

Epigenetic Inactivation of *SFRP1* and *SFRP2* Genes as  
Biomarkers of Invasive Bladder Cancer

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## 1. Abstract

The epigenetic silencing of important tumor suppressor genes through promoter hypermethylation is becoming evident in many cancers. In bladder cancer, it has been proposed that this mechanism causes aberrant signaling in the Wnt developmental pathway through the silencing of important antagonists, the soluble frizzled-related protein (*SFRP*) gene family. A thorough study of such alterations could prove to be useful in determining appropriate therapy in bladder cancer patients and improving their prognosis.

Therefore, using a population-based case series, we sought to investigate three hypotheses, that (1) *SFRP* gene hypermethylation is associated with invasive bladder cancer, (2) smoking is associated with *SFRP* gene hypermethylation and (3) *SFRP* gene hypermethylation and *TP53* mutations act jointly as markers of invasive bladder cancer.

In our main results, we observed a significant association between the methylation of any *SFRP* gene and invasive bladder cancer ( $p < 0.003$ ). Furthermore, we found that methylation of an increasing number of *SFRP* genes was associated with higher odds of invasive disease. The joint effect of *SFRP* methylation and *TP53* alterations (represented by immunohistochemical staining of p53) was significant; patients exhibiting both alterations showed a >7-fold odds of having invasive bladder cancer as compared to those without either alteration ( $p = 0.0001$ ). No conclusive relationship was found between smoking and *SFRP* methylation.

Our results suggest that epigenetic alterations of the *SFRP* genes are highly prevalent in bladder cancer and may be useful not only in the prediction of invasive disease, but also in the development of new cancer therapies.

## **2. Introduction**

### **2.1. Bladder cancer statistics**

Cancer is the second leading cause of death in the United States after heart disease and is estimated to cause one in every 4 deaths (Jemal et al., 2008). In 2008, almost 69,000 new cases of urinary bladder cancer were diagnosed and 14,000 deaths due to it were recorded. Bladder cancers account for 7% of all new cases of cancer among men and 2.5% of cases among women, as well as 3% of cancer deaths among men and 1.5% among women. Mortality is highly dependent on the stage of disease at diagnosis and is usually attributed to higher stage solid tumors that invade the muscular wall of the bladder, or metastasize to nearby organs and lymph nodes (NCI, 2008). Bladder cancer mortality rates have also been shown to vary by geographic location in the United States; rates in both men and women are elevated in the northeastern states (Brown et al., 1995; Michaud et al., 2001). These differences have become more pronounced over time, and cannot be explained entirely by occupational exposures or regional variations in smoking and dietary habits.

### **2.2. Epigenetics**

Although mutations and family history are important factors in the development of cancer, genetics by itself cannot explain the variety of phenotypes observed within a population. It is also not able to explain how, despite identical DNA sequences, monozygotic twins (Fraga et al., 2005) and cloned animals (Humpherys et al., 2001) can have different phenotypes and susceptibilities to disease. Epigenetics offers a partial explanation for these phenomena, and has been defined as heritable changes in gene expression that are not due to any alteration in the DNA sequence (Holliday, 1987).

Metaphorically, epigenetics can be compared to the different ways individuals read a book aloud, the text of the book being the genomic DNA sequence. Three primary mechanisms are used to carry out epigenetic alterations: DNA methylation, histone modification, and RNA interference..

### **2.2.1. DNA Methylation**

The best-known epigenetic marker is DNA methylation, which refers to the attachment of a methyl group to 5-carbon position of a cytosine preceding a guanine (CpG dinucleotide). CpG dinucleotides are not distributed randomly in the genome, but are often found very close together in CpG-rich 'islands' around the promoter end of many genes (Esteller, 2008). Studies have shown gene-specific hypermethylation of the CpG islands in promoter regions of tumor-suppressor genes. Hypermethylation of a gene promoter may result in the silencing of gene, and this mechanism of gene silencing is a major contributor to carcinogenesis. Genes involved in the cell cycle, DNA repair, metabolism of carcinogens, cell interactions, apoptosis and angiogenesis have all been shown to be subject to methylation-induced silencing (Esteller, 2008). Examples of these genes include the retinoblastoma tumor-suppressor gene (*RBI*) (Zeschnigk, 1999), the p16<sup>INK4a</sup> gene (*CDKN2A*) (Kim et al., 2001), breast cancer susceptibility gene 1 (*BRCA1*) (Esteller et al., 2000), as well as the *SFRP* family of genes (Kawano and Kypta, 2003). In each of these cases, DNA hypermethylation results in the silencing of these tumor suppressor genes, such that the growth advantage afforded by inactivation permits clonal selection and outgrowth of malignant cells.

