Table 1: Patient characteristics

Clinicopathological background	
Age at recurrence (years)	
Median	74.5
Range	48–87
Sex	
Male	50
Female	26
Performance status at recurrence	
0	23
1	33
2	13
3	6
4	1
Smoking status	
Non-smoker	23
Light smoker	10
Heavy smoker	40
Unknown	3
Smoking index (pack-years)	
Median	31
Range	6.7–90
Histology	
Adenocarcinoma	53
Squamous cell carcinoma	15
Large cell carcinoma	3
Adenosquamous carcinoma	3
Pleomorphic carcinoma	2
Pathological stage	
IA/IB	14/14
IIA/IIB	6/11
IIIA/IIIB	30/1
Epidermal growth factor receptor mutation status	
Mutation	28
Wild-type	39
Unknown	9
Initial treatment	
Surgical approach	
Thoracotomy	43
Video-assisted thoracoscopic surgery	33
Surgical procedures	
Pneumonectomy	0
Lobectomy	60
Segmentectomy	6
Wedge resection	10
Lymph nodes dissection	
Systematic lymph node dissection	56
Mediastinal lymph node sampling	11
Hilar lymph node sampling	10
Induction chemotherapy	
No	71
Yes	5
Adjuvant chemotherapy	
No	41
Yes	35
Platinum-based	19
Uracil-tegafur	16
p-Stage IA	
Yes (%)	4 (28)
p-Stage IB	
Yes (%)	10 (71)
p-Stage II	
Yes (%)	8 (47)
p-Stage III	
Yes (%)	13 (42)
Recurrent disease	
Symptoms at recurrence	
Yes	25
No	51
Disease-free survival (months)	
Median	12.8
Range	0.9–66.1
Recurrent site	
Local (intrathoracic)	42
Distant (extrathoracic)	16
Both	18
No. of recurrent foci	
Single	29
Multiple	47
Therapy for recurrence	
Systemic chemotherapy	64
Palliative care	12
No. of chemotherapeutic regimen	
Median	2
Range	1–7
First-line therapeutic response	
Complete response	4
Partial response	23
Stable disease	8
Progressive disease	21
Not evaluable	8[a]
EGFR-TKIs for recurrence	
Yes	36
No	40

TKIs: tyrosine kinase inhibitors.
[a]$n = 6$, non-measurable recurrent lesion on the RECIST guideline; $n = 2$, early discontinuation of chemotherapy due to adverse events.

chemotherapy as a standard treatment. Thus, few studies have addressed the effect of preoperative induction therapy and adjuvant chemotherapy on the treatment and prognosis after recurrence [15–19].

The objective of this study was to elucidate the effect of preoperative and postoperative chemotherapy, particularly platinum-based chemotherapy (which is the standard regimen for Stage II and IIIA NSCLC), on the post-recurrence prognosis.

MATERIALS AND METHODS

Patients and methods

Of 430 NSCLC patients who underwent curative surgical resection at Kawasaki Medical School Hospital, Kurashiki, Japan, between January 2004 and July 2011, postoperative recurrences had occurred in 109 patients (25%) as of February 2012. Of these 109 patients, complete information on the post-recurrence treatment and prognosis was available for 76 patients (70%), and they were included in this analysis. We retrospectively evaluated the effect of clinical factors, oncological factors, initial treatment (surgical procedures and whether preoperative or postoperative chemotherapy was used and if so, what regimen), treatment for recurrence (regimen and therapeutic efficacy) and other factors of post-recurrence prognosis.

Data including age, sex, smoking status, histopathological diagnosis (histology and pathological stage), surgical procedures, whether preoperative or postoperative chemotherapy was used, and if so, what regimen, epidermal growth factor receptor (EGFR) mutations, Eastern Cooperative Oncology Group performance status (ECOG-PS), symptoms at the time of recurrence, site of recurrence, post-recurrence treatment and response, and survival were gathered from the patients' medical records. Histology was categorized according to the World Health Organization Classification of Tumours, 3rd edition [20], and the TNM classification was assigned according to the International Union Against Cancer staging system [21]. The response to chemotherapy was

evaluated according to the Response Evaluation Criteria in Solid Tumours (RECIST) guidelines (version 1.1) [22].

All patients had been preoperatively staged using contrast-enhanced CT, FDG-PET and magnetic resonance imaging (MRI) of brain. Routine mediastinoscopy or endobronchial ultrasound with transbronchial needle aspiration to detect occult mediastinal lymph node metastases was not performed.

As a general rule in surgical procedures, we performed a standard surgical procedure, that is, lobectomy with systematic or selective mediastinal lymph node dissection. However, in high-risk patients who were unable to tolerate a lobectomy as assessed by cardiopulmonary function, sublobar resection was selected. Induction therapy was indicated for clinical Stage IIIA patients with resectable mediastinal lymph node metastasis. Adjuvant platinum-based doublet chemotherapy was indicated for pathological Stage II and IIIA patients. On the other hand, adjuvant therapy with oral uracil-tegafur was indicated for pathological Stage IB patients.

In general, a follow-up examination was done every 3 months for the first 2 years, and thereafter every 6–12 months. The follow-up procedures included a physical examination, the serum level of carcinoembryonic antigen and chest radiography. Screening examinations by CT or FDG-PET were done every 6 or 12 months for 5 years.

Recurrent NSCLC was diagnosed based on a physical examination and diagnostic imaging such as CT, MRI and FDG-PET. Histopathological confirmation of the diagnosis was made only when clinically required; as a result, recurrence was histologically confirmed in only 10 of 76 patients.

Written informed consent was obtained from each patient, and the study was approved by the institutional review board of Kawasaki Medical School (IRB no. 1181).

Statistical analysis

The Kaplan–Meier method was used to analyse survival. The χ^2 test was used to compare clinicopathological factors between groups. For univariate analyses of the clinicopathological factors, differences were evaluated using the log-rank test, and a multivariate analysis of independent prognostic factors was conducted using Cox's proportional hazards regression model. Differences were considered significant when the P-value was <0.05.

RESULTS

Patient backgrounds

The patient characteristics are given in Table 1. There were 50 men and 26 women. The median age at the time of recurrence was 74.5 (range, 48–87 years). The median disease-free interval from the initial surgery until recurrence was 12.7 months (range 27 days–66.1 months). Regarding the pathological findings, the most common histology was adenocarcinoma in 53 (70%) patients, and the most common pathological Stage was IIIA in 30 cases (39%). Sixty-seven (88%) patients had been screened for EGFR mutation, and EGFR mutations and wild-type EGFR were found in 28 and 39 patients, respectively.

Initial treatment for NSCLC

The initial treatment was surgery alone in 38 patients; multimodality treatment including surgery and preoperative and/or postoperative chemotherapy was performed in the other 38 (50%) patients. Of these, 5 (7%) patients underwent induction chemotherapy or chemoradiotherapy followed by surgery, and 35 (41%) underwent adjuvant chemotherapy, with 2 patients undergoing both preoperative and postoperative chemotherapy. The induction chemotherapy consisted of platinum-based doublet chemotherapy in all 5 patients, with 2 also undergoing concurrent radiotherapy. The adjuvant chemotherapy consisted of platinum-based doublet chemotherapy in 19 patients and oral uracil-tegafur in 16. Twenty-two (29%) patients had been treated with platinum-based doublet chemotherapy either preoperatively or postoperatively.

Recurrence and post-recurrence therapy

Symptoms were evident in 25 (33%) patients at the time of recurrence. The initial recurrence was intrathoracic in 42 patients, extrathoracic in 16 and a combination of intrathoracic and extrathoracic in 18. Forty-seven (62%) patients developed recurrences in multiple organs.

Treatment for recurrence included systemic chemotherapy in 64 (84%) patients and local therapy only in 2 (stereotactic radiosurgery for brain metastases in 2 patients who had only brain recurrences). The remaining 10 patients received only palliative care because of a poor ECOG-PS, an advanced age and so on. Of the 64 patients who underwent chemotherapy, the response to first-line chemotherapy was a complete response (CR) in 4 patients, a partial response (PR) in 23, stable disease (SD) in 8, progressive disease (PD) in 21 and not evaluable (NE) in 8, with a disease control rate for first-line chemotherapy of 55%. EGFR tyrosine kinase inhibitors (TKIs; gefitinib and erlotinib) were used in 36 (47%) patients; none of the patients were treated with a vascular endothelial growth factor inhibitor (bevacizumab).

