

2.5×10^6 nucleotides, their promoter regions are different, and their transcriptional control in the testis is largely unknown.

ISH demonstrated that the *Mpzl2* mRNA is primarily expressed in the spermatocytes, and IHC further demonstrated that the *Mpzl2* protein is localized in the leptotene/zygotene through to early pachytene spermatocytes in both wild-type and *Cadm1*-deficient mice. It should be noted that *Cadm1*-deficient mice have impaired spermatogenesis accompanied by prominent shedding of elongated spermatids into the lumen of seminiferous tubules, whereas the earlier spermatogenic cells up to early pachytene spermatocytes appeared intact. The presence of *Mpzl2* in *Cadm1*-positive earlier spermatogenic cells in contrast with the absence of *Mpzl2* in *Cadm1*-positive elongated spermatids leads us to the hypothesis that *Mpzl2* and *Cadm1* have functional redundancy in the seminiferous tubules. The deficiency of *Cadm1* may be compensated for by the increased amount of *Mpzl2* in the earlier spermatogenic cells, whereas such compensation may not work in *Mpzl2*-negative elongated spermatids, resulting in their detachment from Sertoli cells.

Elongated spermatids express, besides *Cadm1*, all of N-cad, CAR, Jam-C, and Nectin-3. Among these cell adhesion molecules, Jam-C and Nectin-3, when deficient, cause the shedding of elongated spermatids as well as the malformation of the remaining elongated spermatids, similar to *Cadm1* [6, 8]. Interestingly, Jam-C and Nectin-3, as well as *Cadm1*, have both homophilic and heterophilic binding activities, and interact not only with the same molecules but also with Jam-B, Nectin-2, and Pvr, respectively, that are expressed in Sertoli cells [27]. These adhesion molecules, that are expressed in elongated spermatids and indispensable for spermatogenesis, appear to be unable to compensate for the lack of the others. On the other hand, early spermatocytes have been known to express, besides *Cadm1*, no Jam-C or Nectin-3 but N-cad and CAR [9, 12]. N-cad and CAR are also expressed in Sertoli cells and can be involved in the interaction between spermatogenic and Sertoli cells through homophilic binding. However, considering that N-cad [11] and CAR are unable to compensate for the lack of *Cadm1*, Jam-C or Nectin-3 in elongated spermatids, they are more likely responsible for the interaction between Sertoli cells. Since the present ISH results indicated that *Mpzl2* is not expressed in Sertoli cells, the compensatory function of *Mpzl2* on *Cadm1* may be based on its heterophilic binding with Pvr, the known binding partner of *Cadm1*, or some other yet unidentified adhesion molecule present on Sertoli cells. So far, no molecule has been reported as the heterophilic binding partner of *Mpzl2*. Further studies are required to clarify this issue. On the other hand, the homophilic binding activity of *Mpzl2*, as well as that of *Cadm1*, JamC and Nectin-3, might be responsible for the interaction among spermatogenic cells for their synchronized development.

Spermatogenesis [19] is a process by which spermatogenic stem cells give rise to highly differentiated sperma-

tozoa through the interaction of spermatogenic cells with somatic Sertoli cells. This process may require many cell adhesion molecules expressed on the surface of spermatogenic and Sertoli cells. The present study suggested that there is a complex functional redundancy among these adhesion molecules. In the case of the deficiency of one of these molecules, the presence or absence of compensation of the deficient molecule with another molecule may bring about a variety of phenotypes in seminiferous tubules. The double-knockout study targeting various combinations of these molecules may shed light on the biological significance of each molecule in spermatogenesis. To date only a few cell adhesion molecules have been proven to be essential for spermatogenesis. Although human male infertility is often idiopathic, some infertile patients may have defective spermatogenesis due to dysfunction of some of these cell adhesion molecules. We hope to identify such molecules in the future.

V. Acknowledgments

This work was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan, and by the Honjin Foundation to TW.

VI. References

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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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(Received August 22, 2012/Revised October 30, 2012/Accepted November 6, 2012/Accepted manuscript online November 19, 2012/Article first published online January 4, 2013)

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 adeno-squamous cell line (H596). HCC827, H1650, H1795, 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% CO₂ 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

