et al. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res.* 1993;53:3217–3220.
20. Jungbluth AA, Stockert E, Su Huang HJ, et al. A monoclonal antibody recognizing human cancers with amplification/overexpression of the human epidermal growth factor receptor. *Proc Natl Acad Sci U S A.* 2003;100:639–644.
21. Okamoto I, Kenyon LC, Emlet DR, et al. Expression of constitutively activated EGFRvIII in non-small cell lung cancer. *Cancer Sci.* 2003;94:50–56.
22. Ji H, Zhao X, Yuza Y, et al. Epidermal growth factor receptor variant III mutations in lung tumorigenesis and sensitivity to tyrosine kinase inhibitors. *Proc Natl Acad Sci U S A.* 2006;103:7817–7822.
23. Ohtsuka K, Ohnishi H, Furuyashiki G, et al. Clinico-pathological and biological significance of tyrosine kinase domain gene mutations and overexpression of epidermal growth factor receptor for lung adenocarcinoma. *J Thorac Oncol.* 2006;1:787–795.
24. Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E. Historical Typing of Lung and Pleural Tumors, World Health Organization International Histological Classification of Tumors. Berlin: Springer; 1999.
25. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science.* 2004;305:1163–1167.
26. Mellinghoff IK, Wang MY, Vivanco I, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med.* 2005;353:2012–2024.
27. Ohnishi H, Ohtsuka K, Ooide A, et al. A simple and sensitive method for detecting major mutations within the tyrosine kinase domain of the epidermal growth factor receptor gene in non-small-cell lung carcinoma. *Diagn Mol Pathol.* 2006;15:101–108.

28. Nakamura H, Kawasaki N, Taguchi M, et al. Survival impact of epidermal growth factor receptor overexpression in patients with non-small-cell lung cancer: a meta-analysis. *Thorax.* 2006;61:140–145.
29. Nagai Y, Miyazawa H, Huqun, et al. Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. *Cancer Res.* 2005;65: 7276–7282.
30. Goya T, Asamura H, Yoshimura H, et al. Prognosis of 6644 resected non-small cell lung cancers in Japan: a Japanese lung cancer registry study. *Lung Cancer.* 2005;50:227–234.
31. Toyooka S, Yatabe Y, Tokumo M, et al. Mutations of epidermal growth factor receptor and K-ras genes in adenosquamous carcinoma of the lung. *Int J Cancer.* 2006;118:1588–1590.
32. Yang CH, Shih JY, Chen KC, et al. Survival outcome and predictors of gefitinib antitumor activity in East Asian chemonaive patients with advanced non small cell lung cancer. *Cancer.* 2006;107:1873–1882.

A Simple and Sensitive Method for Detecting Major Mutations Within the Tyrosine Kinase Domain of the Epidermal Growth Factor Receptor Gene in Non–small-cell Lung Carcinoma

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Abstract: The detection of mutations in the epidermal growth factor receptor (*EGFR*) gene impacts therapeutic decision-making for non–small-cell lung carcinoma (NSCLC). Although direct sequencing has been most frequently used to detect *EGFR* mutations, this method displays several disadvantages. We set up simple mutation-specific polymerase chain reaction (PCR) for common delE746-A750 and L858R mutations of *EGFR* using primers specific to these mutations. Both mutation-specific PCR and direct sequencing methods were used to investigate 62 samples of NSCLC, and the results were compared. To evaluate the sensitivity of mutation-specific PCR, DNA mixtures containing various proportions of mutant alleles were analyzed. Mutation-specific PCR revealed delE746-A750 in 8 samples and L858R in 14 samples. All samples with either delE746-A750 or L858R mutation revealed by direct sequencing also displayed positive results for mutation-specific PCR. Conversely, mutations in 3 samples revealed by L858R-specific PCR were barely detectable by direct sequencing. In DNA mixture analysis, DNA mixtures containing 2.5% of delE746-A750 allele or 0.25% of L858R allele yielded positive results with mutation-specific PCR. Our mutation-specific PCR showed satisfactory sensitivity and reliability for detecting major *EGFR* mutations in clinical NSCLC samples. Given the practical availability, this method could be widely applicable to the treatment of lung cancers.

