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GENETIC VARIANTS WITHIN THE WNT/β-CATENIN SIGNALING PATHWAY AS INDICATORS OF BLADDER CANCER RISK

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Abstract

Purpose—The genetic factors that influence bladder cancer risk remain largely unknown. Previous research has suggested that there is a strong genetic component underlying the risk of developing bladder cancer. The Wnt/β-catenin signaling pathway is a key modulator of cellular proliferation through its regulation of stem cell homeostasis. Furthermore, variants in the Wnt/β-catenin signaling pathway have been implicated in the development of other cancers leading us to believe this pathway may play a vital role in bladder cancer development.

Materials and Methods—A total of 230 SNPS in 40 genes in the Wnt/β-catenin signaling pathway were genotyped in 803 bladder cancer cases and 803 healthy controls.

Results—Twenty SNPs were nominally significant for risk. Individuals with two variants of *LRP6*: rs10743980 were associated with a decreased risk of bladder cancer (OR=0.76, 95% CI: 0.58–0.99, P=0.039) in the recessive model in the initial analysis and was also validated using the bladder GWAS chip (OR=0.51, 95% CI: 0.27–1.00, P=0.049) (P value for combined analysis: P=0.007).

Conclusion—Together, these findings implicate variants in the Wnt/β-catenin stem-cell pathway as playing a role in bladder cancer etiology.

Keywords

Urinary Bladder Neoplasms; Wnt Signaling Pathway; Polymorphism; Single Nucleotide

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INTRODUCTION

It is estimated that 54,390 men and 18,300 women will be diagnosed with bladder cancer and 15,580 men and women will die from this disease in the United States in 2014[1]. Multiple environmental and genetic risk factors have been identified for bladder cancer, with smoking and exposure to aromatic amines being the main environmental risk factors[2]. It has also been established that genetics play an important role in risk of developing bladder cancer[3]. Recent genome-wide association studies have identified a total of at least 14 unique genetic loci that have a significant effect on bladder cancer risk in European-descent populations[4–15]. However, these risk factors only represent a small portion of the genetic basis of this disease suggesting that the full spectrum of genetic factors influencing risk of bladder cancer remains undetermined.

Cancer cells and stem cells both possess the ability to self-renew. The cancer stem cell hypothesis states that cancer cells hijack the same processes as stem cells for self-renewal and proliferation. The Wnt/β-catenin, Notch, and Sonic Hedgehog signaling pathways all have been associated with both stem cell regulation and oncogenesis[16,17]. Specifically, the Wnt/β-catenin signaling pathway is a key modulator of cellular proliferation through its regulation of stem cell homeostasis[18]. Signaling through the pathway is complex with multiple layers of regulation. In the absence of binding of the wingless-type MMTV integration site family (Wnt) ligands to the cell surface receptor, β-catenin is destroyed in a destruction complex made up of adenomatous polyposis coli (APC) and AXIN. This results in β-catenin phosphorylation by casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). Phosphorylation by CK1 and GSK3 causes the ubiquitylation and proteasomal degradation of β-catenin. In the normal state, Wnt antagonists such as the secreted Frizzled-related protein (sFRP) and Dickkopf (DKK) family members interact with Wnt-ligands and prevent pathway activation[18]. If the concentration of Wnt becomes greater than the concentration of sFRP and DKK, Wnt can interact with the Frizzled (FZ) family of receptors and LDL-receptor-related proteins 5 and 6 (LRP5/6). This activates disheveled (DSH) phosphoprotein, resulting in degradation of AXIN and the increase in non-degraded β-catenin. β-catenin concentration in the nucleus increases and activates the transcription of target genes through interactions with T-cell Factor (TCF) and lymphoid enhancer-binding protein (LEF)[18].

Recently, it has been shown that the Wnt/β-catenin pathway is involved in the regulation of stem cells in the bladder epithelium, suggesting that alterations in this vital pathway may result in unrestrained proliferation of bladder epithelium resulting in tumor formation[19]. Therefore, we examined the association between genetic variation within the Wnt/β-catenin pathway genes and risk of bladder cancer in NMIBC and MIBC patients using the data from an on-going bladder cancer study at MD Anderson Cancer Center.

