Table 1
Characteristics of the patients ($n = 193$) and differences* in survival rate according to each factor.

	N	5-Year survival (%)	95% CI	p-Value*
Patient and tumor characteristics				
Gender				
Male	96	71.6	0.61–0.80	0.027
Female	97	85.3	0.76–0.91	
Age				
<60	74	81.4	0.70–0.89	0.274
60 or older	119	76.5	0.67–0.83	
Smoking habit				
Never	89	83.2	0.73–0.90	0.151
Ever	104	74.3	0.64–0.82	
Stage				
I	120	94.8	0.89–0.98	<0.001
II–IV	73	48.7	0.36–0.61	
Adenocarcinoma classification				
Pre + minimally invasive**	44	96.5	0.83–0.99	<0.001
Invasive**	149	72.9	0.65–0.80	
Expression status (IHC study)				
pAkt				
–	120	85.3	0.77–0.91	0.007
+	73	68.2	0.56–0.79	
pERK				
–	124	73.8	0.65–0.81	0.058
+	69	86.4	0.76–0.93	
pGSK3B				
–	134	76.7	0.68–0.83	0.289
+	59	82.0	0.69–0.90	
pmTOR				
–	125	75.7	0.77–0.83	0.214
+	68	83.1	0.71–0.90	
pS6K				
–	92	74.1	0.63–0.82	0.260
+	101	82.3	0.73–0.89	
pFKHR				
–	115	85.6	0.77–0.91	0.006
+	78	68.1	0.56–0.77	
TTF-1				
–	39	49.4	0.32–0.65	<0.001
+	154	85.5	0.79–0.90	
Mutation status ($n = 93$)				
EGFR mutation				
–	39	69.2	0.52–0.81	0.175
+	54	84.8	0.72–0.92	
KRAS mutation				
–	86	78.7	0.68–0.86	0.774
+	7	71.4	0.26–0.92	

* Log-rank test (p-value); CI: confidence interval; N: lymph node metastasis.
** Pre + minimally invasive: adenocarcinoma in situ + lepidic pattern predominant adenocarcinoma with minimal invasion (<10% or ≤5 mm invasion); Invasive: adenocarcinoma of papillary (including micropapillary), acinar or solid pattern predominant and other variants; IHC: immunohistochemistry; TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3β: phosphorylated glycogen synthase kinase 3β; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

ubiquitous. The contrast between positive and negative cells was excellent. With the 10% cutoff, 35.8% (69/193) of the tumors were positive for pERK (Fig. 1).

The results of mutation analysis of exons 18–21 of *EGFR* and codons 12, 13 and 61 of *KRAS* are detailed in Suppl. Table 2. *EGFR* mutations were detected in 54 cases (58.1%), among which 90% were in exons 19 and 21. Mutations of *KRAS* were seen in seven cases (7/93, 7.5%), all of which were at codon 12. The *EGFR* and *KRAS* mutations were mutually exclusive except in one case. Of note, both pAkt and pERK were strongly stained in this case with double mutation of *EGFR* and *KRAS*. Types of *EGFR* mutation did not appear to affect the pattern of pathway activation. All but one of the cases with *KRAS* mutation were strongly positive for pERK ($p = 0.009$, Table 4), whereas the *EGFR* mutation did not correlate to pERK ($p = 0.294$) nor pAkt ($p = 0.409$) expression.

3.3. Patient survival

By univariate analyses using log-rank test, gender ($p = 0.027$), stage ($p < 0.001$), adenocarcinoma classification (invasive or not) ($p < 0.001$), expression of TTF-1 ($p < 0.001$) and cytoplasmic staining of pAkt ($p = 0.007$) and pFKHR ($p = 0.006$) were significantly related

Table 2
Multivariate analysis for factors predicting poor prognostic outcome ($n = 193$).

Variable	Relative risk	95% CI	p-Value
Cox regression analysis			
Patient and tumor characteristics			
Female	0.48911	0.25–0.93	0.030
Age (60 or older)	1.43993	0.75–2.78	0.268
Ex or current smoker	1.58799	0.84–3.00	0.147
Stages II–IV	10.96302	5.02–23.92	<0.001
Adenocarcinoma classification (invasive*)	1.81110	1.05–2.73	0.030
Expression status (IHC study)			
TTF-1+	0.24379	0.13–0.45	<0.001
pAkt+	2.28909	1.22–4.29	0.009
pERK+	0.50813	0.25–1.04	0.051
pGSK3B+	0.68778	0.34–1.40	0.289
pmTOR+	0.64731	0.32–1.29	0.204
pS6K+	0.70277	0.38–1.30	0.260
pFKHR+	2.34981	1.26–4.38	0.007
Mutation status ($n = 93$)			
EGFR mutation	0.56279	0.24–1.31	0.180
KRAS mutation	1.23578	0.29–5.29	0.780
Cox regression analysis with step wise selection			
Female	0.54	0.27–1.06	0.074
Stages II–IV	10.542	4.61–24.1	<0.001
pAkt+	2.268	1.17–4.38	0.015
pFKHR+	1.812	0.95–3.48	0.073
TTF-1+	0.282	0.15–0.54	<0.001

* CI: confidence interval; invasive: adenocarcinoma with a frankly invasive region; TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3β: phosphorylated glycogen synthase kinase 3β; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

to survival (Table 1). Multivariate analysis using the Cox's proportional hazard model revealed that stage ($p < 0.001$), expression of TTF-1 ($p < 0.001$) and pAkt ($p = 0.015$) are statistically significant factors for prognosis independent of any other conditions (Table 2).

3.4. Clinicopathological backgrounds of adenocarcinomas with Akt activation or TTF-1 expression

Since pAkt and TTF-1 expression was revealed to be independent prognostic factors, relationships between their positive status and other factors including clinicopathological characteristics and expression of other phosphorylated proteins are summarized in Table 3. pAkt expression was significantly associated with advanced stage (stage II-IV, $p = 0.021$) and lymph node metastasis ($p = 0.002$) but not with expression of any other signal proteins or TTF-1. TTF-1 expression, on the contrary, was significantly associated with never-smoker status ($p = 0.013$) and pre- or minimally invasive nature ($p < 0.001$) as well as with expression of pERK ($p = 0.039$) and pmTOR ($p = 0.014$). Also, TTF-1 expression was related to *EGFR* mutation ($p = 0.017$). Regarding invasiveness of tumor, the pAkt activation frequency tended to be lower in the pre- and minimally invasive adenocarcinomas (18.2%; 8/44) as compared with invasive types (43.6%; 65/149) and this tendency was highly and statistically significant ($p = 0.004$). In contrast, TTF-1 staining was more frequently seen among the pre- and minimally invasive adenocarcinomas ($p < 0.001$).

Correlation coefficients between each factor were shown in Table 4. The general tendency was largely in concert with results of survival analysis (Tables 1 and 2) and expression of Akt and TTF-1. Interestingly, activation of signal proteins of ERK had similar and statistically (or marginally) significant correlation to non-smoking ($p = 0.035$), stage I ($p = 0.029$) and noninvasive status ($p = 0.001$). GSK3B expression was related significantly to activation of ERK ($p = 0.001$), mTOR ($p < 0.001$) and S6K ($p = 0.018$). *EGFR* mutations were significantly associated with female gender ($p = 0.031$), non-smokers ($p = 0.016$), TTF-1 expression ($p = 0.008$) and pS6K expression ($p = 0.008$).

4. Discussion

By IHC applied to primary adenocarcinoma tissues and mouse xenografts of cell lines used as controls, we succeeded in demonstrating Akt activation to be an independent marker of poor prognosis. In addition, TTF-1, which is known to be a marker of type II cell differentiation, was also proved to be a significant favorable prognostic marker in correlation with activation of ERK and mTOR or *EGFR* mutation regardless of tumor stages. The expression of TTF-1 is important in outcome estimation of lung carcinoma at any tumor stage whereas Akt activation is abnormally affected according to the aggressiveness of the tumors regardless of their cell origin. While *EGFR* mutations had no correlation to activation of Akt or ERK pathways, six of seven cases with *KRAS* mutation were remarkably stained for pERK throughout the tumors. Finally, FKHR expression was established to be a marker for poor prognosis.

This study demonstrated that activated Akt was associated independently and significantly with poor prognosis ($p = 0.015$), which was in agreement with previous studies [14–19]. In fact, pAkt expression correlated with factors such as stages II-IV, positive lymph node metastasis and the invasive histology (Table 3), suggesting that Akt activation is an acquired characteristic according to tumor aggressiveness. To cast light on actual regulators and substrates of Akt that might mediate regulatory mechanisms in primary lung adenocarcinoma, we compared the activation status of Akt with upper and downstream components of the signal transduction. However our results somewhat differed from those obtained earlier with cell lines. For example, *EGFR* mutations in NSCLC cell lines were reported to selectively activate the Akt pathway [3], but in our primary tumors, this correlation between Akt and constitutively activated RTK was not significant ($p = 0.409$). Moreover, in our study, expression of pAkt did not correlate with activation of any of the downstream signal proteins such as GSK3B, mTOR, S6K and FKHR in the burgeoning list of Akt substrates implicated in oncogenesis [20,21]. Linkage of Akt with those activated downstream signal proteins has been confirmed in vitro, but the results in clinical samples have been inconsistent [18,22–24]. Certainly, Akt activation is an important factor for development and proliferation of cancer cells and perhaps a marker for targeted therapies, the exploration of this area especially in real tumors has definitely been inadequate and further study is needed.

Our multivariate analysis revealed that TTF-1 expression was also a statistically significant and independent prognostic factor ($p < 0.001$). TTF-1 is a regulator of normal lung development or maintenance of type II pneumocytes [25] and was expressed in 79.8% (154/193) of the cases. Recently, a model of lineage specific dependency on TTF-1 in a subset of adenocarcinoma, that is, the terminal respiratory unit (TRU) type adenocarcinoma, has been proposed [26]. In our study, TTF-1 staining was correlated with never-smoker ($p = 0.013$) and *EGFR* mutation status ($p = 0.039$) independently of tumor stage, which is consistent with the concept of lineage specific tumorigenesis in this subset of lung adenocarcinoma. These insights suggest the importance of the cell lineage that tumors were derived from in outcome estimation of lung carcinoma.

ERK was activated in 35.8% (69/193) of the cases in our study and was associated with pathologically early stages, the pre or minimally invasiveness and TTF-1 expression ($p = 0.039$). Normal tissues, such as type II pneumocytes or interstitial fibroblasts, were also positive with varying intensity (Fig. 1). Generally, ERK activation is known not only as the result of oncogenic dysregulation

Table 3
Backgrounds of patients with expression of a significant prognostic factors; pAkt and TTF-1 (n = 193).

	No. of cases with expression of pAkt and TTF-1 (%)				
	Total	pAkt	p-Value*	TTF-1	p-Value*
	193	73 (37.8)		154 (79.8)	
Patient and tumor characteristics					
Gender					
Male	96	33 (35.1)	0.371	73 (76.0)	0.214
Female	97	40 (42.1)		81 (83.5)	
Age					
<60	74	30 (40.5)	0.760	62 (83.8)	0.357
60 or over	119	43 (37.4)		92 (77.3)	
Smoking habit					
Never	89	37 (42.0)	0.374	78 (87.6)	0.013
Ever	104	36 (35.6)		76 (73.1)	
Stage					
I	120	37 (31.9)	0.021	99 (82.5)	0.269
II–IV	73	36 (49.3)		55 (75.3)	
N					
–	135	41 (31.3)	0.002	112 (83.0)	0.118
+	57	32 (56.1)		42 (73.7)	
Adenocarcinoma classification**					
Pre + minimally invasive	44	8 (18.2)	0.004***	44 (100.0)	<0.001
Invasive	149	65 (43.6)		110 (73.8)	
Pap-pred	118	52 (44.1)		91 (77.1)	
Acinar-pred	19	9 (47.4)		14 (73.7)	
Solid-pred and other variants	12	4 (33.3)		5 (41.7)	
Other IHC results					
pAkt					
–	120			94 (78.3)	0.851
+	73			58 (79.5)	
pERK					
–	124	51 (41.8)	0.275	93 (75.0)	0.039
+	69	22 (32.8)		61 (88.4)	
pGSK3B					
–	134	48 (36.6)	0.421	103 (76.9)	0.331
+	59	25 (43.1)		50 (84.7)	
pmTOR					
–	125	49 (39.2)	0.754	93 (74.4)	0.014
+	68	24 (35.3)		61 (89.7)	
pS6K					
–	92	39 (42.4)	0.370	73 (79.3)	1.000
+	101	34 (33.7)		81 (80.2)	
pFKHR					
–	115	39 (33.9)	0.879	87 (75.7)	0.101
+	78	34 (43.6)		67 (85.9)	
TTF-1					
–	39	15 (38.4)	0.851		
+	154	58 (37.7)			
Mutation status (n = 93)					
EGFR mutation					
–	39	16 (41.0)	0.409	26 (66.7)	0.017
+	54	27 (50.0)		48 (88.9)	
KRAS mutation					
–	86	41 (47.7)	0.445	70 (81.4)	0.148
+	7	2 (28.6)		4 (57.1)	

* Results of Fisher's exact test; pAkt: phosphorylated Akt; TTF-1: thyroid transcription factor-1; N; lymph node metastasis.
** Adenocarcinoma classification; see text for details; -pred: predominant; Pap: papillary pattern including micropapillary pattern; Acinar: acinar pattern; Solid: solid with mucin formation pattern.
*** Comparison between "Preinvasive + minimally invasive" vs other "invasive" carcinoma; IHC: immunohistochemistry; pERK: phosphorylated extracellular signal-regulated kinase; pGSK3B: phosphorylated glycogen synthase kinase 3B; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

Table 4
Correlation analysis of clinicopathological data, expression of phosphoryated proteins and mutation status.

