Carcinogen exposure and gene promoter hypermethylation in bladder cancer

Carmen J. Marsit1, Margaret R. Karagas3, Hadi Danaee1, Mei Liu1, Angeline Andrew3, Alan Schned4, Heather H. Nelson4 and Karl T. Kelsey1,2,*

1Department of Genetics and Complex Diseases and 2Department of Environmental Health, Harvard School of Public Health, Boston, MA 02115, USA, 3Department of Community and Family Medicine and 4Department of Pathology, Dartmouth Medical School, Lebanon, NH 03756, USA.

*To whom correspondence should be addressed. Tel: +1 617 432 3313; Fax: +1 617 432 0107; Email: kelsey@hsph.harvard.edu

Tobacco smoking, certain occupational exposures, and exposure to inorganic arsenic in drinking water have been associated with the occurrence of bladder cancer. However, in these tumors the exposure-associated pattern of somatic alterations in genes is in the causal pathway for disease has been poorly characterized. In particular, the mechanism by which arsenic induces bladder cancer and the effects of lower environmental levels of exposure remain uncertain. Animal and in-vitro studies have suggested that arsenic and other exposures may act through epigenetic mechanisms. We, therefore, examined, in a population-based study of human bladder cancer, the relationship between epigenetic silencing of three tumor suppressor genes, p16INK4A, RASSF1A and PRSS3, and exposure to both tobacco and arsenic in bladder cancer. Promoter methylation of each of these genes occurred in ~30% of bladder cancers, and both RASSF1A and PRSS3 promoter methylation were associated with advanced tumor stage (P < 0.001 and P < 0.04, respectively). Arsenic exposure, measured as toenail arsenic, was associated with RASSF1A (P < 0.02) and PRSS3 (P < 0.1) but not p16INK4A promoter methylation, in models adjusted for stage and other factors. Cigarette smoking was associated with a 2-fold increased risk of promoter methylation of the p16INK4A gene only, with greater risk seen in patients with exposures more recent to disease diagnosis. These results, from human bladder tumors, add to the body of animal and in vitro evidence that suggests a role in epigenetic alterations for bladder carcinogens.

Introduction

Over 60 000 new cases of bladder cancer will be diagnosed in the USA in 2005, and >13 000 deaths will result from this disease (1). Bladder cancer occurs almost three times as often in males than females, and occurs predominantly as transitional cell carcinoma (2). Etiologically, tobacco smoking (3,4), occupational exposures (particularly, aromatic amine and polycyclic aromatic hydrocarbon exposures) are well known to contribute to the occurrence of bladder cancer. Drinking water arsenic exposure in highly exposed populations clearly increases bladder cancer incidence, but the effect at lower levels of exposure remains controversial (5–7). There is also evidence that use of certain hair dyes, exposure to chlorination by-products, individual fluid intake and dietary factors may play a role (8,9).

Sporadic tumorigenesis is driven by clonal somatic alterations of specific cellular pathways. These alterations take the form of activation or functional deregulation of oncogenes and concomitant inactivation of tumor suppressor genes. Numerous somatic alterations have been described in bladder cancer, most notably mutation or functional inactivation of the tumor suppressor TP53 (10,11). Other tumor suppressors are subject to epigenetic silencing, through promoter hypermethylation and in bladder cancer, this silencing has been described for genes such as p16INK4A and RASSF1A (12,13). RASSF1A promoter hypermethylation, in particular, has been shown to be associated with invasive bladder cancer, the more deadly form of the disease (13). These important clinical correlates, as well as the potentially powerful sensitivity and specificity of the approaches used in detection of these alterations have made these good candidates for clinical detection of cancer even in urine specimens (14,15).