Key Words: EGFR, mutation, PCR, lung cancer, methodology

(*Diagn Mol Pathol* 2006;15:101–108)

Lung cancer represents a major challenge for public health all over the world, particularly in developed countries,[1,2] where lung cancer is a primary cause of death in the elderly. The establishment of effective therapeutic modalities is thus required for the treatment of lung cancer. However, chemotherapy for lung cancer remains relatively unsatisfactory, especially for non–small-cell lung carcinoma (NSCLC).[3]

Recently, various mutations within the epidermal growth factor receptor (*EGFR*) gene have been found in NSCLC.[4–14] These mutations tend to cluster in the tyrosine kinase (TK) domain of the gene, and previous studies have demonstrated that some mutations cause alterations in the function of this gene.[4,5,15] The potential impact of *EGFR* mutations in NSCLC has attracted substantial attention from researchers and clinicians, because the presence of mutations may critically influence the effects of TK inhibitor (TKI) drugs against lung cancer. Gefitinib and erlotinib, as specific inhibitors of *EGFR*, are among the drugs for which effects may be altered. Numerous studies have suggested that gefitinib is more effective against tumors harboring *EGFR* mutations than against tumors without such mutations,[4,6,12,14] and similar findings have been reported for erlotinib,[6] although the hypothesis has yet to be confirmed in randomized controlled studies. *EGFR* mutations are widely recognized as occurring more frequently in female patients, nonsmokers, patients with tumors of adenocarcinomatous histology, and Asian populations. Interestingly enough, patients with these features are known to comprise a group susceptible to gefitinib therapy.[6–14] Given these findings, testing for *EGFR* mutations in lung cancer seems desirable.

Many previous studies have utilized direct sequencing to detect *EGFR* mutations, but this method has several disadvantages with regard to clinical use. The most notable of these is a low detection rate when clinical samples are used, presumably because of the presence of high rates of contaminating normal and fibrous tissues in tumor samples. Contaminating wild-type DNA interferes with accurate sequence analysis. In addition, sequence analysis is usually both time consuming and relatively expensive. An easy and reliable method for detecting *EGFR* mutations has thus been awaited for clinical use.

Herein we report a simple and sensitive polymerase chain reaction (PCR)-based method to detect the two most common mutations of the *EGFR* TK domain, and demonstrate the usefulness of this method for detecting such mutations in clinical lung tumor samples.

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MATERIALS AND METHODS

Samples

Previous studies have shown that lung cancer cell line NCI-H1650 carries a 15-bp deletion comprising nucleotides 2481-2495 (delE746-A750), whereas NCI-H1975 includes a T > G point mutation at nucleotide 2819 (L858R).[15] Both cell lines were purchased from American Type Culture Collection (Manassas, VA) and used as positive controls for these mutations. Negative controls comprised 50 peripheral blood samples taken from healthy donors. Clinical samples comprised 62 tumor samples that had been resected from lung cancer patients at the time of operation and immediately frozen at −80C. Written informed consent for DNA analysis was obtained before collecting samples from each individual. Histopathologic diagnosis for these tumors included adenocarcinoma (n = 54), squamous cell carcinoma (n = 5), large-cell carcinoma (n = 2), and adenosquamous carcinoma (n = 1). DNA was extracted from samples using a DNeasy kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. DNA quality was confirmed by PCR of *EGFR* exon 19 using primers 19S4 and 19AS4 (Table 1). Only those DNA products producing a positive band were used for further analyses (data not shown).

Direct Sequence Analysis

Mutations of *EGFR* in lung cancer cluster within exons 18 to 21, and >90% of previously reported mutations reside on exons 19 and 21. We therefore performed direct sequence analysis of *EGFR* exons 19 and 21 for all samples, including the 2 positive-control cell lines. Sample DNA (100 ng) was amplified by PCR under identical conditions to mutation-specific PCR (see below). Amplified DNA was purified using a Qiaquick DNA purification kit (QIAGEN), and then 10 ng of PCR products was applied for the sequencing reaction using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The resulting product was further purified using Centri-Sep spin columns (Princeton Separations, Adelphia, NJ) to be ready for analysis. Sequencing analysis was performed using an ABI Prism 3100-Avant genetic analyzer (Applied Biosystems) according to the protocols of the manufacturer. Primers used for sequence analysis are summarized in Table 1.