MATERIALS AND METHODS

Study Subjects

Bladder cancer cases were recruited from 1999 to 2007 at The University of Texas MD Anderson Cancer Center and Baylor College of Medicine and were all newly diagnosed,

histologically confirmed, and previously untreated. Controls were recruited from Kelsey Seybold Clinic, a large multispecialty physician group in Houston, Texas[20]. There were no restrictions on recruitment for age, gender, or stage of bladder cancer. Cases and controls were matched by sex, age (± 5 years), and ethnicity. Over 90% of our patients recruited to the study were Caucasian; therefore analysis was restricted to this population to limit the confounding effect of population structure. All participants provided written informed consent prior to data and biospecimen collection and the Institutional Review Boards of MD Anderson Cancer Center and Baylor College of Medicine approved this study.

Epidemiologic and Clinical Data Collection

Demographic and risk factor variables were collected in an interview with each study participant. These variables include age, gender, family history, medical history, occupational exposures, and smoking history. Participants were considered “never smokers” if they had smoked less than 100 cigarettes in their lifetime. “Former smokers” were those that had quit smoking greater than one year ago from their diagnosis (cases) or interview (controls). Recent quitters were those who quit smoking greater than one month ago from date of diagnosis (cases) or interview (controls).

DNA Isolation and Genotyping

Each participant provided a 40 ml peripheral blood sample for genetic and molecular analyses. Laboratory personnel were blinded case/control status. Genomic DNA was isolated from peripheral blood samples using the QIAamp DNA Blood Maxi Kit (QIAGEN, Valencia, CA) according to standard protocol. A panel of single nucleotide polymorphisms (SNPs) related to cancer was developed as previously described[21]. This panel was used to create a custom iSelect genotyping array (Illumina, San Diego, CA) and included genes within the Wnt/β-catenin signaling pathway. For each selected gene, tagging SNPs ($r^2 \geq 0.8$ and within 10 kb of the gene) and/or potentially functional SNPs (coding SNPs and SNPs in untranslated regions, promoter, and splicing sites) were identified based on the CEU HapMap population. Genotyping was performed according to the Infinium II assay protocol with genotyping calls and quality control assessment performed using Illumina’s BeadStudio software. The significant SNPs that were identified from the risk analysis were validated using a genome-wide association study (GWAS) of bladder cancer that was previously conducted in our research group. The genotyping for this chip was completed at MD Anderson Cancer Center and was done on the Illumina HumanHap610 chip. Detailed methods for this chip are previously described[5]. After removing duplicated samples, there were 352 samples used.

Statistical Analysis

The Stata 10 statistical software package (Stata Corporation, College Station, TX) was used to perform most of the statistical analysis. For the demographic and clinical variables, Pearson’s $\chi 2$ or Fisher’s exact test was used to analyze the difference in distribution of categorical variables. The Wilcoxon rank sum test or Student’s t test was used to analyze continuous variables. A goodness of fit $\chi 2$ analysis was done to test for Hardy-Weinberg equilibrium in the controls. Bladder cancer risk was estimated using odd ratios (ORs) and 95% confidence intervals (95% CI) which were calculated using unconditional multivariate

logistic regression for the dominant, recessive, and additive models of inheritance, adjusting for age, gender, and smoking status. The false discovery rate (Q Value) was calculated to account for the large number of SNPs and tests included in the analysis using the Q value package implemented in R. Oncomine (version 4.4.3, http://www.oncomine.org), a database of pooled microarray expression data, was queried to search differential expression data for the top significant genes identified for risk, recurrence, and progression. This was completed by examining significant genes from our analysis and comparing differential expression data in normal bladder tissue to the gene expression data in bladder cancer tissue. Variants were validated if the variant was significant in both the discovery phase (SNPs that were on the iSelect genotyping array) and validation phase (SNPs that were on the GWAS chip) of the analysis, that both were statistically significant (P<0.05) in the same model and both OR's were in the same direction. We subsequently evaluated the association between variants in the Wnt/β-catenin pathway by histological subtype.

