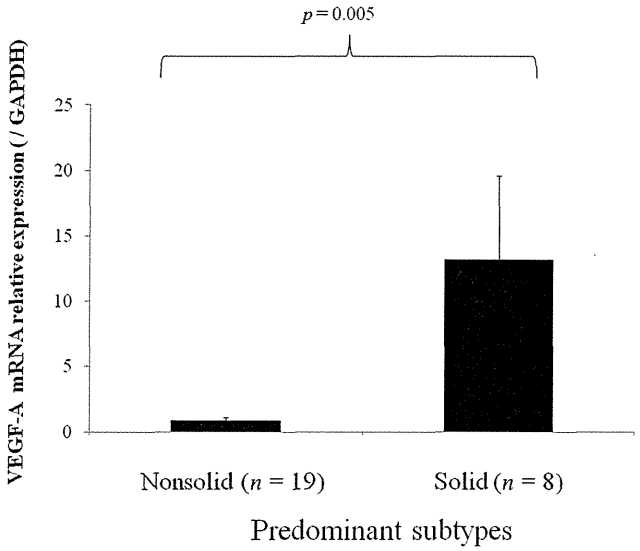

FIGURE 5. Differences in vascular endothelial growth factor (VEGF)-A levels in tumor tissue samples between solid and nonsolid adenocarcinoma patients, as determined using a quantitative real-time polymerase chain reaction. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the existence of a bone marrow reservoir of EPCs and their selective involvement in neovascularization has attracted considerable interest because these cells could be used as surrogate markers to monitor the status of tumor angiogenesis.[16] In this study, the number of EPCs in patients with predominantly solid adenocarcinoma was significantly higher than in patients with predominantly BAC, papillary, or acinar adenocarcinomas. The higher number of circulating EPCs associated with solid adenocarcinoma may indicate the presence of differences in the tumor angiogenic status between early-stage adenocarcinoma histological subtypes.

In addition to the number of circulating EPCs, several reports have also demonstrated a significant correlation between neovascularization assessed using the intratumoral MVD, the tumor angiogenic status, and patient outcome in a variety of tumors.[17–19] In this study, the MVD of the tumor was significantly correlated with the number of circulating EPCs. Regarding the prognostic relevance of angiogenic activity in NSCLC as expressed by the intratumoral MVD, a high MVD has been identified as an unfavorable prognostic factor.[35,36] Yuan et al.[37] reported that the MVD was significantly correlated with the histological types and that a higher MVD was found significantly more frequently in adenocarcinoma than in squamous cell carcinoma, suggesting that adenocarcinomas might have a higher angiogenic potential. However, in this study, no statistically significant differences in the MVD were observed between adenocarcinoma and squamous cell carcinoma. Instead, we found significant differences in the MVD among the adenocarcinoma histological subtypes. A higher MVD was found significantly more frequently in solid adenocarcinomas than in nonsolid adenocarcinomas. This may reflect the aggressive and invasive characteristics of this subtype and may be one of the reasons why patients with solid adenocarcinoma have significantly poorer outcomes than those with other adenocarcinoma histological subtypes.

VEGF is the most important angiogenesis factor, and its expression within tumors is suggested to affect the prognosis of patients.[26,38] Thus far, there have been several reports regarding the association between the level of VEGF-A and MVD.[26,27,36] In addition, bone marrow-derived EPCs are also reported to be mobilized by the stimulation of tumor-derived VEGF-A, inducing them to migrate toward the tumor and to become incorporated into the developing neovasculature.[24,25] In this study, we confirmed that higher levels of VEGF-A are present in solid adenocarcinomas than in nonsolid adenocarcinomas. Recent studies have shown that the addition of antiangiogenic therapy, such as bevacizumab, to paclitaxel and carboplatin improves survival, compared with chemotherapy alone, in patients with previously untreated metastatic nonsquamous NSCLC.[13] Especially among adenocarcinoma patients, those with a solid adenocarcinoma may be the best candidates for the addition of bevacizumab, an antiangiogenic monoclonal antibody that blocks VEGF-A.

In this study, we showed a difference in the number of circulating EPCs or intratumoral MVD, both of which might be potential markers for neovascularization, between adenocarcinoma histological subtypes. Gao et al.[39] reported that circulating EPCs play a major and catalytic role in tumor progression, which may be maximized in metastatic and relapsing disease by the promotion of the progression of avascular micrometastases to vascularized macrometastases. The significantly higher levels of EPCs paralleling clinical severity also suggest the possible relevance of these cells in the metastatic progression of the tumors[16] and point to their potential use as targets in therapy against metastatic sites. Therefore, preoperative or postoperative anti-EPC therapy may be indicated for early-stage adenocarcinoma patients with preoperative high EPC levels to prevent postoperative recurrence after resection. In this study, the number of EPCs in patients with solid adenocarcinoma was significantly higher than that in nonsolid adenocarcinoma patients. This finding may indicate a subgroup of adenocarcinoma patients who may benefit from angiogenesis inhibitors targeted against EPCs.

This study had several limitations. In particular, the study lacked ethnic diversity, as all the patients were Japanese. Another limitation is that the blood mononuclear cells from the PA were isolated from the resected lungs and not directly from the patients preoperatively to avoid unnecessary invasiveness. However, we believe that the level of EPCs in the blood from the PA in the vicinity of the tumor more precisely reflects the effect of the tumors than the samples from the peripheral blood, as previously reported.[16,24] In this study, we first reported the differences in the number of circulating EPCs or MVD between lung adenocarcinoma subtypes. Further clinical studies are needed to confirm the beneficial effects of antiangiogenic therapy against VEGF or EPCs in solid adenocarcinoma patients.

CONCLUSIONS

The number of EPCs and the MVD in patients with predominantly solid adenocarcinomas were significantly higher than those in nonsolid adenocarcinoma patients. In particular, patients with solid adenocarcinoma may be the best candidates for antiangiogenic therapies against VEGF or EPCs among the various adenocarcinoma subtypes.

REFERENCES

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2009. *CA Cancer J Clin* 2009;59:225–249.
2. Yoshida J, Nagai K, Asamura H, et al.; Japanese Joint Committee for Lung Cancer Registration. Visceral pleura invasion impact on non-small cell lung cancer patient survival: its implications for the forthcoming TNM staging based on a large-scale nation-wide database. *J Thorac Oncol* 2009;4:959–963.
3. Devesa SS, Bray F, Vizcaino AP, et al. International lung cancer trends by histologic type: male: female differences diminishing and adenocarcinoma rates rising. *Int J Cancer* 2005;117:294–299.
4. Asamura H, Goya T, Koshiishi Y, et al.; Japanese Joint Committee of Lung Cancer Registry. A Japanese Lung Cancer Registry study: prognosis of 13,010 resected lung cancers. *J Thorac Oncol* 2008;3:46–52.
5. Travis WD, Brambilla E, Muller-Hermelink HK, et al. World Health Organization Classification of Tumors: Pathology and Genetics of Tumors of the Lung, Pleura, Thymus and Heart. Lyon, France: IARC Press; 2004.
6. Higashiyama M, Kodama K, Yokouchi H, et al. Prognostic value of bronchiolo-alveolar carcinoma component of small lung adenocarcinoma. *Ann Thorac Surg* 1999;68:2069–2073.
7. Sakao Y, Miyamoto H, Sakuraba M, et al. Prognostic significance of a histologic subtype in small adenocarcinoma of the lung: the impact of nonbronchioloalveolar carcinoma components. *Ann Thorac Surg* 2007; 83:209–214.
8. Anami Y, Iijima T, Suzuki K, et al. Bronchioloalveolar carcinoma (lepidic growth) component is a more useful prognostic factor than lymph node metastasis. *J Thorac Oncol* 2009;4:951–958.
9. Riquet M, Foucault C, Berna P, et al. Prognostic value of histology in resected lung cancer with emphasis on the relevance of the adenocarcinoma subtyping. *Ann Thorac Surg* 2006;81:1988–1995.
10. Barletta JA, Yeap BY, Chirieac LR. Prognostic significance of grading in lung adenocarcinoma. *Cancer* 2009;116:659–669.
11. Ding L, Getz G, Wheeler DA, et al. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 2008;455:1069–1075.
12. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971;285:1182–1186.
13. Ramalingam SS, Dahlberg SE, Langer CJ, et al.; Eastern Cooperative Oncology Group. Outcomes for elderly, advanced-stage non small-cell lung cancer patients treated with bevacizumab in combination with carboplatin and paclitaxel: analysis of Eastern Cooperative Oncology Group Trial 4599. *J Clin Oncol* 2008;26:60–65.
14. Davidoff AM, Ng CY, Brown P, et al. Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. *Clin Cancer Res* 2001;7:2870–2879.
15. Bolontrade MF, Zhou RR, Kleinerman ES. Vasculogenesis plays a role in the growth of Ewing's sarcoma in vivo. *Clin Cancer Res* 2002;8: 3622–3627.
16. Dome B, Timar J, Dobos J, et al. Identification and clinical significance of circulating endothelial progenitor cells in human non-small cell lung cancer. *Cancer Res* 2006;66:7341–7347.
17. Hasan J, Byers R, Jayson GC. Intra-tumoural microvessel density in human solid tumours. *Br J Cancer* 2002;86:1566–1577.
18. Vermeulen PB, Gasparini G, Fox SB, et al. Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. *Eur J Cancer* 1996;32:2474–2484.
19. Kreuter M, Bieker R, Bielack SS, et al. Prognostic relevance of increased angiogenesis in osteosarcoma. *Clin Cancer Res* 2004;10:8531–8537.
20. Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumours. *J Thorac Oncol* 2007;2:706–714.
21. Chiba H, Ishii G, Ito TK, et al. CD105-positive cells in pulmonary arterial blood of adult human lung cancer patients include mesenchymal progenitors. *Stem Cells* 2008;26:2523–2530.
22. Ishii G, Ito TK, Aoyagi K, et al. Presence of human circulating progenitor cells for cancer stromal fibroblasts in the blood of lung cancer patients. *Stem Cells* 2007;25:1469–1477.
23. Kreuter M, Kropff M, Fischaleck A, et al. Prognostic relevance of angiogenesis in stage III NSCLC receiving multimodality treatment. *Eur Respir J* 2009;3:1383–1388.
24. Nowak K, Rafat N, Belle S, et al. Circulating endothelial progenitor cells are increased in human lung cancer and correlate with stage of disease. *Eur J Cardiothorac Surg* 2010;37:758–763.
25. Spring H, Schüler T, Arnold B, et al. Chemokines direct endothelial progenitors into tumor neovessels. *Proc Natl Acad Sci USA* 2005;102: 18111–18116.
26. Bremnes RM, Camps C, Sirera R. Angiogenesis in non-small cell lung cancer: the prognostic impact of neoangiogenesis and the cytokines VEGF and bFGF in tumours and blood. *Lung Cancer* 2006;51:143–158.
27. Koukourakis MI, Giatromanolaki A, Thorpe PE, et al. Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res* 2000;60:3088–3095.
28. Watanabe Y. Substaging of stage I—a commentary. *Lung Cancer* 2003;42: 59–61.
29. Ahn GO, Brown JM. Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. *Cancer Cell* 2008;13:193–205.
30. Kopp HG, Ramos CA, Rafii S. Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. *Curr Opin Hematol* 2006;13:175–181.
31. Rajantie I, Ilmonen M, Alminaite A, et al. Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. *Blood* 2004;104:2084–2086.
32. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 2001;7:1194–1201.
33. De Palma M, Venneri MA, Roca C, et al. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat Med* 2003;9:789–795.
34. Göthert JR, Gustin SE, van Eekelen JA, et al. Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* 2004;104:1769–1777.
35. Meert AP, Paesmans M, Martin B, et al. The role of microvessel density on the survival of patients with lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer* 2002;87:694–701.
36. Medetoglu B, Gunluoglu MZ, Demir A, et al. Tumor angiogenesis in predicting the survival of patients with stage I lung cancer. *J Thorac Cardiovasc Surg* 2010;140:996–1000.
37. Yuan A, Yang PC, Yu CJ, et al. Tumor angiogenesis correlates with histologic type and metastasis in non-small-cell lung cancer. *Am J Respir Crit Care Med* 1995;152:2157–2162.
38. O'Byrne KJ, Koukourakis MI, Giatromanolaki A, et al. Vascular endothelial growth factor, platelet-derived endothelial cell growth factor and angiogenesis in non-small-cell lung cancer. *Br J Cancer* 2000;82:1427–1432.
39. Gao D, Nolan DJ, Mellick AS, et al. Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. *Science* 2008;319:195–198.

