EGFR that were associated with sensitivity to gefitinib and erlotinib (OSI-774, Tarceva®; another selective EGFR TKI).

On the genomic level, sequence data presented by both groups [33,34] showed that point mutations in exons 18 and 21, respectively, are heterozygous. This contrasts with deletions in exon 19 where, in some of the sequencing traces presented, the normal allele is severely under-represented or absent, indicating a loss of heterozygosity or amplification of the mutant locus. Thus, the point mutations in exons 18 and 21 may be dominant in heterodimers consisting of a normal and a mutant EGFR or an EGFR family member. In contrast, mutations in exon 19 may be functionally recessive and possibly require homodimerization for phenotypic penetrance [36].

Lynch *et al.* went on to show that transfection of the missense EGFR mutant Leu858Arg or the in-frame deletion Leu747–Pro753insSer into cultured cell lines resulted in a two- to threefold increased stimulation of the receptor by EGF and that activation of the mutant receptor lasted up to 12-times longer when compared with the wild-type receptor [33]. Furthermore, the mutant EGFR proteins were approximately 10 times more sensitive to inhibition by gefitinib than the wild-type EGFR. Paez *et al.* investigated a cell line derived from a malignant pleural effusion from a Caucasian female non-smoker with lung adenocarcinoma [34]. The adenocarcinoma had the Leu858Arg mutation in EGFR and was 50 times more sensitive to gefitinib than other adenocarcinoma cell lines. The authors hypothesized that the mutations in EGFR stabilize the interaction between the drug and the kinase, thereby increasing the inhibitory effect of gefitinib. If the association between the *EGFR* mutations and gefitinib sensitivity are confirmed in prospective clinical trials, they will set a standard for approaches to the evaluation and use of targeted therapy for solid tumors.

In the Aichi Cancer Center in Japan, Kosaka *et al.* found that ~ 40% of NSCLC Japanese patients who had undergone resection carried *EGFR* mutations (*Cancer Research*, in press). In their study, RNA was extracted and direct sequencing of the TK domain of the *EGFR* gene was performed after reverse transcription-PCR. The mutations were mostly deletions around codons 746–750 or point mutations, predominantly at codon 858; rarer mutations were also noted. *EGFR* mutations were significantly frequent in females, patients with adenocarcinomas and patients who had never smoked. They also found that *EGFR* mutations and K-*ras* mutations were mutually exclusive, whereas *EGFR* mutations were independent of p53 (*TP53*) mutations. They also studied the patients who were treated with gefitinib for recurrent diseases after surgery. *EGFR* mutations showed good but not perfect correlation with gefitinib effectiveness, even after prior chemotherapy treatment (before the application of gefitinib), as reported previously (Table 1). Patients with *EGFR* mutations survived for a longer period than those without the mutations after initiation of gefitinib treatment (Mitsudomi T *et al.*, submitted for publication).

Tumor mutations that are insensitive to kinase inhibitors, such as those found in human glioblastomas, include extensive deletions or missense mutations in the extracellular domain of the EGFR, or deletions of the regulatory intracellular domain (but not the kinase domain). In contrast, many NSCLCs carrying missense mutations and deletions in *EGFR* are sensitive to kinase inhibitors, such as gefitinib. These mutations are found in three distinct sites in the EGFR kinase domain: the phosphate-binding loop (P-loop), the A-loop, and the αC helix.

It is not clear from these studies whether only tumors with the aforementioned *EGFR* mutations respond to gefitinib. In the Paez *et al.* study [34], 1 out of 61 patients from the USA had *EGFR* mutations in their tumor tissue, which is far lower than the 10% response rate reported by Kris *et al.* in the Phase II study of gefitinib in the USA [17]. In the study by Lynch *et al.* [33], one of nine responding patients had a wild-type receptor. In previous gefitinib studies, there was a group of patients that showed marked symptom improvement and prolonged disease stabilization with no measurable reduction in tumor size. However, the *EGFR* status of such tumors was not reported in either study.

Other molecular correlates of gefitinib response

Akt and STAT phosphorylation

Cappuzzo *et al.* [40] evaluated the two major EGFR signaling pathways (PI3K/Akt and Ras/Raf/MAPK) immunohistochemically, and found that only the PI3K/Akt pathway was significantly associated with gefitinib activity. In their study, Akt was activated in ~ 50% of patients with NSCLC. Furthermore, phosphorylation of Akt (P-Akt) positivity was associated with being female, with never smoking, and with

Table 1. *EGFR* gene mutations and response to gefitinib.

Study	No. of patients with mutations/no. of patients with clinical response	No. of patients with mutations/no. of patients with no clinical response
Paez *et al.* [34], Jänne *et al.* [37]	100% (7/7)	0% (0/6)
Lynch *et al.* [33,38]	89% (8/9)	0% (0/7)
Cappuzzo *et al.* [39]	89% (8/9)	33% (3/9)
Pao *et al.* [35]	70% (7/10)	0% (0/8)

EGFR: Epidermal growth factor receptor.

bronchioloalveolar carcinoma histology. Patients with P-Akt-positive tumors who received gefitinib had a better response rate, disease control rate and time to progression than patients with P-Akt-negative tumors, suggesting that gefitinib may be more effective in patients with basal Akt activation. In the multivariable analysis, P-Akt status was significantly associated with a reduced risk of disease progression (hazard ratio [HR] = 0.58, 95% confidence interval [CI] 0.35–0.94). Importantly, after being adjusted for P-Akt status, performance status and smoking history remained significantly associated with an increased risk of disease progression (HR = 2.65 [95% CI 1.33–5.27] and 1.75 [95% CI 1.08–2.85], respectively), and female gender was immediately removed at the first step of the backward elimination. Recent data indicate that sensitivity to gefitinib therapy requires intact EGFR-stimulated Akt signaling activity and that loss of PTEN (phosphatase and tensin homolog; a phosphatase that negatively regulates Akt by dephosphorylating it) can lead to aberrant Akt activation and, finally, to gefitinib resistance [41-43].

Recently, Sordella *et al.* [44] reported that mutant EGFRs selectively activated Akt and STAT signaling pathways, which promote cell survival, but have no effect on Erk/MAPK signaling, which induces proliferation. EGF-induced phosphorylation of tyrosine residues at codon 1045 (Tyr1045) and Tyr1173 was virtually indistinguishable between the wild-type and mutant EGFRs, whereas phosphorylation of Tyr992 and Tyr1068 was substantially increased in the mutant EGFRs. The increased Akt and STAT phosphorylation following activation of the mutant EGFRs was consistent with the increase in Tyr992 and Tyr1068 phosphorylation. The selective EGF-induced autophosphorylation of C-terminal tyrosine residues within EGFR mutants was well correlated with the selective activation of downstream signaling pathways. Interestingly, Sordella *et al.* suggested that the expression of mutant EGFRs appeared essential for suppression of proapoptotic signals in lung cancers harboring these mutations. Sensitivity to gefitinib may result in large part from its effective inhibition of essential antiapoptotic signals transduced by the mutant receptor in lung cancer cells with EGFR kinase mutations. Thus, mutant EGFRs selectively transduce survival signals on which NSCLCs become dependent, and the inhibition of those signals by gefitinib may contribute to the efficacy of the drug.

EGFR expression

Despite promising clinical results in the treatment of NSCLC, the potential relationship of the sensitivity to gefitinib with various characteristics of NSCLC, such as EGFR expression level, is not clear. In clinical trials, objective responses were observed in lung cancer patients with adenocarcinoma that was less likely to express EGFR than squamous cell carcinoma. In a retrospective analysis from IDEAL, EGFR expression (as detected by immunohistochemistry) was not related to response in patients receiving gefitinib monotherapy [45]. Similarly, in the Phase II study with erlotinib, the intensity of HER1/EGFR staining was not associated with the clinical outcome of metastatic squamous cell cancer in head and neck patients [46]. In this regard, Sirotnak *et al.* demonstrated that the degree of potentiation of gefitinib in a variety of tumor xenografts was not dependent on high levels of expression of EGFR [29]. Using a panel of 19 lung cancer cell lines, Suzuki *et al.* observed the lack of association of gefitinib sensitivity with the expression of EGFR, HER2, HER3, and HER4 [47]. Their results also showed no apparent association between K-*ras* mutations

Highlights

- Gefitinib inhibits the growth of some lung cancers, although this effect is not well correlated with the level of expression of EGFR or related members of the ErbB family of receptors.
- Recently, three studies have identified mutations affecting EGFR in lung cancers from patients. These reports identify somatic mutations in the *EGFR* gene in patients with NSCLC that had clinical responses to the EGFR TKI gefitinib. Another study suggested that the expression of mutant EGFRs appeared essential for the suppression of proapoptotic signals in lung cancers harboring these mutations. Sensitivity to gefitinib may largely result from its effective inhibition of essential antiapoptotic signals transduced by the mutant receptor in lung cancer cells with EGFR kinase mutations. It is, however, not clear from these studies whether only tumors with *EGFR* mutations respond to gefitinib.
- If these data are confirmed in prospective clinical trials, they will set a standard for approaches to the evaluation and use of targeted therapy for solid tumors.

and sensitivity to gefitinib. Although it could be hypothesized that EGFR expression is a prerequisite for a response to the drug, EGFR expression has not turned out to be useful in the selection of patients who respond to gefitinib.

It was reported that the specificity and potency of the signaling output from activated EGFR is highly dependent on the identity of the activating ligand, as well as on the cellular levels of the coreceptors HER2, HER3, and HER4, all of which can oligomerize with EGFR. The sites autophosphorylated in the C-terminal portion of EGFR, as well as the signaling molecules that associate with the receptor, are determined by the heterodimeric partner of EGFR. Thus, other members of the HER network may influence the efficacy of gefitinib.

Gene expression profiling and proteomic analysis

In a microarray analysis, Hirsch *et al.* [48] identified 13 genes for which expression accurately discriminated between gefitinib-sensitive and -resistant NSCLC cell lines. Carbone *et al.* [49] presented results of a proteomic analysis of fresh tumor samples from NSCLC patients resistant or sensitive to gefitinib treatment. This analysis identified six protein peaks that were 95% predictive of response to gefitinib and 88 peaks that were 100% predictive. These observations require prospective validation in large studies.

Outlook

In clinical studies with NSCLC patients, gefitinib has shown modest clinical activity, with higher response rates in females, patients with lung adenocarcinoma, non-smokers, and patients of Japanese origin. In none of these trials were patients selected based on evidence of EGFR kinase dependence. Three studies by Paez *et al.* [34], Lynch *et al.* [33] and Pao *et al.* [35] have revealed some of the molecular underpinnings of the overall low clinical activity of EGFR inhibitors and will almost certainly lead to the identification of subgroups of patients who are likely to benefit substantially from these drugs. It is, however, not clear from these studies whether only tumors with *EGFR* mutations respond to gefitinib. The clear message from studies of *EGFR* mutations is that although these mutations are important in determining a dramatic response to EGFR TKIs, this is not the whole story. In addition, whether these mutant receptors depend on ligands for activation *in situ* is a crucial question. Ligand-independent activation could anticipate therapeutic resistance to the ligand-blocking EGFR antibodies that are currently in clinical development.

