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***BRMS1* transcriptional repression correlates with CpG island methylation and advanced pathological stage in non-small cell lung cancer**

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Abstract

Breast cancer metastasis suppressor gene-1 (*BRMS1*) mRNA and protein expression are significantly decreased in non-small cell lung cancer (NSCLC) and this is a poor prognostic indicator. Given that the *BRMS1* promoter region contains a promoter-associated CpG island (CGI) that encompasses the transcriptional start site, we hypothesized that decreased *BRMS1* mRNA and protein levels in NSCLC was secondary to increased *BRMS1* promoter methylation. Methylation-specific PCR (MSP) of the two known CGIs (−3477 to −2214 and −531 to +608) in the *BRMS1* genome was performed in NSCLC cells. This demonstrated a robust increase in methylation of the promoter-associated CGI (−531 to +608) but not of the upstream CGI (−3477 to −2214). To experimentally verify that methylation contributes to *BRMS1* transcriptional repression, we cloned the *BRMS1* promoter region, including the promoter-associated CGI, into a luciferase reporter gene and found that *BRMS1* promoter activity was dramatically inhibited under methylated conditions. We then assessed the *BRMS1* methylation profile with MSP and bisulphite-sequencing PCR in human NSCLC adenocarcinoma ($n = 20$) and squamous cell carcinoma ($n = 20$) relative to adjacent non-cancerous bronchial epithelium. There was a significant increase in *BRMS1* promoter methylation in all NSCLC specimens relative to non-cancerous tissues, with the most dramatic difference in squamous cell cancer histology. Subsequent immunostaining demonstrated that nuclear *BRMS1* expression is reduced in lung cancer specimens compared to normal bronchial epithelium. The association between *BRMS1* promoter methylation and specific clinical and histopathological variables was examined using a general linear model. Pathological tumour stage was associated with increased *BRMS1* methylation in squamous cell cancers. These observations demonstrate that methylation of the promoter-associated CGI in *BRMS1* results in its transcriptional repression, and highlight the potential clinical relevance of this methylation event with respect to NSCLC tumour histology and pathological stage.

Keywords

lung cancer; *BRMS1*; methylation; methylation-specific PCR; immunohistochemistry; pathological stage; squamous cell cancer

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No conflicts of interest were declared.

Introduction

Lung cancer remains a common cause of cancer-related deaths for men and women in the Western world [1]. Early stage, node-negative lung cancer that has not metastasized has a more favourable prognosis (50% 5-year survival) compared to advanced-stage disease with lymph nodal or other organ dissemination (21% and 3% 5-year survival, respectively) [1]. Therefore, understanding the mechanisms through which lung cancer metastasizes is important if new therapies are to be designed to better treat lung cancer patients.

Welch and colleagues initially identified and described breast cancer metastasis suppressor 1 (*BRMS1*), a gene shown to suppress metastasis in human breast cancer cell lines without affecting primary tumour growth [2], and whose reduced expression has been correlated with poor prognosis in breast cancer patients [3,4]. There are several proposed mechanisms of action for *BRMS1* and its role in the regulation of tumour metastasis; these include suppression of NF- κ B signalling via (a) inhibition of I κ B α or (b) functioning as a co-repressor for the transcriptionally active subunit of NF- κ B, RelA/p65 [5–7]. Other studies have shown that *BRMS1* reduces phosphoinositide signalling [8] and plays a role in cell–cell communication [9–11]. A recent study in breast cancer showed that of the two CpG islands (CGI) in the *BRMS1* genome, only the upstream CGI (–3477 to –2214) was methylated. Importantly, correlation with this CGI methylation and *BRMS1* protein expression were noted, but no direct link to *BRMS1* transcription was described, leaving open the question of the transcriptional significance of this methylation event [12]. Moreover, it is known that CGI methylation profiles are tissue-specific, suggesting that analysis of *BRMS1* promoter methylation profiles in other tissues and cancers merits further investigation [13].