Mutation-specific PCR

Except for a few rare cases, mutations within exons 19 and 21 can be classified as either a deletion mutation of 9 to 24 bp involving nucleotides 2484-2495 within exon 19 or a T > G point mutation of nucleotide 2819 within exon 21. In the present study, specific PCR primers capable of detecting these mutations were designed. For deletion in exon 19, deletion-specific antisense primer 19ASD1 was designed (Table 2, Fig. 1A). This primer was designed to anneal only to the genome in the presence of a deletion of nucleotides 2481-2495 causing delE746-A750, by connecting both ends outside of the deleted sequence. PCR using this primer is referred to as "delE746-A750-specific PCR". For the T > G point mutation of nucleotide 2819 causing the L858R amino acid change, a mutation-specific sense primer 21SM1 was designed (Table 2, Fig. 1B). PCR using this primer is referred to as "L858R-specific PCR". Other PCR primers used are also described in Table 2.

Each 30-μL aliquot of PCR reaction mix contained 3 μL of 10 PCR buffer (Applied Biosystems), 6 nmol each of the 4 dNTPs, 50 nmol of Mg^{2+}, 40 pmol each of sense and antisense primers, 1 U of Taq polymerase (Applied Biosystems), and 100 ng of sample DNA. PCR conditions were as follows: 95°C for 5 minutes, then 32 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds; followed by a final 10 minutes at 72°C. PCR products were electrophoresed in 2% agarose gel containing 1 μL of ethydium bromide, and visualized under UV. Each experiment was performed in triplicate.

To further explore a more convenient application of this technique, "dual-specific PCR" was attempted. This process was intended to detect both delE746-A750 and L858R in one reaction by mixing the 2 sets of primers (20 pmol each) in a single PCR reaction mix.

DNA Mixture Analysis

DNA mixture analysis was performed to investigate the limits of detection of mutation-specific PCR technique using samples containing low levels of mutant DNA. Because we have confirmed by sequence analysis that cell lines H1650 and H1975 contain both mutant and wild-type alleles (Fig. 2), these cell lines were assumed to comprise 50% mutant alleles and 50% wild-type alleles. Conversely, normal peripheral blood samples were considered to have 100% wild-type alleles. Mixing equal amounts of DNA from these cell lines and normal samples would thus result in 25% mutant alleles and 75%

TABLE 1. Primers for Sequence Analysis

	Exon 19		Exon 21	
Primers for PCR				
Sense	19S4	5′-TGCATCGCTGGTAACAT-3′	21S3	5′-TGGTCAGCAGCGGGTTACATCTTC-3′
Antisense	19AS4	5′-AGCTGCCAGACATGAGAA-3′	21AS3	5′-CAATACAGCTAGTGGGAAGGCAGC-3′
Primers for sequence reaction				
Sense	19S5	5′-ACCATCTCACAATTGCCAG-3′	21S4	5′-CTTTGGATCAGTAGTC-3′
Antisense	19AS5	5′-TGAGGTTCAGAGCCAT-3′	21AS4	5′-CTGGCTGACCTAAAGC-3′

TABLE 2. Primers for Mutation-specific PCR

		Primer Sequence	Expected Band Size (bp)
DelE746-A750-specific PCR			
Sense	19S4	5′-TGCATCGCTGGTAACAT-3′	133
Antisense	19ASD1	5′-CGGAGATGTTTTGATAGCG-3′	
L858R-specific PCR			
Sense	21SM1	5′-ATGTCAAGATCACAGATTTTGGGCG-3′	104
Antisense	21AS4	5′-CTGGCTGACCTAAAGC-3′	

wild-type DNA alleles. In a similar manner, DNA samples obtained from mutant cell lines and normal peripheral blood were mixed in various proportions to form DNA mixtures containing 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, and 0.1% mutant alleles. Mutation-specific PCR was subsequently performed using 100-ng samples of these DNA mixtures, to identify the extent to which a low percentage of mutant alleles can be detected using this method.

RESULTS

Mutations of *EGFR* in Lung Cancer Cell Lines

Sequence analysis confirmed that H1650 carries the deletion of nucleotides 2481-2495 and H1975 carries the T > G point mutation of nucleotide 2819, as expected (Fig. 2). In addition, both cell lines were shown to include wild-type sequences at the same time, indicating that these mutations are heterozygous.

