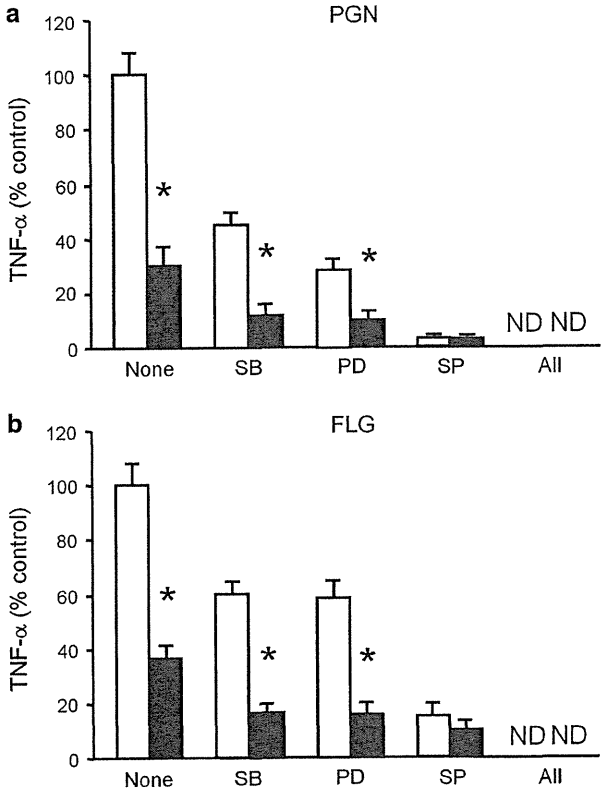

Fig. 5 Preferential inhibition of TNF-α production by JNK inhibitor in TLR2 or TLR5 ligands-stimulated AMs. AMs from pools of control mice (*open bars*) and diabetic mice (*solid bars*) were incubated with p38 inhibitor (SB203580, 10 μM), ERK1/2 inhibitor (PD98059, 50 μM), and/or JNK inhibitor (SP600125, 20 μM) for 30 min followed by stimulation with PGN (10 μg/ml) (**a**) or FLG (1 μg/ml) (**b**). After 18 h, supernatants were collected and levels of TNF-α were measured by ELISA. Data are expressed as mean ± SEM of four separate experiments performed in triplicate. $^{*}P < 0.05$ compared with respective control group; *ND* not detected

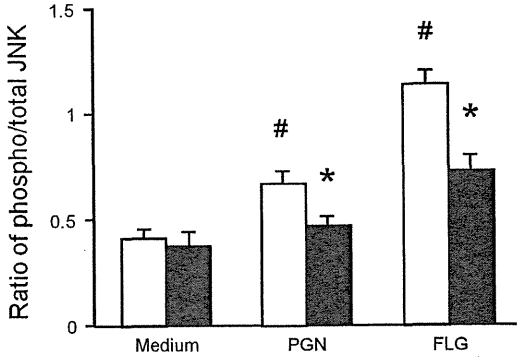

Fig. 6 Downregulation of TLR2 or TLR5 ligands-induced JNK kinase phosphorylation in AMs from diabetic mice. AMs from pools of control mice (*open bars*) and diabetic mice (*solid bars*) were cultured in medium alone or stimulated with PGN (10 μg/ml) or FLG (1 μg/ml) for 30 min. Phosphorylated and total JNK levels were detected by using a specific cell-based ELISA. The ratio of phospho:total JNK is expressed as mean ± SEM of four separate experiments. $^{*}P < 0.05$ compared with respective control group; $^{\#}P < 0.05$ compared with respective medium group

in AMs from diabetic mice, suggesting a contribution to the reduction of pro-inflammatory cytokine production of this pathway. Our results were contrary to reports by Iwata et al. [34] who showed high glucose-enhanced LPS-induced pro-inflammatory cytokine production via upregulation of JNK in THP-1 cells. The particular reason for this discrepancy remains unclear; however, we used primary AMs (as opposed to a monocytic cell line for Iwata et al.) and chose PGN or FLG stimulation (as opposed to LPS for Iwata et al.). Although further examinations are needed, there does not seem to be a question regarding JNK as one of the targets of hyperglycemic state-induced modulation of pro-inflammatory cytokine production.

Diabetes patients may be predisposed to increased morbidity and mortality of certain pulmonary infections, such as those caused by *Staphylococcus aureus*, *Streptococcus pneumonia*, *Mycobacterium tuberculosis*, and *Legionella pneumophila* [35]. TLR2 or TLR5 are indeed important in the recognition of such microorganisms. In fact, experiments using gene-deficient mice for these TLR2 and TLR5 clearly proved their essential role during in vivo pulmonary immune inflammatory response [36–40]. Moreover, recent clinical studies have shown the association of TLR2 and TLR5 polymorphism with susceptibility to infection with *M. tuberculosis* [41] and *L. pneumophia* [42], respectively. While the pathophysiology likely results from a combination of factors, the observed impairment of both TLR2 and TLR5 signaling in AMs may lead to an increased risk of certain pulmonary infections in diabetes. Additionally, recent studies have shown pro-inflammatory and immunostimulatory aspects of hyperglycemic state, leading to the development of many complications of diabetes [43–45]. In regard to host defense against bacterial infection, however, it seems important to understand how the hyperglycemia-stimulated host immune cells respond to further bacterial stimulation. Our study indicates that diabetic patients may not possess the ability in AMs to elicit initial proper cellular activation and inflammatory mediator production when faced with certain pulmonary infections.

In conclusion, we demonstrated that the hyperglycemic state impairs the responsive of AMs to multiple TLR ligands by inhibiting the production of pro-inflammatory cytokines. This effect may result from, at least in part, the alteration of intracellular signaling, including downregulation of JNK kinase phosphorylation induced by hyperglycemic state. The present study has thus delineated a pathogenic mechanism that may mediate the increased susceptibility of diabetic patients to pulmonary infections.

