

## Discovery of BRM Promoter Polymorphisms: Importance in Risk Stratification and Clinical Application

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### Abstract

Lung cancer is the most common cause of all cancer deaths in the United States and worldwide. Despite advances in surgical techniques and therapies, the cure rates for lung cancer have not changed in the last thirty years. Lung cancer typically presents in the advanced stages when the cancer has already spread beyond the chest wall. Therefore, a goal of clinicians is to treat these patients while their lung cancers are at an earlier stage. The screening of smokers and former smokers by computed tomography (CT) is usually the recommended approach in order to detect early-stage lung cancer. However, given that only a small percentage of smokers will get lung cancer, researchers have sought to determine the causes of lung cancer other than tobacco exposure. Throughout the past decade, investigators have attempted to find suitable biomarkers to determine cancer risk so that individuals can be risk-stratified prior to screening. This process would target a much more specific at-risk population and would minimize the risks of CT and other diagnostic modalities in those individuals who are not at the highest risk. The polymorphisms in the promoter of the *Brahma (BRM)* gene are two candidate biomarkers that have been associated with loss of the tumor susceptibility protein BRM, which in turn is correlated with an increase in cancer risk. Much progress has been made since the initial discovery of these polymorphisms in 2011, and the aim of this commentary is to review the discovery of the *BRM* polymorphisms and to discuss their pertinence to clinical practice.

### Introduction

Epigenetic regulation of gene expression involves the activity of histone-modifying enzymes that function in the methylation, acetylation, ubiquitination, phosphorylation and ribosylation (i.e., "marks") of histones [1]. The histone-modifying enzymes cooperate with chromatin remodeling complexes to regulate gene expression, as these complexes are able to recognize the histone marks [1]. The mammalian SWI/SNF (SWItch/Sucrose Non-Fermenting) complex is just one of several chromatin remodeling complexes that functions to shift histones along the chromatin in order to expose or conceal certain segments of the DNA to transcription factor machinery and other important proteins [2, 3]. This complex contains an ATPase/catalytic subunit (i.e., Brahma (BRM) or its homolog Brahma-related gene 1 (BRG1)) that provides the energy required to move the histones along the chromatin, as well as BRG1/BRM-associated factors (BAFs). Some

studies have suggested that each ATPase subunit can compensate for the loss of the other, which may explain why BRG1 and BRM are concomitantly lost in cell lines and primary tumors [3-7]. However, other studies have indicated that BRM and BRG1 maintain completely distinct functions, which may or may not preclude their ability to compensate for each other [8-10].

As a major regulator of gene expression, the SWI/SNF complex functions in growth control, differentiation, DNA repair, development and cell adhesion, and therefore, it is not unexpected that this complex is often a target of cancer initiation and progression [2]. Interestingly, while some subunits of SWI/SNF are targeted by mutations, other subunits are targeted by other means, including epigenetic suppression [11-19]. In fact, *BRM* is rarely mutated in cancer, but is lost in about 15-39% of lung cancers, 15% of breast, 15% of bladder, 16% of head and neck, and 70% of colorectal cancers [11, 20, 21]. In

addition, BRM expression is also lost in 16% of gastric and 35-47% of prostate cancers, and a reduction in expression has been shown in pancreatic cancer [5, 22-24]. *BRM* is silenced in 10-25% of many solid tumor types at a frequency similar to that of *KRAS*, *HER2*, *EGFR* and *ALK* mutations in breast and lung cancers [21, 25]. Since mutations in the *BRM* gene are rare, *BRM* is primarily silenced by some means other than mutations [26, 27].

The *BRM* locus (9p23-24) is also susceptible to loss of heterozygosity, which occurs in a variety of malignancies, including lung cancer [28, 29]. The expression and or function of many anticancer proteins and other key signal transduction proteins, such as RB, p53 and BRCA1, are dependent on a functional SWI/SNF complex [30-34]. The loss of BRM can have wide-ranging effects that have not been completely elucidated. While a conditional knockout of *BRG1* in animal models potentiates carcinogenesis, a knockout of its homolog *BRM* requires the presence of a carcinogen in order to initiate tumor development [35, 36]. For example, when BRM-deficient mice are exposed to carcinogens, they develop 10-fold more tumors [36]. *BRM* is therefore known as a tumor susceptibility gene, as opposed to a tumor suppressor gene.

