

Fig. 3 **a** Unsupervised hierarchical clustering of 64 lung cancer and 30 normal lung samples against 2,803 genes with expression differentially regulated in neuroendocrine tumors. *HGNT* high grade neuroendocrine tumor, *TC/AC* typical carcinoids/atypical carcinoids, *AD* adenocarcinoma, *LCC* large cell carcinoma. **b** Kaplan-Meier

survival curves for patients with HGNTs in group 2 and all other HGNTs and for histopathologically diagnosed SCLC versus LCNEC. SCLC, small-cell lung carcinoma; LCNEC, large-cell neuroendocrine carcinoma

lines [15]. After excluding genes which show clear distinction between fresh and cell-line samples, hierarchical clustering resulted in a large degree of integration of cell lines into four main tumor branches, an SCLC branch, a SCC branch, a cell-line branch, and a branch containing normal tissue, adenocarcinoma and LCC (Fig. 4). As a result, most of SCC cell lines or SCLC cell lines grouped with fresh SCC tumors or fresh SCLC tumors, respectively. In contrast, although none of adenocarcinoma cell lines clustered with fresh adenocarcinoma tumors, some of them clustered with fresh SCC tumors or fresh SCLC tumors. Adenocarcinomas may ultimately progress toward one of two poorly differentiated phenotypes with expression profiles resembling SCC or SCLC. Our observations suggest that adenocarcinoma cell lines either dedifferentiate toward molecular pathologies resembling SCLC or SCC, or that clonal expansion of SCC or SCLC subcomponents occurs frequently. Analysis of larger numbers of adenocarcinoma samples taken at the time of surgery and autopsy will be

required to verify that adenocarcinomas develop similarly in situ.

Comparison of accumulated allele loss between primary lung tumors and lymph node metastases

Sasatomi et al. [16] have compared loss of heterozygosity (LOH) at microsatellites between primary NSCLCs and their lymph node metastases, calculating fractional allele loss (FAL), defined as the ratio of chromosomes affected by LOH in the informative chromosomes, for each sample. With Stage II NSCLCs, the FAL was found to be significantly less in the metastatic sites compared with the primary neoplasms. The authors advanced the theory that this phenomenon was the result of early metastatic spread of the carcinoma, with the primary neoplasm then acquiring additional genetic changes. This concept should be borne in mind when comparing molecular profiles of

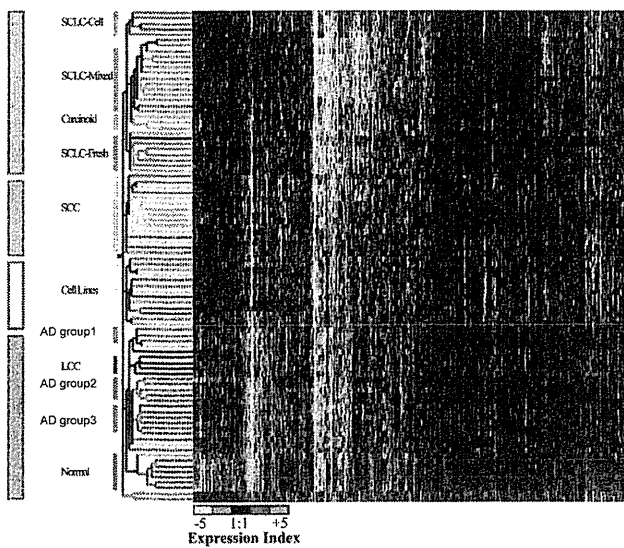


Fig. 4 Dendrogram of the reduced data set of 4,253 genes after filtering for commonly regulated genes in either fresh or cell-line samples. Groupings indicated on the *left* represent distinct clusters of particular carcinoma types. AD, adenocarcinoma; Normal, normal lung tissue

primary neoplasms with those of metastatic or recurrent sites.

Gene expression profile for tissue-specific metastasis

Kang et al. [17] have identified, in a human breast cancer cell line, a specific set of genes that mediates metastasis to bone. They suggested that primary tumors with metastatic capacity possess the poor-prognosis signature but, additional functions, provided by a set of bone metastasis genes, must be expressed in order to achieve an overt, tissue-specific metastasis phenotype. Organ-specific expression profiles for human small-cell lung cancer metastases in mice have also been reported by Kakiuchi et al. [18], but it remains unclear whether these might already be present in the parental cells.

Heterogeneity of primary tumors and metastatic potential

Introduction

Recent microarray experiments have suggested that the majority of cancer cells have the potential to metastasize, with obvious clinical and therapeutic implications, not only in breast cancers [19], but also in lung cancers [7]. Ramaswamy et al. [7] identified a molecular signature of metastatic potential within the bulk of each primary lung cancer, suggesting that metastatic potential is in fact

acquired early and is a feature of the majority of lung cancer cells. To test this hypothesis, we adopted the lung adenocarcinoma, which characteristically shows widespread intratumoral heterogeneity, as a model [3].