### **2.3. Wnt Signaling System**

The Wnt signaling system is a major cellular pathway that plays an important role in the control of embryonic development, among other functions. It operates across cell boundaries through the secretion from cells of one tissue type to activate surface receptors on neighboring cells and tissues, leading to the activation of transcription factors that regulate processes such as cell proliferation, survival, and differentiation. In diseases such as cancer, these processes are dysregulated and aberrant activation of the Wnt pathway occurs, allowing cancer cells to multiply and form tumors (Klaus and Birchmeier, 2008).

### 2.3.1. $\beta$ -catenin

$\beta$ -catenin is an intracellular transcriptional regulator, which has been shown to play an important role in several solid tumors, including bladder cancer. The control of  $\beta$ -catenin levels and stability is integral to the canonical Wnt pathway (Paul and Dey, 2008).

In the absence of Wnt ligands, cytoplasmic  $\beta$ -catenin is usually maintained low levels through phosphorylation by casein kinase 1 $\alpha$  (CK1 $\alpha$ ) and glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) in a

destruction complex and is then subsequently degraded by the ubiquitination-proteasome system (Figure 1; Klaus and Birchmeier, 2008; Jones and Jomary, 2002). Thus, in the

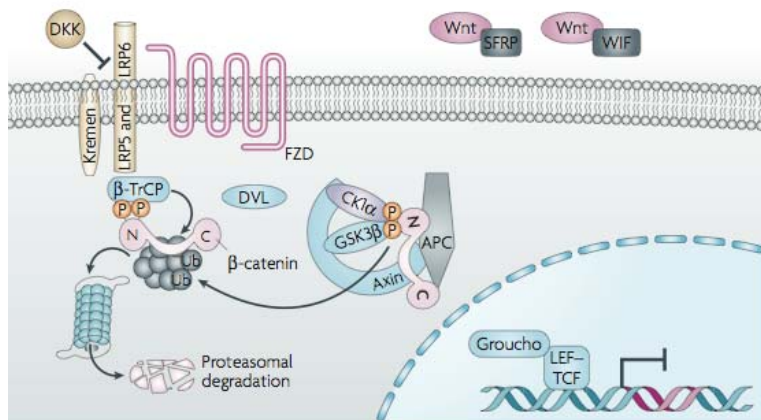


Figure 1. Wnt signaling pathway: the non-activated state. (Klaus and Birchmeier, 2008)

pathway's non-activated state,  $\beta$ -catenin levels remain low and since there is no movement of it into the nucleus, Wnt-specific target genes are not expressed.

However, upon activation, the Wnt proteins bind to the Frizzled (Fz) receptor in complex with Lipoprotein receptor-related protein (LRP) 5 or 6 at the cell surface to activate the pivotal

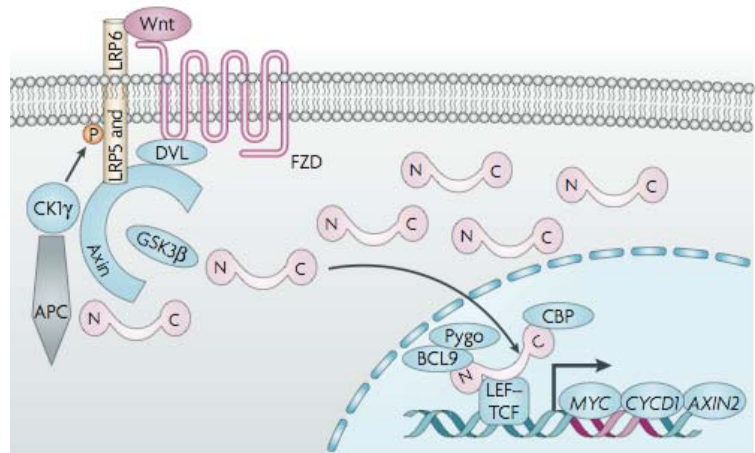


Figure 2. Wnt signaling pathway: the activated state. (Klaus and Birchmeier, 2008)

protein Dishevelled (Dvl) (Logan and Nusse, 2004). Dvl inhibits the phosphorylation of  $\beta$ -catenin through the recruitment of Axin, a part of the destruction complex (Kawano and Kypta, 2003). This inactivates the destruction complex, and allows cytoplasmic stabilization and accumulation of  $\beta$ -catenin (Figure 2). As cytoplasmic levels of  $\beta$ -catenin rise, it is targeted to the nucleus, where it forms a transcriptionally active complex with lymphoid enhancer factor (LEF) and T-cell factor (TCF). Transcriptional activation of target genes that function in cell differentiation, signaling, proliferation and many other genes occurs, including a well-known oncogene, *MYC* (Suzuki et al., 2004).

### 2.3.2. Wnt Pathway and Cancer

Many genes in the Wnt pathway were discovered to act as oncogenes or tumor suppressors when deregulated in human cancer (Klaus and Birchmeier, 2008). Thus, the role of aberrant signaling in the Wnt pathway in the development of cancer is an area that is being widely studied. Disruption of the Wnt pathway can occur in various ways, such

as mutations in components of the pathway, loss of heterozygosity (LOH) in tumors, and epigenetic silencing in specific genes.