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Differences Between Squamous Cell Carcinoma and Adenocarcinoma of the Lung: Are Adenocarcinoma and Squamous Cell Carcinoma Prognostically Equal?

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Objective: We analyzed pulmonary squamous cell carcinoma and adenocarcinoma patient survival in our single institution database, to evaluate the relationship of histologic analysis to survival and tumor aggressiveness.
Methods: We reviewed 1856 consecutive patients with surgically resected pulmonary squamous cell carcinoma or adenocarcinoma regarding their clinicopathologic characteristics, overall survival and recurrence-free proportion.
Results: In squamous cell carcinoma patients, there were more elderly male smokers and more patients with T2–4 tumors, moderately/poorly differentiated tumors, lymph node metastasis or vascular invasion than in adenocarcinoma patients. In all patients and in pN0 patients, patients with squamous cell carcinoma showed significantly poorer overall survival than those with adenocarcinoma, but there were no statistically significant differences in the recurrence-free proportion between the two histologic types. There were statistically significantly more lung cancer-specific deaths in patients with adenocarcinoma than in patients with squamous cell carcinoma ($P = 0.001$).
Conclusions: There were no differences in the development of recurrence between squamous cell carcinoma and adenocarcinoma of the lung, but considerable differences in overall survival were observed between the two histologic types. According to the stage grouping strategy of the TNM Classification for Lung and Pleural Tumours, these two histologic types need to be staged differently. This survival difference, however, may reflect the difference in patient background rather than in biologic aggressiveness between the two histologic types.

Key words: histologic type – prognosis – squamous cell carcinoma – adenocarcinoma – TNM classification

INTRODUCTION

Squamous cell carcinoma and adenocarcinoma are the two major histologic types of non-small cell lung cancer. Patients with adenocarcinoma were known to result in poorer prognosis than those with squamous cell carcinoma (1,2). However, a recent increase in the use of computed tomography (CT) has enabled small adenocarcinoma detection on a screening basis, and many of these small adenocarcinomas are relatively dormant bronchioloalveolar carcinomas and have favorable outcome (3). This may be one reason why patients with squamous cell carcinoma are known today to have a poorer prognosis than those with adenocarcinoma following surgical resection (4).

Squamous cell carcinoma mostly develops in smokers, in whom life-threatening co-morbidities often develop, which may also explain the poorer survival rates of patients with

squamous cell carcinoma compared with those with adenocarcinoma. However, differences in biological aggressiveness between squamous cell carcinoma and adenocarcinoma of the lung are not well understood.

In esophageal cancer staging, squamous cell carcinoma and adenocarcinoma are classified differently in the 7th Edition of the Cancer Staging Manual of the American Joint Committee on Cancer (5–7). In lung cancer, however, prognostic differences in histologic types are not taken into consideration in the latest TNM classification (8).

We retrospectively analyzed the survival differences between squamous cell carcinoma and adenocarcinoma of the lung, in an attempt to identify the prognostic impact of histologic difference and to incorporate it in future staging systems, based on our patient database.

PATIENTS AND METHODS

From July 1992 through December 2006, 1856 consecutive patients with pulmonary squamous cell carcinoma or adenocarcinoma underwent complete resection at our institution. We defined complete resection as segmentectomy or greater, with systematic ipsilateral hilar and mediastinal lymph node dissection but with no evidence of residual cancer either macroscopically or histologically. Patients who had induction chemotherapy, radiotherapy or both, patients with evidence of residual tumor at the surgical margin or patients with malignant effusion or distant metastasis verified intraoperatively or by means of postoperative pathologic examination were excluded from this study.

Cases were pathologically staged based on the 7th Edition of the TNM Classification for Lung and Pleural Tumours (8). Histopathologic studies were done according to the World Health Organization criteria (9). We reviewed the medical records of all patients for the following clinicopathologic factors: age, gender, smoking history (never or ever smoker), pathological differentiation, pathological T stage, pathological N stage, vascular invasion and lymphatic permeation.

Student's *t*-test was used to evaluate the relationships between histologic type (squamous cell carcinoma or adenocarcinoma) and age. Fisher's exact test was used to evaluate the relationships between histologic type and other clinicopathologic factors. We compared overall survival and recurrence-free proportion between squamous cell carcinoma and adenocarcinoma in all patients, in pN0 patients, in pT1N0 patients, in pT2N0 patients and in pT3/4N0 patients. When we analyzed recurrence-free proportion, we excluded 249 cases from this study because their recurrence data were incomplete. The survival rates and recurrence-free proportions were calculated using the Kaplan–Meier method, and univariate analyses were performed with the log-rank test. Multivariate analyses were performed by using the Cox proportional hazards model. Zero time was the date of pulmonary resection. The endpoint of overall survival was defined as the date of death from any cause, and the last follow-up observation was censored when the patient was alive or lost to follow-up. The endpoint of recurrence-free proportion was defined as the date when recurrence was confirmed. We examined patients at 3-month intervals for the first 2 years and at 6-month intervals thereafter on an outpatient basis. The follow-up evaluation included physical examination, chest radiography and blood examination including that of pertinent tumor markers. Further evaluations, including CT scans of the chest and abdomen, brain magnetic resonance imaging and bone scintigraphy, were performed on the detection of any symptoms or signs of recurrence. Since 2004, integrated positron emission tomography and CT have also been performed when appropriate. We diagnosed recurrence based on the findings of physical examination and diagnostic imaging and confirmed the diagnosis histologically when clinically feasible. The date of recurrence was defined as the date of cytohistological proof. However, in cases diagnosed on the basis of clinicoradiological findings, the date of recurrence was defined as the date of identification by a physician. The last follow-up observation was censored when the patient was recurrence-free or lost to follow-up. Patients who died from causes other than lung cancer recurrence were also censored on the date of death.

All P values were two-sided, and P values <0.05 were considered to represent statistically significant differences. Survival analyses were performed on SPSS software (Dr SPSS II for Windows, Standard Version 11.0, SPSS Inc., Chicago, IL, USA).

Data collection and analyses were approved, and the need to obtain written informed consent from each patient in this retrospective study was waived, by the institutional review board in June 2010.

RESULTS

Patient Characteristics

The patient characteristics are shown in Table 1. In squamous cell carcinoma patients, compared with adenocarcinoma patients, there were more elderly male smokers and more patients with T2–4 tumors, moderately/poorly differentiated tumors, lymph node metastasis or vascular invasion. In pN0 patients ($n = 1328$), there were more elderly male smokers and more patients with T2–4 tumors, moderately/poorly differentiated tumors or vascular invasion in squamous cell carcinoma patients.

Overall Survival Differences

Patients with squamous cell carcinoma showed significantly poorer overall survival than those with adenocarcinoma in all patients and in pN0 patients (Figs 1A and 2A). The results of multivariate analyses of the statistically significant characteristics listed in Table 1 are summarized in Table 2. Age, smoking history, pathological T classification, vascular

Table 1. Patient characteristics

Patient characteristics	All patients				pN0 patients			
	AD	SQ	*P*-value	Total	AD	SQ	*P*-value	Total
Age								
Median (range)	65 (32–90)	69 (31–88)	<0.001[a]		65 (32–90)	70 (31–88)	<0.001[a]	
Sex								
Men	731 (52)	418 (90)		1149 (62)	521 (51)	263 (89)		784 (59)
Women	662 (48)	45 (10)	<0.001[b]	707 (38)	510 (49)	34 (11)	<0.001[b]	544 (41)
Smoking history								
Never smoker	617 (44)	12 (3)		629 (34)	485 (47)	9 (3)		494 (37)
Ever smoker	776 (56)	451 (97)	<0.001[b]	1227 (66)	546 (53)	288 (97)	<0.001[b]	834 (63)
Pathological T classification								
T1a, T1b	689 (49)	131 (28)		820 (44)	602 (58)	103 (35)		705 (53)
T2a, T2b, T3, T4	704 (51)	332 (72)	<0.001[b]	1036 (56)	429 (42)	194 (65)	<0.001[b]	623 (47)
Pathological N classification								
N0	1031 (74)	297 (64)		1328 (72)	—	—	—	—
N1, N2	362 (26)	166 (36)	<0.001[b]	528 (28)	—	—	—	—
Pathological differentiation								
Well	491 (36)	21 (5)		512 (28)	454 (44)	17 (6)		471 (36)
Moderately/poorly	892 (74)	440 (95)	<0.001[b]	1332 (72)	569 (56)	279 (94)	<0.001[b]	848 (64)
Vascular invasion								
Absent	818 (59)	150 (32)		968 (52)	732 (71)	128 (43)		860 (65)
Present	575 (41)	313 (68)	<0.001[b]	888 (48)	299 (29)	169 (57)	<0.001[b]	468 (35)
Lymphatic permeation								
Absent	964 (69)	320 (69)		1284 (69)	847 (82)	238 (80)		1085 (82)
Present	429 (31)	143 (31)	1.000[b]	572 (31)	184 (18)	59 (20)	0.444[b]	243 (18)
Total	1393	463		1856	1031	297		1328

AD, adenocarcinoma; SQ, squamous cell carcinoma; T/N classification according to the 7th Edition of the TNM Classification for Lung and Pleural Tumours; numbers in parentheses are percentages.
[a]Student's t-test.
[b]Fisher's exact test.

invasion and lymphatic permeation were significant prognostic factors in all patients and in pN0 patients. Pathological N classification was a significant prognostic factor in all patients. Sex, pathological differentiation and histologic type were not significant prognostic factors in any patients or in pN0 patients.