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Lung cancer with loss of BRG1/BRM, shows epithelial mesenchymal transition phenotype and distinct histologic and genetic features

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BRG1 and BRM, two core catalytic subunits in SWI/SNF chromatin remodeling complexes, have been suggested as tumor suppressors, yet their roles in carcinogenesis are unclear. Here, we present evidence that loss of BRG1 and BRM is involved in the progression of lung adenocarcinomas. Analysis of 15 lung cancer cell lines indicated that BRG1 mutations correlated with loss of BRG1 expression and that loss of BRG1 and BRM expression was frequent in E-cadherin-low and vimentin-high cell lines. Immunohistochemical analysis of 93 primary lung adenocarcinomas showed loss of BRG1 and BRM in 11 (12%) and 16 (17%) cases, respectively. Loss of expression of BRG1 and BRM was frequent in solid predominant adenocarcinomas and tumors with low thyroid transcription factor-1 (TTF-1, master regulator of lung) and low cytokeratin7 and E-cadherin (two markers for bronchial epithelial differentiation). Loss of BRG1 was correlated with the absence of lepidic growth patterns and was mutually exclusive of epidermal growth factor receptor (EGFR) mutations. In contrast, loss of BRM was found concomitant with lepidic growth patterns and EGFR mutations. Finally, we analyzed the publicly available dataset of 442 cases and found that loss of BRG1 and BRM was frequent in E-cadherin-low, TTF-1-low, and vimentin-high cases and correlated with poor prognosis. We conclude that loss of either or both BRG1 and BRM is involved in the progression of lung adenocarcinoma into solid predominant tumors with features of epithelial mesenchymal transition and loss of the bronchial epithelial phenotype. BRG1 loss was specifically involved in the progression of EGFR wild-type, but not EGFR-mutant tumors. (*Cancer Sci* 2013; 104: 266–273)

Lung cancer is the leading cause of cancer death in many developed countries, including the United States and Japan.[1,2] The identification of genetic abnormalities, such as epidermal growth factor receptor (EGFR) mutations, KRAS mutations, EML4–ALK translocation, and MET amplifications has revolutionized our understanding of the molecular mechanisms in lung cancer development.[3] However, it has become increasingly apparent that epigenetic alternations play equally important roles in tumorigenesis, and among them, chromatin remodeling factors have attracted much attention recently.[4] Indeed, identification of mutations of chromatin remodeling factors in cancer has been a major hot topic in the past 2 years.[5–8]

BRG1 and BRM, two core catalytic ATPase subunits in human SWI/SNF chromatin remodeling enzymes, have now emerged as bona fide tumor suppressor genes.[9–12] Inactivating mutations of BRG1 have been identified in 35% of non-small cell lung cancer cell lines and a subset of primary lung cancer.[9] In a mouse model of lung cancer, targeted knockout of BRG1 can affect tumor development.[10] In contrast to BRG1, mutations of BRM have rarely been identified and epigenetic silencing of BRM plays a contributory role in some cancers.[4,11] However, whether loss of BRG1 and BRM affects phenotype and differentiation of lung cancer cells remains unexplored. Furthermore, the previous studies were conducted before the discovery of EGFR mutations, and thus relationship between the EGFR status and loss of BRG1 and BRM is completely unknown.

We have recently demonstrated that lung adenocarcinoma could be classified into two groups based on the patterns of gene expression and genetic abnormalities; bronchial epithelial phenotype tumors and mesenchymal-like phenotype tumors.[13] "Bronchial epithelial phenotype" represents a group of lung adenocarcinomas with high expression of bronchial epithelial markers. This group includes thyroid transcription factor (TTF)-1 positive terminal respiratory unit (TRU) type[14] in addition to TTF-1 negative tumors with high expression of bronchial epithelial markers such as CK7 and MUC1, as detailed in our previous report.[13] Bronchial epithelial phenotype tumor exhibits high phosphorylation of EGFR and MET and frequent mutations or amplifications of EGFR, MET, and HER2. In contrast, mesenchymal-like phenotype tumors were characterized by the absence of the bronchial epithelial phenotype, triple-negative for TTF-1, MUC1, and CK7, showed no or little phosphorylation of EGFR and MET, no mutation or amplification of EGFR, MET, or HER2, and with features of epithelial mesenchymal transition (EMT), such as low E-cadherin and high FGFR1, vimentin, and ZEB1 expressions.[13] The absence of mutations or amplifications of EGFR, MET, or HER2 in mesenchymal-like phenotype tumors suggested to us that other genetic or epigenetic abnormalities may play a role in this group of tumors.

We now show in this paper that loss of expression of chromatin remodeling factors, BRG1 and BRM, correlated with features of mesenchymal-like phenotype with solid predominant histology. In particular, BRG1 loss occurred exclusively in EGFR wild-type tumors.

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Materials and Methods

Cell lines and medium. We used 19 non-small cell lung cancer (NSCLC) cell lines; 15 adenocarcinoma cell lines (H23, H358, H441, H522, H1395, H1648, H1650, H1703, H1795, H2087, HCC827, HCC4006, Calu3, A549, PC-3), three large cell carcinoma cell lines (H661, H1299, Lu65), and one adenosquamous cell line (H596). HCC827, H1650, H1975, PC-3, and HCC4006 were EGFR-mutated cell lines. The sources of the cell lines were described in our previous report.[13] All cell lines were maintained in RPMI1640 supplemented with 10% FCS, glutamine, and antibiotics in a humidified atmosphere with 5% CO_2 and 95% air.

Genetic and protein analysis of cell lines. The DNA, RNA, and cell lysates were prepared from cell lines by standard procedures. Experimental details of sequencing, copy number analyses, and Western blotting are given in Doc. S1. Antibodies used in western blot analysis were summarized in Table 1.

Patients and tumors. Tumor specimens were obtained from 93 patients who underwent lung cancer surgery at the Jichi medical university hospital during the period from October 2005 to June 2008. The demographic and clinicopathologic details of the patients and tumors are provided in Doc. S1.

Immunohistochemistry and evaluation. Formalin-fixed, paraffin-embedded tumor specimens were analyzed by immunohistochemistry using antibodies to BRG1, BRM, E-cadherin, cytokeratin 7, MUC1, TTF-1, p-EGF, and p-MET. The sources of antibodies, staining procedures, and methods of evaluation, are given in Doc. S1.

Mutation analyses of formalin-fixed, paraffin-embedded tissue sections. Details are shown in Doc. S1.

Bioinformatic analyses and statistics. Details are shown in Doc. S1.