Until recently, limited data existed on the role of *BRMS1* in non-small cell lung cancer (NSCLC). We have shown that *BRMS1* mRNA and protein expression are decreased in human NSCLC specimens [6]. Moreover, we have demonstrated that *BRMS1* does function as a metastasis suppressor in a mouse model of lung cancer, and that reduced NSCLC *BRMS1* protein expression strongly correlated with a worse clinical prognosis [14]. Given these observations, we hypothesized that *BRMS1* transcription is decreased in NSCLC through increased *BRMS1* promoter methylation, specifically methylation of the promoter-associated CGI (–527 to +612), which encompasses the transcriptional start site and exon 1 in the *BRMS1* promoter. We also sought to determine whether *BRMS1* promoter methylation correlated with distinct NSCLC histologies and specific clinicopathological variables.

Methods

Cell culture, surgical specimens, and reagents

Human NSCLC cell lines (NCI-H157, NCI-A549, NCI-H1299), normal bronchial human bronchial epithelial cell line (NHBE) and human embryonic kidney cells (HEK 293T) [15] were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown as previously described [6]. Human NSCLC specimens and adjacent non-cancerous lung were obtained from our tissue bank of NSCLC specimens from patients undergoing surgery at the University of Virginia. Informed patient consent and Human Investigations Committee approval was obtained on all cases. The antibodies used in this study were *BRMS1* (Abnova Corp., Taiwan) [6,14] and α -tubulin (Sigma Aldrich, St. Louis, MO, USA).

Western blotting analysis

Whole cell lysates were obtained and western blots were performed as previously described [6]. The primary antibodies were used at 1 : 1000 dilution and secondary antibodies (Promega, Madison, WI, USA) were used at 1 : 5000 dilution.

DNA extraction, sodium bisulphite modification, and bisulphite genomic sequencing

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Austin, TX, USA) and modified by sodium bisulphite (EZ DNA Methylation Kit, Zymo Research, Orange, CA, USA). The modified DNA was amplified by PCR (primers: forward, 5'-ATTAAATTGTTTAATTGTGAGTATTTT-3'; reverse, 5'-AAACTAAAACCTCTAACCTCAC-3') and cloned into pCR2.1 (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA). The constructs were then sequenced (DNA Sciences Core, University of Virginia, Charlottesville, VA, USA).

Methylation-specific PCR (MSP)

Bisulphite-treated DNA was used as a template for PCR with primers designed using MethPrimer (www.urogen.org/methprimer) that were specific to the downstream CGI region (-527 to +612) of *BRMS1*. Quantitative MSPs were performed using iCycler real-time PCR (Bio-Rad iCycler IQ5, Hercules, CA, USA) with SYBR green detection dye (Invitrogen) at an annealing temperature of 57 °C. The internal control was measured using *GAPDH*. The methylation state of each gene was represented as relative methylation. Primers for the upstream CGI region (-3477 to -2214) were designed as previously described [12]; primers for the downstream CGI region (-527 to +612) of *BRMS1* were: methylated region, (-43 from transcription start site, TSS) 5'-TTTTGATGACGTATACGGAAGTATC-3' (forward) and (111 from TSS) 5'-GAAAACTAAAACCTCTAACCTCACG-3' (reverse); unmethylated region, (-42 from TSS) 5'-TTTGATGATGTATATGG AAGTATTGA-3' (forward) and (112 from TSS) 5'-CAAAACTAAAACCTCTAACCTCACA-3' (reverse). In some experiments, the PCR products were separated using 2% agarose gel electrophoresis.

Total RNA isolation and quantitative reverse transcriptase PCR (RT-PCR)

Total RNA isolation and quantitative RT-PCR was performed as previously described [6].

