Genetic Classification of Lung Adenocarcinoma Based on Array-Based Comparative Genomic Hybridization Analysis: Its Association with Clinicopathologic Features

Tatsuhiro Shibata,[1] Satomi Uryu,[2] Akiko Kokubu,[1] Fumie Hosoda,[2] Misao Ohki,[2] Tokuki Sakiyama,[3] Yoshihiro Matsuno,[5] Ryosuke Tsuchiya,[6] Yae Kanai,[1] Tadashi Kondo,[4] Issei Imoto,[7,8] Johji Inazawa,[7,8] and Setsuo Hirohashi[1]

Abstract The array-based comparative genomic hybridization using microarrayed bacterial artificial chromosome clones allows high-resolution analysis of genome-wide copy number changes in tumors. To analyze the genetic alterations of primary lung adenocarcinoma in a high-throughput way, we used laser-capture microdissection of cancer cells and array comparative genomic hybridization focusing on 800 chromosomal loci containing cancer-related genes. We identified a large number of chromosomal numerical alterations, including frequent amplifications on *7p12*, *11q13*, *12q14-15*, and *17q21*, and two homozygous deletions on *9p21* and one on *8p23*. Unsupervised hierarchical clustering analysis of multiple alterations revealed three subgroups of lung adenocarcinoma that were characterized by the accumulation of distinct genetic alterations and associated with smoking history and gender. The mutation status of the *epidermal growth factor receptor* (*EGFR*) gene was significantly associated with specific genetic alterations and supervised clustering analysis based on *EGFR* gene mutations elucidated a subgroup including all *EGFR* gene mutated tumors, which showed significantly shorter disease-free survival. Our results suggest that there exist multiple molecular carcinogenesis pathways in lung adenocarcinoma that may associate with smoking habits and gender, and that genetic cancer profiling will reveal previously uncharacterized genetic heterogeneity of cancer and be beneficial in estimating patient prognosis and discovering novel cancer-related genes including therapeutic targets.

Lung cancer is one of the most lethal and increasing cancers in Western countries as well as in Japan (1). Lung cancer is histopathologically divided in two subgroups, small cell lung carcinoma and non–small cell lung carcinoma, and lung adenocarcinoma comprises >40% of the latter (1).

Previous genetic analyses using allelotyping, comparative genomic hybridization (CGH), or the candidate gene approach revealed many genomic (genetic and epigenetic) alterations of tumor suppressor genes (such as *p53*, *p16^{INK4a}*, *FHIT*, *LKB1*, and *PTEN*) and oncogenes [such as *K-ras*, *B-RAF*, *MYC*, *epidermal growth factor receptor* (*EGFR*), and *ERBB2*] as well as many chromosomal imbalances (such as on 3p, 8p, 9p, 17p, 18q, and 19p) in lung adenocarcinomas (2–10). However, overall understanding of genomic alterations in lung adenocarcinomas is far from complete and analysis of the relationship between the overall profile and combinations of genetic alterations with clinicopathologic parameters is still lacking. Recently, genome-wide gene expression analyses have uncovered a novel dimension of cancer profiling and helped define the nature of the heterogeneous subgroups of lung adenocarcinoma, each of which shows distinct tumor histology and patient prognosis (11–14). However, it is unclear whether there exist multiple genomic pathways in lung adenocarcinoma because of the lack of a genome-wide view of genetic alterations. It is clinically important to examine the correlations of certain molecular-genetic pathways with cancer cell traits relating to patient prognosis or chemotherapy sensitivity because it is possible that genetic alteration profiling may predict tumor recurrence/metastasis or sensitivity to molecular-target therapies as well as mRNA or protein expression profiles do (15–17).

The recently developed array-based CGH method using microarrayed bacterial artificial chromosome clones allows high-resolution analysis of genome-wide copy number changes in various tumors (18, 19). To define and analyze the genetic alterations of lung adenocarcinoma in a more detailed way,

Authors' Affiliations: [1]Pathology Division, [2]Cancer Genomics Project, [3]Center for Medical Genetics, [4]Cancer Proteomics Project, National Cancer Center Research Institute, Tokyo, Japan; [5]Clinical Laboratory and [6]Thoracic Surgery Divisions, National Cancer Center Hospital, Tokyo, Japan; [7]Department of Molecular Cytogenetics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan; and [8]Core Research for Evolutional Science and Technology of Japan, Science and Technology Corporation, Saitama, Japan

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Requests for reprints: Setsuo Hirohashi, Pathology Division, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, 104-0045 Tokyo, Japan. Phone: 81-3-3547-5201, ext. 4101; Fax: 81-3-3248-2463; E-mail: shirohas@ncc.go.jp.

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we used the array CGH technique and laser-capture microdissection of cancer cells, a combination that we have successfully used in other tumor types (20).[9] Using these two powerful methodologies enabled us to detect a considerable number of novel chromosomal numerical alterations in primary lung adenocarcinoma. Unsupervised and supervised hierarchical clustering analyses of genome-wide genetic alterations revealed the presence of heterogeneous groups of lung adenocarcinoma, which are characterized by specific combinations of genetic alterations, varying *EGFR* gene mutation status, and tumor recurrence rates.

Materials and Methods

Patient materials. Surgical specimens of 55 lung adenocarcinoma patients who had been diagnosed and undergone operation between June 2001 and May 2002 at the National Cancer Center Hospital were examined. Fragments of tumor and corresponding normal lung tissue were taken immediately after surgery, fixed with 100% methanol, and embedded in paraffin. This study was approved by the institutional review boards of the National Cancer Center. The clinicopathologic data of the patients are shown in Table 1.

Laser-capture microdissection and whole-genome amplification. Laser-capture microdissection was done using LM200 (Arcuturus, Mount View, CA) as described (21). Only cancer cells were microdissected and lymphocytes, fibroblasts, and endothelial cells were carefully excluded. Corresponding normal lung epithelial cells were similarly microdissected and used as reference. To amplify the genomic DNA fragments, we used an adaptor-ligated whole-genome PCR as previously reported (22).

Array-based comparative genomic hybridization. This study used a custom-made CGH array called "MCG CancerArray-800 ver.2," which consists of 800 duplicated bacterial artificial chromosome clones corresponding to various chromosomal loci that have been reported or considered to be altered in various human cancers (20, 23). Details of hybridization procedures have been previously reported (20). Sixteen-bit fluorescence intensity TIF images were obtained using a scanner (FLA8000, Fuji Film, Tokyo, Japan) and analyzed using GenePix Pro 5.0 (Axon Instruments, Inc., Foster City, CA). Thresholds for chromosomal gain (ratio >1.25) and loss (ratio <0.75) were determined by "normal versus normal experiments" (23, 24). We also validated our array CGH data by other methods. Loss of the *17p13* locus was confirmed by loss of heterozygosity of the *p53* gene, which is located within that bacterial artificial chromosome using a microsatellite marker (TP53CA). Gene amplification of a representative gene, *cyclin D1*, was validated by fluorescence *in situ* hybridization analysis (24). We applied multiplex ligation-dependent probe amplification (MLPA) to validate ourarray data. Copy number alterations of multiple loci were analyzed using MLPA-SALSA kit (MRC-Holland, Amsterdam, the Netherlands) as per the recommendation of the manufacturer (25). Size and quantity of PCR products were calculated by Gene Mapper software (Version 3.5, Applied Biosystems, Tokyo, Japan) and copy number was determined by the ratio to the average of five normal control experiments.

Mutational analysis. We amplified exons 18, 19, 21, and 23 of the *EGFR* gene; exons 2 and 3 (covering codons 12, 13, and 61) of the *K-ras* gene; exons 20 and 21 of the *ERBB2* gene; and exons 10, 14, 16, 17, 18, 19, and 20 of the *MET* gene from microdissected tumor and corresponding normal DNA samples with PCR using High Fidelity Taq polymerase (Roche, Mannheim, Germany) and appropriate primers (primer sequences are available on request). All PCR products were purified and analyzed by sequencing. PCR products showing deletions were subcloned in TA-vector (Invitrogen, Carlsbad, CA) and sequenced.

[9] Unpublished data. Shibata T, Hosoda F, Ohki M, Hirohashi S.

Table 1. The clinicopathologic characteristics and oncogenic mutation profiles of patients and tumors

	No. cases	Frequency (%)
Total no. patient	55	
Mean age (range)	62.3 (35-79)	
Gender		
Male	28	50.9
Female	27	49.1
Smoking history		
Never	27	49.1
Former	10	18.2
Current	18	32.7
Tumor differentiation		
Well	20	36.4
Moderate	25	45.5
Poor	10	18.1
Stage*		
I (IA and IB)	24	43.6
II (IIA and IIB)	6	10.9
III (IIIA and IIIB)	21	38.2
EGFR mutation	26	47
Exon 18 G719S	1	1.8
Exon 19 Del746-750	8	14.5
Exon 19 Del747-752	2	3.6
Exon 19 Del747-752insS	1	1.8
Exon 21 L858R	14	25.5
K-*ras* mutation	6	11
Codon 12	4	7.3
Codon 13	1	1.8
Codon 61	1	1.8

*Clinical stage of four cases was not evaluated.

Immunohistochemical analysis. Four-micrometer sections of formalin-fixed, paraffin-embedded specimens of lung adenocarcinoma were stained with an anti-MET mouse monoclonal antibody (×100 dilution, Zymed, San Francicso, CA) as the suppliers recommended.

Statistical analysis. Two-dimensional hierarchical clustering analysis of the samples and signal ratios was done using the Impressionist (Gene Data, Basel, Switzerland) and GeneMaths (Applied Maths, Sint-Martens-Latem, Belgium) software programs as described (26, 27). Data were standardized by dividing by the root means and dendrograms were produced using the Pearson Correlation algorithm. For supervised clustering, we first selected loci that were significantly different between *EGFR* wild-type and mutated tumors based on the average ratio by Student's t test. We then used a machine-learning method, in which the leave-one-out cross-validation was done with all combinations of loci and multiple independent classifier algorithms, and selected 46 loci that could discriminate *EGFR* mutation status most accurately to classify the tumors. The Kaplan-Meier method was used to estimate the probability of disease-free survival. Cox proportional hazards regression model and multivariate analysis were done to detect the association between the presence of chromosomal alterations and disease-free survival. Log-rank analysis was used to assess the significance of the difference between subgroups.

