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Table 1. Clinicopathologic characteristics on surgically resected primary lung ADC (Cont'd)

	Test cohort ($n = 40$)	Validation cohort ($n = 63$)	P
Platinum + docetaxel	0	4 (6)	
Platinum + pemetrexed	0	2 (3)	
Platinum + vinorelbine	1 (2)	1 (2)	
Tumor response number (%)			
Responder	16 (40)	18 (29)	0.31
CR	1	0	
PR	15	18	
Nonresponder	24 (60)	45 (71)	
SD	21	35	
PD	3	10	

NOTE: The P value from the Fisher's exact test.
Abbreviations: NE, not examined; Platinum, cisplatin or carboplatin.

were subjected to a two-step screening procedure to identify miRNAs whose expression is associated with responses to platinum-based doublet chemotherapy (Fig. 1B).

Differential expression of miRNAs between responders and nonresponders

First, we used microarray analysis to identify miRNAs in test cohort samples that were differentially expressed in LADC tissues from responders and nonresponders to platinum-based doublet chemotherapy. Fifty-nine miRNAs were identified ($P < 0.05$; Welch t test; Supplementary Table S2). Of these, 28 were upregulated in responders and 31 were downregulated.

Next, to identify the limited number of miRNAs that can be used to deduce responsiveness in the clinic, we searched for miRNAs showing highly differential expression between responders and nonresponders. Twelve miRNAs (miR135a*, miR196b, miR1181, miR31, miR31*, miR1290, miR598, miR1, miR144*, miR628-5p, miR449a, and miR34b) showing a >5-fold change in expression were identified as potential candidates and investigated further (Fig. 2A; Supplementary Table S2). The expression of these 12 miRNAs was reanalyzed by qRT-PCR using the same RNA samples used in the microarray experiments. A good agreement between the qRT-PCR (ΔC_t value) and microarray ($\log_2$ signal) data (as indicated by the Pearson correlation coefficient) was observed for 10 of the 12 miRNAs (excluding miR1181 and miR598; Supplementary Fig. S1). qRT-PCR identified three miRNAs (miR1290, miR196b, and miR135a*) as differentially expressed in responders and nonresponders ($P < 0.001$, $P < 0.001$, and $P < 0.008$, respectively; Fig. 2B; Supplementary Table S3). The fold changes observed in the qRT-PCR experiment were lower than those observed in the microarray experiment. This may be due to the higher sensitivity of the former. Several samples that gave no significant signal (calculated as zero) in the microarray experiment yielded ΔC_t values in the qRT-PCR experiment (Supplementary Fig. S1), leading to a reduction in the fold change values (Supplementary Table S3).

We next used linear discriminant analysis to examine these three miRNAs for their potential to discriminate responders from nonresponders. For this, continuous expression values for a single miRNA or a combination of two or three miRNAs, that is, ΔC_t values obtained by qRT-PCR, were included as variables in the analysis. ROC curves were plotted to examine both sensitivity and specificity. The results showed that a combination of all three miRNAs provided the best discrimination, with an AUC of 0.893 (Fig. 2C). This combination of three miRNAs is, henceforth, referred to as the "three-miRNA signature."

Ability of the three-miRNA signature to predict responses to chemotherapy

To validate the findings in the test cohort, we next examined the expression of the three-miRNA signature in the validation cohort (Supplementary Fig. S2 and Supplementary Table S3). We then performed a linear discriminant analysis to evaluate the potential of the three-miRNA signature as a biomarker. The mean expression levels of the three miRNAs were higher in responders than in nonresponders in the validation cohort, although the difference in the expression of miR135a* did not reach a statistical significance. However, a combination of all three miRNAs again was better able to distinguish responders from nonresponders (AUC = 0.837) than a single miRNA or a combination of two miRNAs (Supplementary Fig. S3).

PCA and support vector machine (SVM) analysis showed a predictive response of 37.5% for the test cohort containing 40% responders and a predictive response of 31.7% for the validation cohort containing 28.6% responders. The signature predicts responders and nonresponders in the test and validation cohorts with an accuracy of 82.5% and 77.8%, respectively (Fig. 3). The sensitivity, specificity, and positive and negative predictive values were similar for both cohorts (Supplementary Table S4). There was no significant difference in the clinical characteristics between true responders/

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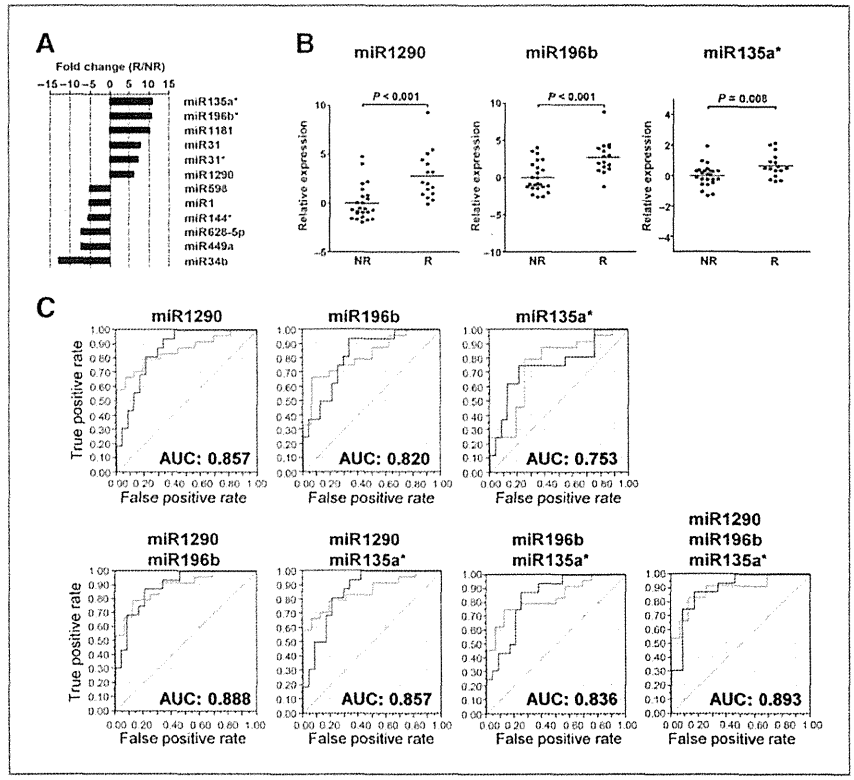

Figure 2. Selection of the three miRNAs whose expression was associated with responses to platinum-based doublet chemotherapy upon LADC recurrence. A, twelve miRNAs differentially expressed in responders (R) and nonresponders (NR) to platinum-based doublet chemotherapy in the test cohort. The diagram depicts miRNAs showing a >5-fold change in expression and with a P value of <0.05. The fold change is represented by the ratio of R to NR derived from the microarray data. B, expression of miRNAs in NR and R in the test cohort, as measured by qRT-PCR. Dot plots, miRNA relative threshold cycle values. Expression was normalized to that of RNU66. Threshold cycle values relative to the mean value in NR are shown on a $\log_2$ scale. Horizontal bars, the mean expression value; P values (Mann–Whitney test) are indicated. C, ROC analysis was performed for miR1290, miR196b, and miR135a* in the test cohort. The AUC value is shown. The blue line, the results for responders and the orange line represents the results for NR.

nonresponders and predicted responders/nonresponders (Supplementary Table S1). Taken together, these results show that the predictive ability of the three-miRNA signature was confirmed in the independent validation cohort, and that the signature is still predictive even if archive FFPE tissues are used for analysis. Specificity and negative predictive values greater than sensitivity and positive predictive values suggest that the three-miRNA signature predicts nonresponders better than responders (Supplementary Table S4).

Combining the three-miRNA signature with the TP53-Arg72Pro polymorphism genotype

We previously showed in the same study population (i.e., the 640 cases shown in Fig. 1A) that the Arg72Pro polymorphism in the *TP53* gene in noncancerous (germline) DNA is associated with responses to platinum-based doublet chemotherapy: The response rate is higher in those harboring the TP53-72Pro polymorphism (35). Therefore, we combined the three-miRNA signature with the *TP53*-Arg72Pro genotype data to ascertain whether the predictive power of the miRNA signature was enhanced. We dichotomized the study cohorts into two subgroups (patients with the TP53-72Pro allele and those without) and examined the predictive accuracy of the three-miRNA signature. We found that the predictive accuracy marginally improved in both the test (85.0%) and validation (82.5%) cohorts (Fig. 1B).