Plasmid construction and antibodies

Human *BRMS1* promoter sequences were amplified by PCR from genomic DNA, using primers (forward) 5'-GAAGAAGATCTCGGGCTGGGCTGGGTAT-3' and (reverse) 5'-CCCAAGCTTCACTGTTAGTATATTCA CTTTG-3' (restriction sites are underlined) located respectively at positions -500 and +601 from the transcription start site. The amplified product was cloned into the *Bgl*II/*Hind*III (New England Biolabs, Ipswich, MA)-digested pGL3 basic reporter gene (Promega) to create the *BRMS1-luc* construct.

Transfection and luciferase assays

Plasmids and reporter genes were transiently transfected using Polyfect reagent (Qiagen) and luciferase reporter activity assays were performed as previously described [6]. CMV- β -galactosidase activities were analysed as controls for the efficacy of transfection.

In vitro methylation assay

The *BRMS1-luc* reporter plasmid (20 μ g) was incubated with CpG methyltransferase M.SssI (20 U; New England Biolabs, Beverly, MA, USA) or buffer only, following the manufacturer's instructions. The efficiency of CpG methylation was tested as follows: plasmid DNA (2 μ g) was digested with restriction enzymes *Bgl*II/*Hind*III (New England Biolabs). The completeness

of methylation was proven by the inhibition of the digestion with 5-methyl-cytosine-sensitive restriction enzyme *Bst*UI (New England Biolabs).

Tissue microarray

Thirty-six cases of non-small cell lung carcinoma had formalin-fixed, paraffin-embedded tissue available for a tissue microarray. Haematoxylin and eosin (H&E)-stained slides were reviewed and areas of viable, well-preserved tumour were selected. Four 0.4 mm cores were taken and set into a single tissue array block (Beecher Instruments, Woodland, USA). Array blocks were incubated (37 °C) for 30 min. The blocks were cut with a microtome. Sections were reviewed and showed tumour in all cores. Control normal lung parenchyma was also used.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using a DAKO Autostainer. Pressure/heat antibody retrieval was performed with DAKO TRS9 buffer. A DAKO dual endogenous enzyme block was used (10 min). Primary BRMS1 monoclonal antibody (1 : 200) was then applied (30 min). The detection agent (DAKO Envision Dual Link) was then applied (30 min). DAKO DAB+ substrate chromagen was used with a haematoxylin counterstain. Scoring was performed by a pathologist blinded to *BRMS1* promoter methylation status and other clinicopathological variables, as previously described [14]. Briefly, the IHC scoring index was a product of the percentage staining (0–4) and the relative intensity (1–3).

Statistical analysis

The results of all experiments represent the mean $\pm$ SD of three separate experiments performed in triplicate. Wilcoxon rank sum and Kruskal–Wallis tests were used to determine significance between variables of two patient populations separated by tumour histology (squamous cell or adenocarcinoma) and to evaluate the methylation profile between the two tumour histologies. A general linear model was used to compare the level of *BRMS1* promoter methylation, *BRMS1* protein expression and specific clinicopathological variables in human NSCLC specimens [16]. $p < 0.05$ was considered significant.

Results

BRMS1 promoter methylation decreases *BRMS1* transcription

To explore the methylation profile of the *BRMS1* promoter region, NSCLC cell lines H157 (squamous cell carcinoma) and A549 (adenocarcinoma) along with NHBE cells were treated or not with 5-aza-2' deoxycytidine (5 μ M), a global demethylating agent, for 5 consecutive days. Using methylation-specific PCR (MSP) primers for the promoter-associated CGI region (–527 to +612) of *BRMS1* (Figure 1A), semi-quantitative MSP (lower panel) and quantitative MSP (upper panel) demonstrated that NSCLC cell lines had increased levels of *BRMS1* methylation at baseline compared to NHBE cells (Figure 1B). After treatment with the demethylating agent, both NSCLC cell lines exhibited methylation levels similar to the NHBE cells. Having identified the promoter-associated CGI (–527 to +612) to be hypermethylated in all NSCLC cell lines, we next examined the upstream CGI (–3477 to –2214) in both the NHBE and NSCLC cells and found no evidence of enhanced methylation of this CGI region (see Supporting information, Figure S1). Collectively, this demonstrates that the *BRMS1* promoter is differentially methylated in normal lung tissue and lung cancer cells. Moreover, a different methylation profile for *BRMS1* appears to exist in lung cancer cells compared to prior studies with breast cancer cells [12], confirming the well-documented observation of CGI tissue specificity.

