Loss of BRG1/BRM in Human Lung Cancer Cell Lines and Primary Lung Cancers: Correlation with Poor Prognosis

David N. Reisman, Janiece Sciarrotta, Weidong Wang, William K. Funkhouser, and Bernard E. Weissman

Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7525 [J. S. W. K. F., B. E. W.]; Laboratory of Genetics, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224 [W. W.]; and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7295 [D. N. R., B. E. W.]

Abstract

A role for the SWI/SNF complex in tumorigenesis based on its requirement for retinoblastoma induced growth arrest and p53-mediated transcription and the appearance of tumors in SWI/SNF-deficient mice. In addition, Western blot data have shown that the SWI/SNF ATPase subunits cell, BRG1 and BRM (BRG1/BRM), are lost in ~30% of human non-small lung cancer cell lines. To determine whether loss of expression of these proteins occurs in primary tumors, we examined their expression in 41 primary lung adenocarcinomas and 19 primary lung squamous carcinomas by immunohistochemistry. These analyses showed that 10% of tumors show a concomitant loss of BRG1 and BRM expression. Moreover, patients with BRG1/BRM-negative carcinomas, independent of stage, have a statistically significant decrease in survival compared with patients with BRG1/BRM. This report provides supportive evidence that BRG1 and BRM act as tumor suppressor proteins and implicates a role for their loss in the development of non-small cell lung cancers.

Introduction

SWI/SNF is a multimeric chromatin remodeling complex important for gene regulation. Like other proteins involved in gene regulation, the SWI/SNF complex is a potential target for disruption during neoplastic progression. Investigations into the chromosomal aberrations in 22q11.2 region led to the discovery of the SWI/SNF component, SNF5/INI1/BAF47, as a tumor suppressor gene in rhabdoid tumors, rhabdomyosarcomas, and chordoid plexus carcinomas (1–3). The finding that SNF5/INI1/BAF47 heterozygous mice develop tumors that closely resemble human rhabdoid tumors further supports this notion (4, 5). In yeast, the functions of the complex require the SWI/SNF is a multimeric chromatin remodeling complex important for gene regulation. Like other proteins involved in gene regulation, the SWI/SNF complex is a potential target for disruption during neoplastic progression. Investigations into the chromosomal aberrations in 22q11.2 region led to the discovery of the SWI/SNF component, SNF5/INI1/BAF47, as a tumor suppressor gene in rhabdoid tumors, rhabdomyosarcomas, and chordoid plexus carcinomas (1–3). The finding that SNF5/INI1/BAF47 heterozygous mice develop tumors that closely resemble human rhabdoid tumors further supports this notion (4, 5). In yeast, the functions of the complex require the functional integrity of the subunits, and loss of any one component is sufficient to alter the function of the complex (6). Thus, disruption of the SWI/SNF complex by the loss of other subunits may also be associated with human tumorigenesis. To this end, investigations have shown an evolving role for the mutually exclusive SWI/SNF ATPases, BRM (Brahma) and BRG1 (BRM-related gene 1), in growth control and tumorigenesis. Transgenic heterozygous BRG1 mice (BRG1+/−) form s.c. tumors resembling breast cancer (7). Although BRM null transgenic mice (BRM−/−) do not form tumors, the loss of expression of BRM causes cells to have an altered proliferation and impaired cell cycle control (8). Moreover, the BRG1 and BRM interact with key regulatory proteins, including RB4 protein, p53, p107, p130 (RB2), BRCA1, and β-catenin (2, 9). Specifically, BRG1 protein is required for RB-induced growth inhibition, p53-mediated transcription and BRCA1-controlled gene expression (10–12). Thus, the loss of BRG1 and BRM expression should impact on a multitude of growth regulatory pathways. These data provide a rationale for BRG1 and BRM to function as tumor suppressor proteins.

Although these and other data appear compelling, establishing BRG1 and BRM as bona fide tumor suppressors also requires demonstrating their loss of expression in primary tumors. Therefore, based on data showing loss of BRG1 and BRM expression in ~30% of human NSCLC cell lines and published data showing loss of heterozygosity surrounding the BRG1 and BRM loci in human lung cancers (13) we conducted immunohistochemical staining for BRG1 and BRM on 60 NSCLC samples taken from patients that underwent curative resections for stage I–IIIA tumors. We observed loss of BRG1/BRM expression in 6 of 60 tumor samples (~10%), suggesting that BRG1/BRM loss in tumor cell lines does not arise as a tissue-culture artifact. Consistent with cell line data, loss of BRG1 expression occurred concomitantly with loss of BRM expression. Clinically, loss of BRG1/BRM expression in NSCLC was associated with a significantly worse patient survival compared with the survival of patients with BRG1/BRM-positive tumors. These data additionally support that BRG1/BRM act as tumor suppressor proteins and that loss of function of these proteins may potentially impact the clinical outcome of NSCLC.