Although these epigenetic alterations occur frequently and are considered critical in the genesis of bladder cancer, little is known about the mechanism of selection of these clones; the manner in which carcinogen exposures might act to contribute to the genesis of these events remains unclear. In lung cancer, where more epidemiologic investigation has addressed similar questions, evidence is building that these alterations may be related to exposures causal for the disease (16). For example, studies of lung cancers have found that the prevalence of p16INK4A promoter methylation is higher in ever smokers (17), and increases with increasing smoking duration (18). Indeed, two separate studies have also found that silencing of RASSF1A in lung tumors was associated with starting smoking at an earlier age (19,20). On the other hand, similar studies of promoter methylation of other genes, such as PRSS3 (trypsinogen IV), which is highly prevalent in lung cancer, have reported no association with smoking (21).

Tobacco smoking, as in lung cancer, is the major known etiologic factor for bladder cancer, although the magnitude of the relative risk is not as high. To date, the relationship of epigenetic alterations with exposure to tobacco smoking or other risk factors important in bladder cancer etiology, such as arsenic exposure, have not been well explored. Arsenic, whose carcinogenic mode of action at low levels is unclear, in animal and in vitro studies does appear to influence gene expression (22–25) and is particularly interesting as a potential agent acting to select epigenetically silenced cells. Understanding the relationship between epigenetic alterations and exposures to bladder carcinogens, including exposure to arsenic, may clarify the underlying carcinogens’ mechanisms and reveal...
important sources of etiologic heterogeneity, ultimately
enabling us to refine the disease classification of these tumors.
We have analyzed the promoter methylation status of three
genes (p16INK4A, RASSF1A and PRSS3) in relation to putative
carcinogen exposures in a population-based case study of
incident bladder cancer in New Hampshire in order to exam-
mine the relationship of epigenetic alterations of these genes
with tobacco and arsenic exposures in bladder cancer patients.

Materials and methods

Study population
This has been previously described (22). Briefly, all patients who were resi-
dents of New Hampshire, ages 25–74 years, and diagnosed with incident bladder cancer from July 1, 1994 to June 30, 1998 were identified by the
rapid reporting system of the New Hampshire State Cancer Registry and were
asked to participate in the study (26). All participants provided informed
consent using appropriate institutional review board protocols. Each particip-
ating patient underwent a personal interview to obtain information on demographic trends and lifestyle factors, such as use of tobacco (including frequency, dura-
tion and intensity of cigarette smoking). Measurement of internal dose of arsenic exposure was assessed through toenail arsenic measurements, as pre-
viously described and validated in this study set (26,27). Pathology reports and paraffin-embedded tumor specimens were requested from the treating physi-
cian/pathology laboratories. Bladder tumors were reviewed by the study pathologist (A.S.) and classified according to the 1973 and 2004 WHO guide-
lines for bladder tumors. Additionally, the proportion of malignant cells in each
sample was recorded for each sample, averaging 69% (median 80%). Excluded
from this analysis were 2 tumors of mixed histology, 10 tumors from non-white patients, and 3 tumors whose diagnosis of cancer could not be confirmed by the
study pathologist. We therefore analyzed a total of 351 tumors.

DNA extraction and sodium bisulfite modification

Three 20 μM sections were cut from each fixed, paraffin-embedded tumor sample and transferred into microcentrifuge tubes. The paraffin was dissolved using
Histochoice Clearing Agent (Sigma-Aldrich, St Louis, MO) followed by two washes with 100% ethanol and one wash with phosphate buffered saline (PBS). The samples were then incubated in SDS–lysis solution [50 mM Tris–HCl (pH 8.1), 10 mM EDTA and 1% SDS] with protease K (Qiagen, Valencia, CA) overnight at 55°C. De-crosslinking was performed by adding
NaCl (final concentration 0.7 M) and incubating at 65°C for 4 h. DNA was recovered using the Wizard DNA clean-up kit (Promega, Madison, WI)
according to the manufacturer’s protocols. Sodium bisulfite modification of the DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer’s protocol, with the addition of a 5 min initial incubation at 95°C prior to addition of the denaturation reagent. The de-crosslinking incubation as well as the 95°C incubation ensure more
complete melting of the DNA and thus more complete sodium bisulfite conversion.

