with gene expression (Fig. 1). As with BRG1, loss of BRM expression was similarly frequent in cell lines with EMT features and loss of the bronchial epithelial phenotype.

These results suggest the following: (i) loss of either or both BRG1 and BRM would be involved in the acquisition of EMT features and loss of the bronchial phenotype; and (ii) loss of BRG1 gene and protein expression correlate with the BRG1 mutation status.

We conducted the same analysis in five EGFR-mutated cell lines (HCC827, H1650, H1975, PC-3, and HCC4006), as shown in Figure S1. Although genetic status of BRG1 was unknown in H1650, H1975, PC-3, and HCC4006, all five EGFR-mutated cell lines showed high expression levels of BRG1, which suggested that loss of BRG1 would be mutually exclusive with EGFR mutations.

Immunohistochemical expression of BRG1 and BRM in primary lung adenocarcinoma tissues. Next, we used 93 cases of primary lung adenocarcinoma cases in our institution to examine the immunohistochemical expressions of BRG1 and BRM and their relationship with (i) histopathological subtypes, (ii) presence or absence of lepidic growth components, (iii) expressions of E-cadherin, TTF-1, CK7, and MUC1, (iv) genetic status of EGFR and KRAS, and (v) activation levels of EGFR and MET.

Overall, in the large majority of cases (>80%), nuclear staining for BRG1 and BRM was observed in cancer cells (Figs 2,3). Stromal cells constantly stained positive for BRG1 and BRM, and thus served as excellent internal positive controls. Using the criteria described in the Methods (Doc. S1), 11 cases (12%) were judged as showing low expression levels of BRG1 and 16 cases (17%) as showing low expression levels of BRM. Five cases (5%) showed low expression levels of both BRG1 and BRM. Most of the BRG1-low cases were either completely negative or showed only scattered positive staining for BRG1. In contrast, BRM showed a more heterogeneous staining pattern, typically showing strong positive staining in lepidic growth components, while showing negative or weak staining in invasive high-grade components (Fig. 4A–C).

Table 2 shows correlations between the expression levels of BRG1 and BRM and histopathological subtypes. All cases of well differentiated adenocarcinomas, that is, adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA), showed positive immunostaining for both BRG1 and BRM (20 of 20, 100%; Figs 2A,3A). Moderately differentiated adenocarcinomas, that is, acinar or papillary adenocarcinoma, also frequently showed positive immunostaining for both BRG1 and BRM (37 of 46, 80%; Figs 2B,3B), while some of them showed loss of either BRG1 or BRM (9 of 46, 20%; Figs 2D,3D). In contrast to these well- to moderately-differentiated tumors, poorly-differentiated adenocarcinomas (solid adenocarcinomas) frequently showed loss of expression of either BRG1 or BRM (12 of 13, 92%; Figs 2C,3C). Most cases (4 of 5, 80%) with loss of both BRG1 and BRM showed solid morphology (Table 2). One of three cases (33%) of invasive mucinous adenocarcinoma showed loss of BRG1.

We also examined correlations between the expression levels of BRG1 and BRM and the presence or absence of lepidic growth components (Table 3). Strikingly, all cases with BRG1 loss were devoid of lepidic growth components, while 6 of 16 cases with BRM loss showed lepidic growth components (Table 3).

Table 4 shows correlations between the expression levels of BRG1 and BRM and that of bronchial epithelial markers (TTF-1, CK7, and, MUC1) and E-cadherin. The expressions of TTF-1, CK7, MUC1 (membranous expression), and E-cadherin were frequently reduced in cases with loss of BRG1 and BRM (shown in Fig. S2). In particular, loss of E-cadherin and TTF-1 was remarkably correlated with loss of BRG1; all but one case of E-cadherin-low tumors showed BRG1 loss and all cases with BRG1 loss showed low expression levels of TTF-1. Depolarized expression of MUC1 was also frequent in cases with loss of BRG1 and BRM.

Table 4 also shows correlations between the expression levels of BRG1 and BRM and genetic status of EGFR and KRAS. Mutually exclusive correlations were observed between EGFR mutations and BRG1 loss ($P = 0.0006$), but no significant correlations between EGFR mutations and BRM loss ($P = 0.3382$). KRAS mutations were sometimes harbored by cases with loss of BRG1 or BRM.

We also examined the expressions of phospho-EGFR and phospho-MET and compared them with the expressions of BRG1 and BRM (Table 4). Low phosphorylation levels of EGFR were significantly correlated with loss of BRG1 and BRM ($P = 0.0003$, $P < 0.0001$, respectively). Phosphorylation

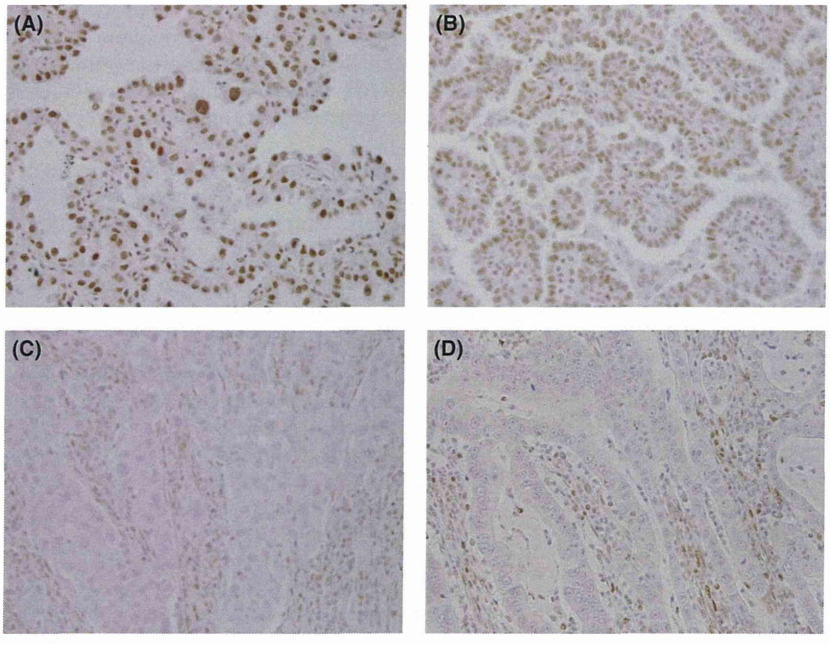

Fig. 2. BRG1 expressions in lung adenocarcinomas. Overall, more than 80% of cases showed positivity for BRG1. (A) Lepidic growth components showed strong immunoreactivity for BRG1. (B) Moderately differentiated adenocarcinomas, such as papillary adenocarcinoma, frequently showed positivity for BRG1. (C) Solid adenocarcinomas with mucin were frequently negatively stained for BRG1. (D) Some cases with papillary or acinar morphology showed negative staining for BRG1. Note BRG1 positivity in stromal cells.