	Gender [Female]	Age [60≦]	Smoking habit [Ever]	Stage [II-IV]	Adenocarcinoma Classification [Invasive]	TTF-1 [+]	pAkt [+]	pERK [+]	pGSK3B [+]	pmTOR [+]	pS6K [+]	pFKHR [+]	*EGFR* mutation
N=193													
Age [60≦]	-0.049	1											
Smoking habit [Ever]	-0.630 (<0.001)	0.011	1										
Stage [II-IV]	-0.063	-0.028	0.079	1									
Adenocarcinoma Classification [Invasive]	-0.021	0.058	-0.191	0.252 (<0.001)	1								
TTF-1 [+]	0.110	-0.058	-0.191 (0.009)	-0.087	-0.153 (0.039)	1							
pAkt [+]	0.083	-0.048	-0.069	0.185 (0.012)	0.107	-0.041	1						
pERK [+]	0.107	0.138	-0.156 (0.035)	-0.161 (0.029)	-0.235 (0.001)	0.178 (0.016)	-0.006	1					
pGSK3B [+]	0.098	-0.014	-0.119	-0.083	0.008	0.073	0.064	0.250 (<0.001)	1				
pmTOR [+]	0.164 (0.026)	-0.107	-0.213 (0.004)	-0.011	-0.160 (0.003)	0.203 (0.006)	-0.036	0.246 (<0.001)	0.234 (0.001)	1			
pS6K [+]	0.279 (<0.001)	0.063	-0.257 (<0.001)	-0.086	-0.134 (0.072)	0.054	-0.049	0.175 (0.018)	0.241 (0.001)	0.233 (0.001)	1		
pFKHR [+]	-0.029	-0.048	0.087	0.140	0.261 (<0.001)	-0.099	0.020	-0.138	0.040	-0.036	-0.096	1	
N=93													
EGFR mutation	0.224 (0.031)	0.040	-0.249 (0.016)	0 (1.000)	0.063	0.272 (0.008)	0.089	0.112	0.111	-0.005	0.274 (0.008)	-0.181	1
KRAS mutation	0.032	-0.024	0.125	-0.115	-0.012	-0.159	-0.101	0.268 (0.009)	0.051	0.095	0.044	0.072	-0.253 (0.014)

Correlation coefficients were calculated assuming the conditions described in the parenthesis ( ) to be observed. For abbreviations, see text. Coefficients values highlighted by underline imply statistical significance. Numbers in ( ) are *p*-values. For details of invasiveness, see text. Darkly shadowed cells imply significantly positive correlation and weakly shadowed cells imply significantly (or marginally) negative correlation. TTF-1: thyroid transcription factor-1; pAkt: phosphorylated Akt; pERK; phosphorylated extracellular signal-regulated kinase; pGSK3B: phosphorylated glycogen synthase kinase 3B; pmTOR: phosphorylated mammalian target of rapamycin; pS6K: phosphorylated ribosomal protein S6 kinase; pFKHR: phosphorylated forkhead transcription factors.

but also as an essential component of epithelial cell development or adaptation to changing circumstances [27–29]. Moreover, ERK regulation depends on very complex mechanisms, involving several intracellular parameters [30], timing or balance of the signals [31–34] and other unknown factors [35]. Considering these arguments, we may conjecture that ERK activation in lung adenocarcinoma mostly reflects the intracellular signal transduction of normal cells, which is still preserved within early-staged adenocarcinoma especially with TTF-1 expression, and that the ERK pathway is gradually switched off as the tumor cells progress to a more malignant phenotype. Also, these results shown here is in line with the recent studies of American cases [36], where the ERK pathway was more activated in earlier stages and the Akt pathway in advanced stages. In further studies, considering significant heterogeneity of ERK activation, we may use more detailed judgment criteria for immunoreactivity and whole sections of tumor, rather than tissue microarrays.

Six of seven cases with *KRAS* mutation were remarkably stained for pERK throughout the tumors, consistent with previous reports [37,38]. It is notable that the clinical impact of such *KRAS* mutation-induced ERK activation is enormous since it has already been shown in mice models that tumors with ERK activation due to *KRAS* or *BRAF* mutations can be successfully treated by an inhibitor of MEK, a signal protein upstream of ERK [39]. Our results imply so far the presence of at least two causes for ERK activation in lung adenocarcinoma, one is the vestige of normal intracellular signal and the other is the impact of *KRAS* mutation.

Two other supplementary implications were obtained from this study. Among downstream proteins of the Akt pathway, only S6K was significantly expressed in cases with the *EGFR* mutation ($p = 0.008$) and may potentially be an alternative marker for *EGFR* mutation. S6K is known to regulate ribosomal biogenesis and to play an important role in progression of G1 phase of the cell cycle [41,42]. This correlation between *EGFR* and S6K suggests again a cross talk between the Akt and ERK pathways and similar result was previously described by Conde et al. [40]. Our results also indicated cytoplasmic localization without intranuclear accumulation of pFKHR protein to be an adverse prognostic factor. FKHR is a member of a transcription factor family and represents a mammalian counterpart of DAF16, first identified at chromosomal breakpoints in human tumors [20]. Subcellular localization of FKHR is known to play an important role by regulating cell cycle and apoptosis in normal cell, which is consistent with our result. Further accumulation of cases will be needed to confirm those possibilities.

As a reference for IHC evaluation we here used mouse xenografts selected from a cell line panel repeatedly used in drug research [12,43]. This resulted in more accurate and reproducible evaluation of protein expression, implying the usefulness of tumor xenografts for clinical researches.

Conflict of interest statement

Kengo Takeuchi is a consultant for DAKO.

Acknowledgements

We thank Dr. Michiyo Okui for discussion, Ms. Kazuko Yokokawa, Ms. Miyuki Kogure, Mr. Motoyoshi Iwakoshi, Ms. Tomoyo Kakita and Ms. Yuki Togashi for their technical assistance, and Ms. Yuki Takano and Ms. Hiroko Nagano for secretarial help.

Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, from the Japan Society for the Promotion of Science, grants from the Ministry of Health, Labour and Welfare, the Smoking Research Foundation, the National Institute of Biomedical Innovation, and the Vehicle Racing Commemorative Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2010.01.001.

References

[1] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
[2] Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial). J Clin Oncol 2003;21:2237–46 [corrected].
[3] Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.
[4] Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
[5] Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153–83.
[6] Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 1997;9:180–6.
[7] Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. Proc Natl Acad Sci USA 2001;98:10983–5.
[8] Burgering BM, Coffer PJ. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 1995;376:599–602.
[9] Virtanen C, Ishikawa Y, Honjoh D, Kimura M, Shimane M, Miyoshi T, et al. Integrated classification of lung tumors and cell lines by expression profiling. Proc Natl Acad Sci USA 2002;99:12357–62.
[10] Sobin LH, Wittekind C, editors. International Union Against Cancer: TNM classification of malignant tumors. 5th ed. New York, NY: Wiley-Liss; 1997. p. 91–7.
[11] Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC. Pathology and genetics of tumours of lung, pleura, thymus and heart. Lyon, France: IARC; 2004.
[12] Yamori T, Matsunaga A, Sato S, Yamazaki K, Komi A, Ishizu K, et al. Potent antitumor activity of MS-247, a novel DNA minor groove binder, evaluated by an in vitro and in vivo human cancer cell line panel. Cancer Res 1999;59:4042–9.
[13] Team RDC. R: a language and environment for statistical computing; 2007. Available from http://www.R-project.org.
[14] Tang JM, He QY, Guo RX, Chang XJ. Phosphorylated Akt overexpression and loss of PTEN expression in non-small cell lung cancer confers poor prognosis. Lung Cancer 2006;51:181–91.
[15] Uddin S, Hussain AR, Siraj AK, Manogaran PS, Al-Jomah NA, Moorji A, et al. Role of phosphatidylinositol 3'-kinase/AKT pathway in diffuse large B-cell lymphoma survival. Blood 2006;108:4178–86.
[16] Chadha KS, Khoury T, Yu J, Black JD, Gibbs JF, Kuvshinoff BW, et al. Erk expression and survival after surgery in pancreatic carcinoma. Ann Surg Oncol 2006;13:933–9.
[17] Kobayashi I, Semba S, Matsuda Y, Kuroda Y, Yokozaki H. Significance of Akt phosphorylation on tumor growth and vascular endothelial growth factor expression in human gastric carcinoma. Pathobiology 2006;73:8–17.
[18] Lin HJ, Hsieh FC, Song H, Lin J. Elevated phosphorylation and activation of PDK-1/AKT pathway in human breast cancer. Br J Cancer 2005;93:1372–81.
[19] Kim TJ, Lee JW, Song SY, Choi JJ, Choi CH, Kim BG, et al. Increased expression of pAKT is associated with radiation resistance in cervical cancer. Br J Cancer 2006;94:1678–82.
[20] Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96:857–68.
[21] Manning BD. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. J Cell Biol 2004;167:399–403.
[22] Balsara BR, Pei J, Mitsuuchi Y, Page R, Klein-Szanto A, Wang H, et al. Frequent activation of AKT in non-small cell lung carcinomas and preneoplastic bronchial lesions. Carcinogenesis 2004;25:2053–9.
[23] Bose S, Chandran S, Mirocha JM, Bose N. The Akt pathway in human breast cancer: a tissue-array-based analysis. Mod Pathol 2006;19:238–45.
[24] Dobashi Y, Suzuki S, Matsubara H, Kimura M, Endo S, Ooi A. Critical and diverse involvement of Akt/mammalian target of rapamycin signaling in human lung carcinomas. Cancer 2009;115:107–18.
[25] Yatabe Y, Kosaka T, Takahashi T, Mitsudomi T. EGFR mutation is specific for terminal respiratory unit type adenocarcinoma. Am J Surg Pathol 2005;29:633–9.
[26] Tanaka H, Yanagisawa K, Shinjo K, Taguchi A, Maeno K, Tomida S, et al. Lineage-specific dependency of lung adenocarcinomas on the lung development regulator TTF-1. Cancer Res 2007;67:6007–11.
[27] Iwagaki A, Choe N, Li Y, Hemenway DR, Kagan E. Asbestos inhalation induces tyrosine nitration associated with extracellular signal-regulated kinase 1/2 activation in the rat lung. Am J Respir Cell Mol Biol 2003;28:51–60.
[28] Yoshida K, Kuwano K, Hagimoto N, Watanabe K, Matsuba T, Fujita M, et al. MAP kinase activation and apoptosis in lung tissues from patients with idiopathic pulmonary fibrosis. J Pathol 2002;198:388–96.