Although patients with squamous cell carcinoma showed significantly poorer overall survival than those with adenocarcinoma in pT1N0 patients and in pT2N0 patients (Fig. 3A and C), no statistically significant differences were observed in pT3/4N0 patients ($P = 0.841$; Fig. 3E).

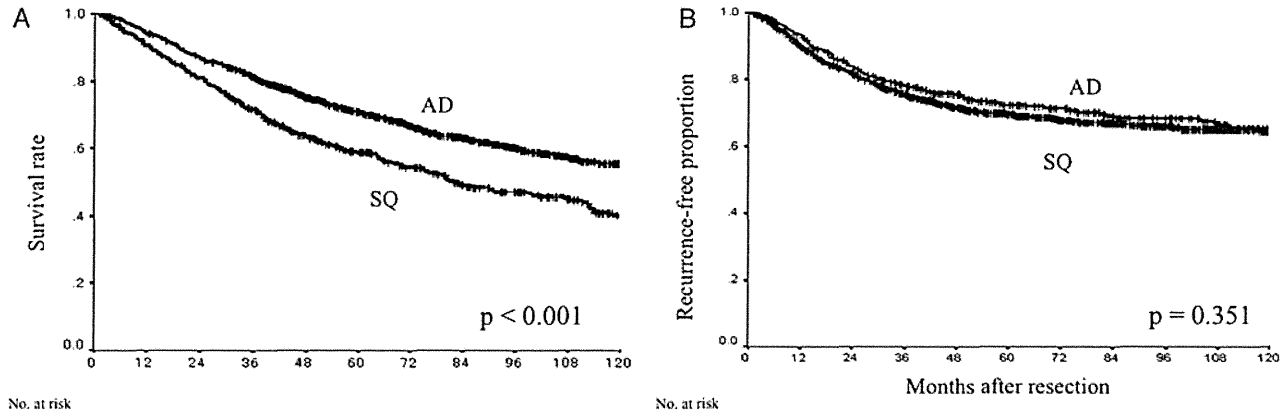
Recurrence-free Proportion Differences

There were no statistically significant differences in recurrence-free proportion between adenocarcinoma and squamous cell carcinoma in any patients ($P = 0.351$; Fig. 1B) or in pN0 patients ($P = 0.715$; Fig. 2B).

In pT1N0 patients, patients with squamous cell carcinoma showed significantly poorer recurrence-free proportion than those with adenocarcinoma (Fig. 3B). In pT2N0 patients, there was no statistically significant difference in recurrence-free proportion between the two histologic types ($P = 0.098$; Fig. 3D). In pT3/4N0 patients, patients with adenocarcinoma showed significantly poorer recurrence-free proportion than those with squamous cell carcinoma (Fig. 3F).

Causes of Death

There were 638 patients whose causes of death were identified in our cohort. There were significantly more lung cancer-specific deaths in adenocarcinoma patients than in squamous cell carcinoma patients ($P = 0.001$; Table 3).



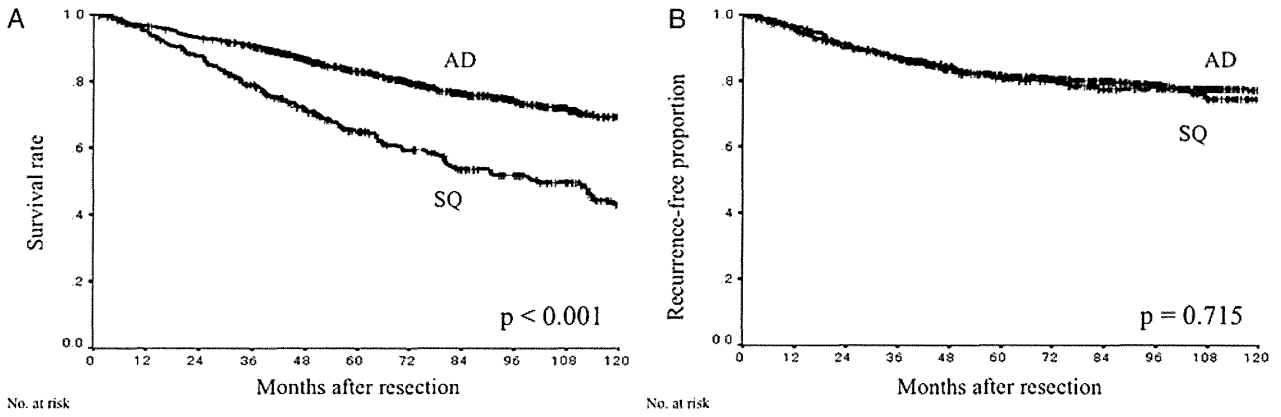
No. at risk

	0	12	24	36	48	60	72	84	96	108	120
AD	1393	1305	1201	1083	897	738	599	475	370	289	228
SQ	463	416	367	316	251	208	168	131	106	84	50

No. at risk

	0	12	24	36	48	60	72	84	96	108	120
AD	1264	1117	990	869	719	607	492	393	310	237	181
SQ	373	324	281	243	203	171	137	108	87	64	34

Figure 1. Overall survival and recurrence-free proportion between squamous cell carcinoma and adenocarcinoma in all patients. (A) Overall survival and (B) recurrence-free proportion curves of squamous cell carcinoma and adenocarcinoma in all patients. AD, adenocarcinoma; SQ, squamous cell carcinoma.



No. at risk

	0	12	24	36	48	60	72	84	96	108	120
AD	1031	992	952	895	759	632	520	413	327	259	205
SQ	297	281	256	223	182	149	120	95	79	63	36

No. at risk

	0	12	24	36	48	60	72	84	96	108	120
AD	942	887	828	761	641	543	444	354	278	214	166
SQ	238	219	197	176	146	122	100	79	64	47	24

Figure 2. Overall survival and recurrence-free proportion between squamous cell carcinoma and adenocarcinoma in pN0 patients. (A) Overall survival and (B) recurrence-free proportion curves of squamous cell carcinoma and adenocarcinoma in pN0 patients.

Table 2. Multivariate analyses of overall survival

Patient characteristics	All patients		pN0	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (>65/≤65)	1.641 (1.414–1.905)	<0.001	2.152 (1.728–2.680)	<0.001
Sex (men/women)	1.023 (0.805–1.300)	0.853	1.054 (0.768–1.446)	0.744
Smoking history (ever smoker/never smoker)	1.429 (1.104–1.848)	0.007	1.661 (1.166–2.365)	0.005
Pathological T stage (T2 + 3 + 4/T1)	1.988 (1.653–2.391)	<0.001	2.267 (1.772–2.900)	<0.001
Pathological N stage (N1 + 2/N0)	2.182 (1.844–2.582)	<0.001	—	—
Pathological differentiation (moderately + poorly/well)	1.185 (0.943–1.490)	0.145	1.180 (0.888–1.567)	0.255
Vascular invasion (present/absent)	1.572 (1.301–1.900)	<0.001	1.811 (1.426–2.301)	<0.001
Lymphatic permeation (present/absent)	1.352 (1.148–1.592)	<0.001	1.375 (1.092–1.731)	0.007
Histologic type (SQ/AD)	0.875 (0.737–1.039)	0.128	1.095 (0.866–1.385)	0.448

HR, hazard ratio for death; CI, confidence interval.

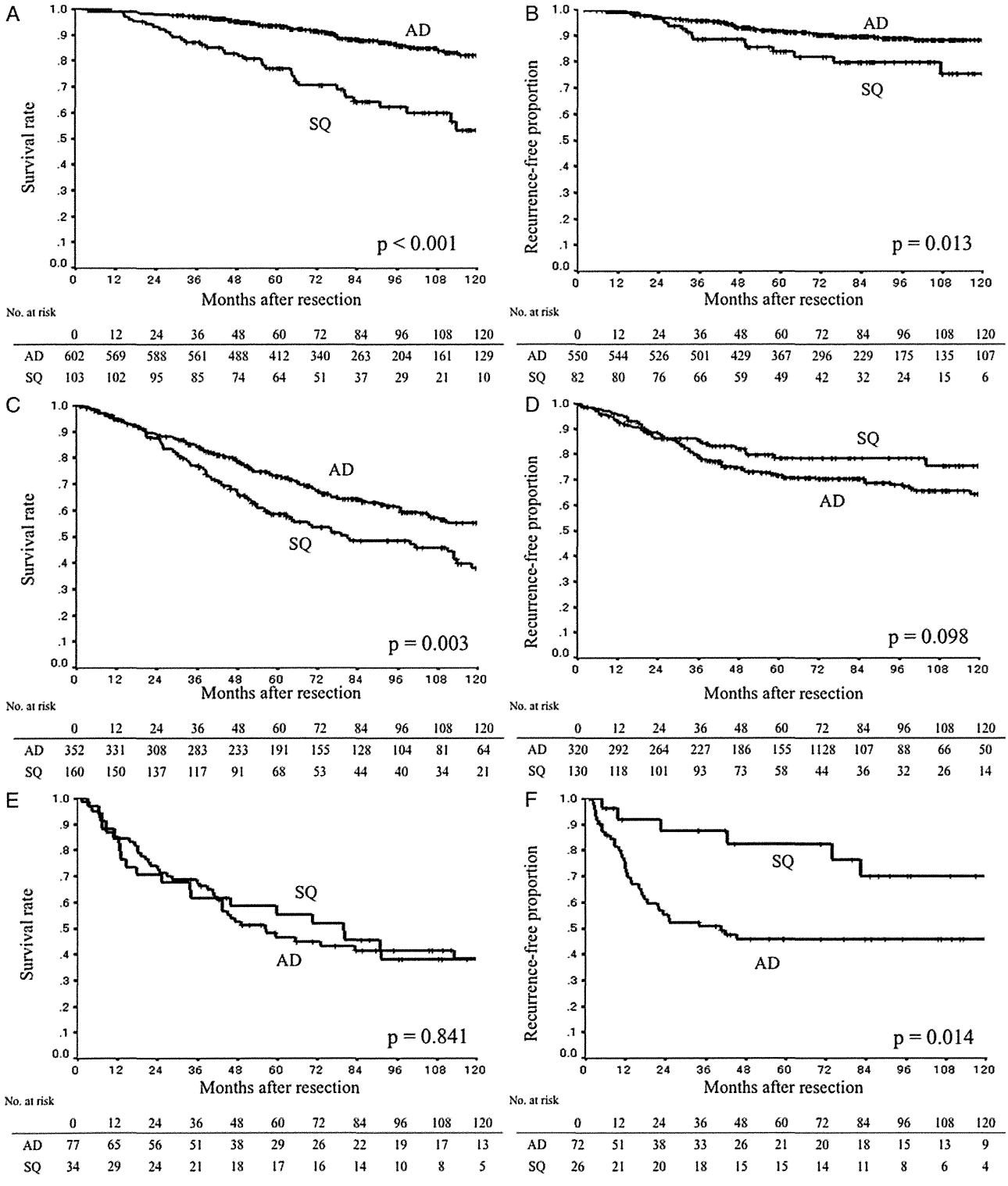


Figure 3. Overall survival and recurrence-free proportion between squamous cell carcinoma and adenocarcinoma in pT1N0 patients, in pT2N0 patients and in pT3/4N0 patients. (A) Overall survival and (B) recurrence-free proportion curves in pT1N0 patients. (C) Overall survival and (D) recurrence-free proportion curves in pT2N0 patients. (E) Overall survival and (F) recurrence-free proportion curves in pT3/4N0 patients.

