A Japanese Lung Cancer Registry Study
Prognosis of 13,010 Resected Lung Cancers

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Purpose: The validation of tumor, node, metastasis staging system in terms of prognosis is an indispensable part of establishing a better staging system in lung cancer.

Methods: In 2005, 387 Japanese institutions submitted information regarding the prognosis and clinicopathologic profiles of patients who underwent pulmonary resections for primary lung neoplasms in 1999 to the Japanese Joint Committee of Lung Cancer Registry. The data of 13,010 patients with only lung carcinoma histology (97.6%) were analyzed in terms of prognosis and clinicopathologic characteristics.

Results: The 5-year survival rate of the entire group was 61.4%. For the small cell histology (n = 390), the 5-year survival rates according to clinical (c) and pathologic (p) stages were as follows: 58.8% (n = 161) and 58.3% (n = 127) for IA, 58.0% (n = 77) and 60.2% (n = 79) for IB, 47.1% (n = 17) and 40.6% (n = 29) for IIA, 25.3% (n = 38) and 41.1% (n = 29) for IIB, 29.0% (n = 61) and 28.3% (n = 60) for IIIA, 36.3% (n = 19) and 34.6% (n = 40) for IIIB, and 27.8% (n = 12) and 30.8% for IV (n = 13). For the non-small cell histology (n = 12,620), the 5-year survival rates according to c-stage and p-stage were as follows: 77.3% (n = 5642) and 83.9% (n = 4772) for IA, 59.8% (n = 3081) and 66.3% (n = 2629) for IB, 54.1% (n = 205) and 61.0% (n = 361) for IIA, 43.9% (n = 1227) and 47.4% (n = 1330) for IIB, 38.3% (n = 1628) and 32.8% (n = 1862) for IIIA, 32.6% (n = 526) and 29.6% (n = 1108) for IIIB, and 26.5% (n = 198) and 23.1% (n = 375) for IV. Adenocarcinoma, female gender, and age less than 50 years were significant favorable prognostic factors.

Conclusion: This large registry study provides benchmark prognostic statistics for lung cancer. The prognostic difference between stages IB and IIA was small despite different stages. Otherwise, the present tumor, node, metastasis staging system well characterizes the stage-specific prognoses.

Key Words: Lung cancer, Surgery, Prognosis, TNM stage, Resection, Cancer registry.

(*J Thorac Oncol.* 2008;3: 46–52)

The newly revised version of the Union Internationale Contre le Cancer tumor, node, metastasis (TNM) staging system is to be promulgated for general use in 2009. The present TNM staging system for lung cancer has been available worldwide since 1978,[1] and the revision process is underway. To establish a more sophisticated, truly prognostic staging system, the validation of the existing system as well as the simulation of the proposed revision based on a large, updated data set are indispensable.

In Japan, the three major societies that deal with patients with lung neoplasms, the Japan Lung Cancer Society, the Japanese Association for Chest Surgery, and the Japanese Respiratory Society, established a task force committee (The Japanese Joint Committee of Lung Cancer Registry) to perform a nationwide registry study on the prognosis and clinicopathologic profiles of lung neoplasms, both retrospectively and prospectively. The prospective follow-up registry study has been underway for all lung cancer patients who newly visited the hospital in 2002. This prospective registry study includes both resected and nonresected cases. Beside this, the committee has periodically performed three separate retrospective studies focused on cases resected in the years 1989, 1994, and 1999 after a 5-year follow-up period. These studies were planned at 5-year intervals to observe changes and trends in the prognosis, staging, histologic distribution, etc. of resected lung cancer patients in Japan. The results of the second study for patients who were resected in 1994 have already been published elsewhere[2] together with our

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Disclosure: The authors declare no conflict of interest.

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ISSN: 1556-0864/08/0301-0046

proposal for possible revisions to the present staging system.[3] The current study deals with third retrospective registry for patients who were resected in 1999.

Therefore, the purpose of the present study was to provide the most up-to-date benchmark statistics on the prognosis of resected lung cancer, and to clarify the appropriateness and insufficiencies of the present TNM staging system for lung cancer.

PATIENTS AND METHODS

Registry

In 2005, the Japanese Joint Committee of Lung Cancer Registry performed a nationwide retrospective registry study on the prognosis and clinicopathologic profiles of resected primary lung neoplasms in Japan. Only primary lung neoplasms that had been resected in 1999 at the certified teaching hospitals in Japan were considered for the registry, which had a follow-up period of at least 5 years. The Committee received the registries of 13,344 patients from 387 teaching hospitals. The questionnaire included 32 items such as gender, age, clinical (c)-T, c-N, c-M, c-stage, preoperative treatment, surgical procedure, extent of lymph node dissection, curability, residual tumor, primary site by lobe, tumor diameter, histology, organ invasion, pathologic (p)-T, p-N, p-M, p-stage, pleural involvement, pleural dissemination, intrapulmonary metastasis, pleural cytology, location of nodal metastasis, survival time, recurrence, and cause of death. Recurrent or multiple lung cancers were not included in this registry. The c-stage and p-stage were based on the 6th edition of the Union Internationale Contre le Cancer-TNM staging system published in 1997.[1] The histology of the tumor was described according to the World Health Organization classification.[4]

Patients

Sixty-nine patients (0.5%) with incomplete descriptions of their tumor histology and 265 patients with low-malignant histology or nonepithelial tumor histology (2.0%) were excluded from the study. Therefore, the present study focused on the remaining 13,010 patients with adenocarcinoma, squamous cell carcinoma, small cell carcinoma, large cell carcinoma, or adenosquamous carcinoma. The surgical resections for these patients were various in terms of surgical mode, level of lymph node exploration, and curability. Especially, the resection was either complete in 11,528 patients (88.6%) or incomplete in 1108 patients (8.5%), and the curability was not clearly described in 374 patients (2.9%). Despite these, the TNM staging of each patient was determined on the basis of best available information before, during, and after surgical resections.

Statistical Analysis

The survival time was defined as the time from the date of surgery to the last follow-up date. The survival curves were estimated by the Kaplan-Meier method, and the difference in survival was tested by the log-rank test in which a p value of less than 0.05 was considered significant.

RESULTS

For 13,010 registered patients with lung cancer, the most common histologic type was adenocarcinoma in 8239 patients (63.3%) followed by squamous cell carcinoma in 3700 patients (28.4%), large cell carcinoma in 474 patients (3.6%), small cell carcinoma in 390 patients (3.0%), and adenosquamous carcinoma in 207 patients (1.6%). The survival curve of the entire registry population is shown in Figure 1, in which the 5-year survival rate was 61.4%. The survival curves according to histologic type of all stages are shown in Figure 2. The 5-year survival rates according to the histologic type were as follows: 67.3% for adenocarcinoma,

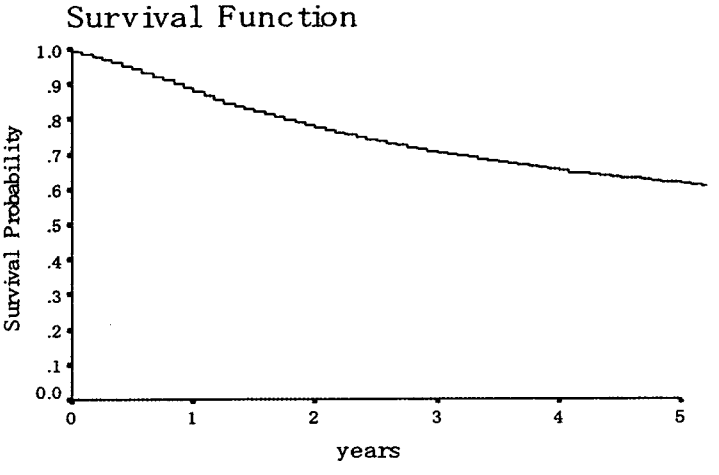

FIGURE 1. A survival curve for all histologic types and all stages (n = 13,010). The 5-year survival rate for the entire group is 61.4%.

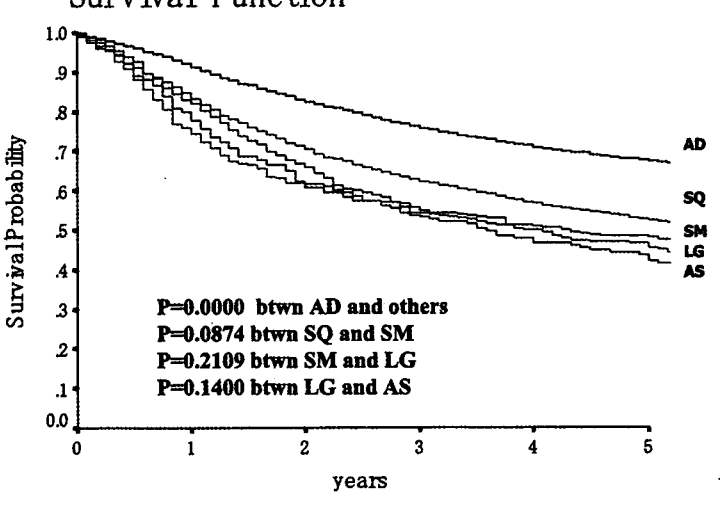

FIGURE 2. Survival curves according to histologic type. The 5-year survival rates according to histologic type are as follows: 67.3% for adenocarcinoma (n = 8239), 52.5% for squamous cell carcinoma (n = 3700), 48.1% for small cell carcinoma (n = 390), 45.5% for large cell carcinoma (n = 474), and 42.1% for adenosquamous carcinoma (n = 207). There is a significant difference in survival between adenocarcinoma and others (p = 0.0000). AD, adenocarcinoma; SQ, squamous cell carcinoma; SM, small cell carcinoma; LG, large cell carcinoma; AS, adenosquamous carcinoma.

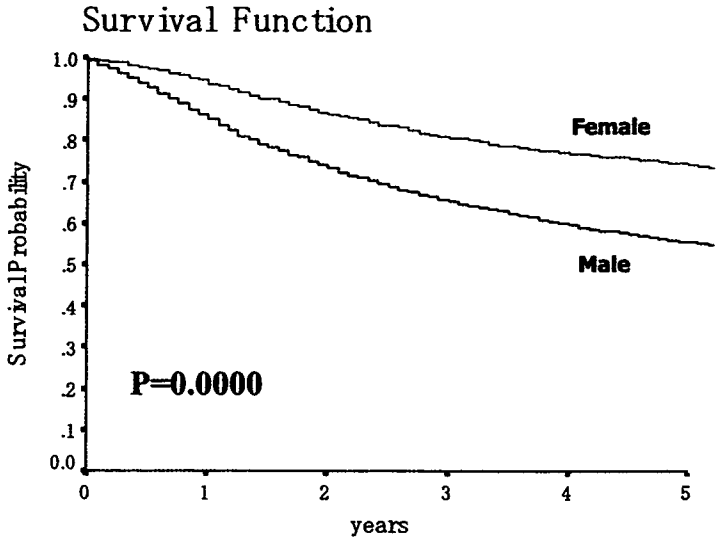

FIGURE 3. Survival curves according to gender. The 5-year survival rates of female (n = 4228) and male (n = 8664) patients are 74.1% and 55.2%, respectively. The survival of female patients is significantly better than that of male patients (p = 0.0000).

52.5% for squamous cell carcinoma, 48.1% for small cell carcinoma, 45.5% for large cell carcinoma, and 42.1% for adenosquamous carcinoma. The adenocarcinoma histology had significantly better survival than other histologic types (p = 0.0000 each). Female patients comprised 32.5% (n = 4228) of the entire registered population, and male patients comprised 66.6% (n = 8664). The 5-year survival rates of the female and male patients were 74.1% and 55.2%, respectively. These survival curves are shown in Figure 3, and the difference in survival between the 2 genders was significant (p = 0.0000). The clinical profiles and stage-specific prognosis were described separately for small cell and non-small cell histologic categories because of the known differences in the pathobiologic nature and response to treatment between these malignancies.

Small Cell Carcinoma

For 390 patients with resected small cell carcinoma of all stages, the 5-year survival rate was 48.6%. The survival curves according to stage are shown in Figure 4. The distribution of c-stage and p-stage, stage-specific 5-year survival rates, and the difference in survival between neighboring stages are presented in Table 1.

Non-small Cell Carcinoma

For 12,620 patients with resected non-small cell histologies of all stages, the 5-year survival rate was 61.8%. The survival curves according to stage are shown in Figure 5. The distribution of c-stage and p-stage, stage-specific 5-year survival rates, and difference in survival between neighboring stages are presented in Table 2. For the c-stage, the difference in survival was significant between all neighboring c-stages except for those between stages IB and IIA and between IIIA and IIIB. For the p-stage, the difference in survival was significant between all neighboring stages, although the difference between p-stages IB and IIA was approaching the marginal significance level.