### Polymorphic Sites in the *BRM* Promoter

Several years ago, during efforts to uncover how *BRM* is silenced in primary lung cancer, both BRM mRNA and genomic DNA from multiple cell lines and primary human tumors were sequenced [21]. No mutations were found in the *BRM* gene. After a confirmation of the transcriptional start site (TSS), Liu et al. examined the sequence of the promoter to determine how *BRM* might be silenced [29]. By Sanger sequencing, no mutations were detected in the *BRM* promoter in multiple BRM-deficient cell lines or in primary tumors; however, two promoter indel sequence variants were found. One is located at -1321 base pairs (bp) upstream from the TSS, and contains a duplicate repeat of the TTTTAA sequence (6 bp), whereas the wild

type genotype contains this sequence only once. The other is located at -741 bp upstream from the TSS and contains a triplicate repeat of the TATTTT sequence (7 bp); in this case, the wild type genotype contains a duplicate of this sequence. The sequences of these insertion variants are actually homologous with MEF2 (myocyte enhancer factor-2) binding sites [37]. Interestingly, MEF is known to recruit histone deacetylases (HDACs), which have been shown to silence genes, including *BRM* [38, 39].

### *BRM* Polymorphisms and *BRM* Silencing

BRM-negative and BRM-positive cell lines were then compared by qPCR-Taqman analysis [29]. All BRM-negative cell lines contained at least one homozygous variant (i.e., at either the -1321 or -741 site), while 42% contained two homozygous variants (i.e., at both -1321 and -741). However, only about 30% of the BRM-positive cell lines demonstrated at least one homozygous variant. When Taqman qPCR was performed on samples of non-small cell lung carcinoma (NSCLC) that did not express BRM protein (as confirmed by Western blot), the majority of the BRM-negative primary tumors possessed genotypes that were homozygous for both variants.

### The Presence of the *BRM* Polymorphisms and Cancer Risk

Liu et al. then conducted a case-control study, whereby they genotyped 484 individuals with lung cancer who also had a history of smoking and 715 control individuals with a history of smoking but no lung cancer [29]. It was determined that the combination of both homozygous variants (-1321 and -741) carried the highest risk; therefore, it was postulated that individuals with genotypes that are homozygous for both insertional polymorphisms are at a greater risk for lung cancer than those whose genotypes are heterozygous or wild type. The adjusted odds ratio (aOR) for the presence of 4 variant alleles versus none of the variant alleles was 2.21 [29]. The presence of both homozygous

variants carried the greatest risk for lung cancer, with an aOR of 2.19 [29].

In Caucasians, these two polymorphisms are in Hardy-Weinberg equilibrium, and they each occur at a frequency of around 20%; the combined frequency of the two polymorphisms is about 6% in a normal population, but is around 16% in a population of lung cancer patients. Research is currently being conducted in other racial and ethnic populations (personal communication).

It was also demonstrated that each insertion variant strongly correlated with the loss of BRM protein expression in both lung-cancer-derived cell lines and human primary lung tumors [29]. Within the past several years, studies have shown a statistically significant correlation of these polymorphisms with a higher risk of lung cancer [29], head and neck cancer [36], hepatocellular carcinoma [40] and upper aerodigestive tract cancers [41]. Interestingly, although initial studies were performed in cell lines and primary tumors, the most recent studies mentioned above were performed using genomic DNA derived from blood lymphocytes. More recently, a correlation was demonstrated between the presence of the -1321 polymorphism and BRM loss in Rhabdoid-derived cell lines [13]. *BRM* is silenced in 10 out of 11 Rhabdoid cell lines and in about 65-70% of primary Rhabdoid tumors according to Western blot and IHC. In short, it was found that *BRM* is regulated in a similar manner in malignant Rhabdoid tumors as it is in lung tumors (i.e., by HDAC9 and HDAC3, GATA3 and MEF2D). Based on these data, it appears that the epigenetic silencing and regulation of *BRM* is conserved among different cell types, at least between lung cancer and malignant Rhabdoid tumors [13]. Additional studies are needed to determine the following: a) if *BRM* is regulated in the same way as in the aforementioned cancer types where the *BRM* promoter polymorphisms have been detected, b) if the correlation between the polymorphisms, BRM loss and cancer risk is sustained across different population samples.