Methylation-specific PCR

Sodium bisulfite modified DNA was used as the template for methylation-specific PCR, as previously described, using primers specific for the methy-
lated promoter of p16INK4A (28), RASSF1A (29) and PRSS3 (30). To control for the presence of modified DNA, primers specific to a modified region of the
ACTB gene containing no CpG sites were used (31). Sodium bisulfite modified circulating blood lymphocyte DNA was used as a negative control for each
run. As a positive control, lymphocyte DNA completely methylated using SssI
DNA methylase and modified with sodium bisulfite was used. For quality assurance, 10% of samples are blindly replicated and there is complete
 Concordance in these replicates.

TP53 immunohistochemistry

The immunohistochemical detection of TP53 in these tumors has been previously described (11).

Statistical analysis

We used multivariate unconditional logistic regression with methylation of
each gene (p16INK4A, RASSF1A and PRSS3) as the dependent variable to examine associations between methylation and patient demographics (i.e.
age and sex), tumor characteristics (i.e. histology, grade and stage), alterations
of TP53 (IHC staining intensity), and exposure history (i.e. smoking history
and arsenic exposure), with adjustment for age and sex in all models. Smoking history
was examined first by status (never, former or current) at time of diagnosis, and
then in sub-analyses of smokers by duration, intensity and pack-years of
smoking. Additionally, in former smokers, the time since quitting was also examined. In our case–control analysis, we observed an elevated odds ratio
(OR) for bladder cancer only in the highest percentiles of arsenic exposure (6),
thus we have dichotomized the arsenic exposure for these analyses by the 95th
percentile, similar to the cut-off where elevated disease risks have been
observed. All P-values represent two-sided statistical tests.

Results

The prevalence of promoter hypermethylation for p16INK4A
was 30.8% (108/351), for RASSF1A was 32.2% (113/351)
and for PRSS3 was 33.0% (116/351) in this population-based
series of bladder cancer. There was no association between
detection of promoter methylation and the percent of tumor
tissue in the sample (data not shown) suggesting that the
amount of malignant tissue (or the amount of non-malignant
tissue) in the sample does not bias this assay. There was no
association between promoter methylation of any of these
genes and patient gender or age (Table I). Although the
number of non-transitional cell carcinoma (TCC) samples was
small, there appeared to be a higher prevalence of promoter
methylation of each of the genes in TCC compared with other
histologies. Both RASSF1A promoter methylation and PRSS3
promoter methylation were related to increasing tumor stage,
with invasive stage tumors being 3.4 times more likely to
have RASSF1A promoter methylation (95% CI 1.8–6.5, P <
0.001), and 1.9 times more likely to have PRSS3 methylation
(95% CI 1.1–3.6, P < 0.04) (Table I). Promoter methylation
of p16INK4A also tended to occur more often in invasive
stage tumors, although this relationship was not statistically
significant.

We have previously demonstrated a relationship between
altered TP53 staining intensity (qualitatively scored on a scale
of 1–3, with 3 being most abnormal) and tumor stage (11), we
therefore also examined the relationship of this alteration with
promoter methylation of these genes, as a potential con-
founder. As shown in Table I, highly intense (3+) TP53 IHC
staining was only border-line significantly associated with
RASSF1A promoter methylation, but not with promoter
methylation of p16INK4A or PRSS3.

