Association of two BRM promoter polymorphisms with head and neck squamous cell carcinoma risk

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The SWI/SNF chromatin remodeling complex is an important regulator of gene expression that has been linked to cancer development. Expression of Brahma (BRM), a critical catalytic subunit of SWI/SNF, is lost in a variety of solid tumors. Two novel BRM promoter polymorphisms (BRM-741 and BRM-1321) have been correlated with BRM loss and elevated cancer risk. The aim(s) of this study were to examine BRM expression in head and neck squamous cell carcinoma (HNSCC) and to correlate BRM polymorphisms with HNSCC risk. BRM expression studies were performed on eight HNSCC cell lines and 76 surgically resected tumor samples. A case–control study was conducted on 668 HNSCC patients (oral cavity, oropharynx, larynx and hypopharynx) and 700 healthy matched controls. BRM expression was lost in 25% of cell lines and 16% of tumors. The homozygous genotype of each polymorphism was significantly associated with increased HNSCC risk [BRM-741: adjusted odds ratio (aOR) 1.75, 95% CI 1.2–2.3, P < 0.001; BRM-1321: aOR 1.65, 95% CI 1.2–2.2, P < 0.001]. Individuals that were homozygous for both BRM polymorphisms had a more than 2-fold increase in the risk of HNSCC (aOR 2.23, 95% CI 1.5–3.4, P < 0.001). A particularly elevated risk was seen within the oropharynx, human papillomavirus-positive subgroup for carriers of both homozygous variants (aOR 3.09, 95% CI 1.5–6.8, P = 0.004). BRM promoter polymorphisms appear to act as susceptibility markers of HNSCC with potential utility in screening, prevention and treatment.

Introduction

As the sixth most common cancer worldwide, head and neck cancer encompasses a broad range of anatomic subites (1). Over 90% of head and neck cancers are squamous cell carcinomas, arising predominantly from the epithelial lining of the oral cavity, oropharynx, larynx or hypopharynx (2). Although smoking and alcohol consumption are considered the main risk factors for head and neck squamous cell carcinoma (HNSCC), only fractions of individuals exposed develop cancer. Genetic variations, therefore, may play an important role in HNSCC susceptibility. Genetic changes in carcinogenesis often involve alterations to oncogenes or tumor suppressor genes (TSG). Studies in the HNSCC literature have focused on the identification of TSGs using methods such as loss of heterozygosity (LOH) analysis (3). LOH in the region of 9p21–24 is the most commonly reported genetic change in HNSCC, occurring in up to 72% of tumors (4,5). The BRM gene mapped to 9p24 was recently recognized as a putative TSG in HNSCC (6). However, evidence demonstrating the importance of BRM and its associated SWI/SNF complex in cancer has been accumulating steadily in other tumor types, particularly lung cancer (7,8). The SWI/SNF complex functions as a chromatin remodeling complex that regulates gene expression by altering nucleosome structure to make DNA more accessible for transcription. This adenosine triphosphate-dependent complex contains multiple subunits, including one of two crucial adenosine triphosphatase subunits, Brahma (BRM) or BRM-related gene 1 (BRG1). It has been shown that inactivation of SWI/SNF through the loss of its adenosine triphosphatase subunits promotes cancer development. Thus, BRM and BRG1 have both been implicated as potential tumor suppressor or susceptibility genes (9).

BRM is of particular interest in translational research, as epigenetic silencing, not mutations, appears to be the major mechanism underlying loss of BRM expression (10). Unlike most TSGs where irreversible mutations have occurred, BRM function could be restored if targeted compounds are able to reverse these epigenetic changes (10). Studies exploring this possibility have begun, with promising findings (10,11). The potential impact of such an agent is significant as loss of BRM expression has been found in at least 10–20% of different solid tumors including lung, breast, colon, esophageal, ovarian, bladder, prostate and gastric tumors (7,10,12,13). In HNSCC, although LOH at the BRM locus has been reported, loss of BRM protein expression has not been specifically examined (6).

