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

Discussion

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

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

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

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

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

Table 5. Clinical outcomes among subgroups of patients

	n	Response rate (%)	P	Median TTP (mo)	P	MST (mo)	P
Total	207	37		3.7		14.5	
Sex							
Women	89	51	<0.001	5.6	0.17	18.3	0.15
Men	118	26		2.3		9.6	
Smoking history							
Never smokers	93	51	<0.001*	6.2	0.073*	16.9	0.22*
Former smokers	38	47		5.2		14.5	
Current smokers	76	14		2.2		9.1	
Histology							
Adenocarcinoma	169	40	0.004	4.3	0.060	15.1	0.10
Others	18	6		1.6		4.9	
EGFR mutations							
DEL/L858R	85	78	<0.001	9.2	<0.001	21.7	<0.001
WT	122	8		1.6		8.7	

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

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

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

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

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References

1. Fukuoka M, Yano S, Giaccone G, et al. A multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small cell lung cancer (The IDEAL 1 Trial). J Clin Oncol 2003;21:2237^46.
2. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003;290:2149^58.
3. Takano T, Ohe Y, Kusumoto M, et al. Risk factors for interstitial lung disease and predictive factors for tumor response in patients with advanced non-small cell lung cancer treated with gefitinib. Lung Cancer 2004;45:93^104.
4. 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;350:2129^39.
5. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497^500.
6. Takano T, Ohe Y, Sakamoto H, et al. Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J Clin Oncol 2005;23:6829^37.
7. 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 postoperative recurrence. J Clin Oncol 2005;23:2513^20.
8. 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;23:2493^501.
9. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 2003;49:853^60.
10. Nomoto K, Tsuta K, Takano T, et al. Detection of EGFR mutations in archived cytologic specimens of non-small cell lung cancer using high-resolution melting analysis. Am J Clin Pathol 2006;126:1^8.
11. Green S, Weiss GR. Southwest Oncology Group standard response criteria, endpoint definitions, and toxicity criteria. Invest New Drugs 1992;10:239^53.
12. Noguchi M, Furuya S, Takeuchi T, et al. Modification formalin and methanol fixation methods for molecular biological and morphological analyses. Pathol Int 1997;47:685^91.
13. Marchetti A, Martella 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;23:857^65.
14. 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;65:7276^82.
15. Pan Q, Pao W, Ladanyi M. Rapid polymerase chain reaction-based detection of epidermal growth factor receptor gene mutations in lung adenocarcinomas. J Mol Diagn 2005;7:396^403.
16. Yatabe Y, Hida T, Horio Y, et al. A rapid, sensitive assay to detect EGFR mutation in small biopsy specimens from lung cancer. J Mol Diagn 2006;8:335^41.
17. Asano H, Toyooka S, Tokumo M, et al. Detection of EGFR gene mutation in lung cancer by mutant-enriched polymerase chain reaction assay. Clin Cancer Res 2006;12:43^8.
18. Janne PA, Borras AM, Kuang Y, et al. A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. Clin Cancer Res 2006;12:751^8.
19. Sasaki H, Endo K, Konishi A, et al. EGFR Mutation status in Japanese lung cancer patients: genotyping analysis using LightCycler. Clin Cancer Res 2005;11:2924^9.
20. Kimura H, Kasahara K, Kawaishi M, et al. Detection of epidermal growth factor receptor mutations in serum as a predictor of the response to gefitinib in patients with non-small-cell lung cancer. Clin Cancer Res 2006;12:3915^21.
21. Endo K, Konishi A, Sasaki H, et al. Epidermal growth factor receptor gene mutation in non-small cell lung cancer using highly sensitive and fast Taqman PCR assay. Lung Cancer 2005;50:375^84.
22. Cappuzzo F, Hirsch 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;97:643^55.
23. Hirsch FR, Varella-Garcia M, McCoy J, et al. Increased epidermal growth factor receptor gene copy number detected by fluorescence in situ hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a Southwest Oncology Group study. J Clin Oncol 2005;23:6838^45.
24. Riely GJ, Pao W, Pham DK, et al. Clinical course of patients with non-small cell lung cancer and epidermal growth factor receptor exon 19 and exon 21 mutations treated with gefitinib or erlotinib. Clin Cancer Res 2006;12:839^44.
25. Jackman DM, Yeap BY, Sequist LV, et al. Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. Clin Cancer Res 2006;12:3908^14.
26. Paz-Ares L, Sanchez JM, García-Velasco A, et al. A prospective phase II trial of erlotinib in advanced non-small cell lung cancer (NSCLC) patients (p) with mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR) [abstract 7020]. Proc Am Soc Clin Oncol 2006;24:369s.
27. Hirsch FR, Franklin WA, McCoy J, et al. Predicting clinical benefit from EGFR TKIs: not all EGFR mutations are equal [abstract 7072]. Proc Am Soc Clin Oncol 2006;24:382s.

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Frequent EGFR mutations in noninvasive bronchioloalveolar carcinoma

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Mutations of the epidermal growth factor receptor gene (EGFR) have been reported to be present in a considerable fraction of lung adenocarcinomas showing dramatic response to EGFR tyrosine kinase inhibitors. To clarify pathogenic significance of the mutations for the development of lung adenocarcinoma, we investigated stage I lung adenocarcinomas for the mutations. First, 107 cases of macrodissected stage I adenocarcinomas were examined for mutations in exons 18–21 of the EGFR gene. EGFR mutations were detected in 36 of the 107 cases (34%). In particular, among the stage I cases, the mutations were detected in 17 of 42 small-sized adenocarcinomas (2 cm in diameter) (40%), including 7 of 11 noninvasive bronchioloalveolar carcinomas (BACs) (64%) and 7 of 25 invasive adenocarcinomas with BAC components (28%). Second, 26 cases of laser capture microdissected small-sized adenocarcinomas, including 9 cases in the first analysis, were examined for the mutations. Reanalysis of microdissected materials in the 9 cases identified the mutations in 2 more adenocarcinomas with BAC components. Moreover, in the analysis of the other 17 microdissected materials, EGFR mutations were detected in 7 of 12 BACs (58%) and in 3 of 5 adenocarcinomas with BAC components (60%). EGFR mutations are present frequently in BACs, and are thus likely to be a critical genetic alteration for the formation of noninvasive lung adenocarcinoma.

Key words: EGFR mutation; small-sized lung adenocarcinoma; bronchioloalveolar carcinoma (BAC)

Epidermal growth factor receptor (EGFR) is a member of a family comprised of 4 homologous receptors, including 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 increase in cellular proliferation, migration, invasion and suppression of apoptosis.[1] In 2004, 2 groups simultaneously reported that most nonsmall cell lung cancer (NSCLC) 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 revealed that EGFR mutations are present in a considerable fraction of NSCLC 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 with adenocarcinoma without smoking histories. This population corresponded to responders to gefitinib. These data indicate that EGFR mutations and/or responsiveness to gefitinib are likely to be associated with a specific subset of lung adenocarcinoma. However, there is still little evidence about when the mutations occur during the development of lung adenocarcinoma and whether the mutations are responsible for a specific phenotype in lung adenocarcinoma.

In 1995, Noguchi et al.[11] classified small-sized lung adenocarcinomas of 2 cm in diameter into 6 histological subtypes, as follows: type A, localized bronchioloalveolar carcinoma (LBAC); type B, LBAC with foci of alveolar structural collapse; type C, LBAC with foci of active fibroblastic proliferation; type D, poorly differentiated adenocarcinoma; type E, tubular adenocarcinoma; and type F, papillary adenocarcinoma with compressive and destructive growth. Types A, B and C grow by replacing the pulmonary alveolar structure (replacing growth type), whereas types D, E and F grow destructively, without such a replacement. In 1999, "bronchioloalveolar carcinoma (BAC)" was newly defined as a subtype of lung adenocarcinoma in the World Health Organization (WHO) classification of lung cancers, in which BAC was defined as being a true noninvasive cancer without evidence of stromal, vascular or pleural invasion.[12] This definition was preserved in the 2004 WHO classification.[13] Among the replacing growth types of adenocarcinomas, types A and B are true noninvasive BAC and type C is an invasive adenocarcinoma with BAC component; thus, types A and B correspond to BAC in the WHO classification. Accordingly, type A tumors are assumed to sequentially progress through type B to type C. Aoyagi et al.[14] reported that accumulation of loss of heterozygosity (LOH) in crucial chromosome regions occurred stepwise during this sequential progression. The results indicate that a fraction of genetic alterations are involved in the progression from noninvasive to invasive lung adenocarcinoma. Since EGFR mutations are present in a considerable fraction of lung adenocarcinomas, it is important to clarify whether EGFR mutations are present in noninvasive stages or in invasive stages of lung adenocarcinoma and to which histological subtypes of lung adenocarcinoma the mutations are associated with.

In the present study, we first investigated 107 cases of stage I lung adenocarcinoma for EGFR mutations. The mutations were detected in 34% of the stage I cases. Then, to address the above unresolved issues, we further analyzed small-sized adenocarcinomas (2 cm in diameter) of stage I cases and compared the mutations with histological subtypes of adenocarcinoma using histological classification reported by Noguchi et al. EGFR mutations were detected in a majority of type A and B adenocarcinomas. Thus, it was indicated that EGFR mutations may contribute to the development of BAC and are one of the early genetic alterations in multistage carcinogenic processes of lung adenocarcinoma.

Material and methods

Patients and tissues

Primary tumors were obtained from 124 patients with stage I lung adenocarcinoma, including 59 patients with small-sized lung adeno-

Abbreviations: BAC, bronchioloalveolar carcinoma; EGFR, epidermal growth factor receptor; LBAC, localized bronchioloalveolar carcinoma; LOH, loss of heterozygosity; NSCLC, nonsmall cell lung cancer; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TK, tyrosine kinase.

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TABLE I – CORRELATION BETWEEN EGFR MUTATIONS AND CLINICOPATHOLOGICAL CHARACTERISTICS IN PATIENTS WITH STAGE I LUNG ADENOCARCINOMA

Clinicopathological feature	Subset	Number of patients	EGFR mutation (%)		p-Value[1]
			1	2	
Gender	Male	61	16 (26)	45 (74)	0.07
	Female	46	20 (43)	26 (57)	
Age	63	54	22 (41)	32 (59)	0.15
	>63	53	14 (26)	39 (74)	
Smoking history	Smoker	62	11 (18)	51 (82)	<0.0001
	Nonsmoker	45	25 (56)	20 (44)	
T stage	T1	70	25 (36)	45 (64)	0.67
	T2	37	11 (30)	26 (70)	
Tumor size	2 cm	42	17 (40)	25 (60)	0.30
	>2 cm	65	19 (29)	46 (71)	
Differentiation	Poorly	15	4 (25)	12 (75)	0.55 (poorly vs well),
	Moderately	37	12 (33)	24 (67)	0.75 (poorly vs mod.)
	Well	55	20 (36)	35 (64)	
KRAS mutation	1	12	0 (0)	12 (100)	0.008
	2	95	36 (38)	59 (62)	
p53 mutation	1	35	9 (26)	26 (74)	0.28
	2	72	27 (38)	45 (63)	

[1]Fisher's exact test.

carcinoma of 2 cm in their maximum diameter. All of the patients underwent curative pulmonary resections at the National Cancer Center Hospital, Tokyo, Japan, from December 1986 to December 2000. None of the patients received neoadjuvant or adjuvant chemotherapy, including treatment with gefitinib and radiotherapy before or after surgery. The tumors were pathologically diagnosed according to the tumor-node-metastasis classification of malignant tumors.[15] In addition, all of the small-sized adenocarcinomas were histologically classified into 6 groups according to the histological classification of small-sized adenocarcinoma of the lung, which was previously reported by Noguchi et al.[11]