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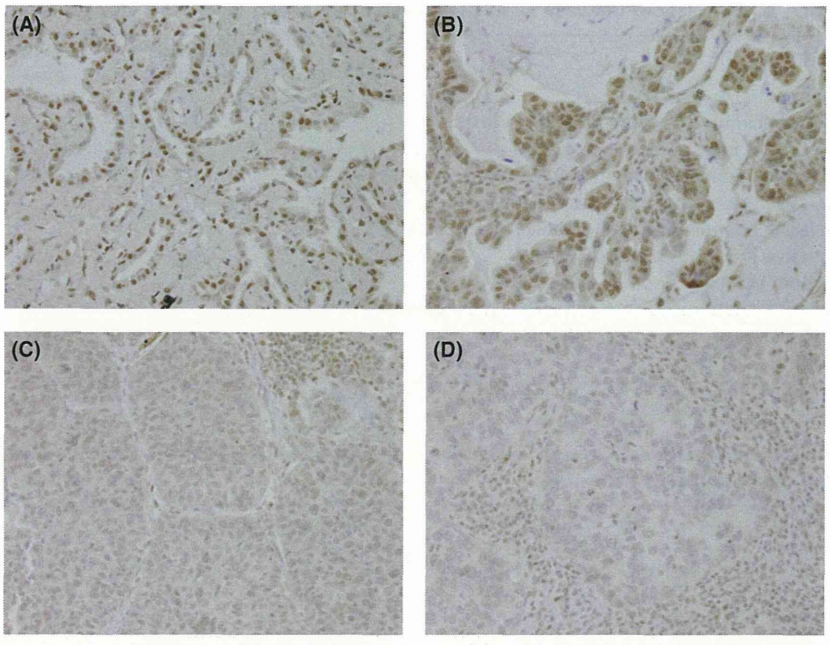

Fig. 3. BRM expressions in lung adenocarcinomas. Overall, more than 80% of cases showed positivity for BRM. (A) Lepidic growth components showed strong immunoreactivity for BRM. (B) Moderately differentiated adenocarcinomas, such as papillary adenocarcinoma, also often show positivity for BRM. (C) Solid adenocarcinoma with mucin frequently showed negative or weak staining for BRM. (D) Some cases with papillary or acinar morphology show negative staining for BRM. Note BRM positivity in stromal cells.

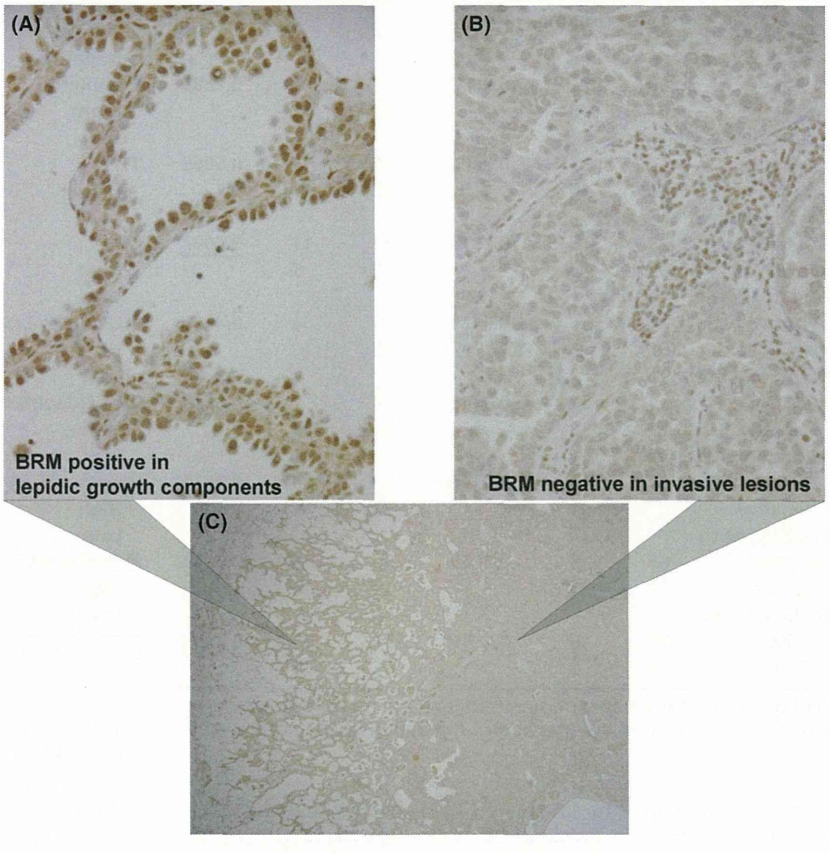

Fig. 4. Heterogeneous BRM expression in lung adenocarcinoma. (A) High-power field of lepidic growth components, which show strong positivity for BRM. (B) High-power field of invasive acinar components, which show negative positivity for BRM. (C) Low-power field of invasive adenocarcinoma with lepidic growth components; left side shows lepidic growth components, and right side shows invasive acinar components.

of MET tended to be low in cases with loss of BRG1 and BRM, but the difference was not significant.

BRM loss was significantly more frequent in heavy smokers and in cases with vessel invasion ($P = 0.0093$ and $P = 0.0002$, respectively; Table 3). BRG1 loss was significantly correlated with pleural invasion and pleural dissemination ($P = 0.0471$ and $P = 0.0449$, respectively; Table 3).