It is clear from these studies that molecular target dependence and patient selection should be central to the development of molecular therapeutics in human cancer. This approach should avoid the spuriously negative or overall weak signals of clinical activity of drugs that are otherwise very active when used in the right group of patients; prevent unnecessary large, costly trials; and limit the exposure of patients to drugs unlikely to produce any clinical benefit. In EGFR-mutant lung cancers that eventually escape gefitinib treatment, it is now important to determine whether the resistance is EGFR-dependent or -independent.

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FAST TRACK

REDUCED EXPRESSION OF CLASS II HISTONE DEACETYLASE GENES IS ASSOCIATED WITH POOR PROGNOSIS IN LUNG CANCER PATIENTS

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HDAC genes are thought to be involved in gene expression through the regulation of chromatin structure, alterations of which may cause abnormal gene silencing in cancers. To clarify the possible role of HDAC genes during tumor development and progression, we studied their expression and influence on clinical features. Expression levels of HDAC class I and class II genes in cancer tissues resected from 72 patients with NSCLC were measured with real-time RT-PCR. Their association with clinicopathologic features was statistically investigated. Reduced expression of each class II HDAC gene was significantly associated with poor prognosis and an independent predictor of poor prognosis. Of all the genes, *HDAC10* was the strongest predictor of poor prognosis. Hierarchical clustering analysis showed that lung cancer tissues could be divided into 3 groups based on the expression level of class I and class II HDAC genes. The group with reduced expression of class II HDACs showed poor prognosis. These results suggest that class II HDACs may repress critical genes and that low expression of these genes may play a role in lung cancer progression. Results of clustering analyses imply that class II HDAC genes may be regulated by a similar mechanism and deregulated during cancer development.

Key words: *lung cancer; histone deacetylase; prognosis*

The accumulation of genetic and epigenetic alterations results in the development of cancer.[1,2] DNA methylation and histone modification are involved in the epigenetic alterations *via* regulating chromatin structure.[3] Histone deacetylation catalyzed by HDACs is the main histone modification in silenced gene promoters.[4] In cancer cells, several genes are silenced by alteration of the chromatin structure with or without DNA methylation,[5–7] suggesting the involvement of abnormal HDAC gene activity.[8,9] Mammalian HDACs consist of 2 groups, classical HDAC genes and the recently identified NAD-dependent SIRT family.[10,11] Based on structural and functional similarities, the classical HDAC family is divided into 2 different phylogenetic classes: class I and class II. Class I HDACs (HDACs 1–3 and 8) are most closely related to yeast RPD3, while class II HDACs (HDACs 4–7, 9 and 10) share domains with similarity to yeast HDA1. The *HDAC11* gene has been identified but not yet assigned to either class. Currently, class I HDACs are thought to be expressed in most cell types, whereas the expression pattern of class II HDACs is more restricted. Class I HDACs mainly exist in the nucleus, while class II HDACs shuttle between the nucleus and cytoplasm, responding to several signals. Nuclear export of class II HDACs is induced by phosphorylation and interaction with an adaptor protein, 14-3-3.[10,11] These fine regulations suggest that class II HDACs might be involved in cellular differentiation and developmental processes and that their dysregulation may be involved in carcinogenesis. However, alteration of the expression level and the involvement in the clinical course have not been clarified. Our study demonstrates that reduced expression of class II HDAC genes is associated with poor prognosis of lung cancer patients.

MATERIAL AND METHODS

Study population and tissue samples

Seventy-two NSCLC tissue specimens (48 adenocarcinomas, 12 squamous carcinomas, 4 adenosquamous carcinomas and 8 large cell carcinomas) were obtained with the approval of our institutional review board from 26 female and 46 male patients who had consecutively undergone potentially curative resection between January 1996 and January 1998 at Aichi Cancer Center Hospital. Twenty-two patients (31%) died (median 23 months), while 50 patients survived and were followed up for 45–75 months (median 63 months). The median age of this cohort was 62 (range 32–80) years. There were 38 pstage I, 14 pstage II and 20 pstage III/IV tumors.

Quantitative RT-PCR

RNA was isolated from tissue specimens, and random-primed cDNA samples were made using 5 μg total RNA, as described previously.[12] Expression of each HDAC gene was measured with the ABI Prism 7900 (Applied Biosystems, Foster City, CA) by quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems) and cDNAs (corresponding to 20 ng of total RNA). Standard curves were made using serially diluted cDNA of one lung cancer cell line. PCR primers were designed within regions that were less similar among HDAC genes as follows: *HDAC1* F, 5′-CCTGGATACGGAGATCCCTA, and R, 5′-CCGCAAGAACTCTTCCAACT; *HDAC2* F, 5′-CCTTGATTGTGAGATTCCCA, and R, 5′-AGGAGGTCGAAGAAATGTGG; *HDAC3* F, 5′-CTCTCTGGGCTGTGATCGAT, and R, 5′-CAATCTTTGAAAACCTGAAGATG; *HDAC4* F, 5′-GAGAGACTCACCCTTCCCG, and R, 5′-CTTGGTTGGTGCAGACCGG; *HDAC5* F, 5′-AGAATGGCTTTACTGGCTCAG, and R, 5′-CATGAGCACATCCTCTATTCC; *HDAC6* F, 5′- CCTCAATCACTGAGACCATC, and R, 5′-GACTAACTCAGAGACAGCTG; *HDAC7* F, 5′-ACCCCTCAGGCTCTCATGC, and R, 5′-

Abbreviations: BRG1, BRM -related gene 1; BRM, brahma human homologue; CI, confidence interval; HDA1, histone deacetylase-A; HDAC, histone deacetylase; HP1, heterochromatin protein-1; HR, hazard ratio; MEF2, myocyte enhancer factor-2; NSCLC, non-small cell lung cancer; pstage, pathologic stage; RPD3, reduced potassium dependence 3; SIRT, homologue of silent information regulator 2.

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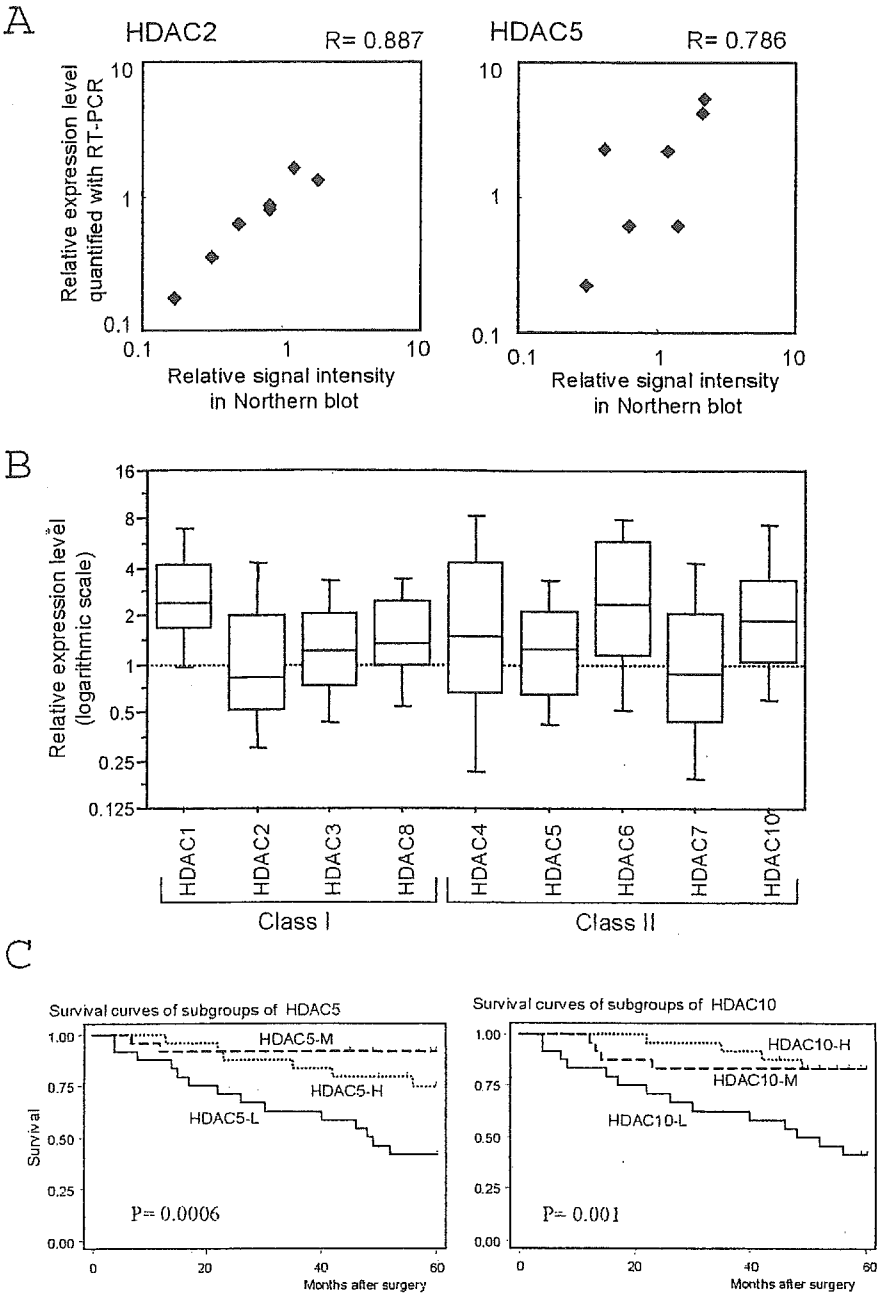

FIGURE 1 – Measurement of the expression of HDAC genes. *(a)* Consistency between Northern blot and real-time RT-PCR analyses. The intensity of Northern blot signals and relative expression level quantified by real-time RT-PCR for *HDACs 2* and *5* are compared with scatter plots. Pearson's correlation for each HDAC gene was also indicated. *(b)* Box-and-whisker plots of the expression of each HDAC gene. Boxes represent the range 25–75%, while bars in the boxes indicate the median level. Areas between lower and upper bars correspond to the 10–90% range. *(c)* Survival curves of equally trisected subgroups, high (H), moderate (M) and low (L) expression, of class II HDAC genes *HDAC5* and *HDAC10* were demonstrated. Survival curves indicate poorer prognosis of the L groups than that of the M or H groups. *p* values of log-rank analyses are also indicated.

CTGGAGCACAGGGAGCTGG; *HDAC8* F, 5′-AGGTGACGTGTCTGATGTTG, and R, 5′-CTCTGAGATCCCAGATCATG; *HDAC10* F, 5′-ATGTGGCTGTTCGGAGAGGC, and R, 5′-CTGCACTCCTGGCTGCAATG. RT-PCR analyses were performed at least twice for each HDAC gene. The expression level of each sample was normalized by the quantity of 18S rRNA and presented as a ratio to a mixed sample of normal lung tissue, the expression level of which was set at 1. Patients were divided into 3 or 2 subgroups based on the expression level of each HDAC gene.