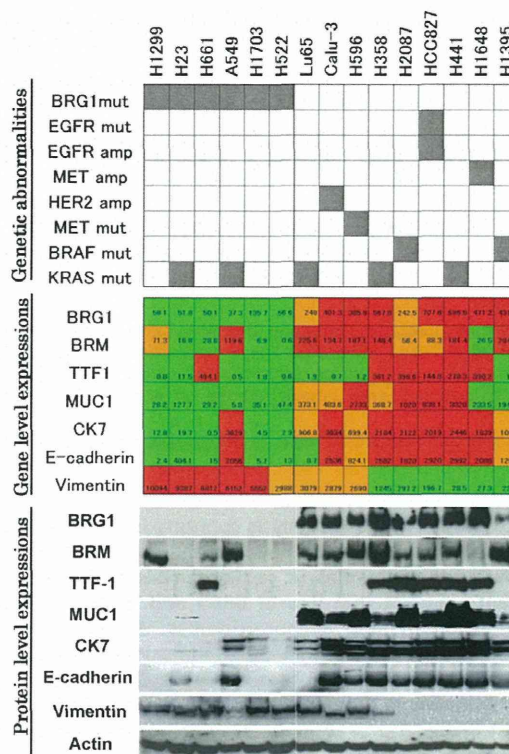


Fig. 1. Genetic status of BRG1, epidermal growth factor receptor (EGFR), MET, HER2, BRAF, and KRAS (upper panel), gene level expressions of BRG1, BRM, TTF1, MUC1, CK7, E-cadherin, and vimentin (middle panel), and protein expression levels of BRG1, BRM, TTF1, MUC1, CK7, E-cadherin, and vimentin (lower 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.

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.

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 Heights, IL, USA)
Anti-mouse IgG peroxidase conjugate		Amersham (Arlington Heights, 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

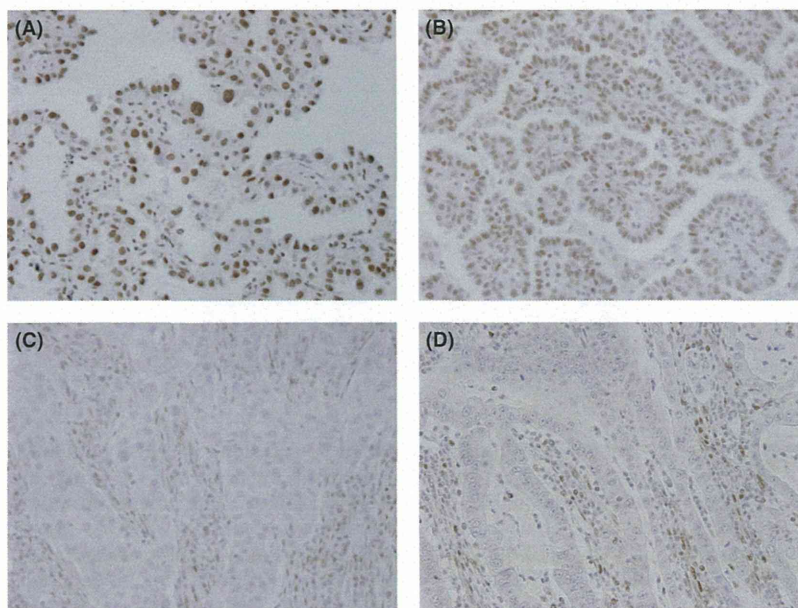


Fig. 2. BRG1 expressions in lung adenocarcinomas. Overall, more than 80% of cases showed positivity for BRG1. (A) Lepidic growth components showed strong immunoreactivity for BRG1. (B) Moderately differentiated adenocarcinomas, such as papillary adenocarcinoma, frequently showed positivity for BRG1.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

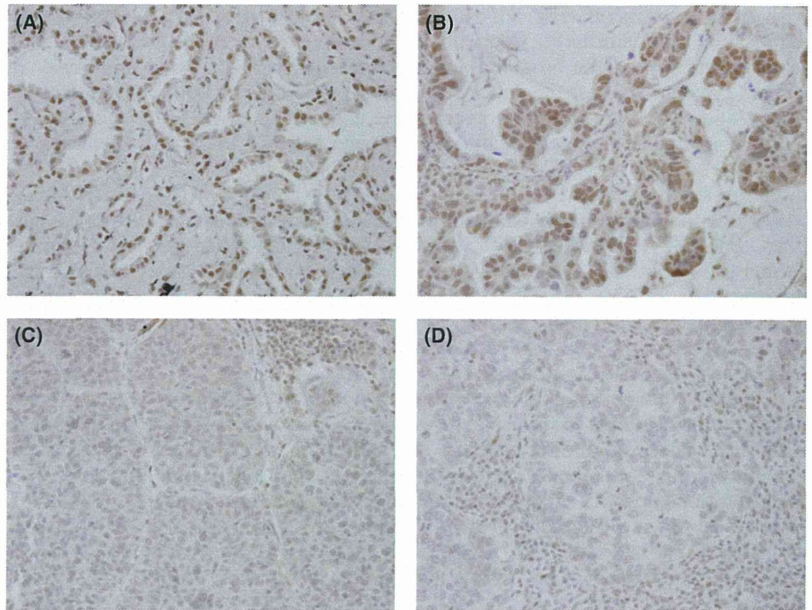


Fig. 3. BRM expressions in lung adenocarcinomas. Overall, more than 80% of cases showed positivity for BRM. (A) Lepidic growth components showed strong immunoreactivity for BRM. (B) Moderately differentiated adenocarcinomas, such as papillary adenocarcinoma, also often show positivity for BRM. (C) Solid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