Two recently identified insertion polymorphisms in the BRM promoter (Figure 1A) could serve as biomarkers to identify individuals who are at increased risk of cancer and may benefit from future targeted interventions. In previous work in lung cancer, homozygous variants of BRM-741 and BRM-1321 polymorphisms were shown to strongly correlate with loss of BRM expression in cell lines and tumors (14). An elevated risk of lung cancer was also associated with these polymorphisms (14). In the present study, we hypothesized that BRM loss also characterizes a subset of HNSCC. Specifically, our objectives were (i) to examine BRM expression in HNSCC cell lines and tumors and (ii) to determine whether the presence of BRM-741 and BRM-1321 is associated with an increased risk of HNSCC.

Materials and methods

Cell lines and human tumor tissue analysis

Cell lines and tumor samples. The head and neck cell lines utilized (UM SCC-6, 9, 10A, 11A, 11B, 14A, 17B, 22B) were established from primary HNSCC samples from patients treated at the University of Michigan. Cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin and were cultured in a humidified 5% CO₂ incubator at 37°C. Tissue microarrays were constructed from HNSCC tumors treated with primary surgical resection at the University of Michigan. Tissue microarray sections were processed by standard deparaffinization with xylene and rehydrated in a descending ethanol series to ddH₂O.

Western blotting and immunohistochemistry. Immunoblotting was performed as described previously (10). Membranes were incubated with affinity purified glutathione-S-transferase–BRM antisera made as described previously and diluted at 1:1000 and glyceraldehyde 3-phosphate dehydrogenase (Chemicon) at 1:5000 (10). Antigen retrieval on tissue microarray sections was done using 10mM Tris buffer, pH 10.0. Slides were then...
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incubated with glutathione-S-transferase-BRM (1:1000) and stained using the BD PharMingen HRP detection kit (BD Biosciences PharMingen, San Diego, CA) according to the manufacturer’s instructions. Finally, sections were counterstained with Harris hematoxylin (Fisher, Middletown, VA), dehydrated and mounted with Permount (Fisher). Tumors were scored both on intensity using a score of 0–3 (absent to highest) and on percent positive. A composite score, which was the product of the intensity score multiplied by the percent positive, was used to rank order the level of BRM expression in each tumor. Based on the composite score, negative was defined as 0–30, weak was defined as 30–100 and positive was defined as 100–300. Mosaics described tumors with absent staining tumor cells juxtaposed to cells with the highest intensity of staining. As this group of tumors encompassed both extremes of BRM expression, a composite score was not utilized to characterize this group.

Case–control analysis

Study population. Head and neck cancer patients treated at the Princess Margaret Hospital in Toronto, Canada, between 2007 and 2010 were prospectively recruited for an institutional head and neck translational study on genetic polymorphisms and cancer risk and prognosis. Controls were recruited from the same hospital in two groups: (i) healthy friends and non-smoking relatives of patients with non-HNSCC solid tumors that were never or light smokers (≤10 pack years) and (ii) self-referred healthy controls using a self-administered questionnaire. Clinicopathologic information of cases and controls (n = 700), WT, wild-type; n, number.

Results

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Margaret Hospital in Toronto, Canada, between 2007 and 2010 were prospectively collected from cases and controls. DNA was extracted from peripheral blood lymphocytes using a commercially available DNA isolation kit (SPrimer, Cat# 2300740) according to the manufacturer’s recommendations. All DNA samples were checked using Nanodrop Spectrophotometer (ND-1000) and plated in 96-well plates at a concentration of 10ng/l in 50l before Taqman assay analysis. Genotyping for BRM-741 and BRM-1321 was completed using two custom-designed Taqman assays (E_BRMpoly1_10 Part #4332077 for BRM-741 and BRMprom-2ND Part #4331349 for BRM-1321). Taqman assay reaction conditions were: 95°C for 10 min and 95°C for 15 s/63°C for 1 min, x40 cycles for BRM-741; and 95°C for 10 min and 95°C for 15 s/60°C for 1 min for BRM-1321. Reaction volume for each sample was 5 l and DNA input was 10 ng (1 l, 10ng/l); PCR master mix was from Thermo Scientific (2x AbsoluteQPCR ROX mix, Cat# AB1139).

Statistical analysis. Wilcoxon rank-sum and chi-squared tests were used to compare frequencies of demographic and environmental risk parameters between cases and controls. Adjusted odds ratios (aORs) with their 95% confidence intervals of the association between polymorphisms and HNSCC risk were estimated by multiple logistic regression models after controlling for age, gender, ethnicity and smoking status. Exploratory analyses were performed for specific clinicopathologic subgroups. Based on previously published findings (14), a codominant genetic model of inheritance was primarily used to study both polymorphisms. All statistical analyses were performed using SAS software (version 9.3, SAS Institute, Cary, NC) with a significance level of <0.05.