Prognostic significance of the expressions of BRG1 and BRM. Lastly, we performed hierarchical cluster analysis using the publicly available data of 442 primary lung adenocarcinoma cases,[15] based on the gene expressions of BRG1, BRM, TTF-1, MUC1, CK7, E-cadherin, and vimentin. Results are shown in Figure 5(A). Principally, primary lung adenocarcinoma cases could be classified into two groups: (i) tumors

Table 2. Correlations between expression levels of BRG1 and BRM and histopathological subtypes of primary lung adenocarcinomas

	BRG1 high BRM high	BRG1 low BRM high	BRG1 high BRM low	BRG1 low BRM low	Total
Non-mucinous adenocarcinoma *in situ*	8	0	0	0	8
Minimally invasive adenocarcinoma	12	0	0	0	12
Invasive adenocarcinoma, lepidic predominant	9	0	0	0	9
Invasive adenocarcinoma, acinar predominant	8	0	2	1	11
Invasive adenocarcinoma, papillary predominant	29	3	3	0	35
Invasive mucinous adenocarcinoma	2	1	0	0	3
Colloid adenocarcinoma	1	0	0	0	1
Invasive adenocarcinoma, micropapillary predominant	1	0	0	0	1
Invasive adenocarcinoma, solid predominant	1	2	6	4	13
Total	71	6	11	5	93

Table 3. Correlations between expression levels of BRG1 and BRM and clinico-pathological factors

	BRG1 expression			BRM expression		
	High	Low	*P*-value	High	Low	*P*-value
Pathological stage						
I	60	7	0.5082	57	10	0.3499
II + III + IV	22	4		20	6	
T-stage						
T1	52	5	0.2508	48	9	0.6492
T2, T3, T4	30	6		29	7	
Nodal involvement†						
Positive	20	1	0.3608	17	4	0.7381
Negative	61	8		58	11	
Lymphatic invasion						
Positive	22	1	0.2004	18	5	0.5066
Negative	60	10		59	11	
Vessel invasion						
Positive	23	5	0.2373	17	11	0.0002
Negative	59	6		60	5	
Pleural invasion						
Positive	21	6	0.0471	21	6	0.4122
Negative	61	5		56	10	
Dissemination						
Positive	3	2	0.0449	4	1	0.8648
Negative	79	9		73	15	
Pulmonary metastasis						
Positive	4	0	0.4540	4	0	0.3514
Negative	78	11		73	16	
Lepidic growth						
Present	65	0	<0.0019	59	6	0.0019
Absent	17	11		18	10	
Smoking Index						
≤600	26	6	0. 1344	22	10	0.0093
>600	56	5		55	6	

†Pathological N-factors were not determined for three cases of stage IV patients with pleural dissemination. Underlined values are $P < 0.05$.

Table 4. Correlations between expression levels of BRG1 and BRM and genetic status of EGFR and KRAS and expression levels of E-cadherin, TTF-1, CK7, MUC1, phopsho-MET, and phospho-EGFR

	BRG1 expression			BRM expression		
	High	Low	*P*-value	High	Low	*P*-value
EGFR mutations						
Positive	45	0	0.0006	39	6	0.3382
Negative	37	11		38	10	
KRAS mutations						
Posivite	5	2	0.1537	4	3	0.0615
Negative	77	9		73	13	
E-cadherin						
High	81	3	<0.0001	72	12	0.0227
Low	1	8		5	4	
TTF-1						
High	62	0	<0.0001	57	5	0.0010
Low	20	11		20	11	
CK7						
High	74	4	<0.0001	67	11	0.0707
Low	8	7		10	5	
MUC1(membranous)						
High	63	3	0.0007	62	4	<0.0001
Low	19	8		15	12	
MUC1(depolarized)						
High	7	3	0.0596	4	6	0.0001
Low	79	8		73	10	
Phospho-EGFR						
High	69	4	0.0003	67	6	<0.0001
Low	13	7		10	10	
Phospho-MET						
High	19	2	0.7102	19	2	0.2892
Low	63	9		28	14	

Underlined values are $P < 0.05$.

showing high expression levels of TTF-1, MUC1 and E-cadherin, and low expression levels of vimentin, and (ii) tumors showing low expression levels of TTF-1, MUC1 and E-cadherin, and high expression levels of vimentin (Fig. 5A). High expression levels of both BRG1 and BRM were frequently seen in the former, while low expression levels of either of or both BRG1 and BRM were frequently seen in the latter (Fig. 5A). These results confirm and reinforce data from cancer cell lines and primary lung adenocarcinoma cases in our institution.

To ascertain the prognostic significance of the expressions of BRG1 and BRM in lung adenocarcinoma, we undertook a survival analysis using the Kaplan–Meier method. We separated 442 lung adenocarcinoma cases into three groups based on the gene expression levels of BRG1 and BRM; (i) cases with high expression (more than or equal to the average); (ii) cases with moderate expression (under the average, and more than or equal to half the average); and (iii) cases with low expression (under half the average). Results are shown in Figure 5(B). High expression of BRG1 and BRM both correlated significantly with better prognosis. Figure 5(C) also shows patient survival curves for the four groups: (i) BRG1-high and BRM-high; (ii) BRG1-high and BRM-low; (iii) BRG1-low and BRM-high; and (iv) BRG1-low and BRM-low. The BRG1-low and BRM-low group showed significantly poorer prognosis than the other groups.

