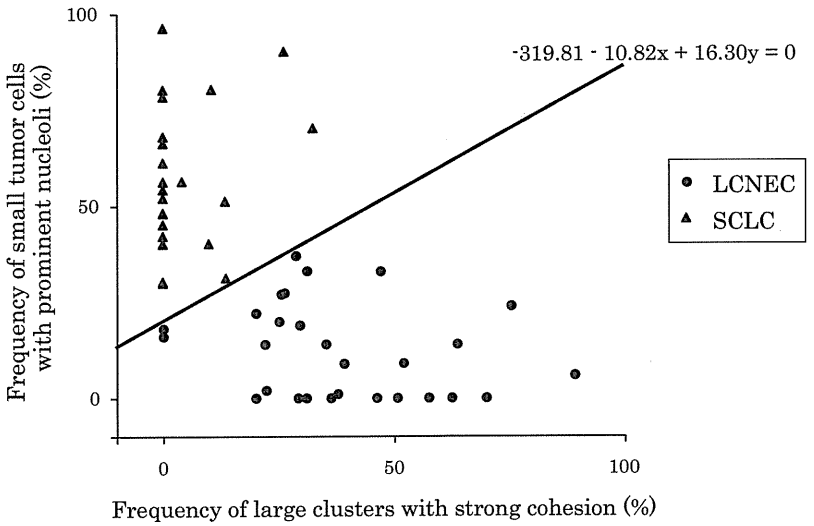

FIGURE 6. Logistic regression for frequencies of small tumor cells without prominent nucleoli and large clusters with tight cohesion. The scatter plot allows clear separation of LCNEC (●) and SCLC cases (▲) by the calculated discriminant line. Therefore, a discriminant model for LCNEC and SCLC, diagnosing 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%.

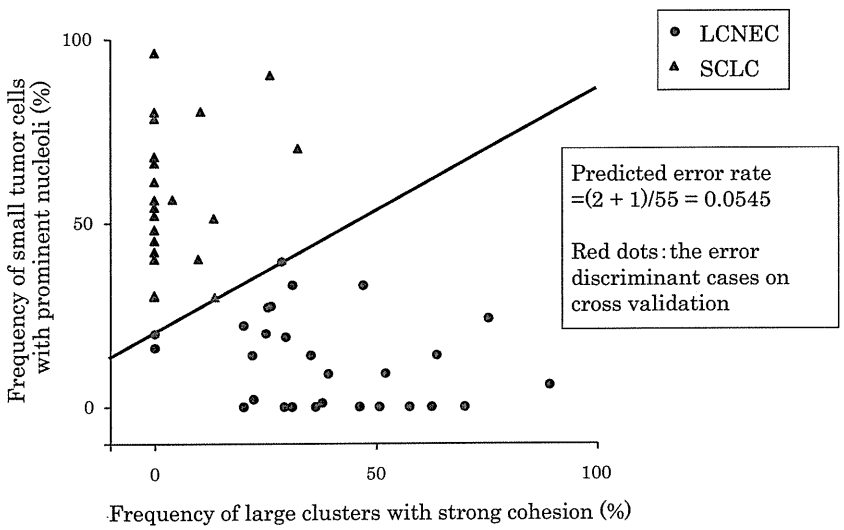

FIGURE 7. Leave-one-out cross validation. Regarding each case as a new case, prediction of the error rate of the discriminant model was analyzed. Red dots show error discriminant cases on cross validation. Two of the LCNEC cases and one SCLC case were discriminated in error with the discriminant model,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

Foundation for Cancer Research, for helpful advice with the statistical analyses.

REFERENCES

1. Cooper WA, Thourani VH, Gal AA, et al. The surgical spectrum of pulmonary neurocrine neoplasms. *Chest* 2001;119:14–18.
2. Garcia-Yuste M, Matilla JM, Alvarez-Gago T, et al. Prognostic factors in neuroendocrine lung tumors: a Spanish multicenter study. *Ann Thorac Surg* 2000;70:258–263.
3. Jones MH, Virtanen C, Honjoh D, et al. Two prognostically significant subtypes of high-grade lung neuroendocrine tumors independent of small-cell and large-cell neuroendocrine carcinomas identified by gene expression profiles. *Lancet* 2004;363:775–781.
4. Travis WD, Linnoila RI, Tsokos MG, et al. Neuroendocrine tumors of the lung with proposed criteria for large cell neuroendocrine carcinoma. An ultrastructural, immunohistochemical, and flow cytometric study of 35 cases. *Am J Surg Pathol* 1991;15:529–533.
5. Asamura H, Kameya T, Matsuno Y, et al. Neuroendocrine neoplasms of the lung: a prognostic spectrum. *J Clin Oncol* 2006;24:70–76.
6. Dresler CM, Ritter JH, Patterson GA, et al. Clinical-pathologic analysis of 40 patients with large cell neuroendocrine carcinoma of the lung. *Ann Thorac Surg* 1997;63:180–185.
7. Fernandez FG, Battafarano RJ. Large cell neuroendocrine carcinoma of the lung. *Cancer Control* 2006;13:270–275.
8. Hiroshima K, Iyoda A, Shida T, et al. Distinction of pulmonary large cell neuroendocrine carcinoma from small cell lung carcinoma: a morphological, immunohistochemical, and molecular analysis. *Mod Pathol* 2006;19:1358–1368.
9. Iyoda A, Hiroshima K, Nakatani Y, et al. Pulmonary large cell neuroendocrine carcinoma: its place in the spectrum of pulmonary carcinoma. *Ann Thorac Surg* 2007;84:702–707.
10. Iyoda A, Hiroshima K, Toyozaki T, et al. Adjuvant chemotherapy for large cell carcinoma with neuroendocrine features. *Cancer* 2001;92: 1108–1112.
11. Jiang SX, Kameya T, Shoji M, et al. Large cell neuroendocrine carcinoma of the lung: a histologic and immunohistochemical study of 22 cases. *Am J Surg Pathol* 1998;22:526–537.
12. Travis WD, Gal AA, Colby TV, et al. Reproducibility of neuroendocrine lung tumor classification. *Hum Pathol* 1998;29:272–279.
13. Wick MR, Berg LC, Herts MI. Large cell carcinoma of the lung with neuroendocrine differentiation: a comparison with large cell "undifferentiated" pulmonary tumors. *Am J Surg Pathol* 1992;97:796–805.
14. Veronesi G, Morandi U, Alloisio M, et al. Large cell neuroendocrine carcinoma of the lung: a retrospective analysis of 144 surgical cases. *Lung Cancer* 2006;53:111–115.
15. Travis WD, Colby TV, Corrin B, et al. *Histological Typing of Lung and Pleural Tumors*. 3rd Ed. *World Health Organization International Histological Classification of Tumors*. Berlin: Springer Verlag, 1999.
16. Colby TV, Koss MN, Travis WD. *Tumors of the Lower Respiratory Tract. Atlas of Tumor Pathology*. 3rd series. Washington DC: Armed Forces Institute of Pathology, 1995:248–255.
17. Hiroshima K, Abe S, Ebihara Y, et al. Cytological characteristics of pulmonary large cell neuroendocrine carcinoma. *Lung Cancer* 2005;48: 331–337.
18. Iyoda A, Baba M, Hiroshima K, et al. Imprint cytologic features of pulmonary large cell neuroendocrine carcinoma: comparison with classic large cell carcinoma. *Oncol Rep* 2004;11:285–288.
19. Jimenez-Heffernan JA, Lopez-Ferrer P, Vicandi B, et al. Fine-needle aspiration cytology of large cell neuroendocrine carcinoma of the lung: a cytopathologic correlation study of 11 cases. *Cancer* 2008;114:180–186.
20. Kakinuma H, Mikami T, Iwabuchi K, et al. Diagnostic findings of bronchial brush cytology for pulmonary large cell neuroendocrine carcinomas. *Cancer (Cancer Cytopathol)* 2003;99:247–254.
21. Nicholson SA, Ryan MR. A review of cytologic findings in neuroendocrine carcinomas including carcinoid tumor with histologic correlation. *Cancer (Cancer Cytopathol)* 2000;90:148–161.
22. Wiatrowska BA, Krol J, Zakowski MF. Large cell neuroendocrine carcinoma of the lung: proposed criteria for cytologic diagnosis. *Diagn Cytopathol* 2001;24:58–64.
23. 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;26:1184–1197.