For the first series of analysis, 107 cases, including 42 with small-sized adenocarcinomas, were used. Clinicopathological characteristics of these patients are summarized in Table I. The median follow-up period of these patients was 63 months (range, 4–110 months). Tumors analyzed in the first series were macrodissected and stored at 280 C until DNA extraction. Their genomic DNAs were prepared by the method described previously[16] or by a QIAamp DNA mini kit (Qiagen, Tokyo, Japan). Cancer cells of 26 cases with small-sized lung adenocarcinoma, which consisted of 9 cases analyzed in the first series and an additional 17 cases, were collected by laser capture microdissection. The tumor specimens obtained from these cases were fixed with methanol and embedded in paraffin. Three or four 8-1m-thick sections from each specimen were deparaffinized and stained with hematoxylin. The stained sections were dried and cancer cells were microdissected using the Pixcell Laser Capture microdissection system (Arcturus Engineering, Mountain View, CA). A total of 1,000–5,000 cancer cells were microdissected from each specimen, and their genomic DNAs were isolated by sodium dodecyl sulfate (SDS)/proteinase K digestion and phenol/chloroform extraction as described previously.[17]

Mutational analysis of the EGFR gene

Exons 18–21 of the EGFR gene were examined for mutations by genomic polymerase chain reaction (PCR) amplification and direct sequencing of PCR products. Ten nanograms of DNA extracted from the macrodissected materials was used for PCR amplification. For the microdissected materials, nested PCR was carried out after initial PCR using 100 pg of DNA. The primer sets for the initial PCR were as follows: exon 18, 5′-CAAATGAGCTGGCAAGTGCCGTGTC-3′ (forward) and 5′-GAGTTTCCCAAACACTCAGTGAAAC-3′ (reverse); exon 19, 5′-GCAATATCAGCCTTAGGTGCGGCTC-3′ (forward) and 5′-CATAGAAAGTGAACATTTAGGATGTG-3′ (reverse); exon 20, 5′-CCATGAGTACGTATTTTGAAACTC-3′ (forward) and 5′-CATATCCCCATGGCAAACTCTTGC-3′ (reverse); exon 21, 5′-CTAACGTTCGCCAGCCATAAGTCC-3′ (forward) and 5′-GCTGCGAGCTCACCCAGAATGTCTGG-3′ (reverse). The internal primer sets for nested PCR were as follows: exon 18, 5′-CAAGTGCCGTGTCCTGGCACCCAAGC-3′ (forward) and 5′-CCAAACACTCAGTGAAACAAAGAG-3′ (reverse); exon 19, 5′-CCTTAGGTGCGGCTCCACAGC-3′ (forward) and 5′-CATTTAGGATGTGGAGATGAGC-3′ (reverse); exon 20, 5′-GAAACTCAAGATCGCATTCATGC-3′ (forward) and 5′-GCAAACTCTTGCTATCCCAGGAG-3′ (reverse); exon 21, 5′-CAGCCATAAGTCCTCGACGTGG-3′ (forward) and 5′-CATCCTCCC-CTGCATGTGTTAAAC-3′ (reverse). Thirty-five cycles (for initial PCR) or 30 cycles (for nested PCR) of 95 C (30 sec) for denaturation, 58 C (1 min) for annealing and 72 C (30 sec) for extension were performed to amplify DNA fragments. PCR products were cycle-sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

Mutational analysis of the p53 gene and the KRAS gene

We previously examined the 107 macrodissected materials for mutations of the p53 gene and the KRAS gene.[16] Briefly, the p53 gene (exons 2–11) was amplified by genomic PCR. PCR products with variant peaks detected by the WAVE DNA Fragment Analysis System and WAVEMAKER Software 4.0 (Transgenomic, Omaha, NE) were purified by a QIAquick PCR Purification kit (Qiagen) for sequencing. The KRAS gene (exons 1 and 2) was also amplified by genomic PCR and directly sequenced. Cycle sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Norwalk, CT) and the ABI PRISM 310 DNA Sequencer (Perkin-Elmer).

Microdissected materials were also analyzed for KRAS mutations. One-hundred picograms of DNA was used for initial PCR amplification. Nested PCR was carried out after initial PCR. The primer sets for initial PCR were as follows: exon 1, 5′-GGTACTGGTGGAGTATTTGAT-3′ (forward) and 5′-ATGGTCAGAGAAACCTTTATCT-3′ (reverse); exon 2, 5′-CCTTTTTTGAAGTAAAAGGTGC-3′ (forward) and 5′-ATCCCCCAAGAACTTCATTTAT-3′ (reverse). The internal primer sets for nested PCR were as follows: exon 1, 5′-TGGTGGAGTATTTGATAGTGTA-3′ (forward) and 5′-ATCTGTATCAAAGAATGGTCCT-3′ (reverse); exon 2, 5′-GGTGCACTGTAATAATCCAGA-3′ (forward) and 5′-ATTACTCCTTAATGTCAGCTTAT-3′ (reverse). Thirty-five cycles (for initial PCR) or 30 cycles (for nested PCR) of 95 C (1 min) for denaturation, 60 C (1 min) for annealing and 72 C (1 min) for extension were performed to amplify DNA fragments. PCR products were cycle-sequenced

using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

Statistical analysis

Fisher's exact test was used to assess the association of EGFR mutations with clinicopathological factors. The association was further examined by multivariate odds ratios adjusted by each other (logistic regression analysis). Overall survivals of the patients with and without EGFR mutations were compared by the Kaplan–Meier curves and the log-rank test. In addition, the effect of the mutations on survival was also investigated using the Cox proportional hazards regression model adjusted by other potential prognostic factors shown in Table I. A p value of <0.05 was considered to be statistically significant. Statistical analysis was performed using the SAS (version 8.02; SAS Institute, Cary, NC).

Results

Correlation between EGFR mutations and clinicopathological characteristics in stage I lung adenocarcinoma

One hundred and seven macrodissected stage I lung adenocarcinomas were examined for mutations in exons 18–21 of the EGFR gene by genomic PCR and direct sequencing. EGFR mutations were detected in 36 cases (34%) (Table I). EGFR mutations were significantly more frequent in nonsmokers (56%) than in smokers (18%) ($p < 0.0001$). Female patients (43%) tended to have EGFR mutations more frequently in their tumors than male patients (26%), although the difference in frequency did not reach the statistical significance (p 5 0.07). There was no significant correlation of EGFR mutations with age, T stage, tumor size (2 cm vs. >2 cm) and differentiation.

All tumors in this series of stage I lung adenocarcinomas had been previously examined for mutations in exons 1 and 2 of the KRAS gene and in exons 2–11 of the p53 gene.[18] KRAS mutations were detected in 12 of 107 cases (11%): 10 in codon 12, 1 in codon 13 and 1 in codon 61. p53 mutations were detected in 37 of the 107 cases. Because 2 of the 37 cases showed a silent p53 mutation, we counted 35 of the 107 (33%) as cases with p53 mutation in this analysis. No EGFR mutation was detected in tumors with KRAS mutations, indicating a mutually exclusive correlation (p 5 0.008). In contrast, there was no significant correlation or inverse correlation between EGFR mutation and p53 mutation (p 5 0.28).

The association of EGFR mutations with the clinicopathological characteristics was also evaluated by logistic regression analysis to account for the effect of the different variables. Nonsmoking was independently associated with EGFR mutations (p 5 0.003; odds ratio, 6.83; 95% confidence interval (C. I.), 1.92–24.32), whereas the other factors shown in Table I were not.

We then analyzed the effect of EGFR mutations on survival of the 107 patients with stage I lung adenocarcinoma. Kaplan–Meier survival estimates showed that EGFR mutations did not affect prognosis of the patients (p 5 0.60; log-rank test) (Fig. 1). A multivariate Cox proportional hazard regression analysis of all of the clinicopathological and molecular factors also showed that EGFR mutations were not an unfavorable prognosis factor independent of other factors (p 5 0.85; hazard ratio, 1.11; 95% C. I., 0.38–3.24).

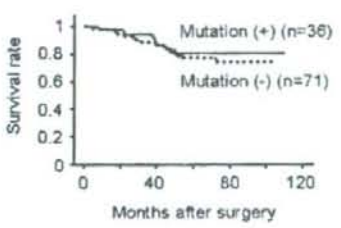

FIGURE 1 – Survival curves for patients with stage I lung adenocarcinoma classified according to EGFR mutations. The resulting curves were compared using log-rank test (p 5 0.60).

EGFR mutations in small-sized adenocarcinoma of the lung

It was noted that the frequency of EGFR mutations in small-sized adenocarcinomas (17/42, 40%) was a little higher than that in stage I cases in total. This result may indicate that EGFR mutations occur early in the development of stage I lung adenocarcinoma. Therefore, according to the histological classification of small-sized adenocarcinoma reported by Noguchi et al.,[11] we further analyzed the frequency of EGFR mutations as well as those of KRAS and p53 mutations (Table II). Among 11 BACs (type A and B tumors), EGFR mutations were detected in 1 of 3 type A (33%) and in 6 of 8 type B tumors (75%), whereas KRAS mutation was detected in only 1 type A tumor and not detected in any type B tumors. Of 25 adenocarcinomas with BAC components (type C tumors), 7 (28%) had EGFR mutations and 4 (16%) had KRAS mutations. All p53 mutations were detected in type C, D and F tumors, whereas none of the type A and B tumors had p53 mutations.

EGFR mutations in noninvasive and invasive adenocarcinomas

It is generally accepted that noninvasive adenocarcinomas further progress to invasive ones by accumulation of additional genetic alterations. However, unexpectedly, the EGFR gene was less frequently mutated in invasive adenocarcinomas with BAC components (type C tumors) than in BACs (type A and B tumors). Since type C tumors contain a large population of fibroblasts and other noncancerous cells, it was possible that EGFR mutations were masked by contaminating noncancerous cells in tumor samples. Therefore, we further analyzed 26 cases of type A, B and C tumors, in which cancer cells were obtained by laser capture microdissection.

Among the 42 cases of small-sized adenocarcinomas in the first series, 9 cases were reevaluated using microdissected materials (Table III). Seven of the 9 cases showed the same results as macrodissected materials in the first analysis, that is, 5 cases had EGFR mutations and 2 cases did not in both materials. However, in the remaining 2 cases, both of which were classified into type C, EGFR mutations were detected only in microdissected materials and not in macrodissected ones. These 2 tumors were rather small, 1.0 and 1.4 cm in size, respectively, and both harbored the leucine to arginine mutation at codon 858 (L858R) in exon 21. Photographs of microdissection and sequence chromatograms obtained from one of these tumors are shown in Figures 2a and 2c, respectively. Among the 5 cases in which EGFR mutations were detected in both macrodissected and microdissected materials, there were 3 cases whose sequence chromatograms showed the increase in the ratio of mutant allele to wild-type allele in microdissected materials compared with macrodissected materials. All of the 3 tumors were <1.5 cm in size, and 1 was classified into type B and the other 2 were into type C. Representative photographs of microdissection and sequence chromatograms are shown in Figures 2b and 2d, respectively. Thus, EGFR mutations were masked in some cases by the contamination of noncancerous cells in the macrodissected materials, in particular, in the small-sized type C tumors.