### The Role of the *BRM* Polymorphisms in the Regulation of BRM Expression

As mentioned above, the sequences of the polymorphic sites are highly homologous to MEF2 binding sites. MEF2 proteins are transcription factors that control gene expression, and because the polymorphisms are more likely to be found in tumors that lack BRM expression, it was believed that perhaps MEF2 is involved in *BRM* silencing. MEF2 proteins are known to silence genes via the recruitment of histone deacetylase complexes (HDACs), and interestingly, HDAC inhibitors that target class I and II HDACs (e.g., FK228 and CHAP31) have been demonstrated to reverse BRM silencing. Liu et al. (2011) treated six BRM-negative cell lines (A427, SW13, H522, C33A, H1299 and H23) and six BRM-positive cell lines (H460, Calu3, A549, H441, Calu6 and H2450) with two different HDAC inhibitors (trichostatin A or CI-994) [29]. A robust induction of BRM was observed at the mRNA level in the BRM-negative cell lines after treatment with either HDAC inhibitor, as determined by qPCR. They demonstrated a nearly 100-fold increase in BRM heterogeneous mRNA induction after treatment of BRM-negative cell lines with either HDAC inhibitor; this suggests that BRM expression is increased by HDAC inhibitors at the transcriptional level.

The knockdown of HDAC3 and HDAC9 via shRNA transfection of BRM-negative cell lines resulted in a strong induction of BRM expression, which demonstrated that these two HDACs are directly involved in the silencing of the *BRM* gene [11]. When the transcription factors MEF2 and GATA3 were sequentially knocked down in BRM-deficient cancer cell lines, BRM was also robustly induced, which indicated that these two transcription factors are also involved in the *BRM* silencing mechanism [11]. Like *BRM*, *HDAC3*, *HDAC9* and *GATA3* were not found to be mutated in cancer, but they are overexpressed in BRM-negative lung tumors and well as in BRM-negative malignant rhabdoid tumors. Gene regulation is likely due to a balance among HDACs and histone acetyltransferases (HATs), which function in the addition of acetyl groups to

histone proteins. It was also demonstrated that ectopic induction of lysine acetyltransferases (KATs)—specifically KAT6A, KAT6B and KAT7—also induces BRM expression [11]. Despite the fact that *KAT* genes are sometimes mutated in certain cancer types [42, 43], no mutations were detected in *KATs* 6A, 6B or 7 [11].

ChIP experiments have shown that MEF2D and HDAC9 bind to the *BRM* promoter only when the polymorphic inserts are present, which further implicates these two transcription factors in the mechanism of *BRM* silencing [13]. Han et al and Bertos et al. have both reported that MEF2D binds to promoter regions and recruits HDAC9, which together, function in the silencing of genes [39, 44].

### **Tumor Suppressors: Can We Turn Them On?**

In recent years, many therapies have been devised that target oncogenes that drive tumor growth, such as epidermal growth factor receptor (*EGFR*), and vascular endothelial growth factor (*VEGF*), among others. Therapies typically consist of small-molecular inhibitors and antibodies. For example, imatinib is a small molecule that blocks the protein product of the *BCR-ABL* oncogene [45], and trastuzumab is a monoclonal antibody used to treat HER2-positive breast cancers [46, 47]. However, the targeting of tumor suppressor genes has been substantially more difficult since most of these genes are either silenced by mutation or are deleted entirely [26]. Nevertheless, *BRM* has been shown to be reversibly and epigenetically suppressed, unlike other tumor suppressor genes that may be mutated or deleted.