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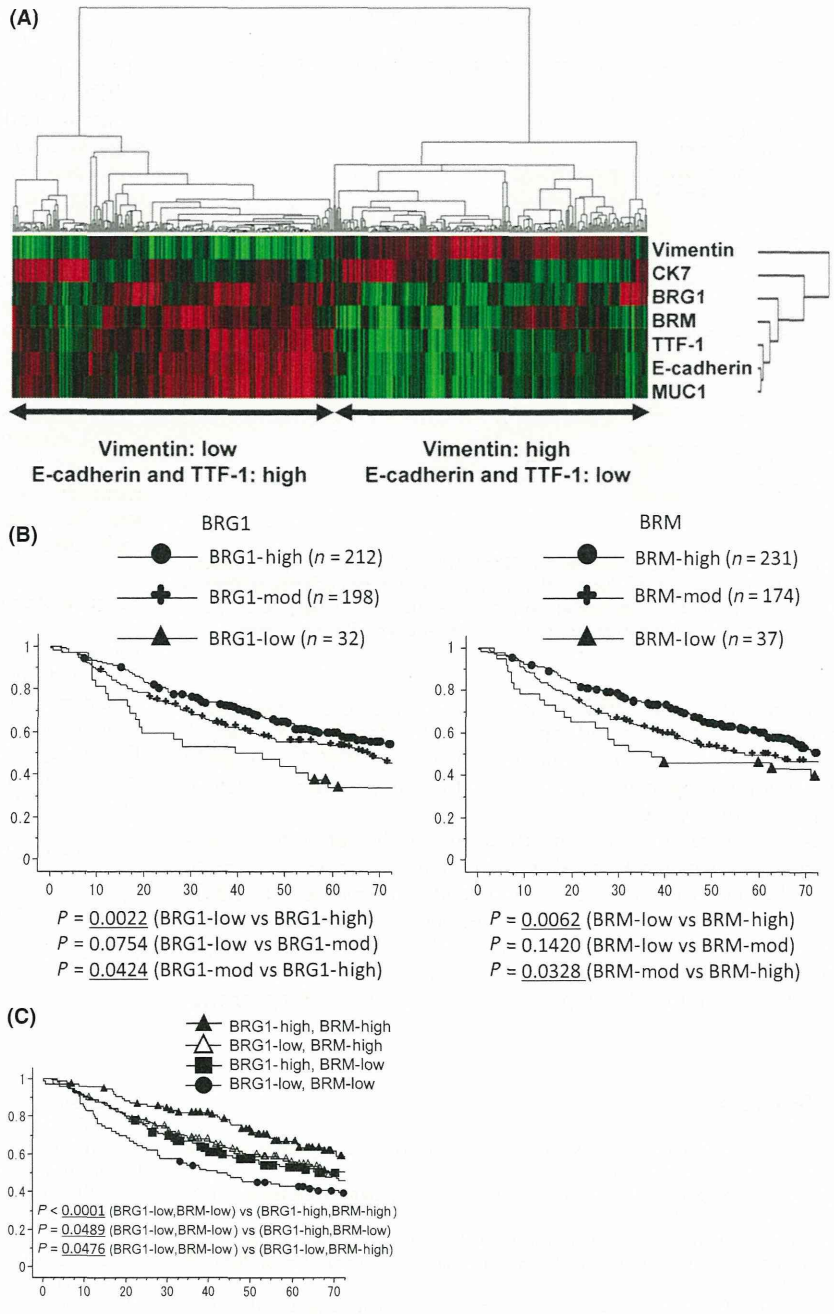

Fig. 5. Analysis of the publicly available data of 442 primary lung adenocarcinoma cases. (A) Hierarchical cluster analysis using the gene expressions of vimentin, CK7, BRG1, BRM, TTF-1, E-cadherin, and MUC1. (B) Patient survival according to the expression levels of BRG1 and BRM. Lung adenocarcinoma cases were separated into three groups based on gene expression levels of BRG1 and BRM: (i) cases with high expression (more than or equal to the average); (ii) cases with moderate expression (under the average, and more than or equal to half the average); and (iii) cases with low expression (under half the average). Left panel shows patient survival curves with high expression levels of BRG1 (BRG1-High), moderate expression levels of BRG1 (BRG1-Mod), and low expression levels of BRG1 (BRG1-Low). Right panel shows patient survival curves with high expression levels of BRM (BRM-High), moderate expression levels of BRM (BRM-Mod), and low expression levels of BRM (BRM-Low). (C) Patient survival according to the expression pattern of BRG1 and BRM. Patients were separated into four groups according to the expression pattern of BRG1 and BRM as follows: cases with high expression levels of both BRG1 and BRM (BRG1-High, BRM-High), cases with high expression levels of BRG1 and moderate or low expression levels of BRM (BRG1-High, BRM-Low), cases with moderate or low expression levels of BRG1 and high expression levels of BRM (BRG1-Low, BRM-High), the cases with moderate or low expression levels of both BRG1 and BRM (BRG1-Low, BRM-Low).

Discussion

This is, to our knowledge, the first report demonstrating the tight correlation between loss of BRG1 and BRM and EMT in cancer. Results of this study also confirm and reinforce our previous data that loss of the bronchial epithelial phenotype occurs in lung adenocarcinomas with EMT features.[13]

Recent studies show that loss of another component of the SWI/SNF chromatin remodeling complex, BAF250A (ARID1A), was frequent in high-grade endometrial carcinomas and clear cell carcinomas of the ovary[16] and that loss of the BAF250A protein correlates with the ARID1A mutation status.[17,18] Interestingly, there appears to be a similarity between loss of BRG1 and that of ARID1A; both tend to occur in high-grade tumors or in tumors with an altered epithelial phenotype.

Another interesting finding of this study was that features of TRU type lung adenocarcinomas,[14] that is, lepidic growth features, high expression levels of the TTF-1 protein, and EGFR mutations were absent in all cases with loss of the BRG1 protein. In tumors with BRG1 loss, BRG1 protein expression was typically absent in almost all cancer cells. These results suggest that loss of BRG1 occurs at an early step of carcinogenesis of lung adenocarcinoma with the mesenchymal-like phenotype, that is, a subset of non-TRU type lung adenocarcinomas with EMT features.

All cases with concomitant loss of BRG1 and BRM were devoid of lepidic growth components, harbored no EGFR mutations, and correlated more with solid adenocarcinoma morphology than a single loss of BRG1 or BRM, which suggested that loss of BRM may also occur in a subset of the mesenchymal-like phenotype, simultaneously with, or subsequently to, loss of BRG1, and may accelerate poorer

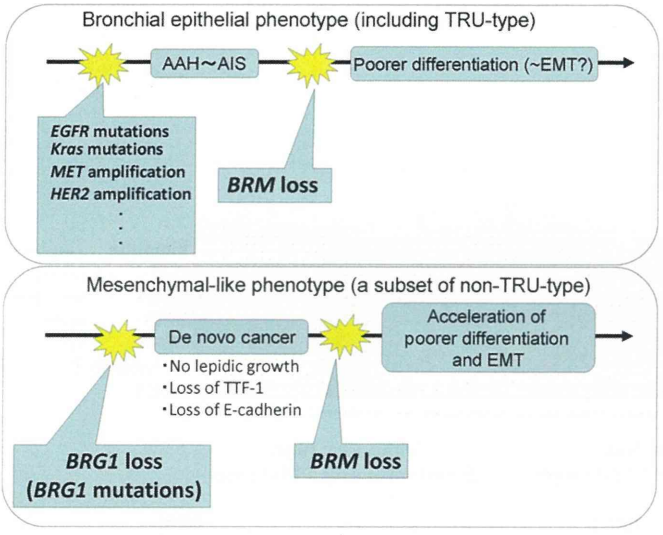

Fig. 6. Hypothetical schemes of BRG1 and BRM loss in the development of two types of lung adenocarcinomas: lung adenocarcinoma with the bronchial epithelial phenotype (upper panel) and lung adenocarcinoma with mesenchymal-like phenotype (lower panel).

differentiation and EMT and lead to the more malignant phenotype. The survival analysis of Shedden's data, which showed that cases with concomitant loss of BRG1 and BRM had poorer prognosis than cases with a single loss of BRG1, supports this hypothesis.

