Epigenetic Inactivation of SFRP Genes and TP53 Alteration Act Jointly as Markers of Invasive Bladder Cancer


Abstract
In the United States each year, almost 13,000 deaths are attributable to bladder cancer, with the majority of these deaths related to higher stage, muscle-invasive solid tumors. Epigenetic silencing of the secreted frizzled receptor proteins (SFRP), antagonists of the WNT pathway, leads to constitutive WNT signaling, altering cell morphology and motility. Identifying alterations in this pathway in bladder cancer may prove useful for defining the invasive phenotype and provide targets for guiding therapy. Using a population-based study of bladder cancer (n = 355), we examined epigenetic alterations, specifically gene promoter hypermethylation, of four SFRP genes in addition to immunohistochemical staining of TP53, which has been previously shown to be a predictor of invasive disease. We observed a significant linear trend (P < 0.0004) in the magnitude of the risk of invasive disease with the number of SFRP genes methylated. Both TP53 alteration and SFRP gene methylation showed significant independent associations with invasive bladder cancer. Strikingly, in examining the joint effect of these alterations, we observed a >30-fold risk of invasive disease for patients with both altered SFRP gene methylation and intense TP53 staining (odds ratio, 32.1; P < 10^{-15}). Overall patient survival was significantly poorer in patients with any SFRP genes methylated (P < 0.0003) and in proportional hazards modeling, patients with methylation of any SFRP gene had significantly poorer overall survival (hazard ratio, 1.78; P < 0.02) controlled for TP53 staining intensity and other survival-associated factors. Classifying tumors based on SFRP methylation status and TP53 protein staining intensity may be a clinically powerful predictor of invasive, deadly disease. (Cancer Res 2005; 65(16): 7081-5)

Introduction
In the United States in 2004, ~60,000 new cases of bladder cancer were diagnosed and almost 13,000 deaths ascribed to this disease (1). The mortality of this disease is attributed to higher stage solid tumors that invade the muscular layers of the bladder (2, 3). Clinically, biological markers that can predict this invasive phenotype would be useful for determining patient prognosis as well as in properly designing treatment regimens. Alteration of the TP53 tumor suppressor, as determined using protein immunohistochemistry, has also been associated with more aggressive, invasive bladder cancer, and has been suggested to clinically motivate more radical surgery or radiotherapy (4, 5). However, the sensitivity and specificity of using TP53 alterations alone in making clinical judgments has been questioned (6) suggesting that additional biomarkers, which can be used alternatively or in conjunction with altered TP53, are necessary. Regulation of cell migration is critical to maintaining a noninvasive phenotype. CTNNB1 (β-catenin), activated by the WNT pathway, acts as a transcription factor, as well as interacts with the cadherins and the cytoskeleton, mediating intercellular adhesion and playing an important role in cellular morphogenesis (7, 8). Disruption of this pathway, through epigenetic inactivation of the secreted frizzled receptor proteins (SFRP), secreted antagonists of WNT signaling, has been shown in colorectal cancer and is responsible for the constitutive activation of WNT signaling in a number of primary colorectal tumors (9). In a hospital-based study of papillary bladder cancer, loss of SFRP1 expression was a marker of higher tumor stage and grade and poorer survival, with the loss of expression attributable to promoter hypermethylation and allelic loss of SFRP1 (10). As appropriate function of the WNT and TP53 pathways may play an important biological role in maintaining a noninvasive phenotype, aberrant activation of these pathways through epigenetic silencing of the SFRPs or alterations of TP53 expression may be important in the determination of this invasive phenotype. Using our population-based bladder cancer case series, we have examined the prevalence and clinical utility of methylation silencing as a marker of invasiveness and survival, limiting the bias that may be introduced in hospital-based studies where low-grade lesions are underrepresented. Additionally, we have analyzed whether these epigenetic alterations are associated with the demographic and exposure histories of these patients to determine the role of methylation-induced silencing of the SFRP genes in the occurrence or progression of bladder cancer.

Patients and Methods
Study population. Residents of New Hampshire ages 25 to 74 years, diagnosed from July 1, 1994 to June 30, 1998 were identified by the rapid reporting system of the New Hampshire State Cancer Registry. As by state law, practitioners are required to provide a report of an incident cancer upon its diagnosis (11). All study participants were consented under the appropriate institutional review board protocols. Study participants underwent a personal interview to obtain information on demographic traits, use of tobacco (including frequency, duration, and intensity of cigarette smoking), and use of hair dyes. Pathology material was complete from a total of 355 patients (see below). Pathology reports and paraffin-embedded tumor specimens were requested from the treating physician/pathology laboratories, and tumor samples were obtained from the
procedure involved in the initial diagnosis. Bladder tumors were reviewed by the study pathologist and classified according to the 1973 and 2004 WHO guidelines for bladder tumors. Additionally, the proportion of malignant cells was recorded, averaging 69% (median, 80%). Patient survival was assessed using clinical records and the Social Security Death Index.