The mixed type adenocarcinoma in the lung, which shows a variety of histological subtypes, is the most frequent subtype in the WHO classification criteria [13], accounting for approximately 80% of resected lung adenocarcinomas [20]. An invasive component with high cellular and structural atypia is often included but peripherally well-differentiated components with low atypia may also be present (Fig. 5). Is the metastatic signature detected only in the aggressive component with high atypia? Or is it present in the entire tumor irrespective of morphological heterogeneity? If the latter is true, then it follows that the metastatic potential is acquired early in tumor progression and the entire tumor, including the morphologically less malignant component, may have metastatic potential.

Using lymph node-positive lung adenocarcinomas, we compared gene expression profiles among moderately-differentiated components with aggressive appearance, peripheral well-differentiated components with less malignant appearance, and patient-matched lymph node metastases. Node-negative lung adenocarcinomas, which are morphologically indistinguishable from node-positive tumors, were included for comparison and differential diagnosis.

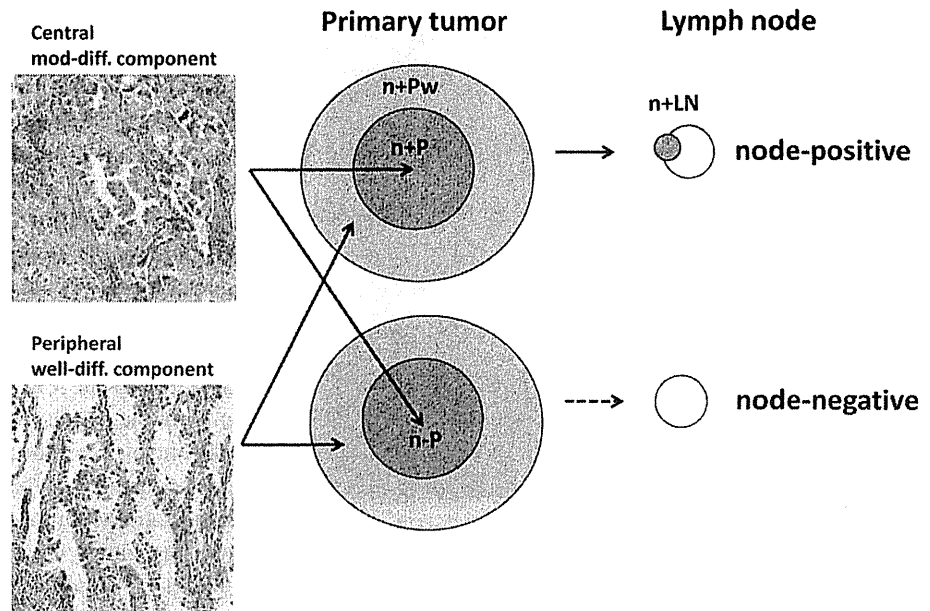
Schematic design

The schematic design for this study is shown in Fig. 5. We analyzed 10 pairs of primary lung adenocarcinomas and their synchronous lymph node metastases and 11 primary lung adenocarcinomas without lymph node or organ metastases. With the 21 primary lung adenocarcinomas, we isolated tumor cells from predominant moderately-differentiated components. In five of the 10 node-positive primary lung adenocarcinomas, we additionally isolated peripheral well-differentiated components. To focus selectively on cancer cells, we applied laser capture microdissection (LCM). For comparison, we included six samples of macrodissected normal lung tissue.

Experiments and results

Firstly, we wished to identify the overall gene expression signature in all 42 samples by unsupervised hierarchical clustering with a set of highly variable genes. Two-way hierarchical clustering was performed using a Pearson correlation (Fig. 6). Eight of the 10 pairs of primary and metastatic tumors clustered next to each other. In these cases, the metastatic tumors had a higher similarity to their matching primary tumors than to all other tumors. In only two of the node-positive cases (cases 5 and 9), the metastatic tumors did

Fig. 5 Schematic design of this study. n + P, node-positive primary tumor; n-P, node-negative primary tumor; n + LN, node-positive lymph node; n + Pw, well-differentiated component of the node-positive primary tumor. Representative microscopic images of primary lung adenocarcinomas used in this study are shown, comprising moderately-differentiated components dominating large portions of the lesions and well-differentiated components evident in peripheral portions



not cluster with their matching primary tumors, but a substantial similarity was still observed. In addition, all the peripheral well-differentiated components showed tight clustering with the predominant moderately-differentiated components from the same primary tumors. In the overall gene expression signatures, the central and the peripheral components from the same primary tumor were strikingly similar to each other.