Statistical analysis and hierarchical clustering

All statistical analyses were conducted with STATA software (StataCorp, College Station, TX). All statistical tests were 2-sided. For comparisons among subgroups, we used Pearson's χ^2 test. Log-rank tests were used to compare survival curves among the various subgroups of patients. Survival probabilities were estimated using the Kaplan-Meier method. HRs for death with 95% CIs were calculated using Cox's proportional hazards regression analysis. Associations between individual clinicopathologic variables, such as tumor histology, age, sex, smoking habit, pstage, primary tumor status (pT), nodal involvement (pN), pathologic grade of differentiation, 5-year survival and expression level of each HDAC gene, were assessed using Cox's proportional hazards regression model. A stepwise variable selection procedure was used in multivariate Cox analysis; a statistical significance level of

TABLE I – χ^2 ANALYSIS OF CLINICOPATHOLOGICAL FEATURES

Variables	HDAC5			
	H-M[1]	L	Total	*p*-value[2]
Smoking History				
Non-smoker	25	5	30	0.019*
Smoker	24	18	42	
Variables	**HDAC7**			
	H-M[1]	L	Total	*p*-value
Disease stage				
I	25	13	38	0.019*
II–III	13	21	34	
Nodal involvement				
pN = 0	29	18	47	0.038*
pN ≥ 1	9	16	25	

[1]High or moderate expression group (H-M) (≥0.8) vs. low expression group (L) (<0.8).–[2]Asterisks indicate significant *p*-values.

0.20 was used as a cut-off to determine whether a variable could be entered into or removed from the regression model. Complete linkage hierarchical clustering was performed using the Cluster program following log transformation and median centering, and the results were visualized using the TreeView program (Cluster and TreeView programs were kindly provided by Dr. M. Eisen, http://rana.lbl.gov).

Northern blot analysis

RNA samples from representative lung cancer tissues were studied with Northern blot analysis, as described previously.[12] To obtain the probes for *HDACs 1, 2, 5* and *10*, cDNA fragments of these genes were amplified by RT-PCR using the primers used for quantification, cloned into pBSSKII plasmid (Stratagene, La Jolla, CA) and sequence-verified. Signal intensity was measured with a phosphoimager BAS-2500 (Fuji, Tokyo, Japan).

RESULTS

At first, to verify the quantification with real-time RT-PCR, the signal intensity of Northern blots and the relative expression level quantified with real-time RT-PCR for *HDACs 1, 2, 5* and *10* were compared using 7 lung cancer cell line samples. Reasonable consistency (Pearson's correlation $R = 0.676–0.887$) was observed in all HDAC genes (Fig. 1*a* for *HDACs 2* and *5*). We then studied the expression of HDAC genes in 72 lung cancer specimens. The expression level of each HDAC gene varied among cancer samples (Fig. 1*b*). In analyses of the *HDAC2, -3, -8, -4, -5, -7* and *-10* genes, 25–56% of cases showed less abundant expression than normal lung. Because the *HDAC1* and *-6* genes frequently showed abundant expression, 11% and 21% of cases had lower expression of these genes than normal lung, respectively. To statistically study the possible effect of HDAC expression level, the cohort was first equally divided into 3 groups based on the expression level of each HDAC gene: high (H), moderate (M) and low (L). We investigated whether the expression level of HDAC genes may affect prognosis. Log-rank analysis indicated statistically significant differences among the survival of trisected groups in all class II HDAC genes examined (*HDACs 4–7* and *10* $p = 0.016$, 0.0006, 0.034, 0.015 and 0.001, respectively) but not class I HDAC genes (*HDACs 1–3* and *8* $p = 0.121$, 0.990, 0.150 and 0.058, respectively). Kaplan-Meier survival curves of trisected groups in all class II HDAC genes indicated significantly poorer prognosis for the L group than either the H or M group in all class II HDAC genes (Fig. 1*c* for *HDACs 5* and *10*).

Following this, we investigated whether reduced expression of HDAC genes may affect prognosis. For each HDAC gene analysis, patients with expression <80% of the level in normal lung tissues were assigned to the L group, while the remaining patients were assigned to the H-M group. The association of these 2 groups (L and H-M) and clinicopathologic features was studied using χ^2 analysis. We found a significant association of *HDAC7* with pathologic stage ($p = 0.019$) and nodal involvement ($p = 0.038$), in addition to the association between *HDAC5* and smoking history ($p = 0.019$) (Table I). Other HDAC genes did not show any significant association with clinicopathologic features, including disease stage and primary tumor status. However, we observed a significant difference in postoperative survival between the L and H-M groups of each class II HDAC gene (Fig. 2). Log-rank analysis indicated statistically significant differences in survival for these 2 groups in all class II HDAC genes examined (*HDACs 4–7* and *10* $p = 0.008$, 0.0007, 0.011, 0.016 and 0.0009, respectively) but not in class I HDAC genes (*HDACs 1–3* and *8* $p = 0.376$, 0.297, 0.710 and 0.194, respectively). Among class II HDAC genes, *HDAC5* and *HDAC10* showed the most significant difference in survival. Figure 2 shows Kaplan-Meier survival curves indicating significantly poorer prognosis for the L group than the H-M group in all class II HDAC genes.

The association of lower expression of each HDAC gene with survival was further studied with Cox's proportional hazards regression analysis (Table II). Univariate Cox analysis showed that, in all class II HDAC genes, classification into the L group was associated with poor prognosis, while class I HDAC genes did not show such an association (Table II). Among class II genes, *HDAC5* and *HDAC10* indicated the strongest association with poor prognosis (HR= 3.92 and 3.94, respectively; $p = 0.002$ for both). To study the interaction among the reduced expression of each class II HDAC gene and clinicopathologic features, multivariate Cox analysis was conducted including all clinicopathologic variables and each class II gene (Table II). These multivariate analyses showed that in all class II HDAC genes classification into the L group was associated with poor prognosis independently of any clinicopathologic feature, including pathologic stage, while class I HDAC genes did not show such an association. Among class II HDACs, the association of *HDAC5* or *HDAC10* with poor prognosis was very significant (HR= 3.91 and 4.12, $p = 0.004$ and 0.003, respectively), similar to the association between pathologic stage and poor prognosis. To study the interaction among the reduced expression of all class II HDAC genes and clinicopathological features, stepwise multivariate Cox analysis was conducted including all HDAC genes and clinicopathologic variables (Table II). In addition to pathologic stage (HR= 6.94, $p < 0.001$), reduced expression of *HDAC10* was an independent predictor of poor prognosis (HR= 7.13, $p = 0.004$), suggesting that reduced expression of *HDAC10* affects the prognosis of lung cancers most significantly and independently. The *HDAC5* gene also showed a trend, though not a significant one, toward an association with poor prognosis (HR = 2.63, $p = 0.054$).

Statistical analysis demonstrated an association between low expression of each class II HDAC gene and poor prognosis but did not provide any information on the whole picture with regard to expression of all HDAC genes in each case. The whole expression profile of all HDAC genes might indicate the number of affected HDAC genes and possibly provide information about the regulation of HDAC gene expression. To obtain the whole expression profile of all HDAC genes, we performed unsupervised hierarchical clustering analysis (Fig. 3*a*). HDAC genes occurred in 2 discrete clusters, clearly corresponding to classes I and II. Lung cancer cases also showed discrete clusters. The top cluster (cluster II, class II predominant) contained lung cancers abundantly expressing class II HDAC genes but few class I genes, while the middle cluster (cluster I, class I predominant) demonstrated the reverse pattern. The bottom cluster (cluster III) did not show any predominance; normal lung tissue belonged to this cluster. Survival among these clusters was significantly different. Kaplan-Meier survival curves in Figure 3*b* demonstrated that cluster I cases had significantly poor prognosis, while cluster II and III cases showed similarly favorable prognosis (log-rank analysis $p = 0.019$). The influence of classification into cluster I was studied with Cox's proportional hazards analysis. Univariate analysis in-

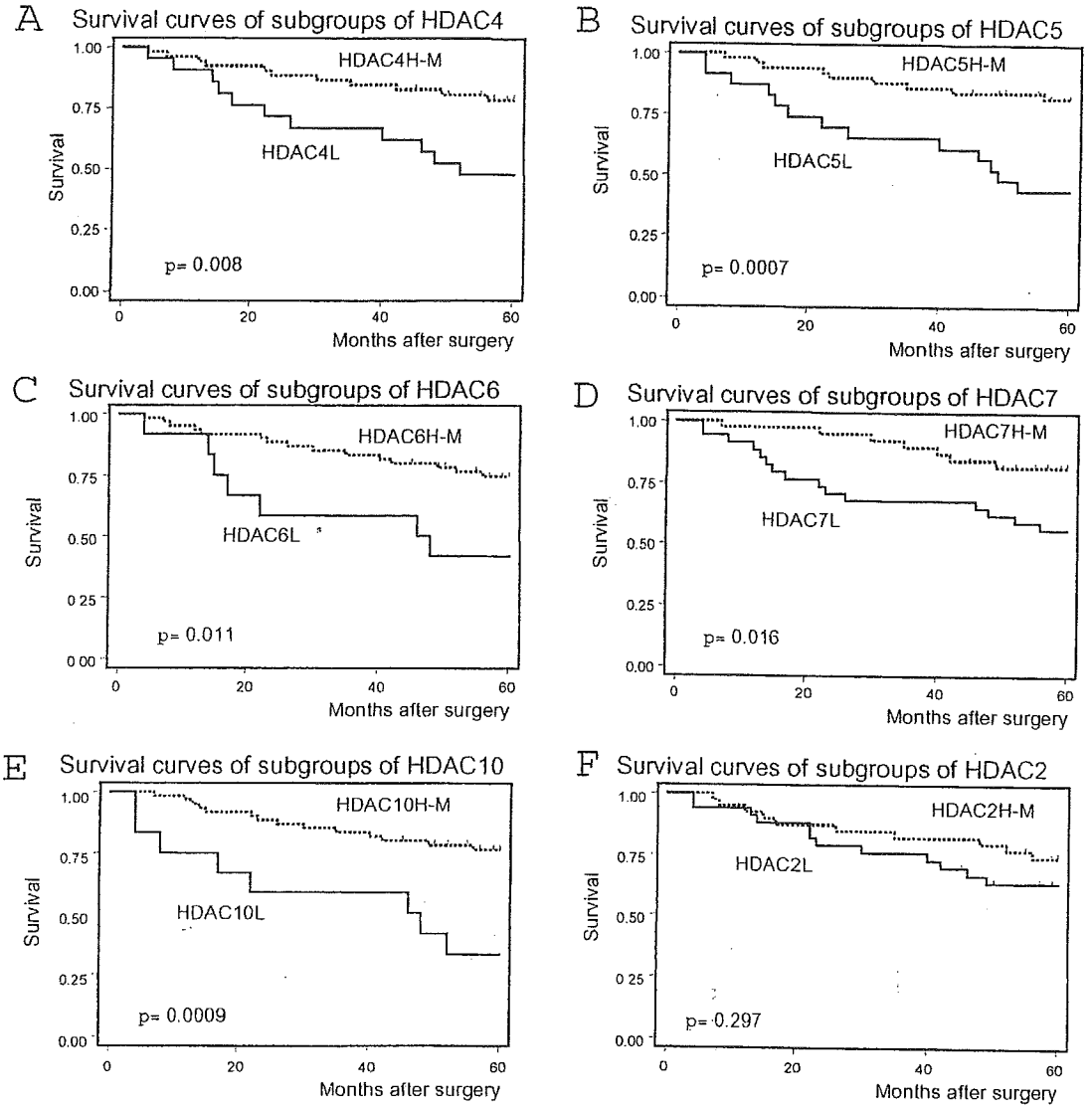

FIGURE 2 – Kaplan-Meier survival curves of subgroups of each HDAC gene. Survival curves of high or moderate expression (H-M) and low expression (L) groups of class II HDAC genes *HDAC4 (a), HDAC5 (b), HDAC6 (c), HDAC7 (d)* and *HDAC10 (e),* as well as a class I HDAC gene, *HDAC2 (f),* were demonstrated. For each HDAC gene analysis, patients with expression <80% of the level in normal lung tissue were grouped into the L group, while other patients were assigned to the H-M group. *(a–e)* Survival curves of all class II HDAC genes indicate poorer prognosis of the L groups than of the H groups. *(f)* Survival curves of the *HDAC2* groups did not show any difference in prognosis. *p* values of log-rank analyses are also indicated.

dicated significantly poor prognosis of cluster I cases (HR = 3.18, $p = 0.008$), while stepwise multivariate analysis still showed a trend, though not a significant one, toward an association between cluster I and poor prognosis ($p = 0.084$) (Table III).