BRM expression was positive in lepidic growth components, but was weak or absent in invasive poorer differentiated lesions, such as solid components. In contrast to BRG1 loss, BRM loss may occur during the progressions of lung adenocarcinomas with the bronchial epithelial phenotype. Figure 6 shows our hypothetical schemes for BRG1 and BRM loss in the development of two types of lung adenocarcinomas: lung adenocarcinomas showing the bronchial epithelial phenotype and those showing the mesenchymal-like phenotype.

BRG1 and BRM regulate a broad range of genetic programs, including cell differentiation and proliferation, and it has been suggested that SWI/SNF complexes may dictate lineage-specific chromatin remodelling functions and act as master regulators of the master regulators.[4] Thus, although the exact mechanism by which loss of BRG1 and BRM leads to tumor development and EMT is unknown, loss of BRG1 and BRM may cause uncontrolled cellular proliferation and disrupt the differentiation program of bronchial epithelial cells,[19] resulting in formation of tumors with loss of expression of CK7, MUC1, and TTF-1. Why BRG1 loss occurs exclusively in the progression of EGFR wild-type tumor is currently unknown. One speculation could be that the simultaneous presence of EGFR mutation and BRG1 loss is for some reason incompatible with survival of cancer cells. Finally, we speculate that epigenetic therapy aiming to restore the functions of BRG1 and BRM would be a possible new therapy for treating tumors with EMT features.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Gene expression data of 19 cell lines.

Doc. S1. Supporting information about materials and methods.

Fig. S1. Gene and protein level expressions of BRG1, BRM, etc. of EGFR-mutated cell lines.

Fig. S2. Immunostaining for BRG1, BRM and E-cadherin in serial sections.

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ORIGINAL RESEARCH

Heterogeneity of the *EGFR* mutation status between the primary tumor and metastatic lymph node and the sensitivity to *EGFR* tyrosine kinase inhibitor in non-small cell lung cancer

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Abstract The purpose of this study was to clarify the distribution of epidermal growth factor receptor (*EGFR*) mutations between primary tumors (PT) and metastatic lymph node (MLN) in patients with resected non-small cell lung cancer (NSCLC) and to identify a better predictive marker of the response to *EGFR* tyrosine kinase inhibitor (EGFR-TKI). We conducted a retrospective review of the data of 70 lung cancer patients with lymph node metastasis who underwent surgical resection. Analysis to detect *EGFR* mutations was performed by a peptide nucleic acid–locked nucleic acid polymerase chain reaction clamp method. *EGFR* mutations were detected in 15.7 % of both the PT and MLN and in 14.3 % of the PT only. The response rate to EGFR-TKI tended to be higher in patients with *EGFR* mutations in the MLN, as all patients with *EGFR* mutations in the MLN showed disease control to treatment with EGFR-TKI. Our results demonstrated that the *EGFR* mutation status of MLN is a predictive marker of the response to EGFR-TKI therapy in patients with recurrent NSCLC after surgical resection.

Keywords Epidermal growth factor receptor mutation · Non-small cell lung cancer · Metastatic lymph node · *EGFR* tyrosine kinase inhibitor

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Introduction

Lung cancer is a leading cause of cancer-related death worldwide. The most effective treatment of early stage (IA–IIIA) non-small cell lung cancer (NSCLC) is surgical resection. In addition, adjuvant chemotherapy after the resection of stage II–IIIA NSCLC is now the "standard of care" based on the results of three large-scale phase III trials and individual patient meta-analyses [1–4]. However, up to 60 % of patients with NSCLC with lymph node metastasis show relapse after surgery [5, 6].

The prognosis of patients with NSCLC is critically dependent on the extent of metastatic spread of the tumor cells at the time of surgery. Tumor cells acquire the metastatic phenotype through the process of clonal evolution occurring during the multistep process of tumor progression [7]. Significant progress in the understanding of the biology and molecular mechanisms of lung cancer has allowed some new molecular targeted therapies to be developed. The most well-known molecular target is mutation of the epidermal growth factor receptor (EGFR). *EGFR* tyrosine kinase inhibitors (EGFR-TKIs) have been shown to have a dramatic clinical effect in a significant proportion of patients with NSCLC [8]. In 2004, such response to EGFR-TKIs was identified to be related to the presence of some type of gene mutation in the tyrosine kinase domain of *EGFR* [9, 10]. *EGFR* mutations associated with clinical sensitivity to EGFR-TKIs have been shown to occur more frequently in lung cancer patients who are females, nonsmokers, Asians, and have adenocarcinoma [11, 12].

Several studies in Asia have shown that patients with *EGFR*-mutated lung cancers show objective response rates of 70–80 % to treatment with EGFR-TKIs, while 20–30 %

of these patients show no response to EGFR-TKI therapy [13–15]. In most studies, the *EGFR* mutation status has been determined based on analysis of the primary tumors (PT). In general, the EGFR-TKIs resistance in a proportion of patients with *EGFR* mutations is explained by the T790M mutation [16]. In addition, it has been speculated that the absence of response to EGFR-TKIs might be due to the discordance of the *EGFR* mutation status between the PT and the metastatic lesions.

Thus, to investigate the heterogeneity of the *EGFR* mutation status between the PT and metastatic lesions, we examined the *EGFR* mutation patterns in PT and the corresponding local metastatic lymph nodes (MLN) using resected samples. The purpose of this study was to clarify the existence of any discordant *EGFR* mutation patterns between the PT and MLN and the clinical usefulness of *EGFR*-targeted therapies in the treatment of patients with NSCLC.