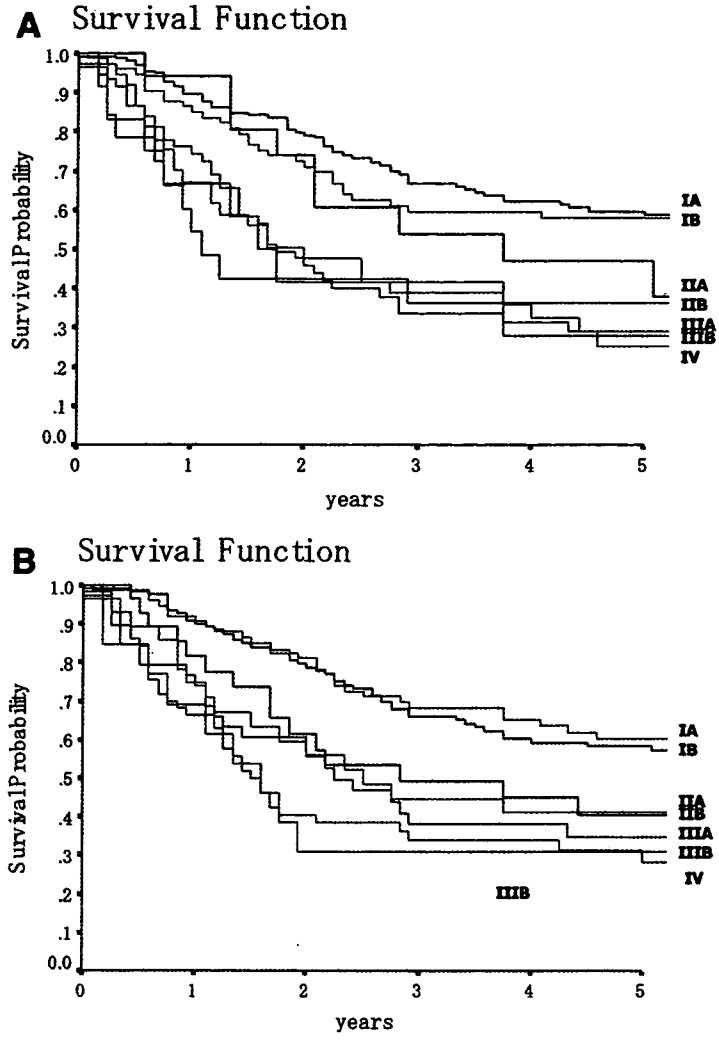

FIGURE 4. Survival curves of small cell lung carcinoma cancers according to c-stage (*A*) and p-stage (*B*) (n = 390). The 5-year survival rates by c-stage are as follows: 58.8% for IA (n = 161), 58.0% for IB (n = 77), 47.1% for IIA (n = 17), 25.3% for IIB (n = 38), 29.0% for IIIA (n = 61), 36.3% for IIIB (n = 19), and 27.8% for IV (n = 12). The 5-year survival rates by p-stage are as follows: 58.3% for IA (n = 127), 60.2% for IB (n = 79), 40.6% for IIA (n = 29), 41.1% for IIB (n = 29), 28.3% for IIIA (n = 60), 34.6% for IIIB (n = 40), and 30.8% for IV (n = 13).

Survival was further analyzed according to patient age. The survival curves according to three age groups, those <50 years (n = 797), those ≥50 years but <70 years (n = 6563), and those ≥70 years (n = 5147) are shown in Figure 6. The 5-year survival rates for the three age groups were 69.9, 66.0, and 54.9%, respectively. The survival of patients aged ≥70 years was significantly worse than those in the other two age groups (p = 0.0000 and p = 0.0000).

Comparison between the 1994 and 1999 Registry Studies

The distribution of histologic types was compared between 1994 and 1999 (Fig. 7). Within the 5-year interval,

TABLE 1. Stage-Specific 5-Yr Survival Rates for Small Cell Carcinoma According to the Clinical and Pathological Settings (n = 390)

	Stage						
Stage Settings	**IA**	**IB**	**IIA**	**IIB**	**IIIA**	**IIIB**	**IV**
Clinical, n (%)	161 (41.3)	77 (19.7)	17 (4.4)	38 (9.7)	61 (15.6)	19 (4.9)	12 (3.1)
5-Yr survival rate, %	58.8	58.0	47.1	25.3	29.0	36.3	27.8
Difference in survival[a]	0.5627	0.4110	0.1577	0.9807	0.7045	0.7265	—
Pathological, n (%)	127 (32.6)	79 (20.3)	29 (7.4)	29 (7.4)	60 (15.4)	40 (10.3)	13 (3.3)
5-Yr survival rate, %	58.3	60.2	40.6	41.1	28.3	34.6	30.8
Difference in survival[a]	0.9331	0.0415	0.8289	0.2300	0.5217	0.6115	—

[a] Significance of the difference in survival between neighboring (lower and next higher) stages (p value).

incidence of adenocarcinoma increased 7%, from 56 to 63%, whereas that of squamous cell carcinoma decreased 5%, from 33 to 28%. The proportion of other histologic types remained almost unchanged. When the overall survival was compared, an improvement of the 5-year survival rate from 52.0 to 61.4% was achieved for all histologic types, and from 52.6 to 61.8% for non-small cell carcinomas. The gender distribution did not change remarkably between 1994 and 1999: female patients comprised 29.9% of the all the registered patients in 1994, and 32.8% in 1999. Nevertheless, the difference in survival according to gender grew within the 5-year interval: the difference in the 5-year survival rate between women and men was 13.2% in 1994, and 18.9% in 1999.

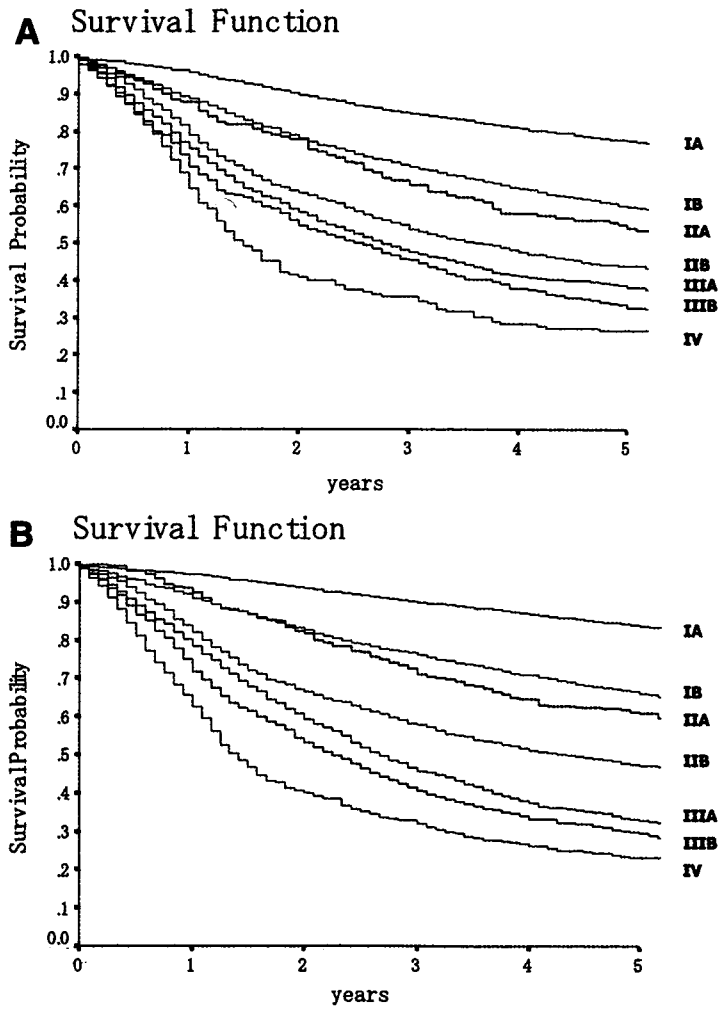

FIGURE 5. Survival curves of non-small cell histologies according to c-stage (A) and p-stage (B) (n = 12,620). The 5-year survival rates by c-stage are as follows: 77.3% for IA (n = 5642), 59.8% for IB (n = 3081), 54.1% for IIA (n = 205), 43.9% for IIB (n = 1227), 38.3% for IIIA (n = 1628), 32.6% for IIIB (n = 526), and 26.5% for IV (n = 198). The 5-year survival rates by p-stage are as follows: 83.9% for IA (n = 4772), 66.3% for IB (n = 2629), 61.0% for IIA (n = 361), 47.4% for IIB (n = 1330), 32.8% for IIIA (n = 1862), 29.6% for IIIB (n = 1108), and 23.1% for IV (n = 375).

The stage distribution was compared in non-small cell lung carcinoma between 1994 and 1999 (Fig. 8). The percentage of stages IA and IB increased 11%, from 59 to 70%, in the c-setting, and 8%, from 51 to 59%, in the p-setting. Stage-specific 5-year survival rates in non-small cell carcinoma were compared between the 1994 and 1999 registry studies for c-stage (Table 3) and for p-stage (Table 4). Although a survival improvement was achieved in all stages, the change in stage IB was remarkable. The 5-year survival rate in stage IB improved from 49.9 to 59.8% in a c-setting, and from 60.1 to 66.3% in a p-setting. Summarizing these, the trends from 1994 to 1999 consisted of an increase in the adenocarcinoma histology and earlier stages, and an improvement in the overall as well as the stage-specific survival.

DISCUSSION

This is a report on the third nationwide registry study conducted by the Japanese Joint Committee of Lung Cancer Registry representing three major Japanese societies that deal with patients with lung cancer, in which the clinicopathologic features and prognosis of the resected lung cancer were studied. Three registry studies have independently and periodically focused on cases that were resected in the years 1989, 1994, and 1999 after a 5-year follow-up period. The details of the second study involving cases resected in 1994 in which 7393 patients with primary lung neoplasms were registered from 307 teaching hospitals in Japan have already been published elsewhere.[2,3] The number of registered patients in the third study (13,344 patients) was almost twice that of the second study (7393 patients) with only a slight increase in the number of participating institutions from 307 to 387. The number of cases registered from each institute ranged from 1 to 212 cases, and 15 institutes registered more than 100 cases. Considering that the total number of lung

TABLE 2. Stage-Specific 5-Yr Survival Rates for Non-small Cell Carcinoma According to the Clinical and Pathological Settings (n = 12,620)

	Stage						
Stage Settings	**IA**	**IB**	**IIA**	**IIB**	**IIIA**	**IIIB**	**IV**
Clinical, n (%)	5642 (44.7%)	3081 (24.4%)	205 (1.6%)	1227 (9.7%)	1628 (12.9%)	526 (4.2%)	198 (1.6%)
5-Yr survival rate, %	77.3	59.8	54.1	43.9	38.3	32.6	26.5
Difference in survival[a]	0.0000	0.1444	0.0022	0.0013	0.0755	0.0111	—
Pathological, n (%)	4772 (37.8%)	2629 (20.8%)	361 (2.9%)	1330 (10.5%)	1862 (14.8%)	1108 (8.8%)	375 (3.0%)
5-Yr survival rate, %	83.9	66.3	61.0	47.4	32.8	29.6	23.1
Difference in survival[a]	0.0000	0.0367	0.0000	0.0000	0.0054	0.0001	—

[a] Significance of the difference in survival between neighboring (lower and next higher) stages (p value).

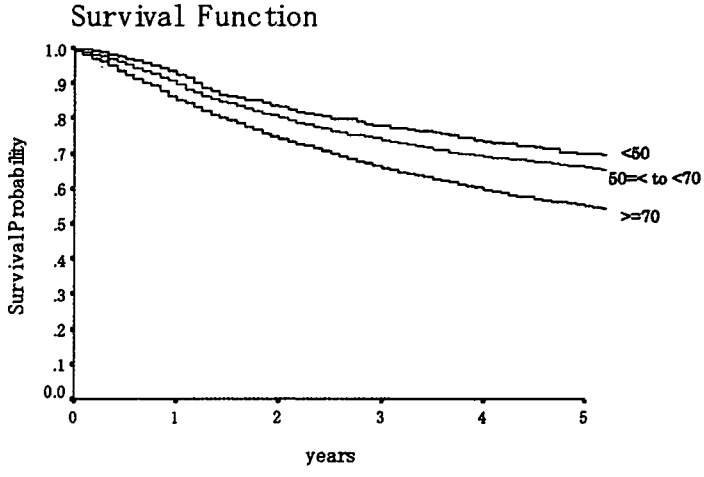

FIGURE 6. Survival curves according age in non-small lung cancer. The 5-year survival rates for the three age groups, <50 years (n = 797), ≥50 years but <70 years (n = 6563), and ≥70 years (n = 5147) are 69.9%, 66.0%, and 54.9%, respectively.

cancer resections in Japan was approximately 30,000, these registered cases are estimated to comprise 30 to 40% of the total. The results of this registry study represent the findings based on the largest series ever published.

There has been remarkable difference in survival between patients resected in 1994 and 1999, where the overall survival rate at 5 years in the registry population improved from 52.6 to 61.4%. The stage-specific survival also improved. Because the survival improvement was achieved not only in all stages but also in the entire population, this improvement should not be interpreted as simply the result of a stage migration phenomenon. The possible reasons for the improvement might be refinements in the evaluation of surgical candidates, advancements and improvement in treatment, and the shift of the registry population toward more curable lung cancer.

Refinement in the preoperative work-up for surgical candidates may better identify patients with distant disease, resulting in a better selection of patients for surgery. Nevertheless, except for an improvement in imaging diagnosis techniques such as computed tomography (CT), the difference in the quality of preoperative work-up between 1994 and 1999 does not seem significant. Even in 1999, positron emission tomography scans were not used as part of a routine preoperative work-up in Japan. Therefore, the difference in preoperative work-up does not seem to account for the difference in survival between the years 1994 and 1999.

When looking at the changes in surgical interventions for lung cancer patients in the 5 years between 1994 and

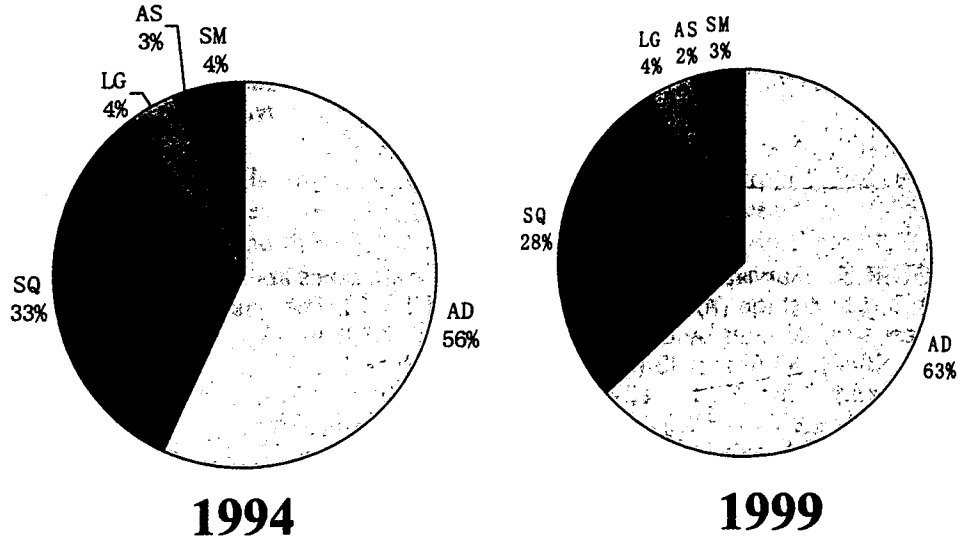

FIGURE 7. Distribution of histologic types in 1994 and 1999. Adenocarcinoma increases 7% (from 56% to 63%) and squamous cell carcinoma decreases 5% (from 33% to 28%).