To identify the gene expression signature of lymph node metastases, we performed a statistical comparison between the 10 pairs of primary and metastatic tumors using a paired *t*-statistic. Only 12 genes were yielded. For 11 of these genes, no significant differences were found between 10 lymph node metastases and 11 node-negative primary tumors, suggesting that they are not part of a metastatic expression signature. Only one gene, *Chromosome 4 open reading frame 7 (C4orf7)* was significantly higher in the lymph node metastases than both node-positive and -negative primary tumors. However, as *C4orf7* is expressed characteristically by follicular dendritic cells found in lymph nodes, the high expression presumably resulted from contamination in the LCM process. It follows that no significant metastatic changes were detected between primary tumors and their lymph node metastases. The marked similarity between primary tumors and their lymph node metastases drove us to consider that the gene expression signature of lymph node metastasis might be acquired by the majority of primary tumor cells.

Next, to identify the metastatic expression signature detected in the primary tumors, we performed a statistical comparison (Welch's *t*-test) between 10 node-positive primary tumors and 11 node-negative primary tumors. This

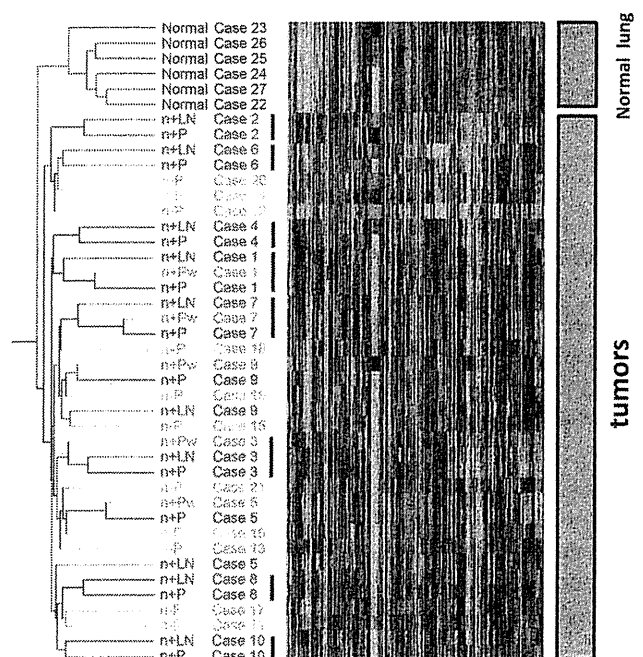


Fig. 6 Unsupervised hierarchical clustering for 2,451 genes against 42 samples comprising 10 pairs of node-positive primary tumors (n + P) and node-positive lymph nodes (n + LN), 11 node-negative primary tumors (n-P), 5 well-differentiated components of the node-positive primary tumors (n + Pw), and 6 normal tissues (Normal). Columns represent gene and rows represent samples. Note that eight of the 10 pairs of primary and metastatic tumors clustered next to each other (bars)

yielded 75 genes, comprising 37 with significantly higher expression in node-positive than node-negative primary tumors and 38 with significantly lower expression. The top discriminating gene was *homeobox B2 (HOXB2)* with

In the 75-gene set we identified, *HOXB2* was the top discriminating gene between node-positive and node-negative primary lung adenocarcinomas. Aberrant expression of *HOX* genes has been implicated in leukemias and various solid cancers, including lung cancers, with likely involvement of the gene products in features of malignant progression, such as invasion and metastasis. Overexpression of *HOXD3* is known to induce coordinate expression of metastasis-related genes in lung cancer cells [26]. A recent study further revealed ectopic *HOXB2* expression in pancreatic cancers and some proportion of precursor lesions, pancreatic intraepithelial neoplasias, possibly associated with a poor prognosis [21]. In our current study, we observed *HOXB2* overexpression not only in the central but also in the peripheral zones of node-positive primary tumors. Malignant potential associated with *HOXB2* expression might thus be acquired early in tumorigenesis.

Our results imply that the metastatic potential might be encoded in the entirety of each primary lung tumor including the morphologically less malignant component. This has profound clinical implications. It offers a rationale for therapeutic applications based on the expression profile of the primary tumor. Even if the peripheral component of the tumor is sampled by bronchoscopic or needle biopsy, the metastatic potential of the tumor could be predicted with accuracy. Such evaluation of metastatic potential would help spare unnecessary lymph node dissection for low-risk patients. However, the 75-gene signature needs to be confirmed using independent samples and further research is required to clarify the included molecular functions.

Very recently, using real time RT-PCR analysis, we investigated the transcriptional levels of the top metastasis-related genes using 96 independent test lung adenocarcinoma samples and investigated their correlations with prognosis [27]. We could document evidence that p-Stage I patients with *HOXB2* up-regulation have a worse prognosis than those with *HOXB2* down-regulation ($P = 0.0065$). Comparing tumors and corresponding normal lung tissue, we confirmed *HOXB2* up-regulated lesions to have much higher *HOXB2* expression than the corresponding normal tissue.

