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Predictive advantage of a cell type classification for pulmonary adenocarcinoma coupled with data for *p53*, K-*ras* and *EGFR* alterations

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We analyzed relationships between histological subtypes of pulmonary adenocarcinomas and three gene alterations (*p53*, K-*ras*, and epidermal growth factor receptor gene), or thyroid transcription factor-1 (TTF-1) expression, and also studied prognoses by the subtypes, with or without combined multiple gene mutation status. Our purpose was to clearly determine pathogenesis, along with the best predictive value for biology and therapy-related traits. A total of 223 consecutively resected pulmonary adenocarcinomas were sub-classified using either the World Health Organization (WHO) or our five-cell type (FCT) classification system (hobnail, columnar/cuboidal, mixed, polygonal/oval, and goblet cell types). DNAs extracted from frozen samples of the adenocarcinomas were examined for gene alterations, and TTF-1 expressions were determined using immunohistochemistry. Next, relationships among the various data and clinicopathological factors were analyzed. The most striking result was: while almost 70% of adenocarcinomas were sub-classified as a mixed subtype by WHO, the FCT classified many of them as other cell subtypes. The FCT closely reflected differences in etiological factors, cellular lineages, and frequencies of gene mutations; and whether the data from combined gene mutations were used or not, differences among the cell types in postoperative survivals appeared. In contrast, subtypes of WHO did not show any association with the gene alteration or prognosis, and the FCT more suitably indicated sensitivity to gefitinib therapy than did WHO. The FCT combined with multiple gene mutation status appears to be useful in indicating pathogenesis and predicting the biological nature of pulmonary adenocarcinomas, and it could facilitate development of new therapies for each subtype. (*Cancer Sci* 2010; 101: 1745–1753)

Adenocarcinomas of the lung are the most common histological type in Japan, and show markedly different biological behavior from case to case.[1] Therefore, if we could predict the malignant potential of an adenocarcinoma and make a prognosis for chemo- or radiation-therapy from cytology, biopsy, and/or operation specimens, it would lead to better treatment options. To better satisfy predictive requirements, sub-classifications by gene expression profiling have been proposed.[2–5] However, emerging evidence showed that gene expression lists selected for these classifications vary considerably from study to study, making it difficult to reconcile findings or reach any definite conclusions.[6,7] Moreover, a recent paper suggested that an integrated approach using gene expression together with associated clinical, pathological, and other available information may be more promising for future work.[8] Thus, the importance of pathological data integration for prognoses has been established.

There is a high correlation between a gene expression profile and tumor histological phenotype. So we suspected that if we analyzed gene alterations by subtypes of histology, it would be possible to get more reliable data for predictive requirements. So far only a few reports have studied prognosis bases on gene mutations by the subtypes,[2,9] and there has been no study on prognosis and other predictive requirements combined with multiple gene mutations.

For histological sub-classification of adenocarcinomas, the 1999 World Health Organization (WHO) classification has been widely used.[10] However, since most cases are actually adenocarcinomas with mixed subtypes, this classification system cannot effectively predict malignant potential and prognosis. Only a few studies using modified WHO sub-classifications have reported correlation between prognosis and subtypes.[9,11]

In sub-classification of lung adenocarcinomas by gene expression profiling analysis, the importance of cellular lineage have been stressed.[12,13] Histologically, the cellular lineage can be determined by looking to the morphologic resemblance of tumor cells to epithelial cells in the pulmonary tissue. It was thus suspected that a sub-classification of adenocarcinomas based on such cytological features would better reflect the cellular lineage. Toward this end, we previously presented a system for sub-classification of adenocarcinomas referring to the cellular lineage based on resemblance to cells constituting the bronchial or bronchiolo-alveolar epithelium.[1]

The *p53*, K-*ras*, and epidermal growth factor receptor (*EGFR*) genes are thought to play important roles in the genesis and progression of lung cancers, and *EGFR* may be related to sensitivity to gefitinib therapy. Furthermore, mutation statuses of those three genes may not always be appropriately identified by expression profiling analysis.

We therefore examined not only relationships between histological subtypes of adenocarcinomas by WHO or our cell type classification system and those three gene alterations, but also the impact on prognosis by subtypes with or without combined multiple gene mutations: we were seeking the best predictive value for biological nature and therapy-related traits.

Materials and Methods

Tumor samples, clinicopathological data, and smoking history. We examined a large number, 223, of lung adenocarcinomas, of which 113 had been examined for *p53* mutation spectra previously.[1] None of the carcinomas were accompanied by

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other primary malignancies and all had been resected consecutively from 1989 to 1995 at the Cancer Institute Hospital, Tokyo, Japan. All patients had undergone operations as described previously.[1] None had received chemotherapy or radiotherapy before surgery, but 66 patients had postoperative chemo- and/or radio-therapy. Histopathological sub-classification of adenocarcinomas was done by three of the authors (E.T., Y.I., and A.O.) according to the 1999 WHO classification of lung tumors,[10] and our original five-cell type (FCT) sub-classification: (i) hobnail; (ii) columnar/cuboidal (col/cub); (iii) polygonal/oval (po/ov); (iv) goblet; and (v) mixed cell (Fig. 1), defined previously.[1] This classification was performed based on the predominant cell type occupying more than 70% of the area, except with the mixed type, for which the cut-off for each cell type was occupation of more than 30% of the area. Polygonal/oval (po/ov) cells were diagnosed only when the areas proliferating in sheets made up more than 95% of the tumor. In the cases classified by WHO, the existence of bronchioloalveolar (BA) spread was also determined.