The three-miRNA signature predicts responses to chemotherapy irrespective of driver oncogene aberrations and clinical characteristics

Driver oncogene aberrations in LADC are a critical factor that determines the therapeutic strategy for each patient. In addition, such aberrations are associated with clinical characteristics, as represented by the predominance of *EGFR* mutation in females and never-smokers. Therefore, we next addressed whether the ability of the three-miRNA signature to predict responses to chemotherapy was affected by driver oncogene alterations or clinical characteristics. The three-miRNA signature failed to predict responses in 21 of 103 cases (21%). The test and validation cohorts contained seven (one with an *EGFR* mutation, one with a *HER2* mutation, and five aberration-negative cases) and 14 (six with *EGFR* mutations, one with a *KRAS* mutation, and seven aberration-negative cases) nonpredicted cases, respectively (Fig. 4). The nonprediction rate was 15% (7/47 cases) for patients harboring the *EGFR* mutation, 10% (1/10) for those harboring the *KRAS* mutation, 33% (1/3) for those harboring the *HER2* mutation, and 32% (12/38) for aberration-negative cases; therefore, there was no significant correlation between driver gene status and nonprediction. Similarly, there was no significant association between clinical factors and nonprediction; thus, the three-miRNA signature may be a useful biomarker for predicting the responses of patients with LADC to chemotherapy,

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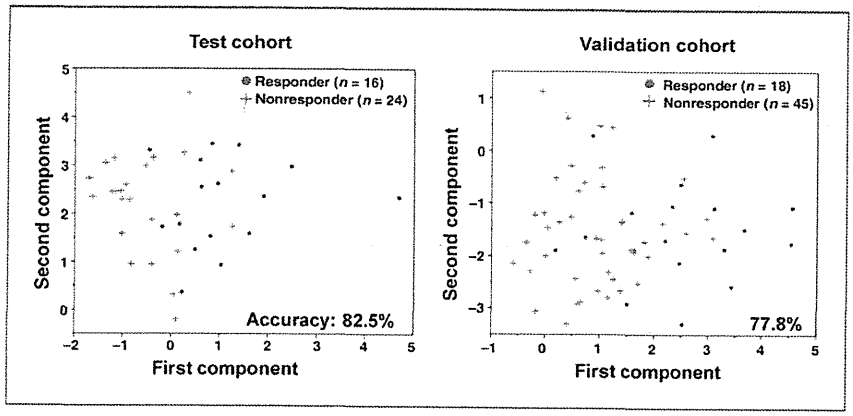

Figure 3. PCA. A PCA–SVM strategy using three miRNAs (miR1290, miR196b, and miR135a*) was used to construct a classifier, which could distinguish responders from nonresponders. The blue dots represent the responders and the orange crosses represent the nonresponders in the test (left) and validation cohorts (right). The classifier had a predictive accuracy of 82.5% for the test cohort and an accuracy of 77.8% for the validation cohort.

irrespective of driver oncogene aberrations and clinical characteristics.

Discussion

Here, we performed miRNA expression profiling of patients who initially underwent surgical resection for primary LADC and were then treated with platinum-based doublet chemotherapy upon recurrence. We identified a three-miRNA signature (miR1290, miR196b, and miR135a*) that predicts whether patients with recurring LADC respond to and, therefore, will benefit from platinum-based doublet chemotherapy. Even patients with LADC harboring druggable oncogene aberrations (that are resistant to treatment with TKIs) may be treated with platinum-based doublet chemotherapy; therefore, platinum-based doublet chemotherapy is a major therapeutic strategy for almost all patients with LADC (39–41). Personalized therapy, in which a drug with the greatest chance of eliciting a response (i.e., tumor shrinkage) is chosen specifically for each patient, is the first critical step toward improved prognosis for patients with LADC with advanced and recurrent disease; indeed, clinical trials examining the effect of new drugs on NSCLC have set improved response rates as their primary endpoint (10); thus, response to treatment according to the RECIST criteria rather than survival was the outcome measure selected for the present study. The three-miRNA signature will facilitate personalized therapy for LADC and will include platinum-based doublet therapy as an option.

Here, we examined the three-miRNA signature of primary tumors to predict the responsiveness of recurrent tumors. It is noteworthy that the biologic characteristics of recurrent tumors are not the same as those of primary tumors due to tumor cell heterogeneity in the primary lesions and the accumulation of additional genetic/epigenetic changes during progression. Thus, at present our findings are applicable to the treatment of recurrent tumors for which corresponding primary tumor tissue samples are available. The finding that the three-miRNA signature is predictive in archived primary tumor tissues is an advantage; patients are spared the additional burden of further tissue sampling for genetic analysis. However, it is also worth analyzing recurrent tumors and inoperable advanced tumors to find out whether the three-miRNA signature is applicable to patients for whom archived surgical tissues are not available. The finding that the signature can be identified in archived FFPE tissues is also an advantage and will facilitate translation to the clinic.

We also examined the combination of the three-miRNA signature with the TP53-Arg72Pro polymorphism genotype to see whether this provided greater predictive accuracy, as blood cells used for genotyping polymorphisms are easily obtained from patients. However, the improvement was only marginal. Therefore, more polymorphisms associated with responses to platinum-based doublet therapy must be identified if we are to achieve any marked improvement over the three-miRNA signature alone. In addition, we used a fold change >5 as a criterion for identifying candidate miRNAs that are differentially expressed between responders and nonresponders. Using less stringent or other statistical criteria may lead to the identification of more miRNAs that are useful for prediction.

The three-miRNA signature predicted responses irrespective of the presence of driver oncogene aberrations. This is important when we consider that LADCs that have acquired resistance to specific TKIs are treated with platinum-based doublet chemotherapy. However, unfortunately, the present study cohort did not include samples from patients with EGFR- or ALK-positive LADC that received TKIs before platinum-based doublet chemotherapy. Such cases should be examined to address this issue.

This study has several limitations. First, it was retrospective in nature, so the ability of the three-miRNA signature to predict responses needs to be further validated using more samples. Here, different types of tumor tissue for the test and validation sets (bulk frozen and microdissected FFPE tissues, respectively), which contained patients that had undergone several different chemotherapeutic regimens, were subjected to analysis; therefore, we may have over- or underestimated predictive value. Thus, further studies that use a larger number of samples obtained according to a defined experimental procedure and take factors such as previous treatment regimen, disease stage, and PS into account are required. In addition, prospective studies, for

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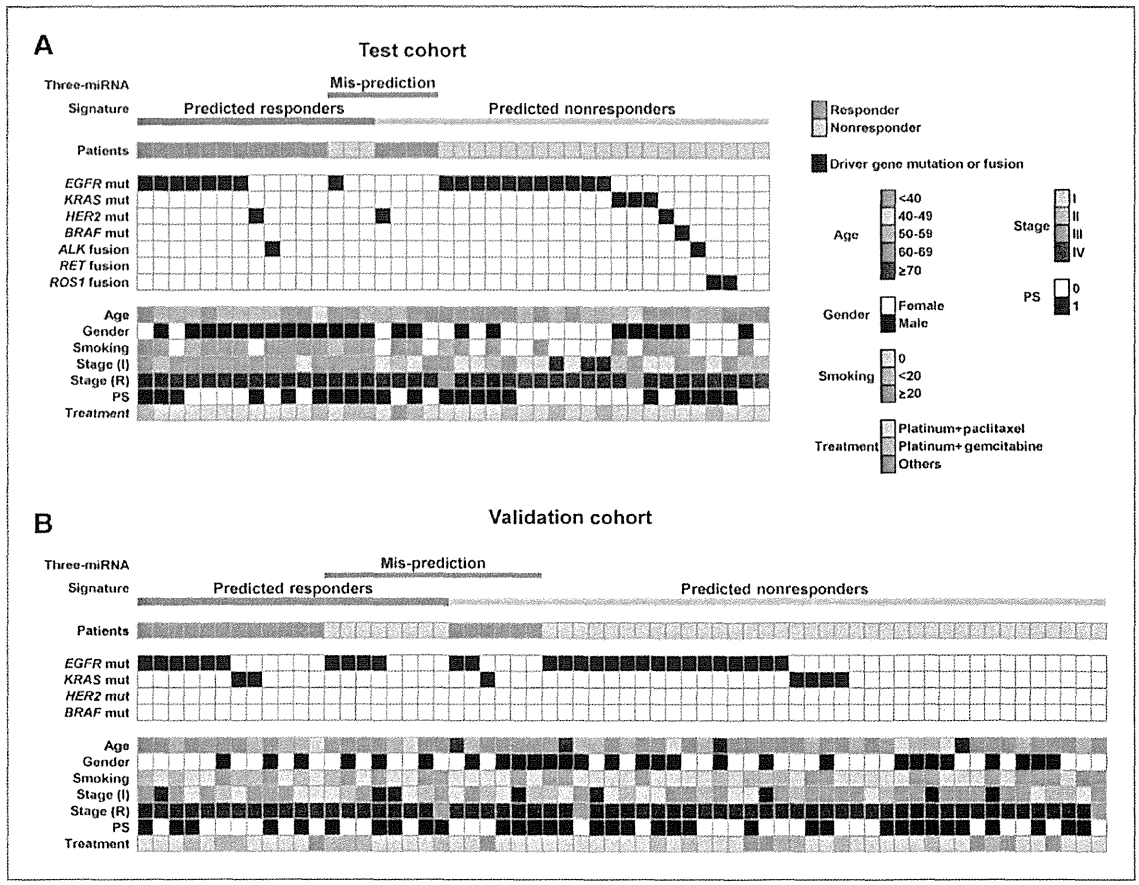