Patients and methods

Study population

Seventy patients of NSCLC with lymph node metastasis who were treated by surgical resection with systematic lymph node dissection at the Kawasaki Medical School Hospital between 2004 and 2010 were enrolled in this study. None of the patients had received radiation therapy prior to the surgery. The histological diagnosis of the tumors was based on the criteria of the World Health Organization, and the TNM stage was determined according to the criteria in 2009. Informed consent was obtained from each patient for the study of tissue samples from the resected surgical specimens. This study was conducted with the approval of the institutional ethics committee of the Kawasaki Medical School.

EGFR mutation analysis

Analysis to detect *EGFR* mutations was performed in the resected, paraffin-embedded lung cancer tissues (the PT and one of their corresponding local MLN) by the peptide nucleic acid–locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp method [17]. In this study, the PNA-LNA PCR clamp assay was performed at the Mitsubishi Kagaku Bio-Clinical Laboratories, Inc.

Drug administration

Sixteen patients received oral gefitinib treatment for disease recurrence, at the dose of 250 mg once daily. These patients were continued on gefitinib therapy until the detection of disease progression or the development of intolerable toxicity.

Assessment of response Tumor recurrences were diagnosed based on the findings on imaging, including thoracic computed tomography, fluorodeoxyglucose positron emission tomography, and brain magnetic resonance imaging, and not necessarily confirmed by histopathology. Objective tumor response and its duration were assessed according to the RECIST criteria, and the response of all the responders was still confirmed to be valid more than 4 weeks after the initial assessment of the response [18].

Statistical analysis

`Statistical analysis was performed for examining the significances of the differences among the groups and any possible correlations between presence/absence of *EGFR* gene mutations and the clinicopathological features using Fisher's exact test or chi-square test, as appropriate. An unpaired *t* test was used for the comparison of continuous data. All the statistical analyses were conducted using the SPSS software (Version 17.0; SPSS Incorporation, Chicago, IL, USA). All statistical

Table 1 The patient characteristics

Characteristics	Number of patients	Percent
Age (years)		
<70	30	42.9
≥70	40	57.1
Sex		
Male	46	65.7
Female	24	34.3
Smoking status		
Smoker	48	68.6
Never smoker	22	31.4
Histology		
Adenocarcinoma	35	50.0
Squamous cell carcinoma	24	32.9
Adenosquamous carcinoma	5	6.8
Large cell carcinoma	4	5.5
Pleomorphic carcinoma	2	2.7
Pathological lymph node status		
pN1	29	39.7
pN2	41	60.3
Neoadjuvant chemotherapy		
Yes	7	10.0
No	63	90.0
Adjuvant chemotherapy		
Yes	41	58.6
No	29	41.4

Table 2 *EGFR* mutation status of the PT and MLN

PT	Lymph node	
	Mutant	Wild type
Mutant	11	10
Wild type	0	49

tests were two-sided, and probability values of <0.05 were regarded as denoting statistical significance.

Results

Clinical characteristics

The characteristics of the patients are summarized in Table 1. The patients ranged in age from 37 to 83 years (mean, 69.1 years), and there were 46 men and 24 women. The majority of patients (35, 50.0 %) had adenocarcinoma, while 24 (32.9 %) had squamous cell carcinoma, 5 (6.8 %) had adenosquamous carcinoma, 4 (5.5 %) had large cell carcinoma, and 2 (2.7 %) had pleomorphic carcinoma. Pathological N1 disease was confirmed in 29 (39.7 %) patients and N2 disease in 41 (60.3 %) patients.

EGFR mutation status of the PT and the lymph node metastases

EGFR gene mutations were found in the PT of 21 (31.5 %) patients and MLN of 11 (15.1 %) patients. All patients with *EGFR* mutations in the MLN also had *EGFR* mutations in the PT. Of 21 patients with *EGFR* mutations in the PT, 10 showed no *EGFR* mutations in the MLN. Of the 49 patients who were *EGFR* mutation-negative in the PT, none showed mutations in the MLN either (Table 2).

Relation between the mutation status of *EGFR* and the clinicopathological characteristics

We categorized the 70 patients into three groups according to the presence/absence of *EGFR* mutations in the PT and MLN, as follows: group 1 (n=11): *EGFR* mutation-positive in both the PT and MLN; group 2 (n=10): *EGFR* mutation-positive only in the PT; group 3 (n=49): *EGFR* mutation-negative in both the PT and MLN. Group 3 had a higher proportion of men ($p<0.001$), current smokers ($p<0.001$), and patients with squamous cell carcinoma ($p<0.001$) than group 1+2, while no significant association of the *EGFR* mutation-

Table 3 Association of *EGFR* mutation and clinicopathological variables

Characteristics	*EGFR* mutation status (PT/MLN)				
	Group1	Group2		Group3	
	Mut/Mut	Mut/WT	p value (G1 vs. G2)	WT/WT	p value (G3 vs. G1+2)
Patients, number	11	10		49	
Age (mean), years	72.2	66.9	0.142	68.9	0.745
Sex			0.149		<0.001
Male	1	4		41	
Female	10	6		8	
Smoking status			0.311		<0.001
Smoker	1	3		44	
Never smoker	10	7		5	
Histology			0.366		<0.001
Adenocarcinoma	10	9		16	
Squamous cell	0	1		23	
Adenosquamous	1	0		4	
Large cell	0	0		4	
Pleomorphic	0	0		2	
Tumor size (mean), mm	34.7	37.3	0.734	37.8	0.657
Pathological nodal status			0.387		0.540
pN1	6	3		20	
pN2	5	7		29	

Mut mutant, *WT* wild type

Table 4 Response to EGFR-TKI: patients

EGFR mutation status					
Case	Age/sex	Histology	PT	Lymph node	Response
1	70/F	AS	Exon 19 del	Exon 19 del	SD
2	60/F	AD	L858R	L858R	PR
3	69/M	AD	L858R	L858R	SD
4	65/F	AD	L858R	L858R	CR
5	73/F	AD	L858R	L858R	PR
6	76/F	AD	L858R	L858R	PR
7	81/F	AD	Exon 19 del	Exon 19 del	PR
8	78/F	AD	Exon 19 del	Exon 19 del	PR
9	59/F	AD	L858R	L858R	SD
10	57/F	AD	Exon 19 del	WT	PD
11	66/F	AD	Exon 19 del	WT	PR
12	75/M	AD	Exon 19 del	WT	CR
13	53/M	AD	Exon 19 del	WT	CR
14	61/F	AD	L858R	WT	PR
15	74/F	AD	L858R	WT	PD
16	77/F	AD	L858R	WT	PD

AD adenocarcinoma, *AS* adenosquamous carcinoma

negative status was observed with age (p=0.745), tumor size (p=0.657), or pathological lymph node status (N1 or N2) (p=0.540) (Table 3). No significant associations with any clinicopathological characteristics were observed in group 1 and group 2 (Table 3).