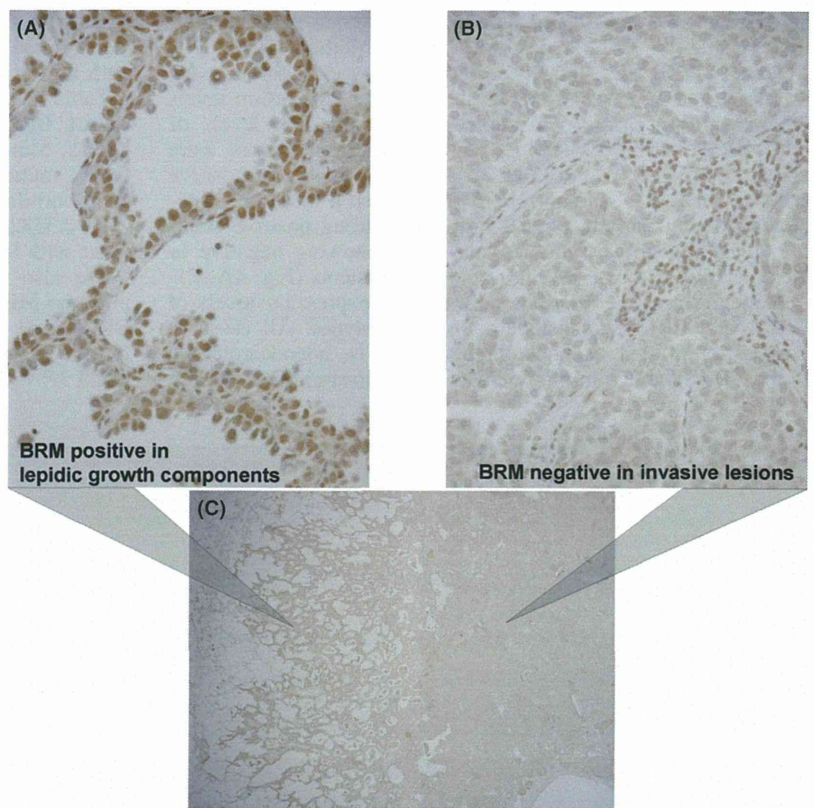


Fig. 4. Heterogeneous BRM expression in lung adenocarcinoma. (A) High-power field of lepidic growth components, which show strong positivity for BRM. (B) High-power field of invasive acinar components, which show negative positivity for BRM. (C) Low-power field of invasive adenocarcinoma with lepidic 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 <i>in situ</i>	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	<i>P</i> -value	High	Low	<i>P</i> -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	<u>0.0002</u>
Negative	59	6		60	5	
Pleural invasion						
Positive	21	6	<u>0.0471</u>	21	6	0.4122
Negative	61	5		56	10	
Dissemination						
Positive	3	2	<u>0.0449</u>	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	<u><0.0019</u>	59	6	<u>0.0019</u>
Absent	17	11		18	10	
Smoking Index						
≤ 600	26	6	0.1344	22	10	<u>0.0093</u>
>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.

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

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, phospho-MET, and phospho-EGFR

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

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.