DISCUSSION

Our study demonstrates that reduced expression of class II HDAC genes is associated with poor prognosis in lung cancer patients and that low expression of *HDAC10* independently affects the prognosis of lung cancer. Clustering analysis indicated a significant association between cluster I and poor prognosis. Clustering also demonstrated clear clusters of class I and class II HDAC genes, suggesting a common mechanism in transcriptional regulation in each HDAC class. The clustering of lung cancer cases demonstrated that, in most cases, expression levels of all class II HDAC genes were altered in the opposite way compared to class I genes. Class-specific expressional regulation of class II HDAC genes might be affected during lung cancer development.

Our study demonstrated statistical significance in several analyses. Generally speaking, multiple comparisons increase the probability of obtaining a statistically significant finding ($p < 0.05$) by chance alone. However, it is unlikely that our statistically significant results were obtained by chance because significant differences were observed in more than half the genes examined (5 class II of 9 HDAC genes). In addition, even if $p < 0.0055$ (= 0.05/9,

TABLE II – UNIVARIATE AND MULTIVARIATE HAZARD RATIO ACCORDING TO EXPRESSION OF HDAC GENES BY COX PROPORTIONAL HAZARDS MODEL ANALYSIS

Univariate analysis Variables	HR[1] [95% CI[2]]	Unfavorable/Favorable	*p*-value[3]
Histology	1.09 [0.37–3.21]	Squamous/Non-squamous	0.882
Age (yr)	1.80 [0.74–4.42]	≥62/<62	0.198
Gender	3.02 [1.02–8.93]	Male/Female	0.046*
Smoking	2.23 [0.87–5.70]	Smoker/Non-smoker	0.094
Disease stage	5.17 [1.90–14.05]	II–III/I	0.001*
HDAC1	1.72 [0.51–5.82]	<0.8/≥0.8[4]	0.383
HDAC2	1.56 [0.67–3.61]	<0.8/≥0,8	0.302
HDAC3	1.19 [0.48–2.91]	<0.8/≥0.8	0.710
HDAC8	1.84 [0.72–4.72]	<0.8/≥0.8	0.202
HDAC4	2.94 [1.27–6.79]	<0.8/≥0.8	0.012*
HDAC5	3.92 [1.67–9.19]	<0.8/≥0.8	0.002*
HDAC6	3.02 [1.23–7.44]	<0.8/≥0.8	0.016*
HDAC7	2.88 [1.17–7.06]	<0.8/≥0.8	0.021*
HDAC10	3.94 [1.65–9.42]	<0.8/≥0.8	0.002*

Multivariate analysis including each class II HDAC gene

Variables	HDAC genes									
	HDAC4		HDAC5		HDAC6		HDAC7		HDAC10	
	HR	*p*-value	HR	*p*-value	HR	*p*-value	HR	*p*-value	HR	*p*-value
Histology	0.65	0.470	0.92	0.897	0.74	0.608	0.72	0.574	0.66	0.511
Age (yr)	1.49	0.395	1.33	0.570	1.31	0.576	1.26	0.623	1.40	0.497
Gender	3.36	0.159	3.50	0.145	2.84	0.201	3.57	0.151	3.19	0.155
Smoking	0.83	0.805	0.60	0.516	0.94	0.932	0.79	0.760	0.86	0.830
Disease stage	3.99	0.007*	4.45	0.004*	4.20	0.005*	3.78	0.011*	4.15	0.006*
HDACs[4]	2.88	0.024*	3.91	0.004*	2.88	0.028*	2.67	0.050*	4.12	0.003*

Stepwise multivariable analysis including all HDAC genes

Variables	HR [95% CI]	Unfavorable/Favorable	*p*-value
Gender	2.87 [0.92–8.93]	Male/Female	0.069
Disease stage	6.94 [2.37–20.37]	II–III/I	<0.001*
HDAC1	0.11 [0.02–0.61]	<0.8/≥0.8	0.012*
HDAC2	2.38 [0.90–6.26]	<0.8/≥0.8	0.080
HDAC5	2.63 [0.98–7.04]	<0.8/≥0.8	0.054
HDAC10	7.13 [1.90–26.67]	<0.8/≥0.8	0.004*

[1]Hazard ratio estimated by Cox proportional hazards regression model.–[2]Confidence interval of the estimated HR.–[3]Asterisks indicate significant *p* values.–[4]Low expression group (<0.8) vs. high or moderate expression group (≥0.8).

i.e., 0.05 was divided by the number of analyzed genes) is considered statistically significant, both *HDAC5* and *HDAC10* demonstrate significant differences (*p*-values were much less than 0.0055 in Table II, Figs. 1*c,* 2*b,e*). Therefore, at least the reduced expression of *HDAC5* and *HDAC10* is associated with poor clinical outcome of lung cancer patients. However, because our cohort was rather small, these findings must be validated using training and test sets in future studies with much larger cohorts.

Class II HDAC genes are abundantly expressed in heart, skeletal muscle and brain tissue, though lung tissue also expresses class II HDAC genes moderately. *HDACs 4, 5* and *7* bind and inhibit MEF2 proteins, which play a significant transcriptional regulatory role in myogenesis.[10,11] In heart, the signal-resistant mutants of class II HDACs render cardiomyocytes resistant to hypertrophic signals, while knockout mice lacking a class II HDAC develop massive cardiac hypertrophy.[13] Therefore, class II HDACs act as signal-responsive suppressors of the transcriptional program governing cardiac hypertrophy. In addition, *in vivo* expression of a signal-resistant form of *HDAC5* resulted in sudden death accompanied by loss and morphologic changes of cardiac mitochondria as well as downregulation of mitochondrial enzymes,[14] suggesting an antiproliferative effect of class II HDAC genes. In contrast, class II HDAC molecules exhibit an antiapoptotic effect through inhibition of Nur77 induction during thymocyte development.[15] These observations suggest that class II HDAC genes may play a critical role in the differentiation and development of several tissues. In addition, class II HDACs may play a role in cancer development. Class II HDACs interact with an oncogenic molecule, Bcl-6,[16] a Kruppel-like zinc-finger repressor that is transcriptionally activated by chromosomal translocation in lymphomas. Class II HDACs also interact with the corepressor protein complex SMRT/N-CoR, which may contribute to leukemogenesis through interacting with the oncogenic chimeric molecules AML-ETO and PML-RARα.[17]

Our study showed that low expression of *HDAC5* and *HDAC10* was an independent predictor of short survival and that the cluster of low expression of class II HDAC genes was associated with poor prognosis. It is conceivable that class II HDAC genes, especially *HDAC5* and *HDAC10,* may also regulate the differentiation and proliferation of lung epithelial cells and that disruption in such regulation may be involved in lung cancer development. In this context, in our preliminary experiments, overexpression of *HDAC5* demonstrated moderate but consistent growth-inhibitory effects on lung cancer cell lines with low expression of class II HDAC genes.

HDAC5 as well as some other class II HDAC molecules, such as *HDACs 4* and *7,* contain a long N-terminal region, which interacts with MEF2.[18] When the appropriate myogenic differentiation signal is delivered, class II HDAC proteins are phosphorylated and dissociated with MEF2 and exported from the nucleus by the adaptor protein 14-3-3.[19] In airway epithelial cells, class II HDACs may also interact with transcription factors, the activity of which might be enhanced by reduced expression of class II HDACs, resulting in deregulation of differentiation and cell growth of lung epithelial cells. Therefore, identification of the transcription-regulating molecules associated with class II HDACs in lung epithelial cells is urgently required.

HDAC10 has an additional leucine-rich putative catalytic domain and is most similar to *HDAC6,* which contains 2 cata-