To determine the effects of *BRMS1* promoter methylation on *BRMS1* transcription and translation, the experiments described above were repeated and *BRMS1* mRNA and protein levels assessed. As shown in Figure 1C, there is a robust rescue of the *BRMS1* mRNA compared to baseline, as measured by quantitative RT-PCR, as well as *BRMS1* protein levels in both NHBE and NSCLC cells following 5-aza-2' deoxycytidine exposure. In order to verify that methylation of the promoter-associated CGI (−527 to +612) contributes to decreased *BRMS1* promoter activity, we cloned this specific region of the *BRMS1* promoter into a luciferase reporter gene, *pGL3* basic. The ability of our cloned *BRMS1* promoter to be methylated *in vitro* was confirmed following treatment with the CpG methyltransferase *SssI*, as described in Materials and methods (Figure 1D, left panel). The *pGL3* basic empty vector and *pGL3*–*BRMS1* were then treated with *SssI* or buffer, transiently transfected into 293T cells and luciferase activity determined. This demonstrates a dramatic four-fold reduction in *BRMS1* promoter activity following methylation of the CGI (−527 to +612) (Figure 1D, centre panel). To confirm the activity of our cloned *BRMS1* promoter construct in NSCLC cells, identical experiments were carried out in H157 and H1299 NSCLC cells (Figure 1D, right panel). Both cell lines demonstrated a two-fold reduction in *BRMS1* promoter activity following CGI (−527 to +612) methylation.

Collectively, the experiments described here confirm that methylation of the promoter-associated CGI (−527 to +612) contributes to *BRMS1* transcriptional repression in NSCLC cells. Inhibition of this methylation event results in a robust increase in *BRMS1* transcript and protein expression levels.

Hypermethylation of the *BRMS1* promoter occurs in human NSCLC and is more predominant in squamous cell cancer histology

To confirm that a distinct and differential methylation profile of the *BRMS1* promoter methylation was also present in human NSCLC specimens, we obtained 40 (20 adenocarcinoma and 20 squamous cell) human NSCLCs and their patient-matched adjacent non-cancerous tissues. Patient demographics are shown in Table 1. Using quantitative MSP, both adenocarcinoma and squamous cell histologies demonstrated a significant increase in the amount of *BRMS1* promoter methylation when comparing tumour specimen to adjacent non-cancerous tissue for both tumour histologies. Representative quantitative MSP results are shown for eight of the 40 matched patient specimens (Figure 2A). Interestingly, the *BRMS1* promoter region was significantly more methylated in squamous cell cancer histologies compared to adenocarcinoma (Table 2).

A potential criticism of methylation-specific PCR is its dependence on annealing temperatures to detect the completely methylated or unmethylated state of these CpG sites. Additionally, as it is PCR-based, small changes in the methylation state of a few CpG sites can occasionally result in large changes in this methylation assay. To experimentally address this concern, we assessed the methylation of endogenous *BRMS1* in human lung cancer specimens using bisulphite sequencing PCR (BSP). Fifteen NSCLC specimens and their matched adjacent non-cancerous lung tissues underwent BSP analysis with primers constructed across the promoter-associated CGI region of *BRMS1* (Figure 2B). Compared to adjacent non-cancerous lung tissues (N), the methylation status of *BRMS1* promoter in tumour (T) exhibited a marked increase in squamous cell and adenocarcinoma lung cancer histologies (squamous cell, T $38.2 \pm 7.2\%$ versus N $6.3 \pm 3.2\%$, $p < 0.001$; adenocarcinoma: T $24.6 \pm 4.1\%$ versus N $4.8 \pm 2.8\%$, $p < 0.001$). Squamous cell cancers also had significantly more methylation of the *BRMS1* promoter compared to adenocarcinoma ($p = 0.006$).