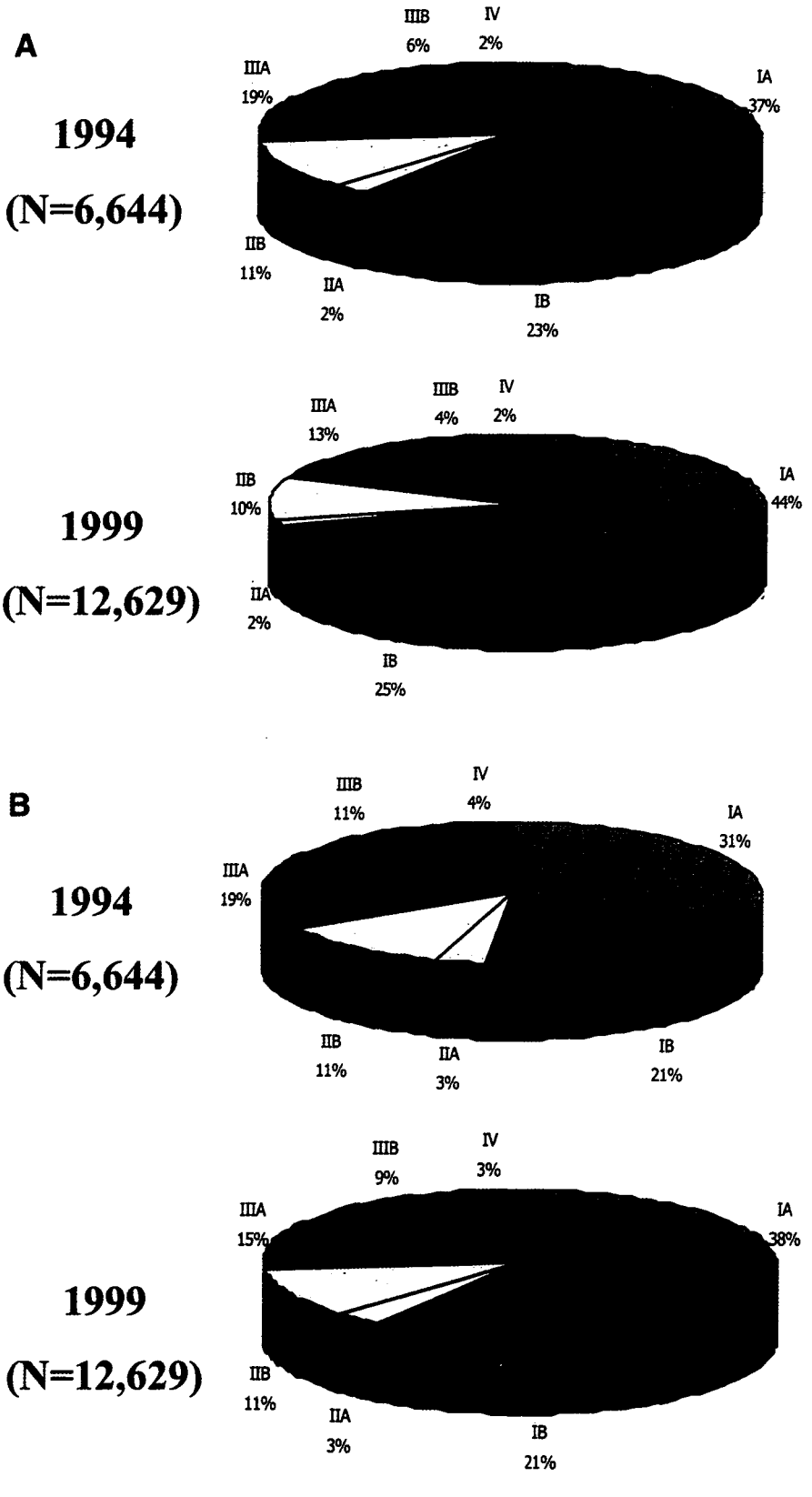

FIGURE 8. Distribution of c-stage (*A*) and p-stage (*B*) for non-small cell lung histologies in 1994 and 1999. The percentage of stage I increased from 60 to 69% (11%) in the c-setting, and from 52 to 59% (7%) in the p-setting.

1999, we recognized that less (or minimally) invasive surgery with or without video assistance had been more generalized.[5] In these minimally invasive techniques, the faster postoperative recovery has been speculated, and this is a present-day trend in oncologic surgery of any sites. Nevertheless, knowing that no one study has ever definitely demonstrated that minimally invasive surgery improves the survival of patients with lung cancer, or the mortality/morbidity, it is unlikely that the improvement in survival of the present registry population was solely because of the advancements in surgical interventions.

Comparing the distribution of histologic types between 1994 and 1999, the 7% increase in adenocarcinomas and the 5% decrease in squamous cell carcinoma were remarkable changes. In this registry study, the noninvasive form of

TABLE 3. Comparison of Stage-Specific 5-Yr Survival Rate (%) between 1994 and 1999 (c-Stage) in Non-small Cell Histologies

	c-Stage							
Year of Survey	IA	IB	IIA	IIB	IIIA	IIIB	IV	All Stages
1994 (n = 6,644)	72.1	49.9	48.7	40.6	35.8	28.0	20.8	52.6
1999 (n = 12,620)	77.3	59.8	54.1	43.9	38.3	32.6	26.5	61.8

TABLE 4. Comparison of Stage-Specific 5-Yr Survival Rate (%) between 1994 and 1999 (p-Stage) in Non-small Cell Histologies

	p-Stage							
Year of Survey	IA	IB	IIA	IIB	IIIA	IIIB	IV	All Stages
1994 (n = 6,644)	79.5	60.1	59.9	42.2	29.8	19.3	20.0	52.6
1999 (n = 12,620)	83.9	66.3	61.0	47.4	32.8	29.6	23.1	61.8

adenocarcinoma, nonmucinous bronchioloalveolar carcinoma, was included in the adenocarcinoma category. These tumors are well known for their characteristic presentation on high-resolution CT images as ground glass opacity and a superb prognosis without recurrence after intervention.[6,7] Considering that the evaluation of these faint, small-sized tumors using high-resolution CT was being generalized in Japan in late 1990s, the increase in bronchioloalveolar carcinoma might have resulted in the inclusion of these earlier, less-aggressive tumors into the registry population. The distribution of the stage of the disease at diagnosis also changed remarkably between 1994 and 1999 as can be seen in Figure 8. The earliest disease, stage IA and IB, comprised 60% of the c-stage and 52% of the p-stage in 1994, and 69% and 59% in 1999, respectively. The shift of the patients' diagnosis toward an earlier staged disease at the time of surgery definitely had a significant impact on the improvement in overall survival.

Based on the second registry study of cases resected in 1994, we proposed a revision of the TNM staging system in which the unification of stages IB and IIA and the division of T1 into T1a and T1b by the cutoff length of a diameter of 2 cm were shown to be necessary. In this latest 1999 data set the prognostic difference in survival between stages IB and IIA was small. In the c-setting, the 5-year survival rates for IB and IIA were 59.8% and 54.1%, and the difference in survival was not statistically significant (p = 0.1444). In the p-setting, the 5-year survival rates for IB and IIA were 66.3% and 61.0%, and the difference in survival was marginally significant (p = 0.0367), probably because of the increase in the overall number of patients. Because the survival improvement in patients with stage IB was so remarkable, the prognostic difference between stages IB and IIA seemed to increase in 1999 compared with that in 1994. Nevertheless, considering the limited number of patients with stage IIA disease, we believe that the unification of stages IB and IIA would well characterize the stage-specific prognosis of both groups.

In the report on the second registry study, the large prognostic difference in survival by gender, age, and histology was addressed. Also, in this third registry study, a significant difference in survival according to these variables was reproduced. Especially, the difference in 5-year survival rate by gender was almost 20% in non-small cell carcinomas, in which the 5-year survival rates for men and women were 55.5% and 74.5%, respectively. It is still unclear what factors account for the large survival difference between men and women. It is necessary to see the relationship between female gender and other significant prognostic variables such as histology and their biologic characteristics. Considering the difference in smoking status between the two genders in Japan, the difference in the biologic nature of cancers in women versus men might have some impact on overall survival.

The present retrospective, nationwide, large-scale registry study provides the most updated benchmark statistics for patients with lung cancer. Further studies to elucidate the factors associated with the improvement of survival and the impact of several prognostic variables is underway.

REFERENCES

1. International Union Against Cancer. Lung tumours. In LH Sobin, CH Wittekind (Eds.), TNM Classification of Malignant Tumours, 5th Ed. New York: Wiley-Liss, 1997. Pp. 91–97.
2. Goya T, Asamura H, Yoshimura H, et al. Prognosis of 6644 resected non-small cell lung cancers in Japan: a Japanese lung cancer registry study. *Lung Cancer* 2005;50:227–234.
3. Asamura H, Goya T, Koshiishi Y, et al. How should the TNM staging system for lung cancer be revised? A simulation based on the Japanese Lung Cancer Registry populations. *J Thorac Cardiovasc Surg* 2006;132: 316–319.
4. Travis WD, Colby TV, Corrin B, et al. Histological Typing of Lung and Pleural Tumors, World Health Organization International Histological Classification of Tumors. Berlin, Germany: Springer, 1999.
5. McKenna RJ, Houck W, Fuller CB. Video-assisted thoracic surgery lobectomy: experience with 1100 cases. *Ann Thorac Surg* 2006;81:421–425.
6. Noguchi M, Morikawa A, Kawasaki M, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995;75: 2844–2852.
7. Sakurai H, Maeshima A, Watanabe S, et al. Grade of stromal invasion in small adenocarcinoma of the lung: histopathological minimal invasion and prognosis. *Am J Surg Pathol* 2004;28:198–206.

Gene expression profiling of epidermal growth factor receptor/KRAS pathway activation in lung adenocarcinoma

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(Received January 29, 2007/Revised March 1, 2007/Accepted March 5, 2007/Online publication April 20, 2007)

We examined the genome-wide expression profiles of 86 primary lung adenocarcinomas and compared them with the mutation status of the four key molecules (EGFR, ERBB2, KRAS and BRAF) in the EGFR/KRAS/BRAF pathway. Unsupervised classification revealed two subtypes (the bronchial type and the alveolar type) of lung adenocarcinoma. Mutually exclusive somatic mutations of the epidermal growth factor receptor (EGFR) gene (36/86, 41.8%), K-ras gene (11/86, 12.8%) and BRAF gene (1/86, 1.1%) were detected. KRAS mutations were observed significantly frequently in bronchial-type tumors, whereas the frequencies of EGFR mutations were similar in both the alveolar and bronchial types. Twenty-seven genes showed increased expression in EGFR-mutated tumors and these included molecules that function in the EGFR/KRAS/BRAF pathway (EGFR, AKT1 and BCR). In particular, expression of BCR, which is required for EGFR protein degradation, was induced by EGF stimulation, suggesting a negative feedback loop in lung cancer. A subgroup of the alveolar type tumors showed significantly better prognosis than other tumors. Integrated analysis of genetic and gene expression profiling aimed to delineate inherent oncogenic pathways in cancer will be valuable not only for the understanding of molecular pathogenesis, but also for discovering novel biomarkers and predicting clinical outcome. (*Cancer Sci* 2007; 98: 985–991)

Lung cancer is one of the leading causes of cancer death worldwide, and lung adenocarcinoma accounts for more than 50% of all lung cancers.[1] Lung adenocarcinoma comprises a heterogeneous group of tumors with broad ranges of histopathology, combinations of genetic alterations and gene expression patterns.[2,3]

Recently, molecular-targeted therapies, which modulate key molecules (such as kinases) of the essential signal pathways that cancer cells exploit for survival, have considerably increased the possibility of personalized cancer therapy.[4,5] It has been discovered that epidermal growth factor receptor (EGFR) gene mutations were closely associated with better responsiveness to EGFR tyrosine kinase inhibitors (EGFR-TKIs).[6–8] Activation of EGFR transfers signals through the ERBB2, RAS and RAF proteins in the downstream pathway, and importantly oncogenic mutations of the erbB2, K-ras or B-raf genes have also been reported in lung cancer.[9] The EGFR/ERBB2/KRAS/BRAF pathway is frequently (more than 50%) altered in lung adenocarcinoma and, consistent with their epistatic roles in the signal pathway, EGFR, ERBB2, KRAS and BRAF mutations exist in a mutually exclusive way.[9] Therefore this pathway is one of the most promising therapeutic targets for lung cancer, and many pharmacological compounds targeting the components of the pathway have been developed and clinically tested.[10]

To achieve personalized therapy by choosing the most effective therapeutic agents and minimizing their undesirable effects, we need to gain a comprehensive view of signal transduction networks and their association with biological phenotypes in cancer. In relation to genetic activation of the EGFR/ERBB2/KRAS/BRAF pathway, many questions still remain, such as whether there exist any molecular or histopathological features of the activated EGFR/ERBB2/KRAS/BRAF pathway and how the pathway interacts with other signal pathways. Such information would be valuable for understanding the molecular pathogenesis of lung cancer as well as for identifying appropriate biomarkers for molecular diagnosis and discovering novel therapeutic targets for individualized therapy.

Materials and Methods

Surgical specimens. Surgical specimens from 86 Japanese patients with lung adenocarcinoma who were diagnosed and underwent surgery at the National Cancer Center Hospital, Tokyo, Japan, between June 1997 and May 2002 were examined. Clinical features of analyzed cases are shown in Table 1. The study protocol was approved by the institutional review board of the National Cancer Center.

RNA extraction and microarray analysis. Each frozen sample was homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted. Biotin-labeled cRNA was synthesized from 5 μg of total RNA using the Superscript Choice System (Invitrogen) and BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). Hybridization to the microarray HG-U95Av2 (Affymetrix, Santa Clara, CA, USA) and detection of the signals were carried out according to the manufacturer's instructions.