We then examined the associations between tobacco smoking,
arSENIC exposure and promoter methylation of these genes in
the case series, using logistic regression models controlling
for age, gender tumor stage, TP53 staining intensity and the
exposures. Patient cigarette smoking status, as well as other
metrics of tobacco use [including intensity (cigarettes per day),
duration (years smoked) and combined measure of duration/ intensity (pack-years)] were examined individually in relation
to methylation of each of the genes studied. We found that
cigarette smoking status was significantly associated only with
p16INK4A promoter methylation (Table II). At the time of
diagnosis, patients who were former smokers having quit for
>10 years (the lower quartile of time since quitting amongst
former smokers in this population) had an OR for p16INK4A
promoter methylation of 2.2 (95% CI 1.0–5.0, P < 0.06),
while those who were former smokers for <10 years were
significantly more likely than never smokers to have p16INK4A
promoter methylation with an OR of 3.7 (95% CI 1.4–9.7, P <
0.007). Those who were current smokers had an OR of
2.4 (95% CI 1.1–5.5, P < 0.04). Looking within smokers, there was no trend in the prevalence of p16INK4A methylation and duration, intensity or pack-years smoked (data not shown).

A significant relationship was identified between arsenic
exposure (as measured by toenail arsenic levels) and promoter

Carcinogen exposure and DNA methylation in bladder cancer
m ethylation of RASSF1A and PRSS3. Arsenic exposure was dichotomized at the 95th percentile of toenail arsenic level in this population, as it is at this level where a main effect of arsenic has been observed in the overall case–control analysis (6). Arsenic exposure in the 95th percentile (≥0.26 µg/g) produced an OR for PRSS3 promoter methylation of 2.8 (95% CI 1.0–7.6, \( P < 0.05 \)), and for RASSF1A methylation of 3.5 (95% CI 1.2–10.0, \( P < 0.02 \)) both controlled for potential confounders (Table II). Examining methylation of any of the three genes compared with no methylation showed no significant association to exposure, suggesting a gene-specific phenomenon (data not shown). Stratified analysis was performed to examine interactive effects between smoking and high arsenic exposure on promoter methylation of these genes, but no interactive effect was observed, although our sample size does not provide sufficient power for examining small interactions.

Discussion

Classically, exposures to carcinogens, such as those in tobacco-smoke, have been thought to act directly, or indirectly (through generation of reactive oxygen species) to damage DNA, and (through incomplete or inaccurate repair) give rise to mutations or larger chromosomal deletion events. More recent work, including this study, suggests that beyond these genetic alterations, carcinogens may also be contributing to the development or selection of epigenetic alterations. M ethylation silencing of RASSF1A and PRSS3 were both significantly associated with an invasive tumor stage, and showed increasing trends in the relative risk estimates of promoter methylation from non-invasive, low grade through these invasive stage tumors, suggesting that these alterations may occur late in the carcinogenesis of the bladder. This selection may be related to their function, as RASSF1A is thought to

| Table I. Associations between promoter methylation (meth.) of \( p16^{INK4A} \), RASSF1A and PRSS3 with patient demographics and tumor characteristics in bladder cancer cases |

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>( p16^{INK4A} )</th>
<th>RASSF1A</th>
<th>PRSS3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No meth. ( N = 243 ) %</td>
<td>Meth. ( N = 108 ) %</td>
<td>Adjusted OR ( 95% ) CI</td>
</tr>
<tr>
<td>Gender Male</td>
<td>57 (74)</td>
<td>20 (26)</td>
<td>1.0 (ref)</td>
</tr>
<tr>
<td>Female</td>
<td>186 (68)</td>
<td>88 (32)</td>
<td>1.4 (0.8–2.5)</td>
</tr>
<tr>
<td>Age &lt;65</td>
<td>117 (69)</td>
<td>53 (31)</td>
<td>1.0 (ref)</td>
</tr>
<tr>
<td>≥65</td>
<td>126 (65)</td>
<td>55 (35)</td>
<td>0.9 (0.6–1.5)</td>
</tr>
<tr>
<td>Histology Non-TCC</td>
<td>9 (82)</td>
<td>2 (18)</td>
<td>1.0 (ref)</td>
</tr>
<tr>
<td>TCC</td>
<td>234 (69)</td>
<td>106 (31)</td>
<td>1.8 (0.9–3.6)</td>
</tr>
<tr>
<td>Tumor stage&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIIS</td>
<td>8 (67)</td>
<td>4 (33)</td>
<td>1.6 (0.4–6.4)</td>
</tr>
<tr>
<td>Non-invasive, low grade</td>
<td>152 (72)</td>
<td>60 (28)</td>
<td>1.0 (ref)</td>
</tr>
<tr>
<td>Non-invasive, high grade</td>
<td>22 (73)</td>
<td>8 (33)</td>
<td>0.9 (0.4–2.2)</td>
</tr>
<tr>
<td>Invasive</td>
<td>58 (62)</td>
<td>36 (38)</td>
<td>1.8 (0.9–3.4)</td>
</tr>
<tr>
<td>TP53 IHC staining intensity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>169 (69)</td>
<td>76 (31)</td>
<td>1.0 (ref)</td>
</tr>
<tr>
<td>&lt;3</td>
<td>56 (66)</td>
<td>29 (34)</td>
<td>0.8 (0.4–1.6)</td>
</tr>
</tbody>
</table>