Data for other clinicopathological parameters, pathological stages (p-stages) and the patient's smoking status are shown in Table 1. The p-stages were determined using the International Union Against Cancer TNM staging system.[14] A patient's smoking history was obtained as described previously.[1] All patients were followed up for more than 5 years. The study was approved by the institutional review board of the Cancer Institute Hospital and Kanagawa Cancer Center Research Institute.

DNA preparation and gene analyses. Genomic DNAs preparation, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), and sequencing for *p53* were performed as described previously.[1,15] The point to emphasize here is that samples which did not show *p53* mutation in our earlier study, as well as those collected after publication of the paper, amounting to one-half of all analyzed cases, were microdissected.

Only point mutations of codon 12 for the K-*ras* gene were analyzed, since more than 90% of K-*ras* gene mutations are reported to involve this codon.[16] The mutant-allele-specific amplification (MASA) method was used for samples documented in a previous paper[16] and for the remaining samples, almost half of all cases, the MASA method with nested-PCR was performed as described previously, with DNAs extracted from microdissected tissue.[17]

We analyzed the *EGFR* hotspot mutation L858R in exon 21 and in-frame deletions of exon 19 that account for approximately 91% of *EGFR* kinase domain mutations using the loop-hybrid mobility shift assay (LH-MSA) developed by Matsukuma *et al.*[18–20] (Fig. S1).

Immunohistochemical staining. Thyroid transcription factor-1 (TTF-1) expression is considered to be a lineage marker of small-sized bronchioles and pneumocytes (SBP), termed the terminal respiratory unit (TRU).[21] Therefore, we examined its expression by immunohistochemistry. Sections (4-μm thick) of formalin-fixed, paraffin-embedded tissue, including large cut surfaces of adenocarcinomas, were immunohistochemically stained by the avidin–biotin peroxidase complex method, according to the manufacturer's instruction. TTF-1 (8G7G3; Dako, Copenhagen, Denmark), a mouse monoclonal antibody, was used as the first antibody. The reaction intensity was evaluated using four categories – none, weak, moderate, and strong – and the latter two categories were considered as positive. The extent of positive cells was also semi-quantitatively categorized

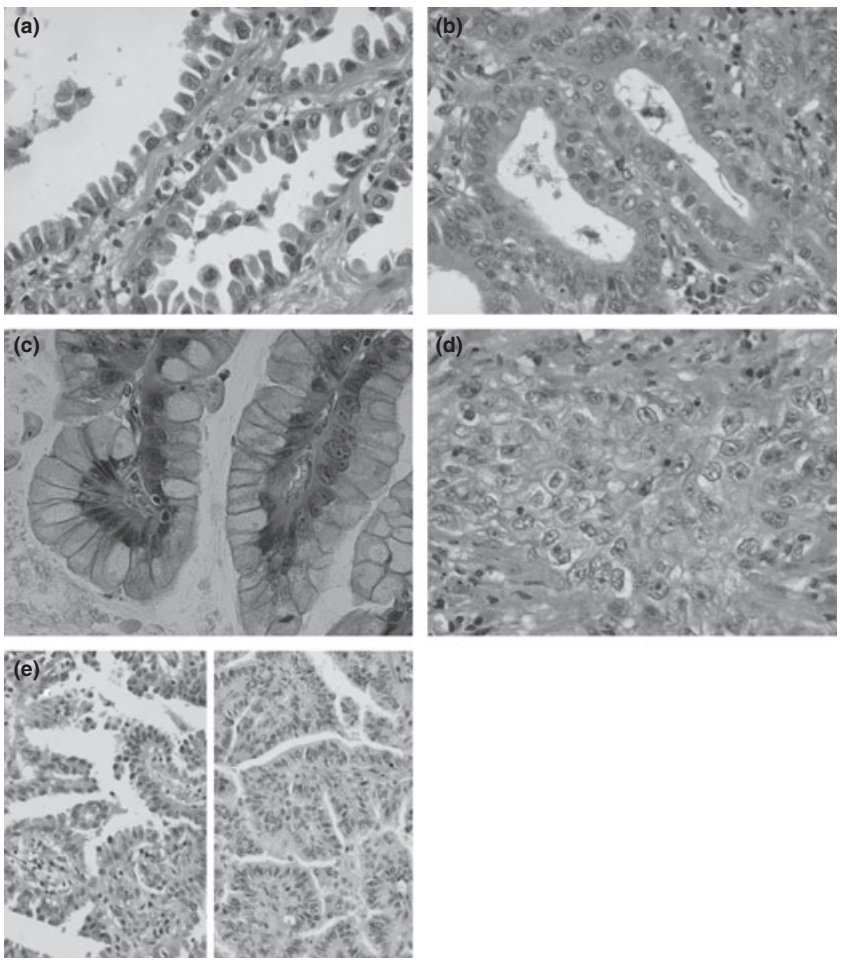

Fig. 1. Cell types of adenocarcinomas. (a) Hobnail cell type: epithelial cells look like Clara or type II pneumocyte cells. Apical portions protrude or bulge into the lumen. Note hobnail- or tadpole-shaped cells. (b) Columnar/cuboidal (col/cub) cell type: characterized by rather large columnar or cuboidal cells with flat apices, resembling ciliated cells of bronchial epithelium; cytoplasmic mucus is usually absent, and even when present, is scanty and located near the free cell surface. (c) Goblet cell type: cells have abundant mucus in the cytoplasm, very similar to goblet cells. (d) Polygonal/oval (po/ov) cell type: composed of polygonal or oval cells with or without mucus in the cytoplasm, proliferating in sheets or nests. (e) Mixed cell type: showing a mixture of hobnail (left) and col/cub cells (right) forming a papillary structure. This type usually consists of two from types (a) to (c). Hematoxylin–eosin staining; original magnification: (a–d) ×400; (e) ×200.

