Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

Research Article

Cancer Prevention Research

Genetic Variations in the Sonic Hedgehog Pathway Affect Clinical Outcomes in Non–Muscle-Invasive Bladder Cancer

Meng Chen[1], Michelle A.T. Hildebrandt[1], Jessica Clague[1], Ashish M. Kamat[2], Antoni Picornell[4], Joshua Chang[1], Xiaofan Zhang[1], Julie Izzo[3], Hushan Yang[1], Jie Lin[1], Jian Gu[1], Stephen Chanock[5], Manolis Kogevinas[6,7,8,9], Nathaniel Rothman[5], Debra T. Silverman[5], Montserrat Garcia-Closas[5], H. Barton Grossman[2], Colin P. Dinney[2], Núria Malats[4], and Xifeng Wu[1]

Abstract

Sonic hedgehog (Shh) pathway genetic variations may affect bladder cancer risk and clinical outcomes. Therefore, we genotyped 177 single-nucleotide polymorphisms (SNP) in 11 Shh pathway genes in a study including 803 bladder cancer cases and 803 controls. We assessed SNP associations with cancer risk and clinical outcomes in 419 cases of non–muscle-invasive bladder cancer (NMIBC) and 318 cases of muscle-invasive and metastatic bladder cancer (MiMBC). Only three SNPs (*GLI3* rs3823720, rs3735361, and rs10951671) reached nominal significance in association with risk ($P \leq 0.05$), which became nonsignificant after adjusting for multiple comparisons. Nine SNPs reached a nominally significant individual association with recurrence of NMIBC in patients who received transurethral resection (TUR) only ($P \leq 0.05$), of which two (*SHH* rs1233560 and *GLI2* rs11685068) were replicated independently in 356 TUR-only NMIBC patients, with P values of 1.0×10^{-3} (*SHH* rs1233560) and 1.3×10^{-3} (*GLI2* rs11685068). Nine SNPs also reached a nominally significant individual association with clinical outcome of NMIBC patients who received Bacillus Calmette-Guérin (BCG; $P \leq 0.05$), of which two, the independent *GLI3* variants rs6463089 and rs3801192, remained significant after adjusting for multiple comparisons ($P = 2 \times 10^{-4}$ and 9×10^{-4}, respectively). The wild-type genotype of either of these SNPs was associated with a lower recurrence rate and longer recurrence-free survival (versus the variants). Although three SNPs (*GLI2* rs735557, *GLI2* rs4848632, and *SHH* rs208684) showed nominal significance in association with overall survival in MiMBC patients ($P \leq 0.05$), none remained significant after multiple-comparison adjustments. Germ-line genetic variations in the Shh pathway predicted clinical outcomes of TUR and BCG for NMIBC patients. *Cancer Prev Res; 3(10); 1235–45.* ©*2010 AACR.*

Introduction

Malignant tumors of the bladder account for approximately 5% of all new primary cancers diagnosed in the United States, with an estimated 70,980 new cases in 2009 (1). Cigarette smoking is the most important etiologic factor for bladder cancer (2), but this disease is multifactorial and involves several environmental and genetic factors. Genetic polymorphisms in pathways controlling essential cellular activities may also play a role in bladder cancer etiology (3–5). Seventy percent to 80% of bladder cancers are non–muscle-invasive bladder cancer (NMIBC; ref. 6). Although NMIBC has an excellent prognosis and has a >80% overall 5-year survival rate, tumor recurrence is a major clinical problem and occurs in up to 70% of NMIBC patients after transurethral resection (TUR; ref. 7). Furthermore, 10% to 20% of such recurrences progress to invasive disease (7).

To reduce the recurrence and progression of NMIBC, intravesical instillation of agents is often administered after

Authors' Affiliations: Departments of [1]Epidemiology, [2]Urology, and [3]Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; [4]Genetic and Molecular Epidemiology Group, Spanish National Cancer Research Center, Madrid, Spain; [5]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland; [6]Centre for Research in Environmental Epidemiology (CREAL); [7]Municipal Institute of Medical Research; [8]CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona, Spain; and [9]National School of Public Health, Athens, Greece

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

M. Chen and M. Hildebrandt are co-first authors and contributed equally to this work. N. Malats and X. Wu are co-senior authors and contributed equally to this work.