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

Yukinori Sakao, MD, PhD, Sakae Okumura, MD, Mingyon Mun, MD, PhD, Hirofumi Uehara, MD, PhD, Yuichi Ishikawa, MD, PhD, and Ken Nakagawa, MD

Departments of Thoracic Surgical Oncology and Pathology, Japanese Foundation for Cancer Research, Cancer Institute Hospital, Tokyo, Japan

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

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

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

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

(Ann Thorac Surg 2010;89:1060–3)

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

Once patients are diagnosed with multilevel N2, they are considered for multimodal treatments as parts of some clinical studies. When we evaluate the therapeutic options for multilevel N2 patients, it is very important to consider the multiple prognostic factors that may exist in these groups, as they are typically very heterogeneous. However, there have been few reports that have considered the prognostic factors focusing on multilevel N2 patients, mainly because of their poor outcomes.

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

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

Accepted for publication Dec 23, 2009.

Address correspondence to Dr Sakao, Japanese Foundation for Cancer Research, Cancer Institute Hospital, Department of Thoracic Surgical Oncology, 3-10-6, Ariake, Koto-ku, Tokyo 135-8550, Japan; e-mail: yukinori.sakao@jfcr.or.jp.

Published by Elsevier Inc

0003-4975/10/$36.00
doi:10.1016/j.athoracsur.2009.12.066

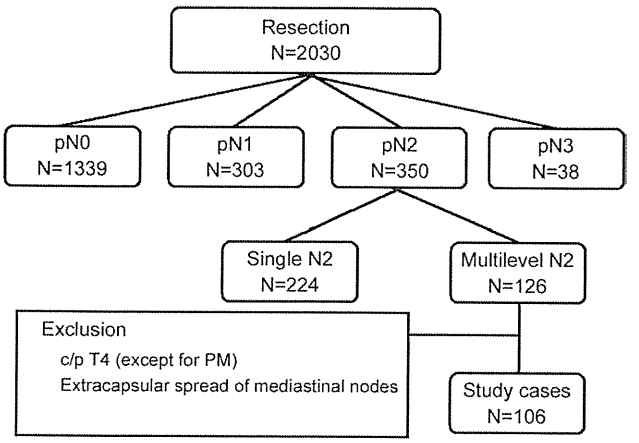

Fig 1. Diagram indicating study group subdivisions. Between 1988 and December 2007, 2,030 patients underwent surgical resections for primary lung cancer at the Cancer Institute Hospital. (PM = intrapulmonary metastases.)

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

Patients and Methods

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

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

For all patients, preoperative staging was assessed according to the TNM classification of the International Union Against Cancer [8], using chest computed tomography (CT), abdominal CT or ultrasonography, brain CT or magnetic resonance imaging, and bone scans. Clinical mediastinal and hilar lymph node status was assessed as positive if the results of the chest CT showed that the shorter axis was longer than 1.0 cm. Clinical T4 (cT4) cases and bulky N2 (shortest mediastinal lymph node diameter >2 cm) were excluded from this study because they were receiving chemotherapy with or without radiotherapy according to the protocol in our institute. Therefore, patients with mediastinal lymph node adenopathy less than 2 cm were considered for surgery. However, mediastinoscopy, fluorodeoxyglucose–positron emission tomography, or endobronchial ultrasonography-guided transbronchial needle aspiration were applied to some patients in this series (shortest mediastinal lymph node diameter ≤2 cm); they were not used for preoperative staging in this series. Furthermore, patients with extracapsular spread of lymph node metastasis were excluded, as they often underwent incomplete resections. Follow-up periods ranged from 2 to 240 months (median follow-up for living patients was 36 months).

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

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

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

Results

Survival Rates for Patients With Multilevel N2

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

Table 1. Patient Characteristics

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

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

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

Prognostic Factors for Multilevel N2

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

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

Survival Rate According to the Prognostic Factors for Multilevel N2

The multilevel N2 patients were categorized as with or without significant prognostic factors determined from multivariate analyses. Patients who had neither cN2 nor PM showed a significantly higher survival rate than patients who had either cN2 or PM (5-year survival rates of 36.5% and 11.2%, respectively; Fig 2).

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

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

CI = confidence interval; PM = intrapulmonary metastases.

Comment

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

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

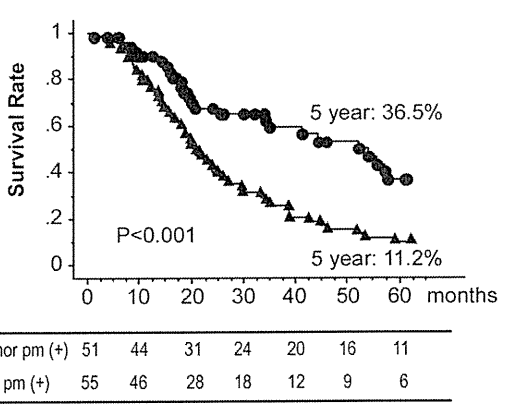

Fig 2. Overall 5-year survival rate for multilevel N2 patients (Pt) depends on cN and intrapulmonary metastases (pm). Survival curves were generated using the Kaplan-Meier method.

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

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

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

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

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

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

In conclusion, multilevel N2 patients can be grouped according to their prognoses by the factors cN and PM. These findings have potential for analyzing the best therapeutic modalities for multilevel N2 patients.