Results

Characteristics of lung adenocarcinoma cell lines with loss of BRG1 and/or BRM. First, we used 15 lung cancer cell lines, for which the mutational status of BRG1 was known, to investigate the molecular features that may characterize lung adenocarcinoma cell lines with loss of BRG1. Of the 15 cell lines, six cell lines harbored BRG1 mutations and nine cell lines did not, according to previous literature,[9,11] and the Sanger COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Figure 1 summarizes (i) the genetic status of BRG1, EGFR, MET, HER2, BRAF, and KRAS (upper panel), (ii) gene level expressions of BRG1, BRM, TTF1, MUC1, CK7, E-cadherin, and vimentin (middle panel), and (iii) protein expression levels of BRG1, BRM, TTF1, MUC1, CK7, E-cadherin, and vimentin (lower panel) of the 15 cell lines (the microarray analysis data of 15 cell lines is located in Data S1). All six BRG1-mutated cell lines showed extreme loss of BRG1 at gene and protein levels, EMT features (low E-cadherin and high vimentin), and loss of bronchial epithelial markers (TTF-1, CK7, and MUC1). In contrast, the nine BRG1-wild type cell lines showed high expressions of BRG1, as well as high expressions of bronchial epithelial markers and E-cadherin and low expression of vimentin, at both gene and protein levels. As for gene abnormalities, BRG1-wild type cell lines showed gene abnormalities for EGFR, MET, HER2, BRAF, or KRAS, but BRG1 mutated cell lines showed no such genetic abnormalities, except for KRAS mutations.

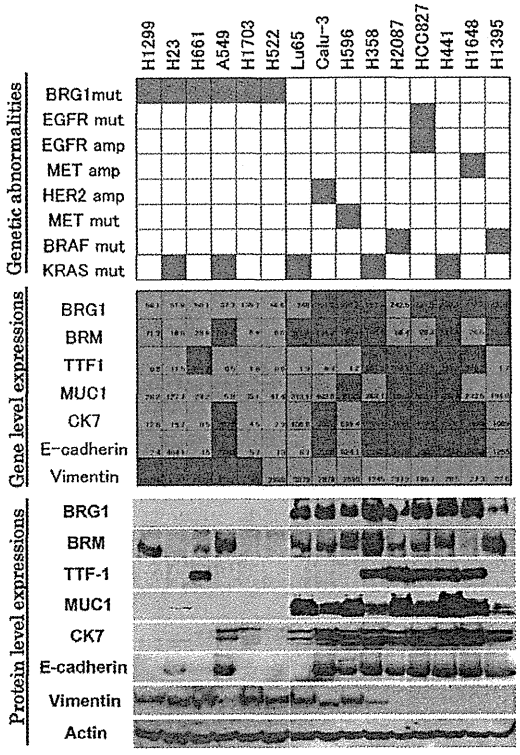

Fig. 1. Genetic status of BRG1, epidermal growth factor receptor (EGFR), MET, HER2, BRAF, and KRAS (upper panel), gene level expressions of BRG1, BRM, TTF1, MUC1, CK7, E-cadherin, and vimentin (middle panel), and protein expression levels of BRG1, BRM, TTF1, MUC1, CK7, E-cadherin, and vimentin (lower panel) of the 15 cell lines. In the upper panel, the grey box means presence of genetic abnormalities and the white box means absence of genetic abnormalities. Color indications in the middle lane are as follows: red means more than or equal to the average of each gene expression; orange: under the average and more than or equal to half the average; and green: under half the average.

We also examined the expressions of BRM in the same cell lines. Of the 15 cell lines, 10 cell lines expressed the BRM protein at modest or high levels, which was largely concordant

Table 1. Antibodies used in western blot analysis

Antibodies	Clone	Sources
BRG1 (sc-17796)	Mouse monoclonal	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
BRM (A301-016A)	Rabbit polyclonal	Bethyl Laboratory (Montgomery, TX, USA)
TTF-1 (clone 8G7G3/1)	Mouse monoclonal	DAKO (Glostrup, Denmark)
Cytokeratin 7 (clone OV-TL 12/30)	Mouse monoclonal	DAKO (Glostrup, Denmark)
Vimentin (clon V9)	Mouse monoclonal	DAKO (Glostrup, Denmark)
E-cadherin (clone 36)	Mouse monoclonal	BD Biosciences (Franklin Lakes, NJ, USA)
MUC1 smaller cytoplasmic subunit	Hamster monoclonal	Lab Vision (Cheshire, UK)
Anti-rabbit IgG peroxidase conjugate		Amersham (Arlington Hights, IL, USA)
Anti-mouse IgG peroxidase conjugate		Amersham (Arlington Hights, IL, USA)
Anti-Armenian hamster IgG peroxidase conjugate		Jackson Immunoresearch (West Grove, PA, USA)