Immunohistochemical analysis. Five-micrometer-thick sections of the formalin-fixed paraffin-embedded tumors were deparaffinized. After heat-induced epitope retrieval, the sections were incubated with mouse monoclonal anticytokeratin 6 (KRT6) antibody (clone D5/16 B4, DakoCytomation, Glostrup, Denmark), mouse monoclonal antimucin 5AC (MUC5AC) antibody (clone 45M1, Zymed, South San Francisco, CA, USA), mouse monoclonal anti-Surfactant Apoprotein A (SP-A) (clone PE10, DakoCytomation) and rabbit polyclonal anti-Clara cell antigen (CC-10) antibody (Santa Cruz, Santa Cruz, CA, USA) at a dilution of 1:100. The sections were incubated with a biotinylated secondary antibody against mouse or rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:200 and then with the Vectastain ABC reagent (Vector Laboratories). Tumors containing more than 10% stained tumor cells in the largest representative section were considered positive.

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doi: 10.1111/j.1349-7006.2007.00483.x

Table 1. Clinicopathological features of analyzed cases

			Cluster A ($n = 42$)	Cluster B ($n = 44$)	P-value
Gender	Male	49	29	20	0.027
	Female	37	13	24	
Age (average)		35–83 (62.4)			
Smoking habit	Never	41	17	24	0.19
	Former	23	10	13	
	Current	22	15	7	
Stage	I	52	23	29	0.29
	II or III	34	19	15	
Differentiation	Well	37	11	26	0.002
	Moderate or Poor	49	31	18	
EGFR mutation	Exon 18	1	1	0	0.25
	Exon 19	18	9	9	
	Exon 20	0	0	0	
	Exon 21	17	5	12	
KRAS mutation	Exon 1	10	8	2	0.019
	Exon 2	1	1	0	
BRAF mutation	Exon 11	0	0	0	ND
	Exon 15	1†	0	1	

†V600E mutation. EGFR, epidermal growth factor receptor.

Laser-capture microdissection, DNA extraction and mutational analysis. Five-micrometer-thick sections of the methanol-fixed paraffin-embedded tumors were subjected to laser-capture microdissection using the LM200 system (Arcturus, Mountain View, CA, USA). Corresponding normal lung epithelial cells were obtained by scraping and were used as a control. DNA was extracted using a standard method. We amplified exons 18, 19, 20 and 21 of the EGFR gene, exons 19 and 20 of the erbB2 gene, exons 1 and 2 (covering codons 12, 13 and 61) of the K-ras gene, and exons 11 and 15 of the B-raf gene by polymerase chain reaction (PCR) using High Fidelity Taq polymerase (Roche, Mannheim, Germany) and appropriate primers (primer sequences available on request). All PCR products were purified and analyzed by sequencing.

Quantitative reverse transcriptase-PCR. Five micrograms of total RNA was isolated from a randomly selected 28 cases and was reverse-transcribed (High Capacity cDNA Archive kit, Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was carried out in triplicate and evaluated using TaqMan probes for each target gene (Applied Biosystems) and the ABI 7500 system (Applied Biosystems). The relative expression of each gene was determined by comparing its expression to that of beta-actin.

Cell culture. EGFR mutated lung adenocarcinoma cell lines (NCI-H1650 and NCI-H1975)[7] were maintained as recommended (American Tissue Cell Collection, Manassa, VA, USA). The cell lines were serum-starved and stimulated with 10 ng/mL epidermal growth factor (Sigma, St. Louis, MO, USA) for the indicated time. To inhibit EGFR activation, the specific EGFR inhibitor AG1478 (Sigma, 10 μM) was added.

Statistical analysis of microarray data. For unsupervised clustering of lung adenocarcinoma, we first selected 685 genes based on two criteria. First, the average difference (which is correlated with gene expression level) exceeded 1000 in more than 5% of the 86 tumors, and second, the average difference varied by at least twofold from the median in at least 25% of the samples. Data analysis was carried out using the Gene Spring (Silicon Genetics, Redwood City, CA, USA) software packages and visualized using the Tree and View software package (Stanford University). We used the significance analysis of microarrays software package (SAM; http://www-stat.stanford.edu/~tibs/SAM/index.html) to select genes differentially expressed in each of the tumor subtypes.[11] The output criteria selected for SAM included a difference of 1.5-fold or more between the two groups and a false discovery rate (FDR) of less than 5%.

Other statistics. The unpaired t-test and the χ^2 test were used for comparisons of proportions. The Kaplan-Meier method was used to estimate survival as a function of time, and log-rank analysis was used to compare the differences between subgroups. Cox proportional hazard modeling was carried out to determine independent prognostic factors.

Results

Gene expression profiling of lung adenocarcinoma revealed histologically and clinically heterogenous subtypes. We analyzed the expression of 12 625 probe targets (representing more than 10 000 unique genes) in 86 primary lung adenocarcinomas using an oligo-nucleotide-based microarray. We selected 685 genes whose expressions were relatively abundant and varied widely among the samples analyzed (Appendix I Table 1) and carried out two-dimensional hierarchical clustering analysis to classify the lung adenocarcinomas according to similarities in their patterns of expression (Fig. 1a).

Unsupervised classification demonstrated that lung adenocarcinomas were largely divisible into two clusters (clusters A and B, Fig. 1a). We compared gene expressions between the two clusters and selected genes that showed significant differences in expression (Appendix I Table 2). Tumors in cluster A preferentially expressed genes associated with the cell cycle/cell proliferation, the extracellular matrix, and those expressed specifically in bronchial epithelium (KRT6 and MUC5AC, Fig. 2a,b).[12,13] In contrast, genes expressed significantly in cluster B included growth factor inhibitors, enzymes involved in lipid metabolism, and alveolar pneumocyte markers (such as CC10 and surfactant pulmonary-associated protein C (SFTPC)).[14] In accordance with their similarities to normal pulmonary epithelium, we named these clusters the bronchial type (cluster A) and alveolar type (cluster B), respectively. To validate our microarray analyses, we carried out immunohistochemical analysis of the classifying molecules. We examined the expressions of KRT6, Mucin 5AC, Surfactant apoprotein A (SFTPA) and CC-10 (Fig. 2c–f). As shown in Figure 1(b) and Table 1, KRT6 (bronchial type 12/42 (28.5%) and alveolar type 4/44 (9%), $P = 0.02$), SFTPA (bronchial type 11/42 (26.2%) and alveolar-type 31/44 (70.5%), $P < 1 \times 10^{-4}$) and

doi: 10.1111/j.1349-7006.2007.00483.x

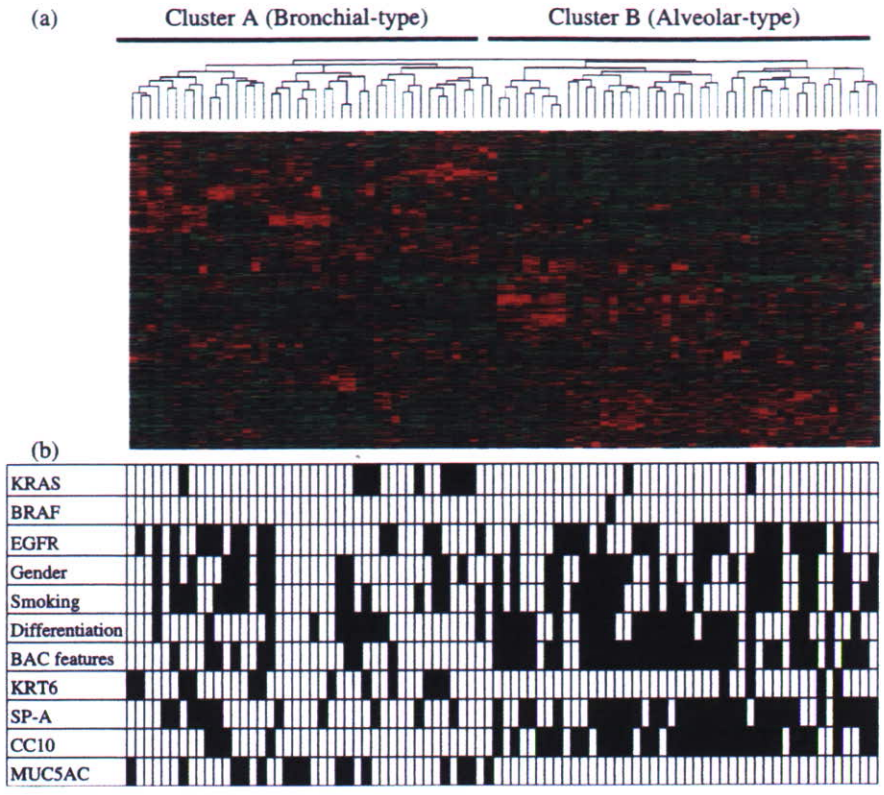


Fig. 1. (a) Unsupervised hierarchical clustering analysis of 86 lung adenocarcinomas. Hierarchical clustering of 86 primary lung adenocarcinomas was carried out based on the expression of 685 transcripts. The normalized expression index for each transcript is indicated by a colored bar. Unsupervised classification revealed two clusters (cluster A, bronchial type, and cluster B, alveolar type) that are shown above the dendrogram of the samples. (b) Clinical features, mutation status of epidermal growth factor receptor (EGFR), KRAS and BRAF, and immunohistochemical expression profile of KRT6, Mucin 5AC, Surfactant apoprotein A (SP-A) and CC10 in 86 lung adenocarcinomas. Clinicopathological features (gender, smoking status, differentiation and bronchioloalveolar carcinoma [BAC] features) of each case are shown. Solid boxes indicate cases in which the patient was female, a non-smoker and the tumor was well-differentiated and contained BAC features, while clear boxes indicate cases in which the patient was male, a smoker, and the tumor was moderately/poorly-differentiated. The presence of mutations in the EGFR, KRAS and BRAF genes is shown by a solid box. Immunohistological positivity for KRT6, SP-A, CC-10 and MUC5AC is shown by a solid box.

CC-10 (bronchial type 4/42 (9.5%) and alveolar type 32/42 (76.2%), $P < 1 \times 10^{-8}$) were preferentially expressed in bronchial-type and alveolar-type tumors, respectively. Mucin 5AC was detected only in bronchial-type tumors (15/42, 35.7%).

To determine whether this gene-expression-based classification had any association with clinical features, we compared clinicopathological factors between these two groups (Fig. 1b). Bronchial-type tumors had a stronger tendency to occur in males ($P = 0.02$) and to show histologically poorer differentiation ($P = 0.002$) than alveolar-type tumors. We also found that alveolar-type tumors frequently ($P < 0.0001$) showed lepidic growth that is characterized as BAC (bronchioloalveolar carcinoma) features, while bronchial-type tumors exhibited solid growth (Fig. 2g, h, Table 1). Therefore these two subtypes represent clinically and histologically distinct subgroups of lung adenocarcinoma.

Comparison of gene expression profiling and EGFR/ERBB2/KRAS/BRAF mutation status in lung adenocarcinoma. We examined oncogenic mutations of the four key molecules (EGFR, ERBB2, KRAS and BRAF) in this pathway simultaneously and attempted to analyze whether any of them were associated with the classification of lung adenocarcinoma described above. To this end, we obtained tumor cells from 86 lung adenocarcinomas by microdissection and determined the mutation status of the EGFR (exons 18–21), ERBB2 (exons 19 and 20), KRAS (exons 1 and 2) and BRAF (exons 11 and 15) genes. We detected somatic mutations of the EGFR gene (36/86, 41.8%), K-ras gene (11/86, 12.8%) and B-raf gene (1/86, 1.1%) in our cohort (Table 1). We were unable to find any mutation in exons 19 and 20 of the erbB2 gene. The occurrence of these mutations appeared to be mutually exclusive. As reported previously,[15] EGFR mutations were frequently observed in female ($P = 0.046$) and non-smoking patients ($P = 0.034$), whereas there was no significant association between the presence of KRAS mutation and any of the clinical background factors examined (age, gender, smoking habit, tumor differentiation and pathological stage).

We then examined whether these alterations had any association with the classification obtained by gene-expression profiling (Fig. 1b). EGFR mutations were more frequent in alveolar-type (21/44, 47.7%) than in bronchial-type (15/42, 35.7%) lung adenocarcinoma, although not to a significant degree ($P = 0.25$). On the other hand, we found that KRAS mutations were significantly more frequent in the bronchial type (9/42, 21.4%) than in the alveolar type (2/44, 4.5%) ($P = 0.019$).

Identification of mRNA expression features associated with the presence of EGFR mutation. Because our unsupervised classification of lung adenocarcinoma based on genome-wide gene-expression pattern failed to detect any specific subgroup of tumors that harbored frequent EGFR mutations and identification of candidate biomarkers to predict EGFR mutation should be clinically valuable, we next attempted to determine whether any expression signature was associated with the presence of EGFR mutation. Comparison of gene expression profiles between EGFR-mutated and EGFR-wild-type tumors detected 26 genes whose expression was significantly increased in EGFR-mutated tumors (Table 2). Interestingly, these included molecules (EGFR and AKT1) that are known to function in the EGFR/KRAS/BRAF pathway. They also included secreted/membrane-associated molecules such as PTK7. To validate these results, we carried out quantitative RT-PCR analysis of these genes in 28 lung tumor samples (14 EGFR-mutated and 14 EGFR-wild-type tumors, and there was no significant difference in clinocopathological features between the two groups). As shown in Figure 3(a), the expressions of these genes differed significantly between EGFR-mutated and EGFR-wild-type tumors.