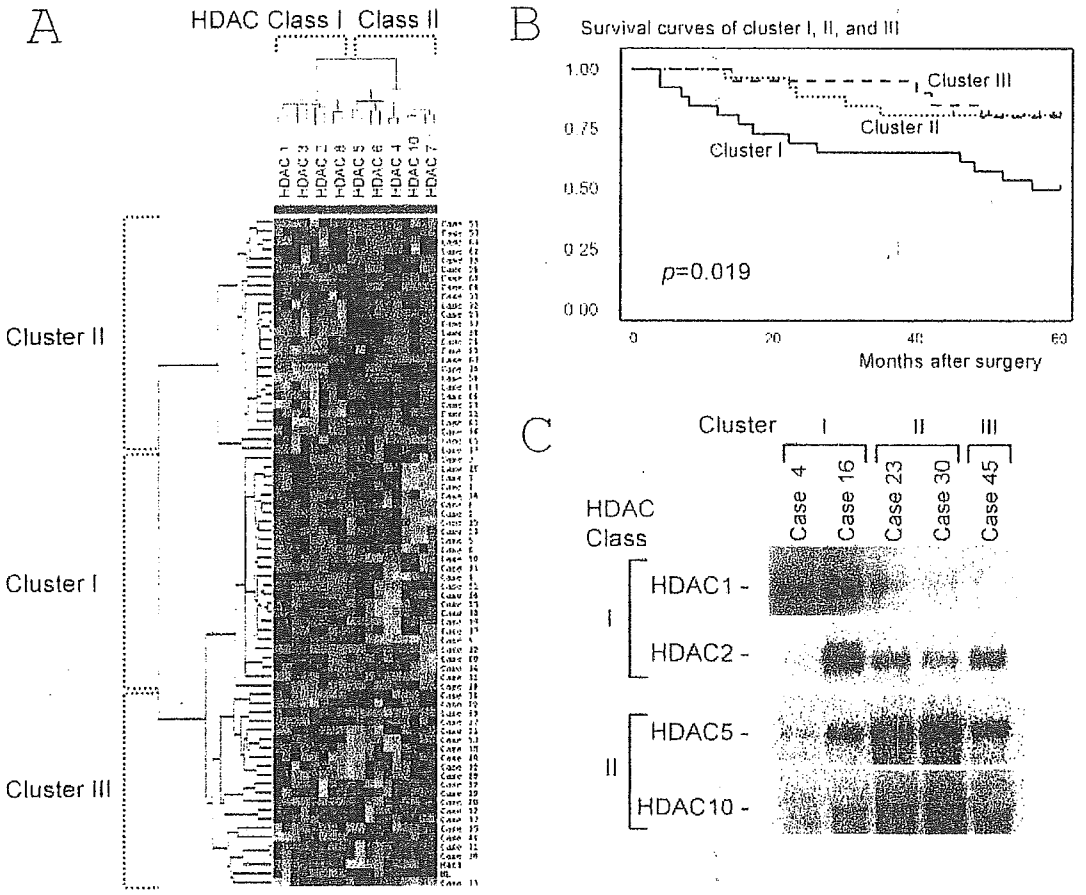

FIGURE 3 – Clustering analysis of HDAC gene expression. *(a)* Unsupervised hierarchical clustering of HDAC gene expression. The dendrogram of HDAC genes shows 2 discrete clusters, class I (*HDACs 8, 3, 2* and *1*) and class II (*HDACs 6, 4, 5, 10* and *7*). The dendrogram of lung cancer patients also indicates 3 clusters. The top cluster (cluster II, class II predominant) contained lung cancers abundantly expressing class II HDAC genes but very few class I genes, while the middle cluster (cluster I, class I predominant) demonstrated the reverse pattern. The bottom cluster (cluster III) did not show any predominance. *(b)* Survival curves of 3 clusters. Cluster I shows significantly poor prognosis, while clusters II and III show similarly favorable prognosis. *(c)* Northern blot analysis of HDAC genes in primary lung cancer specimens. Lung cancer tissues of cluster groups I and II demonstrate high expression of HDAC class I and class II genes, respectively. The lung cancer specimen of cluster III does not show such a class-specific expression pattern.

TABLE III – UNIVARIATE AND MULTIVARIATE HAZARD RATIO ACCORDING TO CLUSTER GROUPS BY COX PROPORTIONAL HAZARDS MODEL ANALYSIS

Variables	HR[1] [95% CI[2]]	Unfavorable/Favorable	*p*-value[3]
Univariate analysis			
Cluster	3.18 [1.357~7.441]	I/II–III	0.008*
Multivariate analysis			
Sex	2.51 [0.843~7.485]	Male/Female	0.098
Disease stage	3.62 [1.270~10.32]	II–III/I	0.016*
Cluster	2.18 [0.900~5.303]	I/II–III	0.084

[1]Hazard ratio estimated by Cox proportional hazards regression model.–[2]Confidence interval of the estimated HR.–[3]Asterisks indicate significant *p* values.

lytic domains. The biologic function of *HDAC10* has not been reported. However, because it interacts with class I HDACs, *HDAC2* and *HDAC3,* low expression of *HDAC10* may affect the activity of class I HDACs and enhance the imbalance of enzymatic activity between classes I and II, which might play a role in the development of cancer.

Other molecules associated with class II HDACs are reported to be involved in cancer development. Class II HDACs were reported to bind HP1α,[20] which interacts with a methyl-lysine of histone H3 and mediates gene silencing. Downregulation of HP1α expression is associated with a metastatic phenotype in breast cancers.[21] Also, subunits of chromatin-remodeling complex SWI/SNF, BRG1 and BRM, were frequently lost in lung cancers.[22] Moreover, patients with BRG1/BRM-negative lung carcinomas, independent of stage, have significantly poorer prognosis. Because HP1α interacts with BRG1[23] in addition to class II HDACs, class II HDACs, HP1α and

BRG1 may compose a large protein complex, which may play a crucial role in the expression of growth- or differentiation-associated genes. Altered expression of any component may contribute to cancer development and progression.

Our study demonstrates that class II HDAC genes might play a crucial role in cancer development/progression. Further studies on the relation of altered expression of class II HDAC genes with expression profiles and epigenetic alterations may clarify the biologic and pathogenetic function of class II HDAC genes. HDAC inhibitors are promising cancer therapeutic reagents. Some therapeutic compounds (FK228[24] and MS-27-275[25]) have demonstrated inhibitory activity, specifically against class I HDACs. These HDAC inhibitors with class I specificity might be desirable as they exhibit anticancer effects.

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Mutations of the *Epidermal Growth Factor Receptor* Gene in Lung Cancer: Biological and Clinical Implications

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ABSTRACT

Recently it has been reported that mutations in the tyrosine kinase domain of the *epidermal growth factor receptor* (*EGFR*) gene occur in a subset of patients with lung cancer showing a dramatic response to EGFR tyrosine kinase inhibitors. To gain further insights in the role of EGFR in lung carcinogenesis, we sequenced exons 18–21 of the tyrosine kinase domain using total RNA extracted from unselected 277 patients with lung cancer who underwent surgical resection and correlated the results with clinical and pathologic features. *EGFR* mutations were present in 111 patients (40%). Fifty-two were in-frame deletions around codons 746–750 in exon 19, 54 were point mutations including 49 at codon 858 in exon 21 and 4 at codon 719 in exon 18, and 5 were duplications/insertions mainly in exon 20. They were significantly more frequent in female ($P < 0.001$), adenocarcinomas ($P = 0.0013$), and in never-smokers ($P < 0.001$). Multivariate analysis suggested *EGFR* mutations were independently associated with adenocarcinoma histology ($P = 0.0012$) and smoking status ($P < 0.001$), but not with female gender ($P = 0.9917$). In adenocarcinomas, *EGFR* mutations were more frequent in well to moderately differentiated tumors ($P < 0.001$) but were independent of patient age, disease stages, or patient survival. *KRAS* and *TP53* mutations were present in 13 and 41%, respectively. *EGFR* mutations never occurred in tumors with *KRAS* mutations, whereas *EGFR* mutations were independent of *TP53* mutations. *EGFR* mutations define a distinct subset of pulmonary adenocarcinoma without *KRAS* mutations, which is not caused by tobacco carcinogens.

INTRODUCTION

Non–small-cell lung cancer (NSCLC) frequently overexpresses receptors of the erbB family including the epidermal growth factor receptor (EGFR) encoded by erbB-1 (HER1; ref. 1, 2). The EGFR is a 170 kilodaltons receptor tyrosine kinases (TK) that dimerizes and phosphorylates several tyrosine residues after binding of several specific ligands (1). These phosphorylated tyrosines serve as the binding sites for several signal transducers that initiate multiple signaling pathways resulting in cell proliferation, migration, and metastasis, evasion from apoptosis, or angiogenesis, all of which are associated with cancer phenotypes (1). Downstream pathways include ras-raf-MEK-ERK (raf-mitogen-activated protein kinase kinase-extracellular signal-regulated kinase), phosphatidylinositol-3 kinase-AKT and PAK-JNKK-JNK (p21-activated protein kinase–c-Jun NH_2 terminal kinase kinase–c-Jun NH_2 terminal kinase; ref. 1). Gefitinib is an orally administered small molecule that specifically inhibits EGFR tyrosine phosphorylation (3). Clinical trials revealed that there was a significant variability in response to gefitinib. Good clinical response has been observed most frequently in women, nonsmokers, patients with adenocarcinomas, and Japanese patients (4, 5). However, it has not been possible to predict gefitinib sensitivity by levels of EGFR overexpression as determined by immunohistochemistry (6) or immunoblotting (7). The factor(s) that determine gefitinib sensitivity has long been an enigma. It has been reported recently that activating mutations of EGFR are present in a subset of pulmonary adenocarcinomas and that tumors with *EGFR* mutations are highly sensitive to gefitinib (8, 9). Furthermore, the incidence of *EGFR* mutations is higher in Japanese than in Caucasian patients (8). In this study, we searched for *EGFR* mutations in a large cohort of unselected Japanese NSCLC to correlate them with clinical and pathologic features including *KRAS* or *TP53* mutations.

MATERIALS AND METHODS

Patients. Primary tumor samples were obtained from 277 unselected patients with lung cancer who underwent potentially curative pulmonary resection at the Department of Thoracic Surgery, Aichi Cancer Center Hospital from May, 2000 through November, 2000 and from January, 2001 through December, 2002, after obtaining appropriate approval from the institutional review and patients' written informed consent. These cases corresponded to 82% of all consecutive cases. Inclusion of the cases into this study was dependent on availability of frozen tumor material. About 20 cases were excluded because tumor cells were too few to sufficiently extract tumor RNA because of inflammation and/or necrosis. There were 159 males and 118 females with an age at diagnosis ranging from 26 to 89 (median 64) years. One hundred fifty-nine patients had stage I disease, 39 had stage II, 74 had stage III and 5 had stage IV diseases. There were 224 adenocarcinomas, 35 squamous cell carcinomas, 9 large cell carcinomas, 5 adenosquamous carcinomas, 3 small cell carcinomas, and 1 carcinoid. There were 115 never-smokers and 162 ever-smokers including current and former smokers. Smoking history was obtained by interviewing each patient at admission or first outpatient visit.

Molecular Analysis of Lung Cancer Specimens. Tumor samples were obtained at the time of surgery, rapidly frozen in liquid nitrogen, and stored at −80°C. Frozen tissue of the tumor specimens were grossly dissected to enrich as much tumor cells as possible by a surgical pathologist (Y. Y.). We isolated total RNA using the RNAeasy kit (Qiagen, Valencia, CA).

The first four exons (exons 18–21) of the seven exons (exons 18–24) that code for TK domain of the *EGFR* gene that includes all of the mutations reported thus far (8, 9) were amplified with primers F1 (5′-AGCTTGTGGAGCCTCTTACACC-3′) and R1 (5′-TAAAATTGATTCCAATGCCATCC-3′), in a one-step reverse transcription-PCR setup with Qiagen OneStep reverse transcription-PCR kit (Qiagen, Valencia, CA). The cDNA sequence of *EGFR* gene was obtained from GenBank (accession number NM005228). Reverse transcription-PCR conditions were available after request. Reverse transcription-PCR products were diluted and cycle-sequenced with the Big Dye Terminator v3.1/1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were electrophoresed on an ABI PRISM 3100 (Applied Biosystems). Both the forward and reverse sequences obtained were analyzed by BLAST and chromatograms by manual review.

***KRAS* and *TP53* Gene Analysis.** We had previously examined the same cohort for *KRAS* mutations and *TP53* mutations (10, 11). Briefly, *TP53* gene (exon 4 through 10) and *KRAS* gene (exons 1 and 2) were amplified and directly sequenced with ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Statistical Analysis. For comparisons of proportions, the χ^2 test or Fisher's exact test were used. The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and survival differences were

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Note: T. Kosaka and Y. Yatabe contributed equally to the present study.

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analyzed by the log-rank test. The two-sided significance level was set at $P < 0.05$. To identify which independent factors jointly had a significant influence on the incidence of EGFR mutations, the logistic regression modeling technique was used. We did all analyses using a StatView (version 5, SAS Institute Inc., Cary, NC) software on a Macintosh computer.