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

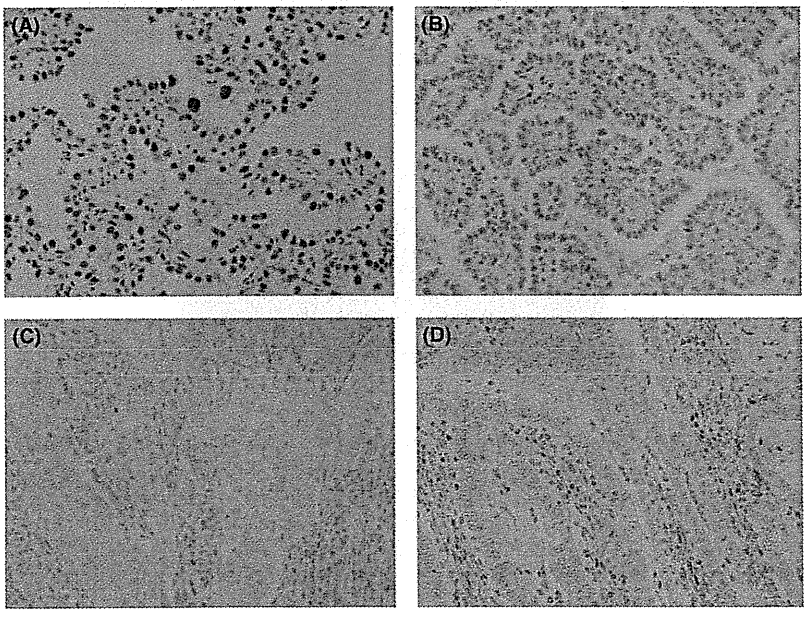

Fig. 2. BRG1 expressions in lung adenocarcinomas. Overall, more than 80% of cases showed positivity for BRG1. (A) Lepidic growth components showed strong immunoreactivity for BRG1. (B) Moderately differentiated adenocarcinomas, such as papillary adenocarcinoma, frequently showed positivity for BRG1. (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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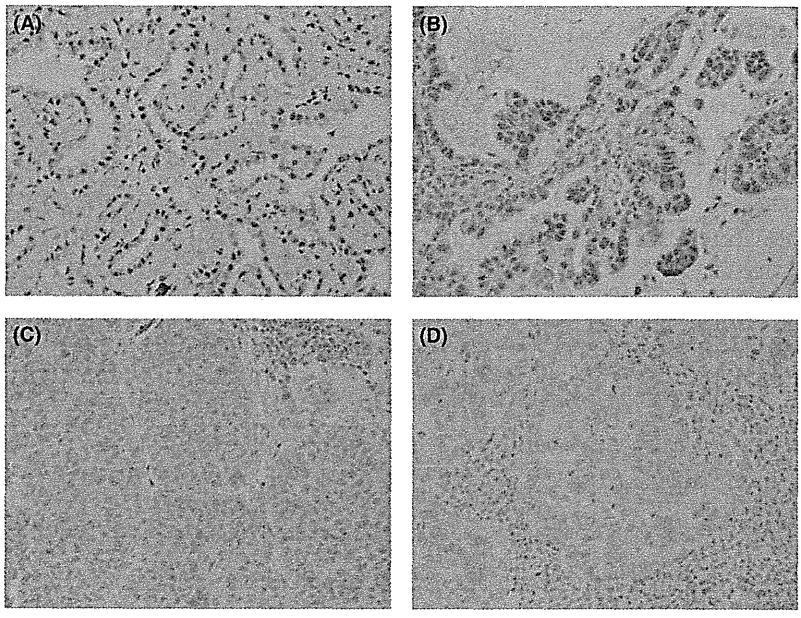

Fig. 3. BRM expressions in lung adenocarcinomas. Overall, more than 80% of cases showed positivity for BRM. (A) Lepidic growth components showed strong immunoreactivity for BRM. (B) Moderately differentiated adenocarcinomas, such as papillary adenocarcinoma, also often show positivity for BRM. (C) Solid 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.