To confirm the results from the macrodissected small-sized adenocarcinomas in the first series, we examined an additional 17 small-sized adenocarcinomas for EGFR mutations using laser capture microdissection. The 17 cases consisted of 5 cases of type A, 7 of type B and 5 of type C. Ten of the 17 cases (59%) had EGFR mutations. As in the analysis of macrodissected materials, EGFR mutations were frequently detected in type A and B tumors (7 of 12, 58%). Moreover, EGFR mutations were also frequently detected in type C tumors (3 of 5, 60%). These results suggest that

TABLE II – FREQUENCY OF EGFR, KRAS, P53 MUTATIONS IN SMALL-SIZED ADENOCARCINOMAS OF THE LUNG

Variable	Frequency (%)						
	Total	Subtype					
		A	B	C	D	E	F
EGFR mutation	17/42 (40)	1/3 (33)	6/8 (75)	7/25 (28)	1/4 (25)	0/0	2/2 (100)
KRAS mutation	6/42 (14)	1/3 (33)	0/8 (0)	4/25 (16)	1/4 (25)	0/0	0/2 (0)
p53 mutation	12/42 (29)	0/3 (0)	0/8 (0)	9/25 (36)	2/4 (50)	0/0	1/2 (50)

TABLE III – FREQUENCY OF EGFR AND KRAS MUTATIONS IN MACRODISSECTED AND MICRODISSECTED SMALL-SIZED ADENOCARCINOMAS OF THE LUNG

Material	Frequency (%)							
	EGFR mutation				KRAS mutation			
	Total	Subtype			Total	Subtype		
		A	B	C		A	B	C
Macrodissected (n 5 27)	9/27 (33)	0/1 (0)	4/6 (67)	5/20 (25)	4/27 (15)	0/1 (0)	0/6 (0)	4/20 (20)
Macro- and Microdissected (n 5 9)								
Macrodissected	5/9 (56)	1/2 (50)	2/2 (100)	2/5 (40)	1/9 (11)	1/2 (50)	0/2 (0)	0/5 (0)
Microdissected	7/9 (78)	1/2 (50)	2/2 (100)	4/5 (80)	1/9 (11)	1/2 (50)	0/2 (0)	0/5 (0)
Microdissected (n 5 17)	10/17 (59)	2/5 (40)	5/7 (71)	3/5 (60)	2/17 (12)	1/5 (20)	0/7 (0)	1/5 (20)

EGFR mutations are present in the majority of small-sized adenocarcinoma, irrespective of noninvasive or invasive ones.

We also examined these 17 microdissected materials for KRAS mutations and found the mutations in 2 tumors without EGFR mutations. One of the tumors with KRAS mutation belonged to type A and the other belonged to type C. No KRAS mutation was detected in 7 type B tumors, consistent with the results of the first series.

In 4 of the microdissected type C tumors, we were able to separately collect cancer cells from the replacing growth (noninvasive) component and those from the central fibrotic area (invasive component) of a single tumor, and examined for EGFR and KRAS mutations in each of the components independently. Three of the 4 tumors had EGFR mutations in the cancer cells from both components, while the remaining 1 tumor had a KRAS mutation also in the cancer cells from both components. There was no tumor that showed mutations only in the invasive cancer cells or in the noninvasive ones (data not shown).

Types of EGFR mutations in stage I lung adenocarcinoma

In a total of 124 stage I lung adenocarcinomas analyzed in the present study, 52 EGFR mutations were detected in 48 cases. All types of mutations identified in the present study are shown in Table IV. There were 28 (54%) in-frame deletions, 4 (8%) in-frame duplications/insertions and 20 (38%) point mutations, but no frameshift mutations and nonsense mutations. Twenty-four of the 28 deletions were simple deletions of 5 amino acid residues from codon 746 to 750, and 1 deletion was a simple deletion of 5 amino acid residues from codon 747 to 751. The remaining 3 deletions were coupled with 1 or 2 amino acid substitutions. Among the 20 point mutations, the leucine to arginine mutation at codon 858 (L858R) in exon 21 was found in 11 tumors. Two tumors with the L858R mutation had other point mutations at codon 709 in exon 18, which were the glutamic acid to lysine mutation (E709K) and the glutamic acid to glycine mutation (E709G), respectively. Two tumors with deletions also had other point mutations in exon 20, which were the serine to isoleucine mutation at codon 768 (S768I) and the threonine to methionine mutation at codon 790 (T790M), respectively.

Discussion

One hundred and seven macrodissected stage I lung adenocarcinomas were initially examined for EGFR mutations, resulting in the detection of the mutations in 36 tumors (34%). In previous studies investigating NSCLC for EGFR mutations, no correlation was observed between EGFR mutations and stage of the disease.[4–7] The frequency of EGFR mutations in our first analysis of stage I adenocarcinomas was also consistent with those in several studies for EGFR mutations in various stages of adenocarcinomas in East Asia, including Japan.[4,5,8,9] These results indicate that EGFR mutations occur early in the development of lung adenocarcinoma. Indeed, we demonstrated here that, even in small-sized adenocarcinomas among stage I cases, 40% (17 of 42) had EGFR mutations. Therefore, we further analyzed the association of the mutations with histological features of small-sized adenocarcinoma. Among replacing growth types of small-sized adenocarcinoma, EGFR mutations were detected in 1 of 3 (33%) type A and 6 of 8 (75%) type B in the first analysis of macrodissected materials. The incidences were reproduced in the second analysis of microdissected materials [2 of 5 (40%) type A and 5 of 7 (71%) type B tumors, respectively]. On the other hand, in type C tumors, EGFR mutations were detected in 7 of 25 (28%) tumors in the first analysis. When 5 cases of type C in the first analysis were reanalyzed using microdissected materials, EGFR mutations were detected in 2 more cases. Moreover, the mutations were detected in 3 (60%) of the additional 5 type C microdissected materials. Thus, the lower frequency of EGFR mutations in type C tumors than in type A and B tumors in the first analysis is likely to be due to masking by the contamination of noncancerous cells in the macrodissected type C tumors. These results suggest that EGFR mutations are present in the majority of replacing growth type of small-sized adenocarcinomas. In addition, given a sequential progression from noninvasive (types A and B) to invasive tumors (type C) in the concept of multistage carcinogenesis of lung adenocarcinoma,[19] EGFR mutations are suggested to be involved in the development of noninvasive adenocarcinoma before progression to invasive adenocarcinoma. Indeed, when we separately examined cancer cells from the replacing growth (noninvasive) component and from the central fibrotic area (invasive component), EGFR mutations were always detected in both components and there was no tumor with the mutation only in the invasive component. These findings further support our suggestion. However, because more than 70% of the type B tumors had EGFR mutations, the mutations might be more strongly associated with the formation of type B tumors, featuring LBAC with foci due to collapse of the alveolar structure. If so, the results may imply that type C tumors are not always progressed sequentially from type A tumors through type B tumors. In other words, it can be said that BACs are preinvasive lesions for a subset of invasive adenocarcinomas.

There were some previous studies evaluating the correlation between EGFR mutations and histological features of lung adenocarcinoma. Yatabe et al.[20] reported that EGFR mutations were

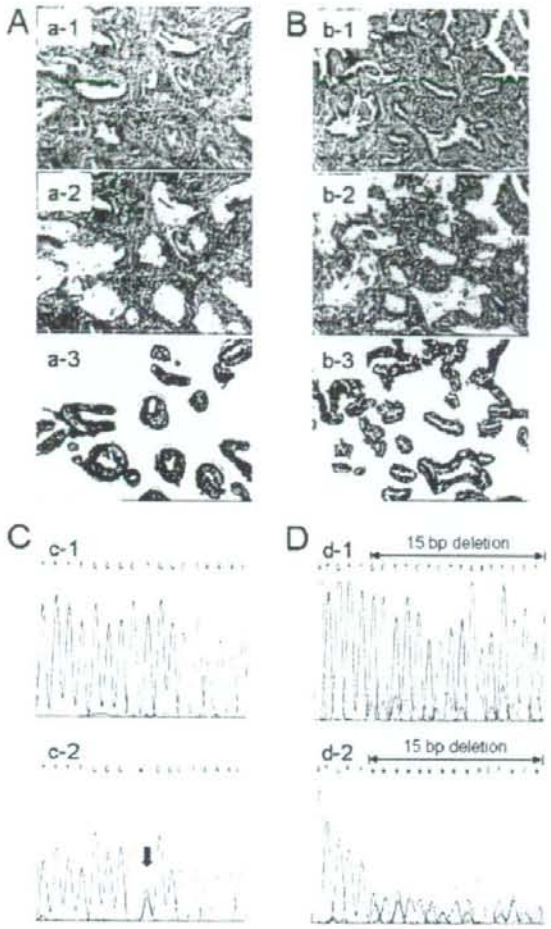

FIGURE 2 – Representative photographs of laser capture microdissection and sequence data showing difference between macrodissected materials and microdissected materials obtained from the same patients. (a) Microdissection of cancer cells from a 14 mm-sized type C adenocarcinoma (magnification 3100). (b) Microdissection of cancer cells from a 10-mm-sized type B adenocarcinoma (magnification 3100). (a-1) and (b-1) show central regions of the tumors in hematoxylin-stained tissue sections. (a-2) and (b-2) show the same sections after microdissection. (a-3) and (b-3) show the cells captured on the transfer films. (c) Sequence data obtained from the type C tumor shown in (a). The sequence chromatogram from macrodissected cancer cells is shown in (c-1), and that from microdissected cancer cells is shown in (c-2). A heterozygous point mutation at nucleotide 2573 (T to G) was detected in microdissected materials (down-arrow) but not in macrodissected ones. (d) Sequence data obtained from the type B tumor shown in (b). The sequence chromatogram from macrodissected cancer cells is shown in (d-1), and that from microdissected cancer cells is shown in (d-2). Although a heterozygous in-frame deletion was detected in both materials, peaks of a mutant allele in macrodissected materials are smaller than those in microdissected materials.

specifically involved in terminal respiratory unit-type adenocarcinoma, which corresponds to the majority of nonmucinous BACs. Furthermore, Marchetti et al.[6] reported that the histologic type of BAC was independently associated with EGFR mutations, in their multivariable analysis. Thus, most of the previous reports demonstrated the preferential occurrence of EGFR mutations in tumors with features of BAC.[2,6,21,22] However, in those studies, adenocarcinomas with BAC features contained various extents of invasive regions and often belonged to advanced stages. Furthermore, BAC and adenocarcinoma with BAC features might be confused. There were a few reports demonstrating the incidence of EGFR mutations in BAC according to the WHO classification. Kosaka et al.[4] reported that 3 of 5 BACs defined by the WHO criteria had EGFR mutations. On the other hand, Shigematsu et al.[7] reported that none of the 7 BACs collected in the United States had EGFR mutation. Thus, it has been controversial whether EGFR mutations are commonly present in strictly defined BAC, although the different incidence between their reports might be, in part, attributable to racial difference. In the present study, type A and B adenocarcinomas, which have absolutely no evidence of invasion, correspond to BAC in the WHO classification. Thus, this is the first report, to our knowledge, to analyze a comparable number of BAC for EGFR mutations and to demonstrate frequent occurrence of them.

The present study also showed a mutually exclusive correlation between EGFR mutations and KRAS mutations, consistent with the results reported previously.[4,6,7] KRAS mutations are known to be associated with exposure to carcinogens in tobacco smoke and to play an important role in the pathogenesis of lung adenocarcinoma.[23,24] In our analysis, 13 of 14 tumors with KRAS mutation were obtained from smokers, whereas EGFR mutations were significantly more frequent in nonsmokers. However, there was no significant difference in tumor size, differentiation and patient prognosis between the tumors with EGFR mutations and those with KRAS mutations (data not shown). In addition, it is of note that in a total of the 59 small-sized adenocarcinomas analyzed, 37 (63%) had either EGFR or KRAS mutations. When limited to the 17 cases of microdissected materials analyzed, 12 (71%) had either mutation. These results indicate that either EGFR or KRAS mutation may be required for the development of the majority of small-sized adenocarcinomas. Furthermore, separate analysis of cancer cells from noninvasive component and from invasive component in microdissected type C tumors showed that KRAS mutation, as well as EGFR mutation, was present in both components. Therefore, KRAS mutations may also contribute to the formation of noninvasive adenocarcinoma. However, it remains unclear whether biological behavior is necessarily equal in the tumors with EGFR mutation and those with KRAS mutation. In this study, we also obtained another interesting result that KRAS mutations were not detected in any type B tumors analyzed. This result was extremely different from the high incidence of EGFR mutations in type B tumors. Although EGFR mutations are likely to contribute to the formation of type B tumors, KRAS mutations may not be essential for it. Moreover, the result raises another possibility that tumors harboring KRAS mutations may not show type B formation, that is, the alveolar structure collapsing, or may sequentially and rapidly progress from type A to type C featuring an invasive phenotype due to acquisition of additional genetic alterations. To verify this assumption, further analyses of a larger number of those subtypes are needed.