[29] Farivar AS, Woolley SM, Fraga CH, Byrne K, Mulligan MS. Proinflammatory response of alveolar type II pneumocytes to in vitro hypoxia and reoxygenation. Am J Transpl 2004;4:346–51.
[30] Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. Trends Biochem Sci 2006;31:268–75.
[31] Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–85.
[32] Cox AD, Der CJ. The dark side of Ras: regulation of apoptosis. Oncogene 2003;22:8999–9006.
[33] Shields JM, Pruitt K, McFall A, Shaub A, Der CJ. Understanding Ras: it ain't over 'til it's over. Trends Cell Biol 2000;10:147–54.
[34] Sun P, Watanabe H, Takano K, Yokoyama T, Fujisawa J, Endo T. Sustained activation of M-Ras induced by nerve growth factor is essential for neuronal differentiation of PC12 cells. Genes Cells 2006;11:1097–113.
[35] Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006;9:189–98.
[36] Sarkaria IS, Zakowski MF, Pham D, Hezel M, Ebright MI, Chuai S, et al. Epidermal growth factor receptor signaling in adenocarcinomas with bronchioloalveolar components. Ann Thorac Surg 2008;85:216–23.
[37] Devereux TR, Holliday W, Anna C, Ress N, Roycroft J, Sills RC. Map kinase activation correlates with K-ras mutation and loss of heterozygosity on chromosome 6 in alveolar bronchiolar carcinomas from B6C3F1 mice exposed to vanadium pentoxide for 2 years. Carcinogenesis 2002;23:1737–43.
[38] Schmitz KJ, Wohlschlaeger J, Alakus H, Bohr J, Stauder MA, Worm K, et al. Activation of extracellular regulated kinases (ERK1/2) but not AKT predicts poor prognosis in colorectal carcinoma and is associated with k-ras mutations. Virchows Arch 2006.
[39] Ji H, Wang Z, Perera SA, Li D, Liang MC, Zaghlul S, et al. Mutations in BRAF and KRAS converge on activation of the mitogen-activated protein kinase pathway in lung cancer mouse models. Cancer Res 2007;67:4933–9.
[40] Conde E, Angulo B, Tang M, Morente M, Torres-Lanzas J, Lopez-Encuentra A, et al. Molecular context of the EGFR mutations: evidence for the activation of mTOR/S6K signaling. Clin Cancer Res 2006;12:710–7.
[41] Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, et al. Phosphorylation and activation of p70s6k by PDK1. Science 1998;279:707–10.
[42] Pearson RB, Thomas G. Regulation of p70s6k/p85s6k and its role in the cell cycle. Prog Cell Cycle Res 1995;1:21–32.
[43] Yaguchi S, Fukui Y, Koshimizu I, Yoshimi H, Matsuno T, Gouda H, et al. Antitumor activity of ZSTK474, a new phosphatidylinositol 3-kinase inhibitor. J Natl Cancer Inst 2006;98:545–56.

ARCHIVES

Pathology & Laboratory Medicine

Official Journal of the College of American Pathologists

Is the Epidermal Growth Factor Receptor Status in Lung Cancers Reflected in Clinicopathologic Features?

Kentaro Inamura, MD, PhD; Hironori Ninomiya, MD; Yuichi Ishikawa, MD, PhD; Osamu Matsubara, MD, PhD

Published by the
College of American Pathologists
Advancing Excellence

Is the Epidermal Growth Factor Receptor Status in Lung Cancers Reflected in Clinicopathologic Features?

Kentaro Inamura, MD, PhD; Hironori Ninomiya, MD; Yuichi Ishikawa, MD, PhD; Osamu Matsubara, MD, PhD

• ***Context.***—**Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are molecular-targeted drugs that are innovatively effective for non–small cell lung carcinomas with *EGFR* mutations. Epidermal growth factor receptor is a transmembrane receptor forming dimers on ligand binding. These then stimulate signals by activating receptor autophosphorylation through tyrosine kinase activity. Autophosphorylation triggers intracellular pathways facilitating malignant conversion. The most clinically advanced EGFR inhibition strategies include small-molecule inhibition of the intracellular tyrosine kinase domain (gefitinib and erlotinib) and monoclonal antibody–mediated blockade of the extracellular ligand-binding domain (cetuximab). Lung cancers with *EGFR* mutations are prevalent among patients who are female, of Asian ethnicity, and nonsmokers; thus, they can obtain benefit from EGFR tyrosine kinase inhibitors.**

Objective.—**To survey histopathologic findings and examine correlations with *EGFR* mutations. We mainly focused on component cell types (hobnail, columnar, and polygonal) and presence or absence of bronchioloalveolar carcinoma elements and a micropapillary pattern. Although *EGFR* mutations can be detected by various methods, including polymerase chain reaction–Invader assay or direct sequencing, these are inconvenient.**

Data Sources.—**Review of the published literature.**

Conclusion.—**Detailed pathologic examination showed significant genotype-phenotype correlations between *EGFR* mutations and presence of a bronchioloalveolar carcinoma component, a micropapillary pattern, and the hobnail cell type. We conclude that these characteristic histologic features are good predictors of *EGFR* mutations, and patients with these features might be good candidates for and could benefit from therapy with EGFR tyrosine kinase inhibitors.**

(*Arch Pathol Lab Med.* 2010;134:66–72)

The epidermal growth factor receptor (EGFR), a receptor of ligands including epidermal growth factor (EGF), is a 170-kDa glycoprotein tyrosine kinase protein that straddles the cell membrane.

Expression of EGFR is prevalent in variant normal cells including cells of epidermal, mesenchymal, and neurogenic origins. When EGF binds to EGFR, signaling pathways are activated that can lead to cell proliferation and differentiation. Epidermal growth factor receptor plays an important role in cell differentiation, development, proliferation, and maintenance. With *EGFR* gene overexpression due to mutation or structural alteration, carcinogenesis, invasion, and metastasis are facilitated.

In recent years there has been substantial interest in developing novel therapeutic agents that specifically target growth factor pathways that are dysregulated in cancer cells. Non–small cell lung cancer (NSCLC) is the most frequent cause of cancer death in the world and targeting EGFR has played a central role in advancing NSCLC research to improve patient outcome during the last several years. With the move to personalized cancer therapy, we need to understand oncologic biology at the molecular and histopathologic levels in individual lesions. In this review article, we focus on clinicopathologic features related to EGFR change and consider their indications for clinical application.

GENE MUTATIONS IN NON–SMALL CELL LUNG ADENOCARCINOMAS

Lung cancer is the leading cause of cancer death in men and women worldwide and identification of activating mutations of *EGFR* is one of the most intriguing recent discoveries in the field of lung cancer research.[1,2] Epidermal growth factor receptor mutations are present in a particular subtype of lung adenocarcinomas, and cancers with this mutation have been shown to be highly sensitive to chemical inhibitors of the kinase activity of EGFR. This subtype is prevalent among patients who are female, of Japanese and other Asian ethnicity, and nonsmokers.[2–4] K-ras is a downstream mediator of EGFR-induced cell signaling, and K-ras mutations confer constitutive activation of the signaling pathways without EGFR activation. Growing evidence indicates that K-ras mutations are also important in the development of lung carcinomas.[5] Very recently, we found a novel transforming fusion gene resulting from linkage between the echinoderm microtubule-associated protein like 4 (*EML4*) and anaplastic lymphoma

Accepted for publication February 20, 2009.

From the Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research, Koto-ku, Tokyo, Japan (Drs Inamura and Ishikawa); and the Department of Basic Pathology, National Defense Medical College, Tokorozawa, Saitama, Japan (Drs Ninomiya and Matsubara).

The authors have no relevant financial interest in the products or companies described in this article.

Reprints: Osamu Matsubara, MD, PhD, Department of Basic Pathology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan (e-mail: matubara@ndmc.ac.jp).

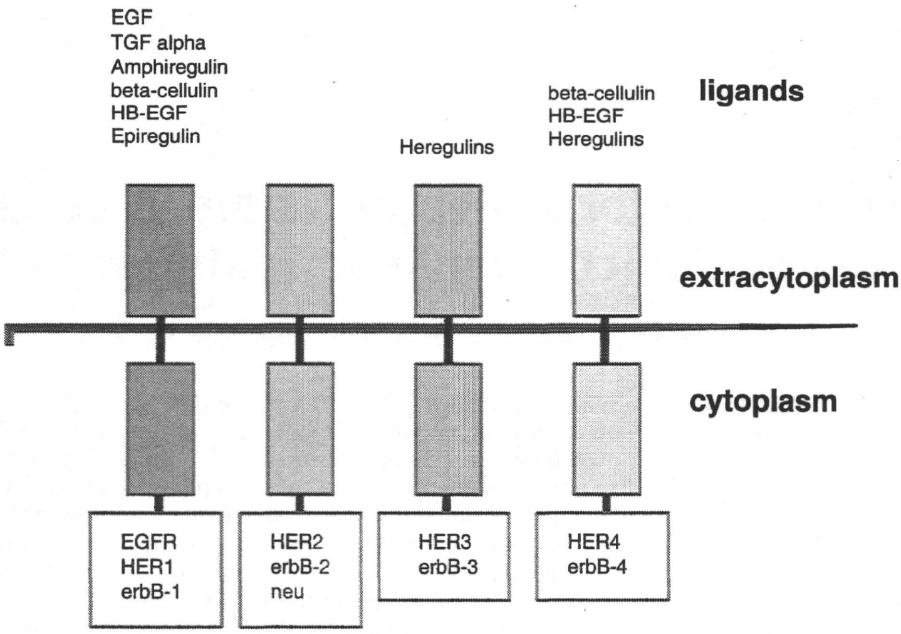

Figure 1. *The epidermal growth factor receptor (EGFR) family proteins and their ligands. TGF, transforming growth factor; HB-EGF, heparin-binding epidermal growth factor–like growth factor; HER, human epidermal growth factor receptor.*

kinase (*ALK*) genes in NSCLCs.[6] This translocation promotes strong tyrosine kinase activity, which is a prominent feature of ALK, leading to intensive oncogenesis in NSCLCs. Cancers featuring *EML4-ALK* fusion thus constitute a subtype of NSCLCs that might be highly sensitive to ALK inhibitors. Interestingly, *EGFR* mutation, K-ras mutation, and *EML4-ALK* translocation are mutually exclusive.[7,8] Furthermore, lung cancers with each of these alterations appear to have their own particular clinicopathologic characteristics.

DISCOVERY OF EGFR

In 1975, the existence of the EGF-specific receptor was first reported on the cell membrane of the fibroblast.[9] Thereafter, from work with the A431 human cancer cell line, EGFR was initially defined as a 170-kDa protein.[10] In 1984, the sequence of v-*erbB*, an oncogene of the avian erythroblastic leukemia virus, was reported to be extremely similar to that of EGFR.[11] Gene products of oncogene *erbB* and *EGFR* subsequently turned out to be identical proteins. Thereafter, it was found that human genes corresponding to v-*erbB* were not only *EGFR* but also human epidermal growth factor receptor 2 (*HER2*), these 2 now being referred to as *ERBB1* and *ERBB2*, respectively.

STRUCTURE AND FUNCTION OF EGFR

Growth factors belong to a family of polypeptides that have been shown to stimulate proliferation and/or differentiation in both normal and malignant cells. One of the first growth factors discovered was EGF. Later studies showed that this protein binds to the cell surface growth factor receptor EGFR, thereby either inducing cell proliferation or differentiation in mammalian cells.

The binding of a ligand to EGFR induces conformational changes within the receptor, which increase the catalytic activity of its intrinsic tyrosine kinase, resulting in the autophosphorylation that is necessary for biologic activity. Epidermal growth factor receptor is a 170-kDa transmembrane glycoprotein that binds to specific ligands. The erbB family cell-signaling process uses EGF-like ligands that include cell-signaling transforming growth factor α (TGF-α), amphiregulin, heparin-binding EGF, epiregulin, heregulin, neuregulin, and betacellulin. Epidermal growth factor receptor is known to bind with particularly high affinity to EGF, amphiregulin, and TGF-α.

As noted above, EGFR is a member of the erbB family of receptor tyrosine kinase proteins, now known to also include HER2/*neu* (erbB2), HER3 (erbB3), and HER4 (erbB4). These receptors are all composed of an extracellular ligand-binding domain, a transmembrane lipophilic domain, and an intracellular tyrosine kinase domain and, with the exception of HER2, all bind to receptor-specific ligands (Figure 1). Phosphorylation of the tyrosine kinase domain followed by homodimerization or heterodimerization between receptors of the same family leads to protein activation on the cell surface. In cancer cells, this is believed to promote signaling cascades, cell growth, differentiation, cell survival, cell cycle progression, and angiogenesis.

GENETIC STRUCTURE OF EGFR

The approximately 200-kb human *EGFR* gene, comprising 28 exons and 27 introns, exists on the short arm of chromosome 7 (7p12). Exons 1 to 16 encode the extracellular domain, while exon 17 codes for the transmembrane domain, and exons 18 to 28 are responsible for the intracellular domains. The tyrosine kinase domain is encoded by exons 18 to 24, while the C-terminal domain is encoded by exons 25 to 28.

ACTIVATION OF EGFR DOWNSTREAM SIGNALING

Receptor tyrosine kinases, such as EGFR, transmit extracellular signals of growth factors into the intracytoplasmic region and transmit their stimulus to the nuclei by signal transduction. As a result, transcriptional upregulation follows, leading to protein synthesis and transformation of cell functions or cellular architecture.

