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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

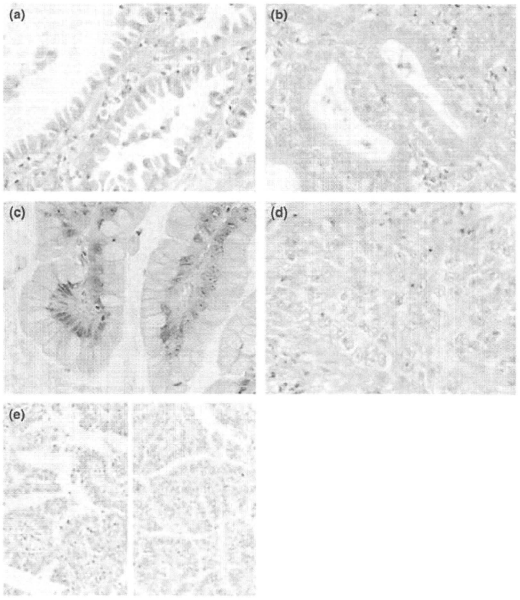

Fig. 1. Cell types of adenocarcinomas. (a) Hobnail cell type: epithelial cells look like Clara or type II pneumocyte cells. Apical portions protrude or bulge into the lumen. Note hobnail- or tadpole-shaped cells. (b) Columnar/cuboidal (col/cub) cell type: characterized by rather large columnar or cuboidal 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

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.

analysis and the goblet cell type in p-stage II–IV analysis were also excluded because the numbers were very small.

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

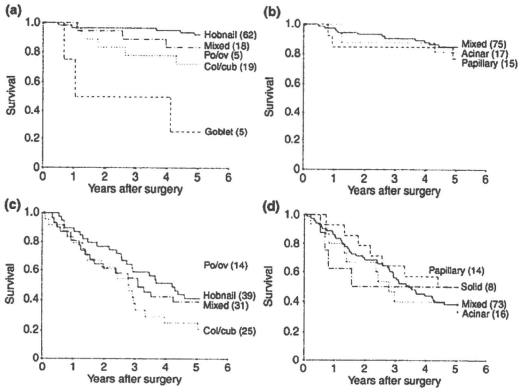

Fig. 2. Disease-specific Kaplan–Meier survival curves with respect to the cell type (a,c) and the World Health Organization (WHO) classifications (b,d) for p-stage I (a,b) and p-stages II–IV cases (c,d). Numbers in parentheses show numbers of patients. (a) The 5-year survival rate of the hobnail type 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.

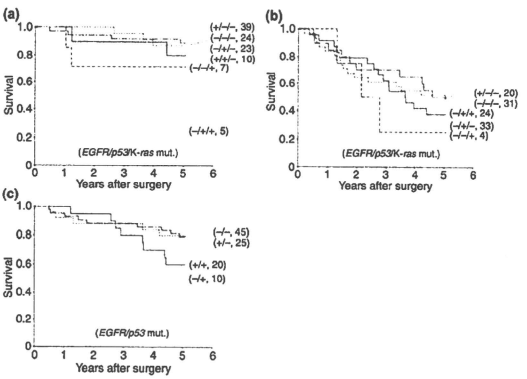

Fig. 3. Disease-specific Kaplan–Meier survival curves with respect to the mutational status for the three genes (*EGFR/p53/K-ras*) in p-stage I (a) and p-stages II–IV cases (b), and for two genes (*EGFR/p53*) in all p-stages for the hobnail cell type (c). The + or − indicate cases with or without mutations, 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.[3] 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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Dlk-1, a cell surface antigen on foetal hepatic stem/progenitor cells, is expressed in hepatocellular, colon, pancreas and breast carcinomas at a high frequency

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Delta-like 1 protein (Dlk-1), also known as preadipocyte factor 1 (Pref-1), is a transmembrane and secreted protein with epidermal growth factor (EGF)-like repeats. Dlk-1 is known to be expressed in foetal liver, but absent in neonatal and adult liver in mice and rats. Dlk-1 is also expressed in a subpopulation of hepatic oval cells, which are considered as stem/progenitor cells in rat adult liver. In this study, we generated monoclonal antibodies against human Dlk-1 (hDlk-1) and investigated hDlk-1 expression in human liver and hepatocellular carcinoma (HCC). Like rodent livers, hDlk-1 was detected in foetal liver, but not in adult liver. In HCC, hDlk-1 was positive for 20.5% of the cases examined and was localized in both cytoplasm and cell membrane, whereas hDlk-1 was undetected in viral hepatitis, nodular cirrhosis. Interestingly, hDlk-1 positive HCC was found more frequently in younger patients and its expression was correlated with alpha-fetoprotein expression. Furthermore, hDlk-1 was also detected frequently in colon adenocarcinomas (58%), pancreatic islet carcinoma (50%), and small cell lung carcinoma (50%). Thus, hDlk-1 is a cell surface protein expressed in many carcinomas including HCC and may be a potential target for monoclonal antibody therapy for carcinomas.

Keywords: cell surface protein/colon adenocarcinoma/hDlk-1/hepatocellular carcinoma (HCC)/small cell lung carcinoma.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence activated cell sorter; His, histidine; MEM, minimum essential medium; mRNA, messenger RNA; PBS, phosphate-buffered saline.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours in the world (*1*). While the occurrence has been unusually high in Asia and Africa, it is recently increasing in United States and the incidence and mortality rates are anticipated to double over the next 10–20 years (*2*, *3*). HCC is often diagnosed at an advanced stage when curative therapies are of limited efficacy. In order to reduce morbidity and mortality of HCC, it is of prime importance to develop a system for early diagnosis, novel systemic therapies for the advanced disease as well as means to prevent HCC development.