We also examined a total of 124 cases used in this study for mutations in exon 20 of the ErbB2 gene, a member of the EGFR family. The mutation was detected only in 1 tumor (data not shown). This tumor was 5 cm in size and the patient with this tumor was male and a smoker. This ErbB2 mutation was a 12-bp in-frame duplication/insertion coding for the amino acids TVMA at codon 776. It was recently reported that ErbB2 mutations were present in 1–4% of lung cancers and that EGFR, ErbB2 and KRAS mutations were never present together in individual tumors.[25,26] In the present study, the tumor with ErbB2 mutation also had no EGFR and KRAS mutations. As a whole, in the first series of analysis, 49 of the 107 stage I lung adenocarcinomas (46%) had either EGFR, ErbB2 or KRAS mutations, suggesting that mutations in those 3 genes contribute to the development of about half of the stage I lung adenocarcinomas.

One of the recent strategies of cancer therapy is to discover mutated oncogenes, which play key roles in tumor growth and progression, and to develop drugs targeted to their protein prod-

TABLE IV – TYPES OF EGFR MUTATIONS IN STAGE I LUNG ADENOCARCINOMA

Type of mutation	Exon	Nucleotide number and sequence	Amino acid change	Case with mutation (%)
In-frame deletion	19	2235–2249 del GGAATTAAGAGAAGC	E746–A750 del	14 (26)
	19	2236–2250 del GAATTAAGAGAAGCA	E746–A750 del	10 (19)
	19	2236–2244 del GAATTAAGA, 2245–2248 GAAG > AGCC	E746–R748 del, E A749–750SP	1 (2)
	19	2237–2248 del AATTAAGAGAAG, 2249–2253 CAACA > TTGCT	E746–E749 del, AT750–751VA	1 (2)
	19	2240–2254 del TAAGAGAAGCAACAT	L747–T751 del	1 (2)
	19	2240–2257 del TAAGAGAAGCAACATCTC	L747–S752 del, P753S	1 (2)
In-frame duplication/ insertion	20	2308–2316 ins GCCAGCGTG	ASV 770–772 ins	2 (4)
	20	2311–2319 ins AGCGTGAAC	SVD 771–773 ins	1 (2)
	20	2327–2338 ins CCTACGTGTGCC, 2328 C > T	PYVC 776–779 ins	1 (2)
Point mutation	18	2125 G > A	E709K	1 (2)
	18	2126 A > G	E709G	2 (4)
	20	2303 G > T	S768I	2 (4)
	20	2369 C > T	T790M	1 (2)
	20	2405 T > G	V802G	1 (2)
	21	2573 T > G	L858R	13 (25)
Total				52 (100)

ucts.[27] In lung cancer, NSCLC with EGFR mutations was revealed to be sensitive to treatment with gefitinib, a TK inhibitor of EGFR. However, the role of this drug in therapeutic strategies of NSCLC has not yet been established. At present, gefitinib is generally applied to pretreated advanced NSCLC as a second or third line of chemotherapy. However, by considering our results that EGFR mutations were present in a majority of BACs, this drug can be used for earlier stage disease rather than advanced stage disease. This suggestion is supported by Dowell and Minna.[28] They indicated the possibility to use relatively nontoxic TK inhibitors as chemopreventive agents, if EGFR mutations are present in preneoplastic lesions.

In addition, recent studies revealed that the threonine to methionine mutation at codon 790 (T790M) of the EGFR gene was a second mutation and was associated with acquired resistance to EGFR TK inhibitors in NSCLC.[29,30] In these reports, this mutation was found in specimens from the patients whose disease progressed during the treatment with these drugs, and not found in untreated cases. Thereafter, Toyooka et al.[4,31] identified the T790M mutations in addition to L858R mutations in tumors before chemotherapy or radiotherapy in 2 NSCLC patients, both of whom later had recurrent diseases and eventually died. Therefore, they suggested that tumors with these double mutations are very aggressive. In the present study, we also detected the T790M mutation with another mutation (deletion from codon 747 to 751 in exon 19) in a patient who had received no chemotherapy or radiotherapy before undergoing surgical resection and died due to disease recurrence 50 months after surgery. This case showed similar unfavorable prognosis to those reported by Toyooka et al. and support their suggestion. Thus, detection of the T790M mutation in addition to other types of EGFR mutations is also important to establish the role of EGFR TK inhibitors in future therapeutic strategies against lung adenocarcinoma and to evaluate the aggressiveness of lung adenocarcinoma.

In summary, frequent EGFR mutations in types A and B of small-sized lung adenocarcinoma strongly indicated that the mutations are a critical genetic alteration for the formation of noninvasive BACs. The present study was performed using stage I lung adenocarcinomas, all of which were obtained from Japanese patients. Given the more frequent occurrence of EGFR mutations in the Japanese than in the Caucasians, type A and B tumors, which correspond to BAC in the WHO classification, may be a relatively specific subtype of lung adenocarcinoma in the Japanese.

References

1. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res 2003;284:31–53.
2. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, 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;350:2129–39.
3. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
4. 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: 8919–23.
5. Huang SF, Liu HP, Li LH, Ku YC, Fu YN, Tsai HY, Chen YT, Lin YF, Chang WC, Kuo HP, Wu YC, Chen YR, 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;10:8195–203.
6. Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Camplese PP, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R, 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;23:857–65.
7. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97:339–46.
8. Han SW, Kim TY, Hwang PG, Jeong S, Kim J, Choi IS, Oh DY, Kim JH, Kim DW, Chung DH, Im SA, Kim YT, 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; 23:2493–501.
9. Qin BM, Chen X, Zhu JD, Pei DQ. Identification of EGFR kinase domain mutations among lung cancer patients in China: implication for targeted cancer therapy. Cell Res 2005;15:212–7.
10. Cortes-Funes H, Gomez C, Rosell R, Valero P, Garcia-Giron C, Velasco A, Izquierdo A, Diz P, Camps C, Castellanos D, Alberola V, Cardenal F, et al. Epidermal growth factor receptor activating mutations in Spanish gefitinib-treated non-small-cell lung cancer patients. Ann Oncol 2005;16:1081–6.
11. Noguchi M, Morikawa A, Kawasaki M, Matsuno Y, Yamada T, Hirohashi S, Kondo H, Shimosato Y. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. Cancer 1995;75:2844–52.
12. Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E, eds. Histological typing of lung and pleural tumours,3rd ed. New York: Springer Verlag, 1999.33–40.
13. Colby TV, Noguchi M, Henschke C, Vazquez MF, Geisinger K, Yokose T, Chori P, Rami-Porta R, Franks T. Adenocarcinoma. In:

Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, eds. Pathology and genetics: tumours of the lung, pleura, thymus and heart. Lyon: IARC Press, 2004;35–44.
14. Aoyagi Y, Yokose T, Minami Y, Ochiai A, Iijima T, Morishita Y, Oda T, Fukao K, Noguchi M. Accumulation of losses of heterozygosity and multistep carcinogenesis in pulmonary adenocarcinoma. Cancer Res 2001;61:7950–4.
15. Sobin LH, Wittekind CH, eds. TNM classification of malignant tumours, 6th ed. New York: Wiley-Liss, 2002;99–103.
16. Yokota J, Wada M, Shimosato Y, Terada M, Sugimura T. Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc Natl Acad Sci USA 1987;84:9252–6.
17. Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 1996;2:1035–7.
18. Tomizawa Y, Kohno T, Kondo H, Otsuka A, Nishioka M, Niki T, Yamada T, Maeshima A, Yoshimura K, Saito R, Minna JD, Yokota J. Clinicopathological significance of epigenetic inactivation of RASSF1A at 3p21.3 in stage I lung adenocarcinoma. Clin Cancer Res 2002;8:2362–8.
19. Yokota J, Kohno T. Molecular footprints of human lung cancer progression. Cancer Sci 2004;95:197–204.
20. Yatabe Y, Kosaka T, Takahashi T, Mitsudomi T. EGFR mutation is specific for terminal respiratory unit type adenocarcinoma. Am J Surg Pathol 2005;29:633–9.
21. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, 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 USA 2004;101:13306–11.
22. Miller VA, Hirsch FR, Johnson DH. Systemic therapy of advanced bronchioloalveolar cell carcinoma: challenges and opportunities. J Clin Oncol 2005;23:3288–93.
23. Slebos RJ, Hruban RH, Dalesio O, Mooi WJ, Offerhaus GJ, Rodenhuis S. Relationship between K-ras oncogene activation and smoking in adenocarcinoma of the human lung. J Natl Cancer Inst 1991; 83:1024–7.
24. Rodenhuis S, Slebos RJ. Clinical significance of ras oncogene activation in human lung cancer. Cancer Res 1992;52:2665s–9s.
25. Stephens P, Hunter C, Bignell G, Edkins S, Davies H, Teague J, Stevens C, O'Meara S, Smith R, Parker A, Barthorpe A, Blow M, et al. Lung cancer: intragenic ERBB2 kinase mutations in tumours. Nature 2004;431:525–6.
26. Shigematsu H, Takahashi T, Nomura M, Majmudar K, Suzuki M, Lee H, Wistuba II, Fong KM, Toyooka S, Shimizu N, Fujisawa T, Minna JD, et al. Somatic mutations of the HER2 kinase domain in lung adenocarcinomas. Cancer Res 2005;65:1642–6.
27. Weinstein IB. Cancer. Addiction to oncogenes—the Achilles heal of cancer. Science 2002;297:63–4.
28. Dowell JE, Minna JD. Chasing mutations in the epidermal growth factor in lung cancer. N Engl J Med 2005;352:830–2.
29. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG, Halmos B. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
30. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med2:e73, 2005.
31. Toyooka S, Kiura K, Mitsudomi T. EGFR mutation and response of lung cancer to gefitinib. N Engl J Med 2005;352:2136.

Hypermethylation of the *TSLC1/IGSF4* Promoter Is Associated with Tobacco Smoking and a Poor Prognosis in Primary Nonsmall Cell Lung Carcinoma

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BACKGROUND. The tumor suppressor gene *TSLC1/IGSF4* on chromosomal region 11q23 is frequently inactivated by promoter methylation in various cancers, including nonsmall cell lung carcinoma (NSCLC). Several studies have demonstrated that the hypermethylation of the CpG islands of genes, including tumor suppressors, is associated with exposure to tobacco smoke. The purpose of this study was to investigate the possible association of *TSLC1/IGSF4* methylation with tobacco smoking as well as with the clinical characteristics of tumors using a large number of primary NSCLC.

METHODS. The promoter methylation of *TSLC1/IGSF4* was analyzed in 103 primary NSCLC. TSLC1/IGSF4 expression was examined by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry, whereas its methylation status was determined by bisulfite single-strand conformation polymorphism (SSCP) coupled with bisulfite sequencing.

RESULTS. The *TSLC1/IGSF4* promoter was methylated in 45 (44%) of 103 primary NSCLC. Methylation was observed in all histologic subtypes of NSCLC, including adenocarcinoma (29 of 68, 43%), squamous cell carcinoma (14 of 26, 54%), adenosquamous carcinoma (1 of 2, 50%), and large cell carcinoma (1 of 7, 14%). The incidence of methylation in tumors was significantly higher in male patients than in female patients (P .027). The *TSLC1/IGSF4* methylation was preferentially observed in heavy smokers (smoking index 800) (P .0054). Furthermore, in smokers the methylation was significantly associated with pack-years smoked (P .034) and cigarettes per day (P .021). The *TSLC1/IGSF4* methylation was also significantly associated with a shorter disease-free survival (P .049), providing an independent prognostic factor (P .038) in adenocarcinoma patients.