Corresponding Author: Xifeng Wu, Department of Epidemiology, Unit 1340, The University of Texas M.D. Anderson Cancer Center, 1155 Pressler Boulevard, Houston, TX 77030. Phone: 713-745-2485; Fax: 713-792-4657; E-mail: xwu@mdanderson.org.

doi: 10.1158/1940-6207.CAPR-10-0035

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

TUR (8). Bacillus Calmette-Guérin (BCG) is the major choice for intravesical therapy, which includes induction BCG (iBCG) in a 6-week cycle and maintenance BCG (mBCG) in 3-week cycles given 3, 6, 12, 18, 24, and 30 months after iBCG (9). Although the NMIBC rate of response to BCG is 60% to 70%, as many as one third of the patients who initially respond will still develop recurrence and progression (10). Furthermore, more than 90% of patients receiving BCG experience side effects such as fever, leukocyturia, and cystitis symptoms, and approximately 5% suffer severe toxicities including sepsis and even death (11). These toxic effects cause a large number of patients to discontinue treatment, especially mBCG (9). In addition, there is evidence that progression and death are less common in association with initial radical cystectomy than with radical cystectomy following failed BCG treatment (12). Therefore, early identification of patients who will fail BCG treatment or experience adverse side effects will not only reduce unnecessary impairment of their quality of life but will also aid physicians in selecting optimal, or personalizing, therapy. Traditional clinical variables have less prognostic value in patients treated with BCG than in patients receiving TUR only (13); therefore, biomarkers that can better predict response to BCG therapy are highly desired.

Cancer stem cells, or tumor-initiating cells, are the putative origin of cancer developing from normal stem or progenitor cells (14, 15). Cancer stem cells play important roles in driving recurrence or metastasis (16–19) and affecting treatment response (20–24). The sonic hedgehog (Shh) pathway is one of the major signaling pathways that regulate cancer stem cells; it also controls cell proliferation, differentiation, and tissue patterning during organ development. Normally inactivated in adult tissues, the Shh pathway is reactivated in a wide range of cancers (25). On activation of the pathway, the secreted ligand sonic hedgehog (SHH) binds to the Patched (PTCH) receptor and activates the transmembrane protein Smoothened (SMO). The activation of SMO initiates a downstream cascade that releases three transcriptions factors (GLI1, GLI2, and GLI3) from the cytoplasm to enter the nucleus to activate specific target genes (Fig. 1; refs. 26, 27).

Uncontrolled activation of the Shh pathway occurs in bladder and many other cancers (28, 29), and Shh signaling is involved in tumor growth, recurrence, metastasis, and stem cell survival and expansion (18). *PTCH1* has been investigated as a potential tumor suppressor in bladder cancer (30–32). The role of *PTCH1* and Shh signaling in bladder cancer risk, however, is still being debated (33, 34). To our knowledge, no previous studies have addressed the association of genetic variations in the Shh pathway with bladder cancer susceptibility and outcome.

In the current study, we determined whether genetic variations, or single-nucleotide polymorphisms (SNP), in core functional components of the Shh pathway were associated with bladder cancer risk. We also evaluated the role of these SNPs in modulating recurrence and the risk of progression in patients receiving or not receiving BCG in our study's NMIBC subpopulation of patients and survival in muscle-invasive and metastatic bladder cancer (MiMBC) patients.

Materials and Methods

Study subjects

The Texas Bladder Cancer Study (TXBCS) recruited bladder cancer cases from The University of Texas M.D.