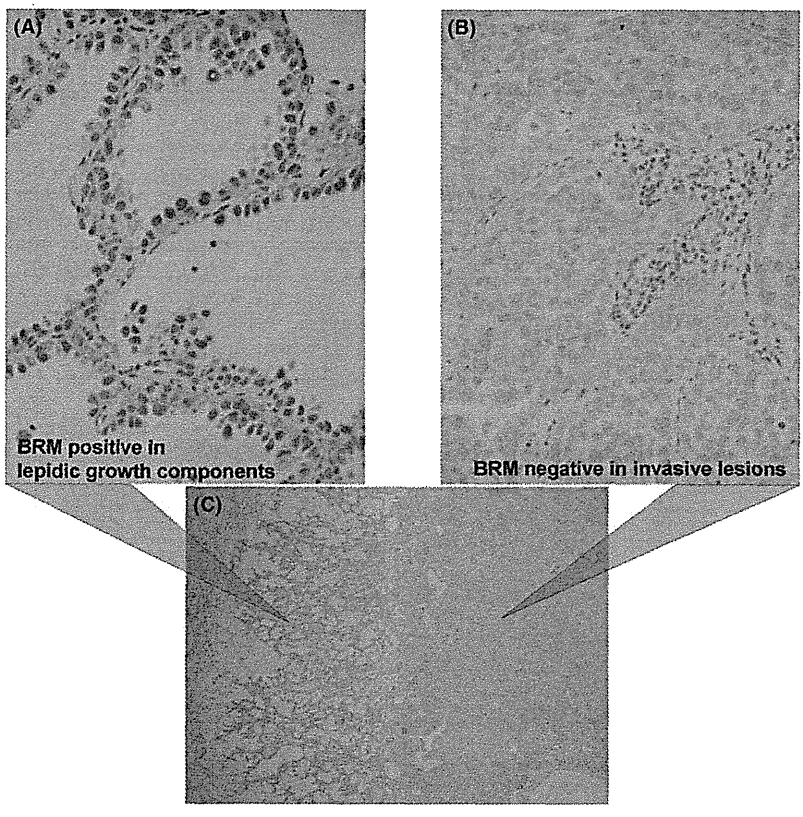

Fig. 4. Heterogeneous BRM expression in lung adenocarcinoma. (A) High-power field of lepidic growth components, which show strong positivity for BRM. (B) High-power field of invasive acinar components, which show negative positivity for BRM. (C) Low-power field of invasive adenocarcinoma with lepidic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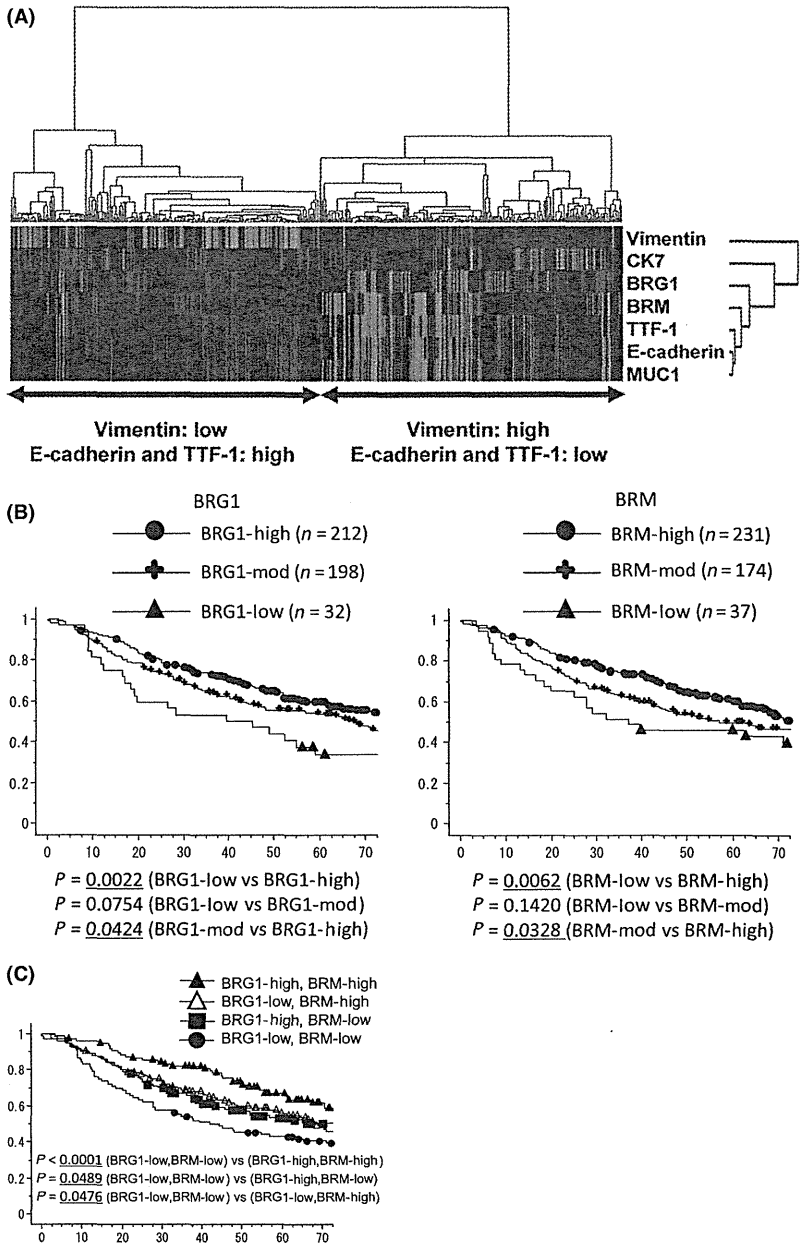

Fig. 5. Analysis of the publicly available data of 442 primary lung adenocarcinoma cases. (A) Hierarchical cluster analysis using the gene expressions of vimentin, CK7, BRG1, BRM, TTF-1, E-cadherin, and MUC1. (B) Patient survival according to the expression levels of BRG1 and BRM. Lung adenocarcinoma 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

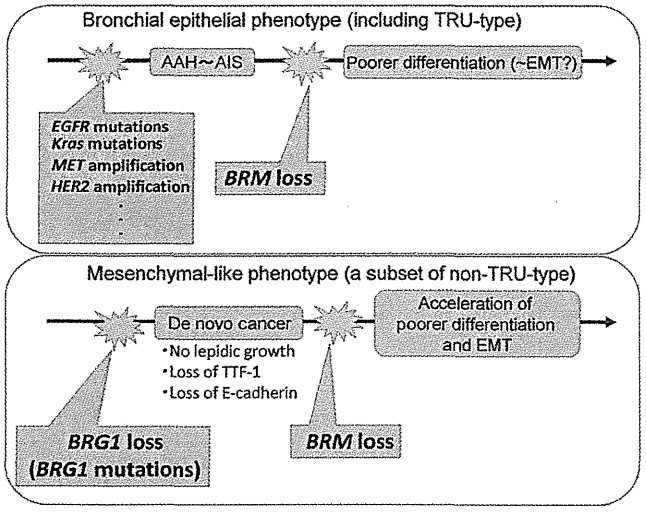

Fig. 6. Hypothetical schemes of BRG1 and BRM loss in the development of two types of lung adenocarcinomas: lung adenocarcinoma with the bronchial epithelial phenotype (upper panel) and lung adenocarcinoma with mesenchymal-like phenotype (lower panel).

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 ARTICLE

Current status of postoperative follow-up for lung cancer in Japan: questionnaire survey by the Setouchi Lung Cancer Study Group—A0901

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Abstract
Purpose. There is no recommended standard follow-up program after resection for lung cancer. Under these circumstances, each doctor establishes his or her own follow-up protocol. This questionnaire survey was conducted to grasp the current status of postoperative follow-up in Japan.
Methods. The questionnaire survey was aimed at determining what examinations were performed and at what frequencies in the setting of postoperative follow-up. Based on these results, examinations performed at a frequency of >50% and the time points after resection at which they were performed were selected and presented as components of an average follow-up program.
Results. Questionnaires were sent to 44 institutions, and 26 doctors responded to the questionnaire. All 26 of the doctors performed physical examinations, blood examinations, chest radiography, and computed tomography (CT) routinely, but their frequencies varied widely among the doctors. The average frequencies of the follow-up examinations as judged from this survey are as follows: Physical and blood examinations are performed three to four times a year for the first 3 years and twice a year during the next 2 years. CT is scheduled at 6 and 12 months after resection and is repeated annually thereafter. Chest radiography is performed three to four times a year for the first 3 years and once a year thereafter, between the CT examinations.
Conclusion. The follow-up programs used in clinical practice vary widely among institutions and doctors in terms of the types of examination performed and the frequencies at which they are performed.

Key words Lung cancer · Postoperative follow-up · Postoperative surveillance · Recurrence

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Introduction

Surgical resection with curative intent is selected for the treatment of localized non-small-cell lung cancer (NSCLC). The 5-year survival rate after complete resection for NSCLC is approximately 60%, and many patients develop recurrences after resection.[1] To detect recurrences, several examinations are performed periodically as part of the postoperative follow-up or surveillance. The purpose of postoperative follow-up is to detect recurrences and/or metachronous tumors, so adequate treatment can be offered in an attempt to improve the survival duration and the quality of life. Some investigators have suggested that the survival duration is greater in patients with asymptomatic recurrences detected by follow-up examinations than in those with symptomatic recurrences, and that follow-up examinations after resection are useful for detecting asymptomatic recurrences.[2,3] At the same time, several investigators have reported that the benefit of postoperative follow-up is questionable from the point of view of efficacy and cost-effectiveness.[4–8] Thus, the benefits and efficacy of postoperative follow-up remains controversial.

