Chromatin Remodeling Factors and BRM/BRG1 Expression as Prognostic Indicators in Non-Small Cell Lung Cancer

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ABSTRACT

We immunohistochemically examined 12 core proteins involved in the chromatin remodeling machinery using a tissue microarray composed of 150 lung adenocarcinoma (AD) and 150 squamous cell carcinoma (SCC) cases. Most of the proteins showed nuclear staining, whereas some also showed cytoplasmic or membranous staining. When the expression patterns of all tested antigens were considered, proteins with nuclear staining clustered into two major groups. Nuclear signals of BRM, Ini-1, retinoblastoma, mSin3A, HDAC1, and HAT1 clustered together, whereas nuclear signals of BRG1, BAF155, HDAC2, BAF170, and RbAP48 formed a second cluster. Additionally, two thirds of the cases on the lung tissue array had follow-up information, and survival analysis was performed for each of the tested proteins. Positive nuclear BRM (N-BRM) staining correlated with a favorable prognosis in SCC and AD patients with a 5 year-survival of 53.5% compared with 32.3% for those whose tumors were negative for N-BRM (P = 0.015). Furthermore, patients whose tumors stained positive for both N-BRM and nuclear BRG1 had a 5 year-survival of 72% compared with 33.6% (P = 0.013) for those whose tumors were positive for either or negative for both markers. In contrast, membranous BRM (M-BRM) staining correlated with a poorer prognosis in AD patients with a 5 year-survival of 16.7% compared with those without M-BRM staining (38.1%; P = 0.016). These results support the notion that BRM and BRG1 participate in two distinct chromosome remodeling complexes that are functionally complementary and that the nuclear presence of BRM, its coexpression with nuclear BRG1, and the altered cellular localization of BRM (M-BRM) are useful markers for non-small cell lung cancer prognosis.

INTRODUCTION

Lung cancer has the highest cancer incidence and mortality in the world (1). Even in localized stages, the prognosis of non-small cell lung cancer (NSCLC) has remained poor over the last decades, despite curative surgery. At present, tumor staging is the strongest prognostic indicator (2, 3), and the 5-year survival rates range from 61% for stage IA disease and 24% for stage IIB disease to <10% for stage III disease (4, 5). Despite intensive research, little has changed in the understanding and management of NSCLC. Detailed histopathological features and known clinical prognostic factors have been of limited help in predicting lung cancer outcome.

Studies have shown that modification of chromatin structure is an essential step in gene regulation primarily mediated by chromatin remodeling proteins. Among these proteins, histone is known to establish a dynamic molecular interface and play an active role in the regulation of transcription (6–8). Often, transcription is also regulated by other cofactors, and the balance of chromatin remodeling activities may be crucial to ensure accurate responses to developmental or environmental cues and to prevent the transition of normal cells into cancer cells (9).

The SWI/SNF complex is a major complex of ATP-dependent chromatin remodeling factors and contributes to the regulation of gene expression by altering chromatin structure (10–14). SWI/SNF complex, originally identified in yeast, is composed of 11 characterized subunits, and SWI2/SNF2 is the central ATPase (15, 16). Human SWI/SNF complexes contain one of the two core catalytic ATPase subunits, BRM or BRG1 (14, 17–19). BRM and BRG1 further diverge in their subunit compositions and are thought to participate in specialized cellular functions (19). Growing genetic and molecular evidence indicates that subunits of the SWI/SNF complex can act as tumor suppressors in human and mice (7, 20–22). Recently, concomitant loss of BRG1 and BRM expression was reported as a poor prognostic indicator in NSCLC (23). Results from biochemical and transfection studies also suggest that SWI/SNF may interact and control the transcriptional activity of a variety of genes involved in cellular growth and transformation (24–30).

The recent development of tissue microarray (TMA) technique allows for high-throughput molecular analysis of a large set of paraffin-embedded surgical specimens and makes it possible to effectively evaluate many proteins in a specific biological pathway (31–35). Here, we focused on ATP-dependent chromatin remodeling and histone-modifying factors by examining their expression status, relationship, and clinical correla-
tion in 300 pulmonary adenocarcinoma (AD) and squamous cell carcinoma (SCC) cases.