Dlk-1 protein, also known as Pref-1, foetal antigen 1 (FA1), pG2 and ZOG, is a transmembrane and secreted protein, which is a member of the epidermal growth factor (EGF)-like family including Notch/Delta/Serrate (*4–8*). Dlk-1 is strongly expressed in foetal tissues such as liver, pancreas and skeletal muscle, but its expression is restricted in adult tissues such as placenta and adrenal grand (*9–12*). As there are many receptors and ligands in the EGF-like family proteins, which regulates cell fate and differentiation during development in many organisms, Dlk-1 may also play a role in development and differentiation (*12–14*). In fact, there are several reports showing the involvement of Dlk-1 in adipogenesis (*15*), hematopoiesis (*16*, *17*) and development of pancreas (*18*, *19*), placenta (*20*) and adrenal gland (*21*, *22*). In addition to the normal tissues, Dlk-1 was also shown to be expressed in several tumours, such as neuroblastoma (*23*), glioma (*24*), small cell lung carcinoma (*25*), myelodysplastic syndrome, acute myelogenous leukaemia (*26*), etc. (*27*, *28*). These results suggest that Dlk-1 may play an important role in tumourigenesis as well as organogenesis.

Previously, we demonstrated that Dlk-1 is strongly expressed on the cell surface of hepatoblasts in murine foetal liver from embryonic day (ED) 10.5–16.5 and Dlk-1+ cells isolated from foetal liver showed high-proliferative activity and bi-potentiality (*10*). Its expression is down-regulated in late gestation and completely absent after birth. In liver injury under conditions that limit proliferation of hepatocytes, immature cells with oval shaped nucleus called hepatic oval cells appear around the portal vein. As they are proliferative and express markers of hepatocytes and cholangiocytes, hepatic oval cells have been considered as adult liver progenitors (*29*). Similar cells were also shown to be present in severe hepatitis and implicated in tumourigenesis (*30*). The expression of Dlk-1 was also observed in a subpopulation of rat oval cells induced by the 2-acetylaminofluorene/partial hepatectomy model (*31*). These data suggest that Dlk-1 is a cell

surface antigen of foetal/adult hepatic stem/progenitor cells.

It has become clear that tumour, in many cases, is a heterogeneous cell population and only a small fraction of the cells possess the potential to self-renew. Cancer stem cell or tumour initiating cell, which was first documented in haematological malignancies, has subsequently been discovered in many solid tumours, including breast, brain, prostate, liver, lung, melanoma, pancreas and colon tumours (*32–36*). While it was shown that CD133, known as a stem cell marker, is expressed in cancer stem cells in many tumours including HCC (*37–41*), the relation between normal tissue stem cells and cancer stem cells is not clear in most of the cases.

In this study, we established many hybridoma clones which produced anti-hDlk-1 monoclonal antibodies (mAb). Among them, we selected three independent clones usable for immunohistochemistry and characterized these antibodies by flow cytometry. Using these mAbs which recognized a different epitope, we investigated the expression of human Dlk-1 (hDlk-1) during liver development by immunohistochemistry. The expression of hDlk-1 showed a pattern similar to mouse Dlk-1 during liver development, suggesting that hDlk-1 is also a marker of hepatic stem/progenitor cells in embryo. We then examined hDlk-1 expression in human neoplastic liver lesions. About a half of HCC specimens from under 40-years-old patients expressed hDlk-1, whereas the positive ratio of hDlk-1 over 50-years-old patients was ~10%. Our study indicates the possibility that hDlk-1 is a common cell surface antigen both in human foetal liver stem/progenitor cells and in a part of HCC. Moreover, hDlk-1 was also frequently expressed in colon, breast, pancreas and lung carcinoma. These observations suggest that hDlk-1 is a potential target for monoclonal antibody-based therapy in those carcinomas.

Materials and Methods

Plasmid constructs

Full length hDlk-1 and its derivatives (EGF1-3 and EGF 4-6) were amplified by PCR. The sequences of primers were as follows: Fw1: 5′-cgcgtccgcaaccagaagccc-3′, Rv1: 5′-aagcttgatctcctcgtcgccggcc-3′ (for full length hDlk-1), Fw2: 5′-gcggccgcgctgaatgcttcccggcc-3′, Rv2: tctagaggcccgaacatctctatcac-3′ (for hDlk-1 EGF1-3), Fw3: 5′-gcggccgcgcctgctcctcggccccc-3′, Rv3: 5′-gcgtatagtaagctctgcgg-3′ (for hDlk-1 EGF4-6). All PCR products were verified by DNA sequencing. Full-length hDlk-1 cDNA was cloned in pcDNA3 vector (Invitrogen, Carlsbad, CA) with Flag tag. hDlk-1 EGF1-3 cDNA was subcloned in pME18SNeo carrying the signal sequence of CD8, His tag, and transmenbrane and cytoplasmic domains of FXYD5, which was kindly gifted by Dr Tanaka, M. (University of Tokyo, Tokyo, Japan). hDlk-1 EGF4-6 cDNA was subcloned in pME18SNeo containing the signal sequence of CD8, His tag.

Antibodies

Mouse monoclonal antibodies against hDlk-1 (clone DI-6, DI-2-20 and DI-4-22) were generated by the DNA immunization method (Nosan Corp., Kanagawa, Japan). To prepare purified monoclonal antibodies, hybridoma clones (3×10^6 cells) were intraperitoneally administered to BALB/c Slc-nu/nu mice (Japan SLC, Shizuoka, Japan), which received 2,6,10,14-tetramethylpentadecane (Sigma Aldrich Japan K.K., Tokyo, Japan) 7 days before injection of hybridoma. After collection of ascites, the antibodies were purified with a protein G column (GE Healthcare, Buckinghamshire, England).