The board of the Japan Lung Cancer Society drew up a clinical practice guideline for lung cancer in 2005, and periodic follow-up after resection is not recommended in this guideline because there was still no clear persuasive evidence to support it.[9] Under these circumstances, each institution or each doctor establishes his or her own postoperative follow-up program in clinical practice. It is suspected that these postoperative follow-up protocols applied in clinical practice vary widely in terms of the examinations performed and their frequency. To grasp the current status of follow-up after resection for NSCLC in Japan, a questionnaire survey of the institutions affiliated with the Setouchi Lung Cancer Study Group was performed to determine the kinds of examination and the frequencies at which they are performed in the setting of postoperative follow-up. In addition, examinations that were performed frequently and the time points after resection at which they were performed were selected, and the average follow-up protocol based on the results of the questionnaire survey is presented.

Methods

A questionnaire designed to obtain information regarding the postoperative follow-up protocol adopted for NSCLC patients was sent by mail to 44 institutions affiliated with the Setouchi Lung Cancer Study Group. The questionnaire consisted of the following questions.

1. Is a standardized follow-up protocol followed at the institution?
2. Does the follow-up schedule differ depending on the disease stage?
3. What are the examination modalities chosen in the setting of postoperative follow-up? At what frequencies are these examinations performed? Please record your answers in Table 1.

Based on the information in this survey, the percentage of the 26 doctors who performed the examinations was calculated for each of the examinations at each time point after the resection. Then, examinations that were performed at a frequency of >50% and the time points after resection at which they were performed were selected and are presented as components of an average follow-up program in this study.

The TNM stage was determined according to the Union for International Cancer Control (UICC) TNM classification of pathological stage, 6th edition.[10]

Results

Questionnaires were sent to 44 institutions affiliated with the Setouchi Lung Cancer Study Group, 17 (38.6%) of which responded to the questionnaire. From these 17 institutions, 26 doctors, comprising 2 oncologists and 24 thoracic surgeons, responded to the questionnaire.

Of the 17 institutions, 7 reported that they followed a standardized institutional follow-up program, whereas the remaining 10 institutions did not (Table 2). Of the 26 doctors, 11 discontinued the follow-up 5 years after the resection, whereas the remaining 15 continued follow-up for >5 years after the resection. Among the 26 doctors, 15 arrange follow-up schedules based on the disease stage; for example, six doctors classified the patients into two groups based on the disease stage (stage IA and other stages), and four classified the patients into three groups based on the disease stage (IA, IB/II, and IIIA). Each of the 26 doctors performed blood examinations, chest radiography, and computed tomography (CT) routinely. Six doctors performed positron emission tomography (PET) or PET/CT, and nine doctors performed brain magnetic resonance imaging (MRI) or brain CT routinely. None of the doctors performed sputum cytology or abdominal ultrasonography (US) in the setting of postoperative follow-up.

Figure 1 shows the frequency of each of the examinations performed after the resection. The Y-axis shows the percentage of doctors who performed the examinations, and the X-axis shows the time of performance of the examination after the resection. More than half of

Table 1 Questionnaire

Procedure	1 Year						2 Years				3 Years				4 Years				5 Years				6 Years		7 Years		8 Years		9 Years		10 Years	
	1	2	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12	6	12	6	12	6	12	6	12	6	12
Physical examination																																
Blood examination (tumor markers)																																
Sputum cytology																																
Chest radiography																																
Abdominal US																																
CT																																
Bone scintigraphy																																
Brain MRI/CT																																
PET/CT																																
Other examinations																																

The numbers underneath the 1–10 years are the months (1, 2, 3, 6, 9, 12) for that year
US, ultrasonography; CT, computed tomography; MRI, magnetic resonance imaging; PET, positron emission tomography

Table 2 Results of the questionnaire

Institutions that responded to the questionnaire	17
Doctors who responded to the questionnaire	26
Standardized follow-up program in the institution?	
Yes	6
No	11
Continued follow-up for more than 5 years?	
Yes	15
No	11
Arrange follow-up schedule by disease stage?	
Yes	15
1A/1B,2,3	6
1A/1B,2/3	4
1/2/3	2
1A/1B/2,3	1
1/2,3	1
1,2/3	1
No	11
Physical examination?	
Yes	26
No	0
Blood examination?	
Yes	26
No	0
Sputum cytology?	
Yes	0
No	26
Chest radiography?	
Yes	26
No	0
Abdominal US?	
Yes	0
No	26
CT?	
Yes	26
No	0
Bone scintigraphy?	
Yes	5
No	21
Brain MRI (CT)?	
Yes	10
No	16
PET/CT?	
Yes	7
No	19

the doctors performed physical examination four times a year during the first 3 years and twice a year during the next 2 years. After 5 years of postoperative follow-up, the frequency of the physical examination decreased gradually, although approximately half of the doctors continued the physical examinations once a year for up to 10 years after the resection. The blood examinations were performed almost at the same time points as the physical examination. CT was performed at 6 and 12 months after the resection and was repeated annually thereafter for the next 4 years. Chest radiography was performed at each visit during the first 2 years and repeated annually thereafter, between the CT