MATERIALS AND METHODS

Clinical Samples. Three hundred lung cancer cases (150 pulmonary AD and 150 SCC cases) were selected from the Armed Forces Institute of Pathology archive, based on the diagnosis and the quality and quantity of the available tissue on the paraffin block. Demographic and clinical data were collected at the time of block acquisition (Table 1). A total of 246 patients had complete clinical information. Survival time and outcome data were available for 211 patients. The tumors were staged at the time of block acquisition (Table 1). A total of 246 patients had complete clinical information. Survival time and outcome data were available for 211 patients. The tumors were staged according to the International Union against Cancer’s tumor-node-metastasis (TNM) classification and histologically subtyped and graded according to WHO guidelines (36).

Lung TMA. The most representative tumor areas were carefully selected based on the matched H&E-stained slides and marked directly on the donor block. The cylindrical tissue sample was cored (diameter, 0.6 mm) from the selected region in the donor block and extruded directly into the recipient block with coordinated wells. One hundred adjacent nondiseased lung tissues from AD and SCC cases, along with 50 nonpulmonary normal tissues, were included in the same array block (Fig. 1). Multiple 4-µm sections were cut with a microtome using an adhesive-coated tape (Instrumedics). Sections attached with adhesive tapes were floated on the water, transferred to charged adhesive-coated tape (Instrumedics). The reaction products were rinsed twice with PBS. When a microwave was used for heat-induced epitope retrieval, endogenous peroxidase was blocked with 0.3% H2 O2 in PBS for 20 min followed by washing twice in distilled water, lightly counterstained with Mayer’s hematoxylin, dehydrated, cleared, and mounted with resinous mounting medium. All procedures were carried out at room temperature.

Immunohistochemical Staining. Immediately after removing the adhesive tapes, the sections were deparaffinized with xylene and rehydrated through graded alcohols into water. Heat-induced epitope retrieval was performed by placing slides in plastic coplin jars in citrate buffer (pH 6.0; BioGenex, San Francisco, CA) for about 30 min. After heat-induced epitope retrieval treatment, endogenous peroxidase was blocked with 0.3% H2O2 in PBS for 20 min followed by washing twice in PBS. When a microwave was used for heat-induced epitope retrieval instead of the decloaking chamber, specimens were heated in a microwave oven for 4 min while keeping the buffer temperature around 95°C. Tissues were placed in the humid chamber and incubated with normal blocking serum for 30 min. The slides were then incubated with the primary antibodies at an optimized titer as shown in Table 2 and diluted using Universal blocking reagent (BioGenex) for 60 min. After washing three times with PBS, each series of sections was incubated for 30 min with biotinylated secondary antibodies (Vector Laboratories) diluted to 1:250 by Universal blocking reagent (BioGenex) for 60 min. After washing three times with PBS, each series of sections was incubated for 30 min with biotinylated secondary antibodies (Vector Laboratories). The reaction products were rinsed twice with PBS, placed in 0.05 M Tris-HCl buffer (pH 7.5) for 5 min, and then developed in liquid 3,3’-diaminobenzidine (Dako) for 3 min. After the development, sections were washed twice with distilled water, lightly counterstained with Mayer’s hematoxylin, dehydrated, cleared, and mounted with resinous mounting medium. All procedures were carried out at room temperature.

<p>| Table 1: Clinical characteristics of all non-small cell lung cancer patients used in this study |
|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
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<th>Variable</th>
<th>Category</th>
<th>No. of patients (n = 230)</th>
<th>Median survival (yrs)</th>
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<tr>
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<tr>
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<td>IV</td>
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<td>Median follow-up (yrs)</td>
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*a Median term of last follow-up.
Western Analysis and Immunohistochemistry of Lung Cancer Cell Lines. To determine the specificity of the antibodies, a panel of nine lung cancer cell lines was obtained from American Type Culture Collection (Manassas, VA) and used for both immunohistochemistry and Western analyses. Briefly, confluent cultures of each cell line were prepared for total cell lysate in SDS-PAGE sample buffer or scraped to obtain the cell pellet and fixed in 4% paraformaldehyde. The pellets were then transferred into 15-ml tubes, dehydrated, and embedded in paraffin. The paraffin blocks were further processed and sectioned for immunohistochemical staining using the protocols described in Table 2. For Western analyses, cell lysates were sonicated and centrifuged to remove the insoluble material, heated at 95°C for 5 min, and stored at −80°C. The protein quantity for each lysate was adjusted based on the γ-tubulin signal, and the proteins of interest were analyzed using standard protocols (40).