Since BCR has recently been shown to regulate the degradation of EGFR protein after ligand stimulation[16] we hypothesized that induction of *BCR* expression in EGFR-mutated tumors might constitute a negative feedback loop. *BCR* overexpression was frequent in EGFR-mutated tumors (16/36, 44.4%) compared to in EGFR-wild-type ones (1/50, 2%). To determine whether *BCR* expression is regulated by EGFR activation in lung

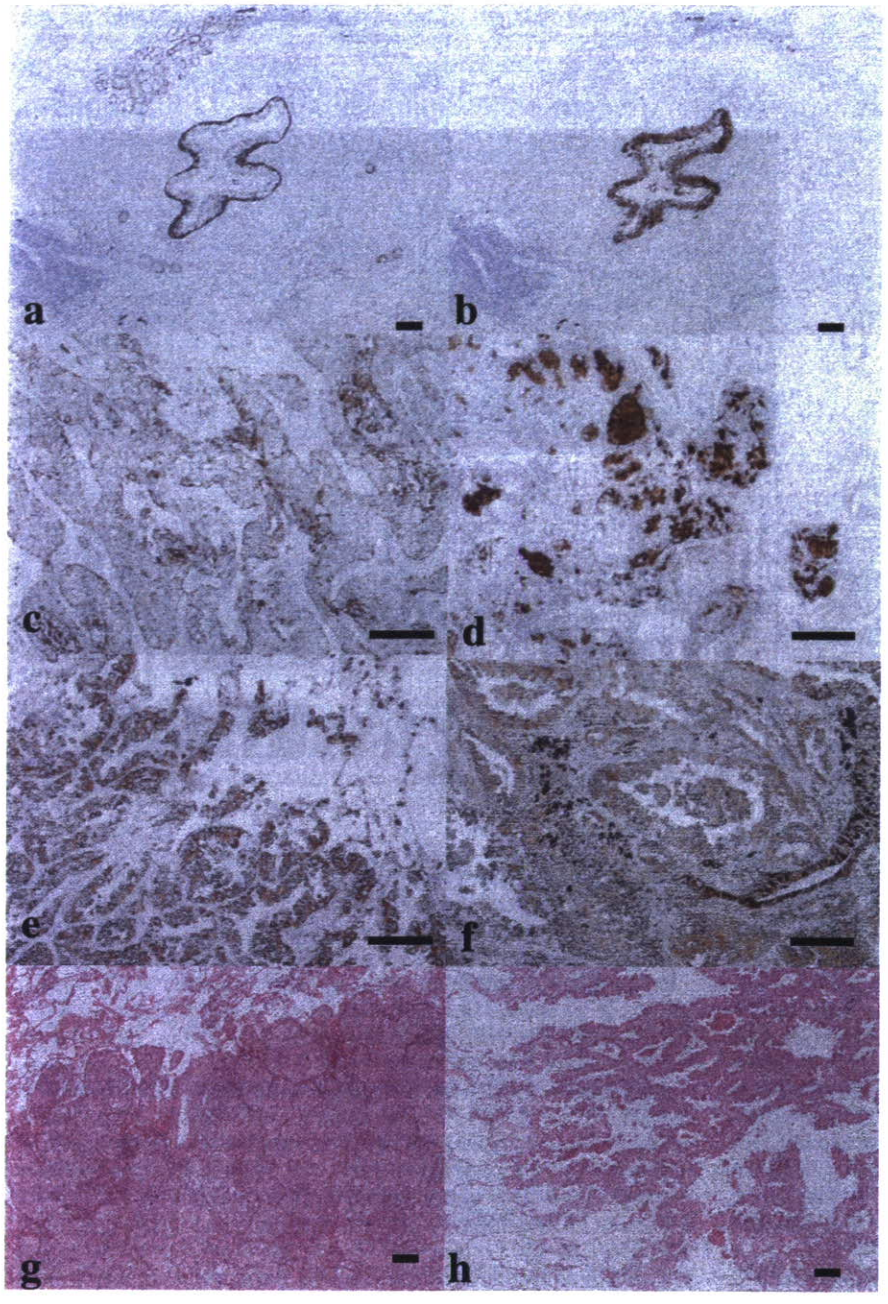


Fig. 2. Immunohistochemical analyses of classifying molecule expression in lung adenocarcinoma and normal lung. Bronchial epithelia but not alveolar pneumocytes were positive for both KRT6 (a, ×100) and MUC5AC (b, ×100). Representative immunohistochemical expression of KRT6 (c, ×200), MUC5AC (d, ×200), Surfactant apoprotein A (SP-A) (e, × 200) and CC-10 (f, ×200) in lung adenocarcinoma is shown. The representative histologies of bronchial-type (left) and alveolar-type (right) tumors (Hematoxylin-eosin stained, ×100). Bar, 200 μm.

cancer cells, we stimulated lung cancer cells with EGF and examined the resulting change in BCR mRNA expression *in vitro*. As shown in Figure 3(b), expression of BCR mRNA was up-regulated (2.2–3.0-fold) in response to EGF stimulation. On the other hand, when we inhibited EGFR activation pharmacologically in EGFR-mutated lung cancer cell lines, we observed a significant decrease (0.6-0.4-fold) of BCR mRNA expression (Fig. 3c).

Prognostic significance of molecular classification of lung adenocarcinoma. We examined whether our molecular classification of lung adenocarcinoma has any association with clinical outcome. The presence of genetic alterations in the EGFR/KRAS/BRAF pathway was not associated with prognosis (data not shown). There was no significant difference in prognosis between the alveolar and bronchial types ($P = 0.07$, Fig. 4b). However, when we divided the alveolar type into two subgroups, we found that a subgroup of the alveolar type showed remarkably better prognosis than the other tumors (Fig. 4c).

Discussion

In this study, we attempted to investigate the biological significance of oncogenic pathway activation in lung cancer. Previous gene expression analyses of lung cancer elucidated heterogenous subgroups with specific gene expression signatures in lung adenocarcinoma.[17–21] Our unsupervised hierarchical clustering based on gene expression profiling revealed two distinct subgroups (bronchial and alveolar types) of lung adenocarcinoma, which are associated with clinical backgrounds and largely consistent with these studies. Especially the TRU (terminal respiratory unit)-type of lung adenocarcinoma[20] shares similar histological and molecular features with the alveolar type. We concurrently analyzed somatic mutations of the components of the EGFR/KRAS/BRAF pathway (ERBB2, EGFR, KRAS and BRAF genes). As reported previously,[8,15] EGFR mutations were frequently observed in female, non-smoking patients, but there was no significant association between the presence of EGFR mutations

doi: 10.1111/j.1349-7006.2007.00483.x

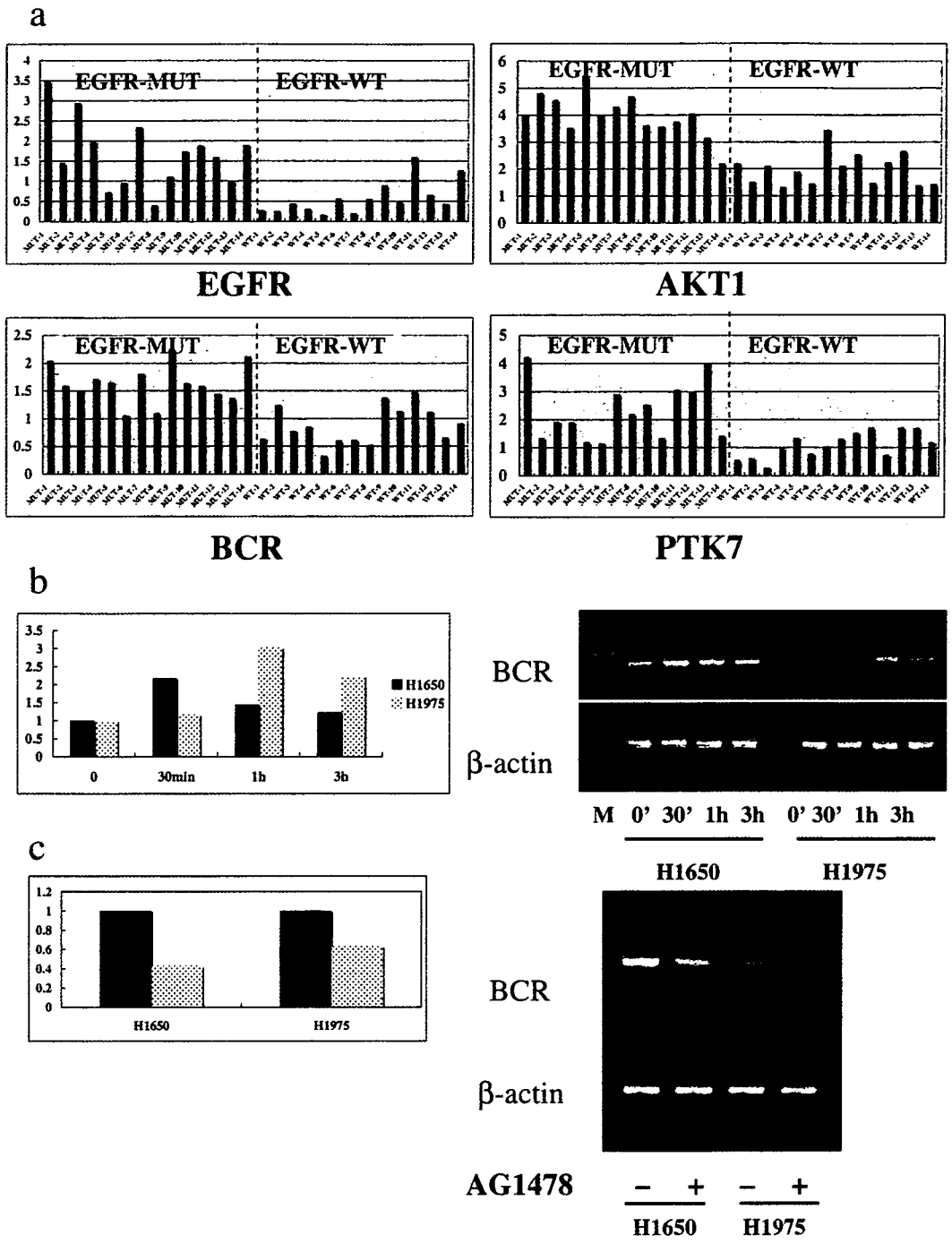


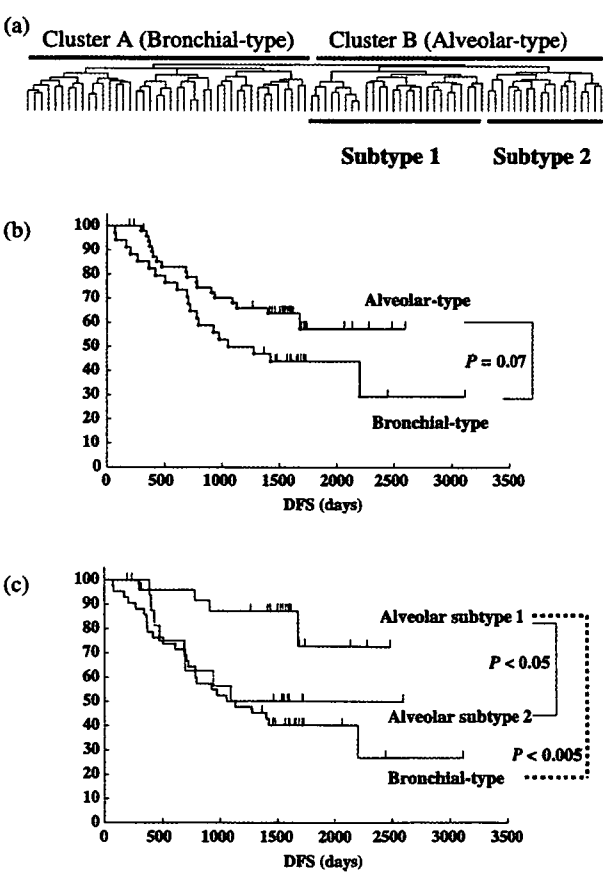
Fig. 3. Validation of distinctive expression between epidermal growth factor receptor (EGFR)-mutated and EGFR-wild-type tumors by quantitative reverse transcription-polymerase chain reaction (RT-PCR). (a) mRNA expression of the EGFR, AKT1, BCR and PTK7 genes was quantified in 14 EGFR-mutated (EGFR-MUT-1~14) and 14 EGFR-wild-type (EGFR-WT-1~14) lung adenocarcinomas. The normalized expressions (relative to beta-actin expression) of the four genes in 28 tumors are shown. (b) BCR mRNA was induced by EGF stimulation in lung cancer cell lines. Relative BCR mRNA expression (compared to the expression at time 0) was subsequently quantified after EGF stimulation (30 min, 1 h and 3 h) in lung cancer cell lines (NCI-H1650: closed bar, NCI-H1975: hatched bar). Gel electrophoresis of PCR products (BCR, upper panel; β-actin, lower panel) at each time point was shown. (c) BCR mRNA expression was reduced by treatment with EGFR kinase inhibitor (AG1478). Relative BCR expression in AG1478 treated lung cancer cell lines was shown (control, closed bar; EGFR inhibitor, hatched bar). Gel electrophoresis of PCR products (BCR, upper panel; β-actin, lower panel) of control and AG1478-treated cells is shown.

and the classification based on the genome-wide gene expression pattern. This suggests that genetic activation of the EGFR gene may confer a growth advantage in both subgroups and that environmental or intrinsic factors might affect the frequency of EGFR mutations. Correlation analysis between gene expression patterns and the presence of EGFR mutations revealed a small group of tumors (the left branch in the bronchial subgroups) that shows frequent EGFR mutation and mixed expression of alveolar and bronchial signatures. This group might represent a transitional subgroup between the two types although further analysis will be required. The frequency of KRAS mutation detected in our study was comparable with that reported previously.[22] In

Table 2. Genes preferentially expressed in epidermal growth factor receptor (EGFR)-mutated lung adenocarcinoma

Ref Seq accession number	Gene symbol	Fold difference	*P*-value
NM_016335	PRODH	3.18	0.0005
NM_001669	ARSD	2.5	0.00078
NM_005228	EGFR	2.31	0.00392
NM_000095	COMP	2.11	0.00515
NM_001012329	CTNNBIP1	2.09	0.000181
NM_004327	BCR	1.89	0.0000272
NM_002885	RAP1GA1	1.83	0.0053
NM_003917	AP1G2	1.82	0.00148
NM_002821	PTK7	1.79	0.000147
NM_025179	PLXNA2	1.73	0.000158
NM_080920	GGTLA4†	1.73	0.000277
NM_004484	GPC3	1.73	0.00295
NM_001015881	TSC22D3	1.72	0.00219
NM_003944	SELENBP1	1.65	0.00252
NM_003317	TITF1†	1.59	0.0000193
NM_014698	TMEM63A	1.54	0.000214
NM_000548	TSC2	1.54	0.00141
NM_004689	MTA1	1.54	0.00362
NM_015316	PPP1R13B	1.53	0.00172
NM_001306	CLDN3	1.53	0.00704
NM_002933	RNASE1	1.53	0.00738
NM_001014431	AKT1	1.52	0.000155
NM_002134	HMOX2	1.51	0.000136
NM_015234	GPR116	1.5	0.0021
NM_004390	CTSH	1.5	0.00593
NM_000802	FOLR1	1.5	0.00761

†Genes previously reported to be preferentially expressed in EGFR-mutated lung cancer.



contrast to EGFR mutations, KRAS mutations were significantly frequent in a specific subgroup of lung adenocarcinoma (the bronchial type) but KRAS mutation status was not associated with any clinical features. Since KRAS mutations have been reported to occur frequently in mucinous-type or goblet cell-type lung adenocarcinoma[23,24] it is possible that bronchial-type adenocarcinoma may contain such histological subtypes.