Tumor-causing mutations can occur in many components of the Wnt pathway, including *APC*, *CTNNB1* (encoding  $\beta$ -catenin), *AXIN1*, and *AXIN2* genes, which often lead to inappropriate stabilization of  $\beta$ -catenin and persistent transcription of genes associated with cell proliferation (Klaus and Birchmeier, 2008; Suzuki et al., 2004). Frequent LOH in multiple chromosomal regions occurs in several tumors, which suggest that a variety of tumor suppressor genes in these arms may be inactivated during carcinogenesis (Shih et al., 2006). In recent years, another mechanism of aberrant activation has been discovered: silencing of genes that encode the inhibitory SFRPs through hypermethylation (Klaus and Birchmeier, 2008; Marsit et al. 2005; Suzuki et al. 2004).

#### **2.4. SFRPs as Antagonists of the Wnt Pathway**

SFRPs are soluble antagonists of the Wnt pathway that bind directly to Wnt proteins, and make up the largest family of Wnt inhibitors (Bovolenta et al., 2008). Bound to the SFRPs, Wnt proteins are impeded from binding to Fz receptors, thus preventing signaling. As a result, regulation of the *SFRP* genes has been shown to be a mechanism of stabilization and activation of  $\beta$ -catenin in the Wnt pathway. This makes the SFRPs an important family for further study.

This family of proteins is comprised of SFRP1, 2, and 5 in one sub-group and SFRP3 and 4 in another subgroup, based on sequence homology (Kawano and Kypta, 2003). The mechanism of how SFRPs impede Wnt from binding with Fz receptors is unclear, although several theories exist. As the SFRPs share a homology with Fz receptors in the

Cysteine-rich domain (CRD), studies have postulated that the SFRP-CRD domain is the site where the Wnt proteins are bound (Kawano and Kypta, 2003; Bovolenta et al., 2008). Other theories of how SFRPs block Wnt signaling include binding at the C-terminal netrin (NTR) domain and alternatively, binding to Fz to form non-functional complexes.

This upstream regulation of the canonical Wnt pathway by SFRPs inactivates the pathway, such that there is a specific loss of  $\beta$ -catenin translocation into the nucleus, and downregulation of Wnt target genes, such as *MYC* (Suzuki et al., 2004). As such, *SFRP* genes can be classified as tumor suppressor genes.

#### **2.4.1. Importance of *SFRP* Silencing in Bladder Cancer**

Direct somatic mutations of *APC* and *CTNNB1*, integral components in the Wnt pathway are uncommon in bladder cancer (Stoehr et al., 2004). However, epigenetic silencing of the *SFRP* genes occurs commonly and is a likely mechanism of  $\beta$ -catenin stabilization and aberrant Wnt signaling.

The hypermethylation of *SFRP* promoters – and subsequent silencing of the *SFRP* genes, responsible for the activation of Wnt signaling – has been shown in colorectal (Suzuki et al., 2004), head and neck (Marsit et al., 2006), hepatocellular (Shih et al. 2006), gastric (Nojima et al., 2007), malignant mesothelioma (Lee et al., 2004) and bladder cancers (Marsit et al., 2005). The results of these studies suggest that the downregulation of SFRPs could enhance or even initiate pre-malignant cell changes (Suzuki et al., 2004; Baylin and Ohm, 2006). Conversely, restoring *SFRP* expression in various cancer cells lessens their tumorigenic behaviour, decreases  $\beta$ -catenin stabilization and promotes cell death, even when downstream components of the Wnt pathway are mutated (Suzuki et al., 2004).

Because SFRPs are soluble proteins and usually act by secretion from cell to cell, they are good candidates to focus on for therapeutic drug research. In fact, antibody-based drugs are already being developed and tested in preclinical tumor models (Klaus and Birchmeier, 2008; Barker and Clevers, 2007). Using SFRPs in directed epigenetic-specific therapy has a lot of promise in comparison with DNA methylation inhibitors as compared to inhibitors of methylation, which may produce non-specific effects with unintended consequences for gene expression profiles, and could even have growth-promoting effects on a tumor (Esteller, 2008). Therefore, more research has to be done on the SFRP family, and its role in cancer development through the Wnt pathway.

Marsit et al. have found a high prevalence of *SFRP* hypermethylation in the tumors of urinary bladder cancer patients, as well as a positive association between methylation and higher stage, invasive bladder cancer. A highly significant trend in the magnitude of odds of having invasive bladder cancer with increasing number of SFRP genes silenced by methylation was also demonstrated, as was a significant synergy between SFRP methylation and p53 alteration on invasive disease risk (Marsit et al., 2005).