CONCLUSIONS. *TSLC1/IGSF4* methylation is associated with tobacco smoking and could be an indicator of poor prognosis. *Cancer* 2006;106:1751–8.

KEYWORDS: promoter methylation, *TSLC1/IGSF4* gene, nonsmall cell lung carcinoma, tobacco smoking, prognosis, *DAL-1/4.1B* gene, bisulfite single-strand conformational polymorphism.

Aberrant methylation of CpG islands in the promoter regions is one of the major mechanisms for the silencing of tumor suppressor genes.[1] In nonsmall cell lung carcinoma (NSCLC), the promoter methylation of various genes, including *p16/CDKN2*[2,3] and *RASSF1A*,[4,5] has been demonstrated to be involved in its development and/or progression. As is well known, the largest etiologic cause of lung carcinoma is tobacco smoking, and it is estimated that 86% of lung carcinoma deaths are related to smoking.[6] Although there is no direct evidence that smoking

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induces the hypermethylation of DNA, recent reports have demonstrated the association of DNA methylation with exposure to tobacco carcinogens. In rats, the de novo methylation of the CpG island of the *p16/CDKN2A* gene occurred frequently in primary lung tumors induced by the inhalation of cigarette smoke.[7] In addition, the methylation of the *p16/CDKN2A* gene was detected in 94% of lung adenocarcinomas induced by the tobacco-specific carcinogenic nitrosoamine, 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK).[3] In human NSCLC, it has also been demonstrated that the hypermethylation of the *p16/CDKN2A*[8,9] and the *RASSF1A* genes[10] was significantly associated with tobacco smoking, suggesting that the carcinogens in tobacco smoke induce the hypermethylation of the DNA.

The *TSLC1/IGSF4* on chromosomal fragment 11q23.2 is another tumor suppressor gene in NSCLC that we have previously identified by functional complementation through the suppression of tumorigenicity in nude mice.[11,12] *TSLC1/IGSF4* encodes an immunoglobulin superfamily cell adhesion molecule.[13,14] Loss of *TSLC1/IGSF4* expression was frequently observed in various cancer cell lines, including those from NSCLC, where the hypermethylation of six CpG sites around the promoter regions was strongly correlated with gene silencing. The hypermethylation of *TSLC1/IGSF4* was also detected in about half of 48 primary NSCLC[15] and 20% to 50% of primary tumors from prostate,[16] breast,[17] pancreas,[18] stomach,[19] esophagus,[20] nasopharynx,[21] and uterine cervix.[22] Furthermore, we have recently demonstrated that the TSLC1/IGSF4 protein is directly associated with an actin-binding protein, DAL-1/4.1B,[23] and that loss of DAL-1/4.1B expression by promoter methylation is also a frequent event in NSCLC, suggesting that DAL-1/4.1B is an additional tumor suppressor candidate in human NSCLC acting in the same cascade as TSLC1/IGSF4.[24]

In the present study, we extensively examined the promoter methylation of the *TSLC1/IGSF4* gene in 103 primary NSCLC by bisulfite SSCP, a sensitive and quantitative method for the detection of the methylation status of the CpG sites within a gene promoter, and investigated the possible association of the *TSLC1/IGSF4* methylation with the smoking parameters of the patients. We also analyzed the clinicopathologic characteristics of NSCLC tumors showing the *TSLC1/IGSF4* methylation. Our results indicate a significant association of *TSLC1/IGSF4* methylation with tobacco smoking as well as poor prognosis of the patients with adenocarcinoma.

MATERIALS AND METHODS

Tumor Samples and Cell Lines

One-hundred and three primary NSCLC tumors and corresponding noncancerous tissues from the same patients were surgically resected and histologically diagnosed at the Department of Diagnostic Pathology, National Cancer Center Hospital, Japan. After surgical removal, all samples were immediately frozen and stored at −135°C. Clinicopathologic data were extracted from the medical records as well as pathology reports. The analyses of human samples were carried out in accordance with institutional guidelines. An NSCLC cell line (SK-LU-1) was purchased from the American Type Culture Collection (Rockville, MD). An NSCLC cell line (RERF-LC-MS) was from the Human Science Research Resources Bank (Osaka, Japan). The cells were cultured according to the suppliers' recommendations.

Bisulfite Sequencing

Bisulfite sequencing was carried out as described previously.[25] Briefly, genomic DNA was denatured with NaOH (0.3 M) and incubated with sodium bisulfite (3.1 M; Sigma, St. Louis, MO) and hydroquinone (0.8 mM; Sigma), pH 5.0, at 55°C for 20 hours, followed by purification and treatment with NaOH (0.2 M) for 10 minutes at 37°C. Modified DNA (100 ng) was subjected to polymerase chain reaction (PCR) using a pair of primers, TSLC1 PRI F (5′-AAGGTGAGTGACGGAAATTTGTAACGT-3′) and TSLC1 PRI R (5′-GTACAAAAACTCGAACTCCAAAAAACGAA-3′) to amplify a 126-basepair (bp) DNA fragment (−464 bp to −339 bp from the first adenine in the initial codon of methionine), including the promoter sequence of *TSLC1/IGSF4*. The PCR products were subcloned to confirm the sequences in at least 6 clones.

Bisulfite Single-Strand Conformational Polymorphism Analysis

For bisulfite single-strand conformational polymorphism (SSCP) analysis, the same 126-bp fragments were amplified by PCR using a pair of primers, TSLC1 PRI F and TSLC1 PRI R, where the former was end-labeled with Texas Red. The PCR product was diluted 10 times with a loading buffer (90% deionized formamide, 0.01% New Fuchsin, and 10 mM EDTA), heat-denatured for 3 minutes at 95°C, cooled on ice for 3 minutes, and then loaded onto the gel (0.5xMDE Gel Solution; BMA, Rockland, ME). Electrophoresis was carried out for 120 minutes at 20°C using SF5200 (Hitachi Electronics Engineering, Tokyo, Japan) with cooling systems. The results were analyzed using a DNA Fragment Analyzer (Hitachi Electronics Engi-

neering). The criterion for hypermethylation was met when the ratio of the methylated fragments to the unmethylated fragments (methylation ratio) was more than 0.20.

Immunohistochemistry

Freshly frozen specimens were fixed with cold 4% paraformaldehyde for 24 hours and then rinsed in a 0.2 M phosphate-buffered saline (PBS, pH 7.4) containing 25% sucrose overnight at 4°C for cryoprotection. The specimens were then cut into 4- m-thick sections and treated with 1% H_2O_2 for 10 minutes to inhibit intrinsic peroxidase activity and with 3% bovine serum albumin (BSA) in PBS for 1 hour to prevent nonspecific antibody binding. The sections were incubated with chicken anti-TSLC1 monoclonal antibody (3E1; 1:500 dilution) in PBS containing 1% BSA as a primary antibody for 12 hours at 4°C. This chicken monoclonal antibody against the extracellular domain of TSLC1/IGSF4 was generated by a member of the research team (A.L.).[26] The sections were subsequently incubated with peroxidase-conjugated antichicken immunoglobulin G (IgG) antibody (MBL, Nagoya, Japan) for 1 hour at room temperature and with a 3 ,3 - diaminobenzidine tetrahydrochloride solution containing H_2O_2 (Dako, Carpinteria, California) for a few minutes. Finally, the sections were counterstained with hematoxylin.

Statistical Analysis

The Wilcoxon rank sum test and Fisher exact tests (or ^{2} test) were used for continuous and categorical variables in univariate analysis, respectively. Kaplan–Meier analysis was performed to estimate a survival function over time for individual covariates. Disease-free survival between tumors with methylation and those without methylation was compared by the log rank test. Cox proportional hazards regression model was conducted to estimate the relation between disease-free survival and the variables that showed statistical significance in Kaplan–Meier analysis. Stat View 5.0 (SAS institute, Cary, NC) was used for the analysis. Differences with P values of less than .05 were considered significant.

RESULTS

TSLC1/IGSF4 Methylation in Primary NSCLC Tumors

The *TSLC1/IGSF4* gene harbors a typical sequence of about 900 bp matching the CpG island that covers the upstream region, exon 1, and intron 1 of the *TSLC1/IGSF4* gene (Fig. 1A). We have previously reported that the hypermethylation of the 6 CpG sites within the promoter region strongly correlates with the loss of TSLC1/IGSF4 expression.[12] To detect the methylation status of this fragment quantitatively, we used bisulfite SSCP coupled with bisulfite sequencing, which can distinguish the completely methylated or partially methylated fragments from the unmethylated fragment, as described previously.[15] When the promoter fragment of the *TSLC1/IGSF4* from NSCLC cells was examined by this method, SK-LU-1 cells lacking TSLC1/IGSF4 expression showed the mobility of hypermethylation, whereas the RERF-LC-MS cells expressing TSLC1/IGSF4 showed that of unmethylation. Moreover, the results of bisulfite SSCP corresponded well with those by bisulfite sequencing (Fig. 1B). These results suggest that the methylation of the 6 CpG sites within the *TSLC1/IGSF4* promoter strongly correlates with loss of expression and can be detected by bisulfite SSCP.

Using this method, we next examined the methylation status of the 6 CpG sites of the *TSLC1/IGSF4* gene in 103 primary NSCLC tumors. Representative results are shown in Figure 1B, where signals corresponding to the methylation were observed in L302C, L255C, and L227C, whereas only unmethylated signals were detected in L299C. The results of bisulfite sequencing of 6 independent clones in each sample, again, coincided well with those of bisulfite SSCP. Similar analyses by bisulfite SSCP revealed that 45 of 103 tumors (44%) showed hypermethylation of the *TSLC1/IGSF4* gene. On the other hand, all 10 samples examined from noncancerous portion of the lung showed unmethylation. Immunohistochemistry of a subset of NSCLC tumors using a specific antibody against TSLC1/IGSF4 confirmed that all 6 tumors with promoter methylation showed loss or marked reduction in the amount of TSLC1 expression, whereas 6 tumors without methylation expressed considerable amounts of TSLC1 along the cell membrane (Fig. 1C,D). These findings suggest that the promoter methylation is also correlated with loss of TSLC1/IGSF4 expression in primary NSCLC.