Figure 4. Response prediction by the three-miRNA signature according to clinicopathologic factors in the test (A) and validation cohorts (B). Driver gene mutations and clinical features are shown: patients (blue, responder; orange, nonresponder); driver gene (black, *EGFR*, *KRAS*, *HER2*, *BRAF* mutation- or *ALK*, *RET*, or *ROS1* fusion-positive; white, negative); age (blue, <40; orange, 40–49; green, 50–59; red, 60–69; and navy blue, ≥70); gender (white, female; black, male); smoking (orange, pack years = 0; green, <20; and red, ≥20); tumor stage at initial diagnosis (I) and at recurrence (R; orange, I; green, II; red, III; and navy blue, VI); PS (white, 0; black, 1); treatment (orange, platinum + paclitaxel; green, platinum + gemcitabine; and red, other).

example, studies using samples from patients treated with a single therapeutic regimen, and the analysis of primary and recurrent tumors and inoperable advanced tumors, should be conducted to confirm the utility of the three-microRNA signature. Second, although the three-miRNA signature was significantly associated with response to chemotherapy, differences in progression-free survival were only suggestive (Supplementary Fig. S4). We chose the response as the primary endpoint of efficacy to identify subgroups for which chemotherapy does work (35). However, treatment is continued after the failure of platinum-based doublet chemotherapy; therefore, clinical response alone would not be enough to improve the outcome. Third, the functional relevance of miR1290, miR196b, and miR135a* to the chemosensitivity of LADC remains unclear. Interestingly, a recent study shows that the expression of miR196b is upregulated in patients with rectal adenocarcinoma that respond to neoadjuvant chemoradiotherapy (capecitabine or 5-fluorouracil), which supports the findings of the present study (42). However, preliminary experiments examining the exogenous expression of the three miRNAs in LADC cell lines did not show increased sensitivity to a platinum agent, cisplatin (CDDP). Therefore, the direct or indirect effects of miRNAs on chemosensitivity should be further investigated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Shiraishi, K. Matsumoto, H. Kunitoh, H. Nokihara, S.-I. Watanabe, K. Tsuta, C.C. Harris
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Saito, A.J. Schetter, H. Ogata-Kawata, N. Tsuchiya, K. Kumamoto

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Writing, review, and/or revision of the manuscript: M. Saito, A.J. Schetter, H. Ogata-Kawata, H. Kunitoh, H. Nokihara, C.C. Harris, T. Kohno
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Shiraishi, S. Takenoshita
Study supervision: J. Yokota, T. Kohno

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A Three-microRNA Signature Predicts Responses to Platinum-Based Doublet Chemotherapy in Patients with Lung Adenocarcinoma

Motonobu Saito, Kouya Shiraishi, Kenji Matsumoto, et al.

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Combined effects of asbestos and cigarette smoke on the development of lung adenocarcinoma: Different carcinogens may cause different genomic changes

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Abstract. The carcinogens in cigarette smoke are distinct from asbestos. However, an understanding of their differential effects on lung adenocarcinoma development remains elusive. We investigated loss of heterozygosity (LOH) and the *p53* mutation in 132 lung adenocarcinomas, for which asbestos body burden (AB; in numbers per gram of dry lung) was measured using adjacent normal lung. All cases were classified into 9 groups based on a matrix of cumulative smoking (CS in pack-years; CS=0, 0<CS<25, ≥25 CS) and AB (AB=0, 0<AB<1,000, ≥1,000 AB). AB=0 indicates a lower level than the detection limit of ~100. LOH frequency increased only slightly with the elevation of CS in the AB=0 groups. In the AB>0 groups, LOH frequency increased as AB and/or CS was elevated and was significantly higher in the ≥1,000 AB, ≥25 CS group (p=0.032). *p53* mutation frequency was the lowest in the AB=0, CS=0 group, increased as AB and/or CS rose, and was significantly higher in the ≥1,000 AB, ≥25 CS group (p=0.039). *p53* mutations characteristic of smoking were frequently observed in the CS>0 groups contrary to non-specific mutations in the CS=0, AB>0 groups. Combined effects of asbestos and smoking were suggested by LOH and *p53* analyses. Sole exposure to asbestos did not increase LOH frequency but increased non-specific *p53* mutations. These findings indicate that the major carcinogenic mechanism of asbestos may be tumor promotion, acting in an additive or synergistic manner, contributing to the genotoxic effect of smoking. Since this study was based on a general cancer center's experience, the limited sample size did not permit the consideration that the result was conclusive. Further investigation with a large sample size is needed to establish the mechanism of asbestos-induced lung carcinogenesis.

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Key words: lung cancer, asbestos, smoking, loss of heterozygosity, *p53* mutation

Introduction

Lung cancer is one of the leading causes of cancer-related death in both men and women worldwide, and adenocarcinoma is the most predominant histologic subtype in many parts of the world. Tobacco smoke is clearly the most important factor associated with the development of lung cancer, accounting for 80-90% of all cases. Asbestos is another significant inhaled carcinogen, contributing to the development of ~5-7% of all lung cancers (1). Many studies on asbestos-related lung carcinogenesis have analyzed the genotoxic effects of asbestos; asbestos fibers induce DNA damage, chromosome aberrations, mitotic disturbances and gene mutations (2). In addition, asbestos fibers can stimulate a range of other effects including cell proliferation, chronic inflammation, enhanced gene expression, such as c-fos and c-jun overexpression, and transformation (3,4). Despite these studies, the efficacy of asbestos-exposure as a complete lung carcinogen, independent of tobacco smoke, has not been demonstrated in humans, since lung cancers of asbestos-exposed individuals frequently occur in smokers and ex-smokers. The majority of asbestos-related lung cancers may result from the combined effects of asbestos and carcinogens in tobacco smoke, with the possibility of a synergistic relationship first proposed by Doll (5). Hence, the mechanism of asbestos-induced lung carcinogenesis still remains unclear.

Both loss of heterozygosity (LOH) and the *p53* mutation are genetic alterations. LOH is frequently noted in cancer cells and is thought to occur through genetic instability at the chromosomal level. On the other hand, the *p53* mutation is a genetic alteration at the nucleotide level. Mutation in the *p53* tumor suppressor gene is the most frequently observed gene

mutation in cancers. As described below, not only *p53* mutations but also LOH spectra differ in different cancer types associated with different etiologies. Previously we compared the frequency of LOH on all autosomal chromosomes among non-small cell lung carcinomas (6,7) as well as *p53* mutation patterns with adenocarcinoma cell morphology (8). The frequency of allelic loss on many chromosomal arms was commonly higher in squamous cell carcinomas than in adenocarcinomas. This result suggested that more cumulative genetic changes are associated with tumorigenesis in squamous cell carcinomas than contribute to adenocarcinomas, a pattern which may reflect a difference in the carcinogenic mechanisms responsible for the two histologies. In addition, we observed high frequencies of allelic losses on chromosomes 9p, 9q and 13q in squamous cell carcinomas, the majority of which were from smokers, and higher frequencies of allelic losses on these arms in adenocarcinomas from smokers than those from non-smokers. This loss of specific chromosomes associated with a particular histology is an example of LOH spectra reflecting etiology. The *p53* mutational spectra differ among cancers of various organs, and its frequency and mutational spectra can be said to reflect carcinogenic patterns characteristic of exogenous or endogenous factors and thus may be helpful for identification of the responsible agents, including, among others, cigarette smoke, aflatoxin B1 and ultraviolet light. Hence, the analysis of *p53* mutation can provide clues to the etiology of diverse tumors and to the function of specific regions of *p53* (9,10). The mutation pattern in smokers shows an excess of G:C to T:A transversions (34.2%), which are relatively uncommon in non-smokers or passive-smokers (16.6%) (11). These transversions often occur at codons 157, 158, 245, 248 and 273, experimentally identified as sites of adduct formation by benzo(a)pyrene, a single polycyclic aromatic hydrocarbon (PAH)-compound found in cigarette smoke. Other PAH-compounds also have a similar preference for adduct formation in these *p53* codons (12,13).

In the present study, to elucidate the combined effects of asbestos-exposure and smoking on development of lung adenocarcinomas, we used 132 lung adenocarcinomas, for which we already obtained all detailed smoking histories, comprehensive LOH data for all autosomal chromosomes (7), and *p53* mutation data.