RESULTS

Subject Characteristics

The demographic variables for the 803 cases and 803 controls used in the discovery phase are shown in Table 1. The population was well matched by sex and age. As expected, controls were more likely to be never smokers (44.2%) than cases (26.4%). Among study participants that ever smoked, cases had a higher mean pack-year (43.0±30.7) than controls (29.9±27.9; P<0.01). The demographic variables for the validation phase are also shown in Table 1. There were 177 cases and 175 controls. There was a significant difference between smoking status between cases and controls.

Genetic Variants Associated with Bladder Cancer Risk

Of the 230 SNPs analyzed, 20 were significantly associated with bladder cancer risk with a P value < 0.05 with four SNPs associated with bladder cancer with a P value < 0.02 (Table 2). The most significant finding was for *AXIN2*: rs12943295 (OR: 1.37, 95% CI: 1.08–1.75) in the dominant model. The other three loci associated with increased risk included: *WNT8A*:rs4835761 (OR: 1.17, 95% CI: 1.01–1.35), *APC*: rs42427 (OR: 1.38, 95% CI: 1.02–1.87), and *WNT3*:rs7218567 (OR: 1.23, 95% CI: 1.00–1.50). However, none remained significant at Q<0.10 when adjusting for multiple comparisons. When validating the bladder cancer risk SNPs with the SNPs from our bladder cancer GWAS, *LRP6*: rs10743980 was significantly associated with a lower risk of bladder cancer (OR: 0.76, 95% CI: 0.58–0.99, P=0.039) in the initial analysis and was also statistically significantly associated with a lower risk of bladder cancer in the GWAS validation (OR: 0.51, 95% CI: 0.27–1.00, P=0.049), in the recessive model (Combined Analysis: OR:0.72, 95% CI: 0.57–0.92, P=0.007) (Table 3). *LRP6* was the most common gene that was associated with significant variants in our analysis. All statistically significant variants located in *LRP6* were associated with a decreased risk of bladder cancer, with ORs ranging from 0.75 to 0.79 (Table 3).

To gain insight into the etiology of NMIBC and MIBC, we performed genetic analyses stratified by MIBC and NMIBC (Supplementary Table 1). There were many different SNPs that were associated with MIBC or NMIBC, which is consistent with the different etiologies

and pathogenesis of MIBC and NMIBC. We also performed an analysis to compare SNPs between MIBC and NMIBC and there was only one overlapping SNP (rs10743980 on LRP6) between MIBC- and NMIBC-predisposing SNPs. We found 16 SNPs that were significantly different between NMIBC and MIBC ($P<0.05$) (Supplementary Table 2), indicating that they may be associated with bladder cancer progression.

DISCUSSION

The Wnt/β-catenin stem cell pathway has been shown to play a role in bladder cancer epithelium stem cell maintenance[19]. In this study, we investigated the effect of common, germline genetic variation within genes functioning in this pathway on bladder cancer risk. Several significant associations were identified for risk. The results of this study have the potential to guide the selection of at risk individuals for bladder cancer.

Multiple molecular studies have found an association between Wnt signaling in the Wnt/β-catenin signaling pathway and bladder cancer. But there are not many studies examining genetic variants in the Wnt/beta-catenin signaling pathway and bladder cancer risk. The variant most associated with risk was *AXIN2*: rs12943295, showing an increased risk of bladder cancer. AXIN is a key component of the destruction complex that degrades β-catenin, and it is possible that this variant or its linked causal SNP(s) disrupt the ability of AXIN to degrade β-catenin, resulting in a build-up of β-catenin[18]. There is limited research on the impact of AXIN and bladder cancer. But research has linked AXIN mutations to other cancers such as hepatocellular carcinoma[22], as well as AXIN epigenetic changes linked to colorectal cancer[23]. The variant rs217259 located in WNT8A and rs135757 located in CSNK1E were both associated with a decreased risk of bladder cancer. It is not clear how WNT8A is associated with a decreased risk of bladder cancer, but research has shown that different isoforms of WNT are involved in tumor pathogenesis[24]. In addition, mutations in CSNK1E have been studied as being synthetic lethal when paired with mutations in TP53 in colorectal cancer patients, which is considered a prognostic marker for stage[25]. We can speculate that the SNP rs135757 or its linked causal SNP(s) negatively impact CSNK1E function, therefore conferring a protective effect on bladder cancer because mutation on this gene is acting in synthetic lethality with TP53 mutations.