Rabbit polyclonal antibodies against hDlk-1 were prepared by immunizing with peptides containing the extracellular domain of hDlk-1 except for the putative signal sequence. Polyclonal antibodies were purified by affinity chromatography using columns conjugated with the peptides used for immunization.

Cell culture, transfection and flow cytometry

COS7 cells, HEK-293 cells, Huh-7 cells and SK-N-FI cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. HepG2 cells and C3A/HepG2 cells were maintained in MEM supplemented with 10% foetal bovine serum. COS7, HEK-293, Huh-7 and HepG2 cells were from Human Science Research Resource Bank (Osaka, Japan). SK-N-FI and C3A/HepG2 cells were purchased from American Type Culture Collection (Rockville, MD). Transfection was performed using Lipofectoamine and Plus reagent (Invitrogen). To establish HEK-293 cells stably expressing hDlk-1 (293-hDlk-1), HEK-293 cells were transfected with pcDNA3 vector containing full-length hDlk-1 cDNA and selected with G418 (Invitrogen). COS7 cells were transiently transfected with expression constructs containing either hDlk-1 EGF1-3 or hDlk EGF4-6, and two days after transfection, these cells were harvested and subjected to fluorescence activated cell sorter (FACS) analysis. 293-hDlk-1 cells were cultured to subconfluency and harvested for flow cytometry by FACSCalibur (Nippon Becton Dickinson, Tokyo, Japan).

Immunohistochemistry

Tissue arrays and sections of tumours used in this study were purchased from Cybrdi (Rockville, MD), Shanghai Outdo Biotech Co. (Shanghai, China), Super Bio Chips (Seoul, Korea), ISU ABXIS (Seoul, Korea), US Biomax (Rockville, MD). Clinical information of patients (age, sex, grade and pathology diagnosis) is described in their homepage and data sheets. Foetal liver specimens were purchased from Biochain (Hayward, CA).

Paraffin embedded tissue sections and arrays were deparaffinized, and then autoclaved for 5 min in citrate buffer (pH 6.0) or TE buffer (pH 9.0). Slides were treated with methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity, and incubated with anti-hDlk-1 mAbs (10 μg/ml) at 4°C over night. After washing with phosphate-buffered saline (PBS), sections were stained with Vectastain ABC Elite kit (Vector, Burlingame, CA) and then counterstained with haematoxylin (Wako, Osaka, Japan). HCC sections with more than 10% immunopositive cells, either cell membrane or cytoplasmic stainin, were considered as positive.

Result

Characterization of anti-hDlk1 monoclonal antibodies

We established over 100 hybridoma clones producing anti-hDlk-1 mAb. Among them, three independent clones usable for immunohistochemistry in paraffin-embedded tissue sections were selected. First, we evaluated the reactivity and specificity of these antibodies by flow cytometry. Three mAbs against hDlk-1, DI-6, DI-2-20 and DI-4-22, specifically recognized HEK-293 cells stably expressing hDlk-1 (Fig. 1B), but not parent HEK-293 cells (not shown). On the other hand, these antibodies failed to recognize mouse Dlk-1, which shares ~90% similarity with hDlk-1 at the amino acid level (data not shown). These results confirmed that anti-hDlk-1 mAbs, DI-6, DI-2-20 and DI-4-22, specifically recognize hDlk-1. We then mapped the region of hDlk-1 to which the antibodies bind using deletion mutants of hDlk-1 (Fig. 1A). As shown in Fig. 1D, DI-6 recognized the EGF repeats 1–3 (amino acid 24–129), whereas DI-2-20 and DI-4-22 recognized EGF repeats 4–6 (amino acid 126–382). DI-2-20 and DI-4-22 recognized the same epitope because they competed each other (results not shown).

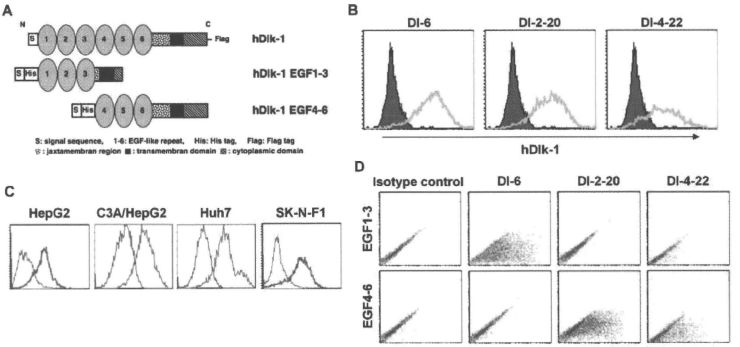

Fig. 1 Characterization of anti-hDlk-1 monoclonal antibodies by FACS analysis. (A) Schematic representation of various hDlk-1 constructs used in this study. (B) Three monoclonal antibodies used in this study specifically recognize hDlk-1 expressing cells. HEK-293 cells stably-expressing hDlk-1 were harvested, dispersed in a single cell suspension, and analysed by flow cytometry using monoclonal antibodies against hDlk-1, DI-6, DI-2-20, DI-4-22 (green line). Blue area: isotype control (mouse IgG1). (C) Anti-hDlk-1 mAb DI-2-20 also recognizes endogenous hDlk-1 in human cancer cell lines. Cancer cell lines indicated here were harvested and subjected to FACS analysis by using DI-2-20, respectively. Blue line: isotype control (mouse IgG1), red line: anti-hDlk-1 mAb (DI-2-20). Another monoclonal antibody, DI-6, showed similar result (data not shown). (D) Identification of the region of hDlk-1 to which anti-hDlk-1 mAbs bind. COS7 cells were transiently transfected with plasmids containing a various domain of hDlk-1 represented in (A). Two days after transfection, these cells were harvested and subjected to FACS analysis with anti-hDlk-1 mAb, DI-6, DI-2-20 and DI-4-22, respectively. DI-2-20 and DI-4-22 recognize EGF repeat 4–6, whereas DI-6 recognizes EGF repeat 1–3.