Finally, to experimentally address the possibility that mutations may exist in the downstream CGI region (−527 to +612) of the *BRMS1* promoter in human NSCLC, we sequenced this

region in all 15 patient NSCLC samples. Analysis of these results revealed no mutations of this region of the *BRMS1* promoter.

***BRMS1* promoter methylation correlates with decreased *BRMS1* protein expression**

Immunohistochemistry for *BRMS1* nuclear and cytosolic staining was performed using our NSCLC tissue microarray (see Supporting information, Table S1). We chose to examine the cytosolic staining of *BRMS1*, given the recent study by Frolova and colleagues [17]. Figure 3 illustrates that *BRMS1* expression is reduced in lung cancer specimens compared to normal bronchial epithelium. Normal bronchial epithelium demonstrated strong and diffuse nuclear immunoreactivity (Figure 3A) when compared to both adenocarcinoma (moderately diffuse, Figure 3B) and squamous cell carcinoma (nearly absent, Figure 3C). Analysis of *BRMS1* promoter methylation and the intensity of nuclear *BRMS1* protein expression demonstrated a strong trend toward a significant correlation ($p = 0.06$).

***BRMS1* promoter hypermethylation and clinicopathological variables**

A general linear model was created to analyse patient (age, gender, smoking packs/year and cancer recurrence) and tumour (pathological tumour stage) variables with respect to NSCLC and tumour histology (Table 3). We then stratified the 40 patient samples by histology and found that none of the variables had an association with *BRMS1* promoter methylation for the adenocarcinoma histology. However, when examining the squamous cell histology, we found that smoking trended towards significance ($p = 0.06$) and pathological tumour stage was significant ($p = 0.04$) with respect to *BRMS1* promoter methylation.

Discussion

CGIs are comprised of CpG dinucleotides clustered in or near promoter regions of nearly half of all protein-coding genes in DNA [18]. Tumour suppressor genes (TSGs) are a well-studied class of genes in which aberrant hypermethylation of normally unmethylated cytosine nucleotides in the CGI of these genes leads to loss of expression and enhanced tumour growth [19,20]. Multiple studies have shown a reduced expression of TSGs in lung cancer secondary to aberrant hypermethylation of CGI, including *p16^{INK4a}*, retinoic acid receptor- β (*RAR β*), E-cadherin (*CDH1*) and the fragile histidine triad gene (*FHIT*) [21–24]. Unlike TSGs, metastasis suppressor genes (MSGs), such as *BRMS1*, function to decrease the formation of spontaneous, macroscopic metastasis [25]. Despite the extensive study of hypermethylation as a mechanism of silencing TSGs in lung cancer, no studies in lung cancer examining the mechanisms through which MSGs, in particular *BRMS1*, are silenced [2,6,14].

In this report we demonstrate that methylation of the promoter-associated CGI in *BRMS1* results in its transcriptional repression, and that this occurs more commonly in NSCLC compared to normal bronchial epithelium tissues. We also show, in lung tissue (cancer and NHBE), that the promoter-associated CGI, not the upstream CGI of *BRMS1*, is preferentially methylated. A previous study involving breast cancer cell lines had likewise identified these two CpG islands (–3477 to –2214 and –531 to +608) in *BRMS1*. In that study, the researchers were unable to identify methylation of specific amplicons in the promoter-associated (–531 to +608) CGI, but were able to demonstrate methylated amplicons across all metastatic breast cancer cell lines for the upstream CGI (–3477 to –2214) [12].