Post-recurrence survival

The post-recurrence follow-up period ranged from 10 days to 50.1 months (median, 15.0 months), and the 1- and 2-year post-recurrence survival rates were 68.3 and 45.8%, respectively; the median survival time (MST) after recurrence was 17.7 months (Fig. 1). Post-recurrence survival was analysed with respect to clinical factors (age at recurrence, sex, ECOG-PS at recurrence and smoking status), pathological factors (histology, pathological stage and EGFR mutation status), initial treatment (surgical procedure, whether induction chemo/chemoradiotherapy or adjuvant chemotherapy was used and their regimens) and factors related to recurrence (symptoms at recurrence, postoperative recurrence-free period, site and type of recurrence, use of systemic chemotherapy or EGFR-TKIs, response to first-line chemotherapy). Univariate analyses showed that the patient outcome was significantly poorer for patients with an age of ≥80 years at surgery, non-adenocarcinoma, wild-type EGFR, no adjuvant chemotherapy, no preoperative or postoperative chemotherapy, an age of ≥80 years at recurrence, a poor ECOG-PS at recurrence (PS 2–4), a postoperative recurrence-free period <12 months

Table 2: Predictors of post-recurrence survival; baseline and primary lung cancer characteristics

	Post-recurrence survival			*P*-value[a]	
	Patients (%)	MST (months)	2 year (%)	Univariate	Multivariate
Sex					
Male	50 (66)	11.7	37.2	0.2110	
Female	26 (34)	21.6	60.5		
Age at surgery (years)					
≤79	66 (87)	17.7	49.5	0.0128	0.460
≥80	10 (13)	9.2	18.0		
Smoking status					
Smoker	50 (66)	11.7	40.2	0.4185	
Non-smoker	23 (30)	21.6	57.5		
Histology					
Adenocarcinoma	53 (70)	19.8	52.9	0.0060	0.159
Non-adenocarcinoma	23 (30)	8.4	26.5		
Epidermal growth factor receptor mutation status					
Mutation	28 (37)	22.8	64.4	0.0378	0.012
Wild-type	39 (51)	10.5	29.8		
Pathological stage					
IA–IB	28 (37)	13.9	43.2	0.9820	
IIA–IIIB	48 (63)	15.5	47.3		
Initial surgery					
Lobectomy	60 (79)	17.7	51.3	0.1290	
Limited resection	16 (21)	9.8	15.5		
Induction chemotherapy					
Yes	5 (7)	17.7	20.0	0.6514	
No	71 (93)	15.5	48.2		
Adjuvant chemotherapy					
Yes	35 (46)	21.6	64.8	0.0021	0.001
No	41 (54)	9.8	29.0		
Chemotherapeutic regimen					
Paltinum-based	22 (29)	19.0	49.9	0.0082[b]	0.291[b]
Uracil-tegafur	16 (21)	21.6	72.2		
No	38 (50)	9.2	32.3		
Platinum-based chemotherapy					
Yes	22 (29)	13.2	47.0	0.3921	
No	54 (71)	19.0	49.9		

MST: median survival time
[a]Log-rank test for comparison of post-recurrence survival among groups.
[b]*P*-value for Paltinum-regimen and uracil-tegafur vs no perioperative chemotherapy.

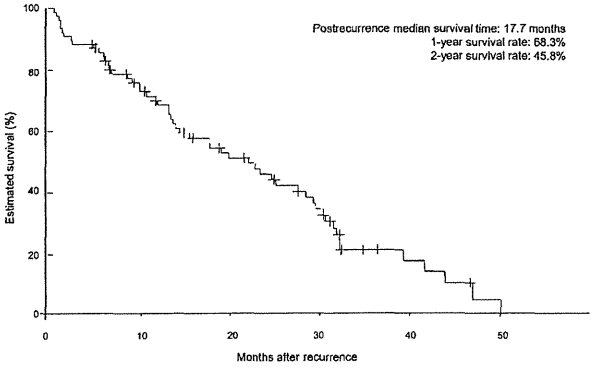

Figure 1: Survival curve illustrating the post-recurrence survival of 76 patients.

and no systemic chemotherapy for recurrence. Multivariate analysis of these factors identified six factors as independent prognostic factors: wild-type EGFR, no adjuvant chemotherapy, an age ≥80 years at recurrence, ECOG-PS 2–4, symptomatic and no chemotherapy for recurrence (Tables 2 and 3, Figs 2 and 3). A multivariate analysis of the 64 patients who underwent systemic chemotherapy showed that the response to first-line chemotherapy for recurrence (P <0.001) and the postoperative recurrence-free period (P = 0.026) were independent prognostic factors (Table 4).

DISCUSSION

In recent years, not only have numerous RCTs established evidence for chemotherapy, but new anti-cancer agents and molecular-targeted drugs have also been introduced, and personalized treatment strategies are now being recommended based on histology and molecular markers [23]. This standardization and personalization of treatment for NSCLC is improving the prognosis of patients with NSCLC. On the other hand, few studies have evaluated treatments for recurrent NSCLC after curative resection. In clinical practice, chemotherapy for recurrence is routinely administered based on the recommended regimen for unresectable advanced NSCLC. However, patients

Table 3: Predictors of post-recurrence survival: recurrent disease characteristics

	Post-recurrence survival			*P*-value[a]	
	Patients (%)	MST (months)	2 year (%)	Univariate	Multivariate
Age at recurrence (years)					
≤79	62 (82)	17.7	49.6	0.0182	0.001
≥80	14 (18)	9.2	20.4		
Performance status at recurrence					
0–1	56 (74)	18.8	58.1	< 0.0001	0.012
2–4	20 (26)	6.3	15.0		
Symptoms					
Yes	25 (33)	6.5	25.1	0.0032	0.003
No	51 (67)	21.5	55.4		
Disease-free interval (months)					
<12	33 (43)	13.3	26.4	0.0084	0.621
≥12	43 (57)	19.8	65.7		
Intra- or extrathoracic recurrence					
Intrathoracic only	42 (55)	15.5	42.8	0.1024	
Extrathoracic only	16 (21)	9.0	23.8		
Both	18 (24)	19.8	56.1		
Initial recurrence					
Single site	29 (38)	18.8	48.0	0.167	
Multiple	47 (62)	13.6	44.7		
Systemic chemotherapy					
Yes	64 (84)	17.7	50.2	<0.0001	0.003
No	12 (16)	1.2	12.5		
EGFR-TKIs (gefitinib and erlotinib)					
Yes	36 (47)	15.5	42.9	0.4205	
No	40 (53)	14.4	48.2		

EGFR-TKI: epidermal growth factor receptor tyrosine kinase inhibitors; MST: median survival time.
[a]Log-rank test for the comparison of post-recurrence survival among groups.

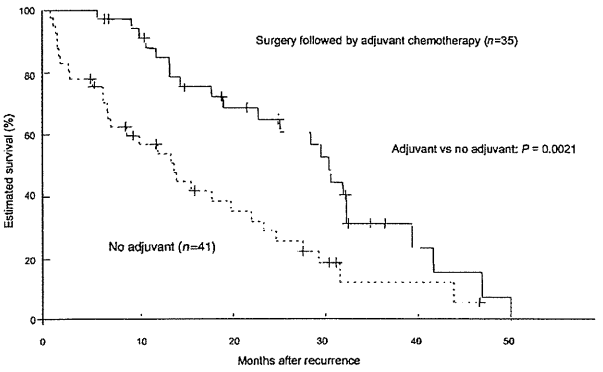

Figure 2: Survival curves illustrating the post-recurrence survival of 35 patients who initially underwent surgery followed by adjuvant chemotherapy and 41 patients who underwent surgery alone. The curves differed significantly for the patients treated with surgery followed by adjuvant chemotherapy vs the patients treated with surgery alone ($P = 0.0021$).

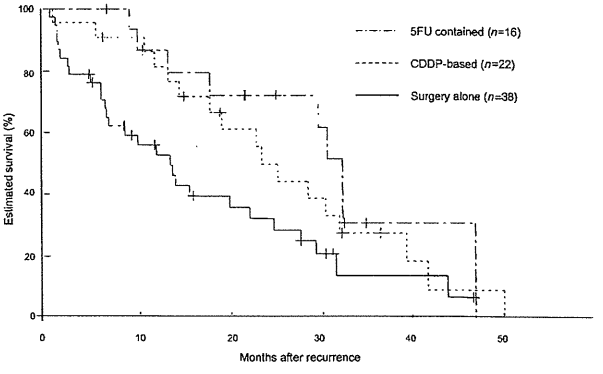

Figure 3: Survival curves illustrating the post-recurrence survival of 22 patients who received platinum-based induction or adjuvant chemotherapy, 16 who received adjuvant chemotherapy with uracil-tegafur and 38 who did not receive pre- or postoperative chemotherapy. The curves differed significantly for patients who received adjuvant chemotherapy with uracil-tegafur vs those who received no perioperative chemotherapy ($P = 0.0089$). However, the curves did not differ significantly for patients who received pre- or postoperative platinum-based chemotherapy vs patients who received no perioperative chemotherapy ($P = 0.1031$) or for patients who received adjuvant chemotherapy with uracil-tegafur compared with those who received pre- or postoperative platinum-based chemotherapy ($P = 0.4231$).

with postoperative recurrent disease can be anticipated to have a poor ECOG-PS and organ function as a result of pulmonary resection or old age. Furthermore, recurrence after pre/postoperative chemotherapy can theoretically be regarded as the proliferation of tumour cells resistant to anti-cancer agents. Therefore, the treatment and prognosis of postoperative recurrence must differ in some aspects from those used for clinical Stage IV NSCLC.

Previous studies of recurrent NSCLC have described a post-recurrence survival rate of 15–20% at 2 years and a post-recurrence MST of 8–13 months [17–19]. In this study, we only investigated patients who had undergone surgery after the mid-2000s, once adjuvant chemotherapy had become established as a standard therapy and found a 2-year post-recurrence

Table 4: Predictors of post-recurrence survival for 64 patients who received systemic chemotherapy

	Univariate *P*-value[a]	Multivariate
Sex		
Male vs female	0.6402	
Age at surgery (years)		
≤79 vs ≥80	0.1273	
Age at recurrence (years)		
≤79 vs ≥80	0.0783	
Performance status at recurrence		
0–1 vs 2–4	0.0010	0.494
Smoking status		
Smoker vs non-smoker	0.7829	
Histology		
Ad vs non-ad	0.2304	
EGFR mutation status		
EGFR mutation vs wild-type	0.1481	
Pathological stage		
IA–IB vs IIA–IIIB	0.4776	
Initial surgery		
Lobectomy vs limited resection	0.4904	
Induction chemotherapy		
Yes vs no	0.8522	
Adjuvant chemotherapy		
Yes vs no	0.0507	0.207
Perioperative chemotherapy		
Yes vs no	0.2780	
Platinum-based chemotherapy		
Yes vs no	0.6443	
Symptoms at recurrence		
Yes vs no	0.0867	
Disease-free interval (months)		
<12 vs ≥12	0.0351	0.026
Recurrent site		
Intra vs extra	0.4756	
Initial recurrence		
Single vs multiple	0.1266	
EGFR-TKIs (gefitinib and erlotinib)		
Yes vs no	0.1566	
Response of initial chemotherapy		
CR/PR/SD vs PD	<0.0001	<0.001