A total of 668 cases and 700 controls were included in the case–control study. Table I summarizes their baseline demographics. Although there was no difference in smoking status as smoking as was performed by frequency distribution, controls with a smoking history had significantly fewer total pack years of tobacco exposure than cases with a smoking history (P < 0.001). Table II presents clinical characteristics of cases. Distribution across anatomic subsites was as follows: 37% oral cavity (n = 248), 35% oropharynx (n = 237), 22% larynx (n = 150) and 5% hypopharynx (n = 31). Two patients presented with synchronous primaries of the oropharynx and hypopharynx and oral cavity/larynx. A majority of patients (53%) presented with overall stage 4 disease (n = 357), but there was a wide distribution across for T and N classifications.

Table III summarizes the genotype frequencies and genotype-specific ORs of the polymorphisms. There were no deviations from the Hardy–Weinberg equilibrium for each polymorphism (P > 0.20 each). The two polymorphisms were in linkage disequilibrium (D = 0.83). Frequencies of the minor insertion allele in the control group were 45 and 42% for BRM-741 and BRM-1321, respectively (Figure 1B). The homozygous genotype of each polymorphism was significantly associated with increased HNSCC risk (BRM-741: aOR 1.75, P < 0.001; BRM-1321: aOR 1.65, P < 0.001). The heterozygous genotype of BRM-1321 (aOR 1.32, P = 0.03), but not BRM-741,
appeared to confer a statistically significant intermediate risk. When the two polymorphisms were analyzed together, individuals that were homozygous for both polymorphisms had the greatest risk of HNSCC (aOR 2.23, \( P < 0.001 \)).

Subgroup analyses are shown in Table IV. Analysis by age (≤50 and >50 years) revealed borderline significant associations in the younger group for both homozygous variants, but the OR was elevated (aOR 2.85, \( P = 0.052 \)) compared with the older group (aOR 1.64, \( P = 0.014 \)). Smoking status (ever versus never) did not appear to significantly impact the association between polymorphisms and HNSCC risk; a strong association was seen for both smokers (aOR 2.24, \( P = 0.008 \)) and non-smokers (aOR 2.93, \( P = 0.006 \)) carrying both homozygous variants.

Stratification by T classification revealed a relatively stronger association in cases with locally advanced (T3 and T4) disease (aOR 2.54, \( P < 0.001 \)) compared with those with T1 or T2 disease (aOR 2.04, \( P = 0.025 \)). However, this trend toward stronger association in advanced T classification was not seen for nodal status.

Analyses by subsite were performed for oral cavity, oropharynx, and larynx. The hypopharynx group was too small for subgroup analysis. In each analyzed subsite, a significant increase in risk was associated with the presence of both homozygous variants. The larynx group had the lowest OR (aOR 2.00, \( P = 0.000 \)) compared with oral cavity (aOR 2.36, \( P = 0.005 \)) and oropharynx (aOR 2.78, \( P < 0.001 \)). The strongest association was observed in the HPV-positive subset of the oropharynx group, where carriers of both homozygous variants had a 3-fold increase in risk compared with the wild-type (aOR 3.09, \( P = 0.004 \)).
Table III. Genotype and allele frequencies of BRM promoter polymorphisms in HNSCC cases and controls and the risk of HNSCC

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Cases (668)</th>
<th>Controls (700)</th>
<th>Adjusted OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRM-741</td>
<td>Wild-type</td>
<td>160</td>
<td>210</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heterozygote</td>
<td>314</td>
<td>350</td>
<td>1.09 (0.8–1.4)</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>Homozygous</td>
<td>194</td>
<td>140</td>
<td>1.75 (1.2–2.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BRM-1321</td>
<td>Wild-type</td>
<td>168</td>
<td>238</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heterozygote</td>
<td>346</td>
<td>336</td>
<td>1.32 (1.0–1.8)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Homozygous</td>
<td>154</td>
<td>126</td>
<td>1.65 (1.2–2.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Combined</td>
<td>Wild-type</td>
<td>94</td>
<td>140</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No homozygous genotypes</td>
<td>330</td>
<td>357</td>
<td>1.28 (0.9–1.8)</td>
<td>0.084</td>
</tr>
<tr>
<td></td>
<td>One homozygous variant</td>
<td>142</td>
<td>140</td>
<td>1.41 (1.0–2.0)</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Both homozygous variants</td>
<td>102</td>
<td>63</td>
<td>2.23 (1.5–3.4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

OR, odds ratio; CI, confidence interval. P-values shown in italics represent a significant difference.