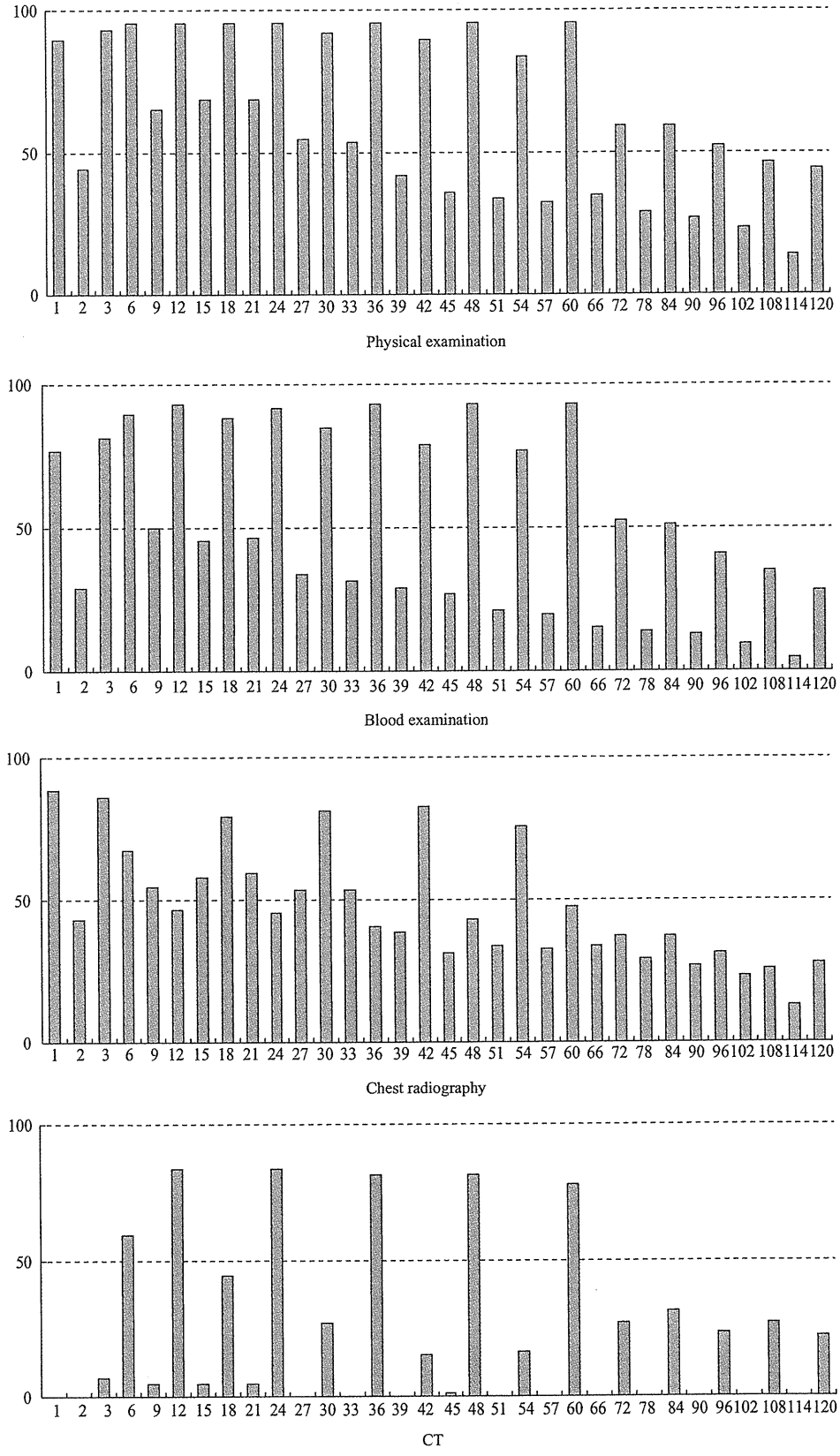
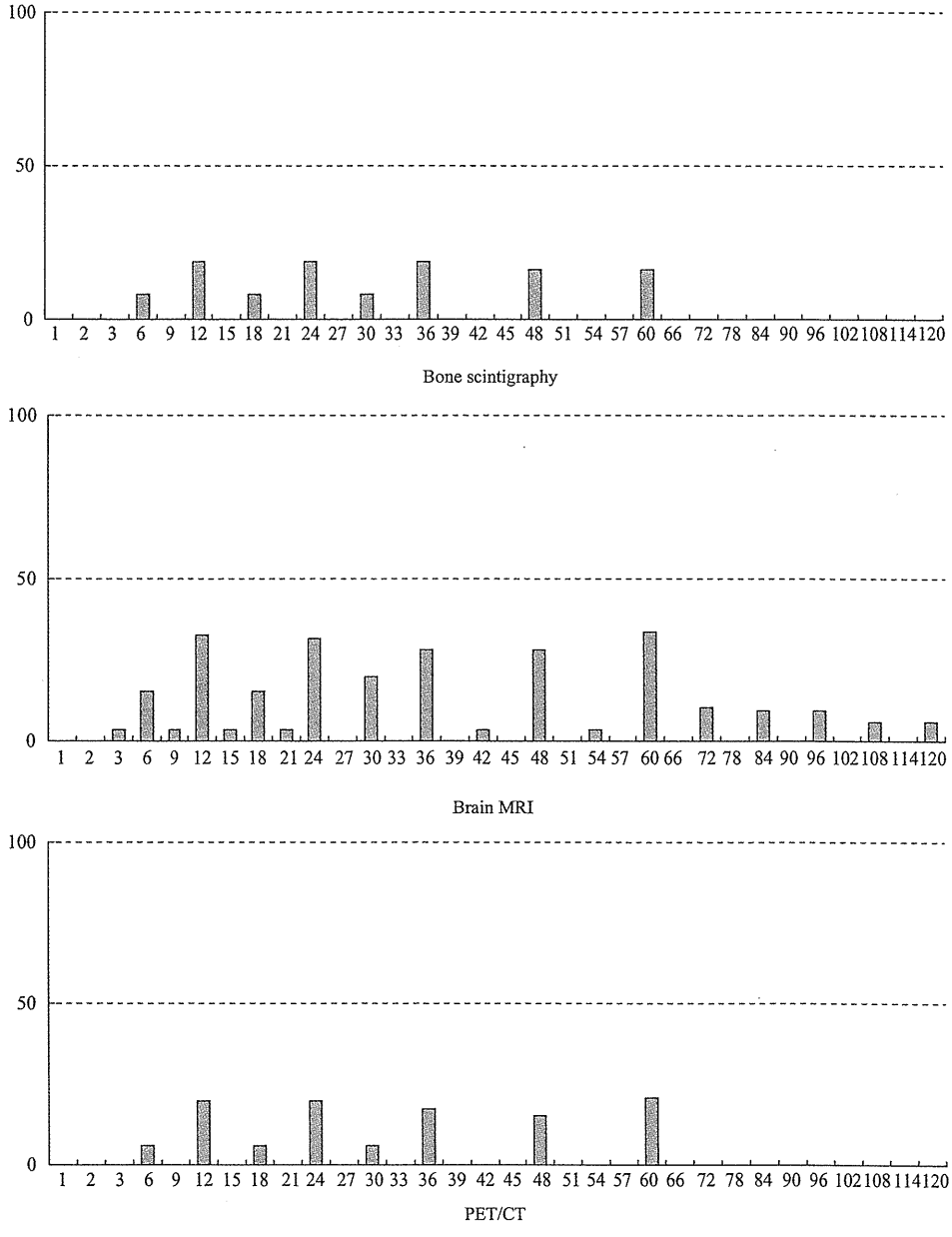


Fig. 1 Frequency of examinations at each time point after resection. *CT*, computed tomography; *MRI*, magnetic resonance imaging; *PET*, positron emission tomography

Fig. 1 Continued



examinations. A few of the doctors routinely performed bone scintigraphy, brain MRI or CT, and PET/CT.