<sup>b</sup>Odds Ratio (OR) are calculated for each gene and are adjusted for all variables in column.

<sup>a</sup>Three tumors were not given stage classification and had no TP53 IHC performed and were entered in the model as missing values.

| Table II. Associations between smoking and arsenic exposure and promoter methylation of \( p16^{INK4A} \), PRSS3 and RASSF1A in bladder cancer cases |

<table>
<thead>
<tr>
<th>Co-variate</th>
<th>( N = 351 )</th>
<th>( p16^{INK4A} ) methylation OR ( 95% ) CI</th>
<th>( P )</th>
<th>PRSS3 methylation OR ( 95% ) CI</th>
<th>( P )</th>
<th>RASSF1A methylation OR ( 95% ) CI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking status at diagnosis&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>55</td>
<td>3.7 (1.4–9.7)</td>
<td>0.007</td>
<td>0.7 (0.3–1.8)</td>
<td>0.5</td>
<td>0.6 (0.3–1.5)</td>
<td>0.3</td>
</tr>
<tr>
<td>Former smoker quit &lt;10 years</td>
<td>42</td>
<td>2.2 (1.0–5.0)</td>
<td>0.06</td>
<td>1.0 (0.5–2.1)</td>
<td>1.0</td>
<td>0.5 (0.2–1.0)</td>
<td>0.06</td>
</tr>
<tr>
<td>Former smoker quit ≥10 years</td>
<td>131</td>
<td>2.4 (1.1–5.5)</td>
<td>0.04</td>
<td>1.4 (0.7–2.9)</td>
<td>0.4</td>
<td>0.5 (0.3–1.1)</td>
<td>0.1</td>
</tr>
<tr>
<td>Toenail arsenic&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.26 µg/g</td>
<td>318</td>
<td>1.0 (ref)</td>
<td>0.9</td>
<td>1.0 (ref)</td>
<td>0.05</td>
<td>3.5 (1.2–10.0)</td>
<td>0.02</td>
</tr>
<tr>
<td>≥0.26 µg/g</td>
<td>18</td>
<td>1.1 (0.4–3.0)</td>
<td>0.9</td>
<td>2.8 (1.0–7.6)</td>
<td>0.05</td>
<td>3.5 (1.2–10.0)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<sup>b</sup>Methylation of each gene is modeled separately, and the model is controlled for age, gender, tumor stage, TP53 IHC staining intensity and both exposure variables in the table.

<sup>c</sup>Six subjects of former smoking status were missing time since quitting information, and were included in the model as missing data.

<sup>f</sup>Fifteen subjects are missing toenail arsenic measurements, and were included in the model as missing data.
interact with microtubule proteins in regulating microtubule dynamics important in cell division (32), while PRSS3, a member of the serine-protease family, may be important for cell motility and adhesion (30).