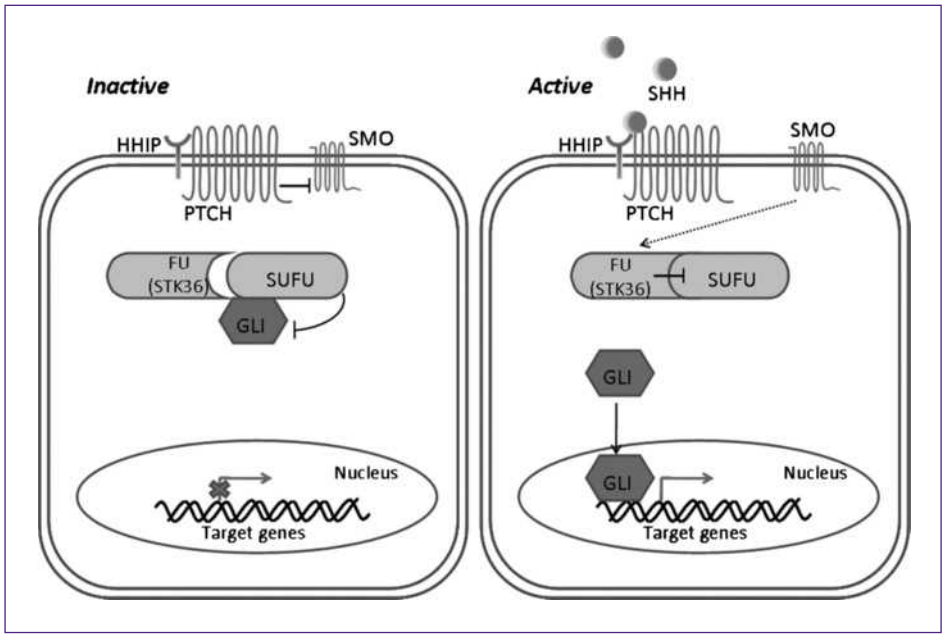

Fig. 1. Sonic hedgehog signaling pathway.

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

Anderson Cancer Center and Baylor College of Medicine through a daily review of computerized appointment schedules as a part of an ongoing project since 1995. Cases were all newly diagnosed within 1 year before recruitment, histologically confirmed, and previously untreated with chemotherapy or radiotherapy. Control subjects with no prior diagnosis of any type of cancer, except nonmelanoma skin cancer, were recruited from Kelsey Seybold, the largest private multispecialty physician group in Houston (35). These participants were matched 1:1 to the cases based on sex, age (±5 years), and ethnicity to evaluate the main effect of the genotype. There were no age, gender, or stage restrictions on recruitment. Because more than 90% of our recruited cases were pure transitional cell carcinoma and the etiology of transitional cell carcinoma differs from that of squamous cell carcinoma, we included patients with NMIBC and MiMBC in this study (Supplementary Table S1). In addition, because 90.6% of the patients in our capture population were Caucasians, we included only Caucasians in this study so as to limit the confounding effect of population structure. Individuals who never smoked or had smoked less than 100 cigarettes in his or her lifetime were defined as never smokers. Cases who had quit smoking at least 1 year before diagnosis and controls who had quit smoking at least 1 year before the interview were defined as former smokers. Individuals who were currently smoking or who had stopped <1 year before being diagnosed (cases) or before interview (controls) were defined as current smokers. Current and former smokers were defined as ever smokers.

An independent validation set for the TXBCS NMIBC patient data was obtained from the Spanish Bladder Cancer (SBC)/Epidemiology of Cancer of the Urothelium (EPICURO) study. All incident NMIBC patients were treated during 1998-2001 in 18 general or university-affiliated hospitals located in five geographic areas of Spain. The replication study included NMIBC patients who received TUR only and excluded NMIBC patients who received BCG mainly because of substantial differences in BCG regimens between the TXBCS and SBC/EPICURO study.

Epidemiologic and clinical data collection

Epidemiologic data of the TXBCS were collected by M.D. Anderson interviewers in a 45-minute interview on demographics, family history, and smoking status. Immediately after the interview, a blood sample was collected for DNA extraction. The clinical data for TXBCS such as tumor size, grade, stage, presence of carcinoma *in situ*, number of tumor foci at diagnosis, intravesical therapy, dates of recurrence and progression events, systemic chemotherapy, radical cystectomy, pathologic findings at cystectomy, and mortality were collected by trained chart reviewers. All patients were followed up with periodic cystoscopic examinations. The end points of outcome assessment in this study included recurrence, defined as a newly found bladder tumor following a previous negative follow-up cystoscopy; progression, defined as the transition from non–muscle-invasive to invasive or metastatic tumors; and overall survival, which was calculated from the date of diagnosis to the date of death or last follow-up, whichever came first. All of the human participation procedures were approved by the University of Texas M.D. Anderson Cancer Center, Baylor College of Medicine, and Kelsey Seybold institutional review boards. Written informed consent was obtained from all patients before interview. Clinical data collection in the SBC/EPICURO study has been described in detail previously (36). Written informed consent was obtained from all participants, and the study was approved by the local institutional ethics committee of each participating hospital and by the institutional review boards of the Institut Municipal d'Investigació Mèdica and U.S. National Cancer Institute.