DISCUSSION

We set out to determine the relationship of histologic analysis to survival and tumor aggressiveness in pulmonary squamous cell carcinoma and adenocarcinoma. Patients with pulmonary squamous cell carcinoma are known today to have a poorer prognosis than those with adenocarcinoma after surgical resection (4). Squamous cell carcinoma mostly develops in smokers

Table 3. Causes of death

Characteristics	Total	AD	SQ	*P* value
Lung cancer-specific deaths	479	355 (79)	124 (66)	
Deaths from other causes	159	96 (21)	63 (34)	0.001[a]
Total	638	451	187	

[a]Fisher's exact test; numbers in parentheses are percentages.

in whom life-threatening co-morbidities also often develop, including atherosclerotic cardiovascular events, chronic obstructive pulmonary disease and cerebral infarction (10), which may explain the poorer survival of patients with squamous cell carcinoma compared with those with adenocarcinoma. In the present study, there were significantly more patients who died of causes other than lung cancer in squamous cell carcinoma than in adenocarcinoma. However, it remains unclear whether biological aggressiveness differs between squamous cell carcinoma and adenocarcinoma of the lung.

In the present study, there were significantly more patients with squamous cell carcinoma than those with adenocarcinoma among smokers. In patients with squamous cell carcinoma, there were significantly more T2–4 patients and patients with lymph node metastases or vascular invasion. There were statistically significant differences in overall survival between adenocarcinoma and squamous cell carcinoma patients in all patients and in pN0 patient cohorts. However, when we analyzed recurrence-free proportion to exclude any possible influence of non-cancer-specific death and to compare biological aggressiveness between squamous cell carcinoma and adenocarcinoma, we found that there were no statistically significant differences in any patients or in pN0 patients. There were significantly more deaths from causes other than lung cancer in patients with squamous cell carcinoma than in those with adenocarcinoma.

These results indicate that although squamous cell carcinoma developed more frequently among smokers and was more advanced and invasive when resected compared with adenocarcinoma, its biological aggressiveness was not significantly different from adenocarcinoma. The poorer overall survival in patients with squamous cell carcinoma than those with adenocarcinoma seemed to be attributable to advanced and invasive cancer status on resection and smoking/age-related co-morbidities.

We also analyzed overall survival and recurrence-free proportion in each pathological T stage in pN0 patients to compare biological aggressiveness between squamous cell carcinoma and adenocarcinoma in each T stage. In pT1N0 patients, the patients with squamous cell carcinoma had significantly poorer survival and recurrence-free proportion than patients with adenocarcinoma. This may partly be explained by the fact that a considerable number of pT1 adenocarcinoma patients had non- or minimally invasive disease, such as bronchioloalveolar carcinoma, thereby resulting in better outcome compared with squamous cell carcinoma patients. In pT3/4 patients, on the other hand, there was no significant difference in overall survival between the two histologic types, but adenocarcinoma patients had significantly poorer recurrence-free proportion than squamous cell carcinoma patients. The poorer recurrence-free proportion of adenocarcinoma patients compared with squamous cell carcinoma patients may be interpreted that adenocarcinoma of this T status has biologically more aggressive nature than squamous cell carcinoma. However, probably because squamous cell carcinoma patients had more smoking/age-related co-morbidities and were more often killed by them than adenocarcinoma patients, there was no significant difference in overall survival.

In the 7th Edition of the TNM Classification for Lung and Pleural Tumours, stage groupings are based on overall survival (8). According to the strategy in this study, and based on our findings, squamous cell carcinoma and adenocarcinoma of the lung need to be staged differently. It is important to note, however, that the difference is likely to be due to advanced and invasive cancer status on resection and smoking/age-related co-morbidities of patients with squamous cell carcinoma, but not to biological tumor aggressiveness of squamous cell carcinoma.

There were several limitations in this study. Although the total number of consecutive patients was large (1856), the study was performed in a single institution using a homogenous Japanese ethnic group. Therefore, a multicenter trial based on various ethnic groups may be valuable. There were more well-differentiated tumors in adenocarcinomas than in squamous cell carcinomas in the present cohort. This may be another reason for the observed better prognosis in adenocarcinoma patients than in squamous cell carcinoma patients.

In conclusion, this study showed that there were no differences in the development of recurrence between squamous cell carcinoma and adenocarcinoma of the lung, but considerable differences in overall survival were observed between the two histologic types in all patients and pN0 patients. According to the stage grouping strategy of the TNM Classification for Lung and Pleural Tumours, these two histologic types need to be staged differently. This survival difference, however, may reflect the difference in patient background rather than the difference in biological aggressiveness between the two histologic types.

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Conflict of interest statement

None declared.

References

1. Ichinose Y, Yano T, Asoh H, Yokoyama H, Yoshino I, Katsuda Y. Prognostic factors obtained by a pathologic examination in completely resected non-small-cell lung cancer. An analysis in each pathologic stage. *J Thorac Cardiovasc Surg* 1995;110:601–5.
2. Suzuki K, Nagai K, Yoshida J, Nishimura M, Takahashi K, Yokose T, et al. Conventional clinicopathologic prognostic factors in surgically resected nonsmall cell lung carcinoma. A comparison of prognostic factors for each pathologic TNM stage based on multivariate analyses. *Cancer* 1999;86:1976–84.
3. Noguchi M, Morikawa A, Kawasaki M, Matsuno Y, Yamada T, Hirohashi S, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer* 1995;75:2844–52.
4. Asamura H, Goya T, Koshiishi Y, Sohara Y, Eguchi K, Mori M, et al. A Japanese Lung Cancer Registry study: prognosis of 13,010 resected lung cancers. *J Thorac Oncol* 2008;3:46–52.
5. Rice TW, Blackstone EH, Rusch VW. A cancer staging primer: esophagus and esophagogastric junction. *J Thorac Cardiovasc Surg* 2010;139:527–9.
6. Rice TW, Blackstone EH, Rusch VW. 7th edition of the AJCC Cancer Staging Manual: esophagus and esophagogastric junction. *Ann Surg Oncol* 2010;17:1721–4.
7. Rice TW, Rusch VW, Apperson-Hansen C, Allen MS, Chen LQ, Hunter JG, et al. Worldwide esophageal cancer collaboration. *Dis Esophagus* 2009;22:1–8.
8. Goldstraw P, editor. *Staging Manual in Thoracic Oncology*. Denver: IASLC 2009.
9. Travis WD, Brambilla E, Müller-Hermelink H, Harris C, editors. *Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press 2004.
10. Doll R, Peto R, Boreham J, Sutherland I. Mortality in relation to smoking: 50 years' observations on male British doctors. *BMJ* 2004;328:1519.

Prognostic Factors After Pulmonary Metastasectomy for Colorectal Cancer and Rationale for Determining Surgical Indications

A Retrospective Analysis

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Objective: We aimed to identify prognostic factors after pulmonary metastasectomy for colorectal cancer and propose the clinical application of them. Furthermore, we endeavored to provide a rationale for pulmonary metastasesectomy.

Background: Several prognostic factors have been proposed, but clinical application of them remains unclear. Moreover, there is no theoretical evidence that pulmonary metastasectomy is indicated for colorectal cancer.

Methods: We retrospectively analyzed 1030 patients who underwent pulmonary metastasectomy for colorectal cancer from 1990 to 2008. Prognostic factors were identified and the relationship of recurrent sites after pulmonary resection to pulmonary tumor size was assessed.

Results: Overall 5-year survival was 53.5%. Median survival time was 69.5 months. Univariate analysis showed tumor number ($P < 0.0001$), tumor size ($P < 0.0001$), prethoracotomy serum carcinoembryonic antigen (CEA) level ($P < 0.0001$), lymph node involvement ($P < 0.0001$), and completeness of resection ($P < 0.0001$) to significantly influence survival. In multivariate analysis, all remained independent predictors of outcome. In patients whose recurrent sites extended downstream from the lung via hematogenous colorectal cancer spread, pulmonary tumor size was significantly larger than in those with recurrent sites confined to the lung and regions upstream from the lung.

Conclusions: We should utilize these prognostic factors to detect patients who might benefit from surgery. Therefore, we should periodically follow up advanced colorectal cancer patients by chest computed tomography to detect small pulmonary metastases before serum CEA elevation. Metastases to the lung or organs upstream from the lung are regarded as semi-local for colorectal cancer. This concept provides a rationale for validating surgical indications for pulmonary metastases from colorectal cancer.

Keywords: colorectal cancer, prognostic factor, pulmonary metastasectomy, pulmonary metastasis, surgical indication

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Colorectal cancer, one of the most common cancers worldwide, frequently metastasizes to the liver and lungs. Recently, the development of chemotherapy for metastatic colorectal cancer has been reported,[1,2] but surgical resection is still believed to be the optimal treatment, if possible, for lung metastasis.[3–7]

Numerous reports have focused on pulmonary metastasectomy for colorectal cancer and several prognostic factors have been proposed. In most cases, however, the number of patients is not large (range: 50–150) and results vary among studies. In addition, clinical application of these prognostic factors has not yet been defined. Moreover, even the essential question of why a local therapy is potentially beneficial for a patient with hematogenous metastatic disease remains unknown.

Herein, we aimed to (1) identify prognostic factors after pulmonary metastasectomy for colorectal cancer and (2) propose the clinical application of any such factors identified, and furthermore, (3) we endeavored to provide a rationale for determining whether surgical intervention is indicated for colorectal pulmonary metastases.

PATIENTS AND METHODS

Patients

The 26-institution Metastatic Lung Tumor Study Group of Japan was established in 1984, and has collected various clinicopathological data from patients undergoing pulmonary metastasectomy with curative intent for malignant tumors. In this registry, we retrospectively reviewed the 1223 patients with colorectal cancer whose pulmonary resections were performed between January 1990 and March 2008 (Table 1). We especially focused on the number of pulmonary metastases, maximum pulmonary tumor size, prethoracotomy serum carcinoembryonic antigen (CEA) level, hilar or mediastinal lymph node involvement, completeness of pulmonary resection, and history of hepatic metastasis. Patients with missing data for these items were excluded. Thus, we ultimately analyzed 1030 patients. This study was approved by the Institutional Review Board of Keio University.