As reported previously, hDlk-1 was expressed early in liver development, but not in adult liver (9). To confirm that selected mAbs were suitable for immunohistochemistry in paraffin-embedded tissue sections, the expression pattern of hDlk-1 protein in liver was examined by immunohistochemical staining using DI-2-20 mAb (Fig. 2). A foetal liver tissue at 22 weeks (22w) showed membrane and cytoplasmic staining of hDlk-1 in hepatocytes. However, hDlk-1 was not detected in foetal liver at 38 weeks (38w) and adult liver. Northern blot analysis showed that hDlk-1 mRNA was strongly expressed in foetal liver from 6 to 12 weeks of gestation (not shown). The expression of hDlk-1 showed a pattern similar to mouse Dlk-1 during liver development, suggesting that hDlk-1 is also a marker of hepatic stem/progenitor cells in embryo. Similar results were obtained with either DI-6 or DI-4-22 mAb, though less sensitive than DI-2-20 (not shown). These results suggest that DI-2-20 was also useful for detecting endogenous hDlk-1 by immunohistochemistry. Therefore, we used mainly DI-2-20 mAb for further analysis.

Expression of hDlk-1 in HCC cells

We then examined the cell surface expression of hDlk-1 in a number of cancer cell lines originated from HCC by flow cytometry by using DI-6 and DI-2-20 antibodies. Among them, significant cell surface expression of hDlk-1 was detected in HepG2, C3A/HepG2 and Huh-7 cells (Fig. 1C). These results suggest that hDlk-1 is the cell surface antigen of HCC

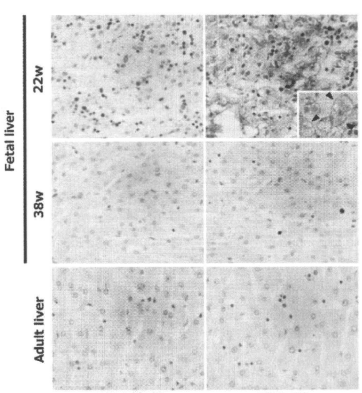

Fig. 2 Immunohistochemical analysis of hDlk-1 in foetal and adult liver. Each specimen was stained with anti-hDlk-1 mAb DI-2-20 (right panels). Foetal liver tissue at 22w (upper) shows membrane (arrow heads) and cytoplasmic staining with DI-2-20 monoclonal antibody. hDlk-1 staining was not observed in foetal liver at 38w (middle) and adult liver (lower). Mouse IgG1 used as isotype control was negative (left panels). Magnification is ×400.

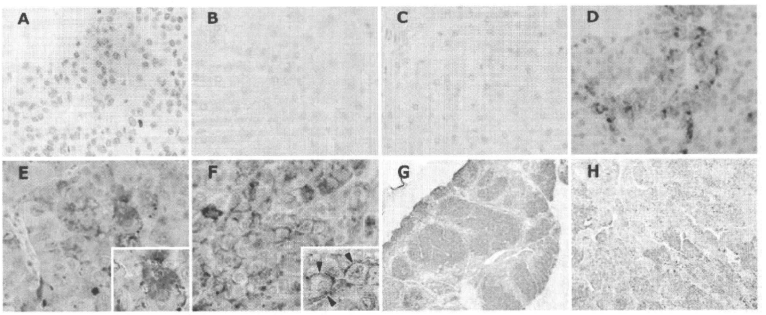

Fig. 3 hDlk-1 expression was observed only in hepatocellular carcinoma. Immunohistochemical staining with anti-hDlk-1 monoclonal antibody DI-2-20 was performed using tissue microarrays of liver tumour (388 malignant and 19 benign), nodular cirrhosis (40), viral hepatitis (11) and normal liver (26). The expression of hDlk-1 was detected only in HCC sections (D–F), not in adult liver (not shown), viral hepatitis (B), nodular cirrhosis (C), cavernous hemangioma (not shown) and intrahepatic cholangioma (not shown). hDlk-1 immunoreactivity in HCC cells was localized in either cytoplasm (E, inset) or cell membrane (F, inset). Membranous staining of hDlk-1 was shown by arrow heads. hDlk-1 expression was also examined in some samples of conventional tissue section corresponding to hDlk-1 positive spots in tissue array (G and H). Mouse IgG1 was used as negative control (A). Magnification is ×400 (A–F), ×1000 (inset in E and F) and ×40 (G and H).

cells. Additionally, hDlk-1 was also expressed in the cell surface of SK-N-F1 cells, a neuroblastoma cell line (Fig. 1C).