The protein filter was incubated for 1 h at room temperature with anti-BRM (n19), BRG1, BAF155, and Ini-1 antibodies at dilutions of 1:20, 1:200, 1:200, 1:200, respectively. Immuno-

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1 J. Fukuoka, A unified approach to standardize immunohistochemical staining using paraffin embedded cell arrays and Western blot, manuscript in preparation.

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Fig. 1 Organization of lung tumor tissue microarray (TMA). A, H&E staining of the entire TMA section. B, scheme of TMA. Tissues samples are represented by small rectangles that are arrayed in groups of 25 each. Tumor types are indicated as light gray (adenocarcinoma) or black (squamous cell carcinoma). The clear areas are control tissues including normal lung from patients with adenocarcinoma (#1), or squamous cell carcinoma (#2), and normal nonpulmonary organs (#3).
blots were washed with TBS [50 mM Tris (pH 7.6) and 0.15 mM NaCl] containing 0.1% Tween 20 for 15 min, incubated with horseradish peroxidase-conjugated secondary antibody (diluted 1:10,000), and visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, UK), and visualized using enhanced chemiluminescence.

**Table 2 Antibodies and protocols used for immunohistochemical staining**

<table>
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<td>IgG2a</td>
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<td>MW</td>
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<td>ABC</td>
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<td>ABC</td>
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<td>n/a</td>
<td>ABC</td>
<td>8000</td>
<td>DC</td>
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</tbody>
</table>

* R, rabbit; DC, Decloaking Chamber; MW, microwave; G, goat; M, mouse; n/a, not available.

For each antibody staining, specimens with questionable signals due to insufficient tumor cells, tumor cells that were difficult to distinguish from other inflammatory or interstitial cells, or tissues with homogeneously weak signals were excluded from further evaluation. Tissue sections with only necrotic tumor cells or tissues with high homogenous background were also excluded. Twenty-seven cases with an intensity score of <3 by all tested antibodies were excluded from all analysis. Furthermore, due to the small size of the tissues in TMA, some positive samples could be scored as negative due to sampling error. In our study, 16 samples showed no AE1/AE3 signal (TS0) in 262 scorable tumors, whereas 9 samples were TS1, despite the fact that all cancer tissues should be positive (42). Therefore, the percentage of positive cases underscored was estimated to be (16 + 9) / (262 - 16 - 9) * 100 = 10.5% of the positive samples. This estimate is also consistent with the staining status of PGP9.5, which was positive in 50% of TMA samples (127 of 253 samples) and 54% in NSCLC cases (54 of 98) when analyzed using conventional pathological sections (43).

**Statistical Analysis.** Comparisons of clinicopathological factors (i.e., age at diagnosis, gender, histological type, or pathological stage) between positive and negative groups were performed using the \( \chi^2 \) test. Because a relatively large number of patients in our cohort died of unknown causes, only overall survival was considered for each antibody staining and prognostic analyses. The log-rank test was used for comparing survival distributions between positive and negative groups of staining, and Kaplan-Meier curves were plotted for the two groups. Clinical factors were accounted for by fitting Cox proportional hazard models. When the number of observations in a group is small (<30), the usual \( P \) value of the log-rank test requiring a large sample size may not be valid. Consequently, a permutation test was used to evaluate the significance of the difference between survival curves. Specifically, the association between the staining status and survival outcome was randomly permuted. A log-rank test statistic was generated with the permuted data. This procedure was repeated 2000 times, and the \( P \) value was the proportion of permutations with the same or larger value of the log-rank test statistic as observed in the actual study. A similar permutation test for the Cox proportional hazards model was used, where the association between staining status and survival outcome along with the clinical factors was randomly permuted. Association of binary staining status between each pair of antibodies was examined using the \( \chi^2 \) test, and the association was considered significant if the \( \chi^2 \) value \( \leq 0.01 \). Association between antibody staining and clinical factors (gender, age at diagnosis, or histological type) was also analyzed using the \( \chi^2 \) test, where \( P \leq 0.01 \) was considered significant.