Recently, methylation-specific gene expression arrays or ‘methylomes’ have shown that CGI methylation can localize to genomic regions distal to the promoters [26], although the majority of evidence supports that it is methylation of the promoter-associated CGI that directly affects gene transcription [18,27,28]. Interestingly, approximately 40% of CGIs display a tissue-specific expression profile [29,30] and differential methylation of specific CGIs can and do

occur across different tissue types. Thus, the different methylation profiles observed in our study, compared to that of Metge and colleagues [12], is likely explained by the tissue specificity of these CGI methylation profiles. Another possibility for the difference between studies could be related to MSP primer design. Given that MSP primer design for the distal CGI was identical in both studies, and that the primers used in this study for the promoter-associated CGI region of *BRMS1* were encompassed by primers used in the Metge study, this seems highly unlikely.

To confirm that methylation of promoter-associated CGI (−527 to +612) is integral for *BRMS1* promoter activity, we cloned the *BRMS1* promoter and performed a luciferase assay that demonstrated methylation dramatically inhibits *BRMS1* promoter activity (Figure 1D). In light of the evidence of the relatively poor correlation between CGI hypermethylation and the transcriptional status of associated genes [31], these studies provide an important, mechanistic link between *BRMS1* promoter-associated CGI methylation and its transcriptional repression.

Recent studies have identified that promoter hypermethylation of specific genes correlate to lung cancer recurrence and overall outcome [32]. Prior to this report, no studies had investigated the correlation between *BRMS1* promoter methylation profiles and clinicopathological variables in NSCLC patients. Examination of our study cohort revealed that *BRMS1* promoter methylation was significantly more robust in squamous cell compared to adenocarcinoma histologies (Table 2). This observation was confirmed using the more stringent BSP analysis, where identical results were obtained. In agreement with our findings, other studies have shown differential methylation profile expression based on tumour histology, particularly with *p16^{INK4a}* [24]. This difference between histologies may be attributed to the resultant inflammation [33] commonly associated with heavy smokers and the squamous cell cancer histology. Finally, using our general linear model we found that when stratified by tumour histology, pathological stage correlated significantly and that smoking trended towards significance with squamous cell histology only (Table 3). These results are supported by prior studies from our group demonstrating a direct correlation between smoking status and *BRMS1* protein expression in NSCLC patients [14]. Smoking is also associated with a loss of both mRNA and protein expression of *RARβ* [34–36] and with aberrant promoter hypermethylation of CGIs in *p16^{INK4a}* [37] and *TSCL1/IGSF4* [38].

In conclusion, we have demonstrated that *BRMS1* promoter-associated CGI methylation results in decreased *BRMS1* promoter activity and transcriptional repression in both NSCLC cells and human NSCLC specimens. Further analysis of *BRMS1* methylation in 40 human NSCLC specimens identified a significant increase in *BRMS1* promoter methylation compared to adjacent non-cancerous tissue. This difference in *BRMS1* promoter methylation was most pronounced in squamous cell cancer histology, where it also correlated with advanced pathological tumour stage and smoking history.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

BRMS1 promoter activity, mRNA and protein levels are regulated by promoter methylation. (A) Schematic illustration of the CpG island in *BRMS1* promoter. Arrow, transcription start site (TSS); empty box, exon 1; small bars, positions of primers for methylation-specific PCR (MSP). (B) Lung cancer (H157 and A549) and NHBE cells were treated or not with 5-Aza (5 μ M) for 5 days. Genomic DNAs were extracted. Quantitative MSP was done by using methylated (M)- and unmethylated (U)-specific primers in the CpG island of *BRMS1* promoter. (C) H157, A549 and NHBE cells were treated or not with 5-Aza (5 μ M) for 5 days; (lower panels) *BRMS1* mRNA levels determined by QRT-PCR; (upper panel) *BRMS1* protein levels detected by western blot. (D) Methylation inhibits *BRMS1* promoter activity. (Left panel) 20 μ g *BRMS1* promoter reporter was treated with CpG methyltransferase M. SssI (20 U) and digested with *Bgl*II/*Hind*III, as described in Materials and methods. The completeness of methylation was visualized by 2% agarose gel electrophoresis after digestion with *Bst*UI. (Centre panel) pGL3 basic empty vector and pGL3-*BRMS1* were treated with SssI or buffer only, as described above, and then were transiently transfected into 293T cells. Luciferase activity was determined. (Right panel) pGL3-*BRMS1* was treated with SssI or buffer only, as described above, and then was transiently transfected into H157 and H1299 NSCLC cells. Luciferase activity was determined.