To evaluate the expression of hDlk-1 in non-neoplastic and neoplastic liver lesions, immunohistochemical analysis was performed by using tissue arrays. Typical staining profiles are shown in Fig. 3 and the results are summarized in Table I. hDlk-1 expression was undetectable at all in normal adult liver (0/26, not shown), non-neoplastic liver lesions, viral hepatitis (0/11, Fig. 3B) and nodular cirrhosis (0/40, Fig. 3C). hDlk-1 was also not found in cavernous hemangioma (0/19, not shown) and intrahepatic cholangioma (0/2, not shown). In contrast, hDlk-1 expression examined by DI-2-20 antibody was specifically observed in HCC and was positive for 79 out of 386 cases (20.5%, Fig. 3D–F). The pattern of hDlk-1 staining varied among individual tumours, e.g. hDlk-1 signal exhibited a uniform distribution within the tumour in one case (Fig. 3E, F), whereas it showed mosaic-like pattern in another case (Fig. 3D). Similar results were obtained with the same tissue array by using DI-6 antibody that recognizes a different epitope from DI-2-20 (not shown). Although hDlk-1 is a type I transmembrane protein, the immunoreactivity in HCC cells was mainly observed in the cytoplasm (Fig. 3E, inset), whereas hDlk-1 was expressed in cell surface in some cases (Fig. 3F, inset). We also examined some of conventional tissue slides corresponding to hDlk-1 positive spots in tissue arrays for hDlk-1 expression. The staining of hDlk-1 was not uniform, but covered more than 10% of the tumour in all tissue sections that we studied (Fig. 3G and H). No immunoreactivity was observed in normal tissues adjacent to the tumour (not shown).

Table I. Summary of immunohistochemical analysis.

	hDlk-1 staining	
	–	+
(A) *hDlk-1 expression in HCC (386 cases)*		
CS03-01-002 (Cybrdi)	40	15
CC03-01-001 (Cybrdi)	43	12
CC03-01-003 (Cybrdi)	46	10
CC03-02-001 (Cybrdi)	14	3
A204 (ISU ABXIS)	29	6
A204(II) (ISU ABXIS)	29	6
BC03013 (Biomax US)	49	10
OD-CT-DgLiv02-002 (Outdo bio.)	25	7
CS3 (HCC only) (Super Biochips)	32	10
Total	307 (79.5%)	79 (20.5%)
(B) *hDlk-1 expression in normal liver, non-neoplastic liver lesions, benign liver tumour and cholangiocarcinoma*		
Normal liver	23	0
Viral hepatitis	11	0
Nodular cirrhosis of liver	40	0
Cavernous hemangioma of liver	19	0
Intrahepatic cholangicarcinoma	2	0

As summarized in Table II, there was no clear correlation between hDlk-1 expression and pathological grade, gender, or aetiology such as HBV or HCV infection ($P > 0.05$ by χ^2 test). In contrast, hDlk-1 expression was clearly correlated with age or expression of alpha-fetoprotein (AFP). Interestingly, hDlk-1 expression was detected at higher frequency in HCC under 50 years old (51 out of 162 specimens, 31.5%), whereas the hDlk-1 positive HCC was dramatically decreased over 50 years old (28 out of 220 specimens, 12.7%). Especially, the hDlk-1 positive HCC was 43.1% (22 out of 51 specimens) under 40 years old. AFP is a well-established marker for HCC and was

Table II. Relationship of hDlk-1 expression and clinical features.

	hDlk-1			
	−	+	hDlk-1+ ratio	
Grade				
I	46	9	16.40%	
II	158	54	25.50%	
III	50	14	21.90%	$P=0.347$
Gender				
Male	245	59	19.40%	
Female	58	20	25.60%	$P=0.225$
Age				
40>	29	22	43.10%	
40–49	82	29	26.10%	
50–60	99	17	14.70%	
>60	93	11	10.60%	$P<0.01$
AFP				
−	179	25	12.30%	
+	26	25	49.00%	$P<0.01$
Aetiology				
HBV+	44	12	21.40%	
HCV+	6	0	0.00%	
−	7	1	12.50%	$P=0.393$

present in 51 of 255 cases (20.0%). hDlk-1 was detected in 25 of 51 AFP-positive HCCs (49.0%), whereas 25 of 204 AFP-negative HCCs (12.3%) were positive for hDlk-1. These results indicated that the hDlk-1 was expressed more frequently in a patient under 50 years old and in AFP-positive HCC.

Expression of hDlk-1 in other carcinomas

We then examined the expression of hDlk-1 in various carcinomas and found that hDlk-1 was highly and frequently expressed in colon adenocarcinoma (58.6%), breast carcinoma (39.0%), pancreatic carcinoma (30.8%) and lung carcinoma (30.2%), but not in ovarian carcinoma (13.2%) and gastric carcinoma (3.33%). Interestingly, in pancreatic carcinomas, hDlk-1+ cells were found more frequently in islet carcinoma (50.0%) than duct carcinoma (28.3%). In the lung carcinoma, hDlk-1 was expressed in small cell lung carcinoma (52.5%), but only few non-small cell carcinoma (8.9%) expressed hDlk-1 (Fig. 4, summarized in Table III). These results suggested that hDlk-1 was expressed in various carcinomas.

As described above, hDlk-1 was more frequently expressed in AFP-positive HCC. Therefore we examined the expression of hDlk-1 in other AFP positive cancer, AFP-producing gastric cancer. hDlk-1 was rarely expressed in gastric carcinoma (3.33%), but was positive for two out of 10 cases in AFP-producing gastric cancer (20.0%, Fig. 4G and H, summarized in Table III). Previously, Dezso *et al.* (*42*) reported that hDlk-1 was highly expressed in hepatoblastoma, AFP-positive liver cancer occurring in childhood. Together with our result, it was suggested that hDlk-1 was frequently expressed in AFP-positive cancers.