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 PBS. The samples were then incubated in SDS-lysis solution [50 mmol/L Tris-HCl (pH 8.1), 10 mmol/L EDTA, and 1% SDS] with proteinase K (Qiagen, Valencia, CA) overnight at 55°C. De-cross-linking was done by adding NaCl (final concentration, 0.7 mol/L) and incubating at 65°C for 4 hours. 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 done using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's protocol, with the addition of a 5-minute initial incubation at 95°C before addition of the denaturation reagent. The de-cross-linking 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 (MS-PCR) as previously described (12) using primers specific for the methylated promoter of SFRP1, SFRP2, SFRP4, and SFRP5 (9). All methylation-specific PCRs are optimized to detect >5% methylated substrate in each sample. To control for the presence of modified DNA, primers specific to a modified region of the ACTB genes containing no CpG sites were used (13). Modified circulating blood lymphocyte DNA and that same lymphocyte DNA completely methylated using MspI DNA methylase and modified by treatment with sodium bisulfite were used as the negative and positive controls, respectively, in each run.

Quantitative methylation-specific PCR. Sodium bisulfite–modified DNA from 10 invasive stage and 10 noninvasive stage tumors (randomly selected from among each category and analyzed blindly) was subjected to quantitative real-time-PCR (RT-PCR) using primers and a Taqman Probe (Applied Biosystems, Foster City, CA) specific to modified ACTB as previously described (13). Bisulfite-modified human sperm DNA served as a positive control and a no template control was also run with the samples. All samples and controls were run in duplicate.

TP53 immunohistochemistry. The immunohistochemical detection of TP53 has been previously described (14).

Statistical analysis. We used multivariate unconditional logistic regression with methylation of any SFRP gene (1 or more versus 0) as the dependent variable to examine associations among patient demographics, exposure history, and tumor characteristics with SFRP gene methylation while controlling for possible confounding. We examined the effects of multiple predictors on tumor invasion (i.e., invasive versus noninvasive) using unconditional logistic regression analysis, with adjustment for potential confounders; in the analysis, the relative risks of invasive disease were estimated for having one, two, or three to four SFRP genes methylated as well as TP53 staining intensity. To examine the joint effect of TP53 alteration and SFRP gene methylation on tumor invasiveness, we conducted an analysis stratified by both SFRP gene methylation and TP53 status with TP53 negative (−3 staining intensity) and no SFRP genes methylated as the reference category, again using unconditional logistic regression with adjustment for multiple covariates (i.e., age and sex).

Patient survival was first examined using Kaplan-Meier survival probability curves, and differences between strata tested using the log-rank test. To control for additional variables related to patient survival, Cox proportional hazards modeling was employed, using the same variables used in the logistic regression analysis. All P values represent two-sided statistical tests.

Results and Discussion

As expression of SFRP1, SFRP2, SFRP4, and SFRP5 has been previously shown in normal bladder epithelium (15), promoter DNA hypermethylation indicative of epigenetic silencing of these genes was determined, using MS-PCR, in a population-based case series study of bladder cancer in New Hampshire. The prevalences of methylation silencing were 18% (64 of 355), 52% (184 of 355), 9% (32 of 355), and 37% (132 of 355), respectively for SFRP1, SFRP2, SFRP4, and SFRP5. Methylation of any of the SFRP genes occurred in 62% (221 of 355) of the tumors examined. To assure that there were no biases in the detection of promoter methylation attributable to the proportion of tumorous tissue in the substrate used for MS-PCR, we examined methylation of any SFRP gene and the proportion of malignant tissue in individual samples, assessed by an experienced urologic pathologist (A.R.S.). There was no relationship between detection of promoter methylation and the percent of tumorous tissue in the sample (P < 0.8). This result is consistent with the previously reported finding (in the same samples) of no relationship between proportion of tumorous tissue and the measured presence of persistent TP53 protein by immunohistochemistry or TP53 mutation (16). To ensure that there was no bias in the amplification of substrate by tumor stage, we also did quantitative RT-PCR on a random subset of samples (10 invasive stage and 10 noninvasive stage) using the primers and probe for modified ACTB. The cycle threshold (Ct) for invasive tumors was 34.3 (SD, 2.3) and for noninvasive tumors was 34.8 (SD, 3.0). There was no significant difference (Student's t test, P > 0.6) in the substrate amplification by tumor stage, confirming the absence of bias in PCR amplifiable substrate.