Figure 2.

BRMS1 promoter is hypermethylated in lung cancer tissue when compared to normal adjacent tissue. (A) *BRMS1* is hypermethylated in lung cancer tissues. Quantitative MSP for *BRMS1* promoter in tumour tissues (T) and patient-matched adjacent lung tissues (N). The data are plotted as the fold over patient-matched adjacent non-cancerous lung tissues (N), where results from N were normalized to 1 for each patient. (B) The *BRMS1* promoter presents higher methylation status in lung cancer compared with adjacent non-cancerous tissue. (Upper panel) Schematic illustration of the bisulphite sequence PCR target sequence in the *BRMS1* promoter. The 18 bars represent CpG dinucleotides; the arrow represents the transcription start site. (Lower panel) Methylation status of the *BRMS1* 5' CpG island in lung cancer samples and adjacent non-cancerous tissues. Each horizontal row of circles represents one patient sample. Each CpG dinucleotide bar from the schematic is depicted by a circle, the fill pattern of which indicates the methylated cytosine and the blank indicates the unmethylated cytosine in CpG dinucleotide. T, tumour; n = adjacent non-cancerous tissue.

Figure 3.

Tissue micro-arrays (TMAs) of nuclear staining for BRMS1 in normal bronchial epithelium and human lung cancer specimens. (A) TMA of human normal bronchial epithelium (arrows) stained with BRMS1 antibody, as described in Materials and methods, demonstrates strong and diffuse nuclear immunoreactivity. (B) TMA of human lung adenocarcinoma tissue stained with BRMS1 antibody, as described in Materials and methods, demonstrates moderate, diffuse nuclear immunoreactivity. (C) TMA of human lung squamous cell carcinoma tissue stained with BRMS1 antibody, as described in Materials and methods, demonstrates an absence of staining.

Table 1

Patient demographics

Variables		Adenocarcinoma (n = 20)	Squamous cell (n = 20)	p
Age (years)	Range	46–82	54–80	0.986
	Median	69	67	
Gender	Male	10	9	1.000
	Female	10	11	
Race	Caucasian	19	19	0.368
	African-American	0	1	
	Asian	1	0	
Albumin	Mean	4.2 ± 0.33	4.3 ± 0.36	0.320
Smoking history	Yes	17	20	0.231
	No	3	0	
Packs/year	Range	0–100	25–100	0.556
	Median	50	48	
Pathological stage	IA	8	6	0.586
	IB	7	5	
	IIA	1	1	
	IIB	1	4	
	IIIA	1	3	
	IIIB	2	1	
Recurrence	No	10	12	0.792
	Yes	8	6	
	Unknown	2	2	

Table 2

Quantitative MSP for *BRMS1* promoter using human lung cancer specimens versus patient-matched adjacent non-cancerous tissues

Histology	No. of patients	Methylation (%)		<i>p</i> (tumour versus adjacent)
		Adjacent tissue	Tumour tissue*	
Adenocarcinoma	20	8.1 ± 7.7	27.3 ± 14.4	0.0001
Squamous cell	20	8.6 ± 5.9	41.0 ± 19.6	0.0114

* $p = 0.03$, squamous cell carcinoma group compared to adenocarcinoma group.

Table 3

General linear model illustrating the relationship between patient variables and *BRMS1* CGI (−527 to +612) methylation in human NSCLC

Variable	Main effect [*]	Main effect stratified by tumour histology [*]	
		Adenocarcinoma	Squamous cell
Age	0.68	0.29	0.67
Gender	0.56	0.83	0.46
Smoking pack/year	0.29	0.74	0.06
Pathological tumour stage	0.42	0.80	0.04
Cancer recurrence	0.45	0.15	0.44

^{*} *p* value.