Results

Array-based comparative genomic hybridization analysis of primary lung adenocarcinoma. We analyzed 55 cases of lung

adenocarcinoma by array-based CGH and the chromosomal alteration profiles of 800 loci are shown in Fig. 1. We identified 32 loci that were lost in >40% of cases (Table 2). Among them, the *9p21* locus containing the $p16^{INK4a}$ gene and the *17p13.1* locus containing the *p53* gene were lost in 54% and 40% of analyzed cases, respectively. We found homozygous deletions of three loci, including two on *9p21* and one on *8p23.3*. We also identified 19 loci that were gained in >50% of cases (Table 3) and recurrent (>4 cases) amplifications (>4 copies) on *12q14-15* (9 of 55, 16.3%) followed by *7p12.3* (5 of 55), *11q13* (5 of 55), *17q12* (5 of 55), *1p36.1* (4 of 55), *1q21* (4 of 55), *5p15* (4 of 55), *7q31* (4 of 55), *8q24* (4 of 55), *14q12* (4 of 55), and *17q21.2* (4 of 55). These included genes previously reported to be amplified in lung cancer, such as the *cyclin D1* (*11q13*), *EGFR* (*7p12.3*), and *ERBB2* (*17q21.2*) genes (28, 29). We further validated copy number alterations on *8q24.3*, *17q21.2*, *3p21*, and *17p13.1* by MLPA method. Chromosomal copy number changes (both gains and losses) detected by array CGH corresponded to those by MLPA (Fig. 1C).

Unsupervised hierarchical clustering of array comparative genomic hybridization data. To examine whether there exist multiple carcinogenesis pathways in lung adenocarcinoma, we attempted two-dimensional hierarchical profiling of the chromosomal alterations detected. We first plotted the number of loci showing various incidences of alterations and found that there exist two peaks (loci altered in 10-15% and 20-25% of cases; Fig. 1D). We assumed that alterations appearing in <20% of cases reflect mostly random alterations as observed in genome-wide allelotyping analyses (30), whereas alterations affecting >20% of cases probably represent nonrandom (cancer-specific) alterations. Therefore, to exclude random changes that may be caused by the intrinsic genetic instability of cancer, we selected the loci that were affected in >25% of analyzed cases (397 loci in total) and did the unsupervised hierarchical clustering analysis. When analyzed by using loci affected in >5% and 15% of cases, we obtained almost the same classification as described below (data not shown).

Our hierarchical clustering yielded three distinct subclasses of primary lung adenocarcinoma (clusters A, B, and C shown in Fig. 1E). Cluster B exhibited significantly fewer genetic alterations (losses and gains) in all examined clones than the other two clusters; the average number of alterations (losses, gains, total) in cluster A was 102, 141, and 244, respectively; in B 43, 81, and 125; and in C 85, 131, and 216 (A versus B: $P < 0.0001$; B versus C: $P < 0.0001$). Frequencies of the various lost or gained loci were significantly different among these three cluster groups ($P < 0.01$). Cluster A was characterized by gains on *1p32-26*, *4p16.3*, *11p15*, *12q13-14*, *16p11.2-13.3*, *17q11.1-25*, *19q13.2*, *20p11*, *20q11.2*, and *22q12.2* and losses on *1p22*, *6q26*, *10q24-26*, *13q22.1-34*, *15q21-25*, and *18p11.2*. Cluster C significantly showed gains on *5p12-14.3*, *7p12.3-21.1*, *7q22*, *7q31*, *8q12-21*, and *14q11-24*, and losses on *1q23.3-41*, *10q22.1*, and *Xq*. Some loci were similarly altered in both clusters A and C, including losses on *3p21-24*, *6q26*, *8q24.3*, *9q21*, *10p15*, *10q11*, *10q26*, *15q21.1*, *15q26.1*, and *19p13.3* (containing *LKB1*), and gains on *5p15*, *6p21*, *7p21-22*, *7q21*, *8q21*, *8q22-24* (containing *MYC*), *9q21-22*, *11q13* (containing *cyclin D1*), and *20q13.1*. Losses on *3p14* (containing *FHIT*), *8p22-23.3*, *9p21* (containing $p16^{INK4a}$), *13q11-34*, *17p13.1* (containing *p53*), *18q21*, and gains on *1q21-23*, *1q42*, *7p15*, *17q12*, *17q21.2*, and *17q25* were observed in all subgroups with similar frequency. Two alterations (a gain on *19q13.1* and a loss on *22q12.2*) were more frequently observed in cluster B than in clusters A and C. The above classification into cluster groups showed significant correlation with the patients' smoking history ($P < 0.01$) and gender ($P < 0.001$); cluster A frequently contained female patients without any smoking history (female: 17 of 20 cases and never smoker: 14 of 20 cases), whereas cluster B included male patients with current or former smoking history (male: 11 of 15 cases and smoker: 11 of 15 cases). Cluster C included more male patients (male: 14 of 20 cases), but showed no significant association with smoking history (smoker: 11 of 20 cases). No significant differences were observed between the groups with regard to other clinical features (histologic differentiation, clinical stage, and disease-free survival). Multivariate analysis revealed that two chromosomal alterations showed significant association with disease-free survival: a loss on 13q14.1 ($P = 0.01$, hazard ratio, 3.21; 95% confidence interval, 1.30-7.91) and a gain on 8q24.2 ($P = 0.02$, hazard ratio, 2.92; 95% confidence interval, 1.16-7.37).

It has been reported that somatic mutations of the K-*ras*, *EGFR*, and *ERBB2* genes are frequent in lung adenocarcinoma (2, 8 - 10, 31 - 33). We attempted to determine the correlation of these oncogenic mutations with the above classification. We sequenced exons covering the kinase domain of the *EGFR* gene and found somatic mutations in 26 cases (47%; Table 1). *EGFR* mutations were more frequently observed in never-smoker patients ($P < 0.001$) and in the A and C cluster groups ($P = 0.01$ and 0.02). We detected K-*ras* activating mutations in six cases (11%; Table 1) and *EGFR* and K-*ras* mutations were mutually exclusive in our cases as reported by others (31, 32). No mutation in the reported exons of the *ERBB2* gene was detected.

Supervised clustering analysis revealed correlation of EGFR gene mutation with specific genetic alterations. Tumors with *EGFR* mutations showed significantly more genetic alterations (losses and gains) than those without *EGFR* mutations; the average numbers of alterations (losses, gains, total) were 68, 110, and 178 in *EGFR* wild-type tumors and 93, 134, and 227 in *EGFR* mutated tumors, respectively (wild-type versus mutated $P = 0.01$, 0.03, and 0.01). We found 58 loci that showed significant differences in the frequency of copy number alterations between *EGFR* wild-type and mutated tumors. To further examine the genetic profile of *EGFR* mutated tumors, we classified lung adenocarcinomas based on their *EGFR* mutation status with the use of supervised hierarchical clustering. We preformed a machine-learning method with leave-one-out cross-validation and selected 46 loci that could discriminate *EGFR* mutation status. *EGFR* wild-type and mutated tumors were clustered in distinct groups using the ratios of 46 selected loci (Fig. 2A). Tumors carrying K-*ras* mutations were segregated from the *EGFR* mutant branch (Fig. 2A). Interestingly, some cases without *EGFR* mutation were clustered with the mutant branch, and tumors carrying *EGFR* mutations were separated in two subbranches (EGFR-MUT-A and EGFR-MUT-B; Fig. 2A). One branch (EGFR-MUT-A) was characterized by amplification of *12q14* or *1p36.1*, whereas the other (EGFR-MUT-B) contained frequent amplification of *7p12.3* (containing the *EGFR* gene), *1q44-23*, *5p12*, *14q31*, and *16p13.3*. Poorly differentiated tumors were significantly ($P = 0.01$) segregated in the EGFR-MUT-B subgroup (EGFR-MUT-A: 1 of 15 cases and EGFR-MUT-B: 5 of 21 cases).

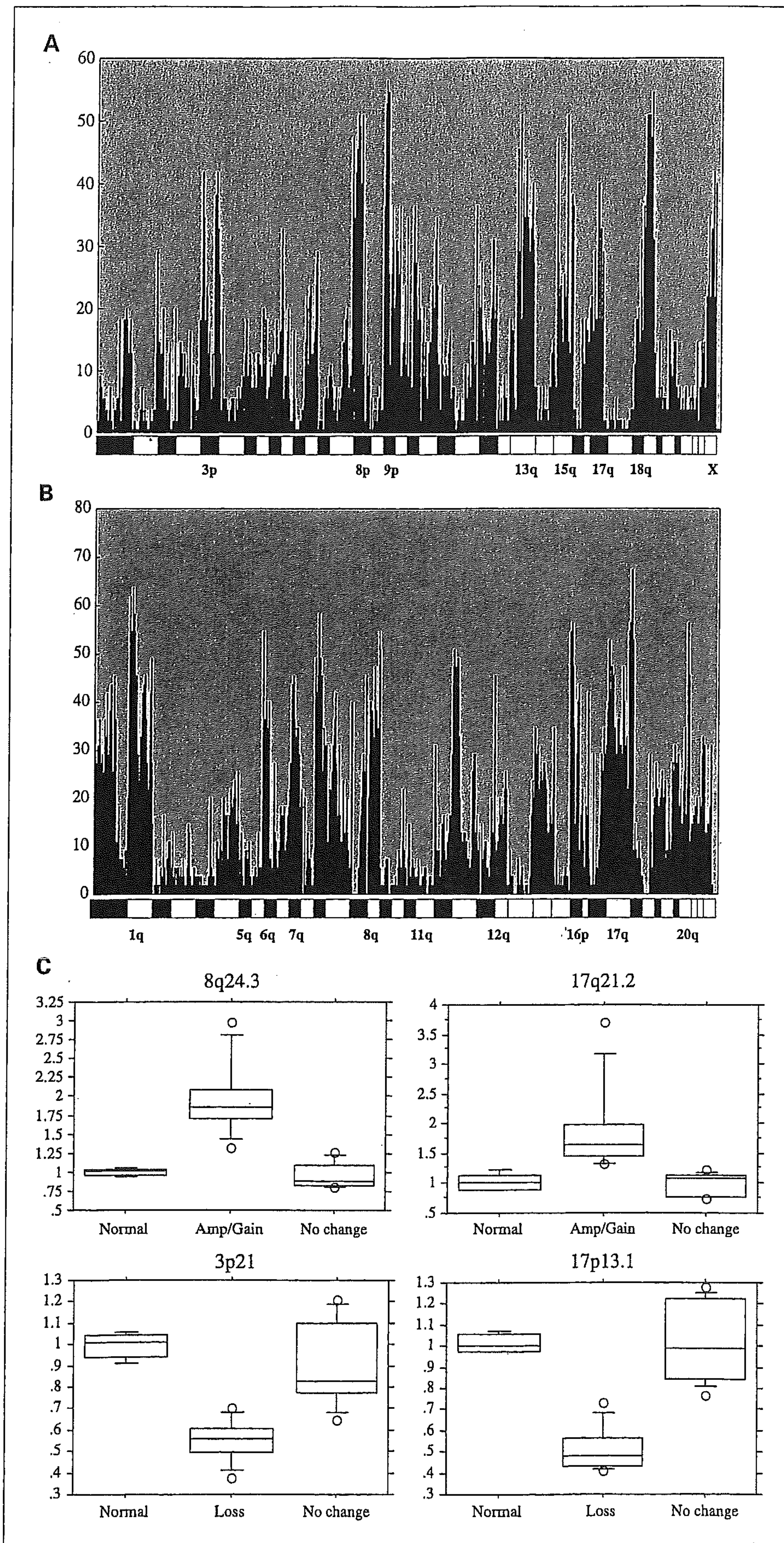

Fig. 1. Copy number alteration profiles of primary lung adenocarcinoma. Frequencies (%) of chromosomal losses (*A*) and gains (*B*) detected by array CGH analyses are plotted from chromosome 1p (*left*) to Y (*right*). Each chromosome is represented by underlining boxes (short and long arms are indicated by closed and open boxes, respectively). Chromosomal arms that contain frequent losses or gains are also indicated. *C*, validation of array CGH analysis. Distributions of copy number on *8q24.3*, *17q21.2*, *3p21*, and *17p13.1* loci detected by MLPA method in five normal samples (*Normal*), tumors with alterations detected by array CGH (*Amp/Gain* or *Loss*), and tumors without alteration by array CGH method (*No change*) were shown.