Materials and methods

Patients and sample preparation. A total of 335 cases of lung adenocarcinoma were surgically removed at the Cancer Institute Hospital (CIH), Tokyo, Japan, between September 1989 and August 1996. Among the cases, fresh tumor tissues and corresponding normal lung and detailed smoking histories were successfully collected from 132 patients, which were used as materials in this study. Hence, they were collected semi-randomly without respect to asbestos-exposure status, and therefore provided a representative population for a cancer center in Japan. The clinicopathological data for these samples are summarized in Table I. We used a differentiation grading that was basically according to the former version of the Japanese Lung Cancer Society (14), as previously performed (15). Smoking history was surveyed intensively from patients and their families and presented as cumulative smoking (CS) in pack-years. The study protocol was approved by IRB of CIH and informed consent was obtained from all patients.

Table I. Clinicopathological data of the patients with lung adenocarcinomas analyzed in this study (n=132).

Clinicopathological features	No. of patients (%)
Age (years ± SD)	61±11
Gender	
Male	74 (56)
Female	58 (44)
Cumulative smoking	
CS=0	54 (41)
0<CS<25	18 (14)
≥25 CS	60 (45)
Asbestos burden	
AB=0	64 (48)
0<AB<1,000	28 (21)
≥1,000 AB <5,000	36 (27)
≥5,000 AB	4 (3)
pStage	
I	63 (48)
II-IV	69 (52)
Differentiation	
Well	35 (27)
Moderately	69 (52)
Poorly	28 (21)
Size (mm)	
<30	75 (57)
≥30	57 (43)

CS, cumulative smoking in pack-years; AB, asbestos burden; pStage, pathological stage. Percentages may not total 100, due to rounding.

Measurement of asbestos-exposure. Asbestos-body burden (AB; in numbers per gram of dry lung tissue) was measured using paraffin blocks of corresponding normal lung tissues by a polarizing microscope (16). The detection limit, which means no AB was found on the measuring filter sample, was ~100 AB/g (dry lung) and expressed as 0 in this study.

A matrix of smoking-exposure and asbestos-exposure. To examine the dose-effect relationship of asbestos-exposure (presented as AB) and smoking-exposure (presented as CS in pack-years) on lung adenocarcinomas, we classified all cases into 9 groups based on a matrix of CS in pack-years: CS=0 (n=54, 41%), 0<CS<25 (n=18, 14%), ≥25 CS (n=60, 45%), and AB: AB=0 (n=64, 48%), 0<AB<1,000 (n=28, 21%), ≥1,000 AB (n=40, 31%). Since the patients were selected consecutively from surgical tumor files in a general cancer center, only 4 cases (3.0%) exceeded 5,000 in AB. To investigate the mechanism of asbestos-induced lung carcinogenesis in

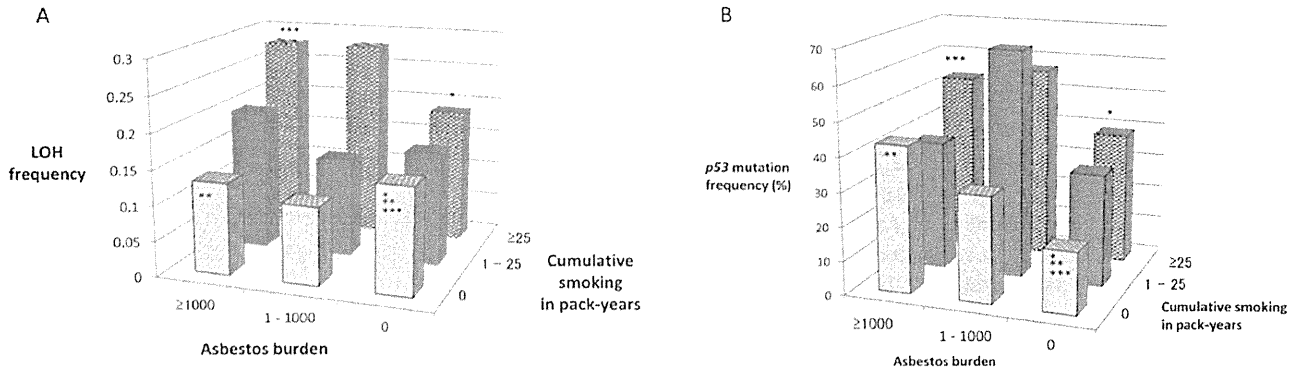

Figure 1. (A) Frequency of the loss of heterozygosity (LOH) in lung adenocarcinomas classified by cumulative smoking (CS) in pack-years (CS=0, 0<CS<25, ≥25 CS) and asbestos burden (AB) (AB=0, 0<AB<1,000, ≥1,000 AB). FAL, fractional allelic loss. *p=0.30 (AB=0, CS=0 vs. AB=0, 25≤CS); **p=0.69 (AB=0, CS=0 vs. ≥1,000 AB, CS=0); ***p=0.032 (AB=0, CS=0 vs. ≥1,000 AB, ≥25 CS). (B) p53 mutation frequency in lung adenocarcinomas classified by CS in pack-years (CS=0, 0<CS<25, 25≤CS) and AB (AB=0, 0<AB<1,000, 1,000≤AB). *p=0.14 (AB=0, CS=0 vs. AB=0, 25≤CS); **p=0.14 (AB=0, CS=0 vs. ≥1,000 AB, CS=0); ***p=0.039 (AB=0, CS=0 vs. ≥1,000 AB, ≥25 CS).

Table II. FAL values (± SD) in lung adenocarcinomas, classified by AB and CS in pack-years.

	AB			
	0	1-1,000	≥1,000	Total
CS				
0	0.15 (±0.13) (n=20)	0.11 (±0.13) (n=16)	0.13 (±0.16) (n=13)	0.13 (±0.12) (n=49)
1-25	0.16 (±0.17) (n=4)	0.14 (±0.04) (n=3)	0.20 (±0.20) (n=7)	0.18 (±0.15) (n=14)
≥25	0.19 (±0.14) (n=28)	0.28 (±0.25) (n=6)	0.28 (±0.22) (n=17)	0.23 (±0.17) (n=51)
Total	0.17 (±0.12) (n=52)	0.15 (±0.16) (n=25)	0.21 (±0.18) (n=37)	0.18 (±0.15) (n=114)

FAL, fractional allelic loss; AB, asbestos burden; CS, cumulative smoking.

a representative population for a cancer center, not a biased population heavily exposed to asbestos, we divided the cases between AB <1,000 and ≥1,000 AB.

LOH analysis. For LOH analysis, we performed Southern blotting. Experimental procedures and probes used were essentially the same as previously described (6,7). To facilitate the comparison, we used a fractional allelic loss (FAL) value, defined as: (number of chromosome arms with LOH)/(number of informative arms) for each case. Of 132 patients with adenocarcinomas, LOH data were available for 114 patients.

p53 mutation analysis. Analysis of *p53* mutation was performed essentially as described elsewhere (8). Genomic DNA from fresh tumor samples was prepared and exons 4-8 and 10 of *p53* were analyzed by polymerase chain reaction and DNA sequencing. Of the 132 patients with adenocarcinomas, *p53* mutation data were available for 123 patients.

Statistical analysis. For statistical analysis, we used the t-test, Fisher's exact test, and Chi-square test, as appropriate. The two-sided significant level was set at $p<0.05$. Data were analyzed with the statistical software Stata version 11 (StataCorp., College Station, TX, USA).

Results

LOH frequency of lung adenocarcinomas classified by CS and AB is shown in Table II and Fig. 1A. LOH frequency increased only slightly correlating with the elevation of CS in the AB=0 groups, whereas, in the AB>0 groups, it increased as AB and/or CS was elevated and was significantly higher in the ≥1,000 AB, ≥25 CS group than in the AB=CS=0 group (p=0.032).

Details of cases with *p53* mutations in lung adenocarcinomas are shown in Table III and summarized in Table IV. The *p53* mutation rates of pathological stage I and II-IV lung adenocarcinomas were 32% (18 of 57) and 44% (29 of 66), respectively, not significantly different by Fisher's exact test (p=0.19). *p53* mutation frequency of lung adenocarcinomas classified by CS and AB are depicted in Fig. 1B. *p53* mutation frequency was the lowest in the AB=CS=0 group (18%), increased as AB and/or CS rose, and was significantly higher in the ≥1,000 AB, ≥25 CS group (53%) than in the AB=CS=0 group (p=0.039). Tobacco smoke, one of the most significant exogenous carcinogenic agents has been shown to frequently cause specific *p53* mutations, especially G:C to T:A transversion (17) at specific codons described as 'hotspots', such as codon 157, 158, 245, 248 and 273 (13). *p53* mutations characteristic of smoking, such as G:C to T:A transversion at the

Table III. Details of the cases with *p53* mutations in lung adenocarcinomas.