Ad: adenocarcinoma; EGFR: epidermal growth factor receptor; TKI: tyrosine kinase inhibitors; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; NE: not evaluable.
[a]Log-rank test for the comparison of post-recurrence survival among groups.

survival rate of 45.8% and a post-recurrence MST of 17.7 months, an improvement in outcome compared with previous reports. This improvement likely reflects the effects of new anti-cancer agents, including EGFR-TKIs. This change in lung cancer chemotherapy can also be expected to have exerted an effect on prognostic factors for recurrent NSCLC. Factors previously reported as affecting the prognosis for recurrent NSCLC include ECOG-PS, whether symptoms are present at the time of recurrence, the postoperative recurrence-free period and pre- or postoperative chemotherapy. Among these, adjuvant chemotherapy has been regarded as a predictor of a poor prognosis for patients with recurrent NSCLC, although this topic has only been addressed by a few studies [15–19]. In our study, 38 (50%) patients received pre- and/or postoperative chemotherapy, with 22 undergoing platinum-based doublet chemotherapy pre- or postoperatively and 16 undergoing adjuvant chemotherapy with oral uracil-tegafur. Among these patients, those who underwent adjuvant chemotherapy had a significantly better outcome than those who did not. This finding differs from that of previous studies. Two possible reasons for this discrepancy can be considered: first, the therapeutic efficacy of EGFR-TKIs and other new anti-cancer agents, and secondly, the use of adjuvant chemotherapy with oral fluorouracil. In Japan, oral uracil-tegafur (referred to as UFT) is routinely used for adjuvant chemotherapy for completely resected pathological Stage IB NSCLC [24, 25]. In our study, oral uracil-tegafur was administered to 16 of the 76 (21%) patients, and the outcomes of these 16 patients were comparatively good (Fig. 3).

Because of the retrospective nature of this analysis, our study has some limitations. First, 33 patients (30% of the recurrent patients) had to be excluded because their post-recurrence course of treatment or outcome could not be analysed. The exclusion of 30% of the patients may have affected our results. Secondly, this study analysed only a small number of patients, and this number was lower than those included in previous studies. So, the conclusions drawn by this study limited the significance of the results obtained. Thirdly, screening for EGFR mutations was not performed in 9 of the 76 (12%) patients. Finally, the response to first-line chemotherapy was not evaluable in 8 of the 64 patients (13%) who underwent systemic chemotherapy for recurrence. These patients discontinued chemotherapy at an early stage because of chemotherapy-induced adverse events or an aggravated general condition caused by disease progression. To resolve these problems, analyse the prognosis of patients with recurrent NSCLC, and establish treatment strategies, multicenter, large-scale, prospective studies are required.

In this study, we investigated the post-recurrence outcome and prognostic factors for patients who had undergone surgery for NSCLC. The prognostic factors included EGFR mutation, adjuvant chemotherapy, ECOG-PS, age, symptoms at the time of recurrence and the use of systemic chemotherapy for recurrence. Although the post-recurrence outcome was better than in previous studies, the outcome for recurrent NSCLC remains poor. The use of adjuvant chemotherapy for initial treatment in accordance with the treatment guidelines is important. Further investigation and standardization of post-recurrence treatment are required.

Conflict of interest: All authors declare that they have no competing interests.

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Serial 3-Dimensional Volumetric Computed Tomography Evaluation of Lung Cancer Growth Rate in Patients With Chronic Obstructive Pulmonary Disease Findings

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Purpose: The objectives of this study were to compare volume doubling time (VDT) of lung cancer with chronic obstructive pulmonary disease (COPD) findings with that without COPD findings using serial 3-dimensional (3D) volumetric computed tomography (CT) and to investigate the association between VDT and COPD findings.

Methods: This study included 45 patients with surgically diagnosed non–small cell lung cancer with serial preoperative follow-up CT. Volume doubling time of the nodule was calculated by using 3D volumetric computer software.

Results: Volume doubling time of lung cancer with COPD findings (n = 26) tended to be shorter than that without COPD findings (n = 19) (998 ± 2178 vs 2226 ± 6748 days; $P = 0.066$). Among COPD findings, severity and pattern of emphysema were significantly correlated with VDT ($P < 0.001$).

Conclusions: Volume doubling time of lung cancer with COPD findings on 3D volumetric CT tended to be shorter than that of lung cancer without COPD findings. Severe or paraseptal emphysema may be associated with short VDT of lung cancer with COPD findings.

Key Word: non–small cell lung cancer, doubling time, COPD, emphysema, 3-dimensional volumetric CT

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Serial computed tomography (CT) follow-up enables the growth rate of pulmonary nodules to be evaluated.[1] Volume doubling time (VDT) is a method of ascertaining lung cancer growth rate, and many studies have previously reported changes in mean tumor diameter or volume.[2–7] Recently, there have also been increasing numbers of VDT studies using 3-dimensional (3D) software.[1,8–10] Yankelevitz et al[11] concluded that volumetric measurement is necessary to evaluate tumor growth, because growth may be asymmetric in not just 2 dimensions, but in all 3.

We often encounter lung cancer with chronic obstructive pulmonary disease (COPD) in clinical settings. A meta-analysis by Wasswa-Kintu and coworkers[12] showed that even a modest reduction in airflow significantly predicted lung cancer. In an editorial, Petty[13] suggested that COPD and lung cancer could arise from the same inflammatory disease process and that they might share common genetic and environmental risk factors. Brody and Spira[14] further postulated that lung cancer and COPD outcomes in response to inflammation caused by smoking might result from individual differences in genes that control genomic integrity and those that control tissue injury. These hypotheses would seem to relate to airflow obstruction and emphysema, 2 overlapping manifestations of chronic lung disease related to smoking.[15] Based on these findings, we hypothesized that VDT of patients with COPD findings would be shorter than in those without such findings.

The purposes of this study were to evaluate whether VDT of lung cancer with COPD findings differed from that of lung cancer without COPD findings, using serial 3D volumetric CT, and to investigate the association between VDT and COPD findings.

MATERIALS AND METHODS

Study Participants

From January 2006 to October 2009, we retrospectively reviewed 273 patients diagnosed with non–small cell lung cancer after surgical resection. We excluded 206 nodules that had not had serial follow-up CT more than twice preoperatively, 14 without thin-section CT imaging, 5 in which volume decreased, 2 causing poor imaging or poor breath-holding, and 1 recurrent nodule. Finally, 45 patients with lung cancer nodules were included in this study. All patients had serial follow-up CT more than twice before surgery to determine VDT of lung cancer. Patients were 28 men and 17 women (age range, 45–83 years; mean, 67.22 years). This retrospective study received institutional review board approval, and informed consent was waived.

Computed Tomography

Chest CT was performed with 2 kinds of scanners: 16-detector-row (LightSpeed 16; GE Healthcare, Milwaukee, Wis) or single-detector-row (Proseed SA Libra; GE Healthcare). Parameters for the 16-detector-row scanner were as follows: exposure settings, 323 to 432 mA, 0.5 s/rotation at 120 kVp; collimation, 16 × 0.625 or 1.25 mm; beam pitch, 1.75:1. Parameters for the single-detector-row scanner were as follows: exposure settings, 130–160 mA, 0.8 s/rotation at 140 kVp; collimation 5 or 1 mm; beam pitch, 1.5:1. Thin-section (0.625–2.0 mm) images were reconstructed with no overlap using the bone convolution kernel and transferred to our research picture archiving and communication system server.

Volume Doubling Time

The 45 lung cancer nodules diagnosed by surgical resection comprised 33 adenocarcinomas (including 5 bronchioloalveolar carcinomas), 10 squamous cell carcinomas (SCCs), 1 adenosquamous cell carcinoma, and 1 pleomorphic carcinoma. Volume measurements of lung cancer nodules were performed

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The authors report no conflicts of interest.

TABLE 1. Clinical Characteristics and Pathological Findings

	CT Findings	
	COPD	**Non-COPD**
Total	26	19
Sex		
Men	20	8
Women	6	11
Age, y	69.6 ± 8.6	67.4 ± 10.8
Brinkman index	838 ± 600	240 ± 381
FEV1.0%	62.8 ± 12.3	73.3 ± 7.8
Pathologic type of lung cancer		
Adenocarcinoma	15 (1)	18 (4)
SCC	10	0
Others	1	1

Data represent the numbers of patients or mean ± SD. Numbers in parentheses are bronchioloalveolar carcinomas.

on the initial and final CT obtained before surgery. On successive several thin-section CT images containing the nodule, we traced manually around the perimeter of the nodule, and initial volume and final volume were automatically calculated based on traced data using 3D volumetric computer software (Xio; CMS, Tokyo, Japan). If the lung cancer nodule contained ground-glass opacities (GGOs), spiculation, or pleural indentation, these were included in the lung cancer volume. Nodules containing secondary obstructive pneumonia had not been included in this study. Using this volume data, we calculated VDT and evaluated lung cancer growth rate. Volume doubling time was obtained using the following equation:

$$\mathrm{VDT} = [t \log 2] / [\log V_t / V_0]$$

where t is the time interval between 2 CT scans, V_t is the final volume of the lung cancer nodule, and V_0 is the initial volume.

CT Findings

Two chest radiologists (with 9 and 6 years of experience) who were blinded to the interval of follow-up CT retrospectively assessed COPD findings and tumor location by consensus. Images of all cancer nodules were viewed at a window setting appropriate for the lung parenchyma (width, 1500 Hounsfield units; level, −700 to −500 Hounsfield units).