Genotyping

Genotyping for the TXBCS was done at M.D. Anderson Cancer Center. Laboratory personnel were blinded to case and control status. Genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood Maxi Kit (Qiagen) according to the manufacturer's protocol. We combined literature search and database mining to select candidate genes in the Shh pathway following a procedure as previously described (4). A total of 177 haplotype-tagging SNPs from 11 Shh pathway genes, including *GLI1*, *GLI2*, *GLI3*, *GLI4*, *HHIP* (*Hedgehog-interacting protein*), *STK36*, *SUFU*, *SHH*, *SMO*, *PTCH*, and *PTCH2*, were selected for genotyping. The genotyping of 150 Shh SNPs in *GLI2*, *GLI3*, *GLI4*, *SUFU*, *PTCH*, *PTCH2*, *SMO*, and *SHH* was done using the Illumina iSelect custom SNP array platform, and 27 Shh SNPs in *GLI1*, *STK36*, and *HHIP* were obtained from our published genome-wide association study using the Illumina Human-Hap610 BeadChips (37), according to the manufacturer's Infinium II assay protocol (Illumina), with 750 ng of input DNA for each sample. All the genotyping data were analyzed and exported using BeadStudio software (Illumina). The average call rate for the SNP array was 99.7%. SNPs selected for replication were genotyped with the Infinium Illumina Human 1M probe BeadChip in SBC/EPICURO patients (38).

Statistical analysis

Most statistical analyses were done using the Intercooled Stata 10 statistical software package (Stata). Pearson's χ^2 test or Fisher's exact test was used to compare the difference in distribution of categorical variables, and Wilcoxon rank sum test or Student's *t* test was used for continuous variables where appropriate. Hardy-Weinberg equilibrium was tested using a goodness-of-fit χ^2 analysis. The effects of genotypes of SNPs on bladder cancer risks were estimated as odds ratios and 95% confidence intervals (95% CI) using unconditional multivariate logistic regression under the dominant, recessive, and additive models of inheritance adjusted for age, gender, and smoking status, where appropriate. For clinical outcome analyses, the main effect of individual SNPs on time to the event of each end point, hazard ratios (HR), and 95% CIs were estimated by multivariate Cox proportional hazard regression, adjusting

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

for age, gender, smoking status, tumor grade, tumor stage, and treatments. The patients who were lost to follow-up or died before the end point were censored. Because many SNPs and tests were done in the analysis, the *Q* value (a false discovery rate adjusted *P* value) was used to adjust the significance level for individual SNPs (39–41). We calculated the *Q* value by the Q value package implemented in the R software. We applied a bootstrap resampling method to internally validate the results. We generated 100 bootstrapped samples for SNPs that remained significant after multiple comparison. Each bootstrap sample was drawn from the original data set, and a *P* value was obtained for each SNP in the dominant, recessive, and additive models. Stratified analysis was used to compare the effects of individual genotypes on different treatment subgroups. In the replication study in SBC/EPICURO patients, HRs and 95% CIs were estimated by a multivariate Cox proportional hazard model, with adjustments for area, sex, stage, T-stage and grade, multiplicity, tumor size, and treatment. The individual effects of all SNPs on recurrence in TUR-only NMIBC patients in the combined TXBCS and SBC/EPICURO were summarized in a meta-analysis. All statistical analyses were two-sided. Kaplan-Meier plots and log-rank tests were applied to compare the difference between the recurrence-free survival time of homozygous wild-type and variant genotypes, which was calculated from the diagnosis date to the end of the follow-up or recurrence.

Results

Subject characteristics

A total of 803 Caucasian patients with transitional cell carcinoma of bladder cancer and 803 Caucasian controls were included in this study (Supplementary Table S1). Cases and controls were perfectly matched on sex (P = 1.00) and no significant difference was observed for cases (63.8 ± 10.9 years) and controls (64.7 ± 11.1 years) on age (P = 0.10). As we predicted, cases were more likely to be current smokers (23.3%) than controls (8.3%, $P < 0.01$), and among ever smoking participants, cases had a significantly higher mean pack-years (43.0 ± 30.7) than did the controls (29.9 ± 27.9; $P < 0.01$).