Classified by CS and AB	Case no.	Gender	Age (years)	Diff.	Size (mm)	pStage	AB	CS	FAL	Mut type	Codon	Base change	Amino acid
CS=0	33	F	49	Mod	27	IIIB	0	0	0.13	ts	273	CGT to CAT	Asp→His
AB=0	70	F	26	W	43	IIIB	0	0	0.53	tv	176	TGC to TTC	Cys→Phe
	73	M	44	P	35	IIIA	0	0	0.35	ts	120	AAG to AGG	Lys→Arg
	74	F	70	W	20	IA	0	0	0.06	ts	248	CGG to CAG	Arg→Glu
CS=0	27	F	77	Mod	38	IIIB	187	0	0.18	tv	237	ATG to ATT	Met→Ile
0<AB<1,000	39	F	51	Mod	24	IIIB	214	0	0.05	ts	245	GGC to AGC	Gly→Ser
	47	F	65	W	24	IA	333	0	0.06	ts	335	CGT to CAT	Arg→His
	81	F	68	Mod	21	IA	671	0	0.05	tv	273	CGT to CTT	Asp→Leu
CS=0	3	F	51	Mod	33	IIIA	1,715	0	0.14	del	341	TTC to T_C	Frameshift
≥1,000 AB	7	F	72	Mod	60	IV	3,939	0	0.58	ts	138	GCC to GTC	Ala→Val
	14	F	57	Mod	40	IIIA	2,305	0	0.29	tv	138	GCC to CCC	Ala→Pro
	84	F	63	Mod	25	IIIB	1,000	0	0.21	ts	282	CGG to TGG	Arg→Trp
	113	F	67	Mod	32	IIIB	1,949	0	0.13	ts	132	AAG to AGG	Lys→Arg
	114	F	49	Mod	33	IB	6,998	0	0.1	ts	213	CGA to TGA	Arg→Stop
0<CS<25	55	F	68	Mod	42	IIIB	0	3.8	0.17	ts	242	TGC to TAC	Cys→Tyr
AB=0	126	M	66	Mod	30	IA	0	8	NA	ts	237	ATG to ATA	Met→Ile
≥25 CS	2	M	73	P	53	IIIB	0	39.4	0.25	ts	259	GAC to AAC	Asp→Ile
AB=0	12	M	69	Mod	28	IA	0	42.3	0.04	del	113-119	Del of 19 bp	Frameshift
	21	M	47	P	39	IIIA	0	32.5	0	tv	245	GGC to TGC	Gly→Cys
	42	M	58	P	24	IIIA	0	80	0.36	ts	273	CGT to TGT	Asp→Cys
	46	M	56	Mod	20	IIIB	0	31	0.1	del	159	GCC to_C	Frameshift
	54	M	54	Mod	25	IIIA	0	48	0.38	tv	198	GAA to TAA	Glu→Stop
	56	M	74	W	17	IA	0	42	0.24	ts	175	CGC to CAC	Arg→His
	58	M	61	P	23	IV	0	80	0.33	tv	135	TGC to TTC	Cys→Phe
	83	M	72	Mod	75	IV	0	126	0.44	del	274	GTT to_T	Frameshift
	88	M	50	Mod	48	IV	0	115.5	0.2	del	189	GCC to G_C	Frameshift
	96	M	54	Mod	27	IA	0	34	0.25	tv	158	CGC to CTC	Arg→Leu
	102	M	56	W	16	IA	0	37.5	0.06	ts	273	CGT to TGT	Asp→Cys
	116	M	50	P	60	IIIA	0	32	NA	tv	245	GGC to TGC	Gly→Cys
0<CS<25	17	M	58	W	27	IA	560	1.3	0.13	ts	234	TAC to TGC	Tyr→Cys
0<AB<1,000	128	F	69	P	60	IB	980	20	NA	ts	245	GGC to GAC	Gly→Asp

Table III. Continued.

Classified by CS and AB	Case no.	Gender	Age (years)	Diff.	Size (mm)	pStage	AB	CS	FAL	Mut type	Codon	Base change	Amino acid
≥25 CS	11	M	64	Mod	20	IA	333	33	0.17	tv	Donor	AGgt to AGtt	Splicing
0<AB<1,000	49	M	41	P	105	IB	446	37.5	0.22	tv	Acceptor	agG to atG	Splicing
	97	M	72	Mod	16	IIIB	929	25.5	0.26	ts	273	CGT to CAT	Asp→His
	131	M	51	P	28	IIIA	339	31	NA	tv	244	GGC to TGC	Gly→Cys
0<CS<25	23	M	59	Mod	24	IA	1,538	24	0.12	tv	274	GTT to TTT	Val→Phe
≥1,000 AB	64	F	74	W	37	IIIB	1,477	12	0.11	tv	209	AGA to TGA	Arg→Stop
	86	M	49	Mod	23	IIA	2,039	1	0.45	tv	238	TGT to AGT	Cys→Ser
≥25 CS	16	M	72	P	35	IIIA	2,490	40	0.31	ts	158	CGC to CAC	Arg→His
≥1,000 AB	53	M	60	P	28	IIA	1,750	40	0.64	ts	158	CGC to CAC	Arg→His
	85	M	65	Mod	28	IA	2,337	45	0.55	ts	275	TGT to TAT	Cys→Tyr
	103	M	67	Mod	28	IIIB	1,293	30.6	0.2	ins	305-306	Ins of 23 bp	Frameshift
	104	M	74	Mod	32	IB	2,378	53	0.05	ts	Donor	AGgt to AGat	Splicing
	105	M	50	Mod	24	IA	2,212	58	0.29	del	179-185	Del of 18 bp	Frameshift
	109	M	47	P	64	IIIA	3,207	81	0.64	tv	158	CGC to CCC	Arg→Pro
	110	M	55	Mod	15	IA	3,881	35	0	ins	46	Ins of 16 bp	Frameshift
	115	M	71	Mod	20	IIIA	5,308	48	0.46	tv	157	GTC to TTC	Val→Phe

CS, cumulative smoking in pack-years; AB, asbestos burden; Diff., differentiation; pStage, pathological stage, FAL, fractional allelic loss; Mut, mutation; F, female; M, Male; Mod, moderately; W, well; P, poorly; ts, transition; tv, transversion; del, deletion; ins, insertion; NA, not analyzed. Specific codons in *p53* mutations characteristic of smoking are underlined.

Table IV. *p53* mutational spectra in lung adenocarcinomas, classified by AB and CS in pack-years.

Classified by CS and AB	No.	With *p53* mutation (%)	Transition				Transversion					Del/Ins (%)
			CpG G:C to A:T	Non-CpG G:C to A:T	A:T to G:C	Total (%)	G:C to T:A	G:C to C:G	A:T to T:A	A:T to C:G	Total (%)	
All cases	123	47 (38)	13	6	3	22 (47)	13	2	2	0	17 (36)	8 (17)
CS=0	49	14 (28)	6	1	2	9 (64)	3	1	0	0	4 (29)	1 (7)
AB=0	22	4 (18)	2	0	1	3 (75)	1	0	0	0	1 (25)	0 (0)
0<AB<1,000	13	4 (31)	2	0	0	2 (50)	2	0	0	0	2 (50)	0 (0)
≥1,000 AB	14	6 (43)	2	1	1	4 (67)	0	1	0	0	1 (17)	1 (17)
0<CS<25	17	7 (41)	1	2	1	4 (57)	1	0	2	0	3 (43)	0 (0)
AB=0	6	2 (33)	0	2	0	2 (100)	0	0	0	0	0 (0)	0 (0)
0<AB<1,000	3	2 (67)	1	0	1	2 (100)	0	0	0	0	0 (0)	0 (0)
≥1,000 AB	8	3 (38)	0	0	0	0 (0)	1	0	2	0	3 (100)	0 (0)
≥25 CS	57	26 (46)	6	3	0	9 (35)	9	1	0	0	10 (38)	7 (27)
AB=0	33	13 (39)	3	1	0	4 (31)	5	0	0	0	5 (38)	4 (31)
0<AB<1,000	7	4 (57)	1	0	0	1 (25)	3	0	0	0	3 (75)	0 (0)
≥1,000 AB	17	9 (53)	2	2	0	4 (44)	1	1	0	0	2 (22)	3 (33)

Percentages may not total 100, due to rounding. Del/Ins, deletion/insertion; AB, asbestos burden; CS, cumulative smoking.