References

1. Mountain CF, Dresler CM. Regional lymph node classification for lung cancer staging. Chest 1997;111:1718–23.
2. Naruke T, Suemasu K, Ishikawa S. Lymph node mapping and curability at various levels of metastasis in resected lung cancer. J Thorac Cardovasc Surg 1978;76:832–9.
3. Watanabe Y, Hayashi Y, Shimizu J, Oda M, Iwa T. Mediastinal nodal involvement and prognosis of non-small cell lung cancer. Chest 1991;100:422–8.
4. Sakao Y, Miyamoto H, Yamazaki A, et al. The prognostic significance of metastasis to the highest mediastinal lymph node in non-small cell lung cancer. Ann Thorac Surg 2006; 81:292–7.
5. Marc R, Jalal A, Patrick B, et al. Skip mediastinal lymph node metastasis and lung cancer: a particular N2 subgroup with a better prognosis. Ann Thorac Surg 2005;79:225–33.
6. Andre F, Grunenwald D, Pignon J-P, et al. Survival of patients with resected N2 non-small-cell lung cancer: evidence for a subclassification and implications. J Clin Oncol 2000;18:2981–9.
7. Paul DL, Lardinoisb D, Van Schilc PE, et al. ESTS guidelines for preoperative lymph node staging for non-small cell lung cancer. Eur J Cardiothorac Surg 2007;32:1–8.
8. Hermanek P, Sobin LH. UICC TNM classification of malignant tumors, 4th ed. Berlin: Springer-Verlag, 1992.
9. Daly BD, Mueller JD, Faling LJ, et al. N2 lung cancer: outcome in patients with false negative computed tomographic scans of the chest. J Thorac Cardiovasc Surg 1993; 105:904–11.
10. Cybulsky IJ, Lanza LA, Ryan MB, Putnam JB Jr, McMurtrey MM, Roth JA. Prognostic significance of computed tomography in resected N2 lung cancer. Ann Thorac Surg 1992:54: 533–7.
11. Vansteenkiste JF, De Leyn PR, Deneffe GJ, et al. Survival and prognostic factors in resected N2 non-small cell lung cancer: a study of 140 cases: Leuven Lung Cancer Group. Ann Thorac Surg 1997;63:1441–50.
12. Sakao Y, Miyamoto H, Oh S, et al. Clinicopathological factors associated with unexpected N3 in patients with mediastinal lymph node involvement. J Thorac Oncol 2007; 2:1107–11.
13. Andre F, Grunenwald D, Pignon JP, et al. Survival of patients with resected N2 non-small-cell lung cancer: evidence for a subclassification and implications. J Clin Oncol 2000;18:2981–9.
14. Suzuki K, Nagai K, Yoshida J, Nishimura M, Takahashi K, Nishiwaki Y. The prognosis of surgically resected N2 non-small cell lung cancer: the importance of clinical N status. J Thorac Cardiovasc Surg 1999;118:145–53.
15. Misthos P, Sepsas E, Athanassiadi K, Kakaris S, Skottis I. Skip metastases: analysis of their clinical significance and prognosis in the IIIA/N2 NSCLC group. Eur J Cardiothorac Surg 2004;25:502–8.
16. Detterbeck F. What to do with "surprise" N2? Intraoperative management of patients with non-small cell lung cancer. J Thorac Oncol 2008;3:289–302.
17. Okumura T, Asamura H, Suzuki K, KondohH, Tsuchiya R. Intrapulmonary metastasis of non-small cell lung cancer: A prognostic assessment. J Thorac Cardiovasc. Surg 2001;122: 24–8.
18. Deslauriers J, Brisson J, Cartier R, et al. Carcinoma of the lung. Evaluation of satellite nodules as a factor influencing prognosis after resection. J Thorac Cardiovasc Surg 1989;97: 504–12.

Cancer Science
The official journal of the Japanese Cancer Association JCA

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

Akira Okada,[1,2] Takuo Shimmyo,[1,3] Takehisa Hashimoto,[2] Yasuhito Kobayashi,[4] Yohei Miyagi,[1] Yuichi Ishikawa,[5] Ken Nakagawa,[6] Junichi Hayashi[2] and Eiju Tsuchiya[1,7]

[1]Laboratory of Molecular Pathology and Genetics Division, Kanagawa Cancer Center Research Institute, Yokohama; [2]Division of Thoracic and Cardiovascular Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata City; [3]Department of Chest Surgery, St. Marianna University School of Medicine, Kawasaki; [4]Department of Pathology, Saitama Cancer Center, Saitama; Departments of [5]Pathology and [6]Chest Surgery, Cancer Institute Hospital, Tokyo, Japan

(Received December 3, 2009/Revised March 20, 2010/Accepted March 28, 2010/Accepted manuscript online April 7, 2010/Article first published online May 19, 2010)

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

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

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

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

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

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

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

Materials and Methods

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

[7]To whom correspondence should be addressed.
E-mail: tsuchiya@gancen.asahi.yokohama.jp

doi: 10.1111/j.1349-7006.2010.01585.x

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

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

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

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

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

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

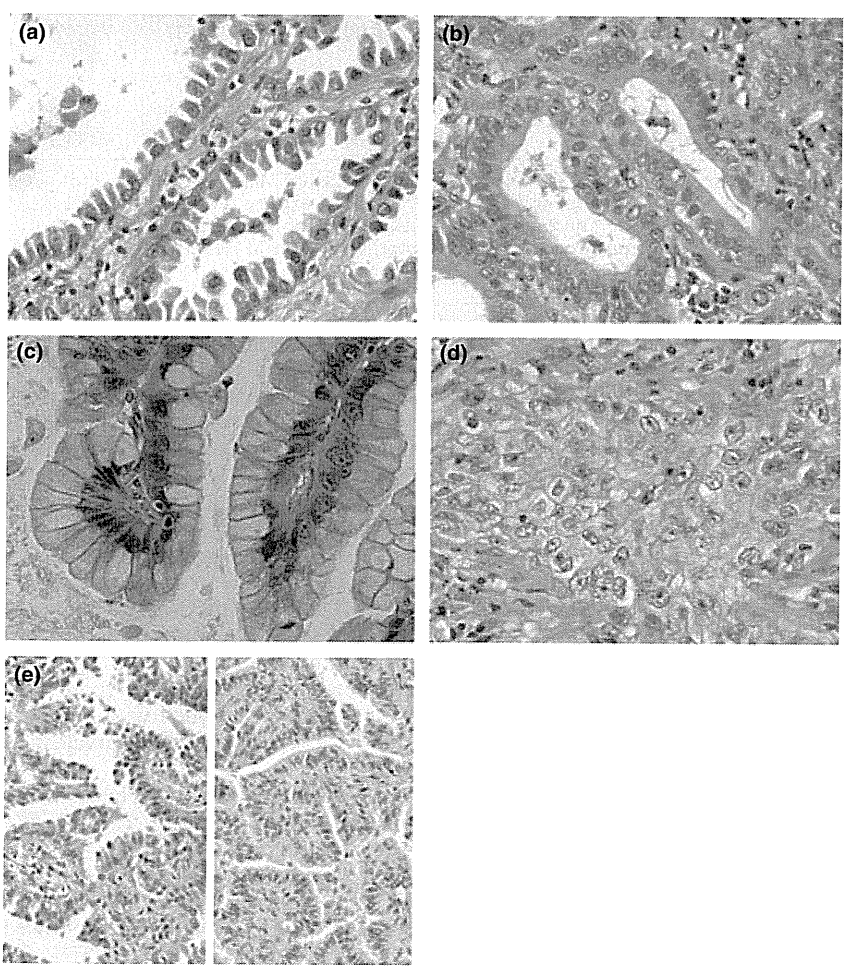

Fig. 1. Cell types of adenocarcinomas. (a) Hobnail cell type: epithelial cells look like Clara or type II pneumocyte cells. Apical portions protrude or bulge into the lumen. Note hobnail- or tadpole-shaped cells. (b) Columnar/cuboidal (col/cub) cell type: characterized by rather large columnar or cuboidal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