In conclusion

Recent studies support the hypothesis that metastatic potential is acquired early in tumorigenesis and that the majority of tumor cells have the potential to metastasize. Although gene expression profiles that can classify cancer patients according to the risk of recurrence have been found in many studies, most of these were retrospective. Very recently, by gene expression profiling, Potti et al. [9] proposed a “lung metagene model” that could identify individuals at increased risk for disease recurrence with

stage IA NSCLC. They are now planning to use this for a prospective randomized clinical trial. In the future, more emphasis needs to be placed on translational research to enable basic research findings to be applied in the clinic.

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Prognostic Heterogeneity in Multilevel N2 Non-Small Cell Lung Cancer Patients: Importance of Lymphadenopathy and Occult Intrapulmonary Metastases

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Background. To evaluate prognostic heterogeneity that may exist in multilevel N2 non-small lung cancer, we attempted to identify clinicopathologic prognostic factors for multilevel N2 patients who underwent standard surgeries.

Methods. We retrospectively evaluated records from 1988 to December 2007 for 106 non-small lung cancer patients diagnosed with multilevel N2 disease by post-operative pathologic examination (49 women, 57 men; median age = 61 years). Patients with clinical T4 (cT4) and bulky N2 (shortest mediastinal lymph node diameter >2 cm) disease were excluded from the study. Follow-up periods ranged from 2 to 240 months (median for living patients = 36 months). Records were examined for age, sex, preoperative nodal status (cN2 versus cN0 or cN1), primary tumor sites, surgical procedure, metastatic stations (distribution and numbers), tumor sizes, histologic features, and adjuvant therapies.

Results. By univariate analysis, cN (cN2), intrapulmonary metastases within the same lobe of the primary tumor (PM), and male sex were significant adverse prognostic factors; smoking only tended toward significance ($p = 0.1$). Other clinicopathologic variables were not significant prognostic factors. By multivariate analysis, cN (cN2) and PM were significant prognostic factors. Patients who had neither cN2 nor PM had significantly higher survival rates than those who had either cN2 or PM (5-year survival rates of 36.5% and 11.2%, respectively).

Conclusions. Multilevel N2 patients can be grouped according to the prognostic factors cN2 and PM. These findings have potential for evaluating the best therapeutic modalities or agents for multilevel N2 patients.

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For non-small cell lung cancer (NSCLC) patients with p-N2, it has been reported that clinical factors, such as c-N (c-N2), skip N2 metastasis (non-skip N2), and the N2 level (multiple station metastases or multilevel N2), were associated with worse prognoses [1-6]. In particular, multilevel N2 is one of the established adverse prognostic factors for N2 patients [4-6]. It has been shown that multilevel N2 patients showed much poorer prognoses (9% to 23% for 5-year survivals) than those with single-level N2 (25% to 60% for 5-year survivals) [1-6].

Once patients are diagnosed with multilevel N2, they are considered for multimodal treatments as parts of some clinical studies. When we evaluate the therapeutic options for multilevel N2 patients, it is very important to consider the multiple prognostic factors that may exist in these groups, as they are typically very heterogeneous.

However, there have been few reports that have considered the prognostic factors focusing on multilevel N2 patients, mainly because of their poor outcomes.

Recently, a new diagnostic modality, real-time endobronchial ultrasonography-guided transbronchial needle aspiration cytology, has enabled the diagnosis of mediastinal lymph node metastasis in a less invasive manner than with previous methods [7]. This new modality has improved the accuracy for the diagnosis of mediastinal lymph node metastasis, even for patients without mediastinal lymph node adenopathy. Therefore, the proportion of NSCLC patients with multilevel N2, which would not have been detected before, has changed. The proportion of multilevel N2 patients without mediastinal lymphadenopathy must be especially increased in the population. It is unclear until now whether multilevel N2 patients without mediastinal lymphadenopathy show the same prognosis as patients with mediastinal lymphadenopathy.

To clarify prognostic heterogeneity that may exist in multilevel N2 NSCLC, we attempted to identify clinicopathologic prognostic factors for patients with pathologically proven multilevel N2 who had undergone standard

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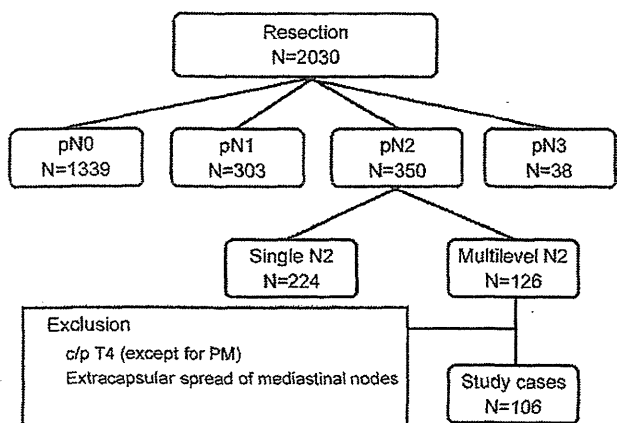


Fig 1. Diagram indicating study group subdivisions. Between 1988 and December 2007, 2,030 patients underwent surgical resections for primary lung cancer at the Cancer Institute Hospital. (PM = intrapulmonary metastases.)

surgeries. These results may provide opportunities to make more relevant evaluations of therapeutic strategies or new agents for multilevel N2 patients.