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TABLE 1. Demographic and Clinical Characteristics of All 1223 Patients

	Study Group (n = 1223)	n (%)
Age, years		
Median (IQR)	64 (57–70)	
Range	26–94	
≤49		106 (8.7)
50–59		284 (23.2)
60–69		452 (37.0)
70–79		307 (25.1)
80+		41 (3.4)
Missing		33 (2.7)
Sex		
Male		722 (59.0)
Female		497 (40.6)
Missing		4 (0.3)
Number of tumors		
Median (IQR)	1 (1–2)	
Range	1–23	
1		717 (58.6)
2		256 (20.9)
3		113 (9.2)
≥4		126 (10.3)
Missing		11 (0.9)
Maximum tumor size, cm		
Median (IQR)	2.0 (1.5–3.2)	
Range	0.1–18.0	
0–1.0		182 (14.9)
>1.0–2.0		423 (34.6)
>2.0–3.0		280 (22.9)
>3.0–5.0		227 (18.6)
>5.0		79 (6.5)
Missing		32 (2.6)
CEA level		
Normal		638 (52.2)
High		438 (35.8)
Not measured		4 (0.3)
Missing		143 (11.7)
Nodal involvement (pathological)		
None		578 (47.3)
Pulmonary/hilar		52 (4.3)
Mediastinal		51 (4.2)
Not resected		529 (43.3)
Missing		13 (1.1)
Completeness of resection		
R0		1117 (91.3)
R1, R2		56 (4.6)
Missing		50 (4.1)
History of hepatic metastasis		
Absent		992 (81.1)
Present		230 (18.8)
Missing		1 (0.1)

Data are median (IQR) or number (%).
IQR indicates interquatile range.

Statistical Analysis

Overall survival was calculated from the date of first pulmonary resection to the date of last follow-up or death. To identify prognostic factors after pulmonary metastasectomy, the probability of survival was estimated by the Kaplan-Meier method and compared among the levels in categorical variables using the log-rank test. Overall survival rates at 5 years and median survival time were calculated. To eliminate the arbitrariness of setting a threshold, the effect of continuous variables on survival was also evaluated with a Cox proportional hazards model. In multivariate analysis for both categorical and continuous variables, a Cox proportional hazards model with the stepwise selection method was used. The correlation between recurrent sites after pulmonary resection and maximum pulmonary tumour size was examined using the Mann-Whitney U test in a case-control analysis and the χ^2 test in a retrospective cohort analysis. A significant difference was defined as a P-value less than 0.01. Statistical analyses were conducted with StatView software (version 5.0).

RESULTS

Patients' characteristics are presented in Table 1. Extensive review of 1223 patients who underwent pulmonary metastasectomy for colorectal cancer revealed that 193 patients (15.8%) had some missing data. We excluded these patients and analyzed the remaining 1030 patients.

Median postoperative follow-up of 624 survivors (60.6%) was 40.3 months (interquartile range : 20.4–71.1). Recurrence after pulmonary resection was noted in 505 patients (49.0%). The estimated overall survival rates at 5 and 10 years were 53.5% and 38.4%, respectively. The median survival time was 69.5 months. Patients were usually followed up by chest X-ray or computed tomography, and additional imaging studies were performed at the discretion of the treating physician. Of 406 deceased cases, the cause of death was obtained from 363. The breakdown was 326 (89.8%) for recurrent colorectal cancer, 7 for other malignancies, and 30 for other diseases.

To identify prognostic factors, we selected eight categorical variables: age (<70 vs ≥70), sex (male vs female), number of pulmonary metastases (solitary vs multiple), maximum tumor size (≤2 cm vs >2 cm), prethoracotomy serum CEA level (normal vs high), nodal involvement (negative vs positive), completeness of pulmonary resection (R0 vs R1, R2), and history of hepatic metastasis (absent vs present). Systematic lymph node dissection was not always performed, and we did not have any guidelines for lymph node sampling. Nodal involvement was assessed pathologically and patients whose lymph node was not resected were excluded. We also examined the use of chemotherapy for pulmonary metastases in the perioperative period. Univariate analysis showed the number of metastases ($P < 0.0001$), maximum tumor size ($P < 0.0001$), prethoracotomy serum CEA level ($P < 0.0001$), nodal involvement ($P < 0.0001$), and completeness of pulmonary resection ($P < 0.0001$) to significantly influence survival (Table 2). Age, the number of pulmonary metastases, and maximum tumor size were also evaluated by a Cox univariate analysis as continuous variables (Table 3). The results were compatible with those of the log-rank analysis.

Table 4 summarizes the multivariate analysis results. All potential prognostic factors described in Tables 2 and 3 except for the use of chemotherapy were entered into a Cox proportional hazards model with stepwise selection. Number of pulmonary metastases (as a continuous variable, $P < 0.0001$), maximum tumor size (as a categorical variable, $P < 0.0001$), prethoracotomy serum CEA level ($P = 0.0008$), nodal involvement ($P = 0.0053$), and completeness of pulmonary resection ($P < 0.0001$) were selected as independent prognostic factors.

There was a significant correlation between recurrent sites after pulmonary resection and maximum pulmonary tumor size. The details of recurrence are shown in Table 5. We divided the recurrent sites after pulmonary resection into 2 areas based on the hematogenous metastatic pathway from colorectal cancer. One was the lung or regions upstream from the lung (ie, locoregional sites or the liver), and the other was downstream from the lung (the brain, bone, and so on). A case-control analysis revealed that in patients whose recurrent sites were downstream, the pulmonary tumor size was significantly larger than in patients whose recurrence were confined to the lung or upstream sites ($P < 0.0001$; Fig. 1). Similarly, a retrospective cohort analysis showed that in patients whose maximum tumor size was

TABLE 2. Univariate Analyses of Potential Survival Prognostic Factors by Using Kaplan-Meier and Log-Rank Tests

	n (%)	5-Year Survival, %	MST, Months	*P*
Age, years				
Median (IQR) 64 (58–71)				
Range 26–94				
<70	725 (70.4)	52.3	63.9	0.3823
≥70	305 (29.6)	56.5	75.9	
Sex				
Male	608 (59.0)	51.1	62.4	0.0437
Female	422 (41.0)	56.9	87.6	
Number of tumors				
Median (IQR) 1 (1–2)				
Range 1–21				
Solitary	597 (58.0)	61.5	99.9	<0.0001*
Multiple	433 (42.0)	42.0	48.1	
Maximum tumor size, cm				
Median (IQR) 2.1 (1.5–3.2)				
Range 0.1–18.0				
≤2cm	505 (49.0)	59.9	100.2	<0.0001*
>2cm	525 (51.0)	48.1	55.5	
CEA level				
Normal	615 (59.7)	60.4	93.0	<0.0001*
High	411 (39.9)	43.4	49.9	
Nodal involvement (pathological)				
Negative	506 (49.1)	59.4	93.1	<0.0001*
Positive	97 (9.4)	37.3	39.0	
Completeness of resection				
R0	979 (95.0)	55.8	75.9	<0.0001*
R1, R2	51 (5.0)	8.3	24.9	
History of hepatic metastasis				
Absent	849 (82.4)	55.3	75.7	0.0156
Present	181 (17.6)	44.6	49.4	
Chemotherapy (neoadjuvant or adjuvant)				
Yes	241 (53.0)	45.2	55.4	0.0152
No	214 (47.0)	60.5	81.9	
Missing	575			

*A significant difference was defined as a *P*-value less than 0.01. Number of tumors, maximum tumor size, prethoracotomy serum CEA level, nodal involvement, and completeness of pulmonary resection significantly influenced survival.

IQR indicates interquatile range; MST, median survival time.

TABLE 3. Univariate Analyses of Continuous Variables by Using Cox Proportional Hazards Model

	Hazard Ratio (95% Confidence Interval)	*P*
Age, year	0.998 (0.988–1.008)	0.7121
Number of tumors	1.215 (1.165–1.268)	<0.0001*
Maximum tumor size, cm	1.123 (1.069–1.179)	<0.0001*

*A significant difference was defined as a *P*-value less than 0.01. Number of tumors and maximum tumor size significantly influenced survival.

TABLE 4. Multivariate Cox Regression Analysis with Stepwise Selection Method

	Hazard Ratio	95% Confidence Interval	*P*
Number of tumors (continuous factor)	1.2	1.148–1.253	<0.0001*
Maximum tumor size, cm			
≤2cm	1		
>2cm	1.577	1.262–1.971	<0.0001*
CEA level			
Normal	1		
High	1.416	1.156–1.734	0.0008*
Nodal involvement (pathological)			
Negative	1		
Positive	1.545	1.138–2.098	0.0053*
Not resected	1.632	1.298–2.051	<0.0001*
Completeness of resection			
R0	1		
R1, R2	2.884	2.035–4.087	<0.0001*

*A significant difference was defined as a *P*-value less than 0.01.

larger than 2 cm at pulmonary metastasectomy, recurrence was more frequent downstream from the lung ($P < 0.0001$; Table 6).

DISCUSSION

The advantage of this study was that the sample size was definitely larger than any other previous studies about colorectal pulmonary metastasectomy. According to a recent systematic review, the

TABLE 5. Details of Recurrence After Pulmonary Resection

Site of Recurrence	Therapy	n	After First Pulmonary Resection 5-Year Survival, %	MST, Months
Lung		318	38.1	47.5
	Surgery	121	56.7	93.0
	Chemotherapy	56	35.2	39.1
	Radiotherapy	11	35.4	37.4
	BSC	15		
	Missing	128		
Liver		118	24.3	31.8
	Surgery	17	38.5	58.0
	Chemotherapy	28	37.4	34.9
	Radiotherapy	6		
	BSC	2		
	Missing	71		
Colorectum*		63	16.1	26.8
	Surgery	10	–	19.3
	Chemotherapy	13	–	26.8
	Radiotherapy	6		
	Missing	28		
Brain		66	10.6	20.7
Bone		46	23.8	23.5
Hilar/mediastinal lymph node		37	15	34.7
Other		33	16.6	38.6
Adrenal gland		8		
Cervical lymph node		7		
Chest wall		5		
Pleural dissemination		4		
Spleen		2		
Pancreas		2		
Kidney		2		
Skin		1		
Larynx		1		
Axillar lymph node		1		

In total, we identified 681 lesion sites of recurrence in 505 patients.
*Including regional lymph node, peritoneal dissemination, and so on.
BSC indicates best supportive care; MST, median survival time.