## **2.5. Smoking and epigenetic alterations**

Cigarette smoking is an established risk factor for bladder cancer. Overall, smokers have two to three times the risk of non-smokers (Silverman et al., 2006). However, the specific carcinogens and the mechanisms responsible for the increased bladder cancer risk are unknown. Many studies have explored the effect of variables such as cessation of smoking, filtration of cigarettes, inhalation, different types of tobacco, environmental tobacco smoke (ETS) on the risk of bladder cancer, with conflicting results. One

proposed mechanism for how smoking translates to tumorigenesis is that it results in epigenetic silencing of important tumor suppressor genes. A positive relationship between smoking and DNA promoter hypermethylation been shown in several studies in lung cancer (Kim et al., 2001, Toyooka et al., 2003), as well as in bladder cancer (Marsit et al., 2007).

Similarly, Marsit et al. have shown a significant correlation between current smokers and *SFRP* gene methylation, as compared to former or never smokers. The prevalence of *SFRP* gene methylation was almost two-fold greater in current smokers (Marsit et al., 2005), which supports the risk figures given above. This suggests that continuous exposure of the cancerous field to tobacco smoke carcinogens is able to select for epigenetic silencing of *SFRPs* (Marsit et al., 2005).

## **2.6. *TP53* gene**

*TP53*, classified as a tumor suppressor gene, is a master transcription factor that under normal conditions is functionally inactive due to rapid degradation (Efeyan and Serrano, 2007). However, upon infliction of cellular stress, p53 protein accumulates in response to DNA-damage and target genes are transcribed (Efeyan and Serrano, 2007; Esrig et al., 1994). Cell cycle inhibitors and pro-apoptotic proteins are activated, resulting in apoptosis or irreversible proliferative arrest. The activation of *TP53* therefore aids in the prevention of tumorigenesis, by allowing time for DNA repair.

### **2.6.1. *TP53* Mutations and Bladder Cancer**

Inactivating mutations of *TP53* result in reduced DNA-repair, producing an environment that is favorable to tumor growth and progression. In fact, about 50% of all human cancers have mutations in *TP53*, and it is among the most well investigated tumor

suppressor genes (Efeyan and Serrano, 2007). A high percentage of invasive transitional-cell carcinomas of the bladder have mutations in *TP53* and these genes are, with little doubt, critical in the multi-step progression of bladder cancer (Esrig et al., 1994). Because most of the observed mutations are missense, they lead to the accumulation of p53 protein in the nucleus (Malats et al., 2005).

### **2.6.2. *TP53* Mutations in Bladder Cancer Prognosis**

Wild-type p53 protein has a short half-life, as compared to its mutant forms, which have a longer half-life, and accumulate in the nucleus (Masters et al., 2003). Thus, researchers have proposed the use of immunohistochemical (IHC) staining to observe over-expression of p53 protein in the nucleus, which acts as a surrogate marker for *TP53* mutation (Malats et al., 2005). Using this technique, studies have shown that the mutation of *TP53* gene has been associated with the development of more aggressive, recurring, invasive cancer, and could be used as a clinical tool to identify susceptible patients who could benefit from more aggressive treatments, such as immediate radiotherapy or cystectomy (Masters et al., 2003; Esrig et al. 1994).

### **2.6.3. Limitations**

The sensitivity and specificity of using p53 immunohistochemistry alone to make clinical judgments has been questioned. A meta-analysis of 117 such studies has also yielded conflicting results; it concludes that changes in p53 are only weakly predictive of recurrence, progression and mortality in bladder cancer (Malats et al., 2005), suggesting that tests for additional biomarkers, which could be used alternatively or in conjunction with measures of altered *TP53* could improve the ability to predict invasive bladder cancer.

## **2.7. Possible Joint Effects of *TP53* Alterations with *SFRP* Methylation**

Normal function of both the Wnt and *TP53* pathways play an important role in maintaining a non-invasive phenotype. Disruption to either pathway has been shown to relate to invasive bladder cancer, but few studies have looked at joint effects of independent disruptions. Using a population-based case series, Marsit et al. showed that the epigenetic inactivation of *SFRP* genes and *TP53* alteration act jointly as markers of invasive bladder cancer, and affect predictions of survival as well as the appropriate modes of treatment (Marsit et al., 2005).

## **2.8. Thesis Objectives**

I aim to confirm parts of the Marsit et al. study with a separate population-based case series (n=204) to determine if results are consistent with the hypotheses that (a) *SFRP* gene hypermethylation is associated with invasive bladder cancer, (b) smoking is associated with *SFRP* gene hypermethylation and (c) *SFRP* gene hypermethylation and *TP53* mutations act jointly as markers of invasive bladder cancer.

The study will investigate promoter methylation in two of the four previously studied *SFRP* genes, *SFRP1* and *SFRP2*, as the possible main actors in bladder cancer. Additionally, I will determine whether these alterations are associated with demographic and exposure histories of these patients to determine any patterns and trends in methylation-induced silencing of these genes in the occurrence of the invasive disease.