The COPD findings were evaluated for the extent of emphysema and its pattern and airway findings on initial thin-section CT images. The extent of emphysema was assessed using a 4-point scale: grade 0 = no emphysema, grade 1 = mild emphysema with less than 25%, grade 2 = moderate emphysema with 25% to 50%, and grade 3 = severe emphysema with more than 50%. Emphysema pattern was classified into 3 groups (centrilobular, panlobular, and paraseptal). If more than 1 pattern was present, the predominant one was selected. Airway findings were evaluated with respect to bronchiectasis, bronchial wall thickening, centrilobular nodule, and mosaic attenuation. Bronchiectasis was defined as present when the diameter of the bronchial lumen was greater than that of the adjacent pulmonary artery, and no tapering of bronchial lumen diameter was seen. Bronchial wall thickening was estimated by measuring the ratio of the thickness of the airway wall to the outer diameter of the corresponding bronchus. Bronchial wall thickening was diagnosed when wall thickness was greater than half of vessel diameter. A centrilobular nodule was defined as a nodule (<10 mm) identified around the peripheral pulmonary arterial branches or 3 to 5 mm away from the pleura, interlobular septa, or pulmonary vein. Mosaic attenuation was defined as a patchwork of regions of differing attenuation.

Regarding the tumor location (upper vs lower, peripheral vs central), the tumor was classified as having an upper location when it was most extensive above the level of the tracheal carina and as having a lower location when it was most extensive below this level. The tumor was defined as peripheral if the center of the nodule was within 2 cm of the interface of the lung and chest wall; otherwise, it was defined as central.

Statistical Analysis

Differences in patient characteristics between the 2 groups were assessed using an unpaired t test for age; a Mann-Whitney U test for the Brinkman index, forced expiratory volume 1.0(sec)% (FEV1.0%), and VDT; and a χ^2 test for the proportion of patients with adenocarcinoma, SCC, and other types of tumor. Spearman rank correlation was used to evaluate the relationship between the VDT and CT findings (such as emphysema score, emphysema pattern, presence or absence of airway findings, upper/lower, and peripheral/central). Statistical analyses were performed using SPSS software (version 17.0J for Windows; SPSS Inc, Chicago, Ill). $P < 0.05$ was considered statistically significant.

RESULTS

Chronic obstructive pulmonary disease findings were observed on the initial CT in 26 (58%) of 45 patients. Three of 26 patients with COPD findings and 2 of 19 patients with non-COPD patients were scanned by single-detector-row scanner. The clinical characteristics and pathologic findings in all 45 patients are shown in Table 1. Patients with COPD findings had significantly higher mean Brinkman index and significantly lower mean FEV1.0% than those without COPD findings ($P < 0.001$ for both). There were no significant differences in age or sex between the 2 groups. Lung cancer patients with COPD findings (n = 26) tended to have a shorter VDT than those without COPD findings (n = 19) (998 ± 2178 vs 2226 ± 6748 days; $P = 0.066$, Table 2).

Table 3 shows the frequency of each COPD finding and the location of the tumor in 26 patients. The most frequently observed COPD findings were emphysema and bronchiectasis (18/26, 69% for each). The severity of emphysema was grade 0 in 8 patients (31%), grade 1 in 9 (34%), grade 2 in 5 (19%), and grade 3 in 4 (15%). In terms of emphysema pattern, centrilobular pattern was observed most frequently (8/18, 44%), followed by panlobular (7/18, 39%) and paraseptal (3/18, 17%) patterns.

Table 4 shows the relationship between VDT and COPD findings. The presence of emphysema finding was significantly

TABLE 2. Results of Volume Measurements and VDT

	CT Findings	
	COPD	**Non-COPD**
Total	26	19
Initial volume, mm^3	3960 ± 4260	3180 ± 5060
Final volume, mm^3	5650 ± 5430	3830 ± 5090
t, d	178	300
VDT, d	998 ± 2178	2226 ± 6748

Data represent the numbers of patients or mean ± SD.
t indicates interval time between 2 CT scans.

TABLE 3. COPD Findings and Tumor Locations in 26 Patients

	Total No. (%)
COPD findings	
Emphysema findings	18 (69)
Score	
Grade 0 (no emphysema)	8 (31)
Grade 1 (mild)	9 (35)
Grade 2 (moderate)	5 (19)
Grade 3 (severe)	4 (15)
Pattern	
Centrilobular	8 (31)
Panlobular	7 (27)
Paraseptal	3 (12)
Airway findings	20 (77)
None	6 (23)
Bronchial wall thickening	3 (12)
Bronchiectasis	18 (69)
Centrilobular nodule	3 (12)
Mosaic attenuation	1 (4)
Tumor location	
Upper	10 (38)
Lower	16 (62)
Central	10 (38)
Peripheral	16 (62)

correlated with VDT ($P < 0.001$). In addition, the severity and the pattern of emphysema were also significantly correlated with VDT ($P < 0.001$ for each). Regarding the emphysema pattern, VDT was shortest in patients with paraseptal pattern. Representative cases of lung cancer with and without COPD findings are shown in Figures 1 to 3. Airway findings including bronchiectasis, bronchial wall thickening, centrilobular nodule, or mosaic attenuation were found in 20 patients. Airway findings and tumor locations were not correlated with VDT ($P = 0.256$, $P = 0.878$, $P = 0.720$, respectively).

DISCUSSION

In day-to-day practice, pulmonary nodules are usually detected and followed up by chest x-ray or CT. Apart from typical cases of lung cancer, it is sometimes difficult to confirm a diagnosis of small nodules, even though suggestive of lung cancer, by clinical examination and radiologic findings alone. In these cases, follow-up studies using CT or pathologic diagnosis such as transbronchial or CT-guided biopsy are often needed. Volume doubling time of a pulmonary nodule is an important factor that can contribute to the diagnosis of lung cancer. Malignant nodules usually grow more rapidly than their benign counterparts, and it is generally accepted that the majority of malignant pulmonary nodules double in volume between 20 and 400 days, although some exceptions to this rule occur.[16] Winer-Muram et al[3] showed that comparison of stage I lung cancer volumes at serial CT examinations reveals a very wide range of growth rates and noted that some stage I lung cancers grow so slowly that biopsy is required to prove they are malignant. A retrospective study examining the relationship between histology and tumor doubling time showed 2 main types of peripheral lung adenocarcinoma.[2] The first type appeared on CT as a localized GGO with slow growth, and the other was a solid attenuation with rapid growth. However, previous studies have obtained tumor volume from measurements of tumor diameter or have calculated tumor volume by considering the tumor as an ellipsoid. Two-dimensional measurement has some limitations in the evaluation of pulmonary nodules, and 3D measurement is recommended to obtain a more precise tumor volume.[17,18] Jennings et al[7] showed that lung tumor growth assessment with diameter or area measurements on serial CT scans is often inaccurate

TABLE 4. Relationship Between VDT and COPD Findings in 26 Patients

	Volume, mm^3			
	Initial	Final	*t*, d	VDT, d
COPD findings				
Emphysema findings	4330 ± 4430	6450 ± 5760	138	309 ± 389*
Score				
Grade 0 (no emphysema)	3110 ± 4000	3860 ± 4410	270	2548 ± 3551
Grade 1 (mild)	4500 ± 4920	5510 ± 5600	114	432 ± 498*
Grade 2 (moderate)	4640 ± 4640	7080 ± 7100	145	235 ± 265*
Grade 3 (severe)	3590 ± 4110	7750 ± 5650	183	127 ± 79*
Pattern				
Centrilobular	4160 ± 5350	4980 ± 6900	118	471 ± 515*
Panlobular	5660 ± 3990	9820 ± 5050	193	204 ± 226*
Paraseptal	1730 ± 1600	2480 ± 1940	62	122 ± 103*
Airway findings	4470 ± 4680	5940 ± 5870	186	1231 ± 2446
Tumor location				
Upper	6070 ± 5340	7780 ± 6760	133	1464 ± 3257
Lower	2640 ± 2890	4320 ± 4100	207	707 ± 1143
Central	4500 ± 4290	6650 ± 5880	256	547 ± 765
Peripheral	3620 ± 4350	5020 ± 5230	130	1280 ± 2708

Data represent the numbers of patients or mean ± SD.

*$P < 0.001$.

t indicates interval time between 2 CT scans.

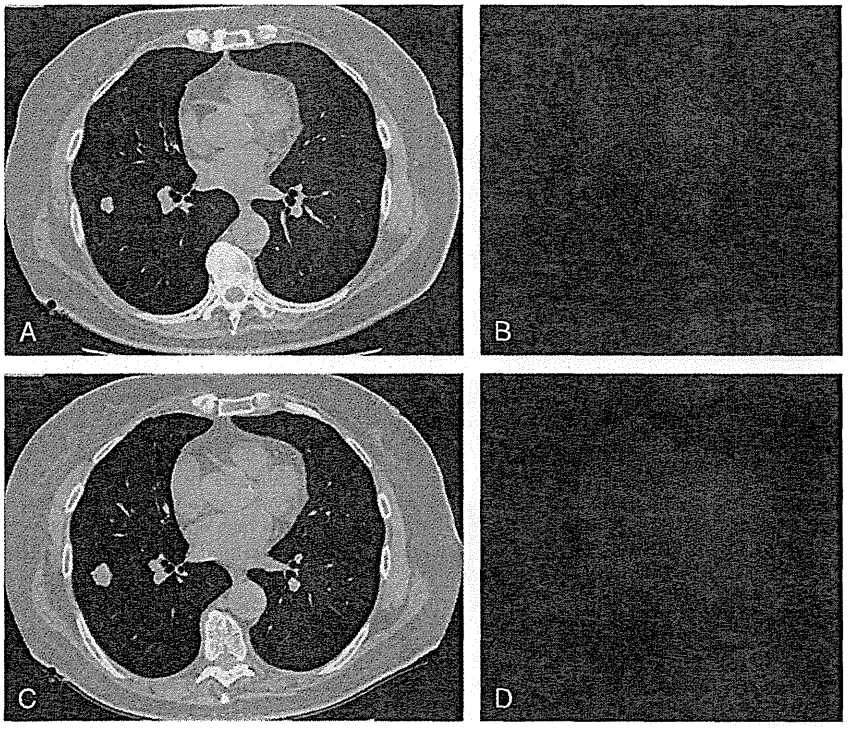

FIGURE 1. Adenocarcinoma of primary lung cancer in a 72-year-old woman without COPD findings. A, Initial thin-section CT image shows a nodule in the right lower lobe. B, Three-dimensional image of the nodule at the initial CT examination. C, After 354 days, the nodule has increased in size. D, Three-dimensional image at the final CT. The volume was 2210 mm^3, and the calculated VDT was 273 days.