Materials and Methods

Immunohistochemical Staining. Normal human tissue and paired NSCLCs were collected from University of North Carolina archives, and 5-μm sections were cut and mounted on Probe On Plus slides (Fisher Scientific). After deparaffinization in xylene, slides were rehydrated and placed in running water. Slides were placed in a MicroProbe slide holder (Fisher Scientific), and the appropriate antigen retrieval buffer was applied. BRG1 in AR-10 (BioGenex) and BRM in Citra (BioGenex). All slides were then placed in a Black and Decker steamer for 30 min and allowed to cool for 20 min. After multiple rinsing in Automation Buffer (Biomeda), endogenous peroxidase was quenched using a 3% solution of hydrogen peroxide. Excess protein was then blocked using normal goat serum (BioGenex), and the sections were then exposed to the primary antibodies, BRG1 1:1000 (a gift from Pierre Chambon) or BRM 1:50 (Transduction Laboratories) for 30 min at 37°C. After rinsing in Automation buffer, slides were then placed in the secondary antibody (BioGenex Super Sensitive Multi Link) for 7 min at 37°C, rinsed in Automation Buffer, and then incubated in the label (BioGenex Super Sensitive HRP) for 7 min at 37°C. After again rinsing in Automation Buffer, detection of the antibody/antigen complex was visualized using 3,3′-diaminobenzidine. Slides were then lightly counterstained in Mayer’s hematoxylin, rinsed, dehydrated, cleared, and mounted.

Tumor Samples. Tumor samples banked at the University of North Carolina from 1997–1999 were randomly selected. The specimens were derived from patients with stage I–IIIA NSCLC. Internal Review Board approval was obtained before our analysis.
Results

To determine whether SWI/SNF complex members other than INI1/SNF5/BAF47, a known tumor suppressor gene, were lost in human carcinomas, we screened a variety of human cell lines derived from different primary carcinomas (13). We found that loss of expression of BRM and BRG1 appears more frequently than the loss of other complex subunits, occurring in 10% (8 of 80) of human tumor cell lines (14). In these cell lines, the down-regulation of BRG1 occurred concomitantly with down-regulation of BRM (15). Also, four of eight BRG1/BRM-deficient cell lines were derived from lung adenocarcinomas, representing 33% (4 of 12) of NSCLCs overall. Wong et al. (16) showed BRG1 has been mutated in 14% (2 of 14) of NSCLC cell lines and 10% (18 of 180) of human tumor cell lines using high throughput DNA sequencing. Subsequent Western blotting analysis of these cell lines showed that only the lung cancer cell lines (A427 and H1299) harboring BRG1 mutations had low to no expression of both BRG1 and BRM (17). Taken together, these cumulative data show that loss of BRG1 and BRM expression occurs in ~30% (6 of 20) of human lung cancer cell lines (Table 1).

Primary Human NSCLC Show Reduced or Absent BRG1 and BRM Expression. To establish that loss of BRG1/BRM occurs in primary tumors and does not arise from the establishment of the cell lines, we examined the expression of BRM and BRG1 in 41 primary lung adenocarcinoma cancers and 19 primary lung squamous cell carcinomas by immunohistochemistry. By using a BRG1-specific antibody, we first detected BRG1-negative tumors. Staining the same BRG1-negative tumors with a second antibody that recognizes a conserved shared BRG1/BRM epitope, allowed us to unambiguously determine the presence or absence of BRM protein. In addition, when staining was not detected with this second antibody, it also confirmed that these tumors were negative for BRG1.

Using the BRG1 specific antibody, we detected loss of BRG1 expression in 6 of 60 tumors (~10%; Figs. 1, A–F, and 2). These 6 tumors also showed no positive signal with the second antibody showing that neither BRG1 nor BRM expression was present (Figs. 1, G–L, and 2). Moreover, these tumors did not show a continuum of staining with either of these antibodies such that the positively stained tumors are distinctly grouped from the negatively stained tumors (Fig. 2).

The other 54 tumors stained positively with both antibodies, indicating they expressed at least BRG1 protein. We previously showed that both antibodies specifically recognize BRG1 and BRG1/BRM, respectively (15). Of the 6 BRG1-negative tumors, 3 were uniformly devoid of either BRG1 or BRM expression, whereas the other 3 tumors had focal and minimally detectable staining. The loss of BRG1/BRM expression in these tumors was not attributable to staining irregularities because the immunohistochemistry on these samples was repeated to confirm the results, and each sample showed internal positive control staining (normal bronchial epithelial and infiltrating lymphocytes).

Although we have previously observed that the loss of expression of both BRG1 and BRM appear limited to adenocarcinoma cell lines (15), we also found reduced or absent BRG1 and BRM expression in adenocarcinomas (3 of 41) as well as squamous cell carcinomas (3 of 19; Fig. 2A). Interestingly, two of these latter carcinomas are adenocarcinoma morphology, and loss of BRG1/BRM expression was noted in the area of adenocarcinoma morphology as well as in the area of squamous carcinoma morphology. These results suggest that loss of BRG1 and BRM may not be restricted to adenocarcinomas, as suggested by our original cell line data. Consistent with our previous studies, tumors demonstrated concomitant loss of BRG1 and BRM expression (Fig. 2A). However, because the second monoclonal antibody (Transduction Laboratories) detects both BRG1 and BRM proteins (Ref. 15; Reisman, unpublished data), we cannot determine whether any of the BRG1-positive tumor samples lack only BRM expression.