These results will influence the possibility of using *SFRP* genes' epigenetic status as a clinical tool for early detection and prognosis, and also as a possible avenue for increased therapeutic drug research and development.

### **3. Patients and Methods**

#### **3.1. Study Population**

The population case series included 405 residents of New Hampshire ages 30 to 79 years with a histologically confirmed carcinoma of the urinary bladder newly diagnosed between January 1, 2002 and July 31, 2004 in the New England Bladder Cancer Study (Karagas). All study participants were consented under the appropriate institutional review board protocols. They underwent a personal interview to obtain information on demographic traits, use of tobacco products, occupational and residential histories, fluid intake, use of hair coloring products, family history of cancer, medication use and dietary factors. Pathology reports and paraffin-embedded tumor specimens were requested from the treating physician/pathology laboratories, and tumor samples 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. Due to the limited amount of extracted DNA available, only 204 of these 405 patients were included in my analysis.

#### **3.2. DNA Extraction and Sodium Bisulfite Modification**

Three 20- $\mu$ m sections were cut from each 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. Briefly, 1 µg of genomic DNA was denatured by incubation with 0.2 M NaOH. Aliquots of 10 mM hydroquinone and 3 M sodium bisulfite were added and the solution was incubated at 50°C for 16 hours. Treated DNA was purified in a Zymo-Spin I column, desulfonated with 0.3 M NaOH, repurified in the column, and resuspended in 15 µL elution buffer.

### **3.3. Methylation-specific PCR**

1 µL of sodium bisulfite-modified DNA was amplified with *SFRP1* and *SFRP2* gene-specific primers in methylation-specific PCR (Polymerase Chain Reaction) (MS-PCR) as previously described (Herman et al., 1996; Suzuki H et al., 2004). All methylation-specific PCRs are optimized to detect >5% methylated substrate in each sample. The controls used in each run were: ~80% methylated and modified control DNA, ~50% methylated and modified control DNA, unmethylated blood DNA, 5% methylated blood DNA and nuclease-free water.

### **3.4. p53 Immunohistochemistry**

This data had been obtained when the tumor specimens were collected; my thesis used this pre-collected data. Because antibodies differ in their sensitivity and specificity, and different techniques often yield highly varied results (Masters et al. 2003), it was important that the IHC techniques used for the patients in my thesis were the same as

those used in the Marsit et al. study. The IHC detection of p53 has been previously described (Kelsey et al., 2004).

IHC staining of paraffin-embedded slides was performed using the avidin-biotin complex technique. For each case, a single representative slide was selected for staining and histologic evaluation. Briefly, slides were de-paraffinized and hydrated in water. Slides underwent antigen revival in citra solution using the Biocare Decloaking Chamber (Biocare Medica, Walnut Creek, CA, USA). Staining of p53 was performed using a monoclonal antibody (BioGenex, San Ramon, CA, USA) at a 1:100 dilution on the Optimax I-6000 Immunostainer (BioGenex). An appropriate positive control was used in each staining run, and each slide was stained with a negative control. The intensity of nuclear staining was graded on a semiquantitative scale (0-3), rating intensity in the dominant pattern within the tumor. In addition, the percentage of positively staining tumor cells was scored (negative, 1-9%, 10-49%, or >50%).

### **3.5. Statistical Analysis**

Similar to the methods utilized by Marsit et al., multivariate unconditional logistic regression with methylation of *SFRP1* gene, *SFRP2* gene and any *SFRP* gene (1 or more versus 0) as the dependent variable was used to examine associations among patient demographics, exposure history and tumor characteristics with *SFRP* methylation while controlling for possible confounding. We examined the effects of multiple predictors on tumor invasion using unconditional logistic regression analysis, with adjustment for potential confounders. 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 p53 status with p53 negative (<3 staining intensity) and no *SFRP*

genes methylated as the reference category, again using unconditional logistic regression with adjustment for multiple covariates.

## 4. Results

### 4.1. *SFRP* Methylation and Patient Demographics

In this study, we observed a prevalence of promoter methylation in the *SFRP1* gene (40%), as well as the *SFRP2* gene (40%) in the 204 cases examined. Promoter methylation in any one of the *SFRP* genes was observed in 58% of the cases (Table 1). There was no association observed between *SFRP* gene promoter methylation and patient's age at diagnosis or gender.

Concomitant methylation of both gene promoters was clearly associated with higher stage disease. In addition, patients with invasive bladder cancer showed significantly higher prevalence of *SFRP1* methylation (OR 1.9, 95% CI 1.0-3.8) and *SFRP2* methylation (OR 3.4, 95% CI 1.7-6.9). However, the association between having any *SFRP* gene methylated and invasiveness of disease is smaller than that for the single *SFRP2* gene promoter (OR 3.1, 95% CI 1.5-6.4).