doi: 10.1111/j.1349-7006.2010.01585.x

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

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

doi: 10.1111/j.1349-7006.2010.01585.x

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

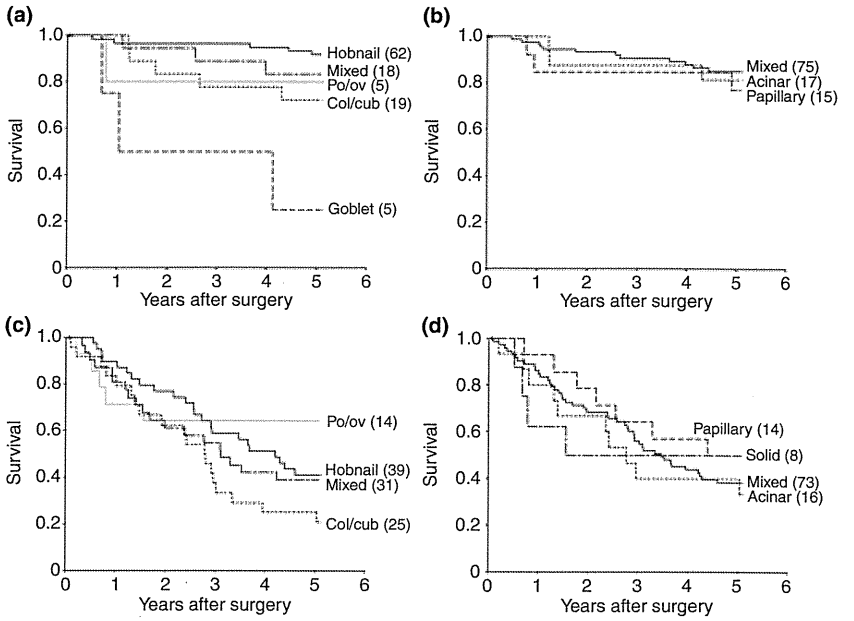

Fig. 2. Disease-specific Kaplan–Meier survival curves with respect to the cell type (a,c) and the World Health Organization (WHO) classifications (b,d) for p-stage I (a,b) and p-stages II–IV cases (c,d). Numbers in parentheses show numbers of patients. (a) The 5-year survival rate of the hobnail type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

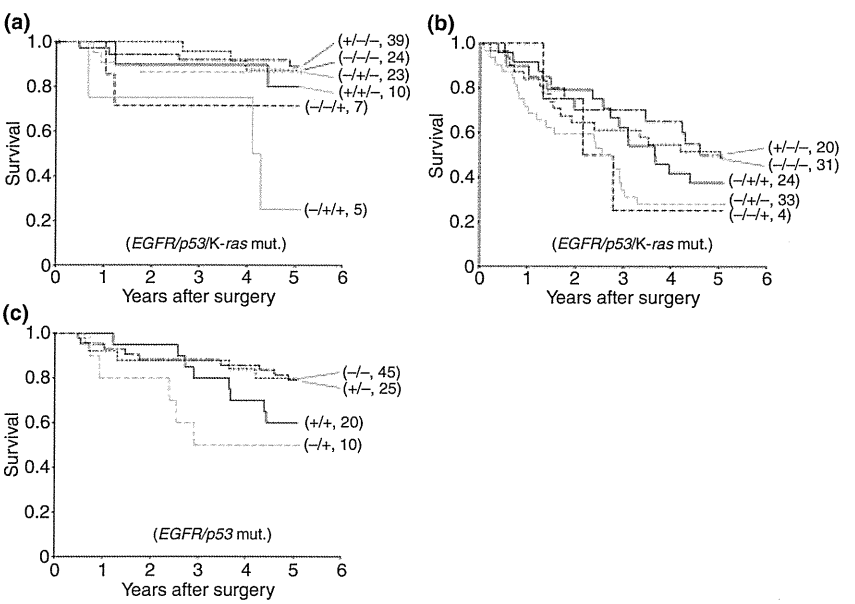

Fig. 3. Disease-specific Kaplan–Meier survival curves with respect to the mutational status for the three genes (*EGFR/p53*/K-*ras*) in p-stage I (a) and p-stages II–IV cases (b), and for two genes (*EGFR/p53*) in all p-stages for the hobnail cell type (c). The + or – indicate cases with or without mutations,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

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

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

Discussion

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

doi: 10.1111/j.1349-7006.2010.01585.x

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgment

This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, Technology and Culture of Japan.

Disclosure Statement

The authors have no conflict of interest.