Patients and Methods

This was a retrospective study. As individual patients were not identified, our institutional review board waived the requirement to obtain patient consent and approval for this study.

Between 1988 and December 2007, 2,030 patients underwent surgical resections for primary lung cancer in the Cancer Institute Hospital. Among these patients, 350 were diagnosed with N2 disease after lung resection and hilar and mediastinal node dissections. Of these 350 patients, 106 were diagnosed as having multilevel N2 disease of NSCLC by postoperative pathologic examination (Fig 1). This subgroup included 49 women and 57 men whose ages ranged from 34 to 78 years (median, 61 years).

For all patients, preoperative staging was assessed according to the TNM classification of the International Union Against Cancer [8], using chest computed tomography (CT), abdominal CT or ultrasonography, brain CT or magnetic resonance imaging, and bone scans. Clinical mediastinal and hilar lymph node status was assessed as positive if the results of the chest CT showed that the shorter axis was longer than 1.0 cm. Clinical T4 (cT4) cases and bulky N2 (shortest mediastinal lymph node diameter >2 cm) were excluded from this study because they were receiving chemotherapy with or without radiotherapy according to the protocol in our institute. Therefore, patients with mediastinal lymph node adenopathy less than 2 cm were considered for surgery. However, mediastinoscopy, fluorodeoxyglucose-positron emission tomography, or endobronchial ultrasonography-guided transbronchial needle aspiration were applied to some patients in this series (shortest mediastinal lymph node diameter ≤2 cm); they were not used for preoperative staging in this series. Furthermore, patients with extra-

capsular spread of lymph node metastasis were excluded, as they often underwent incomplete resections. Follow-up periods ranged from 2 to 240 months (median follow-up for living patients was 36 months).

Mediastinal nodal status was assessed according to modifications of the system by Naruke and colleagues [2], and the mediastinal nodes were classified into seven stations. These were (1) 1; (2) 2 and 3; (3) 4; (4) 5 and 6; (5) 7; (6) 8; and (7) 9. Combinations of numbers 2 and 3 and numbers 5 and 6 were included as they were difficult to separate from each other in clinical practice. When mediastinal nodal involvements were found in two or more stations, cases were classified as multilevel N2.

Patient characteristics are summarized in Table 1. The clinicopathologic records of the patients were examined for age, sex, preoperative nodal status (cN2 versus cN0 or cN1), primary tumor sites, surgical procedure, metastatic stations (distribution and numbers), tumor size, histologic features (cell type, differentiation degree, intrapulmonary metastases), presence of intrapulmonary metastases in the same lobe of the primary tumor (PM), and history of adjuvant therapies.

Survival duration was defined as the interval between surgery and either death attributable to a tumor or the most recent follow-up. Survival rates were calculated using the Kaplan-Meier method. Univariate analyses were performed using a log-rank test, χ^2 test, and logistic regression. Multivariate analyses were performed for variables with probability values less than 0.1 from univariate analysis using the logistic regression test in StatView J 5.0 (SAS Institute, Cary, NC). A probability value less than 0.05 was considered significant.

Results

Survival Rates for Patients With Multilevel N2

The postoperative 5-year survival rate for patients with multilevel N2 was 23%, and the 50% survival period was 26 months.

Table 1. Patient Characteristics

Age (y)	34-78, median age: 61
Sex (male/female)	57/49
c-N	
N0/N1/N2	50/21/35
c-T	
T1/T2/T3	40/54/12
Histologic type	
Adenocarcinoma/others	86/20
Poorly differentiated/others	31/75
Primary site	
Right: upper/middle/lower	36/7/23
Left: upper/lower	25/15
Surgical procedure	
Lobectomy/bilobectomy/ pneumonectomy	74/19/13
Adjuvant therapy	
Chemotherapy: yes/ no	35/71

Table 2. Postoperative Survival According to Clinicopathologic Factors: Univariate Analyses