As signaling pathways of EGFR, the Ras/Raf/MAPK (mitogen-activated protein kinase) pathway, the PI3K (phosphatidylinositol-3-kinase)/Akt pathway, and the Jak (Janus kinase)/ STAT (signal transducers and activator of transcription) pathway are all important. As a result of

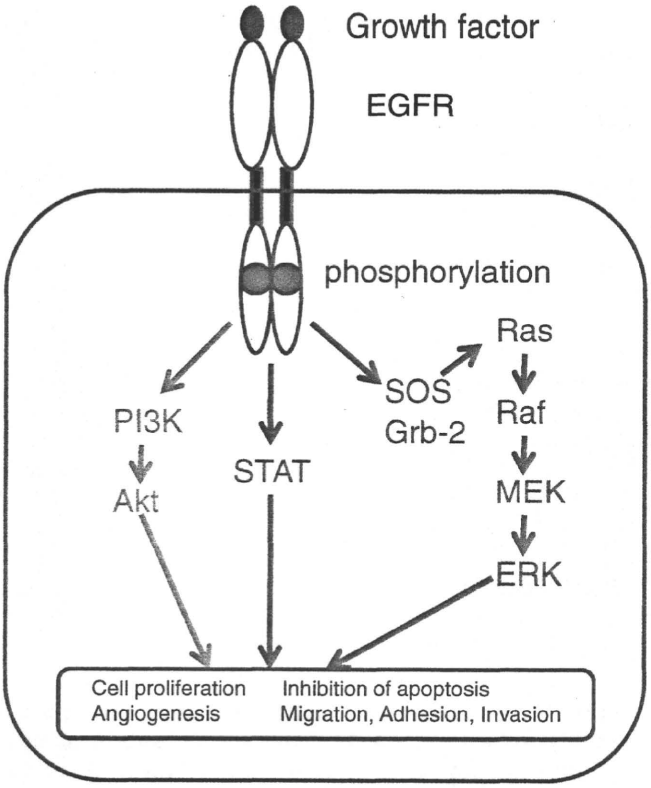

Figure 2. *Schematic illustration of the epidermal growth factor receptor (EGFR) and downstream signaling pathways. Binding of a receptor-specific ligand leads to phosphorylation of EGFR and signaling through the mitogen-activated protein kinase (MAPK) pathway (green), signal transducers and activator of transcription (STAT) pathway (blue), and phosphatidylinositol-3-kinase (PI3K)/Akt pathway (orange). These pathways promote cell proliferation, angiogenesis, migration, adhesion, and/or invasion, while inhibiting apoptosis. SOS, son of sevenless; Grb-2, growth factor receptor–bound protein 2; Ras and Raf are well-known oncoproteins; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase.*

Table 1. Epidermal Growth Factor Receptor (EGFR) Expression in Various Tumor Types

Tumor Type	Tumors Expressing EGFR, %
Head and neck	80–100
Renal	50–90
Lung	40–80
Breast	14–91
Colon	25–77
Ovarian	35–70
Prostate	39–47
Glioma	40–63
Pancreas	30–50
Bladder	31–48

the signal transduction, cell differentiation or cell proliferation are promoted. The Ras/Raf/MAPK pathway mainly promotes cell proliferation and survival, while the PI3K/Akt pathway is mainly associated with cell growth, inhibition of apoptosis, invasion, or migration (Figure 2).

EGFR OVEREXPRESSION

In a wide range of solid cancers, EGFR overexpression has been detected to varying degrees (Table).[12] Reported values are 30% to 38% for gastric adenocarcinomas,[13,14] 30% to 62% for pancreatic cancers,[15,16] and 100% for undifferentiated thyroid carcinomas.[17] Although the prognostic significance of EGFR expression remains unclear, as reports on these issues are contradictory, a retrospective review of EGFR studies determined that EGFR expression levels are highly predictive of clinical outcome for patients with ovarian, cervical, bladder, esophageal, and head and neck cancers. They are of moderate prognostic value for gastric, colorectal, breast, and endometrial cancers and of relatively low prognostic value for NSCLCs.[18]

EXTRACELLULAR MUTATION OF *EGFR*

In 1988, it was found that human glioblastoma multiforme cells carried amplified c-*erbB* genes that bore short deletion mutations within the ligand-binding domain of the EGFR. The products of these mutated c-*erbB* genes were about 30 kDa smaller than the normal 170-kDa EGFR, and cancer cell membrane fractions containing the 140-kDa abnormal EGFR showed a significant elevation of tyrosine kinase activity without any ligand.[19] This mutation type was referred to as EGFRvIII. There is no ligand binding site and the result is constant activation without any ligand binding.[20] EGFRvIII is associated with cell proliferation and malignancy in various neoplasms involving breast cancers, small cell lung cancers, gliomas, and prostatic cancers.[21]

MUTATIONS OF INTRACYTOPLASMIC DOMAIN OF *EGFR* GENE

In 2004, mutations of intracytoplasmic domain of *EGFR* gene were found in NSCLCs, and NSCLCs with such mutations were reduced in size by gefitinib, a chemical inhibitor of the kinase activity of EGFR.[1,2] In the gene coding for the receptor, mutations are divided into 4 major types: point mutations in exon 18, deletions in exon 19, insertions in exon 20, and point mutations in exon 21. Particularly, the 2 most frequent mutations are deletion around codons 746 to 750 of exon 19 and transversion of T to G in codon 858 of exon 21, with an amino acid change from leucine to arginine (L858R). These 2 mutations account for approximately 90% of intracytoplasmic mutations of EGFR (Figure 3).[22] They both cause conformational change in the ATP-binding domain, which results in constant activation of EGFR without ligand binding. However, affinity for gefitinib is upregulated, so that the cancer cells are susceptible to induction of apoptosis by this agent and to reduction in cancer size.[23] The 2 *EGFR* mutations have been found to be present in normal lung tissue around cancers,[24] and mice transgenic for the mutated *EGFR* gene develop lung cancers.[25] The results thus suggest that *EGFR* mutation is involved at an early stage of neoplasia in the lung.

EGFR MUTATIONS GENERATING GEFITINIB TOLERANCE

In addition to the EGFR mutations increasing sensitivity to gefitinib, as mentioned above, secondary mutations can occur so that cancers become tolerant. Substitution in codon 790, with a resulting amino acid shift from threonine to methionine (T790M),[1] or in codon 761, resulting in change from asparaginic acid to tyrosine (D761Y),[26] are reported to be gefitinib tolerance–inducing mutations. T790M has been detected in about half of the NSCLCs exhibiting acquired gefitinib tolerance.[26] Alteration of the gefitinib binding site in the EGFR cytoplasmic domain is presumably involved.

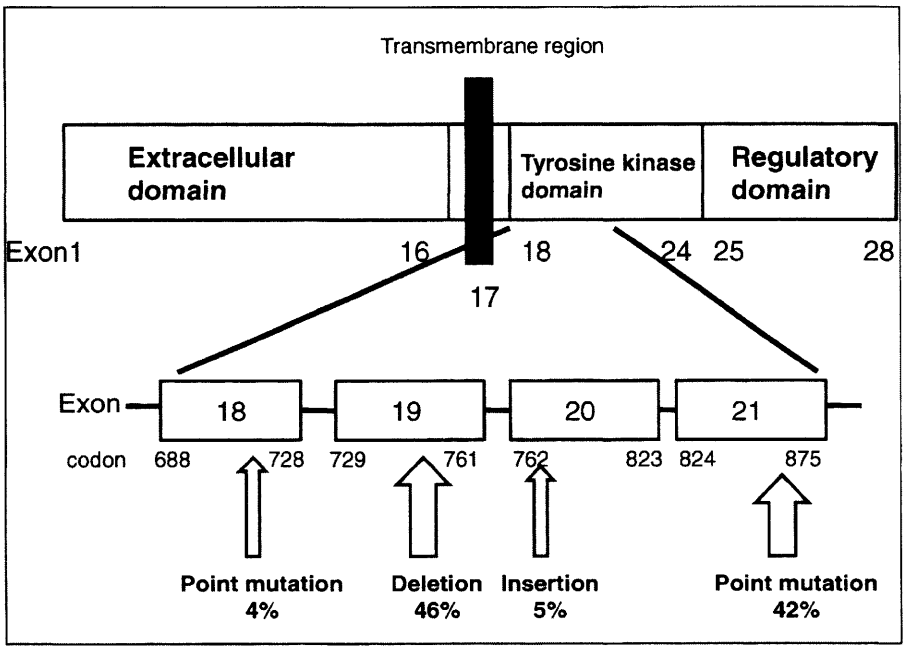

Figure 3. *Distribution of mutations in the epidermal growth factor receptor.*

DRUGS TARGETING EGFR

The most clinically advanced EGFR inhibition strategies include small-molecule inhibition of the intracellular tyrosine kinase domain and monoclonal antibody–mediated blockade of the extracellular ligand-binding domain. Gefitinib and erlotinib are oral anticancer drugs, inhibiting tyrosine kinase domain. Their cytoreductive effects are to some extent dependent on intracytoplasmic mutations of EGFR, as noted above, and they have been found to be useful in the treatment of NSCLCs. Cetuximab is a monoclonal antibody, binding to the ligand-binding site of EGFR and blocking its dimerization and activation. It is also effective for the wild-type EGFR, with applications in the treatment of colorectal as well as head and neck cancers.

EFFECT OF CANCER REDUCTION, COMBINATION USAGE OF CHEMOTHERAPY, AND LIFE PROLONGATION

From 2000 to 2001, 2 phase II studies of pretreated advanced NSCLCs (Iressa Dose Evaluation in Advanced Lung Cancer [IDEAL] 1 study[27] and IDEAL 2 study[28]) were performed. The positive response rate to gefitinib was 9% to 19% and the 1-year survival rate was 21% to 36%. Cancer reduction effects were most prevalent in Asian nonsmoking females with adenocarcinomas. Cancers with EGFR mutations demonstrated significant cytoreductive effects to treatment,[1,2] and this response is predominantly seen in persons with adenocarcinoma, who are nonsmokers, of female sex, and of Asian ethnicity.

From 2000 to 2001, as a first treatment for advanced NSCLCs, gefitinib was given in combination to standard treatment involving the platinum-containing drugs. Although the other drugs included gemcitabine and cisplatin[29] or paclitaxel and carboplatin,[30] significant combination effects were not obtained.

In 28 countries, not including Japan, a phase III study has been performed for 1692 cases of posttreatment advanced NSCLCs (Iressa Survival Evaluation in Lung Cancer).[31] For either all lung cancers or lung adenocarcinomas, gefitinib (versus placebo) could not significantly prolong survival time for patients. However, on subset analysis, gefitinib did significantly enhance survival in Asian persons and nonsmokers.

SIDE EFFECTS OF EGFR TYROSINE KINASE INHIBITION (GEFITINIB)

Major clinical problems caused by gefitinib are acute lung damage and interstitial pneumonia, the latter being the most significant side effect.[32] An epidemiologic investigation by the West Japan Thoracic Oncology Group, which used approximately 2000 cases, revealed an incidence rate of 3.2% to 3.5% and a death rate of 1.2% to 1.4%. Generally, ineffectiveness of steroid therapy makes the condition serious. Male sex, the existence of lung fibrosis before treatment, and a smoking habit were identified as risk factors for development of interstitial lung diseases related to gefitinib therapy. Thus, the effective treatment group for gefitinib and the high-risk group for interstitial lung disease with gefitinib are widely dissociated. This means that it is essential to preselect patients for gefitinib therapy.

EGFR MUTATIONS AND CLINICOPATHOLOGIC FEATURES

Lung cancers with EGFR mutations are prevalent among patients who are young, of female sex, never-smokers, and of East Asian ethnicity.[2–4,33–35]

Correlations between morphology and EGFR mutations in lung adenocarcinomas have been investigated previously. Concerning histopathology, a bronchioloalveolar carcinoma (BAC) histologic feature and well-differentiated to moderately differentiated grades were earlier reported to predict responsiveness to the EGFR tyrosine kinase (TK) inhibitor and the presence of EGFR mutations[33,34] The finding that the hobnail cell type and a micropapillary morphology can predict a higher incidence of EGFR mutations in lung adenocarcinomas has been reported more recently.[36]

EGFR MUTATIONS AND CELL TYPES IN LUNG ADENOCARCINOMAS

Cell type classification of lung adenocarcinomas was originally performed by Hashimoto et al,[37] describing hobnail, columnar, polygonal, goblet, and mixed cell types. They combined the Clara (nonciliated bronchiolar) cell type and type II cell type as the hobnail cell type because these types have the same cytologic features and are usually found to be mixed. This classification was applied with slight modification to a series of lesions in our hospitals. We divided lung cancers into hobnail, columnar, and polygonal cell types, focusing on the most frequent cell type rather than using the mixed cell category. Also, we merged the goblet and columnar cell types because of similarity in histologic and etiologic features and because the goblet cell type is present in minority (Figure 4, A through C).

As a result, the hobnail cell type was found to be significantly more associated with EGFR mutations than any of the other groups ($P < .001$). The cell type classification also relates to differences in mutation frequency and pattern of *TP53* (which codes for p53 protein).[37] The hobnail cell type, characterized by cytoplasmic protrusions and a tadpole or hobnail appearance, shows a low *TP53* mutational frequency, mainly of spontaneous transition type at CpG nucleotides. In contrast, the columnar cell type shows a high *TP53* mutational frequency, with G to T transversions, considered to be caused by exogenous carcinogenic agents like those found in tobacco smoke. We identified a significant difference in EGFR mutation rates between the hobnail cell type and the other 2 types. This finding provided further evidence of differences in the genetic background of EGFR mutations.