There were 419 NMIBC patients and 318 MiMBC patients with full follow-up data among the 803 cases from the case-control study. Of 419 NMIBC patients, 228 cases developed a recurrence. Table 1 shows the distribution of demographic and clinical variables in TXBCS study. The percentage of male patients with recurrence (56.8%) was significantly higher than that of females (43.4%; P = 0.03). There were no statistical differences between the recurrence and nonrecurrence groups in smoking status and clinical factors (tumor stage and grade) except for treatment. We categorized the 419 NMIBC patients into the four following treatment subgroups: TUR only, iBCG (received after TUR), mBCG (received after the TUR and iBCG), and others (such as intravesical chemotherapy but no BCG). Patients receiving mBCG were less likely to develop recurrence than those without mBCG ($P < 0.01$). Among these patients, 71 had progression. Factors associated with progression included sex, age, stage, grade, and treatment. Male patients (18.7%) were more likely to progress than women (9.2%; P = 0.05) and patients who had progression were significantly older at diagnosis (mean age, 66.2 years) than patients without progression (mean age, 62.7 years; P = 0.02). Higher stage and grade are significant risk factors for progression. Patients receiving mBCG treatment were less likely to progress ($P < 0.01$). Because BCG is primarily administered to those with higher risk of recurrence, we compared the stages and grades of NMIBC patients who received TUR only or any type of BCG (iBCG and mBCG). As expected, patients receiving BCG had higher stage and grade than the TUR-only subgroup ($P < 0.001$; data not shown). In the 204 patients who received BCG treatment, there were 65 (32%) stage Ta (4G1, 26G2, 34G3, and one unknown grade), 120 (59%) stage T1 (14G2, 104G3, and two unknown grade), and 18 (9%) stage Tis (11G3 and seven unknown grade; all data not shown). Of the 318 MiMBC patients, 184 (58%) were alive at the end of our study period. There was a significant difference between deceased and alive patients in terms of age, gender, stage, and treatment regimen ($P \leq 0.05$; Supplementary Table S2).

The characteristics of TUR-only NMIBC patients of the TXBCS and SBC/EPICURO study are listed in Table 2. There were 146 such patients in TXBCS, 97 of whom had recurrence. There were no significant differences between TXBCS patients who did and did not have recurrence in gender, age, smoking status, stage, or grade (Table 2). There were 356 NMIBC patients in the SBC/EPICURO study, among whom 133 showed recurrence. There were no significant differences between SBC/EPICURO patients with and without recurrence in gender, age, smoking status, or stage, although grade was higher in patients with recurrence.

Associations between SNPs and bladder cancer risk

Among the 177 individual SNPs we analyzed in relation to cancer risk (Supplementary Table S3), three SNPs on the *GLI3* gene, rs3735361, rs3823720, and rs10951671, reached nominal significance ($P < 0.05$; Table 3); however, none of these associations remained significant after adjusting for multiple testing (data not shown). These same three *GLI3* SNPs were consistently at the top of the list of SNP associations with risk in an analysis restricted to the 419 NMIBC patients (versus 803 controls; Supplementary Table S4) or, albeit nonsignificantly, in an analysis restricted to the 318 MiMBC patients (versus 803 controls; Supplementary Table S5).

Recurrence predictors in TUR-only patients

Nine SNPs had a nominally significant individual association with recurrence in patients receiving TUR only ($P \leq 0.05$) in the TXBCS (Supplementary Table S6). Six of these nine SNPs (i.e., rs17172001, rs1017024, rs1233560, rs2718107, rs2310897, and rs11594179) and rs11677381, which is in strong linkage with *GLI2*