References

1 Hashimoto T, Tokuchi Y, Hayashi M *et al.* Different subtypes of human lung adenocarcinoma caused by different etiological factors. *Am J Pathol* 2000; **157**: 2133–41.
2 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.
3 Garber ME, Troyanskaya OG, Schluens K *et al.* Diversity of gene expression in adenocarcinoma of the lung. *Proc Natl Acad Sci USA* 2001; **98**: 13784–9.
4 Beer DG, Kardia SLR, Huang CC *et al.* Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med* 2002; **8**: 816–24.
5 Yanagisawa K, Tomida S, Shimada Y, Yatabe Y, Mitsudomi T, Takahashi T. A 25-signal proteomic signature and outcome for patients with resected non-small-cell lung cancer. *J Natl Cancer Inst* 2007; **99**: 858–67.
6 Sun Z, Yang P. Gene expression profiling on lung cancer outcome prediction: present clinical value and future premise. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 2063–8.
7 Ein-Dor L, Zuk O, Domany E. Thousands of samples are needed to generate a robust gene list for predicting outcome in cancer. *Proc Natl Acad Sci USA* 2006; **103**: 5923–8.
8 Shedden K, Taylor JM, Enkemann SA *et al.* Gene expression-based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. *Nat Med* 2008; **14**: 822–7.
9 Motoi N, Szoke J, Riely GJ *et al.* Lung adenocarcinoma: modification of the 2004 WHO mixed subtype to include the major histologic subtype suggests correlations between papillary and micropapillary adenocarcinoma subtypes, EGFR mutations and gene expression analysis. *Am J Surg Pathol* 2008; **32**: 810–27.
10 Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E, Sobin LH. *Histological Typing of Lung and Pleural Tumours*, 3rd edn. New York: Springer-Verlag, 1999.
11 Riquet M, Foucault C, Berna P, Assouad J, Dujon A, Danel C. Prognostic value of histology in resected lung cancer with emphasis on the relevance of the adenocarcinoma subtyping. *Ann Thorac Surg* 2006; **81**: 1988–95.
12 Sørlie T, Perou CM, Tibshirani R *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 2001; **98**: 10869–74.
13 Perou CM, Sørlie T, Eisen MB *et al.* Molecular portraits of human breast tumours. *Nature* 2000; **406**: 747–52.
14 UICC. *TNM Classification of Malignant Tumours*, 5th and 6th edn. Geneva: UICC, 1997 and 2002.
15 Shimmyo T, Okada A, Hashimoto T *et al.* Etiologic value of *p53* mutation spectra and differences with histology in lung cancers. *Cancer Sci* 2008; **99**: 287–95.
16 Tsuchiya E, Furuta R, Wada N *et al.* High K-*ras* mutation rates in goblet-cell-type adenocarcinomas of the lungs. *J Cancer Res Clin Oncol* 1995; **121**: 577–81.
17 Hashimoto T, Kobayashi Y, Ishikawa Y *et al.* Prognostic value of genetically diagnosed lymph node micrometastasis in non-small cell lung carcinoma cases. *Cancer Res* 2000; **60**: 6472–8.
18 Matsukuma S, Yoshihara M, Kasai F *et al.* Rapid and simple detection of hot spot point mutations of epidermal growth factor receptor, BRAF, and NRAS in cancers using the loop-hybrid mobility shift assay. *J Mol Diagn* 2006; **8**: 504–12.
19 Oshita F, Matsukuma S, Yoshihara M *et al.* Novel heteroduplex method using small cytology specimens with a remarkably high success rate for analysing *EGFR* gene mutations with a significant correlation to gefitinib efficacy in non-small-cell lung cancer. *Br J Cancer* 2006; **95**: 1070–5.
20 Sakuma Y, Matsukuma S, Yoshihara M *et al.* Distinctive evaluation of nonmucinous and mucinous subtypes of bronchioloalveolar carcinomas in EGFR and K-ras gene-mutation analyses for Japanese lung adenocarcinomas: confirmation of the correlations with histologic subtypes and gene mutations. *Am J Clin Pathol* 2007; **128**: 100–8.
21 Yatabe Y, Mitsudomi T, Takahashi T. TTF-1 expression in pulmonary adenocarcinomas. *Am J Surg Pathol* 2002; **26**: 767–73.
22 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**: 8919–23.
23 Mounawar M, Mukeria A, Calvez FL *et al.* Patterns of *EGFR, HER2, TP53,* and *KRAS* mutations of $p14^{arf}$ expression in non-small cell lung cancers in relation to smoking history. *Cancer Res* 2007; **67**: 5667–72.
24 Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutation in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994; **54**: 4855–78.
25 Shimmyo T, Hashimoto T, Kobayashi Y *et al.* p53 mutation spectra for squamous cell carcinomas at different levels of human bronchial branches. *Int J Cancer* 2006; **119**: 501–7.
26 Cooper CA, Carey FA, Bubb VJ, Lamb D, Kerr KM, Wyllie AH. The pattern of K-ras mutation in pulmonary adenocarcinoma defines a new pathway of tumour development in the human lung. *J Pathol* 1997; **181**: 401–4.
27 Marchetti A, Martella C, Felicioni L *et al.* EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* 2005; **23**: 857–65.
28 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.
29 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.
30 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.
31 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**: 1103–9.
32 Sasaki H, Shimizu S, Endo K *et al. EGFR* and ErbB2 mutation status in Japanese lung cancer patients. *Int J Cancer* 2006; **118**: 180–4.
33 Haneda H, Sasaki H, Shimizu S *et al.* Epidermal growth factor receptor gene mutation defines distinct subsets among small adenocarcinomas of the lung. *Lung Cancer* 2006; **52**: 47–52.
34 Mitsudomi T, Kosaka T, Endoh H *et al.* Mutations of the epidermal growth factor receptor gene predict prolonged survival after Gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. *J Clin Oncol* 2005; **23**: 2513–20.
35 Shigematsu H, Gazdar AF. Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. *Int J Cancer* 2006; **118**: 257–62.
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**: 235–40.
37 Kondo M, Yokoyama T, Fukui T *et al.* Mutations of epidermal growth factor receptor of non-small cell lung cancer were associated with sensitivity to gefitinib in recurrence after surgery. *Lung Cancer* 2005; **50**: 385–91.
38 Taron M, Ichinose Y, Rosell R *et al.* Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. *Clin Cancer Res* 2005; **11**: 5878–85.
39 Huang CL, Taki T, Adachi M *et al.* Mutations of p53 and K-ras genes as prognostic factors for non-small cell lung cancer. *Int J Oncol* 1998; **12**: 553–63.
40 Fukuyama Y, Mitsudomi T, Sugio K, Ishida T, Akazawa K, Sugimachi K. K-ras and p53 mutations are an unfavourable prognostic indicator in patients with non-small-cell lung cancer. *Br J Cancer* 1997; **75**: 1125–30.
41 Mitsudomi T, Hamajima N, Ogawa M, Takahashi T. Prognostic significance of *p53* alterations in patients with non-small cell lung cancer: a meta-analysis. *Clin Cancer Res* 2000; **6**: 4055–63.
42 Slebos RJC, Kibbelaar RE, Dalesio O *et al.* K-*ras* oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* 1990; **323**: 561–5.

doi: 10.1111/j.1349-7006.2010.01585.x

43 Ahrendt SA, Hu Y, Buta M *et al. p53* mutations and survival in stage I non-small-cell lung cancer: results of a prospective study. *J Natl Cancer Inst* 2003; **95**: 961–70.

44 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.

Supporting Information

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

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

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

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

doi:10.1510/icvts.2009.226399

INTERACTIVE CARDIOVASCULAR AND THORACIC SURGERY

Interactive CardioVascular and Thoracic Surgery 11 (2010) 56-59

www.icvts.org

Institutional report - Thoracic oncologic

Surgical resection for oral tongue cancer pulmonary metastases☆

Takahiro Mochizuki[a,b,*], Sakae Okumura[c], Genichiro Ishii[d], Yuichi Ishikawa[e], Ryuichi Hayashi[f], Kazuyoshi Kawabata[g], Junji Yoshida[a]

[a]*Division of Thoracic Surgery, Department of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan*
[b]*Department of Thoracic Surgery, Hamamatsu Medical Center, Hamamatsu, Shizuoka, Japan*
[c]*Department of Thoracic Surgical Oncology, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Koto-ku, Tokyo, Japan*
[d]*Division of Pathology, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa, Chiba, Japan*
[e]*Department of Pathology, Cancer Institute, Japanese Foundation for Cancer Research, Koto-ku, Tokyo, Japan*
[f]*Division of Head and Neck Surgery, National Cancer Center Hospital East, Kashiwa, Chiba, Japan*
[g]*Department of Head and Neck Oncology, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Koto-ku, Tokyo, Japan*

Received 26 October 2009; received in revised form 15 March 2010; accepted 20 March 2010

Abstract

The aim of this study was to evaluate the efficacy of surgical resection of oral tongue cancer (OTC) pulmonary metastases. Between 1977 and 2003, 23 OTC patients who developed 1–3 pulmonary metastases underwent metastasectomy. There were 14 men and nine women with a median age at the time of first metastasectomy of 56 years. All patients had advanced squamous cell OTC with synchronous or metachronous regional lymph node metastases. The median tumor-free interval after the last OTC treatment was 12 months. Five patients underwent pneumonectomy, three bilobectomy, 13 lobectomy, and two wedge resection. Two patients underwent a second pulmonary metastasectomy. One patient continues to survive, without recurrence 19 years after metastasectomy. Another patient was alive with disease at 24 months after metastasectomy but was lost to follow-up. Twenty-two out of 23 patients developed systemic metastases. The median interval to systemic recurrence after lung resection was 4.1 months, and 21 out of 23 patients died of OTC (median, 9.5 months) after metastasectomy. Most patients who underwent pulmonary metastasectomy died of the disease within two years of metastasectomy. Even for patients with a solitary metastasis, surgical metastasectomy is not a recommended treatment option.

Keywords: Lung; Metastasectomy; Outcomes

1. Introduction

The lung is the most frequent site of metastases from head and neck squamous cell carcinomas [1], and this is also true for oral tongue cancers (OTCs). Lung resection for a solitary metastasis from head and neck squamous cell carcinoma has been reported as a valid treatment option [2–4]. However, its role in OTC patients has not been clarified. The anterior two-thirds of the tongue in the oral cavity (oral tongue) and the posterior third in the oropharynx (base of tongue) arise from separate branchial arches, and have separate venous and lymphatic drainages [5]. Because of the embryological and anatomical differences, cancers arising in the oral tongue and those in the base of tongue behave and are treated differently. We reviewed our experiences in OTC patients who underwent lung resection, to determine the efficacy of pulmonary metastases resection.