EGFR MUTATIONS AND MICROPAPILLARY PATTERN OR BAC HISTOLOGY IN LUNG ADENOCARCINOMAS

Additionally, we have focused on the presence of BAC component, as well as micropapillary pattern (MPP), defined as papillary structures with tufts lacking a fibrovascular core (Figure 5, A and B).[38] The micropapillary component belongs to moderately differentiated structures because of the lack of stroma.[38] When a cancer comprises more than 5% MPP, the prognosis has been shown to be poor, even with pathologic stage I disease.[38]

As a result, there was a significant association between the existence of BAC component or MPP and EGFR mutations ($P = .01$ and $P = .04$, respectively). In addition, both BAC component and MPP were significantly associated with the hobnail cell type ($P < .001$ and $P = .01$, respectively), as compared with the combined group of columnar and polygonal cell types. However, there was no association between BAC components and MPP ($P = .75$).[36]

The MPP is a distinct pathologic subtype first reported in lung cancers by Amin et al.[39] Among early stage lung adenocarcinomas, MPP-positive cancers show a significantly poorer prognosis than those that are MPP negative.[38] We speculated that the distinct MPP feature reflects a step of tumor progression from well-differentiated papillary adenocarcinoma of the hobnail cell type to a less differentiated state, unrelated to smoking. From their pathologic presentation and relatively unfavorable outcome, it is suggested that cancers with MPP should be classified as moderately differentiated rather than well dif-

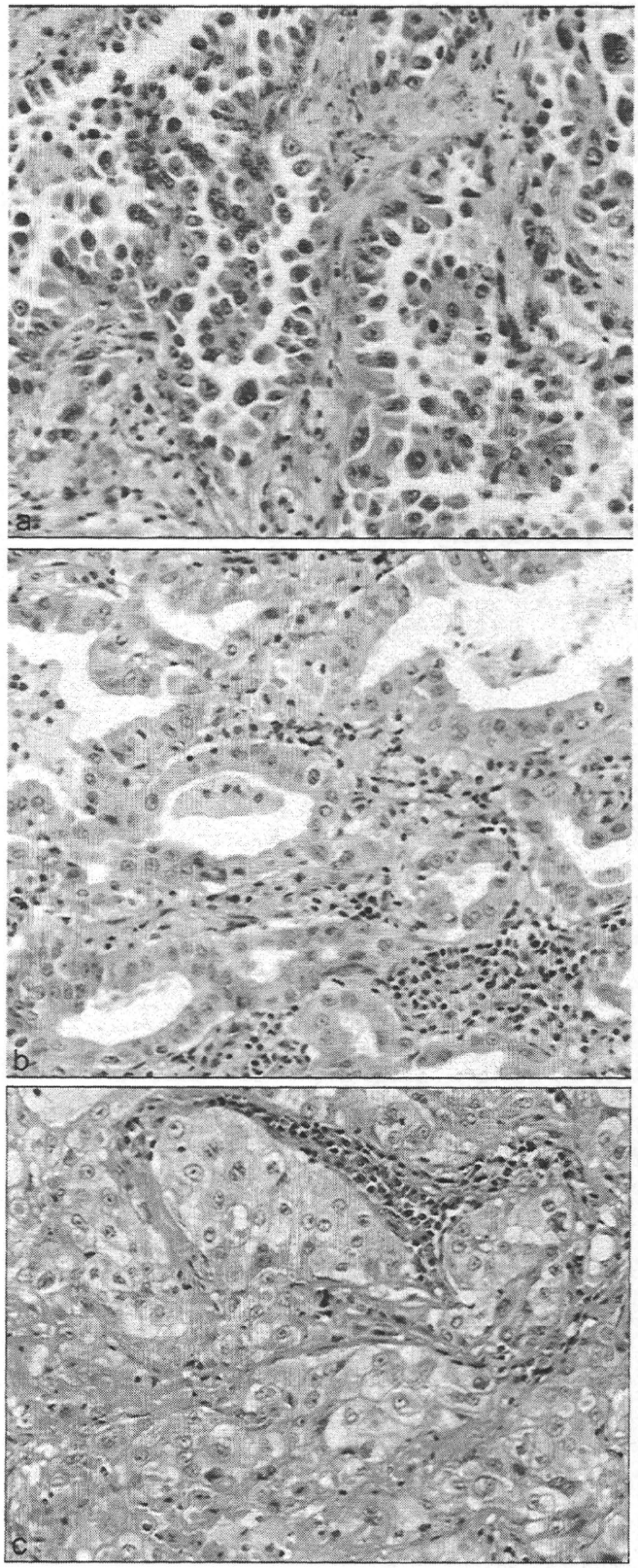

Figure 4. *Microscopic appearance of the 3 cytologic subtypes. A, Hobnail cell type. Apical portions of carcinoma cells containing nuclei protrude or bulge into the lumen. B, Columnar cell type. Nonciliated columnar or cuboidal cells, with or without mucus in their cytoplasm, have flat apical portions. C, Polygonal cell type. Note polygonal cells showing sheet-like growth (hematoxylin-eosin, original magnifications ×400).*

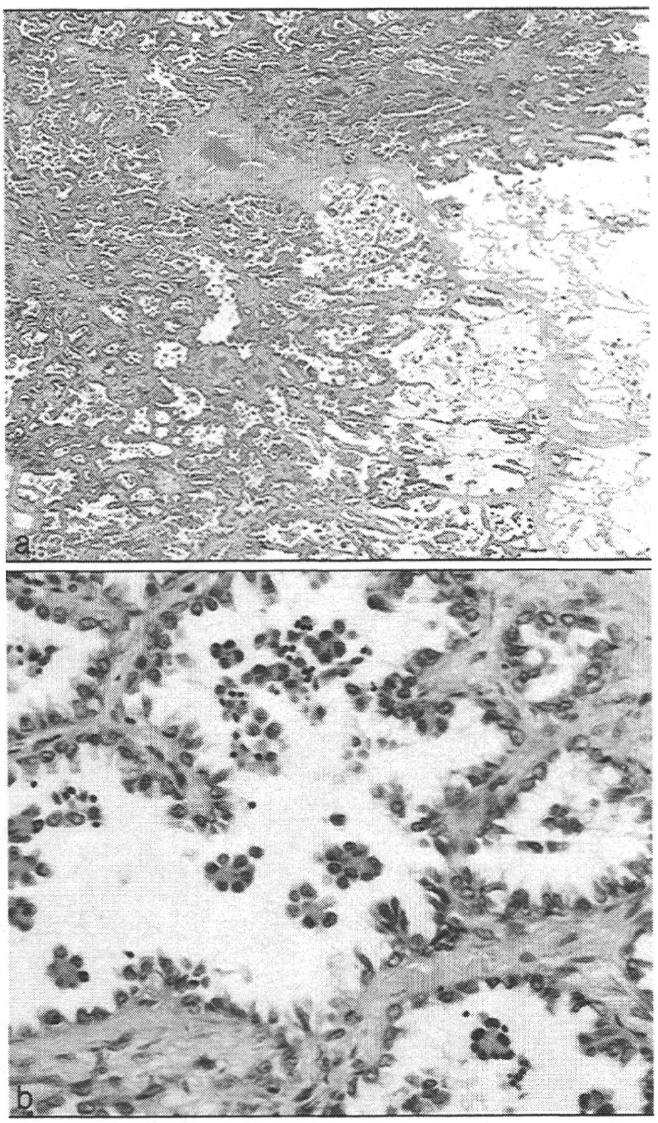

Figure 5. *Histologic features of the micropapillary pattern in pulmonary adenocarcinomas. A, Note diffuse distribution of tufts in alveolar spaces. B, Papillary tufts lack central fibrovascular cores (hematoxylin-eosin, original magnifications ×40 [A] and ×400 [B]).*

ferentiated. This pattern is often observed in nonsmokers and correlates with a high degree of tumor aggression. We also have demonstrated that metastasis to lymph nodes, pleural invasion, intrapulmonary metastases, and nonsmoking status are significantly more frequent in MPP-positive cases with a significantly poorer survival.[38] In our study, the presence of MPP components significantly correlated with EGFR mutations. Kim et al[40] referred to an association between the presence of MPP and tumor sensitivity to an EGFR TK inhibitor, although their analysis was limited to 36 relapsed lung adenocarcinomas. A notable characteristic of MPP is its frequent presence at the periphery of cancers and its predominance in metastatic foci.[39,41] These clinicopathologic observations, accompanied by our findings of a high mutational frequency for EGFR, may explain the dramatic responses to gefitinib in lung adenocarcinomas with diffuse micronodular intrapulmonary metastasis.[42]

COMMENT ON EGFR MUTATIONS AND CLINICOPATHOLOGIC FEATURES

Both BAC components and MPP are prevalent among nonsmokers.[38,43] Considering the etiologic relevance and its correlation with EGFR mutations, we speculate that lung cancers with these features belong to the same lineage characterized by thyroid transcription factor–1[35] and the hobnail cell type. Also, the results imply that lesions featuring MPP may be at a slightly more advanced stage than those with BAC components, because the MPP is an adverse prognostic marker for pathologic stage I disease.[38] Lung cancers in nonsmokers are considered to be less genetically complex than those in smokers[44,45] and, therefore, they may have distinct characteristics depending on simple signaling pathways, such as EGFR/Akt, for maintenance and survival.[2] Consequently, patients with tumors harboring these pathologic features could be good candidates and benefit from EGFR TK inhibitors.

CONCLUSION

There is no doubt that the EGFR TK inhibitors and EGFR monoclonal antibodies offer innovative molecular-targeted drugs, effective for some NSCLCs. However, the possibility of acute lung damage and interstitial pneumonia as negative side effects must be borne in mind. Therefore, the fact that in patients who are young, female, never-smokers, and of East Asian ethnicity, one subtype of NSCLC positively responds to EGFR TK inhibitors—because of the presence of EGFR mutations—is of great importance.

Pathologically, the hobnail cell type, MPP, and BAC components of lung adenocarcinomas are associated with a high incidence of EGFR mutations. Adenocarcinomas with these features form a distinct subtype, a fact suggesting that a genetic background confers susceptibility to EGFR TK inhibitors. The immunohistochemical analysis has a potential vulnerability because different antibodies might yield different results. Hence, these histologic features of lung adenocarcinomas with EGFR mutations, which can be detected by hematoxylin-eosin staining, are meaningful. These findings could provide a clue for selection of patients who might benefit from such treatment, as well as insights into biologic mechanisms of phenotype-genotype correlations.

Parts of this study were supported financially by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of the Japan Society for the Promotion of Science and by grants from the Ministry of Health, Labour and Welfare, the Smoking Research Foundation, the National Institute of Biomedical Innovation, and the Vehicle Racing Commemorative Foundation, as well as a Grant-in-Aid for Young Scientists (B).

References

1. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med.* 2004;350:2129–2139.

2. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science.* 2004;304:1497–1500.

3. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A.* 2004;101:13306–13311.

4. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst.* 2005;97:339–346.

5. Mitsudomi T, Viallet J, Mulshine JL, Linnoila RI, Minna JD, Gazdar AF. Mutations of ras genes distinguish a subset of non-small-cell lung cancer cell lines from small-cell lung cancer cell lines. *Oncogene.* 1991;6:1353–1362.

6. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature.* 2007;448:561–566.
7. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res.* 2004;64:8919–8923.
8. Inamura K, Takeuchi K, Togashi Y, et al. EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. *J Thorac Oncol.* 2008;3:13–17.
9. Carpenter G, Lembach KJ, Morrison MM, Cohen S. Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts. *J Biol Chem.* 1975;250:4297–4304.
10. Carpenter G, King L Jr, Cohen S. Epidermal growth factor stimulates phosphorylation in membrane preparations in vitro. *Nature.* 1978;276:409–410.
11. Downward J, Yarden Y, Mayes E, et al. Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature.* 1984;307:521–527.
12. Herbst RS, Shin DM. Monoclonal antibodies to target epidermal growth factor receptor-positive tumors: a new paradigm for cancer therapy. *Cancer.* 2002;94:1593–1611.
13. Kimura M, Tsuda H, Morita D, et al. A proposal for diagnostically meaningful criteria to classify increased epidermal growth factor receptor and c-erbB-2 gene copy numbers in gastric carcinoma, based on correlation of fluorescence in situ hybridization and immunohistochemical measurements. *Virchows Arch.* 2004;445:255–262.
14. Kimura M, Tsuda H, Morita D, et al. Usefulness and limitation of multiple endoscopic biopsy sampling for epidermal growth factor receptor and c-erbB-2 testing in patients with gastric adenocarcinoma. *Jpn J Clin Oncol.* 2005;35:324–331.
15. Ueda S, Ogata S, Tsuda H, et al. The correlation between cytoplasmic overexpression of epidermal growth factor receptor and tumor aggressiveness: poor prognosis in patients with pancreatic ductal adenocarcinoma. *Pancreas.* 2004;29:e1–e8.
16. Ueda S, Hatsuse K, Tsuda H, et al. Potential crosstalk between insulin-like growth factor receptor type 1 and epidermal growth factor receptor in progression and metastasis of pancreatic cancer. *Mod Pathol.* 2006;19:788–796.
17. Murakawa T, Tsuda H, Tanimoto T, Tanabe T, Kitahara S, Matsubara O. Expression of KIT, EGFR, HER-2 and tyrosine phosphorylation in undifferentiated thyroid carcinoma: implication for a new therapeutic approach. *Pathol Int.* 2005;55:757–765.
18. Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer.* 2001;37(suppl 4):S9–S15.
19. Yamazaki H, Fukui Y, Ueyama Y, et al. Amplification of the structurally and functionally altered epidermal growth factor receptor gene (c-erbB) in human brain tumors. *Mol Cell Biol.* 1988;8:1816–1820.
20. Batra SK, Castelino-Prabhu S, Wikstrand CJ, et al. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. *Cell Growth Differ.* 1995;6:1251–1259.
21. Pedersen MW, Meltorn M, Damstrup L, Poulsen HS. The type III epidermal growth factor receptor mutation: biological significance and potential target for anti-cancer therapy. *Ann Oncol.* 2001;12:745–760.
22. Kosaka T, Endoh H, Yatabe Y, Mitsudomi T. Translational research on lung cancer—EGFR gene mutation [in Japanese]. *Gan To Kagaku Ryoho.* 2005;32:750–758.
23. Choi SH, Mendrola JM, Lemmon MA. EGF-independent activation of cell-surface EGF receptors harboring mutations found in gefitinib-sensitive lung cancer. *Oncogene.* 2007;26:1567–1576.
24. Tang X, Shigematsu H, Bekele BN, et al. EGFR tyrosine kinase domain mutations are detected in histologically normal respiratory epithelium in lung cancer patients. *Cancer Res.* 2005;65:7568–7572.
25. Ji H, Li D, Chen L, et al. The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell.* 2006;9:485–495.
26. Balak MN, Gong Y, Riely GJ, et al. Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. *Clin Cancer Res.* 2006;12:6494–6501.
27. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol.* 2003;21:2237–2246.
28. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA.* 2003;290:2149–2158.
29. Giaccone G, Herbst RS, Manegold C, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 1. *J Clin Oncol.* 2004;22:777–784.
30. Herbst RS, Giaccone G, Schiller JH, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 2. *J Clin Oncol.* 2004;22:785–794.
31. Thatcher N, Chang A, Parikh P, et al. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer). *Lancet.* 2005;366:1527–1537.
32. Inoue A, Saijo Y, Maemondo M, et al. Severe acute interstitial pneumonia and gefitinib. *Lancet.* 2003;361:137–139.
33. Miller VA, Kris MG, Shah N, et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. *J Clin Oncol.* 2004;22:1103–1109.
34. Hsieh RK, Lim KH, Kuo HT, Tzen CY, Huang MJ. Female sex and bronchioloalveolar pathologic subtype predict EGFR mutations in non-small cell lung cancer. *Chest.* 2005;128:317–321.
35. Yatabe Y, Kosaka T, Takahashi T, Mitsudomi T. EGFR mutation is specific for terminal respiratory unit type adenocarcinoma. *Am J Surg Pathol.* 2005;29:633–639.
36. Ninomiya H, Hiramatsu M, Inamura K, et al. Correlation between morphology and EGFR mutations in lung adenocarcinomas: significance of the micropapillary pattern and the hobnail cell type. *Lung Cancer.* 2009;63:235–240.
37. Hashimoto T, Tokuchi Y, Hayashi M, et al. Different subtypes of human lung adenocarcinoma caused by different etiological factors: evidence from p53 mutational spectra. *Am J Pathol.* 2000;157:2133–2141.
38. Miyoshi T, Satoh Y, Okumura S, et al. Early-stage lung adenocarcinomas with a micropapillary pattern, a distinct pathologic marker for a significantly poor prognosis. *Am J Surg Pathol.* 2003;27:101–109.
39. Amin MB, Tamboli P, Merchant SH, et al. Micropapillary component in lung adenocarcinoma: a distinctive histologic feature with possible prognostic significance. *Am J Surg Pathol.* 2002;26:358–364.
40. Kim YH, Ishii G, Goto K, et al. Dominant papillary subtype is a significant predictor of the response to gefitinib in adenocarcinoma of the lung. *Clin Cancer Res.* 2004;10:7311–7317.
41. Makimoto Y, Nabeshima K, Iwasaki H, et al. Micropapillary pattern: a distinct pathological marker to subclassify tumours with a significantly poor prognosis within small peripheral lung adenocarcinoma (</=20 mm) with mixed bronchioloalveolar and invasive subtypes (Noguchi's type C tumours). *Histopathology.* 2005;46:677–684.
42. Kobayashi M, Takeuchi T, Bandobashi K, et al. Diffuse micronodular pulmonary metastasis of lung adenocarcinoma predicts gefitinib response in association with epidermal growth factor receptor mutations. *Anticancer Res.* 2006;26:1621–1626.
43. Okubo K, Mark EJ, Flieder D, et al. Bronchoalveolar carcinoma: clinical, radiologic, and pathologic factors and survival. *J Thorac Cardiovasc Surg.* 1999;118:702–709.
44. Sanchez-Cespedes M, Ahrendt SA, Piantadosi S, et al. Chromosomal alterations in lung adenocarcinoma from smokers and nonsmokers. *Cancer Res.* 2001;61:1309–1313.
45. Powell CA, Bueno R, Borczuk AC, et al. Patterns of allelic loss differ in lung adenocarcinomas of smokers and nonsmokers. *Lung Cancer.* 2003;39:23–29.

Discriminant Model for Cytologic Distinction of Large Cell Neuroendocrine Carcinoma from Small Cell Carcinoma of the Lung

Rira Hoshi, CT, Noriyuki Furuta, CT,* Takeshi Horai, MD,* Yuichi Ishikawa, MD, PhD,† Satoshi Miyata, PhD,‡ and Yukitoshi Satoh, MD, PhD*§*

Background: To establish cytologic criteria for pulmonary large cell neuroendocrine carcinoma (LCNEC), we developed and evaluated a discriminant model for cytologic differential diagnosis between LCNEC and small cell lung carcinoma (SCLC).

Methods: Aspiration cytologic and/or imprint smears from 29 LCNEC cases were reviewed in comparison with 26 SCLC cases. We selected the following parameters for assessment: background, cellular arrangement, cell clusters, cell cohesion, arrangements, cell dimensions areas, the presence of cytoplasm and/or prominent nucleoli, nuclear features, mitosis, naked nuclei, and nuclear streaking. To demonstrate the utility of differences in frequencies of cytologic parameters for LCNECs and SCLCs, a discriminant model was developed and evaluated.

Results: Among the cytologic parameters investigated, large clusters (consisting of $\geq$60 tumor cells) with tight cohesion and small tumor cells (showing $\leq$120 μm^2) without prominent nucleoli on each case were particular focuses of attention, because statistically significant differences with good power were evident between the LCNEC and SCLC groups for their frequencies ($p < 0.0001$). On the basis of variation in plotted location on scatter plots, a discriminant model for LCNEC and SCLC was made and evaluated by logistic discriminant analysis. Sensitivity, specificity, and accuracy were all 100%. With leave-one-out cross validation, the predicted error rate of the discriminant model for new cases was 0.00545.

Conclusion: Our model based on the cytologic features of large cell clusters with tight cohesion and of small tumor cells without prominent nucleoli should be a useful aid for distinction between LCNECs and SCLCs.

From the *Departments of Cytology, Cancer Institute Hospital, Japanese Foundation of Cancer Research, Tokyo, Japan; †Department of Pathology, and ‡Bioinformatics Group, Genome Center, the Cancer Institute, the Japanese Foundation for Cancer Research, Tokyo, Japan; and §Department of Thoracic Surgery, Kitasato University School of Medicine, Kanagawa, Japan.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Yukitoshi Satoh, MD, PhD, Department of Thoracic Surgery, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara-shi, Kanagawa 228-8555, Japan. Email: ysatoh@med.kitasato-u.ac.jp

ISSN: 1556-0864/10/0504-0472

Key Words: Lung cancer, Large cell neuroendocrine carcinoma, Small cell lung carcinoma, Discriminant model, Cytology.

(*J Thorac Oncol.* 2010;5: 472–478)

Large cell neuroendocrine carcinoma (LCNEC) of the lung and small cell lung carcinoma (SCLC) are both now considered as high-grade neuroendocrine carcinomas arising in the lung.[1–4] Based on the large, multiinstitutional study in Japan, Asamura et al.[5] reported that the 5-year survival rates of patients with all stages were 40.3% for LCNEC and 35.7% for SCLC, the differenc not being statistically significant. However, these two tumors are generally thought to have different clinical features[1,4,6–14] and require different treatments.

Currently, surgical resection is advocated for the LCNEC as same as other nonsmall cell lung cancers.[15] However, Iyoda et al.[10] reported that patients with stage I disease treated with either neoadjuvant or postoperative adjuvant chemotherapy had a significantly better prognosis than their counterparts groups receiving surgery alone. Therefore, LCNEC requires a refined histology-specific approach. Conversely, the SCLC is aggressive but chemosensitive, and a standard therapeutic strategy has already been established.[5]

The cytologic diagnosis of SCLC is clear, but criteria for the LCNEC have yet to be established.[5,16–22] Recently, the cytologic features of LCNEC described in several reports are as follows: necrotic background, loose cell aggregates, large cell size (three times as large as mature lymphocytes), rosette and Indian-filing arrangements, abundant cytoplasm, granular nuclear chromatin, clear nucleoli, naked nuclei, and nuclear streaking.[17–22] Because these are also often recognized in SCLC cases,[15,16,23] they are not specific.

The aim of this study was to elucidate the cytologic characteristics of the LCNEC in comparison with SCLCs particularly and evaluate the utility of proposed scoring system for their differential diagnosis.

MATERIALS AND METHODS

Patients

The pathology files of the Cancer Institute Hospital (Tokyo, Japan) between 1990 and 2007 were searched for 29 patients who underwent pulmonary resection for LCNECs.

These LCNEC cases were all confirmed by pathologic examination on surgically resected materials with the World Health Organization (WHO) classification system.[15] The histologic diagnostic criteria of LCNEC proposed by WHO are as follows: neuroendocrine morphologic features (organoid nesting, palisading, rosettes, and trabecular growth pattern); a high mitotic rate (>10 per 10 high-power fields); necrosis (often large zones); cellular features of a nonsmall cell carcinoma (large cell size, a low nuclear/cytoplasmic ratio, polygonal shape, finely granular eosinophilic cytoplasm, coarse chromatin, and/or frequent nucleoli); and neuroendocrine features by immunohistochemistry or electron microscopy or both.[15] For comparison, we randomly extracted 26 cases of SCLCs diagnosed during the same period, 16 of which were diagnosed with surgical materials and the remaining 10 with transbronchial lung biopsy samples. The histologic diagnosis of SCLCs was also based on the WHO classification system.[15] Combined LCNECs and SCLCs and SCLC cases after any treatment were all excluded in this study, which was approved by our institutional review board, each patient giving written informed consent before treatment.

Cytologic Materials

Cytologic specimens obtained by transbronchial aspiration and/or imprint from the resected specimens were fixed routinely in 95% ethanol and stained by the Papanicolaou method. Five to 12 cytologic slides were reviewed for each patient. From previous studies,[17–22] we selected the following parameters for assessment: necrotic background, cellular arrangement, tumor cell clusters, tumor cell cohesion, cell arrangements, cell dimensions areas, the presence of tumor cells with identifiable cytoplasm and/or prominent nucleoli, nuclear features, mitosis, naked nuclei, and nuclear streaking. Cluster size was categorized in the three groups as follows: small clusters, consisting of more than 10 and less than or equal to 20 cells; intermediate-sized clusters, consisting of more than 20 cells and less than 60 cells; and large clusters, consisting of more than or equal to 60 cells. Tight cohesiveness of clusters was defined as a straight cluster border composed of cells lined up and/or arranged in palisades. Cell areas were measured for 50 cells extracted at random in each specimen and calculated as (long diameter + short diameter/$2 \times 2)^2\ \pi$ ($\pi = 3.14$). The diameters of tumor cells were measured using an ocular micrometer (DSM; Olympus, Tokyo, Japan). Cell size was categorized in 2 groups as follows: small tumor cells, less than or equal to 120 μm^2; and large tumor cells, more than or equal to 600 μm^2.