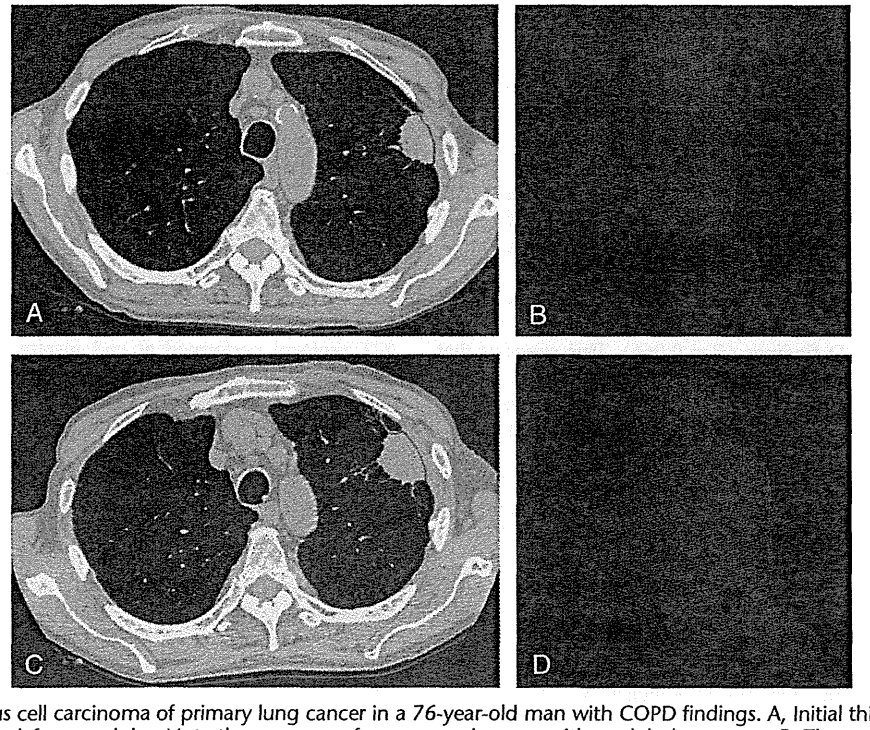

FIGURE 2. Squamous cell carcinoma of primary lung cancer in a 76-year-old man with COPD findings. A, Initial thin-section CT image shows a nodule in the left upper lobe. Note the presence of severe emphysema with panlobular pattern. B, Three-dimensional image of the nodule at the initial CT examination. C, After 30 days, the nodule has increased in size. D, Three-dimensional image at the final CT. The volume was 13,820 mm^3, and the calculated VDT was 60 days.

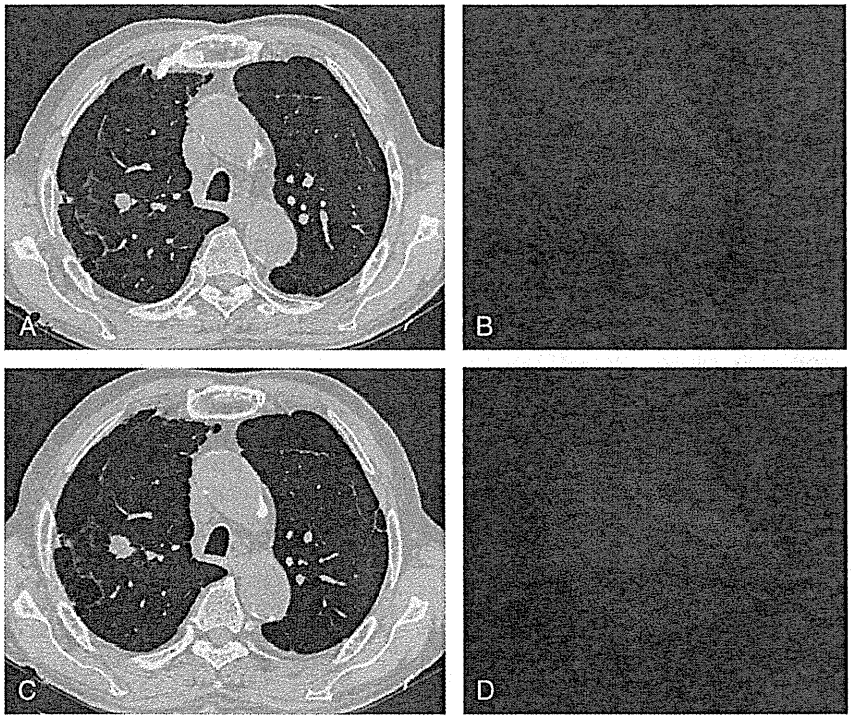

FIGURE 3. Squamous cell carcinoma of primary lung cancer in a 75-year-old man with COPD findings. A, Initial thin-section CT image shows a nodule in the right upper lobe. Note the presence of moderate emphysema with paraseptal pattern. B, Three-dimensional image of the nodule at the initial CT examination. C, After 56 days, the nodule has increased in size. D, Three-dimensional image at the final CT. The volume was 1770 mm^3, and the calculated VDT was 59 days.

compared with volume measurement. Therefore, doubling time in the present study was calculated using 3D volumetric computer software.

Many studies on the relationship between COPD and lung cancer have been published. Moreover, smoking history and pulmonary function tests are essential for patients with suspected lung cancer. In this study, we focused on patients with CT findings of COPD such as pulmonary emphysema or airway lesions separately from criteria of clinical COPD to evaluate the relationship between VDT of lung cancer and CT findings of COPD. To our knowledge, no retrospective studies have examined VDT according to the presence or absence of CT findings of COPD. Our study showed that VDT of lung cancer with COPD findings (n = 26) tended to be shorter than that of lung cancer without COPD findings ($P = 0.066$). This suggests that we may be able to predict lung cancer growth rates for patients who do not undergo pulmonary function testing by evaluating radiologic COPD findings, and we have expected to apply radiologic findings to physiological examinations.

In addition, our results showed that severity and patterns of emphysema were associated with VDT of lung cancer with COPD findings; VDT tended to be shorter as the degree of pulmonary emphysema increased. It is unclear why VDT of nodules with severe emphysema finding tended to be shorter. However, it may be speculated that the patients with pulmonary emphysema had a risk to be exposed to carcinogens such as cigarettes in the long term, compared with patients without pulmonary emphysema. Our results suggest that, when evaluating an indeterminate pulmonary nodule, although suggestive of lung cancer in routine practice, serial CT follow-up is necessary when emphysema findings are more severe. In terms of emphysema pattern, VDT was shortest for lung cancer nodules with paraseptal emphysema. However, there were only 3 nodules showing this pattern, 1 SCC and 2 adenocarcinomas, and none included a GGO component. Therefore, further investigation of larger numbers of lung cancer nodules with paraseptal emphysema is necessary to determine the relationship with VDT.

Regarding airway findings, they did not show a significant correlation with VDT. This may be because airway findings such as bronchial wall thickening and bronchiectasis might be caused by degeneration due to the aging effect other than COPD. Copley et al[19] showed that bronchial dilation and wall thickening were also seen significantly more frequently in older patients than in younger patients. In fact, the average age of patients in our study was 69.6 ± 8.6 years (range, 52–83 years). In this study, mosaic appearance was seen in only 1 patient, possibly because of our CT protocol performed at end inspiration. End-expiratory CT imaging might be better for the visualization of mosaic appearance. Further investigation is therefore necessary with expiratory CT imaging.

The present study had some limitations. First, the study population was relatively small because we have used the strict inclusion criteria for this study. For instance, patients were limited to those who underwent more than 2 follow-up CT examinations preoperatively, to those who did not have chemotherapy or radiotherapy, and to those who had pathologic confirmation by surgical resection, not by transbronchial or CT-guided biopsy. Further investigation of larger numbers of nodules is therefore needed. Second, there were no cases of SCC without COPD findings in this study. Honda et al[1] showed that the median doubling time of SCC is shorter than that of adenocarcinoma, as measured with automated volumetric measurement software. Therefore, the fact that our sample included no SCC cases without COPD findings might have increased the VDT of these lung

cancer nodules. In addition, the association between smoking and SCC is well known. In the present study, the Brinkman index of the patients without COPD findings was less than that of those with COPD (240 ± 381 vs 838 ± 600), and this might be related to a reduced incidence of SCC. However, the purpose of this study was to evaluate VDT of patients with COPD findings, and these patients included those with SCC and those with adenocarcinoma. Third, our study evaluated 2 kinds of scanners, single-detector-row and 16-detector-row. There may be some difference in nodule size detection and software application to a single-detector-row scanner. Further investigation by only a multidetector is needed. In addition, VDT of the nodule was calculated using 3D volumetric computer software after manual tracing around the perimeter of the nodule. This could have introduced measurement errors. However, lung cancer measurements were obtained by consensus of 2 radiologists, which should have mitigated these measurement errors. Finally, we used thin-section CT images of 0.625- to 2.0-mm thickness for volume measurement, depending on the availability of CT scanners. However, the proportion of patients with different section thicknesses among the groups in this study was similar. Therefore, we assumed that variations between individual patients would be averaged and become less critical in group comparisons, although it would be preferable to use the same section thickness for all patients.