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Table 1. ***p53*, K-*ras*, and *EGFR* mutations by clinicopathological parameters**

	Total	No. of cases (%)					
		p53 status		K-*ras* status		*EGFR* status[a]	
		Wild type	Mutated	Wild type	Mutated	Wild type	Mutated
All cases	223	127 (57)	96 (43)	205 (92)	18 (8)	128 (58)	94 (42)
Age at surgery (years)							
Mean ± SD	61 ± 11	61 ± 11	61 ± 11	61 ± 10	63 ± 12	61 ± 10	61 ± 12
Sex							
Male	124 (56)	63 (51)	61 (49)¶**	112 (90)	12 (10)	88 (72)	35 (28)¶*
Female	99 (44)	64 (65)	35 (35)	93 (94)	6 (6)	40 (40)	59 (60)
Pathological stage							
I	110 (49)	72 (65)	38 (35)¶**	97 (88)	13 (12)¶**	59 (54)	50 (46)
II	17 (8)	9 (53)	8 (47)	17 (100)	0	11 (65)	6 (35)
III	90 (40)	43 (48)	47 (52)	86 (96)	4 (4)	54 (60)	36 (40)
IV	6 (3)	3 (50)	3 (50)	5 (83)	1 (17)	4 (67)	2 (33)
Smoking status							
Non-smokers	98 (44)	65 (66)	33 (34)¶**	94 (96)	4 (4)¶***	40 (41)	58 (59)¶*
Smokers	125 (56)	62 (50)	63 (50)	111 (89)	14 (11)	88 (71)	36 (29)
Cell type classification							
Hobnail cell type	102 (46)	72 (71)	30 (29)	101 (99)	1 (1)	36 (36)	65 (64)‡*
Mixed cell type	49 (22)	29 (59)	20 (41)	47 (96)	2 (4)	31 (63)	18 (37)
Columnar/cuboidal cell type	44 (20)	14 (32)	30 (68)○*†*	38 (86)	6 (14)○*	36 (82)	8 (18)†*
Polygonal/oval cell type	19 (8)	7 (37)	12 (63)○*†**	17 (89)	2 (11)○***	16 (84)	3 (16)†***
Goblet cell type	7 (3)	4 (57)	3 (43)	0	7 (100)‡	7 (100)	0†††
Unclassified	2 (1)	1	1	2	0	1	1
WHO classification							
Acinar	33 (15)	9 (27)	24 (73)	30 (91)	3 (9)	28 (85)	5 (15)
Papillary	27 (12)	17 (63)	10 (37)§*#***	26 (96)	1 (4)	15 (56)	12 (44)§**
Bronchioloalveolar carcinoma	2 (1)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)	1 (50)
Solid adenocarcinoma with mucin	10 (4)	3 (30)	7 (70)	9 (90)	1 (10)	7 (70)	3 (30)
Adenocarcinoma with mixed subtypes	149 (67)	95 (64)	54 (36)§*#**	137 (92)	12 (8)	75 (51)	73 (49)§*
Unclassified	2 (1)	2	0	2	0	2	0
Bronchioloalveolar spread[b]							
+	86 (39)	60 (70)	26 (30)¶*	81 (94)	5 (6)	34 (40)	51 (60)¶*
–	137 (61)	67 (49)	70 (51)	124 (91)	13 (9)	94 (69)	43 (31)

○*vs* hobnail cell type; †*vs* mixed cell type; ‡*vs* all other cell types; §*vs* acinar; #*vs* solid adenocarcinoma with mucin; ¶Male *vs* female; pathological stage I *vs* II–IV; non-smokers *vs* smokers; or bronchioloalveolar spread + *vs* –. The number of symbols, *, **, ***, express *P*-values; *$P < 0.01$, **$P < 0.05$, ***$P < 0.1$, respectively, by chi-squared test or Fisher's exact test. [a]*EGFR* mutation was not examined for one case. [b]+, with bronchioloalveolar spread; –, without bronchioloalveolar spread. EGFR, epidermal growth factor receptor; WHO, World Health Organization.

as follows: 0–25%, negative; 26–50%, 1+ positive; 51–75%, 2+; ≥76%, 3+.

Statistical analysis. To search for any correlations between three gene mutation statuses and clinicopathological data, the chi-squared test or Fisher's exact probability test were used. In addition, we used discriminant analysis to estimate which sub-classification could differentiate the presence of the mutation with greatest accuracy. The 5-year survival rates for patients were examined using the Kaplan–Meier method, and differences were determined by the log-rank test for univariate analysis. All statistical analyses were performed with SPSS for Windows (version 10.1; SPSS, Chicago, IL, USA). Differences were considered to be significant with a *P*-value <0.05.