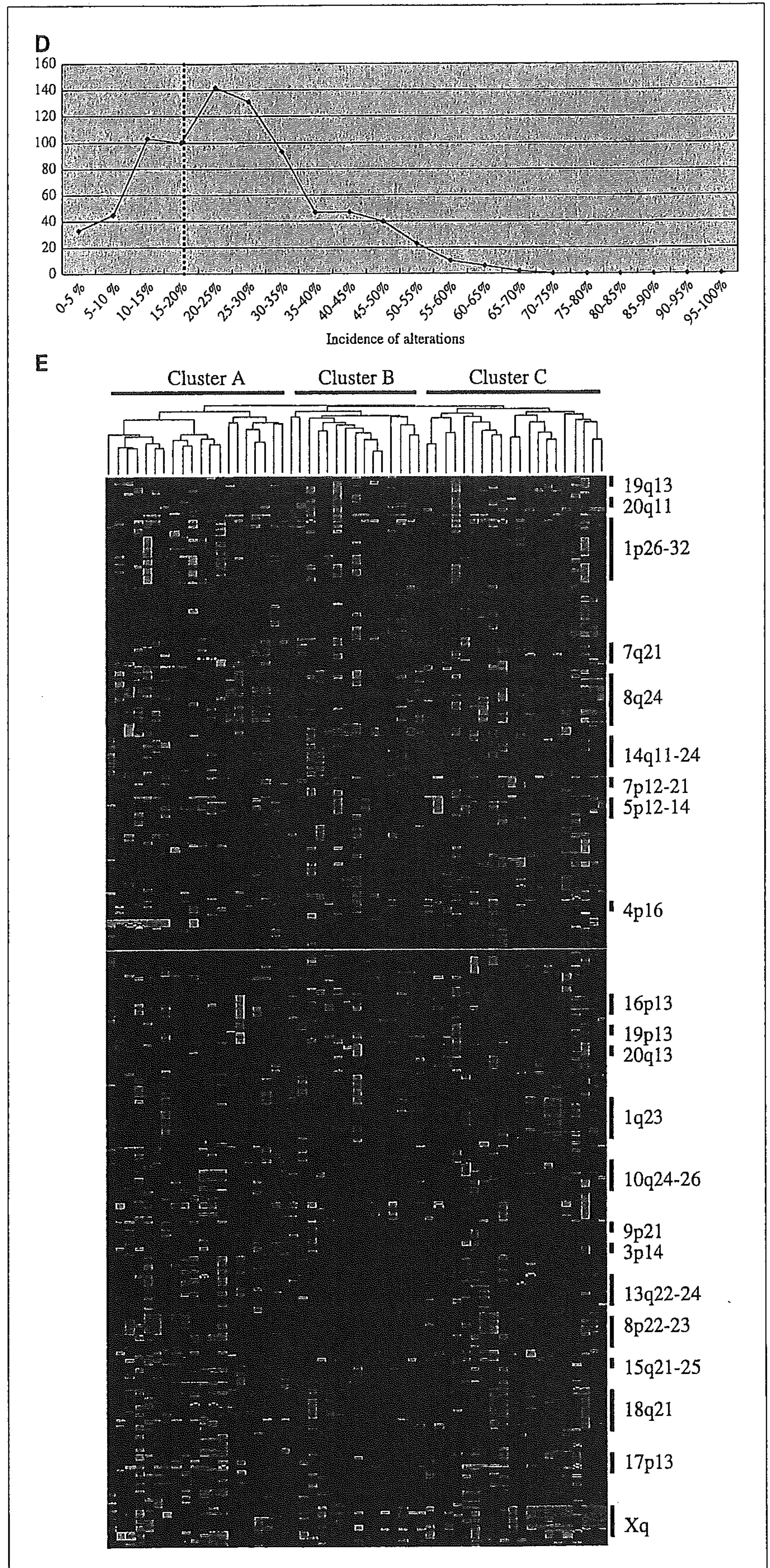

Fig. 1. *Continued*. *D* and *E*, genetic classification of lung adenocarcinoma. *D*, number of loci as a function of the percentage of altered loci in lung adenocarcinoma. Two modes, which can be separated by the vertical dotted line, can be observed. Loci within the first mode (altered in <20% of cases) were considered random, whereas those in the second mode (altered in >20% of cases) were considered pathogenic. *E*, unsupervised genetic profiling of lung adenocarcinoma. Fifty-five lung adenocarcinomas were clustered hierarchically on the basis of copy number changes in 397 loci. Multiple lost (*green*) or gained (*red*) loci are observed in individual tumors. Overall pattern of standardized gene copy numbers and the cluster tree of individual tumors are shown. Tumors were clustered in three subgroups (clusters A, B, and C). Loci that are specific for (*black letters*) or shared with (*blue letters*) each subgroup are indicated on the right side.

The *EGFR* wild-type tumors that were clustered with the EGFR-MUT branch led us to hypothesize that aberrant activation of tyrosine kinases other than *EGFR* have an effect equivalent to that of *EGFR* mutations in these tumors. We examined the copy number changes of loci containing oncogenic receptor-type tyrosine kinases (FGFR1, FGFR2, FGFR3, PDGFR, KIT, MET, ERBB2, FLT3, NTRK1, and NTRK3) detected by our arrays. We found that amplification of a locus containing the *MET* gene (*7q31*) was observed in *EGFR* wild-type tumors (three of four amplified cases; a representative case was shown in Fig. 3A) and that these tumors were clustered with the EGFR-MUT branch (Fig. 2A). Overexpression of MET protein was immunohistochemically detected in 24% (13 of 55) of cases including all cases with amplification of the *MET* gene (Fig. 3B), although there was no significant association between MET overexpression and the clustering. To determine whether somatic mutations of this kinase also occur in lung adenocarcinoma, we sequenced the exons of the *MET* gene, which have been reported to exhibit activating mutations in various tumors (34-36); however, no mutation was found in any of examined 55 cases.

Table 2. Loci frequently lost in primary lung adenocarcinoma

Chromosomal location	Covered candidate gene	Chromosomal loss in lung adenocarcinoma (%)
9p22	*MLLT3*	56.4
9p21	*p16^{INK4a}**	54.5
9p21	*TEK*	54.5
18q23d	*CTDP1*	54.5
9p23	*GASC1*	52.7
9p21.3	*MTAP*	52.7
15q25	*NTRK3*	50.9
13q14.1	*FKHR*	50.9
18q21	*SMAD4**	50.9
8p22	*NAT2*	50.9
18q21.3	*PI5*	50.9
18q21	*GRP*	50.9
18q22	*BCL2*	50.9
8p22	*LZTS1**	49.1
15q12	*SNRPN*	47.3
8p23.3	*D8S504*	47.3
18q21.3	*SCCA1*	47.3
8p22	*N33*†	45.5
13q11-12	*FGF9*	45.5
8p22-11	*NRG1*	43.6
13q22.1	*KLF12*	43.6
Xq28	*MAGEA2*	41.8
3p24.3	*THRB**	41.8
3p14.2	*FHIT**	41.8
8p22-21.3	*DLC1*†	41.8
8p23.1	*AAC1*	41.8
17p11.2	*RH68621*	40
13q14.1	*LCP1*	40
8p22-8p21	*TNFRSF10B*	40
13q33	*EFNB2*	40
17p13.1	*RCV1*	40
18q21.3	*FVT1*	40

*Loss of heterozygosity or mutations, and †aberrant expression was previously reported in lung cancer (3-5).

Table 3. Loci frequently gained in primary lung adenocarcinoma

Chromosomal location	Covered candidate gene	Chromosomal gain in lung adenocarcinoma (%)
17q25	*MAFG*	67.3
1q21	*MUC1**	63.6
1q21	*MCL1**	61.8
7p21	*IL6*	58.2
1q21	*ARHGEF2*	58.2
16p13.3	*ABCA3*	56.4
17q11	*ITGB4*	56.4
20q13	*Livin-2*	56.4
5p15	*TERT*	54.5
8q24	*GLI4*	54.5
16p13.3	IGFALS	54.5
17q24-25	*GRB2*	54.5
1q21	*AF1Q*	54.5
1q23.1	*PMF1*	54.5
12q24	*stSG8935*	52.7
17q12	*PPARBP*	52.7
17q25	*Survivin**	50.9
8q24	RECQL4	50.9
11q12-13	RELA	50.9

*Aberrant expression was previously reported in lung cancer (3-5).

We further examined whether this classification is of clinical significance. Kaplan-Meier plots showed a statistically significant difference in disease-free survival between the two groups (EGFR-WT and EGFR-MUT; log-rank analysis, $P = 0.01$; Fig. 2B) although *EGFR* mutation status alone did not (log-rank analysis, $P = 0.06$; data not shown).

Discussion

This study is the first high-resolution copy number analyses of primary lung adenocarcinoma by array CGH method. To extract common and specific genetic alterations, we collected and analyzed 55 cases of primary lung adenocarcinoma by combining the array-based CGH analysis with laser-capture microdissection of tumor cells. Importantly, our results were validated by the elucidation of frequent alterations of previously reported cancer-related genes in lung adenocarcinoma, including losses on the *p16^{INK4a}*, *p53*, and *FHIT* loci, and amplifications on the *cyclin D1* and *EGFR* loci. Moreover, we elucidated novel frequent alterations in small chromosomal regions such as losses on *13q11-14* and *15q12-25* and gains on *17q25*, *1q21*, and *16p13.3*, which have not been detected by previous studies. Novel recurrent amplification, which may be a landmark for the existence of oncogenes, was also detected on loci, including *1p36.1*, *1q21*, *5p15*, *12q14-15*, and *14q12*. We also found a homozygous deletion on *8p23.3* accompanied with frequent chromosomal loss and identification of candidate tumor suppressor genes in this locus is in progress.