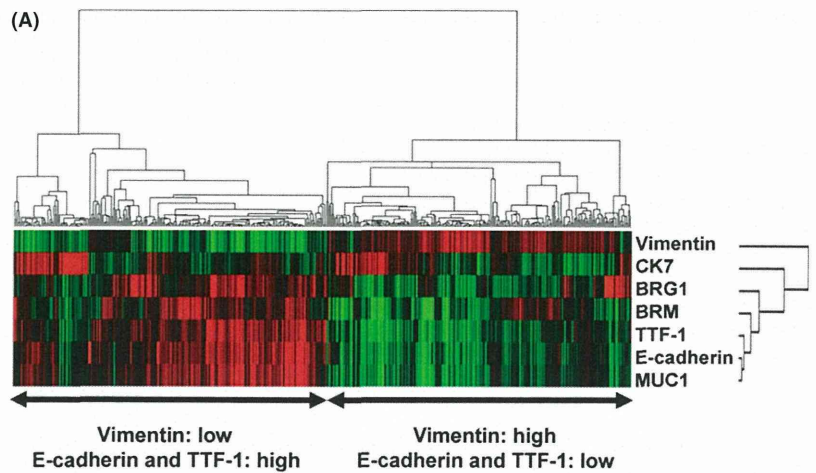
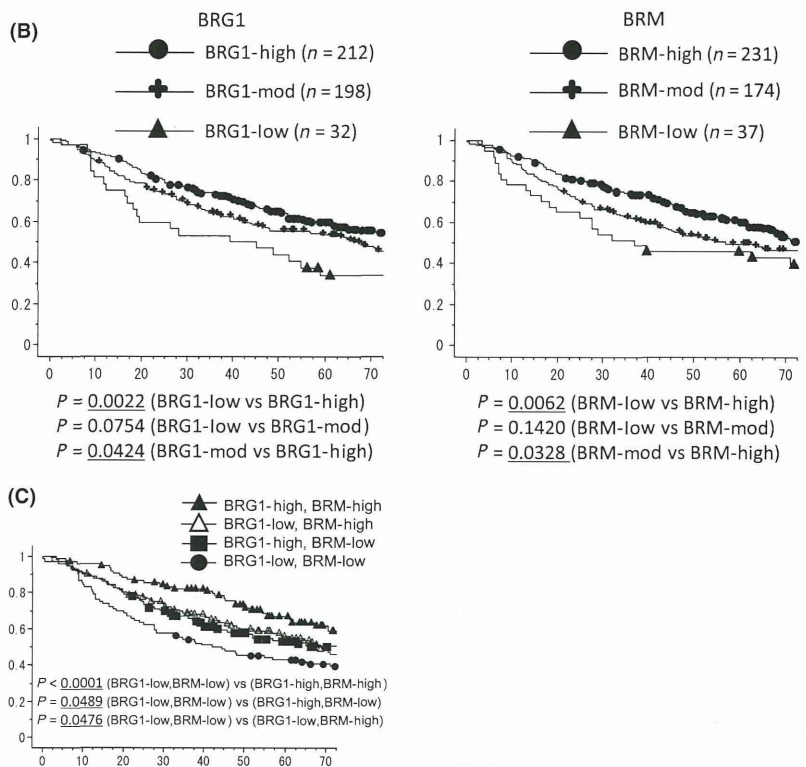


Fig. 5. Analysis of the publicly available data of 442 primary lung adenocarcinoma cases. (A) Hierarchical cluster analysis using the gene expressions of vimentin, CK7, BRG1, BRM, TTF-1, E-cadherin, and MUC1. (B) Patient survival according to the expression levels of BRG1 and BRM. Lung adenocarcinoma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

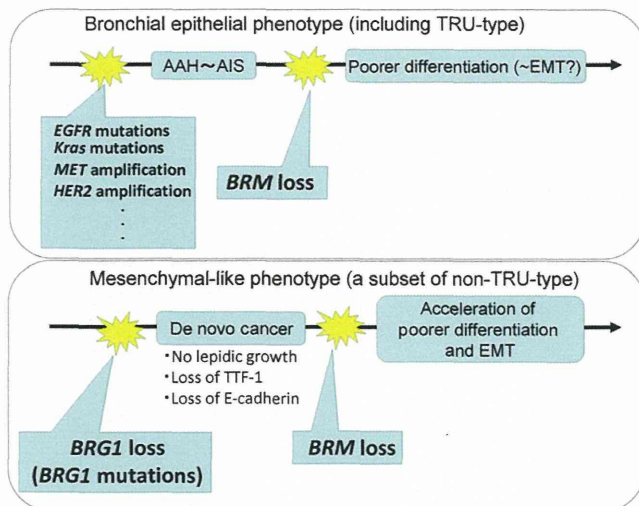


Fig. 6. Hypothetical schemes of BRG1 and BRM loss in the development of two types of lung adenocarcinomas: lung adenocarcinoma with the bronchial epithelial phenotype (upper panel) and lung adenocarcinoma with mesenchymal-like phenotype (lower panel).

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 adeno-

carcinomas: 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.

Acknowledgments

This study was supported in part by the Smoking Research Foundation, the Foundation for Development of Community, the Vehicle Racing Commemorative Foundation, and the Ministry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology of Japan. This publication was subsidized by JKA through its promotion funds from KEIRIN RACE. We thank Dr S. Matsukuma and Dr E. Tsuchiya, Molecular Pathology and Genetics Division, Kanagawa Cancer Center Research Institute, for technical advice on the loop-hybrid method.

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.