Results

Case distributions by WHO and FCT classifications of adenocarcinomas and relationships between the two are presented in Tables 1 and 2, respectively. With the former, almost two-thirds of the tumors were classified as adenocarcinomas with mixed subtypes, while with the latter, about half of the tumors were of hobnail type. Using our system, not only does each cell type show a rather consistent one-on-one correspondence with WHO pure subtypes – such as hobnail to papillary, col/cub to acinar, and po/ov to solid – but cases classified as a mixed subtype can be markedly reduced. There were five exceptional cases which were classified as acinar or papillary subtypes by WHO, but as mixed by FTC, and these consisted of both hobnail and col/cub cells. A representative figure for them is presented in Figure 1(e). Most carcinomas with BA spread (79%) were of hobnail cell type. Both the distribution patterns with the two classification systems and the correlations were almost the same as in our previous study.[1]

Reproducibility using the FCT classification was high. One of the authors (A.O.) was a thoracic surgeon with no experience of histopathological diagnosis of lung carcinomas who had been trained in classification by a veteran pathologist (E.T.): he classified 107 consecutive cases, and 85% coincided with the diagnosis made by the pathologist, a reproducibility equivalent to that in the previous study.[1]

Relationships between TTF-1 staining and FCTs. The distribution of 205 TTF-1 examined cases is shown in Table 3. We then divided TTF-1 expression into two groups – <50% (negative and 1+) and more than 51% (2+ and 3+) – and analyzed relationships of the expression to FCT classification. Almost all hobnail cell cases were ≥51%, followed by mixed, but less than half of the cases were ≥51% for

Table 2. Relationships between cell type and WHO classification or bronchioloalveolar spread of lung adenocarcinomas

	No. of cases (%)					
	Cell type classification					
	Hobnail	Mixed	Col/cub	Po/ov	Goblet	Unclassified
WHO classification						
Acinar	0	2 (6)	26 (79)	5 (15)	0	0
Papillary	22 (81)	3 (11)	0	1 (4)	0	1
Bronchioloalveolar carcinoma	1 (50)	0	0	0	1 (50)	0
Solid adenocarcinoma with mucin	0	0	0	10 (100)	0	0
Adenocarcinoma with mixed subtypes	78 (52)	44 (30)	18 (12)	3 (2)	6 (4)	0
Unclassified	1	0	0	0	0	1
Bronchioloalveolar spread						
+	68 (79)	15 (17)	0	0	3 (4)	0
–	34 (25)	34 (25)	44 (32)	19 (14)	4 (3)	2

+, with bronchioloalveolar spread; –, without bronchioloalveolar spread; Col/cub, columnar/cuboidal; Po/ov, polygonal/oval; WHO, World Health Organization.

Table 3. Relationships between TTF-1 expression and cell type classification system

	No. of cases (%)				Total
	TTF-1 expression				
	Negative	1+	2+	3+	
Hobnail*	1 (1)	5 (5)	8 (8)	83 (86)	97
Mixed*	5 (11)	6 (13)	9 (19)	27 (57)	47
Columnar/cuboidal	18 (46)	4 (10)	6 (15)	11 (28)	39
Polygonal/oval	8 (50)	2 (13)	2 (13)	4 (25)	16
Goblet	5 (83)	0	1 (17)	0	6
Total	37 (18)	17 (8)	26 (13)	125 (61)	205

For statistical analysis, thyroid transcription factor-1 (TTF-1) expression statuses were compiled into two groups, negative and 1+, and 2+ and 3+, and then frequencies of the statuses were compared among the subtypes by chi-squared test or Fisher's exact test. **vs* each other type; $P < 0.01$, respectively.

other types with significant differences between the hobnail and mixed, and between each former type and each other type. Thus, the cell types were divided into three groups: (i) hobnail cells with very high TTF-1 positivity; (ii) mixed type with high positivity; and (iii) col/cub, po/ov, and goblet with rather low positivity.

Smoking status in relation to FCTs. The percentages of smokers with col/cub and po/ov lesions were significantly higher than those with hobnail and mixed cell types. The goblet cell type showed a tendency to be less frequent than that of col/cub cells ($P < 0.1$) (Table 4). By WHO classifications, the acinar and solid adenocarcinomas showed higher frequencies of smokers than the mixed subtypes and the papillary adenocarcinomas, with significant differences.

***p53* mutation.** *p53 mutation frequency.*
Mutations of the *p53* gene were detected in 96 of 223 lesions (43%) (Table 1, Table S1). By FCT classification, the highest frequencies of mutations were observed in the col/cub and po/ov cell types, followed by the goblet, mixed, and hobnail cell types, in order, with significant differences between col/cub or po/ov and hobnail, and between col/cub and mixed cell types. By WHO classification, the frequencies of the mutations were high in acinar adenocarcinomas and solid adenocarcinomas with mucin, and low in papillary adenocarcinomas and adenocarcinomas with mixed subtypes, with statistically significant differences between acinar and papillary or mixed, and between solid and mixed.

p53 mutational spectra (Table 4).
We divided *p53* mutations into: CpG to CpA transitions (CpG → A TS), G:C to T:A transversions (G → T TV), other transversions and transitions, and deletions/insertions. Following the FCT classification, the hobnail type featured many CpG → A TS and fewer G → T TV than the col/cub cell type. Furthermore, we here found that: (i) the mixed type showed fewer CpG → A TS and more deletions/insertions than the hobnail cell type, and fewer G → T TV than the col/cub types, with significant differences; (ii) the po/ov cell type had fewer deletions/insertions in comparison with the mixed type. In contrast, WHO classification revealed no significant links between subtypes and mutation spectra.

K-*ras* mutation. A point mutation of codon 12 was observed in 18 lesions (8%) (Table 1, Table S1). By FCT classification, all cases of the goblet cell type had a point mutation, the 100% incidence being statistically significant compared with the rather infrequent occurrence of mutations in all other cell types. The frequencies in the col/cub and po/ov cell types were low, but still higher than those of the hobnail and mixed cell types, with significant differences between the col/cub and hobnail types. Using WHO classification, all subtypes except BAC showed almost the same low mutation frequencies. No significant differences in frequencies were observed among these groups.