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

Table 1. Demographic and clinical variables for NMIBC patients of the TXBCS

	NMIBC (n = 419)					
	Recurrence, n (%)			Progression, n (%)		
	Yes (n = 228)	No (n = 191)	P*	Yes (n = 71)	No (n = 347)	P*
Sex			**0.03**			**0.05**
Male	195 (56.8)	148 (43.2)		64 (18.7)	278 (81.3)	
Female	33 (43.4)	43 (56.6)		7 (9.2)	69 (90.8)	
Age (y)						
Mean (SD) y	63.0 (11.2)	63.6 (11.4)	0.63	66.2 (9.9)	62.7 (11.5)	**0.02**
Smoking status†			0.63			0.40
Never	64 (52.5)	58 (47.5)		16 (13.2)	105 (86.8)	
Former	117 (56.8)	89 (43.2)		37 (18.0)	169 (82.0)	
Current	47 (51.6)	44 (48.4)		18 (19.8)	73 (80.2)	
Stage			0.28			**<0.01**
Ta	105 (54.4)	88 (45.6)		21 (10.9)	172 (89.1)	
Tis	16 (69.6)	7 (30.4)		7 (30.4)	16 (69.6)	
T1	104 (52.0)	96 (48.0)		42 (21.1)	157 (78.9)	
Grade (G)			0.20			**<0.01**
G1	5 (31.2)	11 (68.8)		1 (6.2)	15 (93.8)	
G2	81 (54.0)	69 (46.0)		12 (8.0)	138 (92.0)	
G3	128 (54.0)	109 (46.0)		53 (22.5)	183 (77.5)	
Treatment‡			**<0.01**			**<0.01**
TUR	97 (66.4)	49 (33.6)		16 (11.0)	129 (89.0)	
iBCG	91 (74.6)	31 (25.4)		37 (30.3)	85 (69.7)	
mBCG	30 (36.6)	52 (63.4)		15 (18.3)	67 (81.7)	
Others	10 (16.7)	50 (83.3)		3 (5.0)	57 (95.0)	

NOTE: The numbers of each variable may not add up to 419 due to missing data.

*P values were derived from Pearson's χ^2 test or Fisher's exact test for categorical variables, and Student's t test for continuous variables.

†Smoking status: individuals who had smoked more than 100 cigarettes in their lifetime were defined as ever smokers; others were never smokers. Smokers included current smokers and former smokers. Individuals who had quit smoking at least 1 y before diagnosis were categorized as former smokers.

‡Treatment: TUR, subgroup who had no further therapy after TUR; iBCG, subgroup who received iBCG after TUR; mBCG, subgroup who further received mBCG after the TUR and iBCG treatment; and others, which included those who received intravesical chemotherapy but no BCG.

rs11685068 (R^2 = 1), were genotyped in the validation data set from the 356 NMIBC patients of SBC/EPICURO (Supplementary Table S6). The SNPs rs1233560 (of *SHH*) and rs11685068 (*GLI2*) were significantly associated with recurrence in both the TXBCS and SBC/EPICURO study (Table 4). The recurrence HR was 2.07 (95% CI, 1.33-3.21; $P = 1.3 \times 10^{-3}$) for *GLI2* rs11685068 and 1.39 (95% CI, 1.14-1.70; $P = 1.0 \times 10^{-3}$) for *SHH* rs1233560 in a meta-analysis of the combined TXBCS and SBC/EPICURO data.

Recurrence predictors in BCG patients

In 204 patients receiving BCG treatment (including 122 patients in iBCG subgroup and 82 patients in mBCG subgroup), nine SNPs located on *GLI3*, *GLI2*, and *HHIP* were associated individually with time to recurrence at $P < 0.05$. After adjustment of multiple testing, two variant genotypes of *GLI3*, rs6463089 and rs3801192, remained significant and associated with a 2.40-fold (95% CI, 1.50-3.84) and 2.54-fold (95% CI, 1.47-4.39) increased recurrence risk, respectively, compared with their corresponding homozygous wild-type genotype (Table 5). Interestingly, variant genotypes of *GLI3* rs6463089 and rs3801192 showed a protective effect on recurrence in the TUR-only subgroup, with HRs of 0.74 (95% CI, 0.42-1.33, P = 0.32) and 0.43 (95% CI, 0.21-0.88, P = 0.02), respectively (Table 5). In the Kaplan-Meier estimates of recurrence-free survival in the BCG treatment group, compared with patients with the homozygous wild-type genotype of rs6463089 (recurrence-free median survival time [MST], 16.3 months; $P_{\text{log-rank}} < 0.01$) and rs3801192 (13.1 months, $P_{\text{log-rank}} = 0.02$), those with at least one variant allele at either of these two SNPs showed a shorter recurrence-free MST of 5.5 months (Fig. 2). Conversely, in patients receiving TUR only, compared with the