Clinicopathologic and Smoking Parameters of Tumors with the Methylated *TSLC1/IGSF4* Gene

The methylation status of the *TSLC1/IGSF4* gene was compared with the clinicopathologic features of each NSCLC tumor (Table 1). *TSLC1/IGSF4* methylation was observed in all histologic subtypes of NSCLC, including 29 of 68 (43%) adenocarcinomas, 14 of 26 (54%) squamous cell carcinomas, 1 of 2 (50%) adenosquamous carcinomas, and 1 of 7 (14%) large cell carcinomas. The incidence of *TSLC1/IGSF4* methylation was slightly higher in squamous cell carcinoma than in adenocarcinoma, although the difference was not statistically significant. Notably, the incidence of *TSLC1/IGSF4* methylation was significantly higher in males

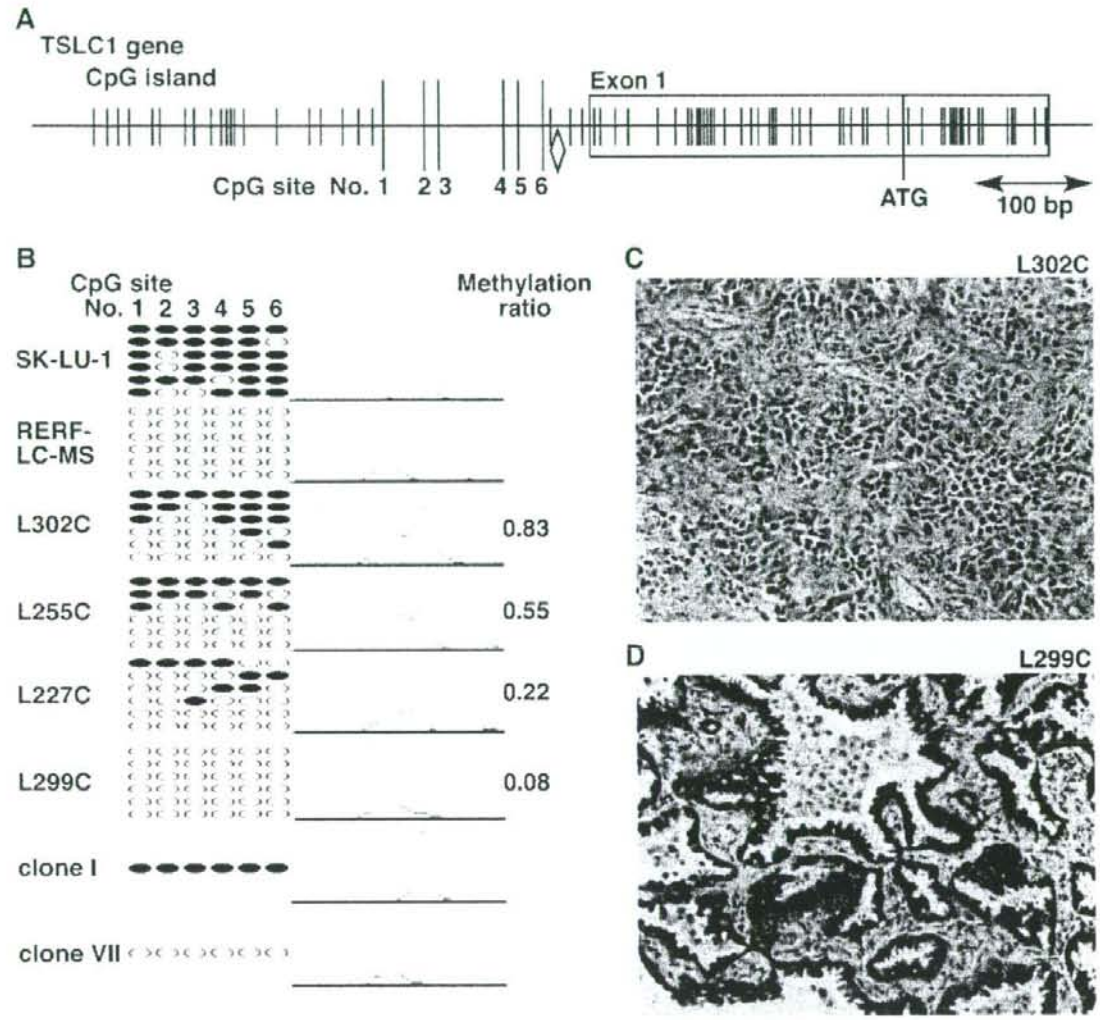

FIGURE 1. Expression and methylation status of the *TSLC1/IGSF4* gene promoter in lung cancer. (A) Schematic representation of the upstream region of the *TSLC1/IGSF4* gene is shown. Vertical bars indicate CpG sites within the CpG island of the *TSLC1/IGSF4* gene, in which long vertical bars indicate the 6 CpG sites examined. An open box and open diamond indicate exon 1 and the predicted TATA box sequence, respectively. ATG indicates the initial codon of methionine. (B) Summary of the bisulfite sequencing and bisulfite SSCP analysis in 2 NSCLC cell lines and 4 primary NSCLC tumors. Bisulfite sequences of 6 independent clones from each cell line or tumor are shown on the left, whereas the signals in SSCP analysis are shown on the right. Black and white circles represent methylated and unmethylated CpG sites, respectively. The results of completely methylated (clone I) and unmethylated (clone VII) fragments are shown at the bottom. (C,D) Immunohistochemical analysis of TSLC1/IGSF4 protein in lung adenocarcinoma. The TSLC1/IGSF4 protein is absent in a poorly differentiated adenocarcinoma, L302C, with the methylated *TSLC1/IGSF4* promoter (C). Conversely, the TSLC1/IGSF4 protein is present in a well differentiated adenocarcinoma, L299C, with the unmethylated *TSLC1/IGSF4* promoter (D). Original magnifications 100.

than in females (P .027). Other clinical characteristics, including age, family history of the patients, and tumor stage, were not correlated with *TSLC1/IGSF4* methylation. The preferential methylation of the *TSLC1/IGSF4* in male patients and in squamous cell carcinomas prompted us to examine the smoking parameters of the patients with NSCLC showing the *TSLC1/IGSF4* methylation.

Of the 103 patients analyzed, 26 had never smoked, 26 were exsmokers (who had ceased smoking

TABLE 1
Promoter Methylation of the *TSLC1* Gene and Clinicopathologic Characteristics in Primary NSCLCs

		TSLC1 gene		
	All subjects	Methylation	Unmethylation	*P*[a]
Subjects, *n*	103	45	58	
Age†	66.5 9.35	65.8 8.45	67.1 10.0	NS
Gender				
Male	74	36	38	
Female	29	9	20	.027
Family history				
Cancer positive	60	23	37	
Cancer negative	43	22	21	NS
Smoking status				
Never	26	9	17	
Exsmoker	26	10	16	
Current	51	26	25	NS
Smoking index				
0 799	46	13	33	
800	57	32	25	.0054
Smokers				
Pack-years†	62.3 39.4	72.3 42.1	53.6 35.1	.034
Years smoked†	40.1 11.4	41.2 10.3	39.2 12.3	NS
Cigarettes per day†	30.3 16.9	34.6 17.3	26.6 15.8	.021
Starting age†	20.8 5.09	20.6 4.22	21.1 5.08	NS
Years quit†	14.2 10.0	13.7 5.62	14.6 12.2	NS
Histologic differentiation				
Adenocarcinoma	68	29	39	
Squamous cell carcinoma	26	14	12	
Adenosquamous carcinoma	2	1	1	
Large cell carcinoma	7	1	6	NS
Stage				
I	51	21	30	
II	27	12	15	
III	22	11	11	
IV	3	1	2	NS

NSCLC: nonsmall cell lung cancer; NS: not significant.
[a] Wilcoxon rank sum test and Fisher exact (or ^{2}) test was used for continuous and categoric variables, respectively.
† Mean SD.

at least 1 yr before the diagnosis), and 51 were current smokers. Promoter methylation of the tumors was more likely to be observed in current smokers (26 of 51, 51%) than in exsmokers (10 of 26, 38%) or in those who had never smoked (9 of 26, 35%), although the difference was not statistically significant. Importantly, the incidence of *TSLC1/IGSF4* methylation in tumors was significantly higher in heavy smokers with a smoking index (the number of cigarettes smoked per day years of smoking) of more than 800 (P .0054). In smokers, the methylation of *TSLC1/IGSF4* was significantly associated with pack-years smoked (P .034) and cigarettes per day (P .021). These results suggest that the methylation of the *TSLC1/IGSF4* promoter would be associated with tobacco smoking, although the duration of smoking and starting age in smokers, as well as the time elapsed since quitting smoking in exsmokers, were not correlated with the *TSLC1/IGSF4* methylation.

Correlation with Methylation and Patient's Prognosis

We finally investigated the correlation of the *TSLC1/IGSF4* methylation in tumors with the disease-free survival of the patients using Kaplan–Meier analysis. The methylation of the *TSLC1/IGSF4* gene was significantly associated with a shorter disease-free survival in adenocarcinomas (P .049) but not in squamous cell carcinomas (P .31) (Fig. 2A). Moreover, the multivariate analysis by the Cox hazard model revealed that the *TSLC1/IGSF4* methylation was an independent prognostic factor to predict poor prognosis for adenocarcinoma patients, as shown in Table 2 (P .038: hazards ratio, 2.52). Because the TSLC1/IGSF4 protein was shown to interact with the DAL-1/

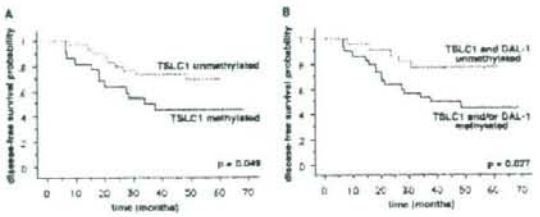

FIGURE 2. Disease-free survival of a patient with lung adenocarcinoma. The Kaplan–Meier log rank test was used to examine the correlation of the methylation in tumors with the disease-free survival of the patients. (A) Disease-free survival of the 56 patients undergoing potentially curative resection of adenocarcinoma. (B) Association between methylation of either the *TSLC1/IGSF4* or the *DAL-1/4.1B* genes and disease-free survival of the 56 patients with adenocarcinoma.

TABLE 2
Prognostic Value of *TSLC1* Methylation and Clinicopathological Characteristics for Disease-Free Survival in 56 Patients with Lung Adenocarcinoma

	Kaplan–Meier Log rank test	Multivariate proportional hazards model		
Variable	*P*	Hazards ratio	95%CI	*P*
Age 70 y vs. 70 y	.915	NS		
Gender, female vs. male	.662	NS		
Smoking Index (800 vs. 800	.077	NS		
Differentiation, well vs. others	.143	NS		
Stage, I vs. others	.037	2.62	1.10–6.23	.030
TSLC1, unmethylation vs. methylation	.049	2.52	1.05–6.03	.038

95%CI: 95% confidence interval; NS: not selected in multivariate proportional hazards model.

4.1 B protein, we further investigated whether the aberrations of the TSLC1-DAL-1 cascade could be associated with the disease-free survival of the patients. The patients with methylation of *TSLC1/IGSF4* and/or *DAL-1/4.1 B* had a significantly shorter disease-free survival than the patients with unmethylation of both genes in adenocarcinoma (*P* .027), as shown in Figure 2B. However, no significant difference was observed between the disease-free survival of the patients with both methylated *TSLC1/IGSF4* and *DAL-1/4.1B* genes and that with methylated *TSLC1/IGSF4* gene alone (*P* .59, data not shown).

DISCUSSION

In the present study, we demonstrated a high incidence of the methylation of the *TSLC1/IGSF4* promoter (45 of 103; 44%) in primary NSCLC by bisulfite SSCP analysis. The advantages of bisulfite SSCP are its high sensitivity and its ability to detect the quantitative as well as the allelic status of methylation. Thus, bisulfite SSCP is especially useful for analyzing the methylation status of genes in primary tumors, as discussed previously.[15,24] By using this method, we detected the methylation and gene silencing of the *TSLC1/IGSF4* in all histologic subtypes of human NSCLC. The incidence of methylation was significantly higher in male patients than in female patients (*P* .027) and slightly higher in squamous cell carcinomas than in adenocarcinomas. These findings are particularly interesting when we consider that the frequency of tobacco smoking is generally higher in males than in females and in squamous cell carcinoma than in adenocarcinoma.[27] As expected, the methylation of the *TSLC1/IGSF4* in tumors was significantly associated with heavy smoking, with a smoking index of more than 800 (*P* .0054). Furthermore, *TSLC1/IGSF4* methylation was associated with pack-years smoked (*P* .034) and cigarettes per day (*P* .021) in smokers. Therefore, it is possible to speculate that tobacco smoking induces aberrant methylation of the *TSLC1/IGSF4* gene in precursor or tumor cells of NSCLC.