In conclusion, VDT of lung cancer with COPD findings on 3D volumetric CT tended to be shorter than that of lung cancer without COPD findings. Severe or paraseptal emphysema may be associated with short VDT of lung cancer with COPD findings.

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Opposing consequences of signaling through EGF family members

Escape from CTLs could be a bait for NK cells

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Keywords: EGF receptors, MICA/B, MHC Class I, NK cell, immune escape

Oncogenes have been traditionally viewed as molecular drivers for tumor growth and survival. Recent evidence indicates that oncogenes may facilitate the escape of malignant cells from immune recognition and elimination. In this article, we discuss the implications of the overexpression of epidermal growth factor receptor (EGFR) family members on immune escape of tumors and immunotherapy.

Tumors targeted with cellular immunotherapy can exhibit an "immune escape" phenotype, potentially rendering immunotherapeutic interventions ineffective. As one of the underlying mechanisms, a variety of oncogenes have been shown to interfere with antigen processing and presentation.[1] The epidermal growth factor receptor (EGFR) family, consisting of four closely related transmembrane tyrosine kinase receptors (EGFR1–4 also known as HER1–4), is particularly important for the etiology of carcinomas and represents an attractive target for immunotherapy. HER receptors undergo homo- or heterodimerization and autophosphorylation in response to the binding of small peptide ligands, which activate downstream signaling pathways. The HER2/HER3 dimer is the most active HER signaling dimer, and is critical for signaling in HER2-overexpressing tumors.[2] We and others have reported that HER2 signaling can lead to the downregulation of MHC Class I molecules and impair MHC Class I-restricted recognition by CTLs.[3-5] This is particularly relevant for therapies targeting breast carcinomas, which frequently overexpressed HER2 together with other members of the EGFR family. We recently confirmed the inverse correlation of HER2 and MHC Class I expression by immunohistochemistry (IHC) in a cohort of 70 patients affected by breast carcinomas (unpublished observations). Moreover, we demonstrated that the administration of inhibitors of the Ras/MAPK pathway enhances MHC Class I expression in breast cancer, suggesting that this pathway is involved in MHC Class I downregulation by HER-overexpressing tumors.

The HER2-induced loss of MHC Class I expression and the resultant decrease in CTL sensitivity have important implications for cancer immunotherapy. One potential approach to circumvent this issue would be to pre-select breast cancer patients with tumors that express low or intermediate levels of HER2 for CTL-based immunotherapy, excluding patients with high HER2 and low MHC Class I expression. At least theoretically, this selection would be of particular importance for patients undergoing vaccination with MHC Class I-restricted CTL epitopes. Benavides et al.[6] reported that patients expressing low levels of HER2 (IHC score: 0 or 1+) respond better to vaccination with the HER2 E75 peptide CTL epitope than patients overexpressing (IHC score: 2+ or 3+) HER2, which substantiates the premise for patient selection. Therefore, patients with tumors expressing low levels of HER2, representing > 50% of breast cancer cases, may have greater clinical benefit from MHC Class I-restricted immunotherapy approaches in comparison to the patients whose tumors present high levels of HER2.

MHC Class I inhibits tumor cell lysis by natural killer (NK) cells. Therefore, it may be speculated that MHC Class I downregulation by oncogenes could make transformed cells suitable targets for direct NK cell-mediated tumor rejection or by NK cell-mediated antibody dependent cellular cytotoxicity (ADCC). The balance between activating and inhibiting signals is known to regulate NK cell-mediated cytotoxicity. Thus it is particularly interesting to understand whether oncogenes signaling also affects the ligands for activating NK cell receptors.

In a recent study, we investigated how signaling through HER2/HER3 regulates the MHC Class I chains A and B (MICA and MICB) in human breast cancer cell lines.[7] MICA/B are examples of ligands for the activating NK group 2, member D (NKG2D) receptor, which

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is expressed not only by NK but also by CD8+ and γδ T cells.[8] While tumor cell lines and primary tumors express the NKG2D ligand (NKG2DL), MICA/B is also expressed—though at lower levels—by some non-malignant cell types. The extent to which oncogenes can regulate NKG2DL expression is not well understood, but the example of the BCR/ABL oncogene, which can induce MICA in chronic myelogenous leukemia (CML) lends credence to these premises.[9] This may relevant for NK cell-mediated tumor surveillance, since tumor cells expressing NKG2DL are often susceptible to killing by NK cells even if they have normal expression of MHC Class I. We tested a number of experimental situations affecting signaling through HER2/HER3 in breast cancer cell lines, including pharmacological and genetic interference, overexpression by transfection, and treatment with the HER3-specific ligand NRG1, and assessed how these manipulations affected MICA/B expression. We concluded that signaling through the HER2/HER3 complex augments the expression of MICA/B, although it appears that HER3 more than HER2 constitutes the rate-limiting factor in this process. Among the multiple pathways activated by HER2/HER3 signaling, the PI3K-AKT pathway was shown to predominantly regulate MICA/B expression. Genotoxic stress, such as ionizing radiation and inhibitors of DNA replication, can induce the upregulation of NKG2DL.[10] We believe, however, that a direct effect on HER2/HER3 is responsible for MICA/B induction in breast cancer cell lines, as inferred from the administration of the ataxia-telangiectasia-mutated (ATM) and ATM and Rad-3-related (ATR) protein kinase inhibitor caffeine. The functional consequences of HER2/HER3 signaling manifested as enhanced NKG2D-MICA/B dependent NK cell-mediated cytotoxicity of breast cancer lines.

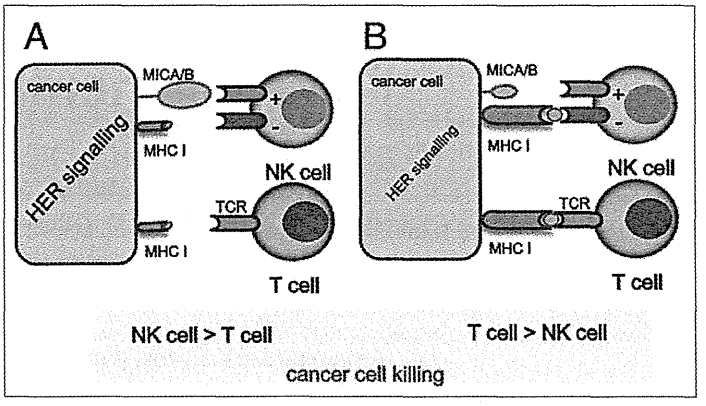

Figure 1. A schematic illustration of how HER2/HER3 signaling might determine whether cancer cells are killed by T cells or NK cells. **(A)** If HER2/HER3 signaling is strong, MHC Class I expression is weak, while the expression of MICA/B will be enhanced. In these conditions, the NK cell-mediated killing of tumor cells will dominate, while cancer cells will be relatively resistant to CTL-mediated killing. **(B)** If HER2/HER3 signaling is weak, MHC Class I expression will be unaffected, a conditions in which cancer cells can be killed by CTLs.

Combinatorial immunotherapies are becoming increasingly diffuse and appear to yield promising results. The findings discussed above should inspire efforts to develop immunotherapy regimens in which adaptive immunotherapy is combined with strategies based on NK cells for the treatment of carcinomas expressing high levels of HER2 and/or HER3. These combinatorial therapies should be tailored to best fit the individual expression of MHC Class I molecules and NK cell activating receptors on each tumor, a true example of individualized immunotherapy.

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Influence of vascular endothelial growth factor single nucleotide polymorphisms on non-small cell lung cancer tumor angiogenesis

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Abstract. Vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis. Several studies have reported that genomic *VEGF* polymorphisms may influence *VEGF* synthesis. To evaluate the role of VEGF single nucleotide polymorphisms (SNPs), we examined the expression of several angiogenesis-related proteins [VEGF, hypoxia-inducible factor-1α (HIF-1α) and delta-like ligand 4 (Dll4)] and the spread of microvessels in resected non-small cell lung cancer (NSCLC). Blood and tumor tissue from 83 patients with NSCLC were examined for *VEGF* -460T/C (rs833061) and *VEGF* +405G/C (rs2010963) SNPs using the SNaPshot method. Immunohistochemical staining was performed to measure protein expression and microvessel density (MVD). *VEGF* -460T/C and +405G/C SNPs showed no association with VEGF or HIF-1α expression and MVD. Patients with *VEGF* -460TT and the TC genotype had significantly higher MVD compared to those with the CC genotypes. Furthermore, patients with the *VEGF* -460TT genotype had significantly higher Dll4 expression compared to those with the TC or CC genotypes, while the *VEGF* +405G/C SNP displayed no association with Dll4 expression and MVD. These findings indicate that the *VEGF* -460T/C SNP may have a functional influence on tumor angiogenesis in NSCLC. We hypothesize that *VEGF* SNPs may influence angiogenesis through Dll4.

Introduction

Angiogenesis plays an important role in tumor progression and metastasis, and vascular endothelial growth factor (VEGF) is a key component. Several studies have demonstrated that VEGF mRNA and protein overexpression are associated with tumor progression and prognosis in non-small cell lung cancer (NSCLC) (1-3).

Several *VEGF* single nucleotide polymorphisms (SNPs) have been recently described (4). *VEGF* is located on chromosome *6p21.3* and is organized into eight exons and seven introns (5,6). The VEGF -460T/C SNP (rs833061) is located in the promoter region and may influence promoter activity (7). Furthermore, the *VEGF* +405G/C SNP (rs2010963) is located within the 5'-untranslated region and may affect transcription factor binding affinity (7,8). These two SNPs have been investigated in different types of cancers, and the association of various *VEGF* SNPs with risk or prognosis of several cancers has been examined (9-12). Recently, *VEGF* +405 and -460 SNPs have been found to be significantly associated with risk and survival in NSCLC (13-15). However, the influence of *VEGF* SNPs on tumor angiogenesis remains unclear. In this study, we examined whether *VEGF* -460 and +405 SNPs may influence VEGF expression and microvessel density (MVD) in NSCLC.