***EGFR* mutation.** From the total 222 patients, 94 *EGFR* mutations (42%) were detected – 38 L858R hotspot mutations in exon 21, 55 in-frame deletions in exon 19, and one duplication/insertion (Table 1, Table S1). Mutation frequencies were highest in the hobnail cell type, followed by mixed, col/cub, po/ov, and goblet, in that order, with significant differences between the hobnail and every other cell type, and between mixed and col/cub types. When the mixed type was further subclassified into two groups, hobnail cells and other cell type predominant, the mutation frequencies were the same (37% each; 11/30 for the former and 7/19 for the latter) in both groups, the same as that of non-sub-classified cases. Using WHO classification, the mutation frequencies for papillary, BAC, and adenocarcinoma with mixed subtypes were very similar, followed by solid adenocarcinoma with mucin, and lastly acinar, with significant differences between acinar and mixed or papillary. The mutation frequency of adenocarcinomas with BA spread was 60%, significantly higher than that without BA spread (31%). On comparison of mutations by discriminant analysis, FCT classification proved more useful to estimate the presence of *EGFR* mutations than the WHO system, as shown in Table 5.

Relationships among *p53*, K-*ras*, and *EGFR* mutations. With one exception, no cases with *EGFR* mutation had a K-*ras* mutation, these mutations being significantly mutually exclusive.

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Table 4. *p53* mutational spectra and smoking status for subtypes by cell type and WHO classifications

Subtypes	No. of cases	No. of *p53* mutations (%)					Smoking status
		All mutations	Point mutation			Deletion/insertion	No. of smokers (%)
			CpG to CpA transition	G to T transversion	Others		
All cases	223	100 (45)	22 (22)	26 (26)	29 (29)	23 (23)	125 (56)
Cell type classification							
Hobnail type	102	30 (29)	14 (47)	6 (20)‡***	5 (17)	5 (17)†**	42 (41)
Mixed cell type	49	20 (41)	1 (5)○*	2 (10)‡**	8 (40)○***	9 (45)	24 (49)
Columnar/cuboidal cell type	44	33 (75)	4 (12)○*	13 (39)	9 (27)	7 (21)	38 (86)○*†*
Polygonal/oval cell type	19	12 (63)	3 (25)	3 (25)	5 (42)○***	1 (8)†**	16 (84)○*†*
Goblet type	7	3 (43)	0	0	2 (67)	1 (33)	4 (57)‡
Unclassified	2	2	0	2	0	0	1 (50)
WHO classification							
Acinar	33	24 (73)	4 (17)	8 (33)	7 (29)	5 (21)	29 (88)
Papillary	27	11 (41)	3 (27)	5 (45)¶***	1 (9)	2 (18)	18 (67)§**¶***
Bronchioloalveolar carcinoma	2	1 (50)	0	0	1 (100)	0	0§**#***
Solid adenocarcinoma with mucin	10	7 (70)	2 (29)	2 (29)	3 (43)	0	8 (80)
Adenocarcinoma with mixed subtypes	149	57 (38)	13 (23)	11 (19)	17 (30)	16 (28)	70 (47)§*#**
Unclassified	2	0	0	0	0	0	0

○*vs* hobnail cell type; †*vs* mixed cell type; ‡*vs* columnar/cuboidal cell type; §*vs* acinar; #*vs* solid adenocarcinoma with mucin; ¶*vs* adenocarcinoma with mixed subtypes. The number of symbols *, **, ***, express *P*-values; **P* < 0.01, ***P* < 0.05, ****P* < 0.1, respectively, by chi-squared test or Fisher's exact test. WHO, World Health Organization.

Table 5. Sensitivity, specificity, and accuracy of the WHO and cell type classification for presence of EGFR mutation by discriminant analysis

Sub-classification	Sensitivity (%)	Specificity (%)	Accuracy (%)
WHO	91.5	28.9	55.4
Cell type	69.1	71.9	70.7*

**vs* WHO classification, *P* < 0.01 (by chi-squared test). EGFR, epidermal growth factor receptor; WHO, World Health Organization.

p53 mutations were less frequent in *EGFR*-mutated cases than in the non-mutated cases with borderline significant difference ($P = 0.068$). In contrast, *p53* and K-*ras* mutations appeared to be independent of each another (Table 6). These results are consistent with earlier reports.[22,23]

Prognosis by FCT or WHO classification system and by mutation status. For case distributions in p-stage I and p-stages II–IV among the cell types, there were significantly more p-stage I lesions of the hobnail type than of other cell types (Table 7). We therefore analyzed prognoses separately for p-stage I and p-stages II–IV in both classifications. For this purpose the two BACs by WHO and their counterparts by FCT classification were excluded because the tumors were ''carcinoma *in situ*.'' The solid adenocarcinomas in p-stage I analysis and the goblet cell type in p-stage II–IV analysis were also excluded because the numbers were very small.