Discussion

Previously, we demonstrated that Dlk-1 is strongly expressed in hepatoblasts in mouse foetal liver, down-regulated in late gestation, and completely disappeared in neonatal and adult liver. Single Dlk-1+ cell isolated from ED14.5 liver exhibited high proliferating activity and was able to differentiate into both hepatocyte and biliary epithelial cell lineages (*10*). These findings suggested that Dlk-1 is a cell surface antigen of foetal hepatic stem/progenitor cells in the mouse. In this study we prepared mAbs against hDlk-1 and showed that the expression pattern of hDlk-1 is similar to mouse Dlk-1/Pref-1 during liver development, i.e. hDlk-1 is expressed in foetal liver but not in adult liver and hDlk-1 is present in both cell membrane and cytoplasm (Fig. 2). Thus, hDlk-1 may be an excellent marker of foetal hepatic stem/progenitor cells in human as well.

In adult liver, hepatic progenitor cells (HPCs) appear around the portal vein when liver is severely injured. These cells are known as hepatic oval cells in rodents and express markers of both hepatocytes and biliary epithelium (*29*). HPCs in chronic liver diseases are suggested to contribute to liver regeneration as well as hepatocarcinogenesis (*43–45*). Dlk-1 is not expressed in normal liver and was found in a subpopulation of hepatic oval cells induced in rats treated with 2-acetylaminofluorene and partial hepatectomy, a well-established rat model of hepatic oval cell induction (*31*). However, in a mouse model of hepatic oval cell induction by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, Dlk-1 is not expressed in hepatic oval cells (*46*). In the present study, hDlk-1 immunoreactive cells were not found in the specimens of viral hepatitis and nodular cirrhosis. In contrast, it was expressed frequently in HCC, but not in intrahepatic cholangioma, cavernous hemangioma and non-neoplastic liver lesions. These results demonstrate that hDlk-1 is expressed in HCC at high frequency, but do not exclude the possibility that Dlk-1 is expressed in some of adult hepatic stem/progenitor cells in chronically injured liver, which can lead to tumourigenesis.

It still remains unclear whether liver tumour is derived from hepatic stem/progenitor cells or mature hepatocytes. Recently, Lee *et al.* (*47*) reported that two subtypes (HB and HC) of HCC were categorized by analysis of gene expression patterns, and suggested that they may reflect the origin of tumour cells. The HB subtype shared a gene expression pattern with foetal hepatoblasts, whereas the HC subtype shared with adult hepatocytes, suggesting that the HB subtype may arise from hepatic stem/progenitor cells. The HB subtype of HCC showed poor prognosis compared to HC subtype. Interestingly, the HB subtype accounts for ~20% of HCC examined, similar to the frequency of hDlk-1 positive cells. In addition, the expression of hDlk-1 was more frequently found in HCC patients younger than 50 years old. Because HCC develops after a long latency period of chronic infection with HBV, HCV or both, the incidence of HCC is relatively high over 50 years old. Therefore, our finding that HCC patients under 50 years old expressed hDlk-1

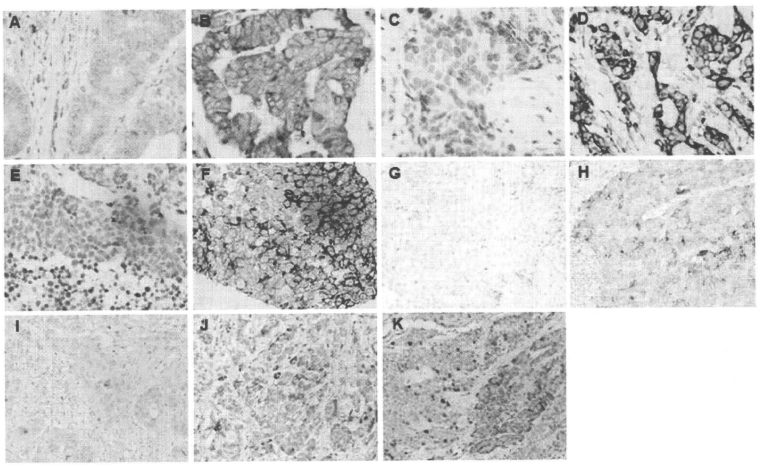

Fig. 4 hDlk-1 expression in various tumours. hDlk-1 expression in various tumours were examined by immunohistochemistry using anti-hDlk-1 mAb, DI-2-20. (A and B) Colon adenocarcinoma, (C and D) breast carcinoma, (E and F) small cell lung carcinoma, (G and H) AFP-producing gastric cancer, (I and J) pancreatic adenocarcinoma, (K) islet cell carcinoma. Tumour cells showed strong staining in cytoplasm (J and K), and cytoplasm and cell membrane (B, D, F and H). A, C, E, G and I showed hDlk-1 negative tumour cells. Magnification is ×200.

Table III. Summary of hDlk-1 staining in various tumours.

Tumour		Dlk−	Dlk+	Total
Colon adenocarcinoma		24 (41.4%)	34 (58.6%)	58
Ovarian carcinoma		59 (86.8%)	9 (13.2%)	68
Pancreatic carcinoma	Duct adenocarcinoma	33 (71.7%)	13 (28.3%)	46
	Islet cell carcinoma	3 (50.0%)	3 (50.0%)	6
Breast carcinoma		36 (61.0%)	23 (39.0%)	59
Lung carcinoma	NSCLC	51 (91.1%)	5 (8.9%)	56
	SCLC	19 (47.5%)	21 (52.5%)	30
Gastric carcinoma		29 (96.7%)	1 (3.33%)	30
AFP-producing gastric cancer	8 (80%)	2 (20%)	10	

more frequently is unexpected and intriguing. However, there was so far no clear correlation between hDlk-1 positive tumours in patients under 50 years old and specific aetiologies such as gender, pathological grade and stage. Recently, Huang *et al.* (*48*) also reported that hDlk-1 expression in HCC showed no significant correlation with HBV infection, tumour size and serology of AFP. Thus, our finding suggests that hDlk-1+ HCC develops in a relatively short latency period and may have an origin different from other HCC with a longer latency period. Alternatively, considering the recent finding that albumin positive hepatocytes can be converted into induced pluripotent stem cells (iPS) by transient expression of c-Myc, Sox2, Oct3/4 and Klf4 (*49*), conversion of mature hepatocytes to an immature stage with hDlk-1 expression may occur during chronic liver injury. Thus, it is tempting to speculate that hDlk-1 may be a hallmark of HCC originated from hepatic or cancer stem/progenitor cells. The origin and mechanism of tumourigenesis of HCC still need extensive investigation.