In our study, the methylation silencing of these genes is also tied to higher level arsenic exposures, thus it is possible that arsenic contributes to the initial development of an epigenetic alteration. Alternatively, arsenic exposure may act to provide the selective pressure needed to allow clones with these alterations to proliferate. The carcinogenic mode of action of arsenic has been difficult to determine; it is not a potent mutagen and it does not produce tumors in animal models. However, arsenite-generated free radicals and reactive oxygen species can lead to genotoxic damage (22). In cell lines, exposure to inorganic arsenic species has been linked to dose-dependent increases in promoter region hypermethylation of Cpg sites, although not to those resulting in altered gene expression. Further, arsenic exposure in vitro is associated with genome-wide hypomethylation (23–25). Mice on methyl-deficient or folate-deficient diets exposed to arsenic in their water supply also showed hypomethylation in portions of hepatic-derived DNA (33), as well as increases in chromosomal aberrations in blood lymphocytes (34). This is thought to be related to depletion of S-adenosyl methionine (SAM, the universal methyl donor) as a result of the metabolism of inorganic arsenic to its methylated forms.

These arsenic-associated increases in overall genomic hypomethylation and increases in genomic instability, demonstrated in these arsenic-exposed animal models, is reminiscent of the well-known characteristic aberrant methylation pattern seen in human solid tumors (35). Our data may suggest that gross alterations in DNA methylation patterns related to arsenic exposure may produce or select for specific gene hypermethylation observed in solid tumors. The precise manner of gene promoter targeting of the hypermethylation events is unclear; our study of bladder cancer suggests it is not a genomic phenomenon, since p16INK4A hypermethylation was not associated with arsenic exposure, nor is looking at any methylation of these three genes versus no methylation. The arsenic, then, may have multiple roles in altering the epigenetic environment of the cell. It may modify the steady-state levels of SAM, contributing to genomic hypomethylation as suggested by animal models. Further, it may generate an environment for selection of cells with epigenetic silencing of specific genes such as RASSF1A and PRSS3.

In lung cancer, the prevalence of p16INK4A promoter methylation increased with increasing duration of smoking (18), yet in bladder cancer we do not observe any duration or dose-dependent trends. Instead, any exposure to tobacco-smoke carcinogens seems to increase the relative risk of p16INK4A promoter methylation. We also see increased odds of this specific alteration in former smokers who have quit <10 years prior to diagnosis compared with those who have stopped smoking for longer periods. Together these results suggest that continued exposure during the development of malignancy may be driving or selecting this alteration. The difference between this finding and the trend seen in lung cancer may be due to the type of and to the level of carcinogens at which these target tissues become exposed. The lung is experiencing a mixture of direct smoke-derived carcinogens as well as oxidative products derived from these carcinogens, while the bladder is more likely to experience the effect of reactive carcinogen metabolites. Again, the mechanism driving the induction or selection of cellular clones with this aberrant methylation remains to be elucidated.

These results indicate that, in a population-based study of bladder cancer, there are specific associations between exposure to arsenic and tobacco-smoke and gene promoter hypermethylation. Animal and in vitro studies have suggested that arsenic’s carcinogenic activity may be tied to epigenetic, rather than genetic toxicity, and this analysis provides evidence, in human tumors, of a potential link between arsenic exposure and epigenetic alterations. It also suggests that tobacco carcinogen exposures may act as selective forces for specific gene silencing, particularly in the period closest to clinical manifestation of the tumor, which may have important clinical consequences for the patient.

Acknowledgements

This work was supported by NIEHS Superfund Center Grant 00002, NCI grant R01 CA100679, and NIEHS toxicology and environmental health sciences training grant T32 ES007155 to C.J.M. and H.D.

Conflict of Interest Statement: None declared.

References


Received May 27, 2005; revised June 20, 2005; accepted June 21, 2005