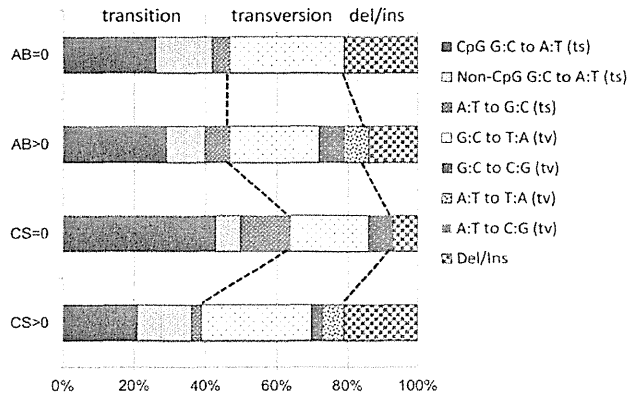

Figure 2. p53 mutation spectra in lung adenocarcinomas. AB=0 (n=19): lung adenocarcinomas from patients without AB (0<CS, n=15; CS=0, n=4). AB>0 (n=28): lung adenocarcinomas from patients with AB (0<CS, n=18; CS=0, n=10). CS=0 (n=14): lung adenocarcinomas from non-smokers (0<AB, n=10; AB=0, n=4). CS>0 (n=33): lung adenocarcinomas from smokers and ex-smokers (0<AB, n=18; AB=0, n=15). CS, cumulative smoking in pack-years; AB, asbestos burden; ts, transition; tv, transversion; Del/Ins, deletion/insertion.

tobacco-specific codons were frequently observed in the CS>0 groups, whereas non-specific mutations were often detected in the CS=0, AB>0 groups (Tables III and IV). In the ≥1,000 AB, CS=0 group, there was only one transversion and no tobacco-specific codons for the six *p53* mutations. In contrast, in the AB=0, ≥25 CS group, there were five G:C to T:A transversions and five tobacco-specific codons among 13 *p53* mutations. Fig. 2 shows *p53* mutation spectra in lung adenocarcinomas, classified as smokers (A, n=33) or non-smokers (B, n=14) and asbestos-exposed (C, n=28) or not (D, n=19). Although *p53* mutation spectra varied depending on the status of smoking history, they showed little difference between asbestos-exposed or non-exposed. Whereas smokers had frequent G:C to T:A transversions, which are smoking-associated *p53* mutations, non-smokers had frequent G:C to A:T transitions at CpG sites associated with spontaneous mutations, consistent with previous reports (9,17).

With respect to tumor differentiation grade, a heavier smoking habit was associated with less-differentiated adenocarcinomas (Fig. 3A, p=0.0010, Chi-square test), in line with a previous study (18). On the other hand, there was no correlation between asbestos deposition and the differentiation grade (Fig. 3B, p=0.75).

Discussion

Both tobacco smoke and asbestos fibers are significant inhaled carcinogens which contribute significantly to lung adenocarcinoma development. We previously revealed that chromosome instability and LOH, rather than minisatellite and microsatellite instability, play major roles in the development of lung adenocarcinomas (19). The LOH and *p53* spectra provide clues concerning the etiology and nature of carcinogenesis. To elucidate the carcinogenic mechanisms of two different inhaled carcinogens, asbestos and cigarette smoke, we investigated LOH on all autosomal chromosomes and measured asbestos burden (AB; asbestos body per gram of dry lung tissue) using corresponding normal lung tissue and investigated *p53* mutation employing fresh tumor samples.

The *p53* mutational spectra may be helpful for identification of the origins of the mutations that give rise to human

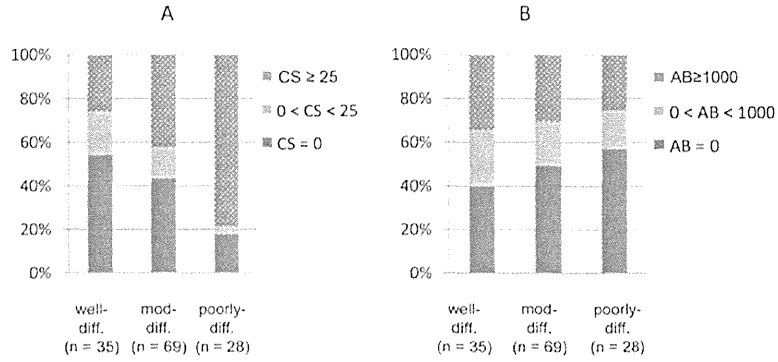

Figure 3. Cumulative smoking (CS) in pack-years (A) and asbestos burden (AB) (B) with reference to the histological differentiation grade. Although there was a significant relationship between CS and the differentiation grade (p=0.0010, Chi-square test), there was no correlation between AB and the differentiation grade (p=0.75). Well-diff., well-differentiated; mod-diff., moderately differentiated; poorly-diff., poorly differentiated.

cancers. For example, aflatoxin B1-associated hepatocellular carcinomas frequently have the specific *p53* mutations: G:C to T:A transversions at the 3rd base of codon 249, AGG to AGT (Arg to Ser) (20). Another example of a clearly characteristic 'finger-print' mutation in *p53* is the CC to TT double mutation in skin cancer (21). Exposure to UV light, a physical mutagen, produces distinctive pyrimidine dimers that, if unrepaired, can produce tandem mutations, most characteristically CC to TT transitions. Similar to these, the *p53* mutational spectra can provide clues to the etiology of cancers.

The possible role of asbestos-exposure in the genesis of *p53* mutations in lung cancers is less well understood. Husgafvel-Pursiainen *et al* investigated *p53* mutation of 105 lung cancers from smokers, comprising 53 squamous cell carcinomas, 39 adenocarcinomas and other 13 carcinomas, focusing on the presence or absence of asbestos-exposure (22). They found *p53* mutations in 39% of asbestos-exposed patients with lung cancer while the percentage was 54% in patients not exposed to asbestos, indicating that the *p53* mutations were less common among the cases with occupational asbestos-exposure than in the non-exposed cases. These results have not been verified yet by another study, and need additional examinations of smoking status.

In adenocarcinoma without asbestos-exposure or smoking-exposure, the *p53* mutation rate was the lowest. It increased in correlation with the elevation of asbestos-exposure and/or smoking-exposure. Adenocarcinomas associated with frequent smoking have characteristic *p53* mutations, especially G:C to T:A transversions (17), at specific 'hotspot' codons (13). However, adenocarcinomas associated only with asbestos-exposure had non-specific *p53* mutations, such as transitions which are thought to be caused by endogenous mechanisms associated with spontaneous events (9,17). Asbestos may work in a promoter-like manner. Production of reactive oxygen species and/or induction of tissue regeneration may be relevant.

Adenocarcinomas have different etiologies from squamous cell carcinomas, which can be reflected also in terms of LOH. As we revealed, LOH frequency was higher in squamous cell carcinomas than in adenocarcinomas (6,7). Poorly differentiated adenocarcinomas, which are often noted in smokers such as squamous cell carcinomas, have higher LOH frequency than differentiated adenocarcinomas, which have a relatively weaker association with smoking (23). Smoking induces complicated genetic changes in lung cancers.

One of the most intriguing recent discoveries in the field of lung cancer research is the identification of new driver mutations in lung adenocarcinomas, such as *EGFR* mutations (24,25) and *ALK* fusion (26). Both lung cancers with *EGFR* mutations or *ALK* translocations are characterized by negative or light smoking history. Lung cancers in non-smokers are considered to be less genetically complex than those in smokers and therefore they often have distinct characteristics developing on simple gene mutations for maintenance and survival. Consequently, patients with tumors harboring such simple oncogenic mutations represent good candidates who may stand to benefit from molecular-targeted drugs. To date, two-thirds of Japanese adenocarcinomas and a little more than half of Caucasian adenocarcinomas have mutually exclusive oncogenic mutations or other genetic alterations including *EGFR*, *KRAS*, *MET*, *ALK* and *HER2* (27). Asbestos-associated alterations in chromosomal regions, such as 19p13 (28), 9q33.1 (29) and 2p16 (30) have been identified. Whereas the smoking status has a significant association with driver mutations in lung adenocarcinomas, the relationship with asbestos-exposure remains unclear.

In adenocarcinomas without asbestos-exposure, the LOH frequency increased only slightly, correlating with the elevation in smoking-exposure. On the other hand, in adenocarcinomas with asbestos-exposure, the LOH frequency increased as asbestos-exposure and/or smoking-exposure was elevated. This suggests that asbestos-exposure in concert with smoking-exposure increases LOH frequency.

In the present study, lung adenocarcinomas, for which asbestos-exposure and smoking-exposure data could be obtained, were examined for LOH and the *p53* mutation. Combined effects of asbestos and cigarette smoke were suggested by these analyses. Asbestos-exposure alone did not increase the LOH frequency but increased non-specific *p53* mutations. These findings suggest that the major carcinogenic mechanism of asbestos in lung adenocarcinomas may be as a promoter, contributing to the genotoxic effect of cigarette smoke. Since this study was based on a general cancer center's experience, the limited sample size does not permit consider-

ation that the result is conclusive. Further investigation with a large sample size is required to establish the mechanism of asbestos-induced lung carcinogenesis.