Our results revealed genes that were specifically expressed in tumors with genetic activation of the EGFR pathway (Table 2). They included genes (TITF1 and GGTLA4) whose expression has been previously reported to be associated with the presence of EGFR mutations.[20,25] Our analysis may also help to identify promising candidate biomarkers for prediction of EGFR activation and sensitivity to EGFR-TKIs in lung cancer. In particular, some of identified molecules (such as COMP, PTK7, PLXNA2, GPC3 and GRP116) are secreted or membrane-associated proteins that could be applicable for proteomic diagnosis using serum or sputum samples.

BCR is a multifunctional protein that forms a chimera protein with ABL in chronic myeloid leukemia.[26] Recently BCR was found to be a component of the endosomal sorting complex and positively regulates lysosomal degradation of the EGFR protein.[16] Ligand-induced down-regulation of growth factor receptors through endocytosis is the major regulatory mechanism that controls the duration and intensity of signal activation.[27] Our analysis revealed that expression of BCR was increased in EGFR-mutated tumors and that EGF stimulation rapidly induced BCR expression in lung cancer cells, supporting the hypothesis that

Fig. 4. Gene expression profiling of lung adenocarcinoma was significantly associated with better disease-free survival (DFS) after surgery. (a) Subclassification of the alveolar type tumors. (b, c) Kaplan-Meier plots of DFS (days) of lung adenocarcinoma stratified according to the gene expression profiling.

doi: 10.1111/j.1349-7006.2007.00483.x

BCR is a component of a negative feedback loop in EGFR signaling. Notably it has been reported that some types of EGFR mutation detected in lung cancer confer resistance to endosome-mediated degradation.[28] It is possible that these EGFR mutations may allow constitutive signal activation by overcoming this negative feedback loop and that destabilization of such oncogenic EGFR protein might be an alternative therapeutic strategy.

In our study cohort, we found that molecular classification of lung adenocarcinoma by gene expression profiling was significantly associated with patient prognosis. We found that a subgroup of the alveolar type was significantly better prognosis than other groups. There was no significant difference in clinicopathological features (gender, smoking habit, clinical stage, the presence of BAC features and EGFR mutation frequency) between the two subtypes (Appendix I Table 3). But this subgroup had more well-differentiated tumors ($P = 0.02$, Appendix I Table 3) and shows characteristic gene expression signatures including several transcriptional factors and secreted molecules (Appendix I Table 4). These gene products might also be useful for molecular diagnosis of lung adenocarcinoma. On the basis of our results, we propose that integrated analysis of genetic and gene expression profiling aimed to delineate inherent oncogenic pathways in cancer will be valuable not only for understanding molecular pathogenesis and identifying therapeutic targets, but also for clinical outcome in patients.

Acknowledgments

This work was supported in part by a grant-in-aid for the Comprehensive 10-Year-Strategy for Cancer Control from the Ministry of Health, Labor and Welfare, Japan and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio), Japan.

References

1 Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC. Pathology and Genetics of tumors of the lung, pleura, thymus and heart. *World Health Organ Classification of Tumors*. Lyon: IARC Press, 2004.
2 Meyerson M, Franklin WA, Kelley MJ. Molecular classification and molecular genetics of human lung cancers. *Semin Oncol* 2004; **31**: 4–19.
3 Osada H, Takahashi T. Genetic alterations of multiple tumor suppressors and oncogenes in the carcinogenesis and progression of lung cancer. *Oncogene* 2002; **21**: 7421–34.
4 Tibes R, Trent J, Kurzrock R. Tyrosine kinase inhibitors and the dawn of molecular cancer therapeutics. *Annu Rev Pharmacol Toxicol* 2005; **45**: 357–84.
5 Baselga J, Arteaga CL. Critical update and emerging trends in epidermal growth factor receptor targeting in cancer. *J Clin Oncol* 2005; **23**: 2445–59.
6 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**: 2129–39.
7 Paez JG, Janne PA, Lee JC *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–500.
8 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 USA* 2004; **101**: 13 306–11.
9 Shigematsu H, Gazdar AF. Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. *Int J Cancer* 2006; **118**: 257–62.
10 Isobe T, Herbst RS, Onn A. Current management of advanced non-small cell lung cancer: targeted therapy. *Semin Oncol* 2005; **32**: 315–28.
11 Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 2001; **98**: 5116–21.
12 Jerome Marson V, Mazieres J, Groussard O *et al.* Expression of TTF-1 and cytokeratins in primary and secondary epithelial lung tumours: correlation with histological type and grade. *Histopathology* 2004; **45**: 125–34.
13 Meezaman D, Charles P, Daskal E, Polymeropoulos MH, Martin BM, Rose MC. Cloning and analysis of cDNA encoding a major airway glycoprotein, human tracheobronchial mucin (MUC5). *J Biol Chem* 1994; **269**: 12 932–9.
14 Nakajima M, Kawanami O, Jin E *et al.* Immunohistochemical and ultrastructural studies of basal cells, Clara cells and bronchiolar cuboidal cells in normal human airways. *Pathol Int* 1998; **48**: 944–53.
15 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**: 339–46.
16 Olabisi OO, Mahon GM, Kostenko EV, Liu Z, Ozer HL, Whitehead IP. Bcr interacts with components of the endosomal sorting complex required for transport-I and is required for epidermal growth factor receptor turnover. *Cancer Res* 2006; **66**: 6250–7.
17 Bhattacharjee A, Richards WG, Staunton J *et al.* Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci USA* 2001; **98**: 13 790–5.
18 Garber ME, Troyanskaya OG, Schluens K *et al.* Diversity of gene expression in adenocarcinoma of the lung. *Proc Natl Acad Sci USA* 2001; **98**: 13 784–9.
19 Beer DG, Kardia SL, Huang CC *et al.* Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med* 2002; **8**: 816–24.
20 Takeuchi T, Tomida S, Yatabe Y *et al.* Expression profile-defined classification of lung adenocarcinoma shows close relationship with underlying major genetic changes and clinicopathologic behaviors. *J Clin Oncol* 2006; **24**: 1679–88.
21 Yatabe Y. Molecular classification of tumors with special reference to EGFR mutation in lung cancer. *Cancer Chemother Pharmacol* 2006; **58** (Suppl 7): 17–23.
22 Noda N, Matsuzoe D, Konno T, Kawahara K, Yamashita Y, Shirakusa T. K-ras gene mutations in non-small cell lung cancer in Japanese. *Oncol Report* 2001; **8**: 889–92.
23 Marchetti A, Buttitta F, Pellegrini S *et al.* Bronchioloalveolar lung carcinomas: K-ras mutations are constant events in the mucinous subtype. *J Pathol* 1996; **179**: 254–9.
24 Yatabe Y, Koga T, Mitsudomi T, Takahashi T. CK20 expression, CDX2 expression, K-ras mutation, and goblet cell morphology in a subset of lung adenocarcinomas. *J Pathol* 2004; **203**: 645–52.
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 Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* 1984; **36**: 93–9.
27 Le Roy C, Wrana JL. Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. *Nat Rev Mol Cell Biol* 2005; **6**: 112–26.
28 Chen YR, Fu YN, Lin CH *et al.* Distinctive activation patterns in constitutively active and gefitinib-sensitive EGFR mutants. *Oncogene* 2006; **25**: 1205–15.

Supplementary Material

The following supplementary material is available for this article:

Table S1.

Table S2.

Table S3.

Table S4.

This material is available as part of the online article from:

http://www.blackwell-synergy.com/doi/abs/10.1111/j.1349-7006.2007.00483.x

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Epidermal growth factor receptor mutation status and clinicopathological features of combined small cell carcinoma with adenocarcinoma of the lung

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(Received June 4, 2007/Revised July 13, 2007/Accepted July 24, 2007/Online publication September 2, 2007)

In lung cancer, somatic mutations of epidermal growth factor receptor (*EGFR*) are concentrated in exons 18–21, especially in adenocarcinoma (Ad), but these mutations have rarely been reported in small cell lung carcinoma (SCLC). Combined SCLC is rare, and the *EGFR* mutation status and its relationship to the clinicopathological features of this tumor type have not yet been elucidated. We retrospectively studied six patients with combined SCLC with Ad components among 64 consecutive patients who underwent resection of SCLC. The clinicopathological features of each patient were reviewed, especially for the distribution pattern of the Ad component and lymph node metastases. *EGFR* mutations were screened by high-resolution melting analysis in each case, and were confirmed by sequencing of each mutation in the microdissected SCLC or Ad components. Regarding *EGFR*, no specific mutation was detected in five of the six patients, whereas one female patient who had never smoked had a missense mutation. In this case, both the SCLC and Ad components shared the same mutation in exon 21 (L858R). We identified a patient with combined SCLC with Ad sharing an identical *EGFR* mutation in both the SCLC and Ad components. In addition to the clinicopathological characteristics of this rare histological type of lung cancer, these findings provide useful information for better understanding the biology, natural history and clinical management of SCLC. (*Cancer Sci* 2007; 98: 1714–1719)

Small cell lung carcinoma (SCLC) accounts for 15–20% of all lung cancers worldwide.[1] SCLC is known to be more sensitive than non-SCLC to chemotherapy, but shows a more aggressive clinical course. The median survival time without treatment is 2–4 months.[2,3] Approximately 20% of patients with limited SCLC achieve a cure, but most patients with SCLC will relapse, and relapsed or refractory SCLC has a uniformly poor prognosis with a 5-year survival rate of less than 5%.[4]

According to the 2004 World Health Organization (WHO)/International Association for the Study of Lung Cancer (IASLC) classification of lung and pleural tumors,[5] 'combined SCLC' is defined as SCLC combined with an additional component that consists of any of the histological types of non-SCLC, usually adenocarcinoma (Ad), squamous cell carcinoma (Sq) or large cell carcinoma. Combined SCLC is rare, and has been reported to account for less than 1–3.2% of all SCLC.[6,7] However, a high proportion (12–26%) of SCLC patients who undergo surgical resection show combination with non-SCLC.[8–12]

In a clinical setting, the distinction of SCLC from non-SCLC is critical because of major differences in patient management and prognosis. Recently, molecular targeted therapy has been developed using agents such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which exerts antitumor activity in patients with advanced non-SCLC (especially Ad) with *EGFR* mutations. High expression of EGFR has been reported in various epithelial malignant tumors, including lung cancer,[13,14] and somatic mutations in the kinase domain of *EGFR* are suggested to be strongly correlated with sensitivity to EGFR tyrosine kinase inhibitor.[15,16] These mutations are concentrated in exons 18–21 of *EGFR*, and approximately 90% of *EGFR*-mutant patients with lung Ad have mutations in two hot spots: in-frame deletion at codons 747–749 (DEL) in exon 19, and a missense mutation at codon 858 (L858R) in exon 21.[17,18] Although these mutations have rarely been reported in SCLC, two recent studies have demonstrated *EGFR* mutation in SCLC.[19,20]

In the present study, we retrospectively investigated six resected cases of combined SCLC with an Ad component to elucidate the clinicopathological features of this rare tumor, especially the ratio of each tumor component, the distribution patterns of the Ad component, and the status of lymph node metastasis. The *EGFR* mutation status in surgically resected specimens was also analyzed for each histological type in the same tumor.

Materials and Methods

Patients and histological diagnosis. A search of our surgical pathology files covering the period January 1982 to December 2004 yielded 64 consecutive patients with SCLC who had undergone surgical resection at the National Cancer Center Hospital, Tokyo, Japan. For the purposes of the present study, we identified six patients with combined SCLC with an Ad component. The research protocol was approved by the Institutional Review Board.

The surgically resected specimens were fixed in 10% formalin. All sections containing both tumor tissues and surrounding lung tissues were embedded in paraffin. Additional consecutive 5 μm-thick sections were cut from the tissue block and stained with hematoxylin and eosin. All histological diagnoses were reviewed by certificated pathologists (K. T., A. M. M. and Y. M.) based on the most recent WHO/IASLC classification of lung and pleural tumors.[5] Both clinical and pathological staging data for each patient have been reported according to the International Staging System for Lung Cancer.[21] Patient survival was calculated as the time between operation and death.