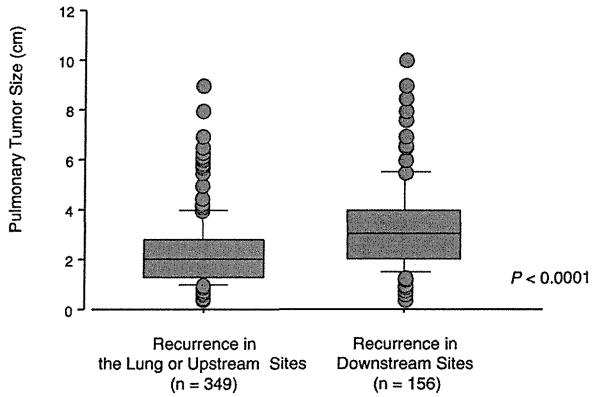

FIGURE 1. Correlation between recurrent sites after pulmonary resection and maximum pulmonary tumor size in a case-control analysis.

TABLE 6. Correlation Between Pulmonary Tumor Size and Recurrent Sites in a Retrospective Cohort Analysis

Pulmonary Tumor Size	Recurrence in the Lung or Upstream Sites	Recurrence in Downstream Sites	Total
≤2cm	197 (39.0)	43 (8.5)	240 (47.5)
>2cm	152 (30.1)	113 (22.4)	265 (52.5)
Total	349 (69.1)	156 (30.9)	505 (100.0)
			$P < 0.0001$

Data are number (%). Statistical analysis was performed using the χ^2 test.

number of patients in most studies ranged from 50 to 150, and the maximum was 378.[3] Thus, the prognostic factors that we pointed out are more precise.

In the univariate analysis, tumor number, tumor size, CEA level, nodal involvement, and completeness of resection significantly influenced survival after pulmonary metastasectomy. The use of chemotherapy was slightly associated with poor prognosis. We believe this may be the result of selection bias. In the multivariate analysis, all of these variables remained independent predictors of outcome. It is noteworthy that maximum tumor size of pulmonary metastases was identified as a prognostic factor. Interestingly, contrary to the widely held clinical impression, only a few studies have shown larger tumor size to correlate with a poor prognosis.[7–9] In fact, among patients with a large and solitary pulmonary metastasis, some have good outcomes. The large sample size of this study eliminated the effect of these anomalous cases on survival.

It is very important to apply these prognostic factors to clinical practice. Almost all previous studies have advocated that prognostic factors be considered in determining surgical indications.[5,10,11] This means that patients with certain poor prognostic factors should not be considered for surgery. However, such exclusion only improves surgical outcomes and does not contribute to the survival of colorectal cancer patients. Furthermore, operative indications should be intrinsically decided by comparing outcomes among patients with the same condition who did or did not undergo pulmonary resection. Thus, we must wait for the results from randomized trials.[12]

Herein, we propose that prognostic factors be utilized not to select but rather to detect patients who might benefit from surgery. From this viewpoint, we should periodically follow up advanced colorectal cancer patients by chest computed tomography and try to detect small pulmonary metastases before serum CEA elevation.

The criteria for resection of metastatic pulmonary tumors were first described by Thomford in 1965 and have been developed into the National Comprehensive Cancer Network guidelines.[13,14] However, the reasons for local therapy possibly being beneficial for hematogenous metastatic disease remain unclear.[8] To provide a rationale for pulmonary metastasectomy for colorectal cancer, we investigated the relationship between maximum pulmonary tumor size and recurrent sites after pulmonary resection.

When considering the hematogenous metastatic pathway from colorectal cancer, tumor cells reach the lung through the liver via the portal vein or directly via the inferior vena cava. In either case, cancer cells that migrate into the blood necessarily arrive at the lung and then spread throughout the body. If cancer cells with metastatic ability pass through the lung and circulate around the body, pulmonary metastasis and metastases to other organs that are located downstream from the lung will be independent. In this case, pulmonary metastasis is one aspect of systemic disease and downstream organ metastasis will not correlate with pulmonary tumor size. However, if cancer cells with metastatic ability are almost certainly trapped within the lung, downstream organ metastasis will only occur after a pulmonary metastasis has grown to some extent and destroyed pulmonary defense mechanisms. In this case, despite distant metastasis from the primary colorectal lesion, metastases to the lungs or other upstream organs can be regarded as semi-local disease.

As a result, pulmonary tumor size tends to be larger in patients whose recurrences extend to sites downstream from the lung (Fig. 1). In other words, our findings suggest that in most hematogenous metastases of colorectal cancer, the lung functions as a filter organ. This is one potential explanation for the pattern of metastases in this disease, and we do not deny that colorectal cancer may have a particular affinity for the lung through some molecules. The possibility that the lung has a filtering action for cancer cells has been hypothesized as an anatomical and mechanical function or to represent a cascade, based on experimental animal studies or autopsy cases.[15–18] However, this is the first evidence of the filtering function of the lung based on clinical data from cases with pulmonary metastasectomy for colorectal cancer. This hypothesis may explain several previously reported results, for example, a history of hepatic metastases and repeated pulmonary resection does not reduce survival after pulmonary metastasectomy,[3,19,20] whereas distant metastases such as those to the brain, bone, adrenal gland, and so on without pulmonary metastases are very rare.[21]

Recently, chemotherapy for colorectal cancer has advanced remarkably,[1,2] but in fact, surgery is still usually chosen in preference to chemotherapy or radiotherapy for localized colorectal cancer.[14] Thus, if pulmonary metastasis is considered to be a semi-local recurrence, pulmonary metastasectomy would logically be supported as long as all lesions are resectable.

Although we recognized several prognostic factors, their utilization is very limited for patient management. The number of pulmonary metastases has essentially been decided at the time of resection of a primary colorectal cancer, and we have no influence over this. We can only detect small pulmonary metastases and resect them as semi-local disease. For this reason, early detection of pulmonary metastases by periodic chest computed tomography is very important.

Our study has some limitations. First, since this is a retrospective study on surgical cases, patients included in the analysis were highly selected and might not be representative of all patients with pulmonary metastases from colorectal cancer. Second, owing to long-standing multicenter registry, there were many cases with incomplete data, such that 193 patients (15.8%) had to be excluded from the analysis due to missing data. For confirmation, we checked that the same results were obtained in the analysis of all 1223 patients. Furthermore, while we consider the liver to be upstream from the lung, some hepatic metastases might arise secondarily from pulmonary metastases. However, patients with localized hepatic metastases downstream from the lung are exceptional and few in number, such that we do not think this factor would have affected our findings.

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REFERENCES

1. Bendell J. Optimum chemotherapy for metastatic colorectal cancer. *Lancet.* 2006;368:2039–2041.
2. Douillard JY, Siena S, Cassidy J, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol.* 2010;28:4697–4705.
3. Pfannschmidt J, Hoffmann H, Dienemann H. Reported outcome factors for pulmonary resection in metastatic colorectal cancer. *J Thorac Oncol.* 2010;5:S172–S178.
4. Pfannschmidt J, Muley T, Hoffmann H, et al. Prognostic factors and survival after complete resection of pulmonary metastases from colorectal carcinoma: experiences in 167 patients. *J Thorac Cardiovasc Surg.* 2003;126:732–739.
5. Onaitis MW, Petersen RP, Haney JC, et al. Prognostic factors for recurrence after pulmonary resection of colorectal cancer metastases. *Ann Thorac Surg.* 2009;87:1684–1688.
6. Borasio P, Gisabella M, Billé A, et al. Role of surgical resection in colorectal lung metastases: analysis of 137 patients. *Int J Colorectal Dis.* 2011;26:183–190.
7. Iizasa T, Suzuki M, Yoshida S, et al. Prediction of prognosis and surgical indications for pulmonary metastasectomy from colorectal cancer. *Ann Thorac Surg.* 2006;82:254–260.
8. Pfannschmidt J, Dienemann H, Hoffmann H. Surgical resection of pulmonary metastases from colorectal cancer: a systematic review of published series. *Ann Thorac Surg.* 2007;84:324–338.
9. Vogelsang H, Haas S, Hierholzer C, et al. Factors influencing survival after resection of pulmonary metastases from colorectal cancer. *Br J Surg.* 2004;91:1066–1071.
10. Hwang MR, Park JW, Kim DY, et al. Early intrapulmonary recurrence after pulmonary metastasectomy related to colorectal cancer. *Ann Thorac Surg.* 2010;90:398–404.
11. Kanemitsu Y, Kato T, Hirai T, et al. Preoperative probability model for predicting overall survival after resection of pulmonary metastases from colorectal cancer. *Br J Surg.* 2004;91:112–120.
12. Treasure T, Fallowfield L, Lees B. Pulmonary metastasectomy in colorectal cancer: the PulMiCC trial. *J Thorac Oncol.* 2010;5:S203–S206.
13. Thomford NR, Woolner LB, Clagett OT. The surgical treatment of metastatic tumors in the lungs. *J Thorac Cardiovasc Surg.* 1965;49:357–363.

14. NCCN Clinical Practice Guidelines in Oncology Version 2.2012: Colon cancer. Available at: http://www.nccn.org/professionals/physician_gls/pdf/colon.pdf. Accessed December 8, 2011.
15. Ewing J. *Metastasis. Neoplastic Disease.* 3rd ed. Philadelphia, Pa: WB Saunders Co; 1928.
16. Zeidman I, McCutcheon M, Coman DR. Factors affecting the number of tumor metastases: experiments with a transplantable mouse tumor. *Cancer Res.* 1950;10:357–359.
17. Weiss L. Comments on hematogenous metastatic patterns in humans as revealed by autopsy. *Clin Exp Metastasis.* 1992;10:191–199.
18. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer.* 2002;2:563–572.
19. Headrick JR, Miller DL, Nagorney DM, et al. Surgical treatment of hepatic and pulmonary metastases from colon cancer. *Ann Thorac Surg.* 2001;71:975–979.
20. Chen F, Sakai H, Miyahara R, et al. Repeat resection of pulmonary metastasis is beneficial for patients with colorectal carcinoma. *World J Surg.* 2010;34:2373–2378.
21. Roth ES, Fetzer DT, Barron BJ, et al. Does colon cancer ever metastasize to bone first? A temporal analysis of colorectal cancer progression [published online ahead of print Aug 7]. *BMC Cancer.* 2009. DOI: 10.1186/1471-2407-9-274.