The association of promoter methylation with smoking has also been reported in various other tumor suppressor genes in NSCLC, including the *p16/CDKN2A* and *RASSF1A* genes.[8–10] Tobacco smoke contains many carcinogens, including NNK, polyaromatic hydrocarbons, chromium, cadmium, plutonium, and nickel.[28] In addition, tobacco smoke is a mucosal irritant that induces inflammation, resulting in the generation of oxygen-free radicals. Furthermore, smoking increases the activity of DNA methyltransferase,[29] which drives the de novo hypermethylation of susceptible loci.[30] These direct or indirect mechanisms could be involved in the enhanced DNA methylation in NSCLC in heavy smokers.

In this connection, we recently examined the methylation status of another tumor suppressor gene, *DAL-1/4.1B*, in the same series of 103 NSCLC and found that 59 (57%) tumors showed hypermethylation. Both TSLC1/IGSF4 and DAL-1/4.1 B proteins act in the same cascade of NSCLC involving cell adhesion. However, *DAL-1/4.1B* methylation was not correlated with a smoking index of more than 800 (*P* .11, data not shown). These findings suggest that the promoter methylation induced by smoking would be rather gene-specific, although the precise molecular mechanism has not been elucidated. It has been reported so far that several factors, including the activity of DNA methyltransferase and locus-specific factors, such as

SP1 transcription factors, chromatin structure, proximity to a methylation center, and the preexisting methylation status of CpG islands, could be involved in the different susceptibility of methylation among the genes.[30] Furthermore, it is expected that the hypermethylation of tumor suppressor genes would be selected and fixed in tumor cells in accordance with the degree of growth advantage caused by their inactivation. Further studies will be required to elucidate the causal involvement of smoking in the epigenetic inactivation of specific genes in smokers.

The TSLC1 /IGSF4 protein is involved in cell adhesion and the formation of epithelial-like cell structures, whereas loss of its function may lead cancer cells to invasion or metastasis.[14] In fact, the restoration of TSLC1 /IGSF4 expression to normal levels not only suppressed tumor formation in the skin but also inhibited metastasis from the spleen to the liver of a lung adenocarcinoma cell line, A549, in athymic nude mice.[12,23] Consistent with these findings, our present study demonstrated that the methylation of the *TSLC1 /IGSF4* gene was significantly associated with a shorter disease-free survival in patients with lung adenocarcinoma (P .049). *TSLC1 /IGSF4* methylation further provides an independent prognostic factor (P .038). A previous immunohistochemical study of 38 lung adenocarcinomas has also demonstrated that the loss or reduction of TSLC1 expression was associated with a poor prognosis.[31] In addition, the expression of TSLC1 protein was down-regulated in the invasive components of lung adenocarcinoma but not in those of bronchioalveolar carcinoma.[26] These findings suggest that TSLC1 /IGSF4 is likely to be involved in the biological aggressiveness of the tumor cells, although the *TSLC1 /IGSF4* methylation was not significantly associated with the disease stage in the present study.

Finally, this study reports that the majority of NSCLC tumors (71 of 103, 69%) show epigenetic inactivation in either the *TSLC1 /IGSF4* or the *DAL-1 /4.1 B* gene. Furthermore, patients with methylation of either gene present a significantly shorter disease-free survival than those with unmethylation of both the *TSLC1 /IGSF4* and the *DAL-1 /4.1 B* genes in adenocarcinoma (P .027). When we consider that the TSLC1 / IGSF4 and DAL-1 /4.1 B proteins act in the same cascade of tumor suppression through cell adhesion, these findings suggest that the disruption of the TSLC1 /IGSF4-DAL-1 /4.1 B cascade plays a critical role in the majority of primary NSCLC.

In conclusion, we detected the promoter methylation of *TSLC1 /IGSF4* in 45 of 103 (44%) primary NSCLC. This methylation is associated with tobacco smoking and could provide an independent factor for poor prognosis. The promoter of the *TSLC1 /IGSF4* gene would be one of the important targets of methylation by smoking in the development or progression of NSCLC.

REFERENCES

1. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med.* 2003; 349:2042-2054.
2. Merlo A, Herman JG, Mao L, et al. 5 CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med.* 1995;1:686-692.
3. Belinsky SA, Nikula KJ, Palmisano WA, et al. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc Natl Acad Sci U S A.* 1998;95:11891-11896.
4. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet.* 2000;25:315-319.
5. Burbee DG, Forgacs E, Zochbauer-Muller S, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst.* 2001; 93:691-699.
6. Shopland DR, Eyre HJ, Pechacek TF. Smoking-attributable cancer mortality in 1991: is lung cancer now the leading cause of death among smokers in the United States? *J Natl Cancer Inst.* 1991;83:1142-1148.
7. Swafford DS, Middleton SK, Palmisano WA, et al. Frequent aberrant methylation of p16INK4a in primary rat lung tumors. *Mol Cell Biol.* 1997;17:1366-1374.
8. Kim DH, Nelson HH, Wiencke JK, et al. p16(INK4a) and histology-specific methylation of CpG islands by exposure to tobacco smoke in non-small cell lung cancer. *Cancer Res.* 2001;61:3419-3424.
9. Jarmalaite S, Kannio A, Anttila S, Lazutka JR, Husgafvel-Pursiainen K. Aberrant p16 promoter methylation in smokers and former smokers with nonsmall cell lung cancer. *Int J Cancer.* 2003;106:913-918.
10. Kim DH, Kim JS, Ji YI, et al. Hypermethylation of RASSF1A promoter is associated with the age at starting smoking and a poor prognosis in primary non-small cell lung cancer. *Cancer Res.* 2003;63:3743-3746.
11. Murakami Y, Nobukuni T, Tamura K, et al. Localization of tumor suppressor activity important in nonsmall cell lung carcinoma on chromosome 11q. *Proc Natl Acad Sci U S A.* 1998;95:8153-8158.
12. Kuramochi M, Fukuhara H, Nobukuni T, et al. TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. *Nat Genet.* 2001;27:427-430.
13. Gomyo H, Arai Y, Tanigami A, et al. A 2-Mb sequence-ready contig map and a novel immunoglobulin superfamily gene IGSF4 in the LOH region of chromosome 11q23.2. *Genomics.* 1999;62:139-146.
14. Masuda M, Yageta M, Fukuhara H, et al. The tumor suppressor protein TSLC1 is involved in cell-cell adhesion. *J Biol Chem.* 2002;277:31014-31019.
15. Fukami T, Fukuhara H, Kuramochi M, et al. Promoter methylation of the TSLC1 gene in advanced lung tumors and various cancer cell lines. *Int J Cancer.* 2003;107: 53-59.

16. Fukuhara H, Kuramochi M, Fukami T, et al. Promoter methylation of TSLC1 and tumor suppression by its gene product in human prostate cancer. *Jpn J Cancer Res.* 2002;93:605-609.
17. Allinen M, Peri L, Kujala S, et al. Analysis of 11q21-24 loss of heterozygosity candidate target genes in breast cancer: indications of TSLC1 promoter hypermethylation. *Genes Chromosomes Cancer.* 2002;34:384-389.
18. Jansen M, Fukushima N, Rosty C, et al. Aberrant methylation of the 5' CpG island of TSLC1 is common in pancreatic ductal adenocarcinoma and is first manifest in high-grade PanINs. *Cancer Biol Ther.* 2002;1:293-296.
19. Honda T, Tamura G, Waki T, et al. Hypermethylation of the TSLC1 gene promoter in primary gastric cancers and gastric cancer cell lines. *Jpn J Cancer Res.* 2002;93:857-860.
20. Ito T, Shimada Y, Hashimoto Y, et al. Involvement of TSLC1 in progression of esophageal squamous cell carcinoma. *Cancer Res.* 2003;63:6320-6326.
21. Hui AB, Lo KW, Kwong J, et al. Epigenetic inactivation of TSLC1 gene in nasopharyngeal carcinoma. *Mol Carcinog.* 2003;38:170-178.
22. Steenbergen RD, Kramer D, Braakhuis BJ, et al. TSLC1 gene silencing in cervical cancer cell lines and cervical neoplasia. *J Natl Cancer Inst.* 2004;96:294-305.
23. Yageta M, Kuramochi M, Masuda M, et al. Direct association of TSLC1 and DAL-1, two distinct tumor suppressor proteins in lung cancer. *Cancer Res.* 2002;62:5129-5133.
24. Kikuchi S, Yamada D, Fukami T, et al. Promoter methylation of DAL-1/4.1B predicts poor prognosis in non-small cell lung cancer. *Clin Cancer Res.* 2005;11:2954-2961.
25. Frommer M, McDonald LE, Millar DS, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A.* 1992;89:1827-1831.
26. Ito A, Okada M, Uchino K, et al. Expression of the TSLC1 adhesion molecule in pulmonary epithelium and its down-regulation in pulmonary adenocarcinoma other than bronchioloalveolar carcinoma. *Lab Invest.* 2003;83:1175-1183.
27. De Stefani E, Boffetta P, Ronco AL, et al. Squamous and small cell carcinomas of the lung: similarities and differences concerning the role of tobacco smoking. *Lung Cancer.* 2005;47:1-8.
28. Rom WN, Hay JG, Lee TC, Jiang Y, Tchou-Wong KM. Molecular and genetic aspects of lung cancer. *Am J Respir Crit Care Med.* 2000;161:1355-1367.
29. Belinsky SA, Nikula KJ, Baylin SB, Issa JP. Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. *Proc Natl Acad Sci U S A.* 1996;93:4045-4050.
30. Vertino PM, Yen RW, Gao J, Baylin SB. De novo methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methyltransferase. *Mol Cell Biol.* 1996;16:4555-4565.
31. Uchino K, Ito A, Wakayama T, et al. Clinical implication and prognostic significance of the tumor suppressor TSLC1 gene detected in adenocarcinoma of the lung. *Cancer.* 2003;98:1002-1007.

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ONCOGENOMICS

Identification of tumor markers and differentiation markers for molecular diagnosis of lung adenocarcinoma

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To identify tumor markers and differentiation markers for lung adenocarcinoma (AdC), we analysed expression profiles of 14 500 genes against three cases of type II alveolar epithelial cells, bronchiolar epithelial cells, and bronchial epithelial cells, respectively, and 10 cases of AdC cells isolated by laser capture microdissection. Hierarchical clustering analysis indicated that AdC cells and noncancerous lung epithelial cells are significantly different in their expression profiles, and that different sets of differentiation markers are expressed among alveolar, bronchiolar and bronchial epithelial cells. Nine genes were identified as being highly expressed in AdC cells, but not expressed in noncancerous lung epithelial cells. Sixteen genes were identified as differentiation markers for lung epithelial cells. Real-time RT–PCR analysis of 45 lung AdC cases further revealed that expression of four tumor markers in AdC cells was significantly higher than that in noncancerous lung cells and that expression of ten differentiation markers was retained in a considerable fraction of lung AdC cases. Five tumor markers and seven differentiation markers were not expressed in peripheral blood cells. Similarities and differences in expression profiles between normal epithelial cells from different lung respiratory compartments and AdC cells demonstrated in this study will be informative for the molecular diagnosis of lung AdC.
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Keywords: lung adenocarcinoma; lung epithelial cells; expression profile; microdissection; micrometastasis; differential diagnosis

Introduction

Lung cancer is the leading cause of cancer death in many countries, and in recent years, adenocarcinoma (AdC) has become the most common type. Since a majority of lung AdC patients are discovered in an advanced stage, difficulties in detection of early stage AdCs and lack of effective therapies for advanced stage AdCs have been recognized as major causes of the high mortality of this disease. Even with the introduction of computed tomographic (CT) screening for lung cancer, the overall 5-year survival rates of patients with small-sized (p 2 cm in diameter) AdC and those with stage IA AdC are approximately 80% (Naruke et al., 2001; Takamochi et al., 2004). Therefore, it has been assumed that a subset of these patients already had occult metastases at the time of primary surgery that were undetectable by current tumor-node-metastasis (TNM) staging methods (Sobin, 2002). For this reason, identification of lung AdC specific molecular markers has been thought to be essential for the development of novel ways of effective screening and more accurate TNM staging (Hosch et al., 2001; Wang et al., 2002). In particular, identification of a set of tumor markers and differentiation markers specific for lung AdC will be highly useful for detection of lung AdC cells in peripheral blood (PB), bone marrow and lymph nodes. Development of a more sensitive and specific method for detection of micrometastasis will lead to a more accurate TNM staging resulting in the more accurate prediction of patients' prognoses and design of postoperative therapeutic approaches.