Direct Sequence Analysis

Sequence analysis revealed deletions within exon 19 in 12 of the 62 NSCLC samples. The types of deletion varied, but 8 of the 12 were of delE746-A750 type, which was also common in previous studies (Table 3). DelE746-A750 included 2 types of deletion: deletion from 2481G to 2495C (19del-type1) and deletion from 2482G to 2496A (19del-type2). Sequence analysis of exon 21 showed that 11 of the 62 NSCLC samples contained the L858R mutation, showing the T > G point mutation at nucleotide 2819.

All samples with sequence mutations in this study also showed concurrent wild-type sequences. This was attributed to heterozygous mutation, but this was difficult to distinguish from homozygous mutation in the presence of large numbers of contaminating normal cells. Most samples with mutation displayed adenocarcinomatous histology (21 of 23 mutant samples), but 2 non-adenocarcinomatous NSCLCs showed mutation of either exon 19 or exon 21.

Mutation-specific PCR

DelE746-A750-specific PCR

To detect deletions in cell line H1650, delE746-A750-specific PCR was performed using primers 19ASD1 and 19S4. H1650 includes a deletion from 2481G to 2495C (19del-type1), generating a sequence exactly

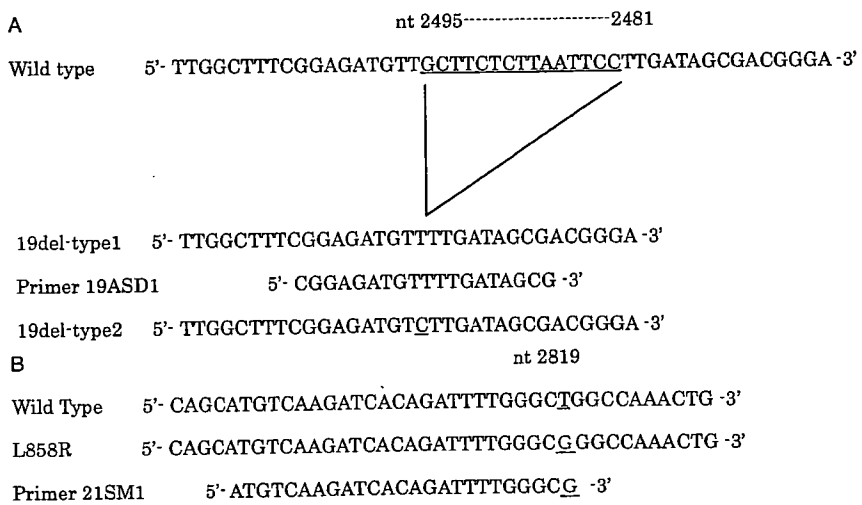

FIGURE 1. Scheme of mutation-specific primers. A, Primer 19ASD1 for delE746-A750-specific PCR, comprising nucleotides homologous to 2472-2480 and 2496-2505 (2481-2495 is deleted). Sequences for 19del-type1 and 19del-type2 are also shown. Note that the 19del-type2 sequence differs from the 19ASD sequence by 1 bp (underlined). All sequences are described in the reverse direction. B, Primer 21SM1 for L858R-specific PCR, comprising nucleotides homologous to nucleotides 2796-2819 of the mutant allele.