RESULTS

***EGFR* Mutations in Unselected Lung Cancer Specimens.** Of 277 unselected patients who underwent surgical resection of their tumors, we found that 111 patients (40%) had mutations in exons 18–21 of the *EGFR* gene. There were 52 deletion mutations, 54 point mutations, and 5 duplication/insertion mutations. In 14 tumors, corresponding cDNA from normal lung tissue far from the tumors was also sequenced, which confirmed that all these mutations were somatic. Details of the resulting changes in EGFR protein as a consequence of these mutations are illustrated in Fig. 1.

All of the 52 deletion mutations occurred around codons 746–750 in exon 19. About half (25 of 52) of deletion mutations were simple deletions of five amino acid residues ELREA from codon 746 to 750. However, 22 deletions were coupled with point mutations or insertions, yielding various changes in amino acid sequences as shown in Fig. 1. It is noted that, in all cases, such alterations were in-frame. Forty-six of the 54 point mutations were from a T to a G transversion at the second nucleotide of codon 858 in exon 21 resulting in substitution of leucine with arginine residue. Four of the point mutations occurred at codon 719 in exon 18. We noted that one tumor with a mutation at codon 719 and three tumors with mutations at codon 858 had another mutation occurring at codons 709, 768, 776, and 790, respectively. For rare mutations (all 5 insertions, E709H, T790M, S768I, R776C, V769L), we resequenced and confirmed that these mutations were actually present. In summary, 52 of the 111 (47%) *EGFR* mutations were deletions around codons 746–750 and 49 (44%) were L858R, altogether accounting for 91% of all of the *EGFR* mutations found. The four major classes of mutations (*i.e.*, deletions, L858R, mutations at codon 719, duplications/insertions) never occurred simultaneously. Furthermore, it is of note that only mutant sequences were present in chromatograms in 19 of 52 deletions, 13 of 46 in L858R, and 1 of 4 codon 719 mutations.

Relationship between *EGFR* Mutations and Clinical-Pathologic Features. *EGFR* mutations were significantly more frequent in females (59%) than males (26%; $P < 0.001$), in never-smokers (66%) than ever-smokers (22%; $P < 0.001$), and in patients with adenocarcinomas (49%) than in those with nonadenocarcinomas (2%; $P < 0.001$). There was only one patient with an *EGFR* mutation of 53 nonadenocarcinoma patients. This patient was a 61-year-old male with adenosquamous carcinoma. Because female patients tended to be never-smokers and were likely to have adenocarcinoma, we did logistic regression analysis to determine which of these three variables independently contributed to the *EGFR* mutations. The result suggested that smoking status and adenocarcinoma histology independently affected *EGFR* mutations whereas female gender did not (smoking status, odds ratio 3.949, $P < 0.001$; histologic type, odds ratio 27.486, $P = 0.0013$; gender odds ratio 0.996, $P = 0.9917$).

Further Analysis of Patients with Adenocarcinoma. *EGFR* mutations were found almost exclusively in adenocarcinomas with only one exception; hence, we did more detailed analysis limited to this subset of patients (Table 1). *EGFR* mutations were also significantly frequent in female, nonsmoking patients. When we divided ever-smokers into 3 categories depending on smoke exposure, there was a trend that the higher the exposure, the lower the incidence of *EGFR* mutations. *EGFR* mutations were significantly more frequent in well to moderately differentiated adenocarcinomas (58%) than in poorly differentiated adenocarcinomas (30%; $P < 0.001$). There were five bronchioloalveolar cell carcinomas (BAC) in our cohort, of which three harbored *EGFR* mutations (60%), according to the World Health Organization classification of lung cancers (which states that BAC is a true noninvasive cancer without stromal or pleural invasion; ref. 12). It seemed that *EGFR* mutations were associated neither with age of the patients nor with stage of diseases. There was no difference in incidence of *EGFR* mutations between both sexes in patients of age 50 (average age of menopause in Japan) or younger, although the number of patients of this age group was small (2 of 7 males, 2 of 7 females).

Our preliminary study indicated that patients with *EGFR* mutations survived for a longer period after gefitinib treatment than those without *EGFR* mutations.[5] However, *EGFR* mutations also might have prognostic impact on patients with pulmonary adenocarcinoma, even when the patients were not exposed to gefitinib because EGFR

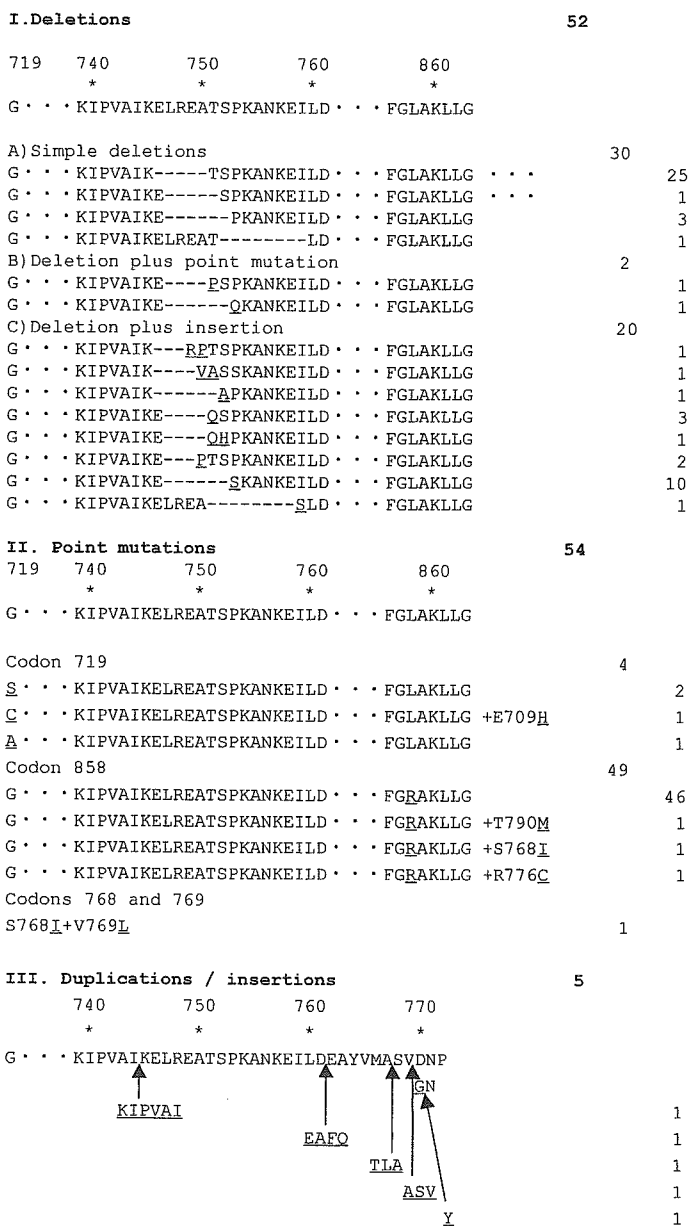

Fig. 1. Analysis of 111 *EGFR* mutations in the TK domain of the *EGFR* gene found in unselected cases with lung cancer.

[5] T. Mitsudomi, T. Kosaka, H. Endoh, Y. Horio, T. Hida, S. Mori, S. Hatooka, M. Shinoda, T. Takahashi, Y. Yatabe, submitted for publication.

Table 1 *Relationship between EGFR mutations and clinical and pathologic features in a subset of patients with adenocarcinoma*

Variables	Category	EGFR			P
		Mutation (%)		Wild-type	
N		110	(49)	114	
Gender	Male	40	(36)	71	<0.001
	Female	70	(62)	43	
Age	≤64	51	(46)	60	0.3481
	> 64	59	(52)	54	
Smoking status	Never-smoker (pack years = 0)	76	(68)	36	<0.001
	Ever-smoker	34	(31)	78	
	Pack years <20	11	(55)	9	
	20–50	15	(27)	40	
	>20	8	(22)	29	
Differentiation	Well to moderately differentiated	89	(58)	65	<0.001
	Poorly differentiated	21	(30)	49	
Stage	IA and IB	69	(50)	70	0.8383
	IIA through IV	41	(48)	44	
Survival	3-year survival rate	86%		91%	0.9933
KRAS mutation	Mutated	0	(0)	26	<0.001
	Wild-type	97	(57)	73	
TP53 mutation	Mutated	37	(47)	42	0.4634
	Wild-type	59	(52)	54	

There were five BACs in our cohort, of which three harbored *EGFR* mutations.

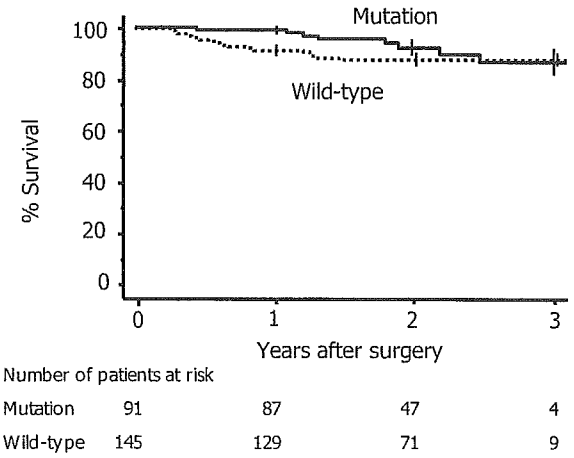

Fig. 2. Effect of *EGFR* mutations on survival of patients with adenocarcinoma calculated from the day of surgery. Patients later treated with gefitinib and those whose surgery was done for recurrent or second primary cancers were excluded.

mutations defined subsets of pulmonary adenocarcinoma with distinct features as described. Therefore, we did survival analysis in patients excluding those who were treated with gefitinib when they had recurrent diseases. The Kaplan-Meier curve (Fig. 2) indicated that EGFR mutations did not affect prognosis of the patients ($P = 0.9933$), although the follow up period was relatively short (median follow up, 788 days)

***KRAS* and *TP53* Gene Mutational Analysis.** Of 224 patients with adenocarcinoma, *KRAS* and *TP53* data were available for 196 and 192 patients, respectively. *KRAS* mutations were present in 26 of 196 patients (13%; 22 at codon 12, 1 at codon 13, and 3 at codon 61). *TP53* mutations were present in 79 of 192 (41%). *KRAS* and *TP53* mutations were significantly more frequent in ever-smokers, respectively [20% *versus* 6% for *KRAS* ($P = 0.0054$) and 54% *versus* 30% for *TP53* ($P < 0.001$)]. Interestingly, *EGFR* mutations were never found in tumors with *KRAS* mutations, showing a mutually exclusive relationship. By contrast, *EGFR* mutations and *TP53* mutations seemed to occur independently. Figure 3 shows the relationship among the three mutations by a Venn diagram in 192 patients in whom information about the status of these three genes was available.

TP53 mutations seemed more widely distributed in tumors without *EGFR* mutations (Fig. 4). Of seven mutations either at codon 157, 248, or 273 in which strong and selective adduct formation of benzo(*a*)pyrene diol epoxide, one of the major tobacco carcinogens, occurs (13), six were in tumors without *EGFR* mutations (Fig. 3). Furthermore, of 16 mutations caused by a G to a T transversions characteristic of mutations caused by aromatic polycyclic hydrocarbons (14), 15 were in tumors without *EGFR* mutations (Fig. 3).