Tumor angiogenesis is influenced by a number of proteins. Hypoxia occurs early in tumor development and results in stable binding of hypoxia-inducible factor-1α (HIF-1α) to DNA and the activation of other angiogenic genes, such as *VEGF* (16,17). Delta-like ligand 4 (Dll4) is a ligand for Notch proteins that is expressed by endothelial cells (18,19) and may be induced by VEGF and HIF-1α (20). It plays an important role in tumor vessel maturation and remodeling (21,22). Therefore, we studied whether these *VEGF* SNPs were associated with the expression of the angiogenesis-related proteins HIF1α and Dll4.

Patients and methods

Study population. Blood and tumor samples were obtained from 83 patients with NCSLC who underwent surgical resection at the Kawasaki Medical School Hospital between October, 2008 and December, 2010. The patients did not receive radio- or chemotherapy before surgery. This study was approved by the Ethics Committee of the Kawasaki Medical School, and informed consent was obtained from all patients for the use of their tissue specimens.

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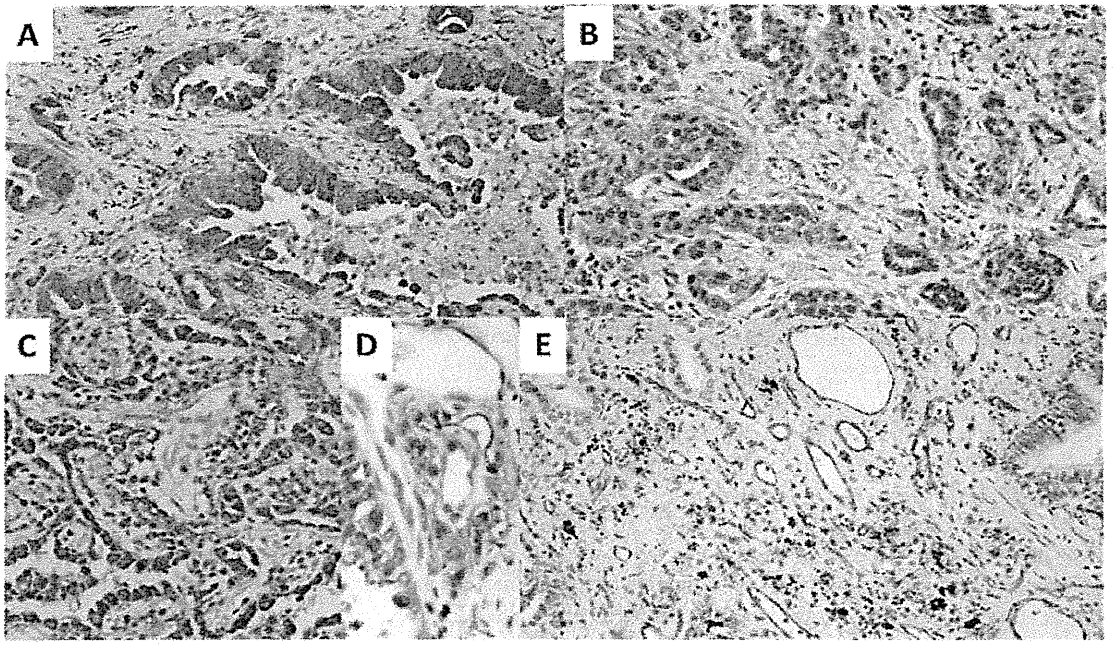

Figure 1. Positive immunohistochemical staining for (A) VEGF, (B) HIF-1α, (C) Dll4 (tumor cells), (D) Dll4 (endothelial cells), and (E) CD31 (for microvessel counting, x200 magnification).

Analysis of VEGF-A -460T/C and +405G/C polymorphisms. Blood samples were collected from all subjects before surgery. Genomic DNA was isolated from peripheral whole blood using the QIAamp™ DNA Blood Mini kit (Qiagen, Hilden, Germany). Genomic regions containing the *VEGF* -460T/C and +405G/C SNPs were amplified by polymerase chain reaction using the following primers: -460T/C, 5'-CGAGAGTGA GGACGTGTGTG-3' (forward) and 5'-ATTGGAATCCTG GAGTGACC-3' (reverse); +405G/C, 5'-GAGAGACGGGGT CAGAGAGA-3' (forward) and 5'-CCCAAAAGCAGGTCAC TCA-3' (reverse). The VEGF SNPs were genotyped by a single-base primer extension assay using the SNaPshot™ Multiplex kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The following primers were used: -460T/C, 5'-ttttttttCTTCTCCCCGCTC-CAAC-3'; +405G/C, 5'-ttttttttttttttGTGCGAGCAGCGA AAG-3'.

DNA sequencing. Polymorphism analysis was performed using the ABI PRISM® 310 Genetic Analyzer, and results were evaluated using GeneMapper® software, ver. 4.1 (all were from Applied Biosystems).

Immunohistochemical staining. VEGF, HIF-1α, Dll4 and CD31 (to measure MVD) expression was analyzed using resected, paraffin-embedded lung cancer tissue. After microtome sectioning (4-μm thick), tissue slides were processed on an automated immunostainer (NexES; Ventana Medical Systems, Tucson, AZ, USA) or manual methods. Streptavidin-biotin-peroxidase detection was performed with diaminobenzidine as the chromogen. The following primary antibodies were used according to the manufacturer's instructions: VEGF (rabbit polyclonal; sc-152; 1:300 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), HIF-1α (mouse monoclonal; ESEE122; 1:1,000 dilution; Novus, Littleton, CO, USA), Dll4 (rabbit polyclonal; ab7280; 1:50 dilution; Abcam, Cambridge, MA, USA), and CD31 (mouse monoclonal; 1:50 dilution; Dako, Carpinteria, CA, USA). The slides were examined by two investigators blinded to the corresponding clinicopathological data. The expression of each protein marker was examined and evaluated according to previously reported protocols (1,23-26).

VEGF staining and scoring. To evaluate VEGF expression, the percentage of positively stained cells and staining intensity were scored as follows: grade 0, negative; grade 1, weak; grade 2, moderate; grade 3, high; and grade 4, very high (23). Grade 0 indicated staining intensity equal to the negative control, grade 3 indicated intensity equal to the positive control, and grade 4 indicated intensity higher than the positive control. Stain intensity in the cell cytoplasm was similarly scored (23). To determine the percentage of cells with the various staining intensities, the number of immunoreactive cells at each intensity was divided by the total number of tumor cells in three fields at x200 magnification (Fig. 1A). The overall VEGF staining score was calculated as follows: VEGF score = 1 x percentage of grade 1 cells + 2 x percentage of grade 2 cells + 3 x percentage of grade 3 cells + 4 x percentage of grade 4 cells. The score was analyzed as a continuous and a dichotomous variable.

HIF-1α staining and scoring. Tumor cells were scored on the intensity and extent of staining as follows: score 1, tumor cells with absent or weak cytoplasmic reactivity and no nuclear reactivity; score 2, tumor cells with moderate/strong cytoplasmic reactivity with a percentage of tumor cells less than their mean percentage and no nuclear reactivity; score 3, tumor cells with moderate/strong cytoplasmic reactivity with

Table I. Characteristics of the patients with NSCLC.

Characteristic	No. of patients	%
Age (years)		
Median	72	
Range	49-89	
Gender		
Male	52	62.7
Female	31	37.3
Smoking		
Never	27	32.5
Former/Current	56	67.5
Stage		
IA	40	48.2
IB	17	20.5
IIA	11	13.3
IIB	9	10.8
III	6	7.2
Histology		
Adenocarcinoma	52	62.7
SCC	19	22.9
Other types	12	14.4

SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer.

a percentage of tumor cells more than their mean percentage; score 4, tumor cells with clear nuclear reactivity (with or without cytoplasmic reactivity regardless of the intensity) (Fig. 1B). Tumors with scores of 1 and 2 were considered to exhibit low HIF-1α expression, whereas those with scores of 2 and 3 were considered to exhibit high HIF-1α expression (24).

Dll4 staining and scoring. Dll4 expression was considered only in endothelial cells, although recent reports have demonstrated its wide cellular distribution beyond vessels (25,26). To evaluate Dll4 staining in tumor cells (Fig. 1C and D), the intensity of expression was scored on a semiquantitative scale in three x200 magnification fields. Negative cores were scored as 0, cores with weak expression were scored as 1 and those with moderate/strong expression were scored as 2. High Dll4 expression was defined as a score greater than 1.5 (26).

Microvessel staining and counting. MVD was assessed by counting the number of microvessels stained for CD31. Vessels with a clearly defined lumen or well-defined linear vessel shape and no single endothelial cells were selected for counting. Microvessels were counted in the three x200 magnification fields with the highest density (Fig. 1E), and the mean MVD was calculated (1).

Statistical analysis. Vascular scores were presented as the means ± standard deviation and the difference between the groups was analyzed using the unpaired Student's t-test. The association of *VEGF* SNPs with clinicopathological parameters and immunostaining results was examined using Chi-squared and Fisher's exact tests, respectively. The level of significance was set at $P<0.05$. All analyses were performed using SPSS software (version 17.0; SPSS, Chicago, IL, USA).

Table II. Relationships between angiogenesis related protein expression as determined by immunohistochemistry.