2. Materials and methods

From May 1977 to April 2003, 23 consecutive patients underwent resection of pulmonary metastases from primary OTC in the thoracic oncology departments of two hospitals (the National Cancer Center Hospital East, Chiba, and the Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokyo, Japan). Demographic, perioperative, pathological, and survival data were retrospectively reviewed. Data collection and analysis were approved and the need for obtaining informed consent from each patient was waived by the institutional review boards of both hospitals in October 2006.

Following primary OTC treatment, patients were followed on an outpatient basis every 3–6 months. Each follow-up visit included a physical examination, chest roentgenogram, and blood examination. If abnormal findings suggestive of recurrence were obtained, further re-staging work-up was performed including whole body computed tomography, brain magnetic resonance imaging, and bone scintigram. Fluorine-18-2-fluoro-D-glucose positron emission tomography was performed for selected patients. To be considered for metastasectomy, the primary OTC had to be controlled, there could be no recurrences other than pulmonary metastases, and the patients had to be able to withstand lung

☆ This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan.
*Corresponding author. Division of Thoracic Surgery, Hamamatsu Medical Center, 328, Tomizuka-cho, Naka-ku, Hamamatsu, Shizuoka, 432-8580, Japan. Tel.: +81-53-453-7111; fax: +81-53-452-9217.
E-mail address: tmochizu@hmedc.or.jp (T. Mochizuki).

Table 1
Clinicopathological characteristics of 23 oral tongue cancer patients

	n=23	%
Sex		
Male	14	61
Female	9	39
Age at metastasectomy (years)		
Median	56	
Range	28–72	
Smoking history		
Former or current smoker	13	57
Never smoker	10	43
Smoking index (pack-years)		
Median	28	
Range	0–70	
Tumor size (cm)		
Median	3.9	
Range	1.3–6.0	
Location site		
Right	16	70
Left	7	30
Procedure		
Pneumonectomy	5	22
Bilobectomy	3	13
Lobectomy	13	57
Wedge resection	2	7
Chemotherapy	10/19	53

resection. The number of nodules or bilaterality was not an exclusion criterion. We performed wedge resection when feasible, but chose extended resection when a metastatic tumor was >3.0 cm, located in the pulmonary hilum, or multiple nodules were found in the same lobe.

We reviewed medical records to determine tumor differentiation grade, based on the World Health Organization (WHO) classification for cell types [6]. The pathological stage of OTC at initial presentation was determined based on the TNM classification of the International Union Against Cancer (UICC) [7].

Survivals were calculated by the Kaplan–Meier method. Zero time was the date of the first metastasectomy, and the terminal event was defined as death due to any cause (overall survival), or as recurrence, identified by serial imaging or biopsy (disease-free survival). An observation was censored at the last follow-up when the patient was alive or lost to follow-up. All statistical analyses were performed using a software package (SPSS, release 11.0; SPSS Inc, Chicago, IL, USA).

3. Results

Patient characteristics of the 23 patients are summarized in Table 1. There were 14 men and nine women (male/female ratio, 1.6:1). The median age at the first treatment was 53 years (range, 26–71 years). In all patients the primary disease were in stage II or higher. The median age at the time of pulmonary metastasectomy was 56 years (range, 28–72 years). Thirteen patients (11 men and two women) had a history of smoking, and the median smoking index was 35 pack-years. The remaining three male and seven female patients were non-smokers. All pulmonary metastases were found by outpatient follow-up examinations. The earliest pulmonary metastasis was detected one month after the initial OTC treatment. The median tumor-free interval after the last OTC treatment was 12 months, with a range of 0–60 months. The metastases arose in the right lung in 16 patients and in the left in seven and all were single-sided.

A total of 28 pulmonary metastatic nodules were resected. In two patients, two nodules were resected, and in two patients, three nodules were resected. At the time of the first metastasectomy, 20 patients had a solitary nodule, one had two, and one had three. One patient, who had two nodules at the first metastasectomy underwent a second thoracotomy to resect a new nodule. The average size of the pulmonary metastases was 3.9 cm (range, 1.3–6.0 cm).

Five patients underwent pneumonectomy, three bilobectomy, 13 lobectomy, and two wedge resection. Fifteen patients underwent ipsilateral mediastinal lymph node dissection, and three hilar node dissection, and one lymph node sampling. Metastasectomy was incomplete in seven patients (30%), because of positive resection margins (four patients), pleural dissemination (two patients), or metastatic lymph nodes (one patient). Of the 19 patients who underwent lymphadenectomy, nine (47%) had lymph node metastasis in the mediastinum, and two (11%) in the hilum. Resected nodules were pathologically confirmed to be compatible with pulmonary OTC metastases and were composed of squamous cell carcinoma with a variable degree of keratinization. Chemotherapy details in two patients were not available, but 10 (48%) among the remaining 21 patients underwent systemic chemotherapy consisting of various regimens before and/or after lung resection.

Within 17 months after lung resection, 22 of the 23 patients developed recurrence: five in the lung, four in the pleural cavity, 12 in the bone, one in the brain, three in the liver, two in the cervical lymph node, and three in the mediastinal lymph node (nine in multiple sites), and unknown in four patients. Twenty of these patients developed recurrence within a year after lung resection (Fig. 1).

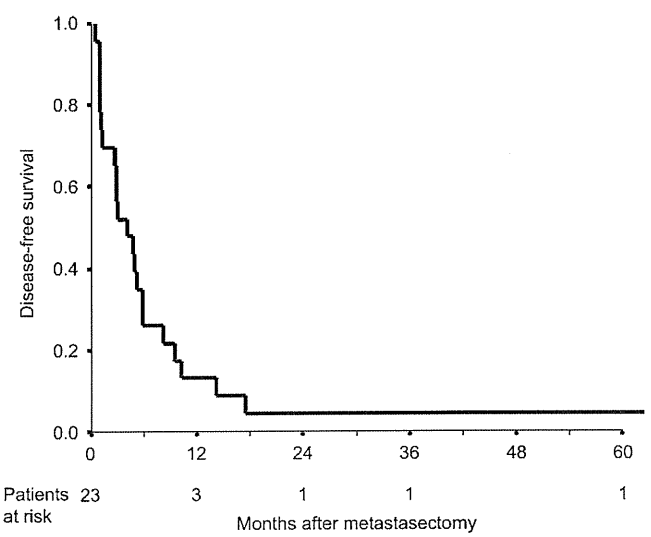

Fig. 1. Disease-free survival curve after first metastasectomy in 23 patients. The median disease-free survival time was 4.1 months.

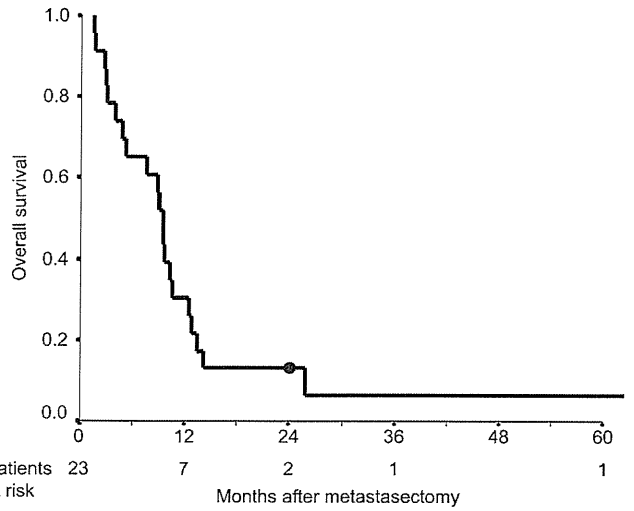

Fig. 2. Overall survival curve after first metastasectomy in 23 patients. The median survival time was 9.5 months, and one-, two-, and three-year survival rates were 30%, 13% and 7%, respectively.