Table 6. Relationships between *p53*, K-*ras*, and *EGFR* mutations

Genes	Mutations	No. of cases (%)			
		K-*ras* mutations		*p53* mutations	
		+	−	+	−
EGFR	+	1 (1)	93 (99)*	34 (36)	60 (64)‡
	−	17 (13)	111 (87)	62 (48)	66 (52)
p53	+	6 (6)	90 (94)		
	−	12 (10)	114 (90)		

**P* < 0.01 (by Fisher's exact test). ‡*P* < 0.1 (by chi-squared test). EGFR, epidermal growth factor receptor.

Table 7. Case distributions of pathological stages by cell types or WHO subtypes

Subclassification	No. of cases (%)			
	Pathological stages			
	I	II	III	IV
Cell type classification†				
Hobnail*	62 (62)	7 (7)	30 (30)	1 (1)
Mixed	18 (37)	4 (8)	26 (53)	1 (2)
Columnar/cuboidal	19 (43)	3 (7)	20 (45)	2 (5)
Polygonal/oval	5 (26)	3 (16)	11 (58)	0
Goblet	4 (67)	0	1 (17)	1 (17)
WHO classification				
Acinar	17 (52)	1 (3)	14 (42)	1 (3)
Papillary	13 (50)	0	13 (50)	0
Solid adenocarcinoma with mucin	2 (20)	2 (20)	6 (60)	0
Adenocarcinoma with mixed subtypes	76 (51)	14 (9)	55 (37)	4 (3)

**vs* mixed, columnar/cuboidal, polygonal/oval, *P* < 0.05, respectively, by chi-squared test (I *vs* II–IV). †One hobnail and one goblet case, both of which were classified into BA carcinoma by the World Health Organisation (WHO) classification, were excluded from original cases.

For p-stage I cases, the 5-year survival rates by FCT classification were highest in the hobnail cell type (92%), followed by mixed (83%), po/ov (80%), col/cub (74%), and goblet type (25%), with significant differences between the hobnail and col/cub or goblet cell types, and between the mixed or col/cub and goblet types (Fig. 2a). In contrast, there were no significant differences among the WHO subtypes (Fig. 2b). In p-stage II–IV cases, the 5-year survival rate was the highest for the po/ov cell type (64%), then hobnail (41%), mixed (39%), and col/cub (24%), with significant differences between po/ov and col/cub (Fig. 2c). However, for WHO subtypes, again no significant differences were observed (Fig. 2d).

Next, prognoses by combined gene mutation status were examined (Fig. 3a,b). In p-stage I, the 5-year survival rate for

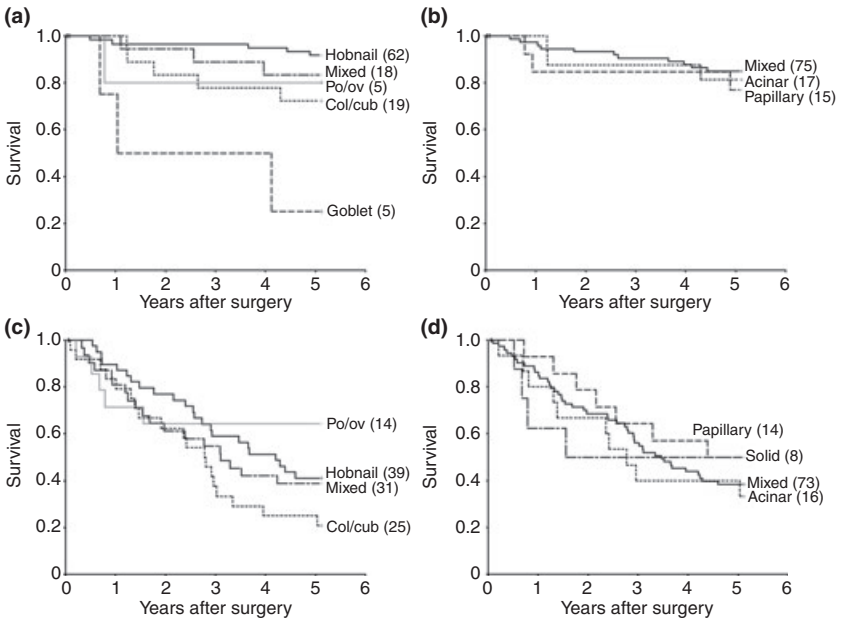

Fig. 2. Disease-specific Kaplan–Meier survival curves with respect to the cell type (a,c) and the World Health Organization (WHO) classifications (b,d) for p-stage I (a,b) and p-stages II–IV cases (c,d). Numbers in parentheses show numbers of patients. (a) The 5-year survival rate of the hobnail type was significantly higher than that of the columnar/cuboidal (col/cub) or goblet cell types ($P < 0.05$ and <0.01, respectively), and survival for the mixed and the col/cub was also higher than for the goblet type ($P < 0.01$ and <0.05, respectively). (b) In contrast, there was no significant variation within the WHO classification. (c) The 5-year survival rate was significantly higher for the polygonal/oval (po/ov) than the col/cub type ($P < 0.05$). (d) Note the lack of variation within the WHO classification.