Variables	5-Year Survival	p Value
Age		
≤ 70 (N = 86)/>70 (N = 20)	23.9%/13.0%	0.84
Sex		
Male (N = 57)/female (N = 49)	17.1%/27.5%	0.05
Tumor diameter		
<30 mm (N = 42)/>30 mm (N = 64)	24.9%/17.0%	0.66
Smoking status		
Never smoker (N = 51)/smoker (N = 55)	28.7%/16.4%	0.12
Histologic subtype		
Adenocarcinoma (N = 86)/others (N = 20)	23.8%/13.3%	0.26
Poorly differentiated (N = 31)/others (N = 75)	20.6%/22.5%	0.88
Primary tumor site		
Upper (N = 61)/others (N = 45)	26.3%/13.2%	0.41
Right (N = 66)/left (N = 40)	25.7%/14.0%	0.28
Surgical procedure		
Lobectomy (N = 74)/others (N = 32)	27.2%/12.9%	0.22
Pneumonectomy (N = 13)/others (N = 93)	8.0%/23.7%	0.37
cN		
cN0 (N = 50)/cN1-2 (N = 56)	30.0%/15.9%	0.12
cN0-1 (N = 71)/cN2 (N = 35)	26.0%/14.3%	0.03
Intrapulmonary metastasis (PM)		
Without PM (N = 77)/with PM (N = 29)	28.6%/7.2%	0.01
Metastatic mediastinal nodal stations		
2 stations (N = 57)/>2 stations (N = 49)	21.3%/22.5%	0.88
Either upper (aortic) or inferior (N = 63)/both (N = 43)	25.4%/16.2%	0.56
Adjuvant chemotherapy		
Yes (N = 35)/no (N = 71)	24.2%/21.7%	0.68
Period when surgery done		
1998-2007 (N = 44)/1988-1997 (N = 62)	15.3%/23.1%	0.52

Prognostic Factors for Multilevel N2

Univariate analyses using the variables listed in Table 2 showed that cN (cN2), PM, and sex (male) were significant adverse prognostic factors, whereas smoking status (smoker) only tended toward significance as an adverse prognostic factor ($p = 0.1$). Metastatic station (distribution and number), adjuvant therapy, the period during which surgery was done, primary tumor site, histologic subtype, tumor diameter, age, and surgical procedure were not significant prognostic factors.

By multivariate analysis ($p < 0.1$ by univariate analysis), cN (cN2) and PM were significant prognostic factors (Table 3).

Survival Rate According to the Prognostic Factors for Multilevel N2

The multilevel N2 patients were categorized as with or without significant prognostic factors determined from

Table 3. Prognostic Factors for Patients With Multilevel N2: Multivariate Analysis Model 1

Variables	Odds Ratio	95% CI	p Value
Sex (female)	0.62	0.34-1.14	0.12
Smoking status (smoker)	0.98	0.54-1.78	0.98
cN (N2)	1.61	1.01-2.58	0.04
PM (positive)	1.99	1.21-3.26	0.007

CI = confidence interval; PM = intrapulmonary metastases.

multivariate analyses. Patients who had neither cN2 nor PM showed a significantly higher survival rate than patients who had either cN2 or PM (5-year survival rates of 36.5% and 11.2%, respectively; Fig 2).

Comment

It is well known that patients who have NSCLC with ipsilateral mediastinal lymph node (N2) involvement are a heterogeneous group [9-12]. This heterogeneity involves multiple factors, such as preoperative detection, susceptibility to neoadjuvant treatment, clinically unsuspected N2 disease, and the level or site and number, or both, of mediastinal lymph nodes that are involved [13, 14]. Examples of factors to be considered include cN2 prognosis worse than the respective unsuspected pN2, single versus different multiple N2 stations, the number of involved lymph nodes, extracapsular spread, the presence of subcarinal node metastasis, and skip metastasis [15, 16]. Each of these subclassifications should be considered as a completely different subpopulation of positive mediastinal lymph nodes.

It has been shown that multilevel N2 patients have much poorer prognoses than those with single-level N2. Thus, there have been few studies that have considered prognostic factors for multilevel N2. Recently, the numbers of pathologically proven multilevel N2 cases before surgery have been increasing owing to the use of new diagnostic modalities, such as endobronchial ultrasonography-guided transbronchial needle aspiration. Further-

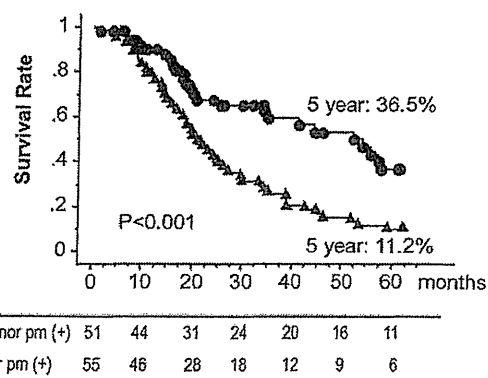


Fig 2. Overall 5-year survival rate for multilevel N2 patients (Pt) depends on cN and intrapulmonary metastases (pm). Survival curves were generated using the Kaplan-Meier method.

more, this multilevel N2 subpopulation of patients without mediastinal lymphadenopathy has been increasing. However, the impact of clinical N status on prognosis for multilevel N2 has been unclear.

We have demonstrated that cN2 and PM are important, poor-prognosis factors for multilevel N2 patients. The 5-year survival for cN0 multilevel N2 patients (N = 50) was much better than that of cN2 multilevel N2 patients (30.0% and 14.3%, respectively). In contrast, the numbers of metastatic stations or the distributions of metastatic stations were not prognostic factors for multilevel N2. Previous reports have shown that for patients with mediastinal lymph node metastases, cN2 was highly associated with unexpected N3, which would not be detected preoperatively by routine evaluations.