We observed that p53 IHC staining is not associated with *SFRP* methylation, suggesting that the epigenetic silencing of *SFRP* genes occurs independently of *TP53* alterations.

Surprisingly, analyses revealed that there was no significant association between *SFRP* methylation and the smoking status of these patients, a previously observed risk factor for bladder disease (Marsit et al., 2005).

**Table 1.** Bladder tumor SFRP gene methylation by patient demographics

Characteristic	SFRP1 Methylation		SFRP2 Methylation		Any SFRP Methylation		Totals		
	Negative n (%)	Positive n (%)	Adjusted OR* (95% CI)	Negative n (%)	Positive n (%)	Adjusted OR* (95% CI)	Negative n (%)	Positive n (%)	Adjusted OR* (95% CI)
Overall Prevalence	122 (60)	82 (40)		123 (60)	81 (40)		85 (42)	119 (58)	
Gender									
Female	35 (63)	21 (37)	1.0 (reference)	39 (70)	17 (30)	1.0 (reference)	26 (46)	30 (54)	1.0 (reference)
Male	87 (59)	61 (41)	1.1 (0.6-2.2)	84 (57)	64 (43)	1.8 (0.9-3.5)	59 (40)	89 (60)	1.3 (0.7-2.4)
Age									
<65	43 (56)	34 (44)	1.0 (reference)	47 (61)	30 (39)	1.0 (reference)	34 (44)	43 (56)	1.0 (reference)
>=65	79 (62)	48 (38)	0.7 (0.4-1.3)	76 (60)	51 (40)	1.1 (0.6-2.1)	51 (40)	76 (60)	1.2 (0.7-2.2)
Tumor Stage									
Non-invasive	91 (65)	50 (35)	1.0 (reference)	98 (70)	43 (30)	1.0 (reference)	69 (49)	72 (51)	1.0 (reference)
Invasive	31 (49)	32 (51)	1.9 (1.0-3.8)	25 (40)	38 (60)	3.4 (1.7-6.9)	16 (40)	47 (60)	3.1 (1.5-6.4)
TP53 Staining									
Score <3	90 (61)	58 (39)	1.0 (reference)	96 (65)	52 (35)	1.0 (reference)	65 (44)	83 (56)	1.0 (reference)
Score 3+	32 (55)	24 (45)	0.9 (0.5-1.9)	27 (48)	29 (52)	1.1 (0.5-2.3)	20 (36)	36 (64)	0.8 (0.4-1.7)
Smoking Status <sup>^</sup>									
Never	22 (71)	9 (29)	1.0 (reference)	21 (68)	10 (32)	1.0 (reference)	16 (51)	15 (49)	1.0 (reference)
Former	57 (53)	49 (47)	2.2 (0.9-5.3)	62 (58)	44 (42)	1.3 (0.5-3.2)	40 (38)	66 (62)	1.6 (0.7-3.8)
Current	41 (64)	23 (36)	1.3 (0.5-3.4)	39 (61)	25 (39)	1.3 (0.5-3.4)	28 (44)	36 (56)	1.4 (0.6-3.4)

\* OR adjusted for all other variables in the table and limited to subjects with complete data for all variables (n= 201).

<sup>^</sup> Three tumors were missing smoking status classification.

#### 4.2. Number of *SFRP* Genes Methylated, Altered *TP53* and Invasiveness

We performed multivariate logistic regression analysis using invasive bladder cancer as the dependent variable in the model. As independent variables, the models included age (<65 or ≥65), gender, number of *SFRP* genes (1 or 2) and p53 staining (3+ or <3).

After controlling for potential confounders, we found a significant trend with increasing number of *SFRP* genes methylated (Table 2). With one *SFRP* gene methylated, the patient is 2.2 times as likely to have invasive disease (95% CI 1.0-4.9), whereas having two *SFRP* genes methylated increases the likelihood of invasive disease to 4.6 (95% CI 1.9-11.3).

The model also shows a strong, significant and independent relationship between p53 staining and invasive disease. After adjusting for the other covariates in the model, patients with intense p53 staining were significantly more likely to have invasive disease (OR 7.1 95% CI 3.5-14.6).

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

Covariate	n (no. of invasive disease)	Invasive bladder cancer * OR (95% CI)^	P
No. of <i>SFRP</i> genes methylated			
0	85 (16)	1.0 (reference)	
1	75 (24)	2.2 (1.0-4.9)	0.0519
2	44 (23)	4.6 (1.9-11.3)	0.0008
TP53 alteration (staining intensity)			
<3	148 (28)	1.0 (reference)	
3+	56 (35)	7.1 (3.5-14.6)	<0.0001

\*Model predicts invasive compared with non-invasive

^Model controls for all covariates, limited to subjects with complete data (n=201)

### 4.3. TP53 and SFRP Joint Effects

Finally, we examined the joint effects of the two independent actors, p53 staining and *SFRP* methylation. Table 3 shows the results of a stratified multivariate logistic analyses using invasive bladder cancer as the dependent variable and p53 staining as the predictor. In tumors with no *SFRP* methylation, p53 positive tumors were significantly more likely to have invasive disease (OR 6.7; 95% CI 2.0-22.6). In addition, tumors with *SFRP* methylation and p53 positive IHC were significantly more likely to have invasive disease (OR 7.7; 95% CI 3.2-18.8;  $p < 0.0001$ ).