For protein association analyses, hierarchical agglomerative clustering was applied to the total score of staining using average linkage and 1 minus the Spearman rank correlation as the distance metric. Clustering reproducibility was assessed by the method proposed by McShane et al. (44).
with the associated clinicopathological information of the tumors are available at the LPG Image Portal. LPG Image was developed leveraging the National Cancer Institute Center for Bioinformatics cancer Images Portal (caIMAGE), which allows researchers to submit and retrieve histology images and annotations. The database is highly interactive and can be searched based on patient’s gender, age, tumor histology, and experimental findings for each of the antibodies described in this study.

RESULTS

Clinicopathological Variables and Their Associations with Patient Survival. Overall, 273 cases had adequately stained tissues for analysis, and clinical information was available for 230 cases. Among them, age at diagnosis was available for 229 cases, with an average of 64.6 years (range, 36–86 years). Gender was available for 230 cases and included 64

Fig. 2 Chromatin remodeling factors in lung cancer cell lines. A, immunoblotting of BRG1, BRM, BAF155, and Ini-1 among nine lung cancer cell lines. The EKVX cell line showed a band reactive to anti-BRM but non-immunoreactive for anti-BRG1 antibody. The apparent sizes of BRG1 and BRM were a little larger than the known molecular size (180 kDa) but are the same as those shown in the data sheet from the supplier (http://www.scbt.com/). BAF155 and Ini-1 showed an immunoreactive band in eight cell lines. H358 appeared to be negative for Ini-1. B, anti-BRM immunohistochemical staining of the same lung cancer cells. Hop92 did not show positive staining by immunohistochemistry but showed a weak positive band on Western blot. Consistent with immunoblotting, immunohistochemical signals of BRM were lower than those of BRG1 in lung cancer cell lines. H520, EKVX, and H2170 showed membranous and cytoplasmic staining, whereas H358 and H226 showed weak and focal nuclear staining.

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female and 166 male patients. Pathological stage was available for 181 cases. Follow-up time or time to death was available in 196 cases, with a median follow-up of 3.2 years. Fifty two patients were alive at the last follow-up.

Female patients had a better prognosis in overall survival with a median survival of 4.6 years in contrast to a median survival of 2.8 years for males (P = 0.028). As expected, stage I and II patients had a significantly better prognosis than stage III and IV patients (P < 0.001). The median survival for stage I/II and stage III/IV patients was 4.2 and 1.3 years, respectively. There was no significant survival difference between patients with AD and those with SCC. The essential clinical information of the study cohort is summarized in Table 1.

**Specificity of the Antibodies.** We compared the protein staining of selected antibodies by Western and immunohistochemical analyses using a panel of nine lung cancer cell lines. BRG1 expression was observed in five of nine cell lines, whereas BRM expression was detectable in six of the nine cell lines, although at much lower levels than BRG1 (Fig. 2A). Furthermore, five of these six cell lines (H520, H358, EKVX, H226, and H2170) had positive immunohistochemical staining, and three of these cell lines (H520, EKVX, and H2170) showed membranous and cytoplasmic staining with no nuclear staining (Fig. 2B). BAF155 showed reactive band in all nine cell lines, whereas one cell line (H266) had a weaker band. Ini-1 expression was generally weak but detectable in eight cells and appeared to be absent in H358 cells (Fig. 2A).

**Expression of Proteins as Detected by Immunohistochemistry.** A summary of the staining features for each antibody is listed in Table 3. As expected, the staining of most proteins including RB, HAT1, HDAC1, HDAC2, RhAP48, BAF170, and mSin3A was located exclusively in the tumor cell nucleus. However, BAF155, Ini-1 BRG1, and BRM showed both nuclear and cytoplasmic or nuclear and membranous staining (Table 3; examples in Fig. 3). Only Tip60 showed mostly cytoplasmic staining. Expression of HDAC2, nuclear BRM (N-BRM), RB, and nuclear BAF155 was significantly higher in SCC than in AD (P values are 0.001, <0.001, 0.005, and <0.001, respectively). No protein was observed more frequently in AD than SCC. No significant staining differences were observed between gender, age (≤60 years versus >60 years), or stage (I/II versus III/IV). In the normal lung, all bronchiolar epithelia showed positive nuclear signal for all tested antibodies, except Ini-1, which showed both nuclear and cytoplasmic staining. Tip60 showed only cytoplasmic staining. For pneumocytes, BRG1 and Ini-1 were positive for most cells; BAF155, BAF170, and HDAC2 were weak to moderately positive in less than half of cells; whereas the remaining antibodies were moderately positive in more than half of the pneumocytes.