Based on the results of the survey, the examinations that were performed at a frequency of >50% and the time points after resection at which they were performed were selected and are the components of an average follow-up program presented in this study (Table 3): Physical examination was scheduled three to four times a year for the first 3 years, twice a year for the next 2 years, then continued once a year for up to 8 years after the resection. Blood examinations were performed approximately at the same time points as the physical examination. CT examination was scheduled at 6 and 12 months after the resection and repeated annually thereafter for up to 5 years after the resection. Chest radiography was scheduled three or four times a year for the first 3 years and repeated once a year thereafter, between the CT examinations.

Discussion

Several issues need to be discussed in relation to the follow-up of NSCLC patients after resection. One of the

Table 3 Average follow-up program based on the results of this survey

Examination	1 Year						2 Years						3 Years						4 Years						5 Years						6 Years				7 Years		8 Years	
	1	2	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12	6	12	6	12	6	12
Physical examination	○		○	○	○	○	○	○	○	○	○	○		○		○		○		○		○		○		○		○		○		○		○		○
Blood examination	○	○	○	○	○	○		○		○		○				○		○		○		○		○		○		○		○		○				
Chest radiography	○	○	○	○	○		○	○	○		○	○	○			○				○				○												
CT				○		○		○		○				○				○				○				○				○						

issues is that optimal examination modalities for the postoperative follow-up have not yet been identified. Several guidelines have been proposed for the follow-up of NSCLC patients after surgery with curative intent, but there is a divergence among the guidelines, especially in relation to the recommendations for imaging examinations such as CT.[11–14] The study presented here demonstrated that follow-up programs applied in clinical practice in Japan also vary widely among institutions and/or doctors in terms of the examinations performed and the frequencies at which they are performed. No optimal or standard examination modalities in the follow-up setting have yet been established.

Another issue pertains to the efficacy and benefits of the follow-up examinations after resection. Several studies have suggested that the survival duration is greater in patients with asymptomatic recurrences than in those with symptomatic recurrences, and that follow-up is useful for detecting asymptomatic recurrences.[2,3] On the other hand, several investigators have reported that the survival benefit of postoperative follow-up is questionable.[4–8] The benefit of follow-up thus remains a controversial subject.

The third issue related to follow-up is cost-effectiveness, which cannot be ignored nowadays when evaluating the efficacy of a certain modality. Several investigators have analyzed the cost-effectiveness of postoperative follow-up and concluded that it is inefficient and that the survival benefit accruing from the follow-up did not justify its cost.[4–6,8] However, these articles were all published from Western countries; and the medical cost for follow-up, social acceptability of the medical cost, and the patients' needs might be different in Japan. Therefore, the cost-effectiveness of the follow-up should be evaluated independently in Japan.

To answer these questions, it would be ideal to conduct a randomized controlled study. However, the lack of standard follow-up modalities makes to it difficult to design a randomized trial. Regarding this point, our survey might give some helpful information for designing such a trial as the survey showed what modalities were commonly used in clinical practice. Another factor that probably makes the randomized trial more difficult to conduct in Japan is ethics. As the first step to evaluate the efficacy of the follow-up, it would be ideal to conduct the randomized trial between a follow-up group and a no follow-up or minimal follow-up group. Follow-up after resection, however, is already commonly performed in clinical practice and no or minimal follow-up would not be acceptable from an ethical point of view—even though there are no recommended follow-up programs and no proven efficacy of follow-up. Considering the present circumstances in Japan, a possible

trial might be comparison between a follow-up group with average modalities and a follow-up group with more intensive modalities, such as the average follow-up modalities + periodical PET/CT.

The follow-up programs identified in this survey seem relatively intensive compared with those that are commonly accepted worldwide. A possible reason might be related to our medical insurance system. All Japanese citizens are covered by public medical insurance, and the cost of the follow-up examinations is not a burden on the patients. This circumstance makes access to hospitals easy and makes the postoperative follow-up examinations relatively intensive. We do not have information about the follow-up programs adopted in other areas of Japan, but we assume that their programs are similar to those presented in this study.

In this survey, one question pertained to "blood examinations (tumor markers)", and it is uncertain what kinds of tumor markers were measured. In the preoperative setting and in cases of advanced/recurrent lung cancer, tumor markers such as carcinoembryonic antigen (CEA) and cytokeratin 19 fragments (Cyfra), among others, are commonly measured as parameters of the tumor aggressiveness or for evaluating the effectiveness of the treatment. In the follow-up setting, therefore, it is assumed that similar tumor markers would be measured.

^{18}F-Fluorodeoxyglucose (FDG)-PET/CT enables examination of the whole body, excluding the brain, in a noninvasive manner; it also can differentiate, if not always definitively, between malignant and benign lesions. Because of these advantages, FDG-PET/CT was applied as one of the follow-up examinations at six of the institutions. Several investigators have reported the usefulness of FDG-PET/CT in the setting of postoperative follow-up for NSCLC.[15–18] However, FDG-PET/CT cannot be recommended commonly in the postoperative follow-up setting because of limitation of availablility in Japan, cost-effectiveness and unknown efficacy.

A total of 15 of the 26 doctors based their follow-up schedules on the disease stage. They performed the examinations less intensively in patients with an early stage of the disease and more intensively in those with more advanced disease. These schedule changes based on the disease stage might be reasonable because recurrence develops more frequently in patients with advanced disease.

Conclusion

A questionnaire designed to obtain information on the follow-up program adopted for NSCLC patients after complete resection was conducted to grasp the current status in our area. The follow-up programs vary widely among institutions and doctors in terms of the examinations performed and the frequencies at which they are performed. The efficacy of the follow-up for NSCLC patients after resection is still unclear, and further studies are needed to answer questions about it.

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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