One patient had no evidence of recurrence ~19 years after metastasectomy, and another patient was alive with pulmonary metastases at 24 months but was lost to follow-up.

The overall survival rates after the first metastasectomy were 30% at one year, 13% at two years, and 7% at three years. The median survival time of the 21 patients who died of OTC was 9.5 months (range, 1–26 months) after the first metastasectomy (Fig. 2). Even in 20 patients whose metastasis was solitary at the first metastasectomy, the one-, two-, and three-year survival rates were 30%, 15%, and 8%, respectively (Fig. 3). There were no significant differences in number or size of pulmonary nodules, disease-free interval, or survival time between patient groups categorized by TNM classification of primary OTC.

The median tumor sizes, positive mediastinal node rates, and median survival times after metastasectomy were 4.5 cm (range, 1.4–6.0 cm), 71%, and 3.1 months (1.4–229 months) in the patients who underwent metastasectomy in the 1980s, and 3.5 cm (1.3–5.5 cm), 56%, and 9.0 months (2.7–26 months) in the 1990s and thereafter. There were no statistical differences in metastatic tumor size, node positive rate, and survival time between these patient groups ($P=0.31$, 0.63, and 0.41, respectively). The median survival time was 3.1 months (range, 1.4–12.4 months) for eight bilobectomy or pneumonectomy patients, and was significantly <9.5 months (2.8–230 months) for 15 wedge resection or lobectomy patients ($P=0.02$). Even among the wedge resection or lobectomy patients, however, 80% died within 1.5 years after resection. The median survival times were 9.5 months in the patients with node metastases, and 7.5 months without metastases, respectively. There was no statistical difference ($P=0.14$).

4. Discussion

Advances in anesthesia and surgical procedures have enabled extensive treatment including lung resection in pulmonary metastases patients. Pulmonary metastasectomy criteria were first proposed by Thomford et al. in 1965, and were modified by McCormack et al. as follows: (1) primary site controlled or controllable; (2) no extrapulmonary metastatic sites demonstrable; (3) good surgical risk; and (4) no effective treatment available by non-surgical means [8]. These criteria have been commonly accepted for the past four decades. At our hospitals, we have actively pursued pulmonary metastasectomy with the following criteria: (1) no metastatic foci outside the lungs; (2) lung resection is tolerable; (3) a good postoperative quality of life is expected; (4) the primary focus is controlled; and (5) complete resection is expected.

Lung resection for a solitary metastasis from head and neck squamous cell carcinoma has been reported as a valid treatment option. Liu et al. analyzed 83 patients with pulmonary metastases from head and neck cancers and reported surgical resection of squamous cell carcinoma metastases resulted in long-term survival with a 32% five-year survival rate [2]. Mazer et al. based on 44 squamous cell carcinoma metastases patient analysis, reported an overall five-year survival rate of 43% [4]. Finley et al. compared 54 head and neck squamous cell carcinoma patients who did or did not undergo resection of their pulmonary metastases, and estimated the five-year survival rates of the 18 patients in surgical group and of the remainder in non-surgical group as 29% and 5%, respectively [3].

To our knowledge, however, there have been no reports on surgical resection only for OTC pulmonary metastases. This study on OTC pulmonary metastases showed outcomes were dismal even in a patient with solitary metastasis. Only a few patients survived more than two years. Despite imaging technique progresses, there were no significant differences in metastatic tumor size, node positive rate, and survival time between patients who underwent metastasectomy in the 1980s and those in the 1990s and on. These findings suggest the poor outcomes were not due to poor pre-metastasectomy staging but due to the aggressive nature of OTC pulmonary metastases. The median survival

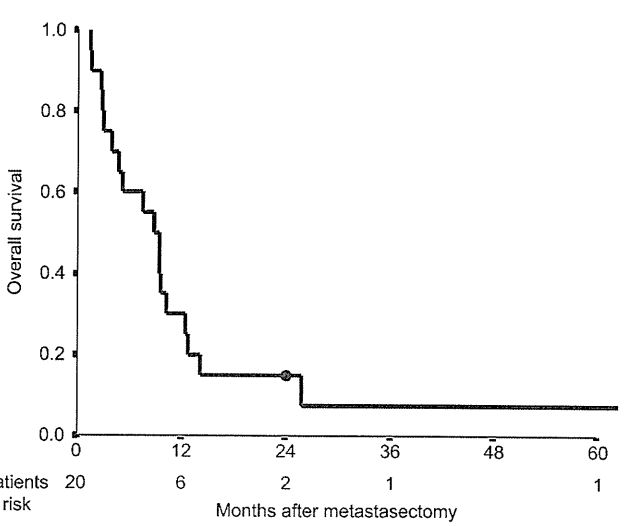

Fig. 3. Overall survival after first metastasectomy in 20 solitary pulmonary metastasis patients. The median survival time was 8.8 months, and one-, two-, and three-year survival rates were 30%, 15% and 8%, respectively.

time in bilobectomy or pneumonectomy patients was significantly shorter than in 15 wedge resection or lobectomy patients. Surgical invasiveness may have played a role in the poorer prognosis. Even among the wedge resection or lobectomy patients, however, 80% died within 1.5 years after resection. Although the median survival time in the patients with node metastasis was shorter than in those without node metastasis, there was no statistical difference. This fact suggests node metastasis did not have a strong impact on outcome. We think OTCs biological aggressiveness had more impact on the poor outcome. We have to conclude that OTC pulmonary metastases resection is of little efficacy.

However, it can be difficult to differentiate OTC pulmonary metastases from primary pulmonary squamous cell cancers. OTC patients are often heavy smokers and are at high-risk for primary pulmonary squamous cell carcinoma [9]. The lifetime incidence of a second primary lung cancer in patients with head and neck cancer has been reported to be 3.3%–5.5% [10, 11]. When multiple pulmonary nodules are found in patients after primary OTC treatment, the reasonable and mostly correct diagnosis is OTC metastases. If a solitary nodule is found, however, it can either be an OTC metastasis or primary lung cancer.

Metastatic squamous cell OTC and primary pulmonary squamous cell carcinoma can be distinguished based on histological disparity, but there are no absolute distinguishing criteria. Because the primary OTC in this series were advanced, we diagnosed the pulmonary lesions as metastases unless there was apparent morphological dissimilarity between the primary tumor and pulmonary lesions. Differential diagnosis can be difficult even in resected specimens, and even more so based on small preoperative biopsy specimens. In order not to miss the possibility of curative surgical resection opportunity for primary lung cancer patients, resection may be indicated in patients with a solitary lung nodule after curative OTC treatment.