**Table 3.** Stratified analysis of the association between TP53 alteration and invasive bladder cancer by methylation of any SFRP gene

	n (no. invasive disease)	Invasive bladder cancer OR (95% CI)*	P
No SFRP methylation			
TP53 WT	69 (7)	1.0 (reference)	0.002
TP53 altered	16 (9)	6.7 (2.0-22.6)	
Positive SFRP methylation			
TP53 WT	83 (21)	1.0 (reference)	0.00001
TP53 altered	36 (26)	7.7 (3.2-18.8)	

\*Models controlled for age and sex, limited to subjects with complete data for all variables (n=201)  
WT = wild type

## 5. Discussion

### 5.1. Observations

The Wnt signaling pathway and its importance in embryonic development has been well studied, and it is also gaining recognition as a key player in tumorigenesis. Mutations and epigenetic alterations of components of the pathway result in the aberrant signaling of the pathway. Several cancers, such as lung cancer (Mazieres et al. 2005) and oral cancer (Lo Muzio, 2001), exhibit aberrant signaling, but mutations in components like *APC* and *CTNNB1* are not common in them (Kok et al., 2002; Shigemitsu et al., 2001). An alternate mechanism of control in the Wnt pathway is the epigenetic alteration of Wnt antagonists, the *SFRPs*. This form of control has been found previously in colon cancer (Suzuki et al., 2004), gastric cancer (Nojima et al., 2007), hepatocellular cancer (Shih et al., 2006) and head and neck cancers (Marsit et al., 2006).

The results of our study were mostly consistent with the results from the Marsit et al. paper that we replicated (Marsit et al., 2005). Our results are thus able to support the validity of their findings and hypotheses. We show that *SFRP* hypermethylation is associated with invasive bladder cancer, and that *SFRP* hypermethylation and *TP53* alteration independently contribute to, and jointly act as markers of invasive bladder cancer.

In this study, we found that methylation of at least one *SFRP* gene occurs in 58% of these bladder cancer patients, and that methylation is strongly associated with invasive disease. This suggests that *SFRP* gene silencing plays an important role in Wnt signaling in bladder cancers, which do not commonly have mutations in other Wnt signaling pathway genes (Stoehr et al., 2004).

Furthermore, the methylation statuses of the two *SFRP* genes are strongly associated with each other ( $p=0.0008$ ) (data not shown). This supports the “multiple hit” hypothesis; several alterations accumulate before tumorigenesis can occur. Recent research suggests that initial epigenetic changes in the early stages of tumor development may ‘addict’ cancer cells to develop more epigenetic and genetic alterations in the same pathway. This gives the cancer cell a selective advantage for tumor progression (Baylin and Ohm, 2006), and has been shown to occur in the Wnt pathway in colon cancer cells (Suzuki et al., 2004). Our results show that pathway addiction may also occur in bladder cancer: methylation of increasing numbers of *SFRP* genes in bladder cancer showed a strong and significant relationship with the invasive disease. As more antagonists are silenced, the pathway increases in aberrant signaling and cancer cell proliferation is more likely to produce the invasive phenotype.

We see that alterations of *TP53* and the epigenetic silencing of the *SFRP* genes both have an independent, significant relationship with invasive bladder cancer. After controlling for *SFRP* methylation, patients with intense p53 staining have an approximately 7-fold likelihood of invasive disease. As we are using p53 staining as a representative of *TP53* alterations, we can conclude that mutations in this gene are significantly associated with invasive disease, and may possibly be used as a clinical tool in determining patient prognosis.

However, p53 staining on its own is not a powerful enough test to be used alone in determining patient prognosis. Marsit et al. suggested that *SFRP* gene methylation be used in conjunction to predict invasiveness, because their results showed that tumors with both *SFRP* methylation and *TP53* alteration had >30 times the odds of being invasive

than tumors without intense p53 staining or *SFRP* gene methylation (Marsit et al., 2005). While we too found that *SFRP* gene hypermethylation enhanced the association between p53 staining and invasive bladder cancer, our results are more conservative. Tumors with both *SFRP* gene methylation and p53 staining had 7.7 times the odds of being invasive. Because the same relationship is maintained in a separate sample, the lower odds estimate reflects a range of odds in the bladder cancer population mean and therefore provides vital information to researchers and clinicians. Nonetheless, classifying bladder tumors according to these two factors could improve clinical estimates of the invasive phenotype, better identify patients in need of more aggressive treatments such as surgery or radiotherapy, and potentially lead to improved patient outcomes.