Table IV. Subgroup analyses by clinical characteristics

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>n</th>
<th>Both homozygous variants, n (%)</th>
<th>Adjusted OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>99</td>
<td>17 (17%)</td>
<td>2.85 (1.0–9.3)</td>
<td>0.052</td>
</tr>
<tr>
<td>&gt;50</td>
<td>569</td>
<td>85 (15%)</td>
<td>1.64 (1.0–2.5)</td>
<td>0.014</td>
</tr>
<tr>
<td>T category</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1–T2</td>
<td>375</td>
<td>51 (14%)</td>
<td>2.04 (1.2–3.4)</td>
<td>0.025</td>
</tr>
<tr>
<td>T3–T4</td>
<td>293</td>
<td>51 (17%)</td>
<td>2.54 (1.4–4.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N category</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Node negative</td>
<td>364</td>
<td>57 (16%)</td>
<td>2.37 (1.4–3.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Node positive</td>
<td>304</td>
<td>44 (14%)</td>
<td>2.17 (1.2–3.6)</td>
<td>0.009</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smokers</td>
<td>173</td>
<td>28 (16%)</td>
<td>2.93 (1.1–7.9)</td>
<td>0.006</td>
</tr>
<tr>
<td>Ever smokers</td>
<td>495</td>
<td>74 (15%)</td>
<td>2.24 (1.4–3.6)</td>
<td>0.008</td>
</tr>
<tr>
<td>Subsite</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>150</td>
<td>22 (15%)</td>
<td>2.00 (1.0–4.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Oral cavity</td>
<td>248</td>
<td>38 (15%)</td>
<td>2.36 (1.2–4.2)</td>
<td>0.005</td>
</tr>
<tr>
<td>Oropharynx</td>
<td>237</td>
<td>38 (16%)</td>
<td>2.78 (1.5–3.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HPV positive</td>
<td>135</td>
<td>24 (18%)</td>
<td>3.09 (1.5–6.8)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Adjusted OR estimates the risk of HNSCC in individuals with both homozygous variants compared to the wild type genotype. P-values shown in italics represent a significant difference between cases and controls.

Discussion

The identification of cancer biomarkers can improve understanding of tumor biology and pave the way for targeted prevention, screening and therapy. In this study, we have shown that loss of BRM expression is found in HNSCC at a similar proportion as other solid cancer types and that BRM promoter polymorphisms, BRM-741 and BRM-1321, are potential susceptibility markers of HNSCC.

In the case–control analyses, although each of the two polymorphisms confers risk, HNSCC risk was the greatest in individuals who were homozygous for both polymorphisms. An intermittently elevated risk was associated with the heterozygous genotype of BRM-1321 in some analyses. This intermediate risk group could reflect selective LOH at the BRM locus in carriers of one allele variant, which essentially renders the individual into a functional homozygote. A similar mechanism of selective LOH could also explain the elevated risk seen in carriers of one homozygous variant.

The importance of BRM loss in carcinogenesis likely relates to SWI/SNF’s involvement in critical cellular processes. This complex participates in a number of signal transduction pathways and interacts with key tumor suppressors including retinoblastoma (Rb) and p53 (9). In particular, BRM binds to and is required for the function of Rb, which has been linked to multiple aspects of HNSCC development (19,20). Loss of BRM has been shown independently to impair Rb-mediated growth regulation (21). The functional link between BRM and Rb suggests that BRM loss may provide an alternative mechanism for cellular dysregulation in the absence of loss-of-function mutations in the Rb pathway.