DISCUSSION

Adenocarcinoma is the most predominant histologic subtype, and its incidence is increasing in Japan. Registration of resected lung cancer in Niigata prefecture, Japan, revealed that the incidence of adenocarcinoma is 71% of 1211 patients operated on from 2001 to 2002 (15). In our institution, adenocarcinoma accounted for 54% of 975 patients who were operated on from 1965 through 1995, 69% of 522 from 1996 through 2000, and 76% of 407 from 2001 through 2003. Considerable evidence indicates that the EGFR pathway also plays an important role in both the pathogenesis and the progression of lung cancer (1).

We found that 40% of 277 unselected patients with lung cancer carried mutations in the TK domain of the *EGFR* gene. More than 90% of the mutations were either deletions around codons 746–750 in

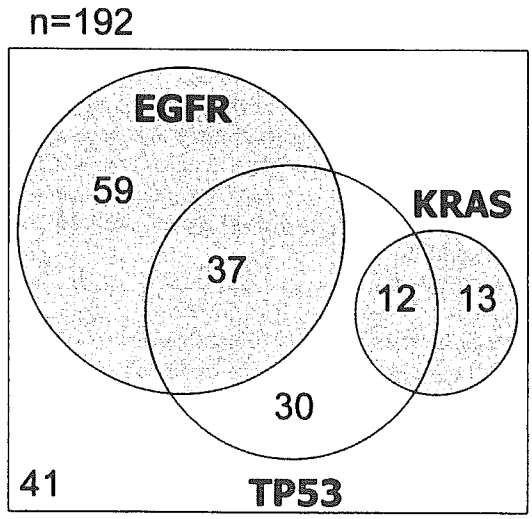

Fig. 3. The Venn diagram illustrating relationship among *EGFR* mutations, *KRAS* mutations and *TP53* mutations in patients with adenocarcinoma ($n = 192$). Diameters of each circle are roughly proportional to the number of mutations.

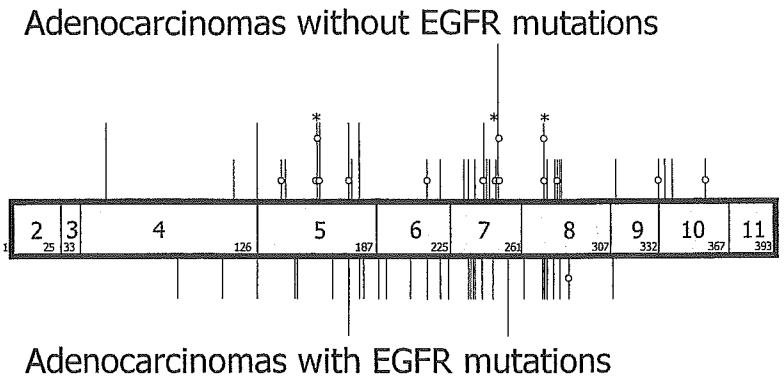

Fig. 4. Distribution of *TP53* gene mutations in adenocarcinomas without *EGFR* mutations (n = 42) or with *EGFR* mutations (n = 32). Numbers below show codons of exon boundaries. *Asterisks* show codons 157, 248, and 273, where strong and selective benzo(*a*)pyrene diol epoxide adduct formation is reported to occur (13). *White circles* indicate where *TP53* mutations were caused by a G to a T transversion.

exon 19 or L858R in exon 21, which all flank the ATP-binding pocket that is important for TK activity (8, 9). We also noted that in about 30% of the cases with *EGFR* mutations, only bands derived from mutant allele were detected on chromatogram. This is somewhat puzzling considering the heterozygous nature of the *EGFR* mutations reported thus far (8, 9) and the presence of stromal cells in resected tumor specimens. This finding may suggest that loss of wild-type alleles or amplification of mutant alleles accompanied with mutations in these cases, as indicated by Minna *et al.* (16).

EGFR mutations were almost exclusively present in adenocarcinoma. Mutations were more prevalent in females and nonsmokers, confirming and extending the results of previous reports (8, 9). It is noteworthy that these characteristics and Japanese ethnicity are all predictors of gefitinib sensitivity at least by univariate analysis (4, 5). Multivariate analysis suggested that nonsmoking status and adenocarcinoma histology independently contributed to *EGFR* mutations but female gender did not. The fact that premenopausal women did not show higher incidence of *EGFR* mutations further suggested that apparent difference between female and male was caused by a difference in lifestyle including smoking habit rather than involvement of sexual environment.

Previously described genetic alterations in lung cancer are almost always more frequent in smokers than nonsmokers. For example, mutations of the *TP53* gene (17), *KRAS* genes (18), or deletion of the short arm of chromosome 3 (19) are known to be more frequent in smokers, as was the case in the present study for the first two. A plausible explanation for the reason why *EGFR* mutations are associated with nonsmoking status are not possible at this time, but it is natural to assume that *EGFR* mutations are caused by carcinogen(s) other than those contained in tobacco smoke. In Taiwan, human papilloma virus type, 16 of 18 infections (20) or cooking oil fume (21) have been investigated as a cause of lung cancer occurring in nonsmoking women. These observations might be relevant with preferential *EGFR* mutations in nonsmoking women. Nevertheless, *EGFR* mutations should provide a clue for pathogenesis of adenocarcinoma occurring in nonsmokers and should ultimately lead to discovery of effective prevention.

We were able to confirm higher incidence of *EGFR* mutations in Japanese patients. Lynch *et al.* found *EGFR* mutation in 2 of 25 unselected United States patients (9), and Paez *et al.* (8) did so in 1 of 61 United States patients and 15 of 58 Japanese patients. The reason for this marked difference between Japanese and United States patients is not very clear. However, difference in incidence of nonsmoking patients between Japanese and American female patients with lung cancer may partly account for this. In our cohort, 83% of female patients and 10% of male patients were never-smokers. This trend is common in Japan. For example, Toyooka *et al.* (22) and Minami *et al.* (23) reported that the proportion of never-smoking women in lung cancer patients is 96% and 75%, respectively. This makes quite a contrast with the fact that only 15% of 706 United States female and 6% of 1,347 male patients with lung cancer are never-smokers (24).

We found that *EGFR* mutations and *KRAS* mutations known to play an important role in pathogenesis of adenocarcinoma of the lung (25) were strictly mutually exclusive, reminding us of a similar exclusionary relationship between retinoblastoma and p16 inactivation in lung cancer (26). This finding may be explained by the fact that the KRAS-mitogen-activated protein kinase pathway is one of the downstream signaling pathways of EGFR (1). Because it has been shown that L858R and delL747-P753ins S are activating mutations that result in markedly increased phosphorylation of EGFR when EGF was added (8, 9), tumors with *KRAS* mutations that already have activated further downstream effectors do not need to have *EGFR* mutations. The high incidence of *EGFR* mutations in lung adenocarcinomas may explain why *KRAS* mutations are lower in Japanese than in Caucasian patients. In the present study, *KRAS* mutations were found in 13% of adenocarcinomas, whereas they were present in 33% of Dutch cases (25). This may be also at least partially attributable to the difference in smoking status, because *KRAS* mutations were more frequent in smokers as reported previously (18). In contrast, the incidence of *TP53* mutations was not associated with *EGFR* mutations, although *TP53* mutations also occurred more frequently in smokers (17). However, *TP53* mutations in tumors without *EGFR* mutations showed characteristics of mutations caused by tobacco carcinogens in terms of sites or base substitution patterns (13, 14).

We also noted that well to moderately differentiated adenocarcinomas had a significantly higher incidence of *EGFR* mutations than poorly differentiated ones. This observation might be relevant to the fact that adenocarcinomas showing BAC feature show higher sensitivity to gefitinib (27). However, when we used the strict criteria as stated by the World Health Organization Classification of lung tumors (12), our cohort included only five BAC, of which three had EGFR mutations. Unfortunately, these strict criteria are not applied by many pathologists, leading to considerable confusion between BAC and adenocarcinoma with BAC features in the literature. Alternatively, we proposed terminal respiratory unit type adenocarcinoma that is characterized by morphological resemblance to type II pneumocytes, Clara cells, and/or bronchioles as well as expression of thyroid transcription factor-1 and surfactant proprotein B (refs. 28, 29). In the World Health Organization classification, most nonmucinous bronchioloalveolar, mixed bronchioloalveolar and acinar subtypes, and some papillary subtypes belong to the terminal respiratory unit type adenocarcinoma (28, 29). We found that most adenocarcinoma with EGFR mutations were categorized into terminal respiratory unit type adenocarcinoma.[6]

[6] Y. Yatabe, T. Kosaka, T. Takahashi, T. Mitsadomi, submitted for publication.

EGFR mutations were not associated with stage of disease, suggesting that EGFR mutations occurs relatively early in clinical course and are associated with pathogenesis of adenocarcinoma rather than progression.

In conclusion, we found a high incidence of EGFR mutations in Japanese patients with pulmonary adenocarcinoma, especially in those who never smoked. EGFR mutations were never present in tumors with KRAS mutations, indicating possibilities of genotype-oriented approach for pulmonary adenocarcinoma.

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Identification of MGB1 as a Marker in the Differential Diagnosis of Lung Tumors in Patients with a History of Breast Cancer by Analysis of Publicly Available SAGE Data

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The risk of developing second primary cancers is increased in patients with breast cancer. The lung is one of the major target organs, and therefore a differential diagnosis between primary and metastatic cancers is required for the treatment of lung tumors in patients with a history of breast cancer. However, biopsy specimens frequently result in small, fragmented tissues containing only a few, degenerated cancer cells. We attempted to find a useful marker for differential diagnosis, using the online SAGE database. We selected three molecules, small breast epithelial mucin (SBEM), prostate epithelium-specific Ets transcription factor (PDEF), and mammaglobin (MGB1), as potential markers for breast cancer. SBEM and PDEF proved of no use for practical differential diagnosis because they are expressed in the normal bronchus. In contrast, expression of MGB1 was detected in all 22 primary breast cancers, but not in 22 normal lung tissues. Furthermore, all 12 metastatic breast cancers examined demonstrated positive MGB1 transcripts, whereas one of 48 primary lung adenocarcinomas expressed MGB1. This suggests that MGB1 can serve as a differential molecular marker. In practice, prospective examination, using the nine cases with a history of breast cancer, confirmed the usefulness of MGB1 in differential diagnosis. *(J Mol Diagn 2004, 6:90–95)*

The lung is a major target of hematogeneous metastases from a variety of cancers. Thus, a diagnosis differentiating between primary and metastatic cancers is always required in clinical practice. In our institute, on average 250 lung biopsies are performed every year, and about two thirds of the tumors are diagnosed as malignant. Metastatic cancers make up 10% to 20% of these. Although this incidence may not be very high, a differential diagnosis of the metastatic cancer is important to determine the therapeutic strategy. For example, in the case of a small solitary lung tumor without any lymphadenopathy, the patient may be treated with chemotherapy or may undergo partial resection of the lung when the lung tumor is diagnosed as a metastatic breast cancer. On the other hand, standard lobectomy may be the treatment of choice when the diagnosis is of a primary non-small cell lung cancer.