Due to a shortage of DNA, and the number of times we had to repeat the MS-PCR to get clear results, we concentrated on two of the *SFRP* genes, *SFRP1* and *SFRP2*. The results that we obtained are similar enough to Marsit et al.'s results to suggest that *SFRP1* and *SFRP2* genes may be the main actors in altering Wnt signaling in bladder cancer. However, this must be verified through future studies.

SFRPs are soluble proteins, and we see that they do play a large role in early determination of invasive bladder cancer. This makes them clear candidates for therapeutic drug research and development. A soluble wnt receptor with the same CRD as the SFRPs has been tested *in vivo* in preclinical trials and has shown success in inhibiting Wnt signaling and the subsequent growth of teratocarcinomas (DeAlmeida et al., 2007). Also, research has shown that re-expression of silenced *SFRP* genes in colorectal cancer cells reduces aberrant Wnt signaling and restores apoptosis, even in the presence of downstream mutations in the Wnt pathway (Suzuki et al., 2004). These

studies, coupled with our findings suggest that drugs or procedures focusing on epigenetic alterations of *SFRP* genes in the Wnt pathway could be developed that would halt, and even reverse, early tumorigenic events in bladder cancer.

If the *SFRP1* and *SFRP2* genes are the key components of the Wnt pathway, a focus of consequent research on these genes could save researchers time and money. More importantly, early-stage therapeutics targeting these specific components of the Wnt pathway may yield significant benefits to bladder cancer patients, especially those with invasive disease.

## **5.2. Smoking**

Cigarette smoking is an established bladder cancer risk factor, with an overall 2- to 3-fold risk in smokers compared to non-smokers (Silverman et al., 2006). This led us to investigate the possibility that carcinogens in cigarette smoke would contribute to higher stage disease through the mechanism of epigenetic silencing.

Surprisingly, we did not find any significant associations between smoking status and *SFRP* methylation status of the bladder cancer patients in this sample. This is contrary to previously completed studies on bladder cancer patients, which show a significantly increased propensity for promoter hypermethylation in current smokers, including those for the *SFRP* genes (Marsit et al., 2005; Marsit et al., 2007). This implies that the original hypothesis, exposure to carcinogens in cigarette smoke results in epigenetic silencing of the *SFRP* genes, may not be valid. Instead, smoking might cause an alternate mechanism of tumorigenesis that is separate from aberrant Wnt signaling.

We also did not find any significant associations between the smoking status and invasive bladder cancer in our case-series study (data not shown). This may indicate that

carcinogens from smoking contribute to early tumorigenesis, but not to processes affecting future cancer cell growth and proliferation.

There could be several reasons for the difference in our results from the Marsit et al. paper. Smoking patterns and views of smoking have changed since the mid-1990s. The patients classified as smokers may be inhaling less vigorously per cigarette, and therefore have lower exposures to carcinogens than expected. This results in smokers exhibiting lower odds of having *SFRP* promoter methylation. Also, there is a small possibility that smokers are not reporting their cigarette usage due to its undesirability, resulting in a weaker relationship.

These results allude to a possibility of other carcinogenic exposures apart from smoking, which are driving the epigenetic silencing of *SFRP* genes and aberrant Wnt signaling.

### **5.3. Survival Data**

Survival data on these patients is now being collected and will enable us to determine if *SFRP* methylation and *TP53* alteration data could predict survival. If so, this would support the usage of these two tests as tools to determine prognosis and treatment.

## **6. Future Directions**

It is still unclear how epigenetic silencing of key genes is triggered, or how it results in invasive tumors. Much research is being done on the role of exposure to carcinogens in the cancer development, but we see many differing results like the smoking results in this study compared with the Marsit et al. study. More research on smoking as a possible exposure must be done in larger case studies to determine if there is indeed a heightened risk in the exposed people of developing invasive and fatal bladder cancer. Furthermore, other exposures, such as toxicants in drinking water, dietary factors, usage of certain drugs etc. should be explored, since there are often several different exposures people are exposed to which may contribute to carcinogenesis.

There is geographic variation in the United States in incidence and mortality of bladder cancer. Reports show elevated mortality rates in the northeastern states (Silverman et al., 2006). The highest incidences of bladder cancer also occur in the northeast, with the highest of 51.6 cases per 100,000 per year for men and 14.5 cases per 100,000 per year for women occurring in Rhode Island, compared with the national average of 38.4 for men and 9.8 for women (Jemal et al., 2008). Future research should focus on possible exposures specific to the northeastern states and particularly Rhode Island in order to determine the reasons behind these elevated rates.

Finally, since epigenetic alterations are important in the development of cancer, future research should be targeted at the epigenome, especially in cancers where mutations in key pathways are not prevalent and epigenetic silencing may provide an alternative mechanism for cancer progression.

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