Response to EGFR-TKI

Of the 21 patients in group 1 and group 2, 16 patients were treated with gefitinib. All the nine patients in group 1 showed disease control (complete response+partial response+stable disease). On the other hand, of the seven patients in group 2, 4 (57.1 %) showed disease control and the remaining three showed progressive disease. The disease control rate tended to be higher in group 1 than in group 2 (p=0.062) (Tables 4 and 5).

Table 5 Response to EGFR-TKI: association of *EGFR* mutation and response to EGFR-TKI

EGFR mutation (PT/MLN)			
Response	Mut/Mut	Mut/WT	p value
CR+PR+SD	9	4	0.062
PD	0	3	

Mut mutant, *WT* wild type

Discussion

This study demonstrated the existence of discordance of the *EGFR* mutation status between the PT and MLN in patients with NSCLC. This is the first report of the investigation of the *EGFR* mutation gene status using the PNA-LNA PCR clamp assay and of the sensitivity of NSCLC patients with postoperative recurrence to treatment with EGFR-TKI in relation to the *EGFR* mutation status.

To date, the heterogeneity of the distribution of *EGFR* mutations is still controversial. Several reports have described the discordance of *EGFR* mutation status in NSCLC patients between the PT and metastatic tumors [19, 20], or even among parts of the PT [21–23]. On the other hand, a few reports have also suggested that such heterogeneous distribution of *EGFR* mutations is rare [24]. Therefore, the issue of discordance of the *EGFR* mutation status between the PT and metastatic sites, including the MLN, in NSCLC patients remains under debate. The reason for the discordant expression of *EGFR* mutations between the primary and metastatic lesions also remain controversial, although recent research suggests the following: (1) presence of intratumoral heterogeneity of *EGFR* mutations, (2) occurrence of changes in *EGFR* mutations during the course of disease progression, and (3) technical limitations in the methods used for the assessment of *EGFR* mutations. As for intratumoral heterogeneity, Yatabe et al. clearly demonstrated, using the Cycleave PCR™ method, that intratumoral heterogeneous distribution of the *EGFR* mutations is extremely rare [24]. As for the occurrence of changes in *EGFR*

mutations during the course of disease progression, Ji et al. demonstrated that *EGFR* mutations represent an early event in the pathogenesis of lung adenocarcinoma [25]. Thus, the discrepancy of the *EGFR* mutation status between the PT and metastatic lesions in NSCLC patients would also seem to depend on the method used for the assessment of *EGFR* mutations.

Over the past 3 years, several methods have been proposed for the detection of *EGFR* mutations. Previously, the direct sequencing method was usually used; however, the sensitivity of direct sequencing was suboptimal for clinical tumor samples and mutation detection by direct sequencing was associated with a higher frequency of false-negative results. In the literature, the PNA-LNA clamp method, Scorpion amplification refractory mutation system method, mutant-enriched PCR method, and Cycleave PCR™ method have been reported to be more sensitive than the direct sequencing method. To date, most reports describing discordance in the *EGFR* mutation status between the PT and metastatic tumors have used the direct sequencing method. For example, Park et al. demonstrated, by using heteroduplex analysis, that a considerable proportion of NSCLC patients showed discrepancy in the *EGFR* mutation status between the PT and MLN [19]. In their series, ten cases were *EGFR* mutation-negative in the MLN while being mutation-positive in the PT and seven cases were *EGFR* mutation-positive in the MLN while being mutation-negative in the PT. However, our results demonstrated that all patients with *EGFR* mutations in the MLN had *EGFR* mutations in the PT. This difference was considered to be a consequence of the method of assessment of the *EGFR* mutation. We believed that our results were highly reliable for the assessment of *EGFR* mutations, as the PNA-LNA clamp assay has been shown as a high-sensitivity detection method.

Our results demonstrated a tendency towards the existence of associations between the *EGFR* mutation status in the PT/MLN and clinical response to treatment with EGFR-TKI. Especially, the response rate to EGFR-TKI tend to be higher in patients with *EGFR* mutations in the MLN, and all patients with *EGFR* mutations in the MLN showed disease control to EGFR-TKI therapy. On the other hand, only half of patients with *EGFR* mutations in the PT but not in the MLN showed disease control to EGFR-TKI therapy. Thus, heterogeneity in the expression of *EGFR* mutations in the PT and MLN would appear to be correlated with the initial response to gefitinib. This is the first report of the investigation of the associations between heterogeneity of the *EGFR* mutation status in the PT/MLN and the clinical response to EGFR-TKIs.

Conclusion

In conclusion, the *EGFR* mutation status is often discordant between the PT and MLN in patients with NSCLC, and this heterogeneity may explain the variable clinical responses of these patients to EGFR-TKI therapy. Our results demonstrated that the *EGFR* mutation status in the MLN may be a better predictive marker of the response to EGFR-TKI therapy in patients with recurrent NSCLC after surgical resection. Further study will be required to characterize the heterogeneity of the *EGFR* mutation status between the PT and metastatic lesions and its correlation with the clinical responses to EGFR-TKI therapy in patients with NSCLC.

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Conflict of interest The authors have no conflicts of interests to declare.