A validated variant located in LRP6 (rs10743980) was shown to be statistically associated with a decreased risk of bladder cancer. Interestingly, this SNP was associated with the risk of both NMIBC and MIBC. LRP6 plays an active role in activation of the Wnt signaling pathway. Specifically, when the concentration of Wnt increases to a certain level, Wnt binds with FZ receptors and LRP5 and 6, which ultimately results in AXIN binding to the tail of LRP5/6 and a reduction in the degradation of β-catenin[18]. LRP6 has been shown to be an important player in tumorigenesis and is over-expressed in many different human cancer tissues. Furthermore, LRP6 over-expression has also been shown to increase cytosolic levels of β-catenin as well as increase activity in transcription factors TCF/LEF[26]. This evidence points to a strong correlation between LRP6 and human malignancies. We can potentially hypothesize that rs10743980 or its linked causal SNP located in LRP6 results in a loss-of-function of LRP6 and therefore the Wnt/β-catenin signaling pathway is not activated, resulting in a lower risk of bladder cancer. Because the selected SNPs in this study were

mostly tagging SNPs, their functional impacts are not clear. The linked to-be identified causal SNP(s) could either positively or negatively affect host gene functions. We speculate that SNPs increasing β-catenin function would be associated with increased risk of bladder cancer, whereas SNPs that decrease β-catenin function would be protective. Nevertheless, the biological mechanisms of the identified significant SNPs and their host genes in regulating β-catenin function and affecting bladder carcinogenesis remain to be studied.

Recent research has uncovered the importance of stem cells in cancer development. A previous study from our laboratory examining genetic variants in the Sonic Hedgehog signaling pathway identified loci that were associated with bladder cancer risk and recurrence[27]. These results combined with the effects seen in this current study lead us to conclude that stem cell signaling pathways are important mediators of bladder cancer tumorigenesis and course of disease. Furthermore, genetic variation in these core pathways could be used for risk assessment A major strength of this study is that it utilized data from one of the largest bladder cancer studies in the United States with detailed patient data. We also applied a comprehensive pathway-based analysis that includes the core components of an important pathway that has been implicated in bladder cancer, yet no previous studies have investigated the link between common, germline variants in the Wnt/β-catenin pathway and bladder cancer risk. The results are intriguing and worthy of replication in an independent population to confirm our findings and further functional analysis.

In conclusion, we identified multiple novel associations between SNPs in the Wnt/β-catenin pathway and bladder cancer risk. Using a bioinformatics approach (through the Oncomine database), several of these relationships were supported by gene expression profile data. Specifically, it was found that casein kinase 1, epsilon (*CSNK1E)* showed over a 3-fold ($P=8.18\times10^{-9}$) increase in expression in NMIBC bladder cancer when compared to normal bladder tissue[28].

Together, these results provide evidence in support of the hypotheses that genetic variants in the Wnt/β-catenin signaling pathway modulate etiology of bladder cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Definition of Abbreviations

NMIBC	non-muscle invasive bladder cancer
SNPs	single nucleotide polymorphisms
GWAS	genome-wide association study

Table 1

Demographic Characteristics of Study Participants

Variables	Discovery			Validation using GWAS Population		
	Cases n=803 (419 NMIBC 318 MIBC)*	Controls n=803	P Value	Cases n=177 (77 NMIBC 79 MIBC)*	Controls n=175	P Value
Age Mean, years (SD)	64.7 (11.1)	63.8 (10.9)	0.05	66.83(11.57)	67.61(11.57)	0.53
Sex (%)						
Male	639 (79.6)	639 (79.6)		152(85.88)	152(86.86)	
Female	164 (20.4)	164 (20.4)	1.00	25(14.12)	23(13.14)	0.79
Smoking Status (%)						
Never	212 (26.4)	355 (44.2)		44(25.00)	61(34.86)	
Former	404 (50.3)	381 (47.5)		89(50.57)	104(59.43)	
Current	187 (23.3)	67 (8.3)	<0.01	43(24.43)	10(5.71)	<0.01
Smoked (%)						
Never	212 (26.4)	355 (44.2)		44(25.00)	61 (34.86)	
Ever	591 (73.6)	448 (55.8)	<0.01	132 (75.00)	114(65.14)	0.04
Mean Pack-Years	43.0 (30.7)	29.9 (27.9)	<0.01	42.71(28.68)	41.19(31.50)	0.69