That is, for patients with mediastinal nodal involvement, more than 70% of patients with cN2 had unexpected N3, resulting in a poor prognosis. However, less than 20% of patients with cN0 or cN1 had unexpected N3 [4, 12]. Furthermore, it is well known that the prognosis for occult N2 metastasis is better than that for patients with clinical N2 disease after surgical resection [13]. Thus, clinical N status evaluated by CT (size criteria) was associated with prognosis, even for multilevel N2 patients.

Because we excluded bulky cN2 from an indication for surgery, and multilevel cN2 cases were also excluded from indications for surgery during the study period, 70% of multilevel N2 was diagnosed as cN0 or cN1 during preoperative examination using CT. Thus, more than a few patients without adenopathy must exist in the multilevel N2 population. Therefore, it is important for us to recognize the difference in prognosis between cN0 or cN1 and cN2 in multilevel N2.

Intrapulmonary metastasis within the same lobe of the primary tumor is an established poor prognostic factor and is classified as T4. The incidence of PM has been reported to be 8% to 9% in NSCLC patients who underwent resection [17, 18]. The incidence of PM according to nodal status was 3.7% in N0, 7.6% in N1, and 14.8% in N2 [17]. The incidence of PM was 27.4% in this series, and that result is compatible with the idea that multilevel N2 is a more advanced stage than single-level N2. Furthermore, PM was an adverse prognostic factor even for multilevel N2 patients. Therefore, PM should be taken into consideration as an important negative factor affecting prognosis when evaluating therapeutic strategies in multilevel N2 patients.

Limitations of the present study include the retrospective nature of the analysis and that routine adjuvant chemotherapy for N2 patients was started in 2006. Therefore, it was difficult to evaluate the effect of adjuvant chemotherapy on prognosis in this study.

In conclusion, multilevel N2 patients can be grouped according to their prognoses by the factors cN and PM.

These findings have potential for analyzing the best therapeutic modalities for multilevel N2 patients.

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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- μ m 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

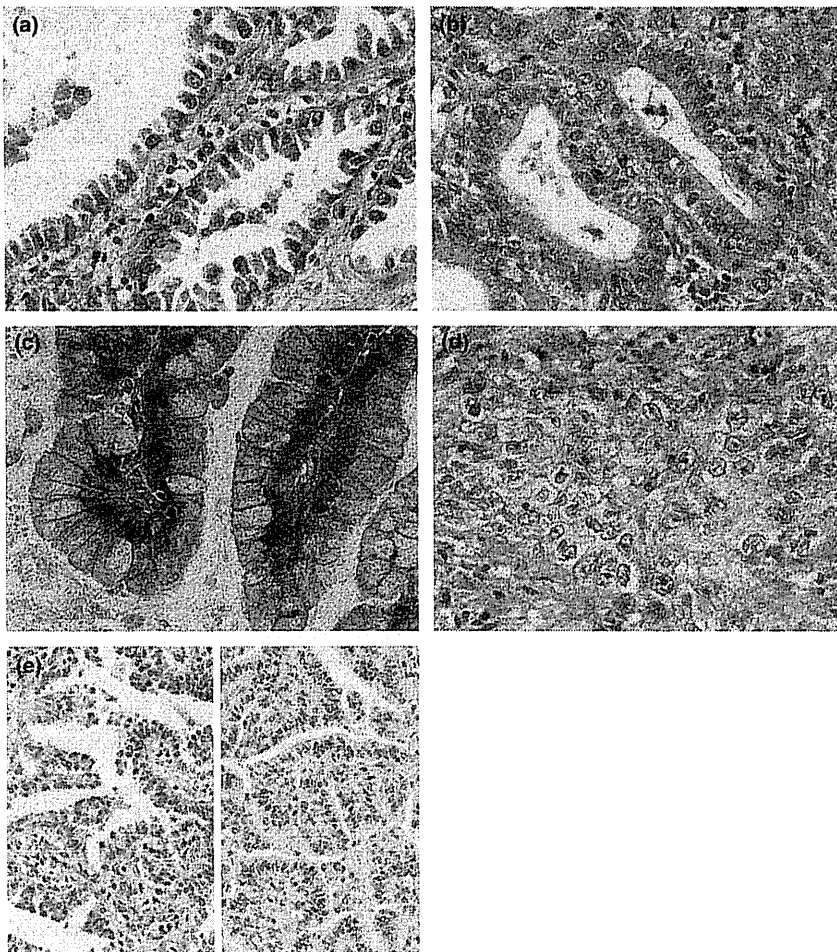


Fig. 1. Cell types of adenocarcinomas. (a) Hobnail cell type: epithelial cells look like Clara or type II pneumocyte cells. Apical portions protrude or bulge into the lumen. Note hobnail- or tadpole-shaped cells. (b) Columnar/cuboidal (col/cub) cell type: characterized by rather large columnar or cuboidal 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) $\times 400$; (e) $\times 200$.