It has been argued that there are distinct subclasses of lung adenocarcinoma by histopathologic observations and recent gene expression profilings (2, 13). Girard et al. (30) reported the possibility of classification of lung cancer by genome-wide allelotyping although their study only examined lung cancer cell lines and could not discriminate between copy number gain and loss. In our study, we analyzed primary lung adenocarcinoma and used unsupervised hierarchical cluster analysis to identify three groups of lung adenocarcinoma based on their distinct genetic changes. Among them, two subclasses (clusters A and C, 20 of

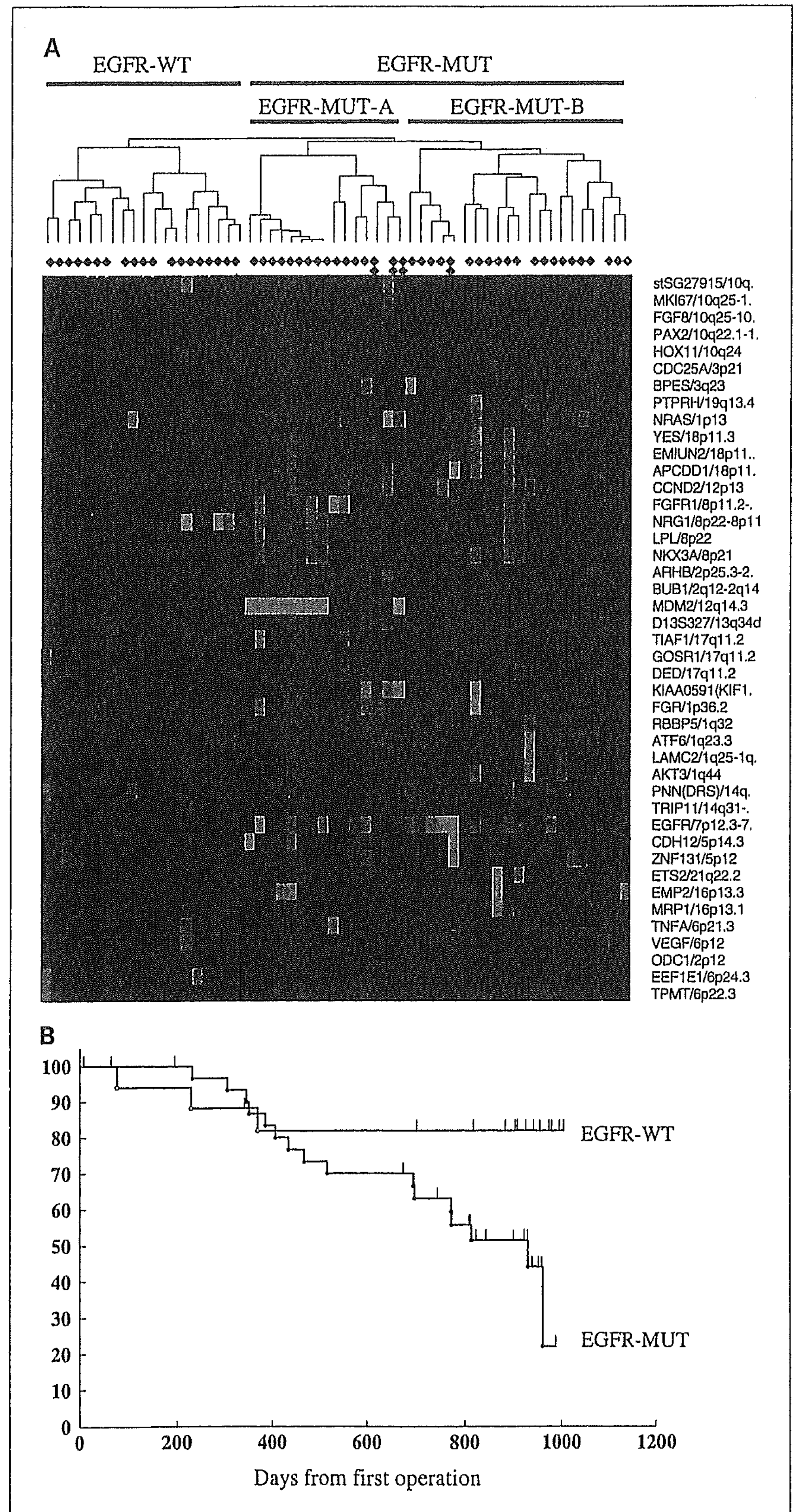

Fig. 2. Supervised genetic profiling of *EGFR* mutation – related loci. *A*, Hierarchical clustering determined copy number change patterns against 46 loci that were identified using the training testing, cross-validation analysis. Lost (*green*) or gained (*red*) loci were indicated in individual tumors. Tumors are classified into two branches (EGFR-WT and EGFR-MUT). All *EGFR* mutated tumors (*red spot*) are clustered in the EGFR-MUT branch. Most tumors without *EGFR* mutations (*blue spot*) are clustered with the EGFR-WT branch, although some are clustered with the EGFR-MUT branch. Six tumors carrying K-*ras* mutations (*yellow spot*) are clustered with the EGFR-WT branch and four MET-amplified tumors (*green spot*) are clustered with the EGFR-MUT branch. The EGFR-MUT branch is subdivided into two subgroups (EGFR-MUT-A and EGFR-MUT-B) with distinctive genetic changes. *B*, genetic profiles and patient disease-free survival. Relationship between patients' disease-free survival and genetic classification based on the *EGFR* mutation – related loci. EGFR-WT and EGFR-MUT groups were significantly different ($P = 0.01$).

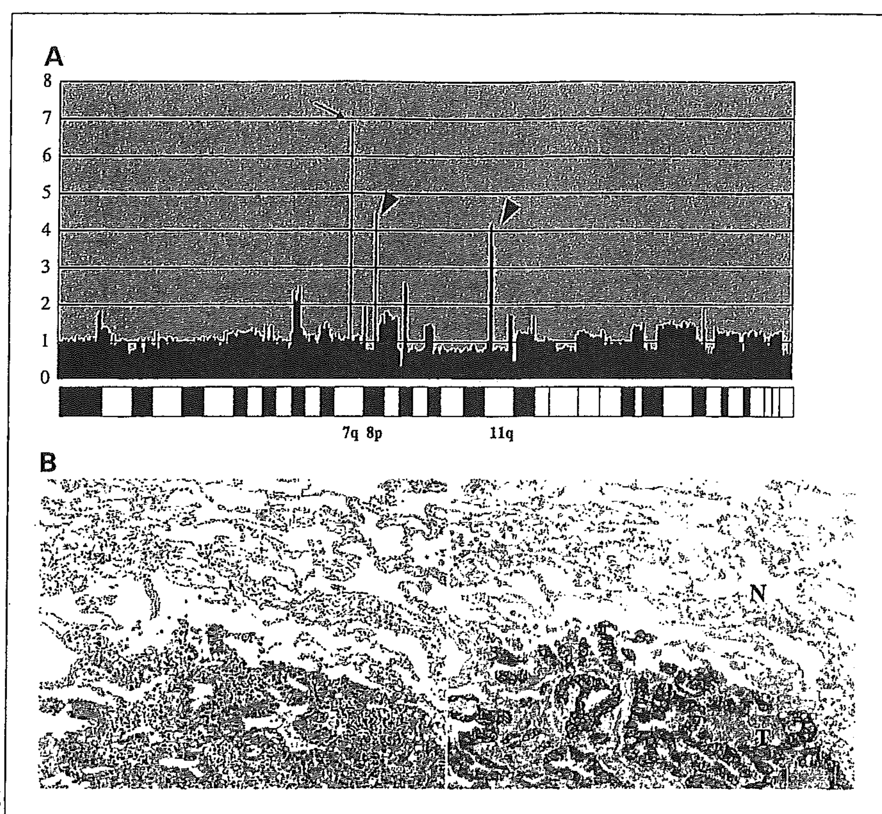

Fig. 3. Amplification and overexpression of MET in lung adenocarcinoma. *A*, a chromosomal copy number alteration profile of a lung adenocarcinoma with an amplification of *MET* locus. Each signal ratio (tumor/normal) of 800 examined loci was plotted from chromosome 1p (*left*) to Y (*right*). Each chromosome is represented by underlining boxes as in Fig. 1A. Chromosomal arms (7q, 8p, and 11q) with high-level amplifications were indicated. More than 6-fold amplification of a bacterial artificial chromosome containing the *MET* gene (*7q21*) was detected (*arrow*). This tumor also showed amplifications on 8p and 11q (*arrowheads*). *B*, immunohistochemical analysis of MET protein expression in the same case (×200). Four-micrometer sections of formalin-fixed, paraffin-embedded specimens were stained with H&E (*left*) and an anti-MET monoclonal antibody (*right*). Overexpression of the MET protein in tumor cells (*T*) was observed compared with the surrounding noncancerous lung tissue (*N*). All MET-amplified tumors exhibited prominent membranous and cytoplasmic expression. *Bar*, 200 μm.

55 cases and 36.3% of total cases, respectively) shared many genetic alterations but also had changes unique to each other. This implied that they may be derived from a common precursor and diverge via the acquisition of specific genetic alterations during tumor development. In contrast, the third subclass (cluster B, 15 of 55 cases, 27.3%) showed characteristically fewer genetic alterations than the other two. Although one would expect this group to consist of tumors of an earlier stage, it contained tumors that varied clinically (from stage I to stage III), and there was no significant correlation of histopathologic features with the above classification. Interestingly, this clustering classification is significantly associated with smoking habits, suggesting that the specific carcinogen exposure may affect overall genetic profile of lung cancer. We propose three possibilities for the carcinogenesis process in the third group; these tumors may predominantly acquire (*a*) genetic alterations not covered by our arrays, although they contain most of the known cancer-related genes; (*b*) genetic alterations that do not involve copy number changes, such as balanced chromosomal translocations or microsatellite instability (37, 38); or (*c*) epigenetic alterations such as aberrant methylation of gene promoters, which have been reported to associate with smoking history (39). Further analysis of this group, focusing on the above mechanisms, will provide a more complete view of lung carcinogenesis.

Because the *EGFR* gene is frequently altered in lung adenocarcinoma and its mutation status is correlated to the sensitivity to the specific inhibitor, Gefitinib (8-10), we assumed that the *EGFR* pathway plays important roles in lung cancer and examined whether *EGFR* mutated tumors have any genetic characteristics in nature. We detected the *EGFR* gene mutations at similar frequency as reported (31, 32, 40) and the presence of somatic mutations was significantly associated with never-smoking history as previous studies reported (8-10, 31, 32, 40). We detected K-*ras* mutations relatively less frequent than previously reported (41) but comparatively to other study (42) probably because our analyzed cases contained more female and nonsmokers. We found that *EGFR* mutation and K-*ras* mutation were mutually exclusive as reported (31, 32, 40) and this finding is consistent with the notion that activation of both *EGFR* and K-*ras* stimulates the same downstream pathway (43).