While Dlk-1, also known as Pref-1, was originally described as an inhibitor of adipogenesis (*8*), the precise function still remains unknown. In this study, we showed that hDlk-1 is expressed in not only HCC but also many carcinomas such as colon, breast, pancreatic and lung carcinomas. As previously reported, colony formation, cell growth and tumourigenicity of HCC cell lines were significantly decreased when the endogenous hDlk-1 was knocked down by RNAi (*48*), and hDlk-1 promoted proliferation of glioblastoma cell line (GBM cells) (*24*) and erythroid leukemia cell line (K562 cells) (*26*). Furthermore, Dlk-1 has been reported to interact with Notch 1, and modulate

Notch signalling as a negative regulator (*50*). Notch 1 signalling prevented HCC cells to proliferate by induction of cell cycle arrest and apoptosis (*51*). Thus, hDlk-1 may contribute to tumourigenesis by enhancing tumour growth. However, precise molecular mechanism of Dlk functions is still unknown, and requires further studies. Because hDlk-1 is a cell surface molecule expressed in many HCCs and also other carcinomas, but neither in normal adult liver nor most of the tissues, it may be an attractive target for antibody therapy. In this study, we established many monoclonal antibodies against hDlk-1, and now we are developing monoclonal antibodies against hDlk-1 that block proliferation of HCC in a xenograft model.

Conflict of interest
None declared.

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Correlating Phosphatidylinositol 3-Kinase Inhibitor Efficacy with Signaling Pathway Status: *In silico* and Biological Evaluations

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Abstract

The phosphatidylinositol 3-kinase (PI3K) pathway is frequently activated in human cancers, and several agents targeting this pathway including PI3K/Akt/mammalian target of rapamycin inhibitors have recently entered clinical trials. One question is whether the efficacy of a PI3K pathway inhibitor can be predicted based on the activation status of pathway members. In this study, we examined the mutation, expression, and phosphorylation status of PI3K and Ras pathway members in a panel of 39 pharmacologically well-characterized human cancer cell lines (JFCR39). Additionally, we evaluated the *in vitro* efficacy of 25 PI3K pathway inhibitors in addition to conventional anticancer drugs, combining these data to construct an integrated database of pathway activation status and drug efficacies (JFCR39-DB). *In silico* analysis of JFCR39-DB enabled us to evaluate correlations between the status of pathway members and the efficacy of PI3K inhibitors. For example, phospho-Akt and KRAS/BRAF mutations prominently correlated with the efficacy and the inefficacy of PI3K inhibitors, respectively, whereas *PIK3CA* mutation and PTEN loss did not. These correlations were confirmed in human tumor xenografts *in vivo*, consistent with their ability to serve as predictive biomarkers. Our findings show that JFCR39-DB is a useful tool to identify predictive biomarkers and to study the molecular pharmacology of the PI3K pathway in cancer. *Cancer Res; 70(12); 4982–94. ©2010 AACR.*

Introduction

Phosphatidylinositol 3-kinases (PI3K) are lipid kinases that phosphorylate phosphoinositide at position D3 of the inositol ring (1, 2). The catalytic subunit of class I PI3K is composed of four isoforms (p110α, p110β, p110δ, and p110γ, encoded by *PIK3CA, PIK3CB, PIK3CD*, and *PIK3CG*). Among these isoforms, *PIK3CA* is often activated in cancer by gain-of-function hotspot mutations (3, 4) and gene amplification (5, 6). On the other hand, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a lipid phosphatase that dephosphorylates PIP3 at position D3 of the inositol ring to generate PIP2. *PTEN* has been shown to be a tumor suppressor gene and is often inactivated by deletion or mutation in cancer (7–9). Activation of PI3K and PTEN loss trigger sequential phosphorylation of the PI3K downstream signal cascade, including Akt and mammalian target of rapamycin (mTOR), and mediates a survival signal, as well as tumor proliferation (9). Therefore, the PI3K pathway is thought to be a promising therapeutic target.

LY294002 and wortmannin are first-generation PI3K inhibitors, but neither has progressed to clinical trials because of cytotoxicity to liver and skin (10, 11). We previously reported a selective PI3K inhibitor, ZSTK474, which has potent antitumor activity and low toxicity *in vivo* (12). Subsequently, several PI3K inhibitors have been developed, and some including NVP-BEZ235 and GDC-0941 have already entered clinical trials (13–15). In addition to PI3K inhibitors, antitumor compounds targeting Akt and mTOR have been developed (15–18).

To develop a molecular-targeted anticancer drug, it is highly desirable to develop predictive biomarkers for stratifying patients susceptible to the drug. In fact, mutations of *EGFR* and *KRAS* are used to predict the efficacy of gefitinib in lung cancer and that of cetuximab in colorectal cancer, respectively (19–22). Moreover, Engelman and colleagues recently showed that tumor growth triggered by KRAS mutant exhibited resistance to NVP-BEZ235 (23). On the other hand, dysregulation of *PIK3CA* and PTEN has been reported to be involved in resistance to EGFR-targeted therapies (24–26). As mentioned above, *PIK3CA* mutation and PTEN loss trigger

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activation of the kinase cascade of the PI3K downstream pathway. However, it is not well understood whether the efficacy of PI3K pathway inhibitors could be predicted by these upstream abnormalities and downstream activities of the PI3K pathway.