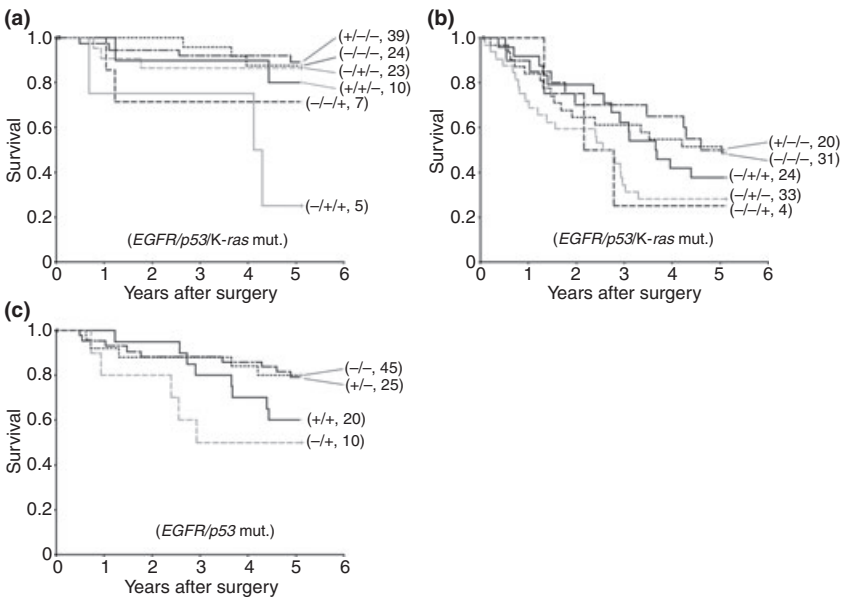

Fig. 3. Disease-specific Kaplan–Meier survival curves with respect to the mutational status for the three genes (*EGFR*/*p53*/K-*ras*) in p-stage I (a) and p-stages II–IV cases (b), and for two genes (*EGFR*/*p53*) in all p-stages for the hobnail cell type (c). The + or – indicate cases with or without mutations, respectively. Numbers in parentheses show numbers of patients. (a) The 5-year survival rate for –/+/+ was significantly lower than those for –/–/–, –/+/–, +/–/–, and +/+/–, respectively ($P < 0.01$, <0.01, <0.01, and <0.05) in p-stage I. (b) In contrast, there were no significant differences between any combinations in p-stages II–IV cases. (c) The survival rate for +/– was significantly higher than that of –/+ with the hobnail cell type ($P < 0.05$).

combined *p53* and K-*ras* mutated cases with no *EGFR* was significantly lower (25%) than those for cases with only *EGFR* (89%), no mutations (88%), only *p53* (87%), or combined *p53* and *EGFR* mutations but no K-*ras* (80%). However, in p-stages II–IV no significant differences in survival rates were found.

We also analyzed effects of the gene mutation status on survival rates in the hobnail cell type, which had sufficient numbers for statistical analysis. As only one case had a K-*ras* mutation, prognoses by *EGFR* and *p53* mutation statuses were examined. Distributions of mutated cases for each gene did not show any significant differences between p-stage I and p-stages II–IV, so these were combined (Table 8). The 5-year survival rates were higher for cases with no *p53* mutation than those with a mutation, regardless of the *EGFR* mutation status (80%, 80%, 60%, and 50%, respectively). There was a statistically significant difference between cases with no *p53* but *EGFR* mutations and with *p53* but no *EGFR* mutations (Fig. 3c).

Discussion

Rate of the mixed cell type in subtypes of adenocarcinomas. A main problem in applying WHO classification is that more than 70% of cases are classified into the mixed subtype. Using our system, a mixed subtype is markedly reduced, from 67% by the WHO to 22%. This may be partly because many cases classified into mixed type by the latter showed one of two histological patterns, that is a combination of (i) bronchioloalveolar pattern at the peripheral, papillary in the middle, and acinar in the central portion of tumor; or (ii) papillary at the peripheral and acinar in the central portion, with increase in fibrous connective tissue toward the central portion. However, tumor cells with each structure were usually classified as the same cell type, mostly hobnail or occasionally col/cub. From these results, use of the FCT classification, or new classification system combining the FCT and WHO classifications, may be effective for reducing the number of cases classified as mixed subtype by WHO.

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Table 8. Case distribution of hobnail cell type by pathological stages and by mutation statuses

Genes	Pathological stages	No. of cases (%)		*P*-values
		Mutation		
		–	+	
p53	I	47 (76)	15(24)	0.13*
	II–IV	24 (62)	15 (38)	
EGFR	I	20 (33)	41 (67)	0.40*
	II–IV	16 (41)	23 (59)	

*By chi-squared test. EGFR, epidermal growth factor receptor.

Cellular lineage of adenocarcinoma subtypes by FCT classification. Yatabe *et al.*[21] reported that in adenocarcinoma cases of the lung, TTF-1 ≥50% positive reactivity was 72%, and that of ≤50% was 28%. These figures were almost the same as ours, 74% and 26%, respectively. Cell types were divided into three groups by positivity: (i) the hobnail cell type with very high positivity; (ii) the mixed, high; and (iii) the col/cub, po/ov, and goblet cells with relatively lower positivity. So the FCT classification also shows differences in cellular lineage expression. In considering histogenesis on the assumption that carcinoma cells imitate inherent characteristics of progenitor cells, almost all the hobnail cell type develop at SBP/TRU, the mixed type develop more distal bronchioles than that of the SBP/TRU, and other cell types develop near the junction of TTF-1-positive and -negative bronchioles or more proximal bronchioles, bronchi and bronchial glands.

Etiological differences of adenocarcinomas by FCTs. The results of this study for relationships of the hobnail and col/cub cell types with *p53* mutations, their spectra (G → T TV attributed to direct mutagenic action of tobacco smoke components, and CpG → A TS ascribed to endogenous mechanisms[15,24,25]), and smoking status, are generally consistent with our previous study.[1] Furthermore, the mixed cell type here showed low frequencies of *p53* mutations and G → T TV and were found in non-smokers, which was quite similar to hobnail cells but significantly different from the col/cub cell type. The mixed cell lesion should thus be classified into the same group as the hobnail type, despite differences in frequencies of CpG → A TS and deletions/insertions. These disparities may be related to differences in endogenous mechanisms underlying development.