While the first HDAC inhibitors that were discovered (e.g., TSA, butyrate) have been shown to induce BRM expression, they also inactivate BRM via acetylation at its C-terminus [26, 27]. These HDAC inhibitors also tend to inactivate a wide range of HDACs (not just specific HDACs), which may have deleterious effects on normal cells. This precludes the use of HDACs in a clinical setting for the restoration of BRM. It has been shown that the inhibition of HDAC1/2 causes the acetylation of BRM, thus inactivating it, whereas

the inhibition of HDAC3 induces functional BRM [11, 26].

In order to determine what other compounds might induce not only BRM expression, but BRM function as well, a luciferase-based BRM functionality assay was conducted in a BRM/BRG1-deficient cell line that has been previously described [26]. After testing the accuracy of this assay and after the initial screening of approximately 5,000 compounds, indoprofen and 4-methoxyflavone were found to robustly induce functional BRM protein [26]. Next, a high-throughput screen of additional compounds in the screening library was conducted. The number of compounds was narrowed to about 500, as these were compounds that demonstrated at least 75% of the activity of the positive controls. Two compounds (RH02032 and GH0037) were discovered that induced luciferase 3-4-fold in this assay [25, 26], and further qPCR experiments to detect BRM-dependent genes verified that these two compounds induce functional BRM protein.

In subsequent studies, it was determined that flavonoid compounds from each of the six structural groups (i.e., Luteolin, Quercetin, Genistein, Hesperidin, EGCG and Delphinidin) readily restored BRM and inhibited growth in BRM-deficient cell lines, which was then demonstrated in an *in vivo* mouse model [48]. It was then shown that this induction is BRM-dependent and that flavonoids can activate BRM by a reversal of its acetylation. It was recently demonstrated that the synthetic flavonoid Flavopiridol induces growth arrest in Rhaboid-derived cell lines [13, 48]. Flavopiridol works, in part, by the induction and reactivation of BRM, which in turn restores RB-mediated growth inhibition [13]. All of these studies illustrate the feasibility that small molecular inhibitors and other such compounds may be used to re-express a tumor suppressor or a tumor susceptibility gene, which in turn would lead to the inhibition of tumor growth.

### **Implications for Clinical Practice**

Despite many advances in treatment and surgical options for lung cancer, cure rates have not changed significantly in the last several decades, and lung cancer continues to kill more people than the next four cancer types combined [49, 50]. One reason why this occurs is because lung cancer often does not manifest in the early stages; therefore, by the time symptoms appear, the cancer has usually already spread beyond the chest wall. Many agree that the key to the reduction in lung cancer deaths lies in the ability to detect lung cancers at earlier stages. Some healthcare companies have presented screening guidelines that essentially call for the screening of individuals (by computed tomography (CT)) who have smoked for a certain period of time [51]. However, only about 10% of smokers will develop lung cancer [52] and about 10-15% of lung cancers are caused by factors other than active smoking [53], which account for as many as 24,000 deaths annually in the United States. Lung cancer in never-smokers is therefore one of the most common causes of cancer-related deaths [53], and thus, there are other factors such as genetic susceptibility that play a vital role in the development of lung cancer [54].

Some clinicians have suggested that such guidelines that recommend the screening of all current and former smokers would entail the screening of far too many individuals, most of whom will never develop lung cancer. This “needle in a haystack” approach is both expensive and it poses additional risks to those who are scanned. According to a recent report, several hundred thousand Americans are diagnosed each year with a pulmonary nodule [55]. These nodules are typically detected by CT scan, which itself may pose risks. Low-dose spiral chest CT scans are useful for the detection of lung cancers in the early stages [56-58], and their use has resulted in a 20% reduction in overall mortality rates in heavy-smokers who were screened [59], most recently in the National Lung Cancer Screening Trial (NLST) [56]. However, CT scans deliver a much higher dose of radiation than other forms of radiography, and therefore, repeated scans can impart a greater risk for the development of leukemia, brain, breast and lung

cancers [60, 61]. A study from 2009 indicated that as many as 29,000 future cancers might be related to CT scans performed in 2007, when approximately 72 million scans were performed in the US alone [61]. Some reports estimate that as many as 2% of cancers in the US can be attributed to radiation from CT scans [61, 62].