One other reason pulmonary metastasectomy was considered to be an option was because there were no effective non-surgical means available [8]. Argiris et al. reported that only a small percentage of patients with recurrent or metastatic head and neck carcinoma can achieve long-term survival after systemic chemotherapy and that a primary hypopharyngeal or oral cavity location was a significant unfavorable prognostic factor [12]. Chemotherapy seems to be an invalid option for OTC metastases patients. As we encountered one long-term survivor after metastasectomy, we may not have to completely exclude surgical resection from treatment options for OTC pulmonary metastases patients.

In conclusion, most patients who underwent OTC pulmonary metastasectomy died of the disease within two years. Even for patients with a solitary metastasis limited to the lung, surgical resection for OTC pulmonary metastases is not a recommended treatment option.

Acknowledgements

We thank J. Patrick Barron, International Medical Communications Center, Tokyo Medical University, for reviewing the English manuscript.

References

[1] Kotwall C, Sako K, Razack MS, Rao U, Bakamjian V, Shedd DP. Metastatic patterns in squamous cell cancer of the head and neck. Am J Surg 1987;154:439–442.
[2] Liu D, Labow DM, Dang N, Martini N, Bains M, Burt M, Downey R Jr, Rusch V, Shah J, Ginsberg RJ. Pulmonary metastasectomy for head and neck cancers. Ann Surg Oncol 1999;6:572–578.
[3] Finley RK 3rd, Verazin GT, Driscoll DL, Blumenson LE, Takita H, Bakamjian V, Sako K, Hicks W Jr, Petrelli NJ, Shedd DP. Results of surgical resection of pulmonary metastases of squamous cell carcinoma of the head and neck. Am J Surg 1992;164:594–598.
[4] Mazer TM, Robbins KT, McMurtrey MJ, Byers RM. Resection of pulmonary metastases from squamous carcinoma of the head and neck. Am J Surg 1988;156:238–242.
[5] Prince S, Bailey BM. Squamous carcinoma of the tongue: review. Br J Oral Maxillofac Surg 1999;37:164–174.
[6] Barnes L, Eveson JW, Reichart P, Sidransky D, editors. World Health Organization classification of tumours. Pathology and genetics of head and neck tumours. Lyon: IARC Press, 2005.
[7] Sobin LH, Wittekind C, editors. TNM classification of malignant tumours, sixth edition. New York: John Wiley & Sons, 2002.
[8] McCormack PM, Bains MS, Beattie EJ Jr, Martini N. Pulmonary resection in metastatic carcinoma. Chest 1978;73:163–166.
[9] Day GL, Blot WJ, Shore RE, McLaughlin JK, Austin DF, Greenberg RS, Liff JM, Preston-Martin S, Sarkar S, Schoenberg JB, Fraumeni JF Jr. Second cancers following oral and pharyngeal cancers: role of tobacco and alcohol. J Natl Cancer Inst 1994;86:131–137.
[10] Shah SI, Applebaum EL. Lung cancer after head and neck cancer: role of chest radiography. Laryngoscope 2000;110:2033–2036.
[11] Dhooge IJ, De Vos M, Van Cauwenberge PB. Multiple primary malignant tumors in patients with head and neck cancer: results of a prospective study and future perspectives. Laryngoscope 1998;108:250–256.
[12] Argiris A, Li Y, Forastiere A. Prognostic factors and long-term survivorship in patients with recurrent or metastatic carcinoma of the head and neck. Cancer 2004;101:2222–2229.

Pathology International

Pathology International 2010; **60**: 575–580

doi:10.1111/j.1440-1827.2010.02565.x

Original Article

Cytokeratin expression profiling is useful for distinguishing between primary squamous cell carcinoma of the lung and pulmonary metastases from tongue cancer

Takehiko Ohba,[1,2] Noriko Motoi,[1] Yukinori Kimura,[1] Sakae Okumura,[3] Kazuyoshi Kawabata,[4] Yasuyuki Yoshizawa,[2] Naohiko Inase[2] and Yuichi Ishikawa[1]

[1]Division of Pathology, The Cancer Institute, Departments of [3]Thoracic Surgical Oncology and [4]Head and Neck Oncology, The Cancer Institute Hospital, Japanese Foundation for Cancer Research and [2]Department of Integrated Pulmonology, Tokyo Medical and Dental University, Tokyo, Japan

It can be difficult to distinguish between primary and metastatic squamous cell carcinoma (SCC) in the lung. Surgical specimens were obtained from two groups of patients, 26 lung SCC patients without histories of any other cancer (the definite primary group) and 17 patients who had undergone surgical removal of SCC emerging in the lung after surgery for tongue SCC (the unknown group). From the former, 26 primary lung SCC were obtained. From the latter, 17 lung tumors and 15 primary tumors of the tongue were obtained. Eleven of the 17 lung tumors from the unknown group were metastatic lung SCC. All specimens were immunostained with cytokeratin (CK)5/6, CK7, CAM5.2, CK19 and p63 antibodies. The frequency of CAM5.2 and CK19 expression was significantly higher in the lung SCC of the definite primary group (21 of 26, 81% and 20 of 26, 78%, respectively) than in the metastatic lung SCC (1 of 11, 9% ($P < 0.001$) and 2 of 11, 18% ($P = 0.003$), respectively) or primary SCC of the tongue (5 of 15, 33% ($P = 0.002$) and 2 of 15, 13% ($P < 0.001$), respectively). CAM5.2 and CK19 are useful for distinguishing between primary SCC of the lung and metastases from tongue cancer.

Key words: cytokeratin, lung cancer, metastasis, squamous cell carcinoma, tongue cancer

The lung is a common site of primary tumors as well as metastatic lesions. Primary lung adenocarcinomas have features that are morphologically and immunohistochemically distinct from those of other organs such as colorectal adenocarcinomas. Therefore, it is not difficult to differentiate between primary lung adenocarcinomas and metastatic tumors from other sites. However, squamous cell carcinomas (SCC) pose a greater challenge. When a patient with a history of tongue SCC develops an SCC in the lung, it can be difficult to determine whether the new tumor is a primary lung cancer or a metastasis from the tongue. This distinction is clinically important for several reasons: SCC of the tongue sometimes recurs as a distant metastasis (4.8–8.0%),[1,2] often in the lung;[3] SCC is one of the major histological subtypes of primary lung cancer; and therapeutic strategies and prognoses are quite different between cases with primary and metastatic tumors. Similarities in the etiologies and histological features of these tumors contribute to the difficulty in determining the tumor's origin.

Cytokeratin (CK) polypeptides are the major cytoskeletal proteins in epithelial cells. CK has been separated by molecular weights into at least twenty subtypes, which are expressed in various combinations depending on the epithelial cell type. Roughly speaking, relatively high molecular weight CK is characteristic of squamous epithelium, whereas lower molecular weight CK typifies simple columnar epithelium.[4,5] SCC arising from tissues with true squamous epithelium usually express CK characteristics of squamous epithelium. On the other hand, primary lung SCC express CK characteristics of columnar epithelium, such as CK8, 18 and 19, more abundantly than do SCC of tissues with true squamous epithelium, including the oral cavity, esophagus, vulva and skin.[6] CK19 is also expressed more frequently and intensely in lung SCC than in oral SCC.[7] These reports suggest that primary lung SCC arising from columnar epithelium through a metaplastic process may have a somewhat different CK profile from those arising from true squamous

Correspondence: Yuichi Ishikawa, MD, PhD, Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan. Email: ishikawa@jfcr.or.jp

Received 11 January 2010. Accepted 24 April 2010.