Statistical Analysis

The clinicopathologic factors analyzed in this study included age (<65 or ≥65 years), gender, and smoking habits, evaluated by the χ^2 test. Differences in cell areas and the frequency of the cytologic features between LCNEC and SCLC cases were analyzed by an unpaired Student t test and χ^2 test; $p < 0.05$ was considered significant.

Logistic Discriminant Analysis

To demonstrate the utility of differences in frequencies of cytologic parameters for LCNECs and SCLCs, a discriminant model was developed and evaluated. The frequencies of two cytologic features, in which differences were statistically significant, were regarded as two variables for a set of data, displayed as a scatter plot. By logistic discriminant analysis based on the scatter plots, a discriminant model for LCNEC and SCLC was made. When two variables for frequency of cytologic features were regarded as x_1 and x_2, the probability of an SCLC was calculated as follows.

$$P(SCLC) = \frac{\exp(-319.81 - 10.82x_1 + 16.30x_2)}{1 + \exp(-319.81 - 10.82x_1 + 16.30x_2)}$$

And the discriminant line was as follows.

$$-319.81 - 10.82x_1 + 16.30x_2 = 0 \Leftrightarrow x_2 = 19.62 + 0.6641x_1$$

We regarded a point on upper part of the line as true (SCLCs) and a point on lower part of the line as false (LCNECs). Furthermore, we analyzed prediction of error discrimination for new cases by leave-one-out cross validation. A discriminant model for LCNEC and SCLC was made except in one case. The excepted case was predicted by the discriminant model, and the discrimination confirmed whether it was correct. For all SCLC and LCNEC cases, the same analyses were performed repeatedly.

RESULTS

Clinical Findings

Clinicopathologic findings for the 29 LCNEC patients are summarized in Table 1. There were 26 men and 3 women, ranging in age from 48 to 80 years, with a median of 67 years. Lobectomy was performed on all. Mean follow-up time was 2.4 years (range, 0.33–9 years); 14 were dead, and 15 were alive at the time of this analysis. All patients had a smoking habit, ranging from 3 to 206.5 pack years. Of the 26 SCLC patients, 19 were men and 7 women, ranging in age from 58 to 80 years, with a median of 69 years. Eight were treated with surgical resection and eight with surgical resection after chemotherapy. In these 16 cases, no combination of SCLC with other histologic types was identified on resected materials. The remaining 10 underwent chemotherapy and/or radiotherapy, but again no admixture of other types was noted in biopsy specimens. Mean follow-up for the 26 patients was 2.6 years (range, 0.08–8 years); 14 were dead, and 12 were alive at the time of this analysis. All patients also had a smoking habit. A comparison of data for LCNEC and SCLC groups revealed no statistically significant differences in age, gender, and smoking status (Table 1).

Cytologic Findings

The initial cytologic diagnoses of 29 LCNEC patients were 4 LCNECs, 5 SCLCs, 2 combined SCLCs and adenocarcinomas, 5 neuroendocrine carcinomas, 1 atypical carcinoid, 7 poorly differentiated adenocarcinomas, 3 poorly differentiated squamous cell carcinomas, and 2 nonsmall cell carcinomas. In the LCNEC group, the unanimity in diagnosis between pathology and cytology was 21.1%. The cytologic

TABLE 1. Clinicopathologic Findings for LCNEC and SCLC Cases

Characteristic	No. of Patients	LCNEC (n = 29)	SCLC (n = 26)	p
Age (yr)				
<65	14	9	5	0.32
≤65	41	20	21	
Gender				
Male	45	26	19	0.11
Female	10	3	7	
Smoking status				
Nonsmoker	0	0	0	1.00
Smoker	55	29	26	
Cytologic materials				
TBAC	34	16	18	0.56
IC	5	3	2	
TBAC and IC	16	10	6	
Cytologic diagnosis				
LCNEC	4	4	0	<0.0001
LCNEC > SCLC	2	2	0	
NE	4	4	0	
SCLC	31	5	26	
SCLC + NSCLC	2	2	0	
NSCLC	12	12	0	
Tumor location				
Right lung	5	—	5	—
RUL	15	12	3	
RLL	12	7	5	
Left lung	2	—	2	
LUL	14	9	5	
LLL	7	1	6	
Type of location				
Central	11	3	8	0.09
Peripheral	44	26	18	
Tumor size (cm)				
≤3.0	25	12	13	—
>3.0	24	17	7	
NA	6	0	6	
Pathologic stage (pTNM)				
IA	14	8	6	—
IB	12	11	1	
IIA	5	1	4	
IIB	4	3	1	
IIIA	8	4	4	
IIIB	2	1	1	
IV	6	1	5	
NA(LD)	4	—	4	
Survival after surgery				
Dead	28	14	14	0.68
Alive	27	15	12	
Mean ± SD		2.39 ± 2.27	2.63 ± 2.33	

TBAC, transbronchial aspiration cytology; IC, imprint cytology; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung carcinoma; NE, neuroendocrine carcinoma; NSLC, nonsmall cell lung carcinoma; RUL, right upper lobe; RLL, right lower lobe; LUL, left upper lobe; LLL, left lower lobe; NA, not available; pTNM, from Ref. 15; LD, Limited disease.

TABLE 2 Cytologic Comparison Between LCNEC and SCLC

Cytologic Parameters	LCNEC (n = 29)	SCLC (n = 26)	p value
Necrotic background	25/29 (86.2%)	23/26 (88.5%)	0.802
Predominant cellular arrangement			
Cluster	26/29 (89.7%)	5/26 (19.2%)	
Single cells	3/29 (10.3%)	21/26 (80.7%)	<0.0001
Presence of characteristic clusters			
Large sized	27/29 (93.1%)	4/26 (15.4%)	<0.0001
Strong cohesion	27/29 (93.1%)	3/26 (11.5%)	<0.0001
Presence of tumor cell arrangement			
Rosette	28/29 (96.6%)	21/26 (80.7%)	0.061
Molding	26/29 (89.7%)	26/26 (100 %)	0.092
Pair cells	12/29 (41.3%)	17/26 (65.4%)	0.075
Palisading	27/29 (93.1%)	3/26 (11.5%)	<0.0001
Mean tumor cell size	178.1 μm^2	127.2 μm^2	<0.0001
Presence of characteristic tumor cells			
Large sized	18/29 (62.0%)	20/26 (76.9%)	0.224
Evidently identifiable cytoplasm	27/29 (93.1%)	20/26 (76.9%)	0.089
Prominent nucleoli	24/29 (82.8%)	20/26 (76.9%)	0.589
Small sized without prominent nucleoli	15/29 (51.7%)	26/26 (100 %)	<0.0001
Chromation pattern			
Finely granular	10/29 (34.5%)	11/26 (42.3%)	
Finely granular to granular	14/29 (48.3%)	15/26 (57.7%)	0.085
Granular	5/29 (17.2%)	0/26 (0 %)	
Presence of characteristics			
Mitoses	25/29 (86.2%)	25/26 (96.2%)	0.200
Nuclear streaking	26/29 (89.7%)	25/26 (96.2%)	0.354
Naked nuclei	24/29 (82.8%)	10/26 (38.5%)	0.0007

diagnoses for the 26 SCLC patients were all SCLCs, with statistically significant unanimity ($p < 0.0001$).

In a preliminary study, we evaluated any cytologic differences between aspiration smears and touch preparations in pilot groups consisting of 10 cases each of LCNEC and SCLC. In these groups, aspiration preparations and imprints showed no significant differences in any of the parameters chosen for assessment (data not shown). Comparisons between LCNEC and SCLC for each cytologic parameter are shown in Table 2. Cytologic parameters with statistically significant differences were as follows: cellular arrangement, presence of large clusters, tumor cell cohesion, palisading arrangement of tumor cells, mean of cellular areas, and presence of small cells without prominent nucleoli and naked nuclei. With regard to cellular arrangement, single cells were evident in all SCLC cases, whereas tumor cell clusters were frequently observed in LCNECs (Figures 1, 2). In the LCNEC group, although single cells were evident, many of them had naked nuclei. In particular, large clusters consisting of more than 60 cells were characteristic in LCNEC group

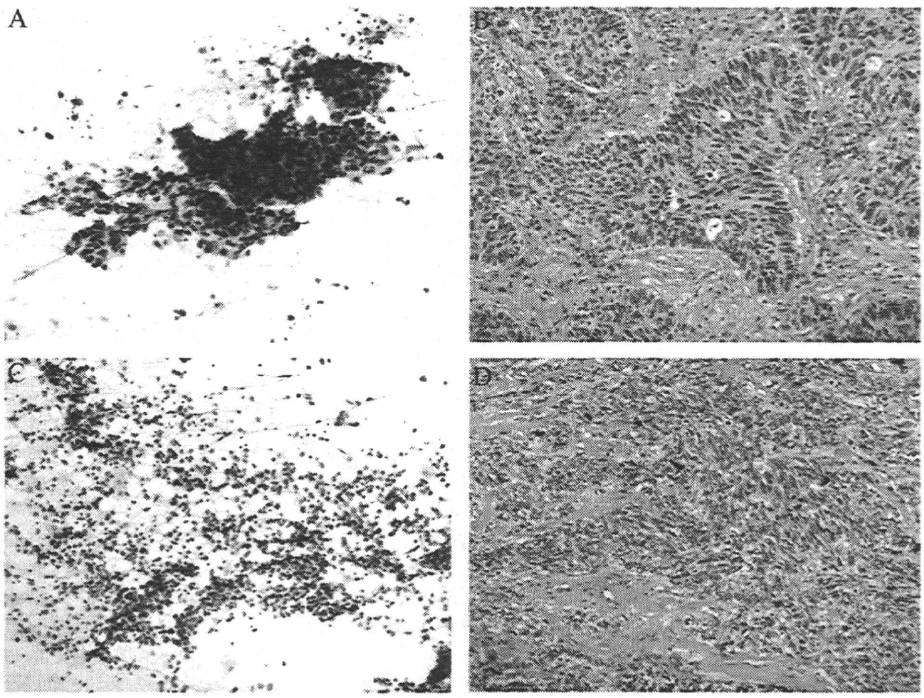

FIGURE 1. Photomicrographs illustrating cellular arrangement in transbronchial aspiration or histology specimens of LCNEC and SCLC cases. *A*, Large and three-dimensional clusters are conspicuous in a cytologic smear of an LCNEC case (Papanicolaou stain, ×20); *B*, tumor nests of an LCNEC case show palisading and Rosette-like formations in a histology specimen (hematoxylin and eosin stain, ×40); *C*, single cells are conspicuous in the cytologic smear of an SCLC case (Papanicolaou stain, ×20); and *D*, tumor cells of an SCLC case comprise irregular nests in a histology specimen (hematoxylin and eosin stain, ×40).

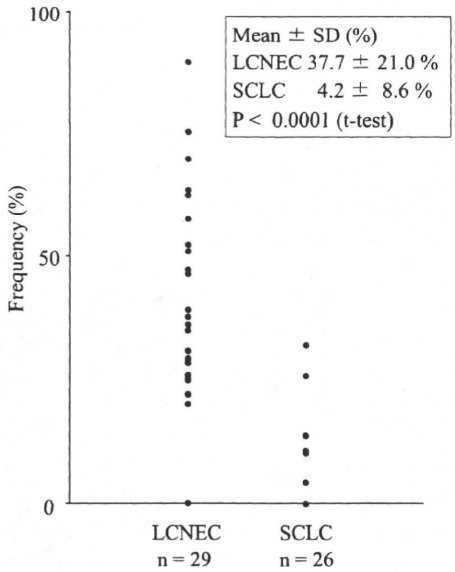

FIGURE 2. Frequencies of large clusters with tight cohesion in the LCNEC and SCLC groups (n = 55).

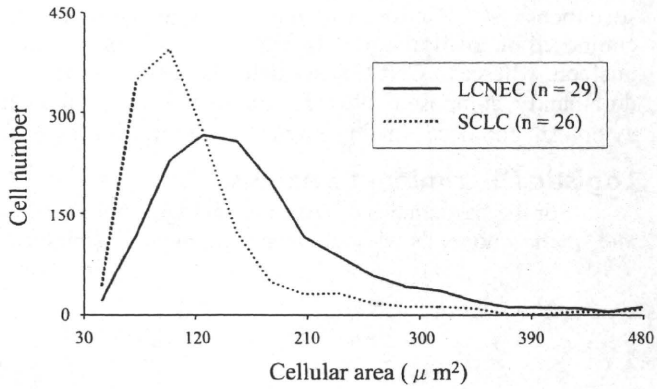

FIGURE 3. Histograms of cell areas in LCNEC and SCLC groups. Note that about 60% of the SCLC cells fall in the range of less than 120 μm^2, as compared with about 25% for LCNEC cells ($p < 0.0001$).