Immunohistochemistry and evaluation. For phenotypic analysis, paraffin section immunohistochemistry was carried out using the primary antibodies listed in Table 1, followed by subsequent labeling with the Envision+ horseradish peroxidase (HRP) system (DAKO, Carpinteria, CA, USA). For heat-induced epitope retrieval, sections stained for p63 were treated with 1.0 mmol/L

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doi: 10.1111/j.1349-7006.2007.00600.x

Table 1. Results of immunohistochemistry

Patient no.	SCLC component (%)	Immunoreaction					Non-SCLC component (%)	Immunoreaction					No. tumor embolism cells per slice† (%)		
		CgA	SYN	NCAM	TTF-1	p63		CgA	SYN	NCAM	TTF-1	p63	SCLC	Ad	Sq
1	95	2+	3+	3+	3+	0	Ad, 5	1+	1+	1+	3+	0	30 (97)	1 (3)	–
2	80	3+	3+	3+	3+	0	Ad, 10	0	1+	1+	2+	1+	21 (84)	3 (12)	1 (4)
							Sq, 10	0	0	0	0	3+			
3	70	1+	3+	3+	3+	0	Ad, 30	0	1+	0	3+	0	38 (93)	3 (7)	–
4	55	2+	3+	3+	3+	0	Ad, 45	0	0	1+	1+	0	24 (92)	2 (8)	–
5	35	3+	3+	3+	3+	0	Ad, 60	1+	1+	1+	3+	1+	17 (100)	0 (0)	0 (0)
							Sq, 5	0	1+	0	0	2+			
6	5	Not done					Ad, 95	Not done					Not done		

CgA, chromogranin-A; NCAM, neural cell adhesion molecule; SCLC, small cell lung carcinoma; SYN, synaptophysin; TTF-1, thyroid transcription factor-1. Semiquantitative assessments of the percentage of positive tumor cells (0 = none, 1+ = 1–33%, 2+ = 34–66%, 3+ = 67–100%) were made.
†We counted the number of lymph vessels with tumor embolisms confirmed by staining for D2-40 for a representative slide.

Table 2. Clinical characteristic of the patients with combined small cell lung carcinoma (SCLC) with adenocarcinoma (Ad)

Patient no.	Age/Sex	ECOG PS	Smoking status	Smoking index	Tumor location	Size (mm)	Stage (cTNM)	Preoperative diagnosis	Surgical procedure
1	74/Male	0	Current	2160	Peripheral	31	IIb (210)	Unknown	RLL†
2	66/Male	0	Ever	900	Peripheral	38	IIb (210)	Unknown	RM/LL‡
3	62/Female	0	Never	0	Peripheral	31	Ib (200)	SCLC	LUL
4	77/Male	1	Current	570	Peripheral	15	Ia (100)	Unknown	Left pneumonectomy
5	75/Male	0	Ever	1000	Peripheral	30	Ia (100)	Non-SCLC	RUL
6	76/Male	0	Current	1120	Peripheral	28	Ia (100)	Ad	RUL

Smoking index: (number of cigarettes smoked per day) × years. Adjuvant chemotherapy: †cyclosphosphamide + doxorubicin + vincristine × 1 cycle. ‡Cisplatin + etoposide × 1 cycle followed by cisplatin + irinotecan × 3 cycles. LUL, left upper lobectomy; RLL, right lower lobectomy; RM/LL, right middle and lower lobectomy; RUL, right upper lobectomy.

ethylenediaminetetraacetic acid buffer (pH 8.0). Sections stained for chromogranin A (1:500, polyclonal; DAKO), synaptophysin (1:100, polyclonal; DAKO), neural cell adhesion molecule (NCAM) (1:200, Lu243; Nihon Kayaku, Tokyo, Japan), thyroid transcription factor (TTF)-1 (1:100, 8G7G3/1; DAKO), p63 (1:100, 4A4; DAKO) and D2-40 (1:50, D2-40; DAKO) were treated with 0.02 mol/L citrate buffer (pH 6.0). The slides were incubated overnight with each primary antibody. Diaminobenzidine was used as the chromogen, and hematoxylin as the counterstain.

Positive staining was defined as distinct linear membrane staining for neural cell adhesion molecule, cytoplasmic staining for chromogranin A and synaptophysin, and nuclear staining for TTF-1 and p63. Immunostaining of each of the SCLC and non-SCLC components was graded on a scale of 0–3+ according to the percentage of positive tumor cells (0 = none; 1+ = 1–33%; 2+ = 34–66%; 3+ = 67–100%). We then carried out immunohistochemical identification of lymph vessels with or without tumor embolisms for a representative slide.[22] After independent evaluation by two of us (T. F. and K. T.), judgment consensus was obtained by joint viewing of the slides using a multiheaded microscope.

Analysis of *EGFR* mutational status. In our previous study, we established a practical and precise non-sequencing method for detecting *EGFR* mutations involving high-resolution melting analysis (HRMA) using LCGreen I dye (Idaho Technology, Salt Lake City, UT, USA).[23] First we screened for the *EGFR* mutations, DEL and L858R, using the HRMA method in formalin-fixed paraffin sections obtained from surgically resected combined SCLC with Ad. Human genomic DNA (Roche Diagnostics, Basel, Switzerland) was used as a control sample with wild-type *EGFR*. Second, we used 10% formalin-fixed, paraffin-embedded surgical specimens of primary combined SCLC from patients demonstrating DEL or L858R by HRMA, and the DNA was extracted from each of the SCLC and Ad components, respectively, the areas of which were clearly determined morphologically after laser capture microdissection (Arcturus Engineering, Mountain View, CA, USA) of the tumor tissue.[24] Nested polymerase chain reaction (PCR) was carried out to amplify exons 19 and 21 of *EGFR* using previously described primers.[17] The PCR products were electrophoresed on 2% agarose gels and subcloned into the TA vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA), then the sequences were determined with M13 primers using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Results

Clinical characteristics. The clinical characteristics of the six patients are shown in Table 2. All patients were Japanese, aged between 62 and 77 years (mean 71.7 years). Five patients were male and one was female. Five patients were smokers whereas the remaining patient had never smoked. The median survival time of the six patients was 16.8 months (range 0.4–27.4 months); one patient died of heart failure 13 days after left pneumonectomy.

All six tumors were located in the peripheral portion of the lung. On clinical evaluation, three patients were staged as Ia (T1N0M0), one as Ib (T2N0M0) and two as IIb (T2N1M0). Preoperative pathological diagnoses were obtained in three patients and comprised one case each of SCLC, non-SCLC and Ad.

Pathological findings. Among six patients with combined SCLC with Ad, histological examination demonstrated that four had

Table 3. Histological findings of primary tumor and lymph node metastases, and epidermal growth factor receptor (*EGFR*) mutation

Patient no.	Stage (pTNM)	Ratio of each component (%)			Histological type of lymph node metastasis		BAC-like extension	*EGFR* mutation
		SCLC	Ad	Sq	Mediastinal	Hilar		
1	IIa (110)	95	5	0	Non†	SCLC	Absent	Wild type
2	IIIa (220)	80	10	10	SCLC	SCLC	Present	Wild type
3	IIIb (410)	70	30	0	Non†	Ad	Present	L858R
4	IIIb (420)	55	45	0	Ad	SCLC or Ad‡	Present	Wild type
5	IIIa (220)	35	60	5	Ad	SCLC or Ad‡	Present	Wild type
6	Ib (200)	5	95	0	Non†	Non†	Present	Wild type

†The patient had no mediastinal or hilar lymph node metastasis. ‡The patient had lymph node metastasis only from the SCLC component, and another lymph node showing metastasis only from the Ad component. Ad, adenocarcinoma; BAC, bronchioloalveolar carcinoma; hilar, hilar lymph node; L858R, mutation at codon 858 of *EGFR*; medical, mediastinum lymph node; pTNM, pathological TNM; SCLC, small cell lung carcinoma; Sq, squamous cell carcinoma.

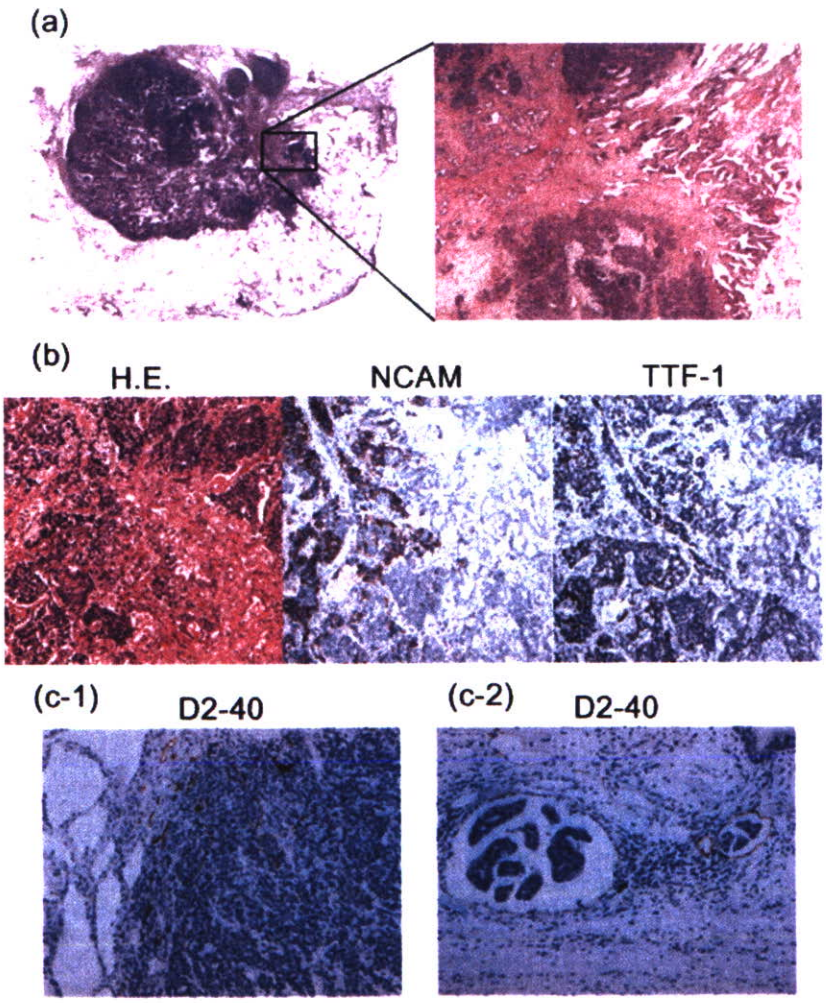

Fig. 1. Combined small cell lung carcinoma (SCLC) with adenocarcinoma (Ad). (a) The periphery of this tumor consisted of a non-mucinous bronchioloalveolar carcinoma-like extension (patient no. 3). (b) The transitional zone between the SCLC and Ad components had poorly differentiated cells, shown by the immunohistochemical studies (patient no. 1). (c) D2-40 with a membranous staining pattern of the lymph vessels. Tumor embolism of lymph vessels was confirmed by D2-40 staining (patient no. 3). (c-1) SCLC cell embolisms increased in number around the primary lesion. (c-2) Ad cell embolisms invaded the lymph vessels.

SCLC combined only with an Ad component (ratio of Ad in the tumor: 5, 30, 45 and 95%), whereas two had both Ad and Sq components (ratio of Ad/Sq: 10%/10% and 60%/5%, respectively). On pathological staging, one patient was staged as Ib (T2N0M0), one as IIa (T1N1M0), two as IIIa (T2N2M0) and two as IIIb (T4N1M0 and T4N2M0). In five of the six patients, the Ad components were observed in the peripheral part of the tumor showing a lepidic extension pattern, simulating bronchioloalveolar carcinoma. In the remaining one patient, Ad formed a minor component comprising approximately 5% of the tumor (Table 3). The Ad components in two patients showed a micropapillary growth pattern, whereas mucin production was not detected in any patient (Fig. 1a). The boundary between the SCLC and Ad components was not clear, and showed an

doi: 10.1111/j.1349-7006.2007.00600.x

indeterminate component that suggested gradual morphological transition from one to the other (Fig. 1b). In the two patients who also had combined Sq, the Sq component showed keratinization and was distinct from the SCLC component, but the border between the Ad and Sq components was unclear.

The results of immunohistochemical studies carried out in five cases are shown in Table 1. The specimen from patient no. 6 was not available. All of the SCLC components showed positive staining for at least one neuroendocrine marker. In addition, the Ad components in all five patients examined showed positive staining for at least one neuroendocrine marker, although semiquantitative assessments of the percentage of positive Ad cells were lower than those for SCLC cells in the same tumor. Also, the Ad components showed positive staining for TTF-1 in all five patients. TTF-1 staining of the SCLC component tended to be similar to that of the Ad component in terms of the percentage of positive cells. p63 immunostaining served as a good marker of Sq differentiation.

Status of lymph node metastasis. Five patients had pathologically confirmed hilar lymph node metastases, and three of them also had histologically proven mediastinal lymph node metastases, which had not been evident at the time of preoperative clinical evaluation (Table 3). Among these five patients with hilar lymph node metastases, two showed only SCLC in the metastatic lesion, one showed Ad only, and two showed SCLC or an Ad component that had developed separately in each lymph node. Among the three patients with mediastinal lymph node metastases, one had only SCLC in the nodes, and two had an Ad component only. Metastatic Ad components were found only in patients with a primary tumor in which Ad accounted for more than 30% of the total volume.

In the six patients, we identified tumor embolism of the lymph vessels immunohistochemically with D2-40 staining. There were approximately 800–1000 lymph vessels in each of these tumors per representative slide. The major component invading the lymph vessels around the tumors was SCLC cells. Even in the two patients who had mediastinal lymph node metastases with an Ad component, the SCLC cells tended to spread to the lymph vessels rather than the Ad cells (Table 1).

***EGFR* mutational status.** First, we analyzed 10 surgically resected samples from six patients with combined SCLC and Ad by HRMA. Analysis of exon 19 demonstrated curves identical to those of the control (wild type) in all samples, as shown in Fig. 2a. In the analysis of exon 21, thorough melting curves were obtained for two samples from patient no. 3, showing a different curve from the control, whereas the other eight samples demonstrated curves identical to the control (wild type), as shown in Fig. 2b. The normal lung tissue from patient no. 3, who was a female non-smoker, showed a wild-type curve, and therefore we judged that this patient had L858R in exon 21 of *EGFR*.

Next we confirmed this mutation in the SCLC and Ad components in patient no. 3. DNA was extracted from each SCLC and Ad component separately using laser capture microdissection or by manual microdissection, which was carried out for each clearly determined component on paraffin-embedded sections. Sequence analysis of subcloned PCR products obtained from the separate components was carried out. Examination of both SCLC and Ad components showed an identical mutation (L858R) in exon 21 (Fig. 3), confirming the results obtained by HRMA.