*NMIBC – Non-Muscle Invasive Bladder Cancer, MIBC – Muscle Invasive Bladder Cancer

Table 2

Significant Associations between SNPs in the Wnt/β-catenin Pathway and Bladder Cancer Risk

***Gene*: SNP**	**Case (WW/WV/VV)**	**Control (WW/WV/VV)**	**Model**	**OR[**] (95% CI)**	**P Value**
AXIN2: rs12943295	602/190/11	648/144/11	Dominant	1.37 (1.08–1.75)	0.011
WNT8A: rs217259	407/330/66	383/323/97	Recessive	0.65 (0.46–0.91)	0.012
CSNK1E: rs135757	467/299/37	423/323/53	Additive	0.81 (0.68–0.96)	0.013
LRP6: rs10845493	613/173/17	568/215/19	Dominant	0.75 (0.59–0.94)	0.013
LRP6: rs11054704	606/180/17	564/218/21	Dominant	0.76 (0.61–0.96)	0.019
CSNK1E: rs6001093	538/238/27	499/278/26	Dominant	0.78 (0.63–0.96)	0.020
FZD4: rs713065	315/394/94	309/369/125	Recessive	0.71 (0.52–0.95)	0.022
LRP6: rs7136900	384/340/79	331/386/86	Dominant	0.79 (0.64–0.97)	0.023
FZD1: rs12537425	344/358/101	303/386/114	Additive	0.84 (0.72–0.98)	0.023
LRP6: rs12823867	566/211/26	521/253/29	Dominant	0.78 (0.63–0.97)	0.023
LRP6: rs12816278	604/180/19	565/219/17	Dominant	0.78 (0.62–0.98)	0.030
LRP6: rs2302685	557/219/27	515/256/32	Dominant	0.79 (0.64–0.98)	0.031
WNT8A: rs4835761	204/405/194	239/395/169	Additive	1.17 (1.01–1.35)	0.033
LRP5: rs640569	474/287/42	465/281/57	Recessive	0.64 (0.41–0.98)	0.037
APC: rs42427	317/374/112	327/386/90	Recessive	1.38 (1.02–1.87)	0.039
[*]*LRP6*: rs10743980	280/392/131	256/391/156	Recessive	0.76 (0.58–0.99)	0.039
TCF7: rs151822	489/275/39	454/305/44	Dominant	0.81 (0.66–0.99)	0.044
AXIN2: rs7224837	646/151/6	616/169/17	Dominant	0.78 (0.61–1.00)	0.045
WNT8B: rs3793771	513/241/42	480/274/46	Dominant	0.81 (0.65–1.00)	0.045
WNT3: rs7218567	358/376/69	398/319/86	Dominant	1.23 (1.00–1.50)	0.048

Abbreviations: WW-Wildtype Wildetype, WV- Wildtype Variant, VV-Variant Variant, OR – Odds Ratios, 95% CI – 95% Confidence Interval

[*] Validated in GWAS Chip

[**] Adjusted for sex, age, and smoking status

Table 3

Validated Association between *LRP6*: rs10743980 in the Wnt/β-catenin Pathway and Bladder Cancer Risk

***Gene*: SNP**	**Case (WW/WV/VV)**	**Control (WW/WV/VV)**	**Model**	**OR (95% CI)**	**P Value**
Discovery Phase					
LRP6: rs10743980	280/392/131	256/391/156	Recessive	0.76 (0.58–0.99)	0.039
Validation Phase					
LRP6: rs10743980	55/94/28	59/77/39	Recessive	0.51 (0.27–1.00)	0.049
Combined Analysis					
LRP6: rs10743980	335/486/159	315/468/195	Recessive	0.72 (0.57–0.92)	0.007

Abbreviations: OR – Odds Ratios, 95% CI – 95% Confidence Interval

** Adjusted for sex, age, smoking status