We identified 58 loci whose alterations significantly correlated with the presence of *EGFR* mutations. It is interesting to note that amplification of the *EGFR* gene itself is significantly observed in *EGFR* mutated tumors, indicating that both somatic mutation and amplification of the *EGFR* gene simultaneously occur in part of lung adenocarcinoma. Using these selected loci, we classified the tumors by supervised hierarchical clustering. This classification revealed two groups: one containing only *EGFR* wild-type tumors (EGFR-WT) and the other (EGFR-MUT) containing all *EGFR* mutated and some *EGFR* wild-type tumors. Because the *EGFR* wild-type tumors that were grouped with the EGFR-MUT group shared similar genetic alterations with the *EGFR* mutated tumors, we hypothesized that they may have unknown genetic alterations complementary to *EGFR* activation and subsequently examined loci containing oncogenic receptor-type tyrosine kinases in our arrays. We found that a locus (*7q21*) containing the *MET* gene was amplified in part of these *EGFR* wild-type tumors and immunohistochemically validated overexpression of MET protein in these tumors. MET was shown to be implicated in *ras*-mediated tumorigenicity (44, 45) and activated in many tumors (34-36). Although the number of cases with MET amplification is small in this study, it is tempting to speculate that amplification of the *MET* gene may play a role similar to *EGFR* mutation in lung adenocarcinoma. Recently, somatic alterations of the *MET* gene were detected in lung cancer and pharmacologic inhibitors specific to the MET kinase have been

reported (46 – 48). Our results also support the idea that the MET oncoprotein is a potent new candidate for therapeutic target in lung adenocarcinoma although there was no somatic mutation in the analyzed exons of our cases. In our cases, there are seven tumors without either *EGFR* mutations or *MET* amplification in the EGFR-MUT group. Somatic mutations in the kinase domain of *ERBB2* were reported in *EGFR* wild-type lung adenocarcinomas (7, 33). Therefore, we searched for *ERBB2* mutations in all 55 cases and found no somatic mutations, suggesting that other oncogenic kinases might be involved in these tumors.

EGFR mutation status could not predict tumor recurrence, which is consistent with a previous report on the insignificant relationship between *EGFR* mutation and patient prognosis (40). However, we found that EGFR-MUT group, which is revealed by genetic classification, showed significantly shorter disease-free survival than EGFR-WT group. Our results imply the possibility that specific combinations of genetic alterations (genetic code) selected by genome-wide analysis could evaluate tumor characteristics and estimation of such codes would be applicable for diagnostic purposes. Our classification also revealed that there are two genetically distinctive subgroups in the *EGFR* mutated lung adenocarcinoma, which were associated with tumor histologic differentiation. Because Gefitinib is one of the most promising molecular target drugs against lung cancer and molecular mechanisms determining its efficacy are still unclear (49), further analysis of a larger cohort is warranted to determine any possible relationship of genetic profiling with sensitivity to chemotherapeutic agents, including tyrosine kinase inhibitors.

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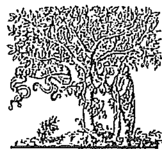
Lung Cancer (2005) 50, 1–8



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Mutations of the epidermal growth factor receptor gene in atypical adenomatous hyperplasia and bronchioloalveolar carcinoma of the lung

Yukihiro Yoshida[a], Tatsuhiro Shibata[a], Akiko Kokubu[a], Koji Tsuta[a], Yoshihiro Matsuno[b], Yae Kanai[a], Hisao Asamura[c], Ryosuke Tsuchiya[c], Setsuo Hirohashi[a,*]

[a] *Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[b] *Diagnostic Pathology Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*
[c] *Thoracic Surgery Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan*

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K-ras

Summary A hypothesis of multistep carcinogenesis of lung adenocarcinoma from atypical adenomatous hyperplasia (AAH) to invasive adenocarcinoma through bronchioloalveolar carcinoma (BAC) has been proposed. However, the genetic alterations that play a role during these processes are not yet clear. Recently, somatic mutations of the epidermal growth factor receptor (EGFR) gene were found in lung adenocarcinoma. We examined the status of EGFR mutations in AAH and BAC to elucidate the role they play during multistage of lung adenocarcinoma. We found somatic EGFR mutations in 3% (1/35) of AAH, 10.8% (4/37) of BAC and 41.9% (13/31) of invasive adenocarcinoma. Sixteen of 18 EGFR mutations were found in exon 19 and two were in exon 21. Among the 16 EGFR mutations in exon 19, 13 were deletions of 15 bp and one was an insertion/duplication of 18 bp. Mutations of the K-ras gene were detected in 26.7% (8/30) of AAH, 16.7% (5/30) of BAC and 10% (3/30) of invasive adenocarcinoma. None of the tumors with EGFR mutations had K-ras mutation simultaneously. Patients who had invasive adenocarcinoma with EGFR mutations were younger than those without mutations (60.6 versus 67.4 years, $p = 0.03$). These results suggest that tumors with EGFR mutations may progress more rapidly and develop into invasive cancer faster than those without mutations. Alternatively it is also possible that some invasive adenocarcinomas with EGFR mutations may not follow the AAH-adenocarcinoma sequence. We analyzed 24 patients with multiple lung lesions and

* Corresponding author. Tel.: +81 3 3547 5201x4101; fax: +81 3 3248 2463.
E-mail address: shirohas@ncc.go.jp (S. Hirohashi).

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13 patients had at least one lesion that had either an EGFR or K-ras mutation. In all cases each lesion had a different mutation status. This finding suggests that the genetic alterations responsible for the development of lung adenocarcinoma occur randomly even under exposure to the same carcinogen.
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1. Introduction

Lung cancer is one of the leading causes of cancer deaths worldwide [1]. About 80% of lung cancers are non-small cell lung cancer (NSCLC) and the frequency of adenocarcinoma has recently overtaken that of squamous cell carcinoma in many countries [1,2]. As a result of improved computed tomography (CT) images with higher resolution and the prevalence of CT screening for lung cancer detection, small and early adenocarcinomas in the peripheral lung are found more frequently than in the past [3,4].

A hypothesis has been proposed of multistep carcinogenesis in which lung adenocarcinoma develops from atypical adenomatous hyperplasia (AAH) to invasive adenocarcinoma through bronchioloalveolar carcinoma (BAC) [5]. In this context, AAH is considered to be a precursor lesion of lung adenocarcinoma [6]. The WHO classification scheme revised in 1999 defines BAC to be non-invasive [6]. Patients with BAC tend to be females and those who have never smoked previously [7–9].

Gefitinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor and radiographic regressions of tumors were observed in 10–19% of chemo-refractory NSCLC patients in clinical trials [10,11]. Such responses were frequent among Japanese, females, those who had never smoked and tumors with an adenocarcinoma histology [10–12]. Recently, three groups reported somatic EGFR mutations within exons 18, 19 and 21 encoding a tyrosine kinase domain in lung adenocarcinoma which subsequently responded to gefitinib [13–15].

Since the clinicopathological characteristics of BAC and lung adenocarcinoma that respond positively to gefitinib are similar in terms of the prevalence of females and patients who have never smoked before [10–12,15], it is possible that EGFR mutations may occur in BAC that eventually progresses to invasive adenocarcinoma. Recent large-scale analyses demonstrated that EGFR mutations were frequent in females, those who had never smoked and lung adenocarcinoma with BAC features and support our hypothesis [16–18]. Since these mutations may be an early event in lung adenocarcinoma development, we analyzed EGFR mutations in both AAH and BAC to elucidate the role they play in the multistep carcinogenesis of lung adenocarcinoma.

2. Materials and methods

2.1. Patients

A total of 134 lesions surgically resected from 74 patients (58 AAH, 43 BAC and 33 invasive adenocarcinoma) during the period from June 1999 to February 2004 were studied. This study was approved by the institutional review board of the National Cancer Center, Japan. All cases of BAC in this study were nonmucinous subtype. Fifteen patients had synchronous multiple primary lung cancers (invasive adenocarcinoma or BAC). We used the criteria of Martini and Melamed for the diagnosis of multiple primary lung cancer [19]. Forty cases of AAH were concomitant with invasive adenocarcinoma and nine were concomitant with BAC. Pathological diagnosis was made as a consensus of at least two staff pathologists including two of us (KT and YM) in accordance with the WHO classification as revised in 1999 [6]. BAC was defined as "an adenocarcinoma with a pure bronchioloalveolar growth pattern and no evidence of stromal, vascular or pleural invasion." Twenty-three of the 58 AAH cases, six of the 43 BAC cases and two of the 33 invasive adenocarcinoma cases were excluded because we could not obtain enough PCR products for subsequent direct sequence in at least one exon. Thus, the EGFR mutation status of 103 lesions in 69 patients was analyzed in exons 18, 19 and 21. Clinicopathological information was obtained by reviewing the medical chart. We defined "never-smokers" as patients who had never smoked in their lives and "former-smokers" as those who had quit smoking at least one year before the operation.

2.2. DNA extraction

Surgically resected specimens were fixed in formalin and embedded in paraffin. All sections were

cut from paraffin blocks at a thickness of 5 μm, deparaffinized with xylene and graded alcohols and stained with hematoxylin and eosin. Tumor cells were microdissected using a Pixcell laser-capture microscope (LCM) with an infrared diode laser (Arcturus Engineering, Santa Clara, CA). Samples were incubated in 200 μl of lysis buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA, 0.5% SDS) with 40 μg of glycogen and 2 μl of proteinase K (20 mg/ml) and incubated at 56 °C overnight. DNA was purified by phenol and chloroform extraction and dissolved in 20 μl TE (10 mM Tris-HCl pH8.0, 1 mM EDTA).

2.3. PCR protocol

Each PCR reaction contained 4 μl of DNA, 2 μl of buffer, 4 μl of dNTP (2.0 mM each), 2 μl of dimethylsulfoxide (DMSO), 0.2 μl of forward and reverse primers (100 pM), and 0.2 μl of DNA Taq polymerase (Expand Hi-Fidelity PCR system, Roche Diagnostics) in a reaction volume of 20 μl. Primers used to amplify each exon of the EGFR and K-ras genes were as follows: Exon 18 of EGFR, 5′GAGGTGACCCTTGTCTCTGTGTTC3′ (sense) and 5′GGCTCCCCACCAGACCATGAGAGG3′ (antisense); Exon 19 of EGFR, 5′GGCACCATCTCACAATTGCCAGTT3′ (sense) and 5′AGGTGGGCCTGAGGTTCAGAGCC3′ (antisense); Exon 21 of EGFR, 5′ACTACTTGGAGGACCGTCGCT3′ (sense) and 5′GTGTCAGGAAAATGCTGGCTG3′ (antisense); Exon 2 of K-ras, 5′GGCCTGCTGAAAATGACTGA3′ (sense) and 5′GTCCTGCACCAGTAATATGC3′ (antisense). PCR cycling parameters were one cycle of 96 °C for 1 min, 50 cycles of 94 °C for 30 s, 56 °C for 30 s and 68 °C for 2.5 min and finally one cycle of 72 °C for 5 min.