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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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Key words: polymorphisms, angiogenesis, vascular endothelial growth factor, delta-like ligand 4

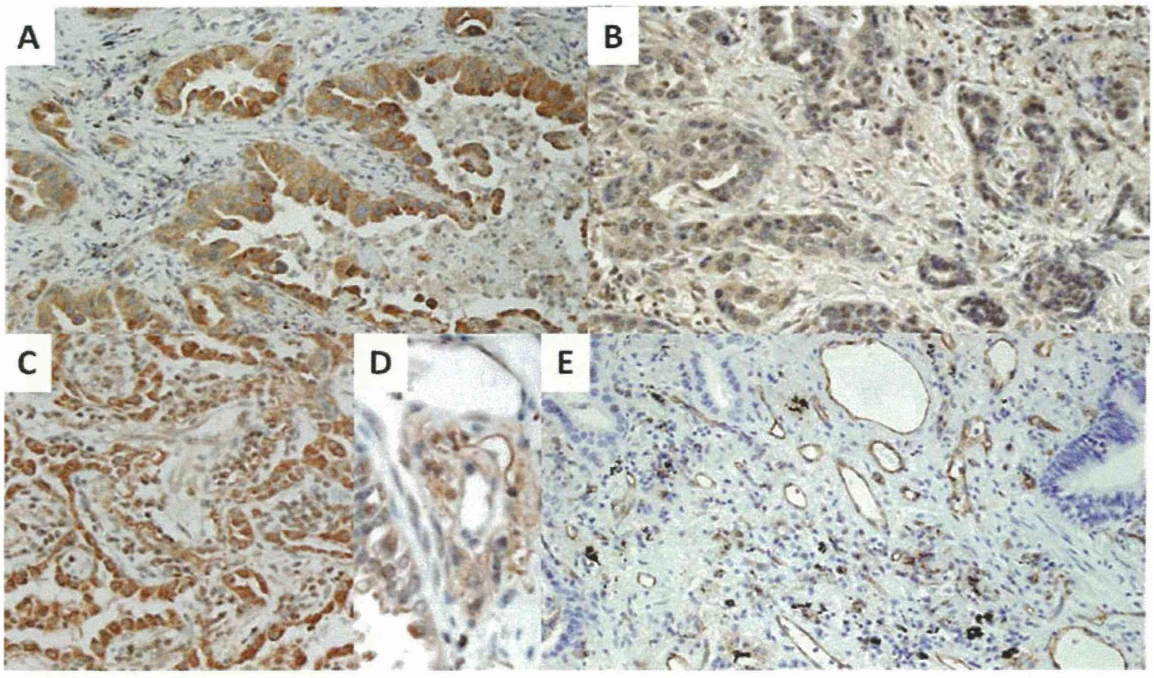

Figure 1. Positive immunohistochemical staining for (A) VEGF, (B) HIF-1α, (C) Dll4 (tumor cells), (D) Dll4 (endothelial cells), and (E) CD31 (for microvessel counting, x200 magnification).

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.

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.

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).

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.

Characteristic	*VEGF* +405 genotype				*VEGF* -460 genotype			
	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 Genotype	VEGF			HIF-1α			Dll4		
	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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ORIGINAL ARTICLE - THORACIC

Post-recurrence survival of patients with non-small-cell lung cancer after curative resection with or without induction/adjuvant chemotherapy

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Abstract

OBJECTIVES: Recently, the prognosis of patients with non-small-cell lung cancer (NSCLC) has improved, thanks to the standardization of adjuvant chemotherapy and the introduction of molecular-targeted drugs, notably epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors and other new anti-cancer agents. However, the survival characteristics and prognosis of patients with recurrent NSCLC after curative resection are not well understood.

METHODS: Of the 430 consecutive patients with NSCLC who underwent complete surgical resection at our institution between January 2004 and July 2011, we included 76 patients with recurrence whose post-recurrence treatment and outcome could be confirmed. We then retrospectively evaluated the effect of prognostic factors on post-recurrence survival.

RESULTS: There were 50 men and 26 women, and the median age at recurrence was 74.5 years. The median time from surgical resection to recurrence was 12.7 months. Thirty-eight of the 76 (50%) patients underwent multimodality treatment with surgery and preoperative and/or postoperative chemotherapy as their initial treatment. For recurrence, systemic chemotherapy was administered to 64 (84%) patients, and the disease control rate for first-line chemotherapy was 55%. The 1- and 2-year post-recurrence survival rates were 68.3 and 45.8%, respectively, and the median post-recurrence survival time was 17.7 months. Six independent prognostic factors were identified: wild-type EGFR, no adjuvant chemotherapy for the primary lung cancer, age ≥80 years at recurrence, a poor Eastern Cooperative Oncology Group performance status at recurrence, symptomatic at recurrence and no systemic chemotherapy for recurrence, which significantly decreased the post-recurrence survival.

CONCLUSIONS: The prognosis of patients with NSCLC recurrence after surgery is currently improving. Our results suggested two new prognostic factors, adjuvant chemotherapy and EGFR mutations, neither of which have been previously reported. Treatment strategies for postoperative recurrence should be established based on a more detailed subdivision of factors, such as histology and molecular markers, in the future.

Keywords: Non-small-cell lung cancer • Post-recurrence survival • Adjuvant chemotherapy

INTRODUCTION

The 5-year survival rates after curative resection for non-small-cell lung cancer (NSCLC) have improved remarkably from 52.6% for patients who underwent resection in 1994 [1] to 61.4% in 1999 [2] and 69.6% in 2004 [3]. This improvement is believed to be a consequence of the increase in the detection of small-sized lung cancers, thanks to improvements in diagnostic imaging, such as computed tomography (CT) and ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET), as well as other factors, such as the standardization of adjuvant chemotherapy. Several randomized controlled trials (RCTs) were reported in the first half of the 2000s, demonstrating the efficacy of adjuvant chemotherapy followed by complete surgical resection; now, this regimen has been accepted as the standard treatment for pathological Stages II and IIIA NSCLC [4–6]. Preoperative induction chemo- or chemoradiotherapy for superior sulcus tumours [7, 8] and resectable clinical Stage IIIA NSCLC with mediastinal lymph node metastasis [9–12] are also being proactively tested. Thus, attempts are now underway to improve the prognosis of Stage II or more advanced NSCLC with a high risk of recurrence using multimodality treatment, including surgery.

Although the rate of recurrence after the total resection of NSCLC varies according to the pathological stage, it is relatively high at 30–75%, and the prognosis remains poor [13, 14]. Most studies of the treatment for recurrent NSCLC after curative resection and its prognostic factors have investigated patients from before 2000, prior to the establishment of adjuvant

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