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Clinicopathological features and EGFR gene mutation status in elderly patients with resected non–small-cell lung cancer

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Abstract

Background: The rapid aging of the population in Japan has been accompanied by an increased rate of surgery for lung cancer among elderly patients. It is thus an urgent priority to map out a treatment strategy for elderly patients with primary lung cancer. Although surgical resection remains standard treatment for early stage non–small-cell lung cancer (NSCLC), it is now essential to confirm the status of epidermal growth factor receptor (EGFR) gene mutations when planning treatment strategies. Furthermore, several studies have reported that *EGFR* mutations are an independent prognostic marker in NSCLC. However, the relations between age group and the molecular and pathological characteristics of NSCLC remain unclear. We studied the status of *EGFR* mutations in elderly patients with NSCLC and examined the relations of *EGFR* mutations to clinicopathological factors and outcomes according to age group.

Methods: A total of 388 consecutive patients with NSCLC who underwent complete tumor resection in our hospital from 2006 through 2008 were studied retrospectively. Formalin-fixed, paraffin-embedded tissue sections were used to isolate DNA from carcinoma lesions. Mutational analyses of EGFR gene exons 19, 20, and 21 and KRAS gene exons 12 and 13 were performed by loop-hybrid mobility shift assay, a highly sensitive polymerase chain reaction-based method.

Results: *EGFR* mutations were detected in 185 (47.7%) and *KRAS* mutations were detected in 33 (8.5%) of the 388 patients. *EGFR* mutations were found in a significantly higher proportion of patients younger than 80 years (younger group; 178/359, 49.6%) than in patients 80 years or older (older group; 7/29, 24.1%) (P = 0.008). In contrast, *KRAS* mutations were more common in the older group (6/29, 20.7%) than in the younger group (27/359, 7.5%) (P = 0.014). The older group showed a trend toward a higher rate of 5-year overall survival among elderly patients with *EGFR* mutations (100%) than among those with wild-type *EGFR* (66.2%), but the difference was not significant.

Conclusions: Our results suggest that the *EGFR* status of patients with NSCLC differs between patients 80 years or older and those younger than 80 years. *EGFR* mutation status might be a prognostic marker in elderly patients with completely resected NSCLC.

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Background

Primary lung cancer remains the leading cause of the death from malignant tumors worldwide [1]. Non–small-cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer [2]. Although surgical resection remains the standard treatment for early NSCLC, several molecular pathways have been shown to have prognostic significance in NSCLC. The epidermal growth factor receptor (EGFR) pathway is considered particularly important. EGFR is a membrane glycoprotein with an extracellular ligand-binding domain, a transmembrane lipophilic segment, and an intracellular domain that has tyrosine kinase activity. When a growth factor binds to EGFR, EGFR is self-phosphorylated by tyrosine kinase, and phosphorylated EGFR activates cell-signaling pathway involved in the regulation of cell cycle, apoptosis, angiogenesis, and cellular proliferation. Specific mutations of *EGFR* induce constant phosphorylation of EGFR, and increased levels of phosphorylated EGFR activate downstream signals that induce carcinogenesis [3,4]. *EGFR* mutations predict the effect of EGFR tyrosine kinase inhibitors (EGFR-TKI) [5,6]. It is now essential to confirm *EGFR* mutation status when planning treatment strategies for advanced or recurrent NSCLC.

The population of Japan is aging rapidly. In 2011 the average life-span in Japan was 83 years (males 79 years, females 86 years) [7]. Aging of the population is accompanied by a rapid increase in the incidence of primary lung cancer as well as the number of operations for lung cancer among elderly patients. Since 2009 persons 80 years or older have accounted for more than 10% of all patients in Japan. In 2011, patients 80 years old or older accounted for 11.5% of all patients [8-12]. Aging will become a global problem in the future, and knowledge acquired in Japan may contribute to solving related problems. Previous studies have suggested a relation between *EGFR* mutations and several clinicopathological factors, but whether *EGFR* status differs according to age group remains unclear. The present study assessed the status of *EGFR* mutations in elderly patients with NSCLC and examined the relations of *EGFR* mutations and clinicopathological factors to outcomes.

Methods

Patients

We retrospectively studied 388 consecutive patients with NSCLC who underwent complete tumor resection at Kanagawa Cancer Center Hospital (Yokohama, Japan) from 2006 through 2008. This study was approved by the ethics committee of the Kanagawa Cancer Center, and informed consent was obtained from all patients. The pathological diagnoses were independently made by 2 pathologists (T.N., T.Y.). Discrepancies in diagnoses were resolved by mutual agreement. The median follow-up time was 1981 days.

Assessments

Formalin-fixed, paraffin-embedded tissue sections of the resected tumors were used for DNA extraction. Mutational analyses of *EGFR* gene exons 19, 20, and 21 and *KRAS* gene exons 12 and 13 were performed by loop-hybrid mobility shift assay (LH-MSA), a highly sensitive polymerase chain reaction–based method, as described previously (Additional file 1: Table S1) [13].

Statistical analysis

Relations between *EGFR* status and categorical data were evaluated with the chi-square test. Continuous variables were compared by Student's t-test. Survival curves were plotted using the Kaplan-Meier method, and differences in survival rates were assessed using the log-rank test. $P < 0.05$ was considered to indicate statistical significance. Statistical manipulations were performed using the IBM SPSS Statistics 20 for Windows software system (IBM Corp, Armonk, NY, USA).

Results

Relations between *EGFR, KRAS* status and clinicopathological features

The patients' characteristics are summarized in Table 1. Of the 388 patients, 228 (58.8%) were men, and 160 (41.2%) were women. The mean age was 66.6 years (range, 35–90). *EGFR* mutations were detected in 185 patients (185/388, 47.7%) and *KRAS* mutations were detected in 33 (33/388, 8.5%). *EGFR* mutations were found more frequently in women (110/185, 59.5%), adenocarcinoma (183/185, 98.9%), and non-smokers (106/185, 57.3%) ($P < 0.001$). Patients with *EGFR* mutation had fewer pre-existing cardiopulmonary comorbidities than patients with wild-type ($P = 0.028$). The mean tumor diameter was smaller in patients with *EGFR* mutations (2.68 ± 0.92 cm) than in those with wild-type *EGFR* (3.35 ± 1.71 cm; $P < 0.001$). The rate of pathological T1 disease was significantly higher among patients with *EGFR* mutations (114/185, 61.6%) than among those with wild-type *EGFR* (83/203, 40.9%; $P < 0.001$). In contrast, *KRAS* mutations were not significantly related to gender, histopathological type, or smoking status. Although *KRAS* status did not correlate with pathological T factors, mean tumor diameter was larger in patients with *KRAS* mutations (3.46 ± 1.99 cm) than in those with wild-type *KRAS* (2.99 ± 1.36 cm; $P = 0.001$).

Relations between age group and clinicopathological features

We divided the patients into two groups according to whether they were 80 years or older (older group) or

Table 1 Correlations between *EGFR* mutations and clinicopathological features

Characteristics	Total	No. of patients					
		EGFR status		p^a	*KRAS* status		p^a
		Mutation	Wild-type		Mutation	Wild-type	
	(*n* = 388)	(*n* = 185, 47.7%)	(*n* = 203, 52.3%)		(*n* = 33, 8.5%)	(*n* = 355, 91.5%)	
Mean age, yr ± SD[b]	66.6 ± 10.0	65.1 ± 10.3	67.9 ± 9.57	0.462	68.6 ± 9.11	66.4 ± 10.1	0.553
Gender				<0.001			0.552
Male	228	75	153		21	207	
Female	160	110	50		12	148	
Histological type				<0.001			0.059
Adenocarcinoma	302	183	119		30	272	
Others	86	2	84		3	83	
Vascular invasion							
Ly -	314	155	159	0.172	25	289	0.429
Ly +	74	30	44		8	66	
V -	261	151	110	<0.001	23	238	0.756
V +	127	34	93		10	117	
p-stage				<0.001			
I	293	155	138		22	271	0.217
II / III	95	30	65		11	84	
T-factor				<0.001			
T1	197	114	83		14	183	0.316
T2 / 3	191	71	120		19	191	
Tumor diameter (cm)	3.03 ± 1.43	2.68 ± 0.92	3.35 ± 1.71	<0.001	3.46 ± 1.99	2.99 ± 1.36	0.001
N-factor				0.348			
N0	322	157	165		29	293	0.435
N1 / 2	66	28	38		4	62	
Smoking status				<0.001			0.107
Non-smoker	157	106	51		9	148	
Smoker	231	79	152		24	207	
Pre-existing cardiopulmonary comorbidity	203	86	117	0.028	20	183	0.319

[a]$p < 0.05$ statistically significant.
[b]SD, standard deviation.
EGFR, epidermal growth factor receptor; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; ND, lymph node dissection.

younger than 80 years (younger group) and compared *EGFR* status and clinicopathological features between these age groups (Table 2). The younger group comprised 359 patients (92.5%), and the older group comprised 29 (7.5%). The proportion of patients with *EGFR* mutations was significantly higher in the younger group (178/359, 49.6%) than in the older group (7/29, 24.1%; P = 0.008). In contrast, *KRAS* mutations were more common in the older group (6/29, 20.7%) than in the younger group (27/359, 7.5%; P = 0.014). The proportion of smokers was significantly lower in the younger group (208/359, 57.9%) than in the older group (23/29, 79.3%; P = 0.024). Elderly patients had more pre-existing cardiopulmonary comorbidities than younger patients (P = 0.024). Gender, histopathological type, vascular invasion, pathological stage, and tumor diameter did not differ significantly between the groups. We omitted lymph-node resection in the older group (P < 0.001). Table 3 shows the region of *EGFR* mutation according to age group. Although the study group was small, there were no exon 20 mutations in the older group.