Although repeated scans for the purpose of detecting early stage lung cancer would likely be conducted at lower doses, these scans would likely be coupled to higher-dose diagnostic scans if a lesion were found [51]. In addition, more than 90% of pulmonary nodules observed by CT scan are found to be benign after a biopsy [63]. Transthoracic needle biopsies also carry an additional risk. Complications such as pneumothorax occur in anywhere from 9-54% of patients (average: 20%). Other less common complications include hemoptysis, hemothorax, air embolism and infections [64]. Although there are additional risk factors for these complications, such as advanced age, smoking status and COPD [55], many patients with noncancerous lesions might experience unnecessary morbidities.

As recently as 2011, Evans et al. claimed that we do not yet understand how to “optimally define the at-risk population, when to start screening, what screening interval to use, and for how long” [49]. Since screening strategies often use smoking as the sole criterion, lung cancer screening in the US is more expensive than screening for colon cancer or breast cancer [49, 65-67] and may pose additional risks to those who are screened. Many clinicians and investigators have called for better biomarkers to detect lung cancer risk. Numerous population-based studies have been conducted in recent years that have found numerous genetic polymorphisms that may be associated with lung cancer risk [51, 54, 68-70].

### **Relevance of Biomarkers for Lung Cancer**

Researchers have tried for decades to find suitable biomarkers for the early detection of lung and other types of cancers. The urgency to identify biomarkers of lung cancer is reflected in

the morbidity and mortality of this disease. Nearly 1.4 million people worldwide die from lung cancer every year, which indicates that lung cancer continues to be a growing public health concern [71]. According to one report, the 5-year survival rate for patients with lung cancer is only 15% [72]. Another recent report states that 5-year survival rate of patients with early operable non-small cell lung cancer is as high as 70%, whereas the 5-year survival rate of patients who were diagnosed with NSCLC that has already spread to distant sites is only 2-5% [73]. While CT screening has demonstrated effectiveness in the reduction of mortality from lung cancer, these scans pose additional risks to those who are screened and often result in many false-positives [72].

According to Cipriano et al. [74], the average monthly cost for a 72-year old with lung cancer ranged from \$2687 (no active treatment) in the first few months of care to \$9360 (receiving chemo/radiotherapy). In order to reduce the substantial cost of screening large segments of the population, many of whom are current or former smokers, and to minimize any health risks from biopsies or unnecessary radiation exposure, a risk stratification strategy is urgently needed. While BRM is not expressed in every tissue type, it has been demonstrated to be lost in a variety of cancer types. The BRM promoter polymorphisms not only potentially explain how they contribute to BRM silencing, they also have proven to be important germline risk factors for

the development of several different cancer types, including lung cancer.

Unfortunately, none of the other germline polymorphisms associated with lung cancer risk that have been validated in multiple large sample sets have not been translated into clinical practice.

Since these polymorphic inserts are present in the germline, they can actually be detected in DNA obtained from peripheral blood lymphocytes. The studies by Wang et al., Gao et al. and Wong et al. all used purified DNA from blood samples obtained in the clinic for other purposes [36] [40] [41]. Therefore, since all of these case-control studies were conducted using blood-derived DNA, the only tissue required to risk-stratify individuals is blood. This poses much less of a risk than invasive biopsies or CT scans. If biomarkers such as the *BRM* polymorphisms can be used to identify which individuals are at the highest risk for lung cancer, those individuals can then be recommended for CT screening. Since cancers themselves are heterogeneous and because specific genetic changes occur in a fraction of cancer patients [26], drug treatments of the future that target specific patient populations that would best respond to those treatments, will likely be the most effective. As the dawn of personalized medicine comes to fruition, risk stratification of those at the highest risk for disease will become increasingly important so that individuals can be targeted for preventive and early treatment options.

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