Loss of BRM/BRG1 Correlates with a Worse Prognosis in NSCLCs. We compared the survival of the 6 patients who had BRG1/BRM-negative tumors to the 54 patients with BRG1-positive tumors (Fig. 3, B and D). During a median 36-month follow-up period, the BRG1-positive patients with stage I, II, and III disease had a survival of 79, 66, and 44%, respectively, consistent with previously published data (Fig. 3; Ref. 18). However, the patients with BRG1-negative tumors had 0% survival during this period (Fig. 3, A and C). The Kaplan-Meier curves show this survival difference between patients with BRG1-positive and BRG1-negative tumors (Fig. 3B).

### Table 1 Expression of BRG1 and BRM in human lung cancer cell lines

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Statistical Analyses. The Kaplan-Meier (or product limit) method was used to estimate the survivorship function. We used the log-rank test to identify possible differences between estimated survival curves. All analyses were performed with SAS statistical software, version 8.2, from SAS Institute (Cary, NC).
cancer patients with stage I, II, and IIIA disease have progressively worse survival despite best therapy (curative surgical resection). Although the patients with BRG1-negative tumors had primarily stage I (2 of 6) and stage II (3 of 6) disease, they had a statistically worse survival ($P < 0.03$) than patients with the clinically more advanced stage IIIA (BRG1 positive) tumors (Fig. 3C). This difference in survival is not because of difference in treatment or therapies as all 60 patients were treated with surgery alone without any neoadjuvant or}

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**Fig. 1.** BRG1 and BRM expression in primary NSCLCs. Forty-one adenocarcinomas and 21 squamous cell carcinomas were stained for BRG1 expression using a BRG1-specific monoclonal antibody. Six tumors were devoid of BRG1 expression, whereas the normal bronchial epithelium and/or infiltrating lymphocytes within these samples showed specific nuclear staining (as illustrated in the 3 tumors B, D, and F). In comparison, 54 of the tumors showed preferential nuclear staining as illustrated in A, C, and E. The same 6 tumors, which were devoid of BRG1 expression, were stained with a BRM/BRG1 antibody that detects both proteins. These tumors were also found to have minimal to no BRM expression as illustrated in the 3 tumors H, J, and L. Internal positive controls can be seen in these samples [bronchus (J) and lymphocytes (H and L)]. The other 54 tumors show specific nuclear staining as exemplified in tumors shown in G, I, and K.
adjuvant therapies. As might be expected, patients with BRG1-negative NSCLC also had worse survival than patients with stage-matched BRG1-positive NSCLC.

Discussion

SWI/SNF complex subunits were first implicated in tumorigenesis when INI1/hSNF5/BAF47 was recognized as a tumor suppressor. The expression of this protein is lost in pediatric rhabdoid tumors and certain central nervous system tumors secondary to inactivating mutations and gene deletions (1, 2, 19). We show that like INI1/hSNF5/BAF47, the SWI/SNF ATPase subunits, BRG1 and BRM proteins, are also lost in a subset of primary human tumors. This finding, together with transgenic animal data showing that loss of BRG1 is tumorigenic and that BRM is involved in...
proliferation, supports that BRG1 and BRM are tumor suppressor proteins. Mechanistically, cell line data additionally supports this concept by showing that BRG1 and BRM expression is required for RB-mediated growth inhibition (12, 15, 20). Thus, the loss of BRG1/BRM may serve to abrogate the RB pathway similar to the loss of p16 and/or RB function in human tumors.

The loss of function of the SWI/SNF complex will likely produce major phenotypic changes within tumors because of its interaction and requirement in many signal transduction pathways in addition to the RB pathway. BRG1 and BRM have been shown to interact with cyclin E, Myo-D, p53, p107, and p130 (RB2; Ref. 2, 11, 21, 22). Their function is also required for the function of estrogen, glucocorticoid, progesterone, and retinoic acid receptors (19). Another affect of BRG1/BRM loss may come from the SWI/SNF complex’s contribution, via BAF60a, to the induction of AP1 responsive genes (23). The number of genes actually regulated by the SWI/SNF complex is at least 80 and in yeast the SWI/SNF complex regulates 5–7% of the yeast genome (24). Thus, the broad changes in the signaling pathways that must occur with loss of BRG1/BRM must have an impact on the tumor phenotype. Our data suggest that this loss may be an important indicator of a patient’s overall survival.

As with human cancer cell lines, primary tumors appear to have concomitant loss of both BRG1 and BRM protein expression. Because experiments with human tumor cell lines show that BRG1 and BRM have redundant functions (12, 15, 25), their concomitant loss may be required to completely inactivate the SWI/SNF complex. However, biochemical analyses by several groups have shown that BRM and BRG1 are contained in different complexes that, in turn, have apparently different biochemical functions (26, 27). Whether the concomitant loss of BRG1 and BRM expression is required to abrogate separate SWI/SNF functions or occurs because BRG1 and BRM share...
redundant functions important for tumorigenesis, warrants additional investigation.

Acknowledgments

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References


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