Up to the present, several tumor markers, such as carcino-embryonic antigen (CEA) and cancer testis (CT) antigens, have been studied for detection of lung AdC (D'Cunha et al., 2002; Egland et al., 2002; Kufer et al., 2002; Takamochi et al., 2004). Several differentiation markers, such as cytokeratin (CK) 19, have been also studied for detection of lung AdC. However, most of them are not specific for lung AdC but for epithelial cells or cancers of epithelial cell origin in general. Even for tissue-specific differentiation markers, such as the surfactant pulmonary-associated proteins A, B and C (SFTPA2, SFTPB and SFTPC, respectively) and thyroid transcription factor-1 (TTF-1), sensitivity as well as specificity is not high enough to use for molecular diagnosis (Chhieng et al., 2001; Zamecnik and Kodet, 2002). Thus, it is absolutely necessary to search for more specific and highly sensitive markers that will be useful for molecular diagnosis of lung AdC.

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Recently, GeneChip oligonucleotide microarray analysis has been introduced to identify many additional molecular markers (Segal et al., 2004), and several groups have reported microarray-based subclassifications of lung AdC (Bhattacharjee et al., 2001; Garber et al., 2001; Miura et al., 2002; Virtanen et al., 2002; Tomida et al., 2004). However, it is still unclear what genes are useful for molecular diagnosis of lung AdC in clinics. The origins of AdC cells are thought to be alveolar, bronchiolar and bronchial epithelial cells (Ten Have-Opbroek et al., 1997; Otto, 2002; Borczuk et al., 2003). Thus, we thought it was important to elucidate similarities and differences of AdC cells in comparison with several types of lung epithelial cells for gene expression profiling for the identification of novel and useful molecular markers specific for lung AdC. For this purpose, a panel of type II alveolar epithelial cells, bronchiolar epithelial cells and bronchial epithelial cells as well as that of lung AdC cells isolated by laser capture microdissection (LCM) (Bonner et al., 1997; Player et al., 2004) was subjected to oligonucleotide microarray analysis of 14500 genes. By combination of LCM and microarray analyses and by confirmation with quantitative RT–PCR analysis of candidate genes, we identified several genes that will be useful for molecular diagnosis of AdCs.

Results

Laser capture microdissection of lung adenocarcinoma cells and noncancerous lung epithelial cells

Ten cases of AdCs were microdissected by the LCM method, and histologically, four of them were classified as being well differentiated (WD1 – WD4), three were moderately differentiated (MD1 – MD3), and the remaining three were poorly differentiated (PD1 – PD3). Three cases of type II alveolar epithelial cells (Alv1 – Alv3), bronchiolar epithelial cells (Bio1 – Bio3) and bronchial epithelial cells (Bia1 – Bia3), respectively, were also obtained by the LCM method as representatives of noncancerous epithelial cells from three different lung respiratory compartments. Pairs of Alv1/Bio1, Alv2/WD1, Alv3/PD1 and Bio3/Bia1 were obtained from the same patients, respectively, while other samples were obtained from different patients. Microscopic visualization of representative tissue sections are shown in Figure 1. Type II alveolar epithelial cells and bronchiolar epithelial cells as well as AdC cells were microdissected as previously described (Kobayashi et al., 2004). Bronchi are encircled with cartilage, while bronchioles are not. Thus, bronchial epithelial cells were microdissected from epithelia neighboring cartilage. Nonciliated columnar cells are called Clara cells and line the distal bronchi and the bronchioles. Ciliated bronchiolar or bronchial epithelial cells were microdissected together with Clara cells.

Total RNA was extracted from the cells, and the quality, quantity and purity of RNAs were assessed by RT–PCR analysis of the surfactant pulmonary-associated protein B (SFTPB), Clara-cell-specific 10-kDa protein (SCGB1A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (Kobayashi et al., 2004). SFTPB mRNA is known to be expressed in both type II alveolar epithelial cells and bronchiolar epithelial cells, whereas SCGB1A1 mRNA is known to be expressed in Clara cells in bronchiolar and bronchial epithelia but not in type II alveolar epithelial cells (Broers et al., 1992; Xu et al., 1998; Barth et al., 2000; Borczuk et al., 2003). As expected, SFTPB specific PCR products were detected in both type II alveolar epithelial cells and bronchiolar epithelial cells, and SCGB1A1 specific products were detected in both bronchiolar and bronchial epithelial cells (Figure 2a). The quality and quantity of RNAs extracted from all samples, including 10 AdC samples, were confirmed by RT–PCR of the GAPDH gene. The results indicated that the quality of RNA from epithelial cells as well as AdC cells was well preserved after LCM and good enough for microarray analysis.

Oligonucleotide microarray analysis of lung adenocarcinoma cells and noncancerous lung epithelial cells

RNAs were amplified by the TALPAT method (Aoyagi et al.,. 2003), and hybridized to the U133A geneChip that contain 22283 probe sets corresponding to 14500 genes. The percent of probe sets judged as 'present calls' ranged from 25.6 to 35.7% in noncancerous lung epithelial cells and from 27.0 to 41.7% in lung AdC cells, indicating that the number of genes expressed is not significantly different between noncancerous epithelial cells and AdC cells (Table S1). Signal intensities for gene expression were then scaled to a target intensity of 1000 by Affymetrix Microarray Suite 4.0 software. Figure 2b shows microarray data for the expression of the SFTPB, SCGB1A1 and GAPDH genes in the corresponding samples used for RT–PCR analysis. The results of microarray analysis were well compatible with those of RT–PCR analysis. We then classified all 19 samples by hierarchical clustering analysis to examine how gene expression profiles were different among the samples. For the analysis, expression signals were first filtered by absolute calls, and among 22283 probe sets, 7446 sets that showed 'absent calls' for all 19 samples were excluded. The analysis using the remaining 14837 probe sets classified the samples into several subgroups (Figure 3). Nine noncancerous epithelial cell samples and 10 AdC cell samples were first separated to different branches. Among the nine epithelial cell samples, type II alveolar epithelial cells were then branched from bronchiolar and bronchial epithelial cells. Finally, three bronchiolar epithelial cell samples and three bronchial epithelial cell samples were divided into two different groups. However, 10 AdC cell samples did not evenly branch according to the differentiation status of pathological classification. These results indicated that differences in gene expression profiles were more evident between noncancerous epithelial cells and AdC cells than among alveolar, bronchiolar and bronchial epithe-

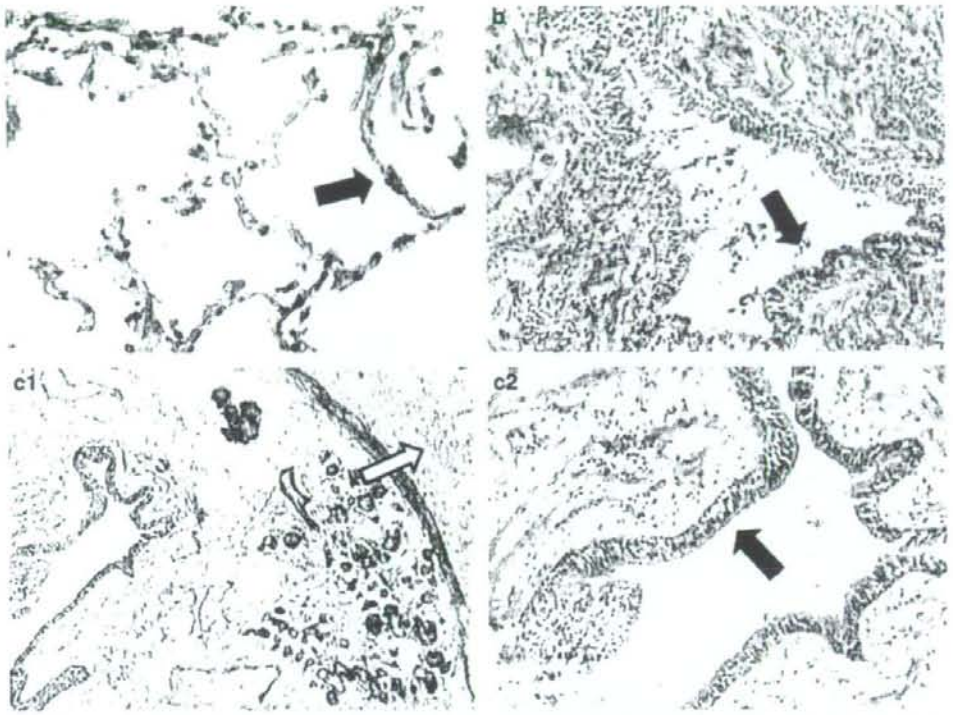

Figure 1 Microscopic visualization of representative tissue sections, stained with hematoxylin and eosin, used for microdissection. (a) Alveolus (magnification 200). (b) Bronchiole (magnification 200). (c1) Bronchus (magnification 100). (c2) Bronchial epithelium (magnification 200). Closed arrows indicate epithelial cells microdissected, and an open arrow indicates cartilage neighboring bronchial epithelium.

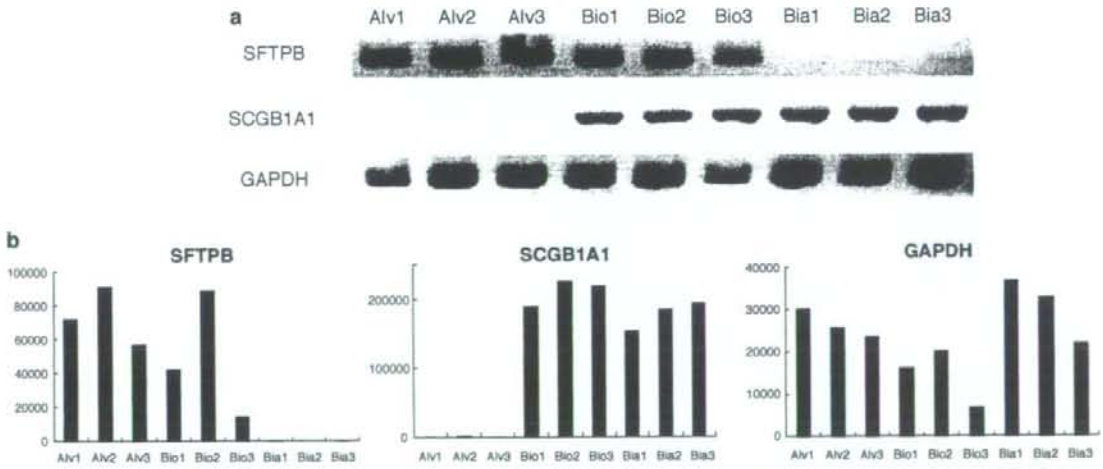

Figure 2 RT–PCR and microarray analyses of the SFTPB, SCGB1A1 and GAPDH genes in noncancerous lung epithelial cells. Alv1 B Alv3 are type II alveolar cells, Biol B Bio3 are bronchiolar epithelial cells, and Bial B Bia3 are bronchial epithelial cells.

lial cells, that gene expression profiles among alveolar, bronchiolar and bronchial epithelial cells are significantly different to each other, and that expression profiles do not always correlate with pathological classification of AdC cells.

Identification of tumor markers for lung adenocarcinoma

To identify genes whose expression is high in lung AdC cells and not detected in noncancerous lung epithelial cells, we compared gene expression profiles of lung AdC cells with those of alveolar, bronchiolar and bronchial