The resulting PCR products were electrophoresed on 2% agarose gel and bands of the expected molecular weight were excised. The DNA was extracted using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then directly sequenced using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. PCR products that showed mutations were subcloned in TA-vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) and their sequences were determined as above with M13 primers. EGFR mutations were found only within exon 18 through 21 of the kinase domain in the mutational analysis of the entire coding sequence so far [13,14]. We analyzed only three exons in this study because the amount of DNA extracted from AAH was small and most mutations were clustered in exon 18, 19 and 21 [16].

2.4. Statistics

Associations between clinical factors and EGFR mutations were evaluated by the chi-square test. Mean age differences between two groups were assessed with the t-test. All statistical analyses were performed using Statview 4.5 (SAS Institute, Cary, NC). A p value of less than 0.05 was considered to be statistically significant.

3. Results

The clinicopathological characteristics of 103 lesions in 69 patients are listed in Table 1. There were 35 cases of AAH, 37 cases of BAC and 31 cases of invasive adenocarcinoma. Female patients and never-smokers were predominant in this cohort. Twelve patients had synchronous multiple primary lung cancers (invasive adenocarcinoma or BAC). Twenty-one cases of AAH were concomitant with invasive adenocarcinoma and 6 were concomitant with BAC. EGFR mutation status is shown in Table 2. One sample of AAH (3%) (Fig. 1), 4 cases of BAC (10.8%) and 13 cases of invasive adenocarcinoma (41.9%) harbored EGFR mutations. All mutations were somatic. Sixteen of 18 EGFR mutations were found in exon 19 and 2 were in exon 21. Among the 16 EGFR mutations in exon 19, 13 were deletions of 15 bp and one was an insertion/duplication of 18 bp (Fig. 2). In the 31 cases of invasive adenocarcinoma, EGFR mutations were not correlated with gender or smoking history (current- and former-smoker versus never-smoker) (chi-square test, $p = 0.35$ and 0.21, respectively). The mean age of patients without EGFR mutations (67.4 years) was significantly greater than that of patients with EGFR mutations

Table 1 Clinicopathological characteristics of 103 lesions in 69 patients

Parameters	Number of cases
Gender	
Male	20
Female	49
Age (years)	42–82 (mean 62.2)
Smoking history	
Current	9
Former	9
Never	51
Histology	
AAH	35
BAC	37
Invasive adenocarcinoma	31

Table 2 Clinicopathological characteristics of AAH, BAC and invasive adenocarcinoma with EGFR mutations

Age	Gender	Smoking history	Exon	Nucleotide	Amino acid
AAH (1/35, 3%)					
45	F	Never	19	2236_2250delGAATTAAGAGAAGCA	delE746_A750
BAC (4/37, 10.8%)					
71	F	Never	19	2240_2254delTAAGAGAAGCAACAT	delL747_T751
49	M	Current	19	2235_2249delGGAATTAAGAGAAGC	delE746_A750
49	F	Never	19	2240T>C and 2249_2263delCAACATCTCCGAAAG 2240T>C and 2250_2264delAACATCTCCGAAAGC[a]	L747S and delA750_K754
62	M	Former	19	2240_2257delTAAGAGAAGCAACATCTC	delL747_P753insS
Invasive adenocarcinoma (13/31, 41.9%)					
66	M	Never	19	2236_2250delGAATTAAGAGAAGCA	delE746_A750
51	F	Never	21	2573T>G	L858R
61	F	Never	19	2235_2249delGGAATTAAGAGAAGC	delE746_A750
65	F	Former	19	2236_2250delGAATTAAGAGAAGCA	delE746_A750
75	F	Never	21	2573T>G	L858R
64	F	Never	19	2235_2249delGGAATTAAGAGAAGC	delE746_A750
59	F	Never	19	2235_2249delGGAATTAAGAGAAGC	delE746_A750
47	M	Never	19	2235_2249delGGAATTAAGAGAAGC	delE746_A750
55	F	Current	19	2219_2236ins/dupTTCCCGTCGCTATCAAGG	ins745VPVAIK
70	F	Never	19	2240_2254delTAAGAGAAGCAACAT	delL747_T751
54	F	Never	19	2236_2250delGAATTAAGAGAAGCA	delE746_A750
68	M	Former	19	2236_2250delGAATTAAGAGAAGCA	delE746_A750
53	F	Never	19	2236_2250delGAATTAAGAGAAGCA	delE746_A750

[a] Substitution and 15 bp deletion, two patterns can be possible.

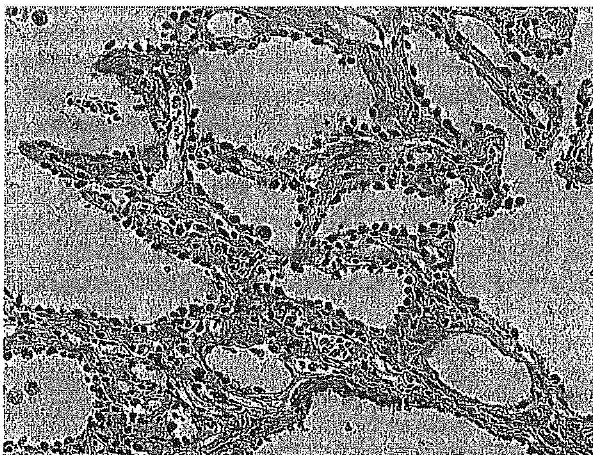

Fig. 1 A case of AAH showing an EGFR mutation (hematoxylin and eosin staining, original magnification: ×200).

(60.6 years) (t-test, $p=0.03$). When we exclusively analyzed patients with pathological stage I in each group, the mean age of patients without EGFR mutations was 66.7 years ($n=11$) and that of patients with EGFR mutations was 59.9 years ($n=10$), but this difference was not statistically significant (t-test, $p=0.1$). The mean age of patients who had invasive adenocarcinoma with K-ras mutation was 64.3 years, while that of patients who had invasive adenocarcinoma without K-ras mutation was 64.7 years. There was not significant difference in age between mutation status of K-ras gene.

Mutations of the K-ras gene at codons 12 and 13 were found in eight of 30 AAH cases (26.7%), five of 30 BAC cases (16.7%) and three of 30 invasive adenocarcinoma cases (10%) (Table 3). None of the tumors with K-ras mutations had EGFR mutations. Of the 16 cases with K-ras mutations, 12 harbored a transversion that changed G to T at codon 12 and 2 showed a transition that changed G to A at

Table 3 Frequencies (%) of EGFR and K-ras mutations in AAH, BAC and invasive adenocarcinoma

Genes	Lesions		
	AAH	BAC	Invasive adenocarcinoma
K-ras	8/30 (26.7%)	5/30 (16.7%)	3/30 (10%)
EGFR	1/35 (3%)	4/37 (10.8%)	13/31 (41.9%)

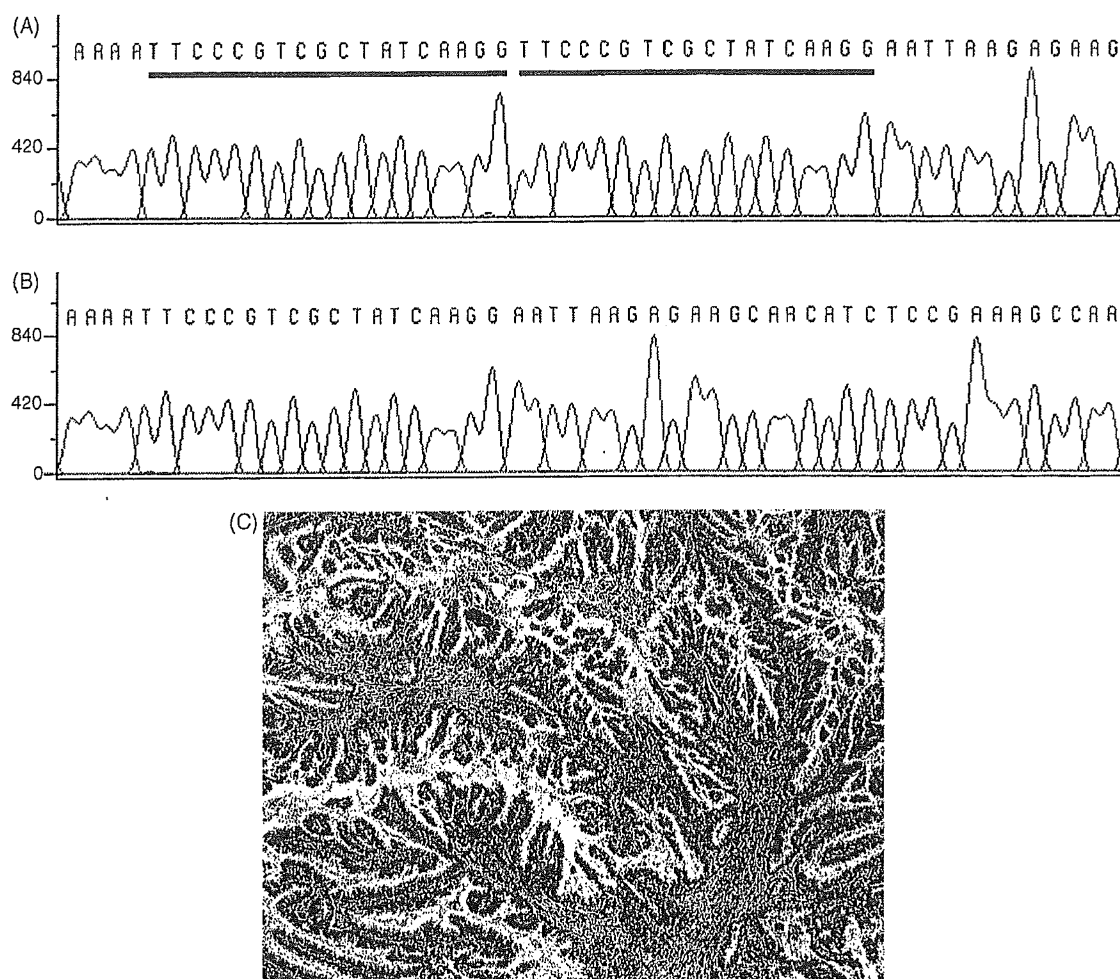

Fig. 2 An 18-nucleotide insertion (underlined) was observed in exon 19 of the EGFR gene from an adenocarcinoma. (A) Sequence analysis of the subcloned mutant allele of the tumor revealed an insertion. (B) Sequence analysis of the wild allele of the same tumor. (C) The tumor showed a complex papillary proliferation (original magnification: ×100).

codon 12. One invasive adenocarcinoma and one AAH harbored heterogeneous K-ras mutations that showed both transversion and transition in a single tumor. With regard to smoking status of patients whose lesion harbored K-ras mutations, 10 cases were never-smokers, four were former-smokers and two were current-smokers.