In the po/ov cell type, the frequencies of *p53* mutation and smokers were high, very similar to those of the col/cub cells, and the frequencies of other transitions and transversions or deletions/insertions were significantly different from those of hobnail or mixed cell types. So the po/ov cell type should be classified into the same group as the col/cub cell type. The goblet cell type was intermediate among them in relation to smoking. Thus, considering etiological factors, adenocarcinomas were divided into three groups by FCT classification: the col/-cub and po/ov cell types probably caused by tobacco smoke, the hobnail and mixed cell types possibly due to endogenous mechanisms but weak association with tobacco carcinogens, and the goblet cell type intermediate among them. On the other hand, although the subtypes by WHO classification may reflect the *p53* mutation frequency and smoking status to a certain extent, we could not find any distinct differences in the mutation spectra among the subtypes. It is thus relatively more difficult to use WHO subtypes to connect with etiological factors than using cell types.

Remarkable gene mutations by FCTs. *p53* mutations and the mutation spectra and K-*ras* mutations showed characteristic patterns depending on the cell type.[1,16,26] In contrast, only *p53* mutations rates were different among the subtypes of WHO classification. As for *EGFR* genes, frequencies of mutations in adenocarcinomas of the lung are higher for Japanese people (40–65%) than for those in Western countries (≤13%),[9,27–29] being especially high in carcinomas with bronchioloalveolar features (over 50%).[20,22,30–36] Our results showed similar mutation frequencies for Japanese, 42% for all cases and 60% for carcinomas with BA spread. We found that the hobnail cells were more closely associated with *EGFR* mutations compared with other cell types, with high significance. The same results were reported using different adenocarcinoma cases by Ninomiya.[36] The variation we found between cell types with regard to *EGFR* mutations again points to the superiority of FCT classification over the WHO classification based on results of discriminant analysis. Since the presence of *EGFR* mutations significantly correlated with tumor sensitivity to tyrosine kinase (TK) inhibitors,[28,29,37,38] FCT classification is more useful in selecting cases for TK inhibitor therapy than is the WHO classification.

Prognoses by morphological subtype and gene mutation status. Considering the WHO classification, only a few studies using modified WHO sub-classifications have reported prognostic differences among subtypes.[9,11] Using the FCT classification, however, significant differences in 5-year survival rates are apparent. For example, prognosis with the hobnail cell type was better than for col/cub or goblet cell types in p-stage I. As for differences between the hobnail and goblet cell types, all goblet cell tumors were localized with papillary, acinar, and/or BA spreading patterns and no intrapulmonary microscopic metastasis, so the differences may be partly due to the presence or absence of *p53* and K-*ras* mutations, both of which are considered to give aggressive growth potential to tumors, as noted below and already indicated in many papers.[39,40]

For the po/ov cell type, the prognosis of stages II–IV was comparable to that of stage I. To clarify the reason, we examined differences of case distributions between stages I and II–IV by sex, age, and smoking status, and *p53, K-ras*, and *EGFR* mutation status: we found no significant differences between them in any category (data not shown). Furthermore, in p-stages II–IV, the po/ov cell type had a better prognosis than did the col/cub. This contrasts with papers where patients with tumors having solid carcinoma with mucin component showed significantly worse survival compared with nonsolid subtypes in cases sub-classified by the modified WHO classification.[9,11] There are some differences in the histological criteria used and p-stages of analyzed cases between our paper and other papers, but the precise reasons for these differences remain unclear. Therefore, further examination of the prognosis of the po/ov cell type is warranted.

So far, the number of reports on the influence of multiple gene mutations on prognosis has been limited. In this study, considering six kinds of combinations of three genes, only one – *p53* and K-*ras* but not *EGFR* mutated – showed a worse prognosis, with significant differences, than most other combinations in p-stage I, though this difference disappeared in more advanced stages. Furthermore, since the prognosis differed by cell type, we examined the effects of concurrent gene mutations in the hobnail cell type, and found the *p53* mutation to be clearly associated with a worse prognosis. Taken together, we can hypothesize that *p53* and K-*ras* mutations in carcinomas result in a worse prognosis for patients, but may be obscured in advanced cases by many other factors associated with prognosis.[41–43] The *EGFR* mutation status was not linked with survival in this study or any other papers,[22,32,44] although a significant association was detected between poor prognosis and the presence of *EGFR* mutations in TRU-type adenocarcinomas.[2] Therefore, further studies restricted to subtypes are certainly warranted.

Application of a new TNM staging system (NTNM) for lung cancer is planned in 2010. For N categories, however, a consensus on the handling of isolated tumor cells in a lymph node has not yet been reached among Japanese pathologists. So we

revised only the T and M categories according to NTNM, and found that only eight cases converted from p-stage I to p-stage II. When prognoses by FTC or mutation status were analyzed with the present TNM and the NTNM, no differences were found between them. We suspect that cases for which we must change N categories would also be a small number. All results considered, we believe that the FTC combined with multiple gene mutation status appears to be useful in predicting the biological nature of pulmonary adenocarcinomas even in NTNM.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Analysis of epidermal growth factor receptor (*EGFR*) exon 21 point mutation. (a) A loop-hybrid band with exon 21 point mutation (arrow). (b) An electropherogram image of re-amplified DNA extracted from the mutation band in (a). The upper band is due to heteroduplexes by normal alleles and internal deletion alleles from the loop-hybrid-genorator (LH-G) probe, the middle band to homoduplexes of mutant alleles (arrow), and the lower band to homoduplexes of internal deletion alleles. (c) DNA sequence electropherogram by direct sequencing of DNA extracted from the middle band in (b), illustrating an L858R mutation.

Table S1. *p53*, K-*ras*, and epidermal growth factor receptor (*EGFR*) mutations in lung adenocarcinomas.

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