BRM likely represents a tumor susceptibility gene, where carcinogenic exposure is required for tumor development in addition to genetic aberration. Knockout mice studies found that BRM null mice (BRM−/−) exhibited altered cellular proliferation and cell cycle control, but did not develop cancer (22). However, when exposure of carcinogen urethane was combined with BRM loss, there was a 10-fold increase in lung tumors, indicating that presence of other environmental risk factors is needed (10). Although BRM promoter polymorphisms were originally linked to lung cancer risk in smokers only (14), the strength of their association with HNSCC risk was similar in both smokers and non-smokers. Sensitivity analysis of alcohol use (results not shown) also did not reveal differential risk profiles for never and ever drinkers although power of the analysis was limited by missing data.

A novel finding of this study was the relatively substantial elevation in the risk associated with BRM polymorphisms in HPV-positive oropharyngeal cancer, which may partially explain the strong associations seen in non-smokers. This finding has a plausible biological basis since both BRM and HPV are known to target the Rb pathway in tumorigenesis (19,23). It is possible that an initial aberration in the Rb pathway due to BRM loss makes it more vulnerable to further impairment by oncoprotein E7 produced by high-risk HPV strains. The HPV-positive oropharynx cancer group is of increasing importance in HNSCC with rising incidence rates (24). Oral HPV infection and/or seropositivity can be detected in only a portion of patients with HPV-positive tumors (25). As such, there is need for additional screening markers in this subpopulation. Despite a subgroup analysis of limited size, our results pointed toward potential utility of BRM polymorphisms for identifying individuals with behavioral risk.
factors who are at high risk for oncogenic HPV infection and oro-pharyngeal cancer.

The exact mechanism underlying BRM loss remains unknown. The discovery of BRM promoter polymorphisms elucidates one plausible mechanism for BRM loss. Previously, it has been shown that BRM-741 and BRM-1321 were strongly associated with complete loss of BRM expression in lung cancer tumors and cell lines (14). It was also found that insertion variants of both polymorphisms created DNA sequences that were highly homologous (92%) to myocyte enhancer factor-2 (MEF2) transcription factor-binding sites (14,26). A similar binding site did not exist in the wild-type (14). The MEF family of transcription factors has been shown to recruit histone deacetylase (HDAC), and HDAC inhibitors have been shown to upregulate BRM expression in vitro (9,27). Recently, we have found that HDAC3 and HDAC9 regulate BRM, as silencing of either HDAC induces BRM expression. Moreover, GATA3 and MEF2D also regulate BRM expression and drive HDAC9 overexpression only in BRM negative cell lines (unpublished data from Reisman lab). How these proteins cooperate to precisely regulate BRM is not yet known. However, we surmise that MEF2 or other transcription factors recruit HDAC3 and HDAC9 to the BRM promoter, whereby BRM expression is then silenced via HDAC recruitment (14).

These findings come at an exciting time where HDAC inhibitors are being assessed as targeted agents in cancer treatment (28). Since BRM loss is found in a wide variety of tumor types and BRM polymorphisms have been shown to predict cancer risk in both HNSCC and lung cancer patients, it is possible that these polymorphisms serve as global markers for an aberrant cancer-associated epigenetic mechanism that silences BRM and other important genes. As such, reversal of epigenetic changes could reactivate other cellular regulatory genes in addition to BRM. In addition to their potential use as biomarkers for screening in HNSCC, BRM polymorphisms may also identify subsets of patients with BRM loss who could benefit from such targeted therapeutics. Moreover, the polymorphisms may enable development of targeted preventative strategies for individuals carrying both homozygous variants.

A current limitation of this study is a lack of comprehensive genotyping data on HNSCC cell lines and tumors to correlate with BRM loss, replicating the association demonstrated in lung cancer (14). From the available cell line genotyping data, although a trend was observed demonstrating a relationship between presence of BRM polymorphism and loss of BRM expression in HNSCC, there was not enough sample size for meaningful statistical analysis. Genotyping of HNSCC tumor tissue could not be performed in this study due to lack of institutional access to adequate samples. In the near future, we plan to acquire tumor tissue from a variety of HNSCC subsites by collaborating with other institutions in order to enable complete examination of BRM polymorphisms from germline DNA to cell lines to tumors tissues. We also plan to evaluate the significance of weak or mosaic BRM expression in HNSCC and other tumor types as well as correlating BRM polymorphism genotypes with LOH in tumor samples. Future directions for clarifying the value of BRM polymorphisms as a biomarker in HNSCC will also include further exploration of the effect of HPV in larger samples as well as examination of their effect on treatment response and prognosis.

Supplementary material

Supplementary data can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

References


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