The risk of developing second primary cancers is increased in patients with breast cancer, and the lung is one of the major sites involved.[1–3] Some articles have described an increased risk of primary lung cancers in association with radiation therapy following mastectomy.[4,5] Furthermore, the long latent period before identification of metastasis makes a differential diagnosis challenging. Indeed, a latent period of more than 10 years is not rare in patients with breast cancer. Histologically, a differential diagnosis between metastatic breast cancer and primary lung adenocarcinoma is difficult. Cytoplasm with secretory feature and stromal fibrosis were frequently observed in both adenocarcinomas. Moreover, metastatic breast cancer can grow along with the alveolar septa, in a similar manner to bronchioloalveolar carcinomas.[6] Difficulty is also caused by the need to carry out a differential diagnosis on biopsy specimens. Often, lung biopsies produce small amounts of fragmented tissue, which contain only a few degenerated cancer cells. Therefore, the differential diagnosis has to draw on auxiliary analysis, such as immunohistochemistry.

There are a limited number of immunohistochemical markers to identify breast cancers. Gross cystic disease fluid protein-15 (GCDFP-15) is one such marker; it is positive in only a few normal breast epithelia, but frequently expressed in breast carcinomas showing apo-

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crine features. Estrogen receptor (ER) is also commonly used for differential diagnosis. The combined application of GCDFP-15 and ER are very helpful for differential diagnosis, but each marker is not complete. About 50% to 60% of breast cancers express one or both, but the remaining tumors are negative for both markers. Indeed, Perry et al[7] reported that GCDFP-15 and ER are specific, but insensitive, for breast origin through the differential diagnosis of 68 metastatic adenocarcinomas to the brain. Currently, extensive human genome data have been accumulated, and the data contain much information that can be directly used in clinical practice. In the present study, we searched for molecules of potential breast-specific expression using the online database of serial analysis of gene expression (SAGE) from the National Center for Biotechnology Information (NCBI). Our results demonstrated that one of the molecules examined was specifically expressed in breast cancers, indicating that the molecule can serve as a differential marker.

Materials and Methods

Patients

Using a database of the Department of Pathology and Molecular Diagnostics of Aichi Cancer Center Hospital (Nagoya, Japan) , we first analyzed the incidence of lung biopsies that required a differential diagnosis of primary or metastatic lung cancer. For RT-PCR studies, 70 primary lung cancers, 51 metastatic lung cancers, and 22 normal lung tissue samples, as well as 22 invasive breast cancers were analyzed. All these tissues were obtained immediately after surgery, snap-frozen, and stored at −80°C until use. In addition, for prospective analysis, nine touch-imprint slides were prepared from fine-needle biopsy specimens.

Reverse Transcription and PCR (RT-PCR) Analysis

Total RNA was extracted using a standard acid guanidinium thiocyanate-phenol-chloroform method,[8] and was digested with DNase I, followed by conversion to cDNA with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers. After confirmation of RNA integrity and negative contamination of genomic DNA by RT-PCR for β-actin (275 and 369 bp products for cDNA and genomic DNA, respectively), cDNA was subjected to PCR analysis. Gene-specific amplification was performed using Amplitaq Gold (Applied Biosystems, Foster City, CA). The nucleotide sequences of MGB1, SBEM and PDEF were obtained from GenBank (NM 002411, NM 058173, and NM 012391, respectively) and primers were designed to span an exon junction as follows: MGB1, 5′-ACCATGAAGTTGCTGATGGTC-3′ and 5′-AAACACCTCAACATTGCTCAGA-3′; SBEM, 5′-GTATCCAGCTACTGGTCCTGCT-3′ and 5′-CAATTGCAGAAGACTCAAGCTG-3′; PDEF, 5′-CGGTCATTGACAGCCAAG-3′ and 5′-AGGAGCCACTTCTGCACATT-3′.

Products were analyzed by electrophoresis on 2.5% high-resolution gels (NuSieve GTG agarose, BioWhittaker Molecular Applications, Rockland, ME). Some products were directly sequenced using an ABI 310 Genetic Analyzer and BigDye Primer Cycle Sequencing Kits (ABI, Foster City, CA), to confirm the amplified sequences. To determine the precise location of the mRNA expression of the gene of interest, parts of the tissues were isolated with a laser-captured microdissection system (Arcturus, Mountain View, CA). Extraction of RNA and RT-PCR were carried out as described above.

Results

Incidence of Lung Biopsies Requiring Differential Diagnosis

For the years 2000–2002, 834 lung biopsies were submitted to our department; 564 were diagnosed as malignancies or suspicious malignancies. A differential diagnosis between primary and metastatic cancers was required for 174 (21%) of the specimens submitted, because these patients had histories of cancer before the identification of lung tumors. The primary sites of the previous cancers included lung (26%), head and neck (22%), breast (13%), stomach (13%), colon (7%), and others (19%). We have reported on the differential diagnosis of metachronous[9] and synchronous lung cancers,[10] and, therefore, this study focused on the differential diagnosis of primary lung cancer and metastatic breast cancer.

Searching for Sensitive Markers of Metastatic Breast Cancer

To obtain markers that are sensitive and specific to breast cancers, we searched the online SAGE database of the NCBI (http://www.ncbi.nlm.nih.gov/SAGE/). Three candidates, mammaglobin (MGB1, Hs.46452), small breast epithelial mucin (SBEM; Hs.348419), and prostate epithelium-specific Ets transcription factor (PDEF; Hs.79414), were selected. Although only a few articles have described these molecules, they support breast-specific expression.[11–15] Detailed results from the SAGE database and the literature are summarized in Table 1. We next examined whether expression of these molecules could be used as practical distinguishing markers between primary lung cancers and metastatic breast cancers. In the 22 primary breast cancers, MGB1 was expressed in all 22, SBEM in 20, and PDEF in 20. In contrast to the results from the SAGE database, SBEM and PDEF transcripts were detected in 21 and 20 of 22 normal lung tissues, respectively, whereas MGB1 was not expressed in any (Table 1). SBEM has also been reported to be expressed in salivary glands,[14] which histologically resemble bronchial glands. We therefore determined the precise location of expressions in isolated bronchial glands, bronchial surface epithelium, and peripheral lung tissue, using a laser-capture microdis-

Table 1. Expression of MGB1, SBEM, and PDEF in Breast Cancers and in the Normal Lung

	MGB1	SBEM	PDEF
SAGE database*			
Normal tissues	9:2	376:0	4:0
Cancer tissues	17:4	32:0	126:0
Reported description			
Tissue-specific expression	Yes	Yes	Yes
Expressing organ(s)	Breast†	Breast and salivary glands	Breast and prostate
Expression in breast cancers	Yes	Yes	Yes
References	11, 16	14	12, 13
RT-PCR			
Primary breast cancer (n = 22)‡	22	20	20
Normal lung (n = 22)	0	21	20

*Ratio of total breast counts to lung counts; libraries used were GSM692, 677, 691, 760, and 780 for normal breast tissue; GSM 670, 671, 672, 673, and 694 for breast cancer tissue, and GSM762 for normal lung tissue.
†One article reported low-level of expression in gynecological malignancies, using nested RT-PCR.
‡All are invasive ductal carcinoma.

section device. SBEM expression was detected both in bronchial glands and bronchial surface epithelium, but not in parenchyma without bronchioles (Figure 1). The same experiment was carried out on PDEF, which was also expressed in bronchial cells.

Expression of MGB1 in Primary and Metastatic Cancers of the Lung

The lack of expression of MGB1 in the normal lung prompted us to examine whether MGB1 could be used in differential diagnosis between primary lung cancers and metastatic breast cancers. All of the 14 metastatic breast cancers of the lung were confirmed to express MGB1, whereas only seven of 70 primary lung cancers (10%) were positive (Table 2). No MGB1 expression could be detected in the 47 cases of adenocarcinoma of the lung

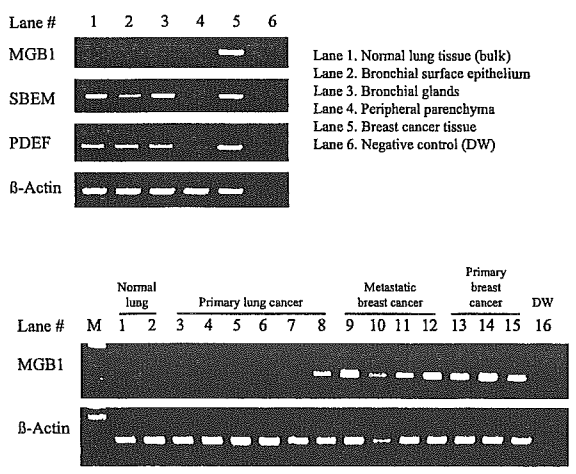

Figure 1. Expression analysis of potential breast-specific molecules (**A**) reveals that bulk tissue of normal lung (**lane 1**) expressed SBEM and PDEF, but not MGB1. Detailed examination using laser capture microdissection (**lanes 2** to **4**) demonstrated that bronchial surface epithelium and bronchial gland cells, but not the peripheral lung, were the source of the expression. In tumors (**B**), MGB1 expression was specific to breast cancers (**B, lanes 9** to **15**), with the notable exception of small-cell lung carcinomas (**B, lane 8**). DW indicates distilled water.

except for one, which interestingly metastasized to the breast 1 year after the lung operation. Detailed immunohistochemical analysis of this case revealed that both lung and breast tumors were positive for thyroid transcription factor-1 (TTF-1) and surfactant apoprotein A, and negative for estrogen and progesterone receptors. The morphological features were that of an ordinary lung adenocarcinoma. Five of the remaining six tumors with unexpected MGB1 expression were three small cell lung cancers, two large cell neuroendocrine carcinomas and a squamous cell carcinoma expressing neuroendocrine markers (CD56 and synaptophysin) in parts. Conversely, for high-grade neuroendocrine tumors of the lung, half the small cell lung cancers and large cell neuroendocrine carcinomas were positive for MGB1 expression.

We also examined the expression status of MGB1 in various other metastatic cancers. All 15 colon cancers and 12 sarcomas were negative for MGB1 expression, whereas three of four salivary gland carcinomas (adenoid cystic carcinomas) and one each of metastatic esophageal and endometrial cancers showed MGB1 expression. All of the PCR products of MGB1 in the non-breast metastatic cancer cases were confirmed by direct sequencing of the products. The other metastatic cancers, including one each of thyroid, tongue, gastric, pancreas and uterine cervix cancers, were negative for MGB1 expression. Because MGB1 transcripts were detected in the metastatic tumors, 17 primary esophageal cancers, and 10 primary salivary tumors were examined: the esophageal cancers were all negative for MGB1, and six of the 10 salivary gland tumors expressed MGB1.

Practical Application of MGB1 in Differential Diagnosis

These results indicated that MGB1 could serve as a marker of breast cancers, and thus we prospectively evaluated MGB1 expression in touch-imprint specimens of fine-needle biopsies from nine lung tumors with a breast cancer history. In six of the nine, MGB1 expression was detected, and detailed immunohistochemical results