**Patterns of Association between Antibodies.** We examined the association of staining status between each pair of the tested proteins using χ2 test. Among a total of 128 comparisons(16 × 16/2), we observed 12 and 11 significant associations in AD and SCC, respectively. Of these associations, six were common for both SCC and AD and included associations between HDAC1 and RB, mSin3A, and HAT1; mSin3A and nuclear Ini-1 and HAT1; and HDAC2 and nuclear BAF155. Among the AD cases, staining of BAF170 was positively associated with HDAC2, nuclear BRG1 (N-BRG1), and RhAP48; N-BRG1 was also positively associated with RhAP48, whereas HDAC1 expression was correlated with nuclear Ini-1. For SCC, N-BRG1 staining was positively associated with HDAC2, nuclear BAF155, and Tip60, whereas staining of the RB protein was correlated with mSin3A and HAT1 (Fig. 4A).

To gain insight into the potential interaction of the proteins, we organized the expression patterns of all tested antibodies using unsupervised clustering analysis (Fig. 4). Two major groups of nuclear protein staining were observed (Fig. 4 and C): nuclear staining of BRM, Ini-1, RB, mSin3A, HDAC1, and HAT1 clustered as one group; whereas nuclear staining of BAF155, HDAC2, BAF170, RhAP48, and BRG1 clustered as a second group. Application of the reproducibility measures of McShane et al. (44) showed that these two groups of protein associations were highly reproducible (robustness index > 90%). All of the nonnuclear signals branched outside these two clusters further support the reliability of the nuclear protein associations (Fig. 4C).

**Prognostic Significance.** When the expression of each of the 12 proteins was associated with clinical survival, only BRM staining demonstrated prognostic significance. Positive N-BRM staining was observed in 51 cases among 193 cases with survival outcomes (Fig. 5A). Kaplan-Meier analysis indicated that the overall 5-year survival for patients whose tumors were N-BRM positive was 53.5% compared with 32.3% for those whose tumors were negative for N-BRM (P = 0.015). In contrast, M-BRM staining was observed in 30 of 268 cases, and only four of these tumors also had nuclear staining. Of the 18

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**Table 3 Immunohistochemical staining of chromatin remodeling factors using TMA**

The actual number of cases is shown for each scoring, whereas percentages are given for − and + cells. N, nuclear, C, cytoplasmic, M, membranous. Total scores 2 and 3 were designated as positive, whereas scores 0 and 1 were recognized as negative.

<table>
<thead>
<tr>
<th>Total score</th>
<th>BRG1</th>
<th>BRM</th>
<th>BAF155</th>
<th>Ini-1</th>
<th>BAF170</th>
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* TMA, tissue microarray.
cases with survival data, M-BRM correlated with a poorer survival in AD with a 5-year survival of 16.7% for those whose tumors were membrane positive compared with 38% for those whose tumors were negative for M-BRM staining (\( P = 0.016 \)). The number of the M-BRM-positive cases (six) with SCC did not allow meaningful statistical evaluation.

Previous study has shown that concomitant loss of BRG1 and BRM was a poor prognostic indicator (23). Consistent with this observation, our study also showed that patients whose tumors were positive for both nuclear BRG1 and BRM (\( n = 15 \)) had a 5-year survival of 72% compared with 33.6% for the rest of the cases (\( P = 0.013 \)). However, after adjusting for gender and stage, the \( P \) value was 0.068 for N-BRM and 0.072 for N-BRM/N-BRG1. In contrast, the predictive value of M-BRM

Fig. 3 Examples of immunohistochemical staining by BRG1 and BRM antibodies in primary lung tissues. A, BRG1 in normal lung. Most of the cells including pneumocytes and interstitial cells were positively stained in the nucleus. B, a lung tumor negative for BRG1 [TS0]. C, a tumor positive for BRG1 (TS3). D, BRM in normal lung. Most of the cells including pneumocytes and other interstitial cells are positive in the nucleus. E, tumor with negative BRM staining (TS0); the surrounding inflammatory cells were positive. F, tumor positive for BRM (TS3). G and H, membranous BRM staining in tumor cells. I, high magnifications (×400) of images shown in G and H. Diameter of tissue core = 0.6 mm.
remained strong after adjustments, with a \( P \) value of 0.0065. The associated Kaplan-Meier curves are shown in Fig. 5B.