Discussion

The present study using microdissected tumor tissue is the first to report a patient with combined SCLC with Ad showing the *EGFR* mutation in both the SCLC and Ad components. *EGFR* mutations, especially DEL and L858R, have been reported in

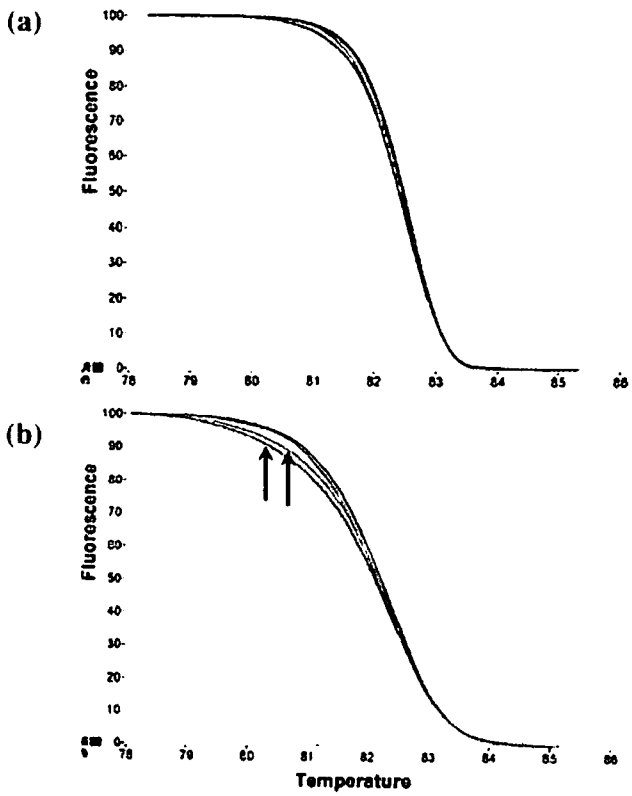

Fig. 2. Results of high-resolution melting analysis (HRMA). Adjusted melting curves obtained by HRMA of combined small cell lung carcinoma (SCLC) with primers designed to detect mutations in (a) exon 19 or (b) exon 21 of epidermal growth factor receptor (*EGFR*). Two samples from patient no. 3 were identified as containing the L858R mutations (↑). The DNA extracted from normal lung tissue of patient no. 3 was identified as wild type (not shown).

Ad of the lung. These somatic mutations in the kinase domain of *EGFR* have been shown to be predictive molecular markers for sensitivity to kinase inhibitors such as gefitinib (Iressa; AstraZeneca, Osaka, Japan). However, these mutations have rarely been demonstrated in SCLC. To our knowledge, there have been two reported cases of metastatic SCLC harboring DEL in exon 19 of *EGFR* showing responsiveness to EGFR tyrosine kinase inhibitors.[19,20,25] Considering that the diagnosis of SCLC is often based on small biopsy specimens that may not be sufficiently representative of the total tumor, there is a possibility that any combined component may be overlooked.

In a clinical setting, the distinction of SCLC from non-SCLC is critical because of major differences in management and prognosis between the two cancers. SCLC is well known to be more common in men and smokers, but so far SCLC with *EGFR* mutations has been detected only in female patients who have never smoked,[19,20] as was the case in our present female patient. Thus it seems reasonable to suggest that in clinically unusual SCLC patients, for example those who are non-smokers and female, showing peripheral nodular lesions and histological combination with Ad, *EGFR* mutation status should be analyzed because previous studies have shown that EGFR tyrosine kinase inhibitors are effective in patients with metastatic SCLC with *EGFR* mutations.

The present study is considerably informative with regard to the origin and histogenesis of SCLC. *EGFR* mutation is detected in patients with pre-invasive adenocarcinomatous lesions such as atypical adenomatous hyperplasia and bronchioloalveolar

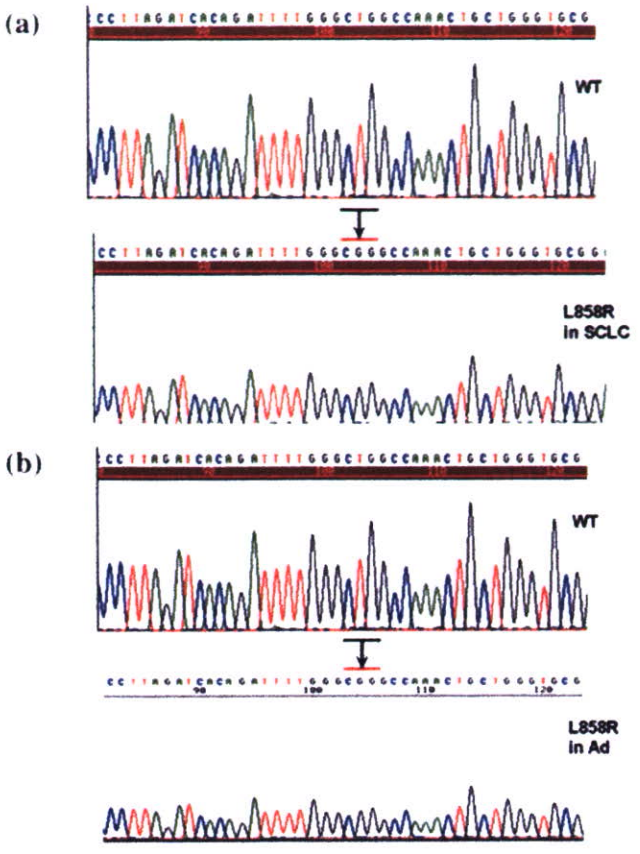

Fig. 3. Results of DNA sequencing from patient no. 3. The tumor of patient no. 3 was microdissected into the small cell lung carcinoma (SCLC) and adenocarcinoma (Ad) components. (a) Sequence analysis of the subcloned polymerase chain reaction (PCR) products from the microdissected SCLC component. (b) Sequence analysis of the subcloned PCR products from the microdissected Ad component. The patient had a tumor with L858R of EGFR, which was in both the SCLC and Ad components.

carcinoma, which eventually progress to invasive lung Ad.[26] In addition, *EGFR* mutations are also linked to Ad with a bronchioloalveolar carcinoma component.[27] Thus it is suggested that *EGFR* mutation occurs and plays a critical role in the early developmental stage of lung Ad. The mutation is detected more frequently in Ad in female non-smokers than in male smokers. In the present study, the only patient with SCLC harboring an *EGFR* mutation was female and a non-smoker, and the combined Ad component also harbored the same mutation. Moreover, as mentioned above, the two SCLC patients with *EGFR* mutation reported previously were also female and non-smokers. These findings imply that the mutations are an early genetic event in carcinogenesis of the lung and at least a certain proportion of SCLC may originate as a result of progression or transformation of Ad harboring *EGFR* mutation.

This phenomenon can also be linked to pathological features. The histological patterns of lymph node involvement showed that Ad components spread to mediastinal lymph nodes in the patients with hilar lymph node involved by SCLC or Ad component. Considering the status of tumor embolism of the lymph vessels observed using D2-40 staining, SCLC cell embolisms, but not Ad, increase in number around primary lesion in these tumors. It is suggested that a common uncommitted stem cell might differentiate into each component after involvement in a lymph node. Furthermore, positive staining for TTF-1, which is a highly specific immunohistochemical marker identifying carcinomas of pulmonary origin (especially non-mucinous Ad and SCLC),[28] was shown in the SCLC and Ad components, but not Sq. Previous studies have demonstrated TTF-1 expression in 83–100% of SCLC, but low expression in Sq.[29,30] These findings could be interpreted as being compatible with the hypothesis that SCLC and Ad originate from a common uncommitted stem (or precursor) cell originally expressing TTF-1.[31] It is possible to postulate that a fraction of SCLC possessing stem (or precursor) cell properties might have the potential to form an Ad component. In fact, in the present cases, there were some areas comprising morphologically indeterminate tumor cell components at the border of the SCLC and Ad components.

The rarity of patients with combined SCLC makes it difficult to determine the optimal management and biological characteristics of this tumor. However, the present findings suggest that the classical classification of lung cancer might provide insufficient management for a specified subpopulation in molecular targeted therapy. Although this retrospective study examined only a very limited number of lung carcinoma cases, we consider that the findings provide useful information for understanding the biology of this lung cancer and devising more effective forms of clinical management.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology, and for the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare, Japan and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, Japan. We thank Karin Yokozawa and Kiyoaki Nomoto for their technical support.

References

1 Stupp R, Monnerat C, Turrisi AT 3rd, Perry MC, Leyvraz S. Small cell lung cancer: state of the art and feature perspectives. *Lung Cancer* 2004; **45**: 105–17.
2 Chua YJ, Steer C, Yip D. Recent advances in management of small-cell lung cancer. *Cancer Treat Rev* 2004; **30**: 521–43.
3 Aisner J. Extensive-disease small-cell lung cancer: the thrill of victory, the agony of defeat. *J Clin Oncol* 1996; **14**: 658–65.
4 Simon GR, Wagner H. Small cell lung cancer. *Chest* 2003; **123**: 259S–71S.
5 Travis WD, Coby TV, Corrin B, Shimosato Y, Brambilla E. *World Health Organization International Histological Classification of Tumours; Histological Typing of Lung and Pleural Tumours*, 3rd edn. Berlin: Springer, 1999.
6 Fraire AE, Johnson EH, Yesner R, Zhang XB, Spjut HJ, Greenberg SD. Prognostic significance of histopathologic subtype and stage in small cell lung cancer. *Hum Pathol* 1992; **23**: 520–8.
7 Mangum MD, Greco FA, Hainsworth JD, Hande KR, Johnson DH. Combined small-cell and non-small-cell lung cancer. *J Clin Oncol* 1989; **7**: 607–12.
8 Hage R, Elbers JR, Brutel de la Riviere A, van den Bosch JM. Surgery for combined type small cell lung carcinoma. *Thorax* 1998; **53**: 450–3.
9 Nicholson SA, Beasley MB, Brambilla E *et al*. Small cell lung carcinoma (SCLC): a clinicopathologic study of 100 cases with surgical specimens. *Am J Surg Pathol* 2002; **26**: 1184–97.
10 Lucchi M, Mussi A, Chella A *et al*. Surgery in the management of small cell lung cancer. *Eur J Cardiothorac Surg* 1997; **12**: 689–93.
11 de Antonio DG, Alfageme F, Gamez P, Cordoba M, Varela A. Results of surgery in small cell carcinoma of the lung. *Lung Cancer* 2006; **52**: 299–304.
12 Deslauriers J. Surgery for small cell lung cancer. *Lung Cancer* 1997; **17**: S91–8.
13 Ozanne B, Richards CS, Hendler F, Burns D, Gusterson B. Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. *J Pathol* 1986; **149**: 9–14.
14 Cerny T, Barnes DM, Hasleton P *et al*. Expression of epidermal growth factor receptor (EGF-R) in human lung tumours. *Br J Cancer* 1986; **54**: 265–9.

doi: 10.1111/j.1349-7006.2007.00600.x

15 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**: 2129–39.
16 Paez JG, Janne PA, Lee JC *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–500.
17 Takano T, Ohe Y, Sakamoto H *et al.* Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. *J Clin Oncol* 2005; **23**: 6829–37.
18 Pao W, Miller VA. Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J Clin Oncol* 2005; **23**: 2556–68.
19 Okamoto I, Araki J, Suto R, Shimada M, Nakagawa K, Fukuoka M. EGFR mutation in gefitinib-responsive small-cell lung cancer. *Ann Oncol* 2005; **17**: 1028–9.
20 Zakowski MF, Ladanyi M, Kris MG. EGFR mutations in small-cell lung cancers in patients who have never smoked. *N Engl J Med* 2006; **355**: 213–15.
21 Mountain CF. Revisions in the international system for staging lung cancer. *Chest* 1997; **111**: 1710–17.
22 Evangelou E, Kyzas PA, Trikalinos TA. Comparison of the diagnostic accuracy of lymphatic endothelium markers: Bayesian approach. *Mod Pathol* 2005; **18**: 1490–7.
23 Nomoto K, Tsuta K, Takano T *et al.* Detection of EGFR mutations in archived cytologic specimens of non-small cell lung cancer using high-resolution melting analysis. *Am J Clin Pathol* 2006; **126**: 608–15.
24 Emmert-Buck MR, Bonner RF, Smith PD *et al.* Laser capture microdissection. *Science* 1996; **274**: 998–1001.
25 Araki J, Okamoto I, Suto R, Ichikawa Y, Sasaki J. Efficacy of the tyrosine kinase inhibitor gefitinib in a patient with metastatic small cell lung cancer. *Lung Cancer* 2005; **48**: 141–4.
26 Yoshida Y, Shibata T, Kokubu A *et al.* Mutations of the epidermal growth factor receptor gene in atypical adenomatous hyperplasia and bronchioloalveolar carcinoma of the lung. *Lung Cancer* 2005; **50**: 1–8.
27 Blons H, Cote JF, Le Corre D *et al.* Epidermal growth factor receptor mutation in lung cancer are linked to bronchioloalveolar differentiation. *Am J Surg Pathol* 2006; **30**: 1309–15.
28 Stahlman MT, Gray ME, Whitsett JA. Expression of thyroid transcription factor-1 (TTF-1) in fetal and neonatal human lung. *J Histochem Cytochem* 1996; **44**: 673–8.
29 Jerome Marson V, Mazieres J, Groussard O *et al.* Expression of TTF-1 and cytokeratins in primary and secondary epithelial lung tumours: correlation with histological type and grade. *Histopathology* 2004; **45**: 125–34.
30. Kalhor N, Zander DS, Liu J. TTF-1 and p63 for distinguishing pulmonary small-cell carcinoma from poorly differentiated squamous cell carcinoma in previously pap-stained cytologic material. *Mod Pathol* 2006; **19**: 1117–23.
31 Sturm N, Lantuejoul S, Laverriere MH *et al.* Thyroid transcription factor 1 and cytokeratins 1, 5, 10, 14 (34βE12) expression in basaloid and large-cell neuroendocrine carcinomas of the lung. *Hum Pathol* 2001; **32**: 918–25.