We previously established a panel of 39 human cancer cell lines (JFCR39) derived from various organs (27–29). JFCR39, as well as NCI60 developed by National Cancer Institute, has been used as an *in vitro* tool to measure "fingerprints" of cytotoxic compounds. Fingerprints are defined as the patterns of differential drug efficacy across a panel of cell lines and have been found to reflect mechanisms of drug action (27–30). ZSTK474 is a compound that we identified as a new PI3K inhibitor based on the similarity with the fingerprint of LY294002 (12). In other words, each of the JFCR39 cell lines showed similar responses to ZSTK474 and LY294002, and the responses varied from cell line to cell line. This suggested that the status of biological pathways determining a cancer cell's response to PI3K inhibitors (which may include the PI3K pathway) is heterogeneous across these cell lines.

In the present study, taking advantage of JFCR39, we examined the mutation and/or expression of upstream regulators of the PI3K and Ras pathways, including four PI3K isoforms (*PIK3CA, PIK3CB, PIK3CD*, and *PIK3CG*), *PTEN*, the ERBB family receptor tyrosine kinases (RTKs), *KRAS* and *BRAF*, and the phosphorylation of downstream pathway members including Akt, TSC2, GSK-3, mTOR, S6K1, mitogen-activated protein kinase (MAPK)/extracellualr signal–regulated kinase (ERK) kinase (MEK)-1/2, and ERK1/2. Next, we examined the fingerprints of 25 compounds targeting the PI3K pathway as well as other conventional anticancer drugs. We then combined the PI3K and Ras pathway database and the drug database to develop an integrated database of pathway status and drug efficacies (JFCR39-DB). Using this database, we first evaluated the functional relationships among these pathway inhibitors and those among drugs by comparing their fingerprints. Second, we evaluated the correlation between PI3K pathway members and PI3K pathway inhibitors to identify candidate biomarkers for predicting their efficacy.

Materials and Methods

Cell lines and cell culture

A panel of 39 human cancer cell lines, termed JFCR39, were previously described (27, 28, 31). Cells were grown in RPMI 1640 (Wako Pure Chemical Industries Ltd.) supplemented with 1 μg/mL kanamycin and 5% (v/v) fetal bovine serum (Moregate Exports) and incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. Authentication of cell lines was done by short tandem repeat analysis using PowerPlex16 Systems (Promega; data not shown).

Amplification of genomic DNA fragments for sequencing

Extraction of genomic DNA was done using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Amplification of genomic DNA fragments was done using Pfu Ultra High-Fidelity DNA polymerase (Agilent Technologies), FastStart High Fidelity PCR System (Roche Applied Science), or AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen). Polymerases and primer sequences used in each reaction are shown in Supplementary Table S1.

Nucleotide sequence analysis

Sequencing reactions were done using BigDye Terminator v3.1 and dGTP BigDye Terminator v3.0 (Applied Biosystems) according to the manufacturer's instructions. Primers were shown in Supplementary Table S1. Nucleotide sequences were analyzed using a 3130 Genetic Analyzer (Applied Biosystems) and sequence files were edited using 4 Peaks software (Mekentosj B.V.).

Detection of lipid kinase activities of PI3K p110α mutants

Determination of lipid kinase activities of PI3K p110α was described as previously (32). In brief, HEK293T cells were transfected with pFLAG-PIK3CA (with or without mutation) and pc-PIK3R1 using Lipofectamine 2000 (Invitrogen). After 48 hours of incubation, cells were harvested and the lysates were immunoprecipitated by using FLAG-Tagged Protein Immunoprecipitation Kit (Sigma-Aldrich). To detect kinase activity, we used the PI3K-HTRF assay kit (Millipore) and EnVision 2103 Mutilabel Reader (Perkin-Elmer).

Preparation of total cell extract

Cells were resuspended in lysis buffer [10 mmol/L Tris-HCl (pH 7.4)], 50 mmol/L NaCl, 0.5% w/v NP40, 0.1% w/v SDS, 50 mmol/L sodium fluoride, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium orthavanadate, 5 mmol/L EDTA, 0.1 trypsin inhibitor unit/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride] and lysed by sonication in an ice bath. Concentrations of proteins in the extracts were determined using a protein assay kit (Pierce).

Immunoblot analysis

Equal amounts of protein were subjected to SDS-PAGE and the separated proteins were transferred onto an Immobilon FL polyvinylidene difluoride membrane (Millipore). The membrane was incubated with a primary antibody. The antibodies for PI3K p110α, Akt, phospho-Akt (T308, S473), phospho-GSK-3 (S9), phospho-TSC2 (T1462), phospho-mTOR (S2448), phospho-S6K1 (T389), phospho-MEK1 (S217/S221), and phospho-ERK1/2 (T202/Y204) were purchased from Cell Signaling Technologies. The antibodies for PI3K p110β, p110γ, and p110δ were purchased from Millipore. The antibody for PTEN was purchased from BD Biosciences Pharmingen. Bound antibody was quantitatively detected using an appropriate antimouse or rabbit immunoglobulin secondary antibody labeled with Alexa Fluor 680 (Invitrogen) and the Odyssey Infrared Imaging System (LI-COR). Data shown are median values of three independent experiments.