**DISCUSSION**

Chromatin remodeling factors have been the subject of strong interest in human cancers. However, little is known about their role in lung cancer. Here, we investigated the expression of 12 chromatin remodeling factors in NSCLC by immunohistochemical staining of a TMA containing 300 primary tumor samples.

**Association between Antibodies.** Chromatin remodeling factors form complexes, and SWI/SNF is one of the most well-examined complexes (8, 10). In our study, we observed many statistically significant associations between pairwise comparisons of protein expression. Among these associations, about 50% were shared between AD and SCC. The remaining 50% were unique to each tumor type.

The SWI/SNF complex is known to be composed of one catalytic ATPase containing core protein (either BRG1 or BRM) and the other BRG1-binding proteins, *i.e.*, BAF170, BAF155, Ini-1, BAF47, BAF60a, BAF60b, BAF60c, BAF250, and BAF50 (10, 17, 19). It is also known to associate with some HDACs, such as HDAC1 and HDAC2, RbAP48, and RB (7, 10, 45, 46). There are increasing numbers of associated proteins known to interact with this complex. Because the role of SWI/SNF complex in transcription is broad, variation of the associated proteins in the complex is expected for different cellular functions. Among the associations observed in the current study, association between nuclear signals of BRG1 and HDAC2, BAF155, nuclear BAF155, and RbAP48, as well as HDAC1 and mSin3A, Ini-1, and RB have already been reported (10, 26, 45–47). Using \( \chi^2 \) test and clustering analysis, we showed that there were two distinct groups among these proteins (Fig. 4, B and C). Indeed, most of the known interactions were consistent with our \( \chi^2 \) test. Significantly, correlations between RB and HAT1 and mSin3A seen in this study have not been reported previously. HAT complexes and SWI/SNF complexes interact with each other for transcription. To stabilize the SWI/SNF on the promoter region, HAT complexes are thought to increase the retention of SWI/SNF on the promoter (38). However, our study only revealed the coexistence of potentially interacting proteins and cannot address whether these associations are due to direct
interaction within one complex or merely coexist in the same cell.

Prognostic Significance of BRM and BRG1. BRG1 and BRM are two highly homologous ATPases that are functionally equivalent and form the core of the mammalian SWI/SNF chromatin remodeling complex (17). Recently, concomitant loss of BRG1 and BRM was reported as a poor prognostic indicator by Reisman et al. (23). In their study, 6 cases that were negative for both BRG1 and BRM had a poorer survival rate compared with the other 54 NSCLC cases (41 AD and 19 SCC cases). The antibody used could not distinguish the two proteins. Here, we used antibodies that appeared to be specific for BRG1 and BRM, respectively, based on Western analyses and immunohistochemistry (Figs. 3A and 5A). Expression of BRM in lung cancer cells was generally low but detectable as a single band at the expected size and showed nonoverlapping expression with BRG1 by immunohistochemistry analysis. In lung cancer cell lines, three of five immunopositive cell lines had membranous staining, whereas two others showed only nuclear staining (Fig. 3B). Taken together, these findings suggest the following: first, the anti-BRM antibody used in this study did not cross-react with BRG1; second, both membranous and nuclear staining
represented the same BRM antigen; and third, membranous staining of BRM is present in primary lung cancers and may be more common in lung cancer cell lines.

We also compared all combinations of BRG1 and BRM staining with survival. Consistent with those reported by Reisman et al. (23), coexpression of N-BRG1/N-BRM correlated with a trend of better prognosis than any other combinations of these two markers. Significantly, BRG1 and BRM belonged to different clusters based on their expression status among the tested tumors in our study. Kadam and Emerson (48) showed that BRG binds to zinc finger proteins though its NH₂-terminal domain and is not present in BRM complex, whereas BRM interacts with two ankylin repeat proteins that are a part of the Notch signal transduction pathway. Our results are consistent with the knowledge that those two proteins do not exist in the same complex (26, 49) and indicate that BRG and BRM may participate in distinct but complementary cellular processes that are required for cell regulation.