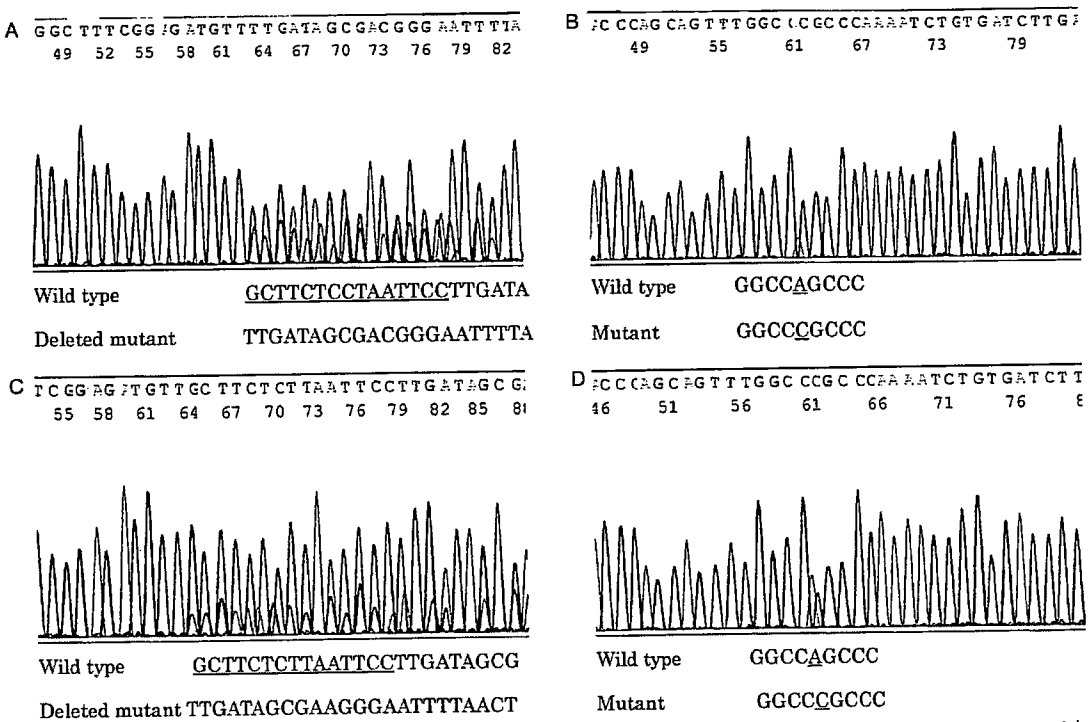

FIGURE 2. Results of sequence analysis. A, Sequence for exon 19 of the H1650 cell line in the reverse direction, with deletion of 15 bp (nucleotides 2481-2495). Note that the deleted allele is dominant, and reading of the sequence (top of the figure) is mostly mutant. Wild-type and mutant sequences are described at the bottom. The 15-bp deletion is indicated by underlining in the wild-type sequence. B, Sequence for exon 21 of the H1975 cell line in the reverse direction, with nucleotide 2819 changed from A to C. Note that the mutant allele is dominant, and reading of the sequence (top of figure) is the mutant version. Wild-type and mutant sequences are described at the bottom. Mutated base is indicated by underlining. C, Sequence of a sample with delE746-A750. D, Sequence of a sample with L858R mutation. In both these cases, the mutated signal is lower than the wild-type.

matching that of primer 19ASD1. A single 133-bp band was detected by delE746-A750-specific PCR, as expected. In contrast, delE746-A750-specific PCR for 50 normal samples yielded no positive bands.

Mutation-specific PCR was then performed for tumor samples. DelE746-A750-specific PCR revealed positive bands for 8 of the 62 NSCLC samples (Fig. 3). Although 5 of these 8 samples displayed the expected 19del-type1 sequence, the remaining 3 samples exhibited the 19del-type2 sequence (Fig. 1). Although the 19ASD1 primer (CGGAGATGTTTTGATAGCG) and the actual 19del-type2 sequence (CGGAGATGTCTTGATAGCG) differ by 1 bp, PCR presumably generated a positive band with the same size as that for 19del-type1 because of the

TABLE 3. Summary of Previously Reported Mutations in *EGFR* TK Domain

Exon 18	Exon 19		Exon 20	Exon 21		Authors
	delE746-A750	Others		L858R	Others	
1	1	3	0	2	1	Lynch et al[4]
2	10	5	0	3	0	Paez et al[5]
0	7	2	1	13	0	Pao et al[6]
4	25	27	1	46	3	Kosaka et al[7]
0	9	4	3	20	2	Huang et al[11]
1	11	8	0	18	0	Tokumo et al[9]
4	2	4	0	6	1	Han et al[13]
3	12	5	0	12	1	Mitsudomi et al[24]
3	12	6	0	18	1	Marchetti et al[8]
6	42	20	12	52	8	Shigematsu et al[10]
24 (5.2%)	131 (28.3%)	84 (18%)	17 (3.7%)	190 (41%)	17 (3.7%)	Total (463) (Percentage of total)