Relations between *EGFR* status and outcomes

Kaplan-Meier curve analysis showed that *EGFR* mutation status was significantly associated with survival (Figure 1). The 5-year overall survival rate was significantly higher in patients with *EGFR* mutations (90.2%) than in those with wild-type *EGFR* (75.2%) in the younger group (P < 0.001; Figure 1A). The 5-year overall survival rate was slightly, but not significantly higher

Table 2 Correlations between age group and clinicopathological features, including *EGFR* status

Characteristics	No. of patients			
	Total (*n* = 388)	≥80 years (*n* = 29, 7.5%)	<80 years (*n* = 359, 92.5%)	*p*[a]
Mean age, yr ± SD[b]	66.6 ± 10.0	82.6 ± 2.41	65.3 ± 9.29	<0.001
Gender				0.246
Male	228	20	208	
Female	160	9	151	
Histology				0.034
Adenocarcinoma	302	18	284	
others	86	11	75	
Biomarker				
EGFR wild type	203	22	181	0.008
EGFR mutation	185	7	178	
KRAS wild type	355	23	332	0.014
KRAS mutation	33	6	27	
Vascular invasion				
Ly -	314	26	288	0.214
Ly +	74	3	71	
V -	261	18	243	0.535
V +	127	11	116	
p-stage				0.080
I	293	18	275	
II / III	95	11	84	
T-factor				0.506
T1	197	13	184	
T2/3	191	16	175	
Tumor diameter (cm)	3.03 ± 1.43	3.00 ± 1.44	3.40 ± 1.24	0.629
N-factor				0.584
N0	322	23	299	
N1/2	66	6	60	
Operation				0.155
Limited resection (wedge/segmentectomy)	80	3	77	
Standard surgery (lobectomy, pneumonectomy)	308	26	282	
Lymph node resection				<0.001
ND0/1/sampling	109	25	156	
ND2	278	4	203	
Smoking				0.024
Non-smoker	157	6	151	
Smoker	231	23	208	
Pre-existing cardiopulmonary comorbidity	203	21	182	0.024

[a]$p < 0.05$ statistically significant.
[b]SD, standard deviation.
EGFR, epidermal growth factor receptor; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; ND, lymph node dissection.

in patients with *EGFR* mutations (100%) than in those with wild-type *EGFR* (66.2%) in the older group (P = 0.226; Figure 1B).

Discussion

In the present study, we first evaluated *EGFR* mutations in resected NSCLC tissue by LH-MSA. LH-MSA is a

Table 3 Region of *EGFR* mutation according to age group

	No. of patients		
EGFR mutations	Total	≥80 years	<80 years
Exon 19	73	3	70
Exon 20	13	0	13
Exon 21	97	4	93
Combined	2	0	2

highly sensitive polymerase chain reaction-based method. Sakuma *et al.* previously evaluated *EGFR* mutations by LH-MSA in our hospital. *EGFR* mutations were detected in 53.2% of NSCLCs and were significantly associated with adenocarcinoma, female sex, and no smoking history [14]. In the present study, we detected EGFR mutations in 47.7% of NSCLCs (Table 1). The presence of an *EGFR* mutation is closely linked to several clinicopathological factors, such as gender, smoking history, and pathological findings. Our results are consistent with those of recent studies reporting that the rate of EGFR mutations is higher among Asians (including Japanese), females, nonsmokers, and adenocarcinomas [14,15]. Although LH-MSA yet has not been generally performed, it is known to be a sensitive and low cost method in scanning the known gene mutation. Furthermore, we can treat many samples in a short time by LH-MSA. Nakajima et al. analyzed *EGFR* mutations using LH-MSA, and confirmed the results by direct sequencing. They concluded that LH-MSA has a high detection capability compared with direct sequencing [16]. Guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology indicate that LH-MSA compares favourably with the other method [17].

We then studied the relations between *EGFR* status and clinicopathological factors according to age group (Table 2). Past report suggested the impact of age on *EGFR* mutation, and concluded that age was associated with *EGFR* mutation in lung cancer [18]. In this study, if we analyze the *EGFR* status using the median age of 66 years old as a cutoff, there is no difference between younger and elderly group. Next, we divided the cohort in every ten years old, and we found that the rate of *EGFR* mutation suddenly decreased in a group 80 years or older. Because aging of the population is a global problem, the average life-span older than 80 years old in Japan was worthy of mention to the world. Due to the above reasons, we thought that the age of 80 years old is turning point in consideration of gene profile change, and divided the patients into two groups at 80 years of age. The older group (≥80 years) of patients with NSCLC included significantly higher rates of non-adenocarcinoma, wild-type *EGFR*, *KRAS* mutations, and smokers. There was no difference between the older group and younger group in tumor size, T-factor, or pathological stage. Moreover, in Japan, females outlive males (males 79 years, females 86 years). Of the 29 elderly patients, 9 are females include 7 adenocarcinomas and 4 smokers. *EGFR* mutations were detected in 3 females. The 5-year overall survival rate was 100% regardless of *EGFR* mutation or wild type. When we examined the region of *EGFR* mutation according to age group (Table 3), no exon 20 mutations were found in the older group. Although our study group was small, our results suggest that *EGFR* mutation status might differ

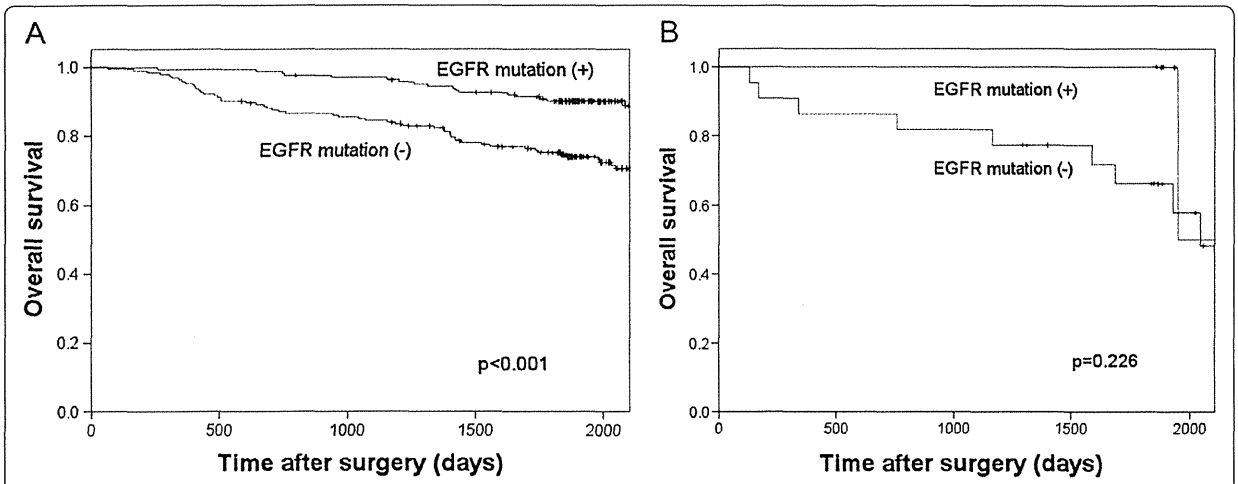

Figure 1 Relations between *EGFR* mutations and outcomes. Kaplan-Meier curve analysis showed that *EGFR* mutation status was significantly associated with survival. **(A)** The 5-year overall survival rate was higher in patients with *EGFR* mutations (90.2%) than in those with wild-type *EGFR* (75.2%) in the younger group ($P < 0.001$). **(B)** In the older group, the 5-year disease free survival rate was 100% among patients with *EGFR* mutations; however, the difference between the two groups was not significant.