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Accumulation of Activated Invariant Natural Killer T Cells in the Tumor Microenvironment after α-Galactosylceramide-Pulsed Antigen Presenting Cells

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Abstract
Purpose The intravenous administration of α-Galactosylceramide (α-GalCer)-pulsed antigen presenting cells (APCs) is well tolerated and the increased IFN-γ producing cells in the peripheral blood after the treatment appeared to be associated with prolonged survival. An exploratory study protocol was designed with the preoperative administration of α-GalCer-pulsed APCs to clarify the mechanisms of these findings, while especially focusing on the precise tumor site.
Methods Patients with operable advanced lung cancer received an intravenous injection of α-GalCer-pulsed APCs before surgery. The resected lung and tumor infiltrating lymphocytes (TILs) as well as peripheral blood mononuclear cells were collected and the invariant NKT (iNKT) cell-specific immune responses were analyzed.
Results Four patients completed the study protocol. We observed a significant increase in iNKT cell numbers in the TILs and augmented IFN-γ production by the α-GalCer-stimulated TILs.
Conclusion The administration of α-GalCer-pulsed APCs successfully induced the dramatic infiltration and activation of iNKT cells in the tumor microenvironment.

Keywords Invariant NKT cell · antigen presenting cell · immunotherapy · tumor infiltrating lymphocyte · non-small cell lung cancer

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Introduction

Vα24 invariant natural killer T (Vα24 iNKT) cells are a unique innate lymphocyte subpopulation characterized by the expression of a canonical invariant T cell receptor with a specific α-chain gene rearrangement (Vα24-Jα18) and pairing mostly with a Vβ11 β-chain in human. Synthetic glycolipid, α-Galactosylceramide (α-GalCer) is a mouse and human iNKT cell ligand, presented by a monomorphic class I-like antigen presenting molecule CD1d [1–3]. Ligand activated iNKT cells exhibit both direct and indirect potent anti-tumor activity.

Patients with malignant diseases show either a decreased number or functionally impaired Vα24 iNKT cells in human peripheral blood mononuclear cells (PBMCs) [4–9]. Head and neck cancer patients with poor circulating iNKT cell number show significantly worse clinical outcomes, suggesting an important contribution of iNKT cells to anti-tumor responses [10]. In addition, the ability to produce IFN-γ from circulating iNKT cells in cancer patients is preserved even though the absolute number of iNKT cells decreases, and thus, residual iNKT cells might still have a good competence to exert anti-tumor responses. Therefore, the expansion and activation of these cells in vivo may be therapeutically meaningful in patients with severely decreased or functionally deficient Vα24 iNKT cells. Clinical studies of α-GalCer-pulsed antigen presenting cells (APC)s have been conducted to recover a functionally sufficient number of Vα24 iNKT cells [11–14]. A phase I/II study ofα-GalCer-pulsed APCs in patients with advanced or recurrent non-small cell lung cancer (NSCLC) found that the treatment elicits Vα24 iNKT cell-dependent immune responses, which are correlated with prolonged overall survival time [13]. The mechanisms that underlie this positive clinical outcome are still unclear.

The current clinical trial focused on the iNKT cell-specific immunological responses in the tumor microenvironments to investigate further anti-tumor mechanisms of Vα24 iNKT cells after α-GalCer-pulsed APC treatment. Therefore, in this exploratory study, the preoperative administration of α-GalCer-pulsed APCs was performed to clarify the iNKT cell specific immune responses at the tumor site more precisely. The results indicated that α-GalCer-pulsed APCs successfully induced the activation of tumor infiltrating Vα24 iNKT cells in the lung.

Material and Methods

Patient Eligibility Criteria

The study included patients between 20 and 80 years of age, with a diagnosis of clinical stage IIB or IIIA NSCLC that was to be treated surgically. Further inclusion criteria were a performance status of 0, 1, or 2; normal or near normal renal, hepatic and hematopoietic function; and no chemotherapy or radiotherapy received for at least 4 weeks before enrollment. $V\alpha24^{+}V\beta11^{+}$ iNKT cells were detected by flow cytometry in the enrolled patients at a level of >10 cells in 1 ml peripheral blood. The exclusion criteria were a positive response to HIV, hepatitis C virus, or human T-cell lymphotrophic virus antibodies; positive for hepatitis B antigen; the presence of active inflammatory disease or active autoimmune disease; a history of hepatitis; pregnancy or lactation; concurrent corticosteroid therapy and evidence for another active malignant neoplasm. The α-GalCer-pulsed APC non-treatment cases were investigated as a control group to elucidate the effects of α-GalCer-pulsed APC treatment. The inclusion and exclusion criteria of the control group were the same as for the treatment group. The histological type, tumor-node-metastasis classification and the anti-tumor effect of treatment were classified according to the general rules for the clinical and pathologic recording of lung cancer as described by the Japan Lung Cancer Society.

Clinical Protocol and Study Design

The study was carried out in the Department of Chest Surgery, Chiba University Hospital, Japan, according to the standards of Good Clinical Practice for Trials on Medicinal Products in Japan. The protocol was approved by the Institutional Ethics Committee (No. 1972). In addition, this trial underwent ad hoc reviews by the Chiba University Quality Assurance Committee on Cell Therapy.

The study design is illustrated in Fig. 1. Written informed consent was obtained from all of the patients before undergoing a screening evaluation to determine eligibility. Clinical and laboratory assessments were conducted once a week, including of a complete physical examination and standard laboratory values. Any adverse events and changes in laboratory values were graded according to the National Cancer Institute Common Toxicity Criteria version 4.0.

Preparation of APCs from Peripheral Blood

All procedures were carried out according to the Good Manufacturing Practice standards. Eligible patients underwent peripheral blood leukapheresis (COBE Spectra, Gambro BCT,

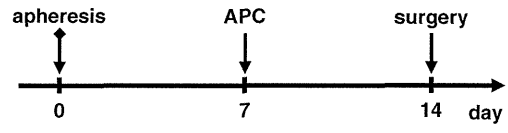

Fig. 1 Study design of α-GalCer-pulsed APC administration. The patients received α-GalCer-pulsed APCs. The timing for both apheresis and α-GalCer-pulsed APC administration are shown. APC, α-GalCer-pulsed APC administration

Inc., Lakewood, CO) and PBMCs were collected and further separated by density gradient centrifugation (OptiPrep, Nycomed Amersham, Oslo, Norway). Thereafter, whole PBMCs were cultured with GM-CSF and IL-2, as previously described [11, 15]. Briefly, PBMCs were washed three times and resuspended in AIM-V (Invitrogen Corp., Carlsbad, CA) with 800 units/ml of human granulocyte macrophage colony-stimulating factor (GeneTech Co., Ltd., China) and 100 Japanese reference units per milliliter of recombinant human IL-2 (Imunace, Shionogi, Osaka, Japan). The cultured cells were pulsed with 100 ng/ml of specific ligand, α-GalCer (KRN7000; Kirin Brewery, Gunma, Japan) on the day before administration. Whole cells were harvested after 7 days of cultivation, washed 3 times and resuspended in 100 ml of 2.5 % albumin in saline. The patients received an intravenous injection of the cultured cells once (Fig. 1). The criteria for α-GalCer-pulsed APC administration included a negative bacterial culture 48 h before APC injection, cell viability >70 % and an endotoxin test 48 h before APC injection with a result <0.7 Ehrlich units/ml. The patients were injected with 1×10^9 cells/m^2/injection of APCs.

Phenotype Evaluation of APCs

The phenotypes of α-GalCer-pulsed APCs were determined using a FACSCalibur flow cytometer (BD biosciences). The monoclonal antibodies (mAb) used were FITC-labeled anti-HLA-DR, CD83, CD14; phycoerythrin-labeled anti-CD86, CD1d; and allophycocyanin-labeled anti-CD11c, CD40 (Becton Dickinson, San Diego, CA). Isotype-matched control mAbs were used as negative controls.

Preparation of Tumor Infiltrating Lymphocytes, Tumor Cells, Normal Lung Mononuclear Cells and Lymph Nodes Mononuclear Cells

Fresh tumor tissue specimens were obtained from the surgical specimens and the tissue was cut into small pieces with scissors. The tissue specimen was placed in a flask with a mixture of 0.1 mg/ml DNase type I, 1 mg/ml collagenase type IV and 0.5 mg/ml hyaluronidase type V (all from Sigma, St. Louis, MO) in RPMI 1640 and stirred at room temperature for 1 h. The resultant cell suspension was washed in HBSS and subjected to two-layered (75 and 100 %) Ficoll-Hypaque discontinuous density gradient centrifugation at 1200 g for 20 min. The cells from the 100 % interface and 75 % interface were used as tumor infiltrating lymphocytes (TILs) and tumor cells, respectively. Normal lung tissue and lymph nodes were excised from the surgical specimen, cut with scissors in RPMI 1640 containing enzymes and passed through a gauze filter. The resultant cell suspension was washed in HBSS and subjected to Ficoll-Hypaque gradient centrifugation. The interface was collected and used as either a normal lung or lymph node.

Immunological Monitoring

PBMC samples were obtained at least twice before APC administration and 1 week after APC injection.

Flow Cytometric Analysis of $V\alpha24^+V\beta11^+$ Inkt Cells in the Peripheral Blood and TILs

The cell concentrations of $V\alpha24^+V\beta11^+$ iNKT cells in PBMCs, TILs and mononuclear cells from normal lung tissue or lymph node were assessed by flow cytometry. Mononuclear cells were three-color stained with FITC-conjugated anti–T-cell receptor (TCR) Vα24 mAb (C15; Immunotech, Marseilles, France), phycoerythrin-conjugated anti-TCR Vβ11 mAb (C21, Immunotech) and APC-conjugated anti-CD3 mAb (UCTH1; BD Bioscience). The stained cells were subjected to flow cytometry and the percentages of $V\alpha24^+V\beta11^+CD3^+$ cells among mononuclear cells were calculated. Thereafter, the number of iNKT cells (counts/ml) was estimated based on the PBMC counts.

Single-Cell Enzyme-Linked Immunospot Assay

PBMCs, TILs and cells from normal lung tissue or lymph nodes were washed 3 times with PBS and then were stored in liquid nitrogen until use. IFN-γ-secreting cells were assayed in 96-well filtration plates (Millipore, Bedford, MA) coated with mouse anti-human IFN-γ (10 μg/ml; Mabtech, Nacka Strand, Sweden). The cells (5×10^5 per well) were incubated for 16 h with or without α-GalCer (100 ng/ml) in 10%FCS containing RPMI. Phorbol 12-myristate 13-acetate (10 μg/ml) plus ionomycin (10 nmol/l) was used as a positive control. After culture, the plates were washed and incubated with biotinylated anti-IFN-γ (1 μg/ml; Mabtech). Spot-forming cells were quantified by microscopy.

Quantitative Real Time PCR of Vα24 Invariant TCR and CD1d Expression

Total RNA was extracted from the tumors, normal lung tissue and lymph nodes using TRIzol Reagent (Sigma Aldrich) and reverse transcribed using Superscript II RT (Invitrogen Life Technologies) and oligo (dT12–18) primers (Invitrogen Life Technologies). The primers specific for the constant region of TCR α chain (Cα) (sense, CGCCTTCAA CAACAGCATTA; antisense, ACCAGCTTGACATCA CAGGA), TCR Vα24 (sense, GCAAAGCTCTCT GCACATCA; antisense, CCAGGGTTGAGCCTCTGTC), CD1d (sense,gtcagggaagtcggaactga; antisense, atcctgaga-catggcacacc) were used with 5 μg of sample cDNA and

amplified with *Taq* polymerase (Promega). Quantitative real-time PCR was performed using real-time Taq-Man technology and an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA). The expression was normalized using the Cα signal for Vα24 and GAPDH for CD1d.