(Figures 1, 2). Also, on those histologic specimens, cell adhesion between tumor cells of LCNEC cases was conspicuous, whereas it was indistinct in SCLC cases (Figures 1*B*, *D*).

Furthermore, tumor cell cohesion was weak in SCLC cases, whereas in LCNEC cases tightly cohesive clusters predominated (Table 2). Frequencies of large clusters with tight cohesion are shown in Figure 3. The mean frequency was 37.7 ± 21.0% in the LCNEC cases and 4.2 ± 8.4% in SCLCs, the difference being statistically significant ($p < 0.0001$). LCNEC cases featured discrete cell nests divided by fibrous stroma with frequent peripheral palisading, whereas SCLC cases were characterized by cell nests, frequently infiltrating adjacent fibrous stroma (Figure 1).

Mean cell areas were 178.1 ± 84.8 μm^2 (range, 45.3–808.9 μm^2) for LCNEC cases and 127.8 ± 69.3 μm^2 (range, 36.8–699.5 μm^2) for SCLCs, the difference being statistically significant ($p < 0.0001$). The distributions are shown graphically in Figure 3. Some 58.2% of the SCLC cells (756/1300) were less than 120 μm^2, as compared with only 24.6% for LCNEC cells (357/1450; $p < 0.0001$). Furthermore, small tumor cells lacking prominent nucleoli in SCLC cases were observed more frequently than in LCNEC cases ($p < 0.0001$; Table 2 and Figure 5). Frequencies are shown in Figure 4. The mean values were 11.9 ± 12.1% in LCNEC and 55.8 ± 18.9% in SCLC cases, the difference being

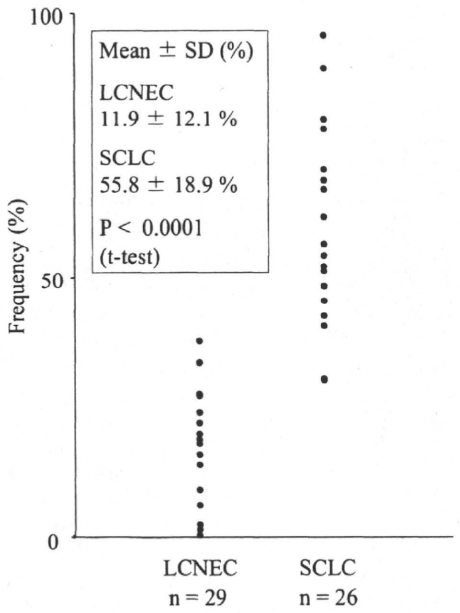

FIGURE 4. Frequencies of small tumor cells without prominent nucleoli in the LCNEC and SCLC groups (n = 55).

statistically significant ($p < 0.0001$). Also, in histologic specimens, SCLC cases had the cell nests predominantly composed of small tumor cells with scant cytoplasm without nucleoli, whereas LCNEC cases demonstrated cell nests predominantly composed of large tumor cells with abundant cytoplasm and occasional prominent nucleoli (Figure 5*B*, *D*).

Logistic Discriminant Analysis

For the frequencies of large clusters with tight cohesion and small tumor cells without prominent nucleoli, statistically significant differences with strong power was evident between LCNEC and SCLC groups. Therefore, these two cytologic parameters were considered as the two variables for the scatter plots. The dots for LCNEC cases are located on the lower right, whereas those of SCLC cases were located on the upper left, with clear differences between the two for the majority. The results of logistic discriminant analysis are shown in Figure 6. Because all SCLC and LCNEC cases were cytologically discriminated accurately, sensitivity, specificity, and accuracy were all 100%. Moreover, the results of leave-one-out cross validation, shown in Figure 7, gave a predicted error rate of $(2 + 1)/55 = 0.00545$.

DISCUSSION

In this study, the large cell cluster with tight cohesion was confirmed to be a valuable cytologic feature, allowing distinction between LCNECs and SCLCs. Although other reports on cytologic features of LCNECs described that cell cohesion of LCNECs was reduced as in SCLC,[17–22] the difference was highly significant in our series. However, palisade arrangement was described as a one point for cytologic distinction of LCNEC from SCLC,[17–22] and it was considered to be easy to detect tight cell cohesion by light microscope. Therefore, it should be emphasized that focusing on large clusters with tight cohesion is most important for cytologic discrimination between LCNECs and SCLCs.

Several authors showed that tumor cells of LCNECs had similar morphologic features to SCLCs except for cell size, this being significantly larger for LCNECs than SCLCs.[17–22] In these series, a majority of the SCLC cells were less than 120 μm^2 in size, statistically significant as compared with LCNEC cells ($p < 0.0001$). Another characteristic was that most of small cells in SCLC cases had no

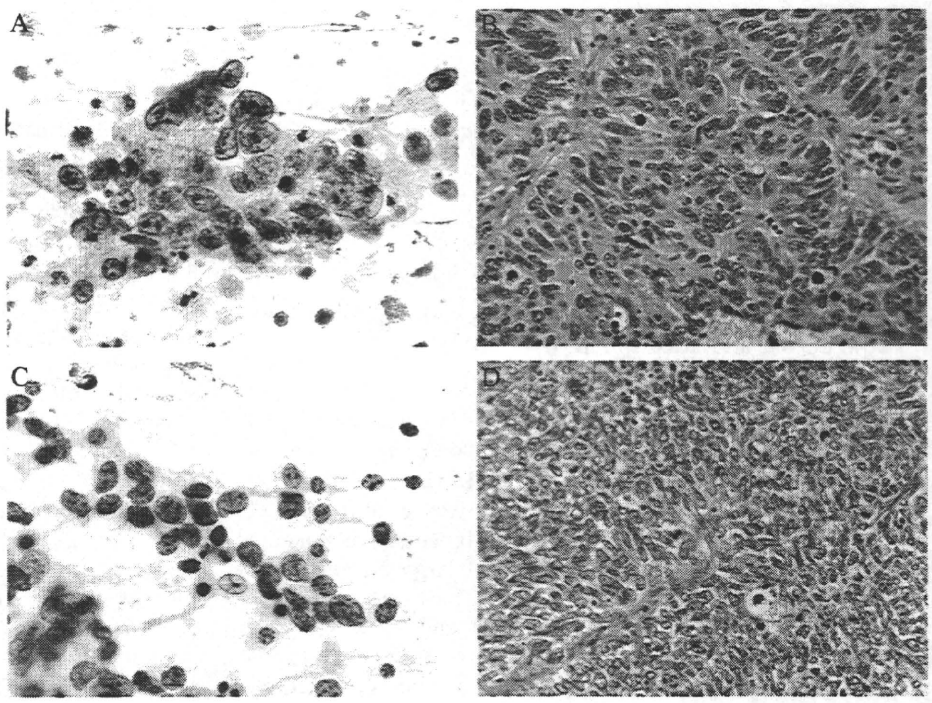

FIGURE 5. Photomicrographs illustrating single cells and tissue architecture in LCNEC and SCLC cases. *A*) Tumor cells ≥120 μm^2 and/or with prominent nucleoli are evident in a cytologic smear of an LCNEC case (Papanicolaou stain, ×100); *B*, nests of LCNEC cells are predominantly composed of large tumor cells with abundant cytoplasm and occasional prominent nucleoli (hematoxylin and eosin stain, ×40); *C*, tumor cells <120 μm^2 without prominent nucleoli are evident in a cytologic smear of an SCLC case (Papanicolaou stain, ×100); *D*, nests of SCLC cells are predominantly composed of small tumor cells with scant cytoplasm without nucleoli (hematoxylin and eosin stain, ×40).

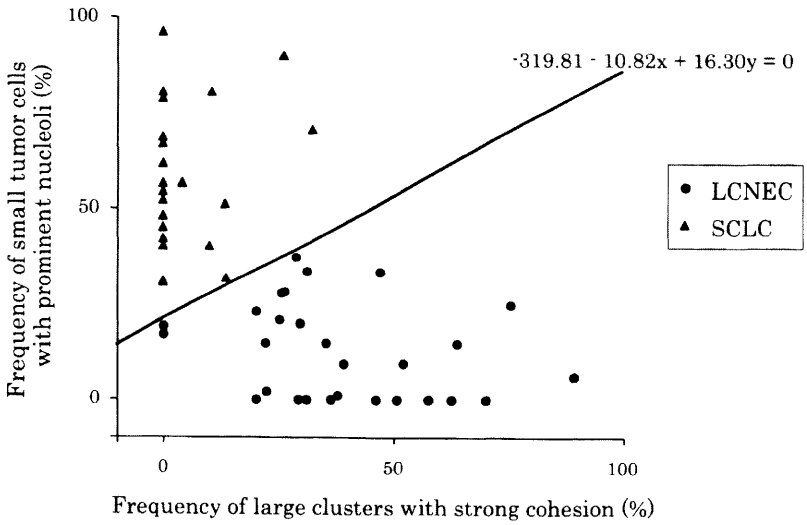

FIGURE 6. Logistic regression for frequencies of small tumor cells without prominent nucleoli and large clusters with tight cohesion. The scatter plot allows clear separation of LCNEC (●) and SCLC cases (▲) by the calculated discriminant line. Therefore, a discriminant model for LCNEC and SCLC, diagnosing as SCLCs if dots exist in the field above the line and as LCNECs if below the line, was made. All SCLC and LCNEC cases were discriminated correctly with the discriminant model based on logistic regression from cytologic frequencies, and sensitivity, specificity, and accuracy were all 100%.

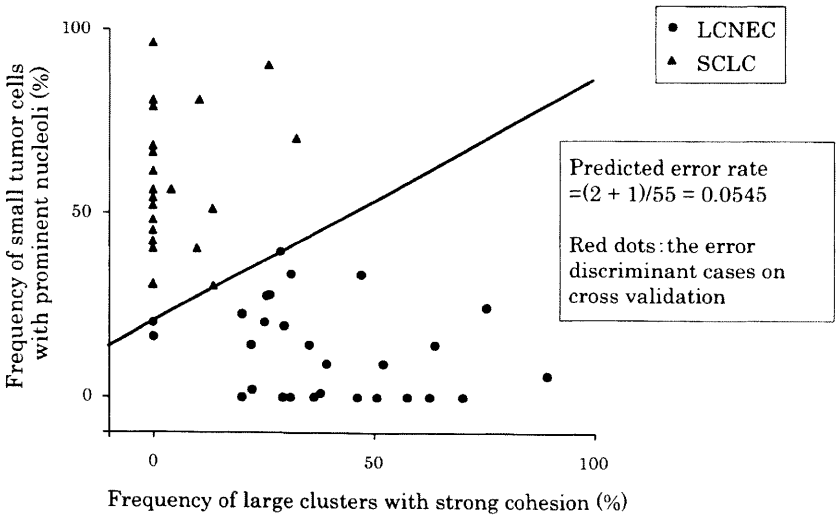

FIGURE 7. Leave-one-out cross validation. Regarding each case as a new case, prediction of the error rate of the discriminant model was analyzed. Red dots show error discriminant cases on cross validation. Two of the LCNEC cases and one SCLC case were discriminated in error with the discriminant model, so that the prediction of error rate of the model was 0.00545.

prominent nucleoli, again being significantly different from LCNECs ($p < 0.0001$). Moreover, although naked nuclei appear to be a significant distinguishing attribute between the two tumor types, it was considered to be inadequate for inclusion in the discriminant model for the following reasons: it is rather difficult to perceive cytoplasm in intact large cells compared with small cells, and naked nuclei was not found in more than 60% of SCLC cases. Therefore, only the frequency of the small cells without prominent nucleoli contributed to cytologic discrimination between LCNEC and SCLC.

To establish accurate cytologic diagnosis of LCNEC using the two cytologic parameters, we established a discriminant model that gave exceedingly good sensitivity, specificity, and accuracy. The current discriminant model, however, does have some problems with routine cytology as follows: complicated procedures for obtaining the two cytologic parameters and necessity of uniform diagnostic criteria among cytopathologists. However, with greater experience of LCNEC cases and grasp of detailed cytologic features, it should be possible to overcome these problems.

In conclusion, our discriminant model based on the cytologic features of large cell clusters with tight cohesion and of small tumor cells without prominent nucleoli should prove a useful aid for distinction between LCNECs and SCLCs particularly. Prospective large-sized studies including other nonsmall cell lung cancers are now required to assess the diagnostic impact of this model with routine cytology.

ACKNOWLEDGMENTS

Supported by a Grant-in-aid from the Ministry of Education, Sports, Culture, Science and Technology grant 20591676, Ministry of Health, Labor and Welfare grant 19-12, and the Vehicle Racing Commemorative Foundation grant.

The authors thank Drs. Masaru Ushijima and Masaaki Matsuura, Bioinformatics Group, Genome Center, Japanese