	VEGF		HIF-1α	
Variable	High	Low	High	Low
HIF-1α				
High	29	15		
Low	13	26		
P-value	P=0.003			
DLL4 (T)				
High	27	23	34	16
Low	15	18	10	23
P-value	P=0.446		P<0.001	

VEGF, vascular endothelial growth factor; Dll4, delta-like ligand 4; HIF-1α, hypoxia-inducible factor-1α; T, tumor cells.

Results

Clinical characteristics. Characteristics of the patients with NSCLC are summarized in Table I. Patients ranged in age from 49 to 89 years (median, 72 years), with 52 men and 31 women. Fifty-six (67.5%) patients were former/current smokers. There were 40 (48.2%) stage IA, 17 (20.5%) stage IB, 11 (13.3%) stage IIA, 9 (10.8%) stage IIB, 6 (7.2%) stage III. Fifty-two (62.7%) patients had adenocarcinoma, 19 (22.9%) had squamous cell carcinoma, and 12 (14.4%) had other histological malignancies.

Immunohistochemistry of angiogenesis-related proteins. Forty-two patients (50.6%) exhibited a marked increase in VEGF immunoreactivity of tumor cells. The mean VEGF staining score was 2.79±0.67, and the median score of 2.90 was used to distinguish between low and high VEGF staining. VEGF expression was correlated with HIF1α expression (P=0.003), but not with Dll4 expression (P=0.446) (Table II).

VEGF SNPs and clinicopathological characteristics. For the *VEGF* +405G/C SNP, 50.6% of patients had the GC genotype, 25.3% had CC and 24.1% had GG. For the *VEGF* -460T/C SNP, 50.6% had the TT genotype, 38.6% had TC and 10.8% had CC. No significant association was observed between *VEGF* SNPs and clinicopathological characteristics such as gender, pathological stage, lymphatic invasion, vascular invasion, histological type, and smoking status (Table III).

VEGF SNPs and angiogenesis-related proteins. Both SNPs displayed no association with VEGF or HIF-1α expression; however, Dll4 expression was significantly higher in patients with the *VEGF* -460TT genotype (P=0.031) (Table IV).

Table III. *VEGF* SNPs and clinicopathological characteristics.

	VEGF +405 genotype				*VEGF* -460 genotype			
Characteristic	CC	GC	GG	P-value	TT	TC	CC	P-value
No. of patients	21	42	20		42	32	9	
(%)	(25.3)	(50.6)	(24.1)		(50.6)	(38.6)	(10.8)	
Gender								
Male	15	23	14	0.321	23	21	8	0.143
Female	6	19	6		19	11	1	
Stage								
IA	11	19	10	0.807	21	14	5	0.481
IB	5	8	4		7	8	2	
II	3	11	6		9	10	1	
III	2	4	0		5	0	1	
Lymphatic invasion								
+	5	10	3	0.707	10	6	2	0.871
-	16	32	17		32	26	7	
Vascular invasion								
+	10	15	8	0.661	19	10	4	0.455
-	11	27	12		23	22	4	
Histology								
Adenocarcinoma	12	27	13	0.522	26	21	5	0.688
SCC	8	8	3		8	8	3	
Other types	1	7	4		8	3	1	
Smoking								
Never	7	14	6	0.962	17	6	4	0.102
Former/current	14	28	14		25	26	5	

VEGF, vascular endothelial growth factor; SCC, squamous cell carcinoma.

Table IV. *VEGF* SNPs and angiogenic-related protein expression.

	VEGF			HIF-1α			Dll4		
VEGF Genotype	High	Low	P-value	High	Low	P-value	High	Low	P-value
VEGF +405									
CC	12	9	0.735	10	11	0.739	12	9	0.741
GC	21	21		24	18		27	15	
GG	9	11		10	10		11	9	
VEGF -460									
TT	19	23	0.448	21	21	0.289	31	11	0.031
TC	19	13		16	16		14	18	
CC	4	5		7	2		5	4	

Dll4, delta-like ligand 4; HIF-1α, hypoxia-inducible factor-1α; *VEGF*, vascular endothelial growth factor.

Angiogenesis-related proteins and MVD. MVD ranged from 2.0 to 80.0, with a mean value of 29.9±15.9 and a median score of 29. High MVD was significantly associated with high VEGF (P<0.001) and Dll4 (P=0.026) expression, but not with HIF-1α expression (P=0.235) (Table V).

VEGF SNPs and MVD. Patients with the *VEGF* -460TT and TC genotypes had significantly greater MVD compared to those with the CC genotype (TT/TC vs. CC; P=0.027) (Table VIA). Moreover, in a group of tumors with high VEGF expression, patients with the VEGF -460TT genotype

Table V. Angiogenesis-related protein expression and MVD.

Protein marker expression	MVD	
	Mean ± SD	P-value
VEGF		
High	37.2±18.0	<0.001
Low	24.3±11.7	
Dll4 (T)		
High	33.9±17.4	0.026
Low	26.1±13.9	
HIF-1α		
High	32.9±16.5	0.235
Low	28.5±16.3	

VEGF, vascular endothelial growth factor; Dll4, delta-like ligand 4; HIF-1α, hypoxia-inducible factor-1α; MVD, microvessel density; SD, standard deviation; T, tumor cells.

Table VI. *VEGF* SNPs and MVD.

A, *VEGF* SNPs and MVD

VEGF Genotype	MVD	
	Mean ± SD	P-value
VEGF +405		
CC	27.3±17.0	CC/GC vs. GG 0.426
GC	31.9±16.4	GG/GC vs. CC 0.961
GG	28.8±14.0	
VEGF -460		
TT	31.9±18.1	TC/CC vs. TT 0.550
TC	31.4±16.0	TT/TC vs. CC 0.027
CC	23.9±7.8	

B, *VEGF* SNPs and MVD in the high VEGF expression group

VEGF Genotype	MVD	
	Mean ± SD	P-value
VEGF +405		
CC	36.75±19.16	CC/GC vs. GG 0.392
GC	39.48±18.14	CC vs. GG 0.615
GG	32.67±17.29	
VEGF -460		
TT	40.05±19.77	TT/TC vs. CC 0.032
TC	36.63±17.54	TT vs. CC 0.033
CC	26.75±6.85	

MVD, microvessel density; SD, standard deviation; VEGF, vascular endothelial growth factor.

had significantly higher MVD compared to those with the CC genotypes (P=0.033) (Table VIB).

Discussion

Angiogenesis is important for tumor progression and utilizes several factors, with VEGF being the key factor. Recently, several *VEGF* SNPs have been identified, and their effect has attracted a great deal of attention. An *in vivo* study by Stevens *et al* (7) discovered that *VEGF* -460/+405 SNPs significantly altered VEGF promoter activity in response to phorbol esters. Recent literature has reported the association of *VEGF* SNPs with risk or prognosis of various types of cancers (9-12). A large case-control study in Caucasians demonstrated that male patients with NSCLC and the *VEGF* +405CC+CG genotype had a higher risk of lung adenocarcinoma, while those with the -460T/+405G/936C haplotype had a reduced risk. (14). The C allele of the *VEGF* +405G/C SNP significantly improved survival in early-stage NSCLC (13), whereas the -460CC genotype decreased overall survival in advanced-stage NSCLC (15). Other studies have suggested a lower survival rate for the *VEGF* +405CC genotype in gastric and ovarian cancers (27,28). The reason for these conflicting results is currently unclear, and the influence of *VEGF* SNPs remains uncertain and controversial.

However to date, few studies have focused on the association between *VEGF* SNPs and VEGF expression. Therefore, we conducted a study with NSCLC patients to examine the functional activity of *VEGF* SNPs and their possible role in VEGF expression and angiogenesis.

The genotype frequencies for *VEGF* +405G/C (GG, CC, and GC) and *VEGF* -460T/C (TT, CC and TC) SNPs in this study were equivalent to previous reports involving Japanese patients (4,15). In our current study, there was no association between *VEGF* SNPs and VEGF expression. Koukourakis *et al* (29) reported that *VEGF* SNPs were associated with VEGF expression in NSCLC tumor cells and tumor angiogenic activity. They discovered that the *VEGF* -2578CC, +405GG (also referred to as -634GG) -1154AA and GA genotypes were associated with low VEGF expression in 36 patients with NSCLC (29). The vascular density of patients with the *VEGF* -2578CC and +405GG genotypes was also significantly lower compared to that in patiens with other genotypes. This result is not in agreement with our findings, which may be due to variations in genotype function related to racial differences between the patient groups.

We discovered that patients with the *VEGF* -460TT and TC genotype had significantly higher MVD compared to those with CC genotypes. In general, as in our study, high VEGF expression is associated with high vascular density. However, there was no association between the *VEGF* -460T/C SNP and VEGF expression in tumors. Furthermore, even in high VEGF expression cases, the -460TT genotype was associated with significantly higher MVD compared to CC genotype. This result suggested that high MVD in -460TT genotype was not caused by VEGF expression. The *VEGF* -460TT genotype was associated with significantly higher Dll4 protein expression, which demonstrated a significant association with high MVD. From these results, we concluded that Dll4, induced by the *VEGF* -460TT genotype, influenced the spread of microves-

sels. Dll4 is generally upregulated by VEGF, which in turn acts as a negative feedback regulator of VEGF. Our results suggest that *VEGF* SNPs may influence VEGF downstream signaling to Dll4, although potential mechanisms have not been examined in this study. Dll4 is associated with tumor vessel maturation and remodeling (21,22). Thus, high Dll4 expression should theoretically lead to fewer but larger vessels, and Dll4 overexpression or inhibition may consequently impair tumor angiogenesis. However, further study of this visceral function is warranted.

In conclusion, the *VEGF* -460T/C SNP may have a functional influence on tumor angiogenesis in NSCLC. Although *VEGF* SNPs were not associated with VEGF expression in tumor cells, they are considered to regulate the response to Dll4 signaling through functional changes in VEGF.

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