Table 1 examines the association between having any SFRP gene methylated and demographics of the cases or tumor characteristics. Men had a significantly higher prevalence of any SFRP gene methylation compared with women, although there seemed to be no significant difference in the prevalence of any SFRP gene methylation by patient age or by histology; this analysis is limited to the small number of nontransitional cell carcinomas available. Methylation of the SFRP genes was positively associated with advanced tumor stage [adjusted odds ratio (OR) for SFRP methylation among invasive tumors, 2.8; 95% confidence interval (95% CI), 1.4-5.6]. The prevalence of SFRP gene methylation was almost 2-fold greater in current smokers compared with never or former smokers. When the metrics of smoking were examined in current smokers, there was no significant association between SFRP gene methylation and the intensity or duration of smoking.

We have previously reported that TP53 immunohistochemical staining intensity is associated with invasive disease in these tumors (14). After controlling for potential confounders, TP53 staining was not significantly associated with SFRP gene methylation, suggesting that epigenetic silencing of the SFRP genes occurs independent of alterations to TP53.

As TP53 alterations and SFRP gene methylation are unrelated, we examined their association with invasive bladder cancer. We used invasive disease as the dependent variable and included variables for the number of SFRP genes methylated, TP53 staining intensity (3+ versus -3), age (<65 versus ≥65 years), and gender in the multivariate logistic regression model. Current cigarette smoking was initially included in the model, due to its association with SFRP gene methylation. Tests for confounding showed that smoking was not significantly associated with
invasiveness and its removal from the model had no effect on the point estimates. Table 2 shows a highly significant trend in the magnitude of the odds of having invasive bladder cancer with increasing numbers of SFRP genes silenced by methylation (P < 0.0004). This result indicates a very strong and significant relationship between methylation of these genes and invasive disease. Having one SFRP gene methylated imparts an almost 2-fold relative risk of invasive disease, whereas having three to four SFRP genes methylated imparts a 4.2-fold relative risk of invasiveness (controlled for potential confounders in the model). This model also shows a strong, significant, and independent association between TP53 staining intensity and invasive disease. After adjusting for SFRP gene methylation as well as age and gender, patients with intense TP53 staining were almost 17 times as likely to have invasive disease, compared with patients with less intensely staining tumors. The significant trend, showing that as the number of SFRP genes methylated increases the likelihood of the tumor to be invasive increases, suggests this pathway is crucial for facilitating this invasive phenotype. That is, as more of these antagonists become silenced, the pathway can become more constitutively active and the clone more likely to become invasive. However, Salem et al. (17) have previously noted that invasive bladder tumors have greater numbers of methylated CpG islands, suggesting these tumors may have a methylator phenotype. Interestingly, the methylation of the individual islands studied by Salem et al. (17) occurred independently, whereas the methylation of SFRP loci is significantly correlated (data not shown); this is more suggestive of selection of silencing of the Wnt pathway, rather than action of a less targeted, global epigenetic mechanism.

Next, we investigated whether SFRP gene methylation status may enhance the association between TP53 staining intensity and invasive disease in a more than additive fashion. Table 3 shows the results of stratified multivariate logistic analyses, again using invasive bladder cancer as the dependent variable, and TP53 staining intensity as the predictor. In tumors having no SFRP genes methylated, high TP53 staining intensity imparted a 9.4-fold risk of invasive bladder cancer (95% CI, 2.8-31.8) compared with low TP53 staining intensity, controlled for age and gender. On the other hand, in tumors with one or more SFRP genes methylated, high TP53 staining intensity had an OR for invasive bladder cancer of 32.1 (95% CI, 12.9-79.8; P < 10^{-13}). This striking result suggests that tumors with SFRP methylation and TP53 pathway alteration have >30 times the odds of being invasive than tumors with no or low TP53 staining intensity.

Finally, as silencing of the SFRP genes was associated with invasive disease, we examined the effect of these alterations on overall patient survival. Figure 1 shows the Kaplan-Meier survival probability plots stratified by methylation of any SFRP gene. The log-rank test indicated a significant (P < 0.0003) difference in survival between the strata. The covariate-adjusted hazard ratios and 95% CIs for the association between these variables and patient overall survival is given in Table 4. Patient age and carcinoma in situ histologic stage showed significant associations with patient survival, whereas being male and having invasive stage disease also showed elevated instantaneous risks of death.