Statistical Methods

Statistical analyses were performed using Student's *t*-test.

Results

Patient Characteristics

A total of 4 patients met the inclusion criteria and were enrolled in the study. The patient characteristics are summarized in Table I. The study included one patient with adenocarcinoma and three patients with squamous cell carcinoma. Two patients were stage IIb and two were stage IIIa primary lung cancer. No patients had received any previous treatments.

In addition, a total of 6 patients who had not received α-GalCer-pulsed APC injection were enrolled as the control group. Fresh tumor tissue, normal lung tissue and lymph nodes were excised from the surgical specimens. The patient characteristics of the control group are also listed in Table I.

Phenotypes of αGalCer-Pulsed APCs

The phenotypes of αGalCer-pulsed APCs prepared for administration were analyzed by flow cytometry. All profiles for each patient are shown in Fig. 2. The percentages of HLA-DR$^+$, CD11c$^+$, CD86$^+$, CD40$^+$, CD83$^+$ and CD1d$^+$ cells were determined by the overtone subtraction test using the population comparison platform in the FlowJo software package. More than 50 % of the cultured cells were HLA-DR$^+$ cells, 10 % to 50 % were CD11c$^+$ cells and 50 % to 80 % were CD86$^+$ cells. Interestingly, the majority of the cultured cells were CD3$^+$ T cells or CD56$^+$CD3$^-$ NK cells, indicating the expression of HLA-DR$^+$, CD11c$^+$ or CD86$^+$ on human T cells or NK cells (data not shown). Some variations were observed in the expression of CD83 (23.9–55.4 %), CD40 (8.5–17.1 %) and CD1d (23.1–69.9 %; Fig. 2).

Adverse Events

No serous (grade >2) toxicity or severe side effects were observed in any patients.

Immunological Monitoring of PBMCs and Resected Specimens

Immunological assays were conducted for all patients. The frequency of peripheral blood Vα24 iNKT cells in all patients was measured by FACS. Figure 3 shows that two patients (cases 002 and 004) showed an increased number of circulating Vα24 iNKT cells after the α-GalCer-pulsed APC administration. No clear relationship was found between the number of circulating Vα24 iNKT cells and the α-GalCer-pulsed APC administration in the remaining two patients (cases 001 and 003).

Immunological assays were also performed for TILs and mononuclear cells (MNC)s from normal lung and lymph node tissues. TILs from all 4 cases in the α-GalCer-pulsed APC administration group contained a high percentage of Vα24 iNKT cells in comparison to the normal lung MNCs (TILs; 1.86 %, 0.32 %, 0.15 % and 0.39 % vs. lung MNCs; 0.031 %, 0.013 %, 0.003 % and 0.01 %, Fig. 4a). The frequency of Vα24 iNKT cells in the TILs in case 001 was 60 times higher than the normal lung MNCs. Though the content of Vα24 iNKT in the normal lung MNCs was extremely low in case 003, the Vα24 iNKT cells were found to have accumulated in the TILs. The average percentage of Vα24 iNKT cells in the TILs was 50 times higher than that

Table I Patient characteristics of α-GalCer-pulsed APC group and control group

Case	Treat[a]	Age/Sex	Histology	c-stage	Operation method
001	APC[b]	75/M	Ad[d]	T2N1M0 (stage IIB)	Lobectomy+LND[g]
002	APC	76/M	Sq[e]	T2N1M0 (stage IIB)	Lobectomy+LND
003	APC	74/M	Sq	T1N2M0 (stage IIIA)	Lobectomy+LND
004	APC	68/M	Sq	T3N1M0 (stage IIIA)	Pneumonectomy+LND
c-01	cont[c]	71 M	Sq	T2N1M0 (stage IIB)	Lobectomy+LND
c-02	cont	55 M	large[f]	T2N1M0 (stage IIB)	Lobectomy+LND
c-03	cont	70 M	Sq	T3N0M0 (stage IIB)	Lobectomy+LND
c-04	cont	72 M	Sq	T3N1M0 (stage IIIA)	Bilobectomy+LND
c-05	cont	56/M	Sq	T2N1M0 (stage IIB)	Lobectomy+LND
c-06	cont	63/M	Ad	T2N2M0 (stage IIIA)	Lobectomy+LND

[a]Treat, Treatment; [b]APC, α-GalCer-pulsed APC administration; [c] cont, control; [d]Ad, Adenocarcinoma; [e] Sq, Squamous cell carcinoma; [f] large, large cell carcinoma; [g] LND, Lymph Node dissection

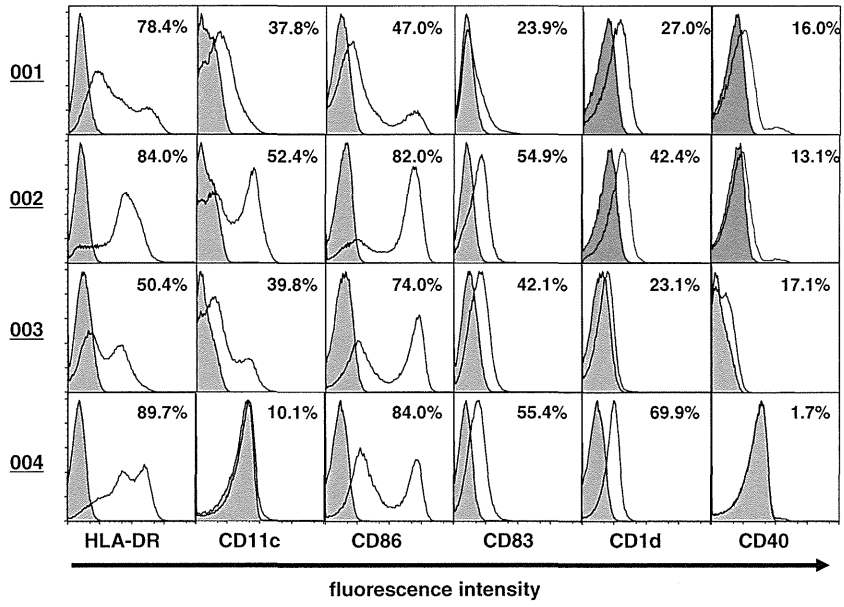

Fig. 2 Flow cytometric analysis of α-GalCer-pulsed APCs. The expression levels of HLA-DR, CD11c, CD86, CD83, CD1d and CD40 were assessed by flow cytometry. Shaded areas: background staining with an iso-type control. Solid lines: staining profiles of the indicated molecules. Values represent the percentages of positive cells

in normal lung MNCs. The Vα24 iNKT cell frequency in the draining lymph nodes of each case was almost the same as that in the normal lung MNCs (Fig. 4a).

The proportion of Vα24 iNKT cells in the control group showed a relatively high percentage of TILs in comparison to the normal lung MNCs (TILs; 0.031 %, 0.058 %, 0.13 %, 0.47 %, 0.18 % and 0.12 % vs. lung MNCs; 0.034 %, 0.011 %, 0.004 %, 0.039 %, 0.014 % and 0.02 %, Fig. 4b). The average percentage of Vα24 iNKT cells in the TILs was only 8 times higher than that in the normal lung MNCs.

Normal lung MNCs in the control group demonstrated a trend toward a higher Vα24 iNKT cell rate in comparison to the treatment group (Fig. 4c). On the other hand, the proportion of Vα24 iNKT cells in TILs tended to increase in the α-GalCer-pulsed APC injected group in comparison to the

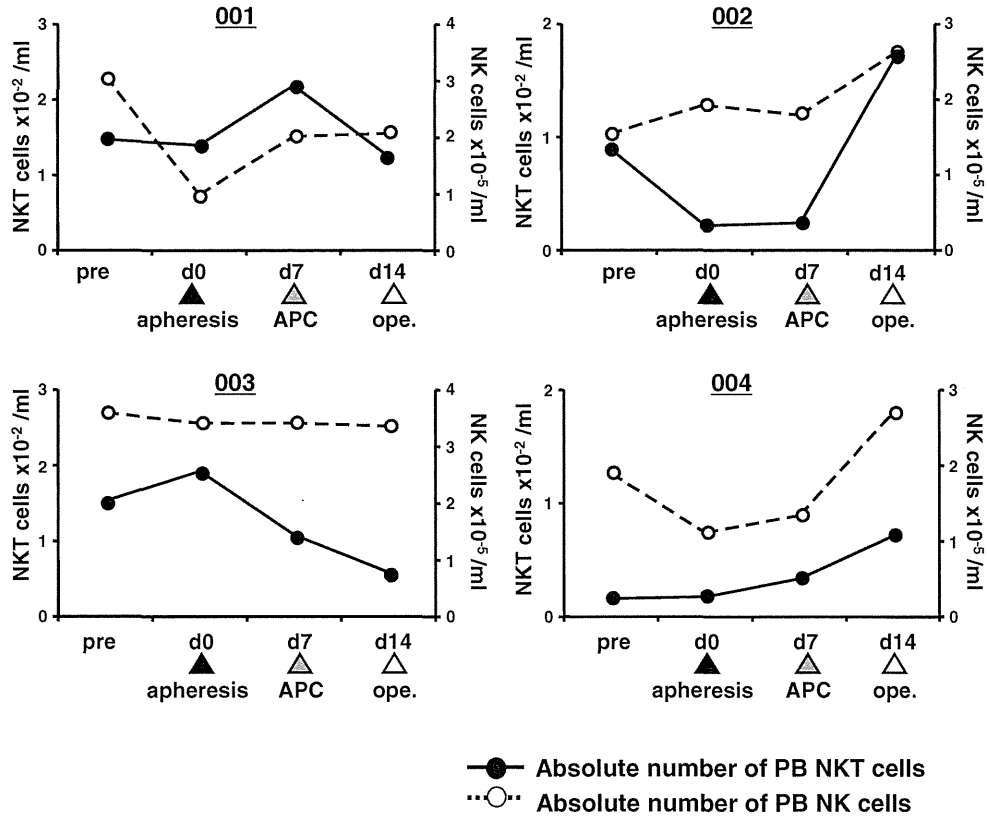

Fig. 3 Immunological monitoring of PBMCs of patients with α-GalCer-pulsed APC administration. The absolute number of peripheral blood iNKT cells ($V\alpha24^+V\beta11^+$ cells) and NK cells ($CD56^+CD3^-$ cells). Flow cytometric analysis and automated full blood counts (Chiba University Hospital) indicated the absolute number of Vα24 iNKT cells and NK cells. APC, α-GalCer-pulsed APC administration; ope., operation