Twenty-four patients had multiple synchronous lesions that could be evaluated in this study. Mutation status in synchronous multiple lung lesions is shown in Table 4. Thirteen patients had at least one lesion that had either an EGFR or K-ras mutation. In each patient, each lesion had a different mutation status. Among them, four patients had invasive adenocarcinoma with EGFR mutation and AAH with K-ras mutation. Five patients had invasive adenocarcinoma with EGFR mutation, while neither EGFR nor K-ras mutations were found in AAH. The tumors in the 11 patients with synchronous multiple lesions had neither EGFR nor K-ras mutations.

4. Discussion

AAH is predominantly found in patients with adenocarcinoma, and WHO classification proposes AAH as a precursor lesion of lung adenocarcinoma [5,6]. Although many studies have demonstrated that AAH has certain characteristics, genetic alterations in AAH are still poorly understood [5,20]. The WHO classification defines BAC as adenocarcinoma showing bronchioloalveolar extension without invasion [6]. Due to the recent prevalence of CT screening in Japan, this type of tumor is found more commonly than in the past. The clinicopathological characteristics of BAC include its good prognosis and the prevalence of females and patients who have never smoked before [7—9]. In analogy with ''adenoma-carcinoma sequence'' proposed in colon cancer development, a hypothesis of multistep carcinogenesis has been proposed where lung adenocarcinoma develops

Table 4 K-ras and EGFR mutation status in patients with multiple lung lesions

Case	Lesions		
	AAH	BAC	Invasive adenocarcinoma
Case 1	◍		●
Case 2	○		●
Case 3	◍		●
Case 4		○○	◍
Case 5	○		●
Case 6	◍		○
Case 7	◍		●
Case 8	○		●
Case 9	◍		●
Case 10	○		●
Case 11	○		●
Case 12[a]		○●[a]	
Case 13		○○◍	

◍ K-ras mutation; ● EGFR mutation; ○ wild type of K-ras and EGFR.

[a] Analysis of K-ras mutation in a BAC in this case was not done. Tumors in 11 patients with synchronous multiple lesions had neither EGFR nor K-ras mutation.

from AAH to invasive adenocarcinoma through BAC [5].

Recently, EGFR mutations in lung adenocarcinoma have been reported to be frequently found in Japanese, females, and patients who have never smoked before [16–18]. Therefore, we speculated that EGFR mutations occur in early lesions such as BAC or AAH which eventually progress to invasive adenocarcinoma. If this hypothesis is correct, significant proportions of AAH and BAC should have EGFR mutations as invasive adenocarcinoma. EGFR mutations in BAC have been reported in three studies and the result was 3/5, 0/8 and 0/3, although the number of analyzed cases is small [16,18,21]. And their frequency in AAH has not been reported so far. In the present study we found EGFR mutations in one of 35 AAH cases and four of 37 BAC cases.

Our results showed that while EGFR mutations occurred in the early stage of lung adenocarcinoma development, their frequencies were lower than those of invasive adenocarcinoma. There are at least three possible explanations for this result. First, the acquisition of EGFR mutations may also occur in the relatively late stage in multistep carcinogenesis of lung adenocarcinoma. Second, tumors with EGFR mutations may progress more rapidly and develop into invasive cancer faster than those without mutations (Fig. 3). Third, some invasive adenocarcinomas with EGFR mutations may not follow the AAH-adenocarcinoma sequence. Among 31 cases of invasive adenocarcinoma, patients with EGFR-mutated invasive adenocarcinoma were significantly younger than those without EGFR mutations. This finding may support the second hypothesis, although these hypotheses are not mutually exclusive. However, if we only consider patients with pathological stage I ($n = 21$), there was no significant difference between the two groups ($p = 0.1$), although patients with EGFR mutations

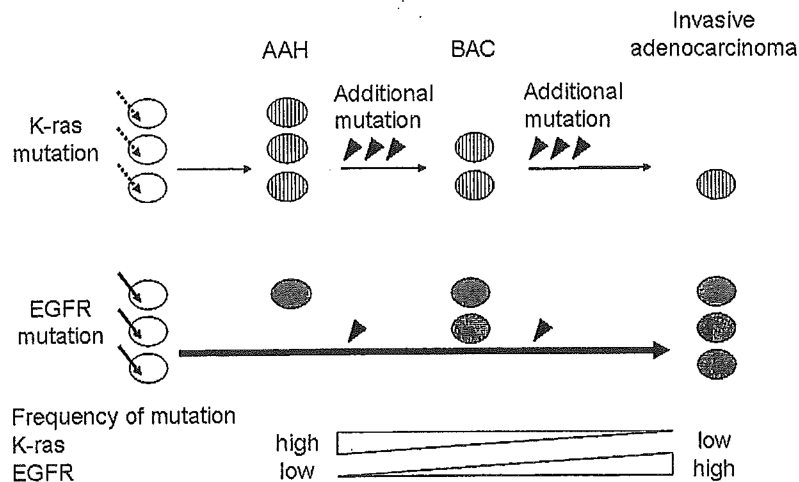

Fig. 3 A scheme of the distinct pathways followed by tumors with K-ras and EGFR mutations in the AAH-adenocarcinoma sequence. K-ras mutation (shown as dotted arrow) and EGFR mutation (shown as solid arrow) can occur in the early stage of this process. Tumor cells that acquire either K-ras (shown by oval with vertical line) or EGFR mutation (shown by oval with mesh) may develop into invasive adenocarcinoma. But tumors with EGFR mutation (lower pathway) could develop into invasive adenocarcinoma with fewer genetic alterations (shown as arrowhead) and consequently shorter time than tumors with K-ras mutation (upper pathway). Existence of two mutually exclusive pathways may result in the different frequencies of the two oncogenic mutations during lung adenocarcinoma development.

were 6.8 years younger than patients without mutations. Therefore, further analysis of a larger number of cases will be necessary to prove the second hypothesis.

Recently Marchetti et al. reported the superiority of SSCP (single-strand conformation polymorphism) analysis in the detection of mutations (especially point mutations in exon 21) compared to direct-sequencing and that 21% of mutations were reported to be missed by direct-sequencing [17]. In our study, we found two cases of point mutations in exon 21 out of 31 invasive adenocarcinoma and its frequency seems to be lower than other reports, even though the total frequency of EGFR mutations in invasive adenocarcinoma (13/31cases, 41.9%) was in the range of previous results from other groups (44-49%) [16,18]. Therefore it is possible that the quality and quantity of DNA extracted from formalin-fixed samples might give false negative results in the analysis of exon 21.

K-ras mutations are the most extensively investigated genetic alterations in AAH and previous reports have demonstrated that K-ras mutations are found in 15—50% of AAH [5,22—25]. In our study, the frequency of K-ras mutations was 26.7%, while that of K-ras mutations in BAC and invasive adenocarcinoma was 16.7 and 10%, respectively. Our results confirmed other studies which found that K-ras mutation is an early event in the multistep carcinogenesis of lung adenocarcinoma [5,22—25]. K-ras mutations in lung adenocarcinoma have been reported to be frequently observed in smokers and mucinous-type histology [17,26—29]. However, in our study, 10 of 16 cases of K-ras mutations were found in patients who had never smoked. Similar results were recently reported by other groups and the relationship between smoking habit and K-ras mutation will need to be examined further [30,31].

In our study, none of the tumors with EGFR mutations also had K-ras mutations as the past reports [16—18]. More recent reports demonstrated that HER-2 mutations never presented together with mutations of EGFR, K-ras, N-ras, or B-raf genes [32,33]. Thus, these oncogenic mutations may be exclusive of each other and may play a similar role in the development of lung adenocarcinoma. Consistent with this notion, a recent study demonstrated that K-ras mutation activated the Akt signal pathway which was also activated in cell lines with EGFR mutations [34,35].

The close relationship between AAH and adenocarcinoma suggests that exposure to some carcinogen may initiate genetic alterations to cause AAH, which subsequently progresses to adenocarcinoma. In our study, out of 35 AAH cases, 21 were concomitant with invasive adenocarcinoma and six were concomitant with BAC. We analyzed 24 patients with multiple lung lesions and found that in all cases each lesion had a different mutation status. This finding suggests that the genetic alterations responsible for the development of lung adenocarcinoma occur randomly even under exposure to the same carcinogen. Interestingly, four patients had invasive adenocarcinoma with EGFR mutation and AAH with K-ras mutation. This result also supports the above hypothesis that tumor cells with EGFR mutations may progress rapidly to invasive adenocarcinoma, while tumor cells with K-ras mutation remain indolent until other genetic alterations accumulate (Fig. 3).

5. Conclusions

We found that EGFR mutations in AAH and BAC were less frequent compared to those detected in invasive adenocarcinoma. None of the tumors with EGFR mutations had K-ras mutation simultaneously. Oncogenic activation of EGFR may also contribute to the early step of lung adenocarcinoma development as K-ras does so.

Acknowledgement

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Adenocarcinoma of the thymus: mucinous subtype

Fumiyuki Takahashi MD[a,b,*,†], Koji Tsuta MD[e,†], Yoshihiro Matsuno MD[e], Kazuhisa Takahashi MD[a,b], Michie Toba MD[a,b], Koichi Sato MD[a,b], Toshimasa Uekusa MD[f], Hiroshi Izumi MD[c], Kazuhiro Nakamura MD[d], Sachiko Hirose MD[d], Yoshinosuke Fukuchi MD[a,b]

[a]*Department of Respiratory Medicine, Juntendo University, School of Medicine, Tokyo 113-8421, Japan*
[b]*Research Institute for Disease of Old Ages, Juntendo University, School of Medicine, Tokyo 113-8421, Japan*
[c]*Department of Pathology I, Juntendo University, School of Medicine, Tokyo 113-8421, Japan*
[d]*Department of Pathology II, Juntendo University, School of Medicine, Tokyo 113-8421, Japan*
[e]*Division of Clinical Laboratory, National Cancer Center Hospital, Pathology Division, National Cancer Center Research Institute, National Cancer Center Hospital, Tokyo 104-0045, Japan*
[f]*Department of Laboratory, Kanto Rousai Hospital, Nakahara-Ku, Kawasaki-Shi, Kanagawa 211-0021, Japan*

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Summary Primary thymic adenocarcinoma, mucinous subtype, is extremely rare with only one case reported to date. We describe herein a case of thymic mucinous adenocarcinoma. A 59-year-old man was identified to have an anterior mediastinal tumor and was diagnosed as mucinous adenocarcinoma. Clinical and radiographic examinations disclosed no evidence of tumor elsewhere. The patient received radiotherapy, but the general condition deteriorated and died 11 months after tumor detection. Thoracic autopsy revealed an anterior mediastinal tumor measuring greater than 10 cm, uncapsulated, and white. The tumor had clear margins and was clearly isolated from the lung. Histologically, the tumor demonstrated papillary, acinar, and cribriform structure and produced abundant extracellular mucin. Immunohistochemically, most tumor cells were positive for cytokeratin 7, were partially positive for CD5, and were negative for TTF-1, Sp-A, CDX-2, MUC2, napsin A, and cytokeratin 20. Collectively, the diagnosis of the tumor was primary mucinous adenocarcinoma of the thymus. We propose that the mucinous subtype should be recognized as one of the histopathological entities of thymic adenocarcinoma.
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1. Introduction

Thymic carcinomas of various histological types have been reported [1]. Among them, primary adenocarcinoma of the thymus is rare with only 9 cases having been reported [2-4]. Papillary carcinoma is the only histological subtype described in detail among the primary thymic adenocarcinoma [3]. In contrast, only one case of mucinous subtype has

* Corresponding author. Department of Respiratory Medicine, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-Ku, Tokyo 113-8421, Japan.
E-mail address: fumiyuki@med.juntendo.ac.jp (F. Takahashi).
† The first 2 authors contributed equally to this study.