### Table 1. Bladder tumor SFRP gene methylation by patient demographics and tumor characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Any SFRP methylation</th>
<th>Adjusted OR* (95% CI)</th>
<th>Totals (n = 355)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No methylation (n = 134, n (%))</td>
<td>Any methylation (n = 221, n (%))</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Female 37 (27.6) 40 (18.1) 1.0 (reference) 77</td>
<td>Male 97 (72.4) 181 (81.9) 2.0 (1.1-3.5) 278</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>&lt;65 68 (50.7) 106 (48.0) 1.0 (reference) 174</td>
<td>≥65 66 (49.3) 15 (22.0) 1.3 (0.8-2.0) 181</td>
<td></td>
</tr>
<tr>
<td>Histology†</td>
<td>Transitional cell carcinoma 130 (97.7) 210 (96.3) 1.0 (reference) 340</td>
<td>Nontransitional cell 3 (2.3) 8 (3.7) 0.7 (0.07-8.1) 11</td>
<td></td>
</tr>
<tr>
<td>Tumor stage‡</td>
<td>Noninvasive 108 (82.4) 134 (61.7) 1.0 (reference) 242</td>
<td>Carcinoma in situ 6 (4.6) 6 (2.8) 0.5 (0.1-1.9) 12</td>
<td></td>
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<tr>
<td></td>
<td>Invasive 17 (13.0) 77 (35.5) 2.8 (1.4-5.6) 94</td>
<td>Score &lt;3 102 (82.3) 143 (69.4) 1.0 (reference) 245</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Score ≥3 22 (17.7) 63 (30.6) 1.3 (0.6-2.6) 85</td>
<td>Current smoker No 101 (75.4) 135 (61.1) 1.0 (reference) 236</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes 33 (24.6) 86 (38.9) 1.9 (1.1-3.3) 119</td>
<td></td>
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</tbody>
</table>

*OR adjusted for all other variables in the table and limited to subjects with complete data for all variable (n = 330).
†Four tumors were missing histologic classification.
‡Seven tumors were missing stage classification.
§TP53 immunohistochemistry was done on 330 tumors.
Methylation of any SFRP gene imparted a 1.78-fold increased risk of death (95% CI, 1.08-2.92), yet TP53 staining intensity, in this model, has no significant association with patient survival.

Although direct somatic mutations of APC or CTNNB1, integral components of the WNT pathway, are uncommon in bladder cancer (18), epigenetic silencing of pathway antagonists, the SFRPs, occurs commonly. Our data shows that silencing of these genes occurs in 60% of tumors and occurs more often in men.

We and others have previously reported associations between smoking exposure and DNA methylation in lung cancer (19, 20). The current results, showing an association between SFRP gene methylation and smoking at the time of diagnosis, suggest that continuous exposure of the cancerous field to tobacco smoke carcinogens is able to select for epigenetic silencing of these genes.

Importantly, alterations of TP53 and epigenetic silencing of the SFRPs are both independently significantly related to the invasive phenotype of bladder cancer. As the majority of mortality in bladder cancer is associated with invasive disease, we observe a significant association between SFRP gene methylation and overall patient survival. It is of interest that TP53 staining is not significantly associated with survival in the adjusted analysis but may indicate its colinearity with tumor stage. Our study suggests that classifying tumors based upon both SFRP gene methylation status and TP53 immunohistochemistry would provide significantly improved clinical estimates of an aggressive and potentially fatal phenotype, better identifying patients in need of more aggressive treatment of their disease.

Table 2. Methylation of multiple SFRP genes and altered TP53 status are independently associated with invasive bladder cancer

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Hazard ratio (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>Number of SFRP genes methylated</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>1</td>
<td>1.9 (0.8-4.5)</td>
</tr>
<tr>
<td>2</td>
<td>3.5 (1.5-8.4)</td>
</tr>
<tr>
<td>3 or 4</td>
<td>4.2 (1.7-10.2)¹</td>
</tr>
<tr>
<td>TP53 alteration (staining intensity)</td>
<td></td>
</tr>
<tr>
<td>&lt;3</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>3+</td>
<td>16.8 (8.7-32.4)</td>
</tr>
</tbody>
</table>

¹Model predicting invasive disease compared with noninvasive. Carcinoma in situ were excluded from the model.

Table 3. Stratified analysis of the association between TP53 alteration and invasive bladder cancer by methyla-

<table>
<thead>
<tr>
<th>TP53 status (Immunohistochemistry intensity)</th>
<th>Invasive bladder cancer, OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No SFRP methylation</td>
<td></td>
</tr>
<tr>
<td>TP53 WT</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>TP53 altered</td>
<td>9.4 (2.8-31.8)</td>
</tr>
<tr>
<td>Positive SFRP methylation</td>
<td></td>
</tr>
<tr>
<td>TP53 WT</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>TP53 altered</td>
<td>32.1 (12.9-79.8)</td>
</tr>
</tbody>
</table>

NOTE: Both models are controlled for age and sex. Carcinoma in situ were excluded from the models.

Abbreviation: WT, wild type.
methylation in serum is being proposed as a tool for cancer detection and follow-up, and detection of tumor-associated methylation in urine-derived DNA also holds promise for clinical application (21, 22). Our results suggest that the SFRP genes are strong candidates for clinical use in these types of serum or urine diagnostics, as they may greatly aid in determining which patients will develop invasive disease, and we believe further research into using methylation of these genes as screening tools is warranted.

Acknowledgments

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