In our study, 30 of 268 NSCLC cases showed a distinctive membranous BRM signal, whereas only 4 of those had coexpression of nuclear signal as well. Staining with COOH terminus and NH₂ terminus anti-BRM antibody (clone C-20 and N-19; Santa Cruz Biotechnology) showed a consistent membranous staining pattern (30 of 268 versus 26 of 263 countable samples). The correlation efficiency $P$ value between two antibodies is $10^{-14}$. In our study, N-BRM was associated with better prognosis in both SCC and AD tumors, whereas M-BRM was associated with a poorer prognosis only in AD. Although the exact mechanism is unclear, this difference in BRM cellular location may reflect the abnormal translocation of BRM in the cancer or indicate that BRM also participates in cellular processes other than chromatin remodeling. Because concomitant expression of N-BRG1/N-BRM correlated with a better prognosis, we reasoned that M-BRM status may be negatively correlated with N-BRG1 as well. However, 15 of 28 M-BRM-positive tumors were also N-BRG1 positive. Therefore, this change of localization more likely reflects BRM alterations in lung cancer cells independent of BRG1.

**Antibody Specificity and Staining Quality Control.** It is worth noting that tissue samples placed on TMA represent only a small portion of the whole tumor. Some samples will demonstrate negative staining due to tissue sampling or heterogeneity. Furthermore, some of the antibodies used here have not been extensively studied by immunohistochemical methods. To ensure specificity, we used Western analysis and immunohistochemistry on lung cancer cell lines to evaluate and optimize the tissue staining conditions. We also relied on well-established antigens, AE1/AE3 and PGP9.5, to evaluate the true positive rate of each antibody and used stringent criteria to exclude samples with uniformly weak stainings. In our pilot tests, we estimated that a number approximately equal to 10% of the positive samples may be falsely negative for each staining. Therefore, antibodies with a high percentage of positively stained samples will have a higher amount of false negative cases, making it less likely to identify significant clinical association. On the other hand, although each chromatin remodeling factor is expected to be present in the majority of cancer cells, all proteins investigated here have portions of negatively stained cases that far exceed the estimated number of false negative tumors (Table 3). Some of the negative staining reported here may represent a down-regulation of these proteins in the tumors or the lower sensitivity of the antibody. Our consideration of TS1 staining as negative may also have contributed to the lower estimation of antibody staining reported. For instance, our immunoblotting data showed that BRG1 was detected in a similar proportion of lung cancer cell lines as observed in TMA, whereas BAF155 and Ini-1 were only positive in <50% of the primary tumors compared with the data on lung cancer cell lines. Furthermore, because the tumor blocks were collected from pathologists worldwide, the quality and processing history of the blocks could vary greatly, and the clinical pathological and survival information for each case are less than perfect. These factors may also have contributed to the marginal statistical significance observed for some proteins, particularly after adjusting for other clinical factors.

Nonetheless, our study is one of the largest series of lung cancer analyses using an immunohistochemical method to examine the expression status of chromatin remodeling factors (35). We showed that BRM appeared to be a central protein whose cellular location and staining status appeared to correlate with overall survival in NSCLC patients. Furthermore, we propose that BRM may participate in distinct cellular processes that control growth, depending on the type of lung cancer cells. We also showed that the expression patterns of the chromosome remodeling factors branched into two distinct and reproducible clusters and argue that these clusterings may correspond to the true biological interactions of these factors. Clearly, the uniqueness and biological significance of these associations will have to rely on further analyses in other tissues and tumor types as well as validations by biological and biochemical assays. The prognostic trend of BRM reported here will also await confirmation using cohorts with more complete clinical pathological information.

**ACKNOWLEDGMENTS**

We thank Drs. Michael Emmert-Buck and John Gillespie for critical comments regarding this study and Anne Wenzel and Craig Hicks for editing the manuscript. We also express our gratitude to the pathology follow-up division at the Armed Forces Institute of Pathology for making follow-up and clinical information available for this study.

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