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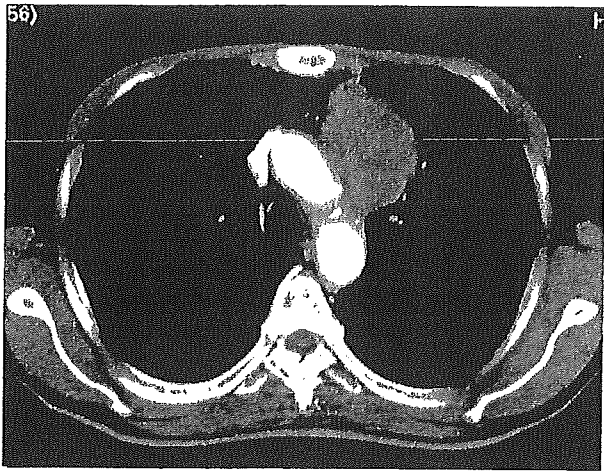

Fig. 1 Computed tomography of the chest revealing a localized mass in the anterior mediastinum.

been reported [4]. In this paper, we describe a case of thymic adenocarcinoma with presence of abundant mucin. This is the second documented case of the mucinous subtype for primary thymic adenocarcinoma. We conducted immunohistochemistry with various antibodies and discussed the histopathological findings.

2. Case report

A 59-year-old male patient was admitted to Juntendo University Hospital. His medical history was unremarkable except for gallbladder stone. Chest radiograph and computed tomography revealed a localized mass in the anterior mediastinum (Fig. 1). There were no tumorous lesions identified in both lung fields. The serum levels of α-fetoprotein, β-human chorionic gonadotropin, and carcinoembryonic antigen were within normal limits. Comprehensive clinical and radiographic examinations disclosed no evidence of tumor elsewhere. Confirmatory diagnosis was not established because the patient denied further examinations.

Table 1 Antibodies used in the current study

Antibody	Clone	Dilution	Pretreatment	Source	Positivity
34βE12	34βE12	25	Pro K	DAKO	+
AFP	poly	5000	–	DAKO	–
Bcl-2	124	40	MW	DAKO	–
CA19-9	116-NS-19-9	50	–	DAKO	++
CA125	OC125	20	MW	DAKO	+++
Calretinin	poly	100	MW	Zymed, San Francisco, Calif	–
CD5	4C7	100	MW	Novocastra, Newcastle upon Tyne, UK	+
CD56	Lu-243	200	MW	Nihonkayaku, Tokyo, Japan	–
CD99	013	50	MW	Signet Laboratories, Dedham, Mass	– (inflammatory cells)
CDX-2	Cdx-2-88	200	MW	BioGenex, San Ramon, Calif	–
CEA	CEM010	200	–	Mochida, Tokyo, Japan	+
Chromogranin A	poly	500	MW	DAKO	–
CK5/6	D5/16B4	400	TRS	Novocastra	–
CK7	OV-TL12/30	50	MW	DAKO	+++
CK20	Ks20	25	MW	DAKO	–
c-kit	poly	50	MW	DAKO	–
EMA	E29	100	–	DAKO	+++
HCG-β	M94138	dil	MW	BioGenex	–
HER-2	CB11	dil	MW	BioGenex	–
Ki-67	MIB-1	100	MW	DAKO	30%
Napsin A	TMU-Ad02	400	–	IBL, Gunnma, Japan	–
MUC 2	Ccp58	100	MW	Novocastra	–
MUC 5 AC	CLH2	100	MW	Novocastra	+++
MUC6	CLH5	100	MW	Novocastra	+
M-GGMC-1	HIK1083	10	–	Kanto Kagaku, Tokyo, Japan	–
p53	DO-7	100	MW	DAKO	++
p63	7JUL	50	TRS	Novocastra	–
PLAP	poly	1000	–	DAKO	–
S-100	poly	200	–	DAKO	–
Sp-A	PE10	100	–	DAKO	–
Synaptophysin	poly	100	MW	DAKO	–
TdT	poly	100	MW	DAKO	– (inflammatory cells)
TTF-1	8G7G3/1	100	MW	DAKO	–
Vimentin	V9	200	MW	DAKO	–

AFP indicates α-fetoprotein; dil, diluted; Pro K, proteinase K; MW, microwave with citrate buffer; TRS, microwave with target retrieval solution.

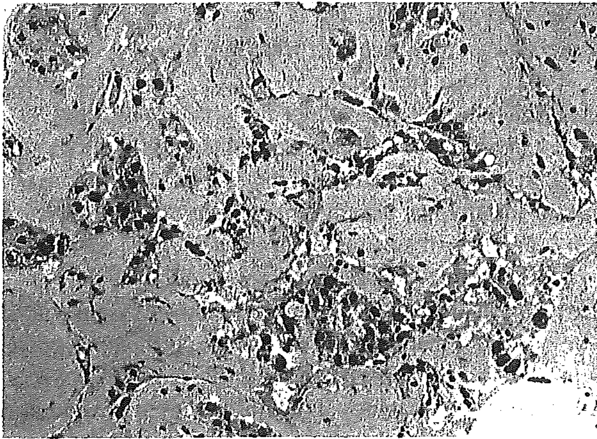

Fig. 2 Biopsy specimen before treatment showed that the tumor exhibited features of mucinous (colloid) carcinoma, being comprised of islands and strips of columnar cells floating in large pools of extracellular mucin (×20 HE). The tumor cells showed eosinophilic granular cytoplasm and partial intracellular mucin formation.

Nine months later, he was readmitted because of increase in anterior mediastinal mass size. The patient underwent echogram-guided biopsy. Histological findings demonstrated features of mucinous adenocarcinoma. His general condition was poor; therefore, only localized thoracic irradiation of 40 Gy was given. He died 2 months later, although minor response to the treatment was obtained. Consent for partial thoracic autopsy was obtained from his family.

3. Materials and methods

The tissue specimens were routinely fixed in 10% buffered formalin and then were embedded in paraffin. Consecutive 3-μm-thick sections were cut from a selected tissue block and stained with hematoxylin and eosin (HE), and alcian blue–periodic acid-Schiff. Immunohistochemical staining was performed with the LSAB (DAKO, Carpinteria, Calif) method, and the primary antibodies are listed in Table 1. The grading of the immunostaining was performed on a sliding scale of 1+ to 3+ according to the percentage of immunoreactive cells (negative, <1%; 1+, 1%-25%; 2+, 26%-50%; 3+, >51%).

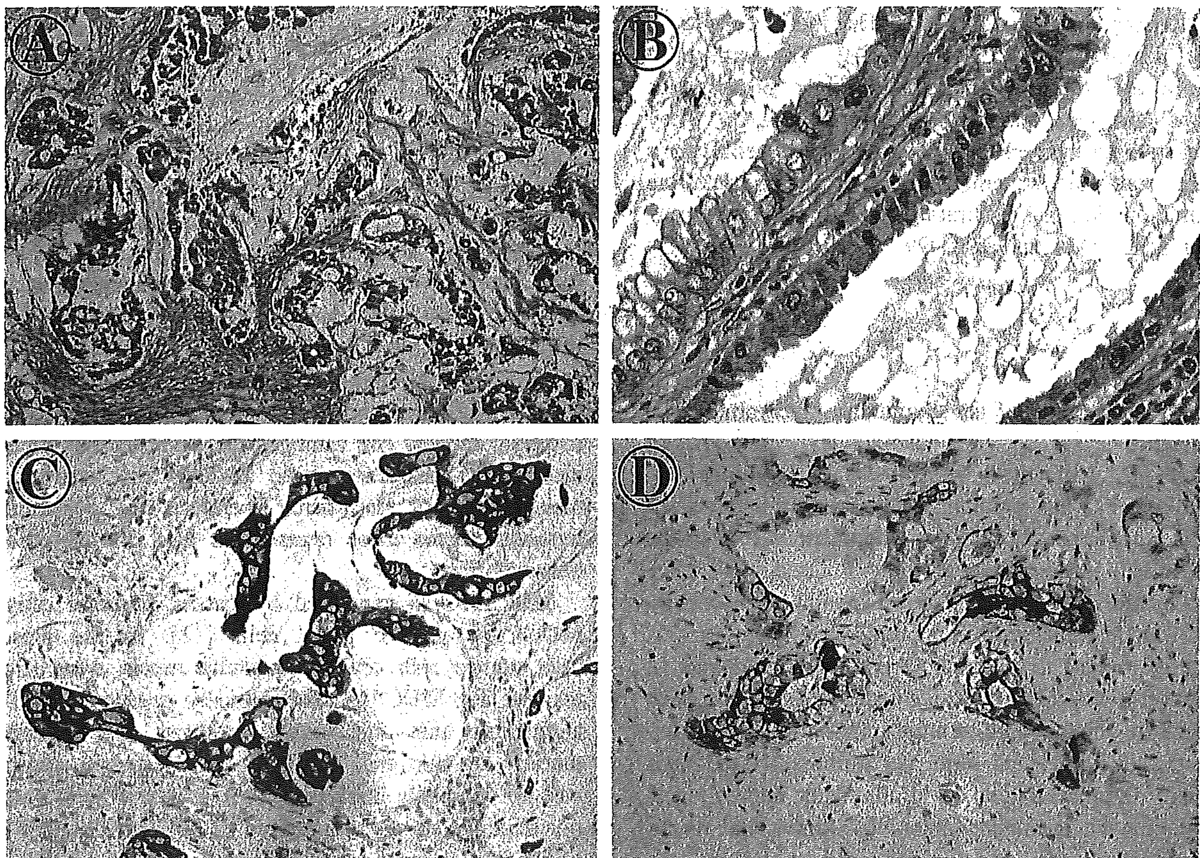

Fig. 3 The specimen obtained from autopsy. (A) The tumor cells produced abundant extracellular mucin (×10 HE). (B) The tumor epithelium was composed of tall columnar cells, with varying amounts of cytoplasmic mucin, and columnar cells with eosinophilic granular cytoplasm with apical snoutlike features (×40 HE). (C) Most tumor cells showed diffuse cytoplasmic staining for CK7 (×20). (D) Tumor cells partially showed cell membrane staining for CD5 (×20).