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. ^aEGFR 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 sub-classified 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.

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

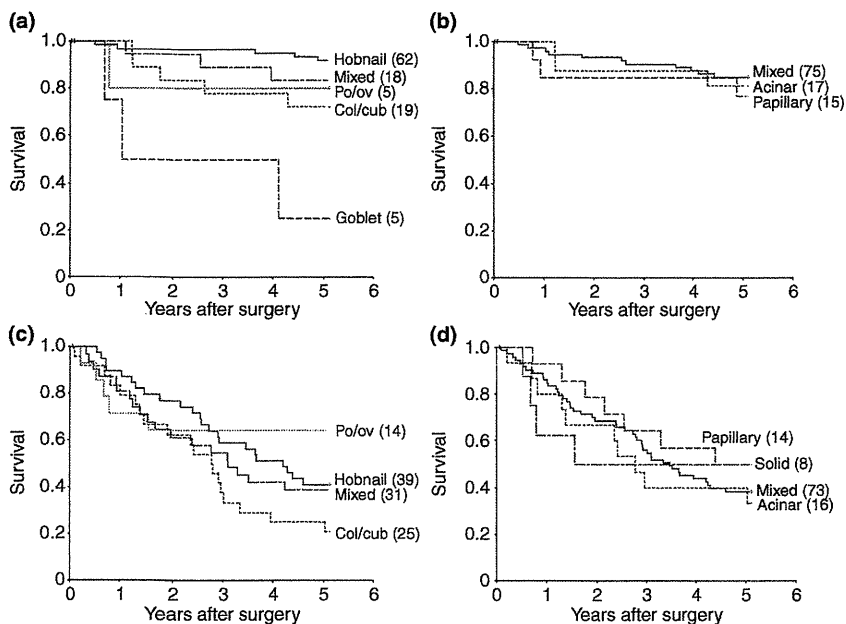


Fig. 2. Disease-specific Kaplan-Meier survival curves with respect to the cell type (a,c) and the World Health Organization (WHO) classifications (b,d) for p-stage I (a,b) and p-stages II-IV cases (c,d). Numbers in parentheses show numbers of patients. (a) The 5-year survival rate of the hobnail type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

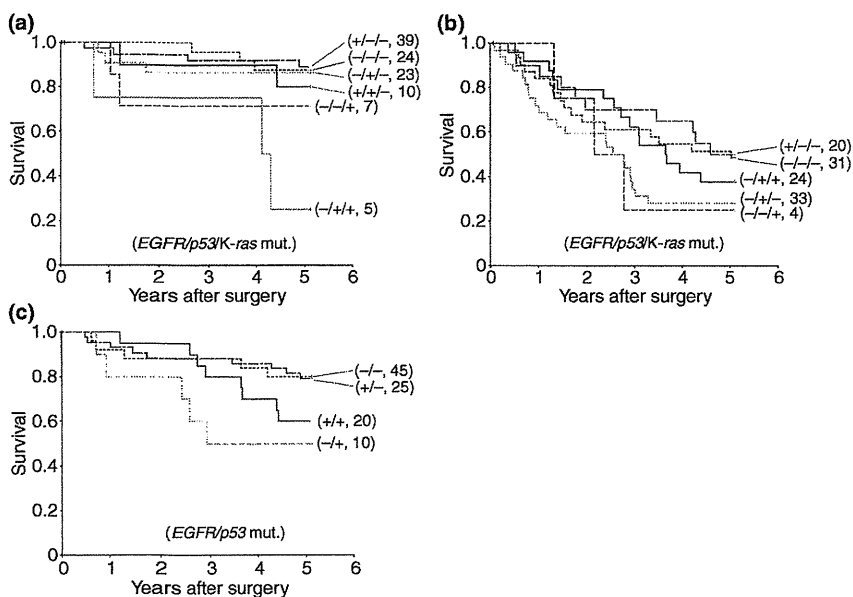


Fig. 3. Disease-specific Kaplan-Meier survival curves with respect to the mutational status for the three genes (*EGFR/p53/K-ras*) in p-stage I (a) and p-stages II-IV cases (b), and for two genes (*EGFR/p53*) in all p-stages for the hobnail cell type (c). The + or - indicate cases with or without mutations, 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) bronchioalveolar 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.

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

Genes	Pathological stages	No. of cases (%)		P-values
		Mutation		
		-	+	
<i>p53</i>	I	47 (76)	15 (24)	0.13*
	II-IV	24 (62)	15 (38)	
<i>EGFR</i>	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 $\geq 50\%$ positive reactivity was 72%, and that of $\leq 50\%$ 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.

Biological 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 \rightarrow T TV attributed to direct mutagenic action of tobacco smoke components, and CpG \rightarrow 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 \rightarrow 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 \rightarrow 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 ($\leq 13\%$),^(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 NTN, 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 NTN, 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 NTN.

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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-generator (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 gland (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