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

Table 2. Host characteristics for NMIBC patients receiving TUR only in the TXBCS and SBC/EPICURO study

	TXBCS			SBC/EPICURO study		
	Recurrence, *n* (%)			Recurrence, *n* (%)		
	Yes (*n* = 97)	No (*n* = 49)	*P**	Yes (*n* = 133)	No (*n* = 223)	*P**
Sex			0.18			0.59
Male	77 (69.4)	34 (30.6)		118 (36.9)	202 (63.1)	
Female	20 (57.1)	15 (42.9)		15 (41.7)	21 (58.3)	
Age (y)						
Mean (SD) y	62.5 (12.2)	60.6 (13.7)	0.39	65.69 (10.07)	66.14 (10.39)	0.69
Smoking status†			0.46			0.17
Never	28 (60.9)	18 (39.1)		17 (43.6)	22 (56.4)	
Former	48 (71.6)	19 (28.4)		52 (32.7)	107 (67.3)	
Current	21 (63.6)	12 (36.4)		64 (42.1)	88 (57.9)	
Stage			0.50			0.85
Ta	62 (63.9)	35 (36.1)		123 (37.7)	203 (62.3)	
Tis	31 (68.9)	14 (31.1)		1 (50)	1 (50)	
T1	2 (100)	0 (0.0)		9 (32.1)	19 (67.9)	
Grade (G)			0.55			**<0.01**
G1	4 (50)	4 (50)		57 (29.8)	134 (70.2)	
G2	55 (64.7)	30 (35.3)		57 (48.3)	61 (51.7)	
G3	34 (69.4)	15 (30.6)		19 (40.4)	28 (59.6)	

*P values were derived from Pearson's χ^2 test or Fisher's exact test for categorical variables, and Student's t test for continuous variables.

†Smoking status: individuals who had smoked more than 100 cigarettes in their lifetime were defined as ever smokers; others were never smokers. Smokers included current smokers and former smokers. Individuals who had quit smoking at least 1 y before diagnosis were categorized as former smokers. Six patients without recurrence had missing data for smoking status variable in the SBC/EPICURO study.

homozygous wild-type genotype (rs6463089 recurrence-free MST, 6.4 months; rs3801192 recurrence-free MST, 6.2 months), those with the variant alleles at either of these two SNPs had longer recurrence-free MST (for rs6463089, 10.6 months, $P_{\text{log-rank}} = 0.22$; for rs3801192, >109.9 months, $P_{\text{log-rank}} = 0.01$; Fig. 2). Although we did not conduct validation assessments of SNP association in BCG patients because of BCG differences between the SBC/EPICURO study and TXBCS (also stated in Materials and Methods), we performed bootstrap sampling to internally validate associations in the primary analysis. The overall HRs and 95% CIs generated by bootstrapping were consistent with our initial results. Table 5 lists the number of times that the bootstrap-generated P value was 0.05, 0.001, or 0.0001 for each SNP. The significant results of *GLI3* rs6463089 and rs3801192 in the BCG group reached significance at $P = 0.05$ in >90% of 100 bootstrap samplings. The bootstrap findings indicate that the results for these SNPs in the primary analysis were unlikely due to chance alone.

Table 3. Association between selected Shh pathway–related SNPs and bladder cancer risk

Gene	SNP	Genotype	Case/control	OR (95% CI)*	*P*
GLI3	rs3735361 (G>A)	GG/GA	708/733	Reference	
	Flanking 3′ UTR	AA	95/70	1.42 (1.01-1.99)	0.04
	rs3823720 (G>A)	GG/GA	704/738	Reference	
	3′ UTR	AA	99/65	1.57 (1.11-2.20)	0.01
	rs10951671 (G>A)	GG/GA	748/769	Reference	
	Intron	AA	55/34	1.75 (1.11-2.74)	0.02

Abbreviations: OR, odds ratio; UTR, untranslated region.

*Adjusted by age, gender, and smoking status.

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

Table 4. Significant SNP associations with recurrence in TUR-only NMIBC patients in the TXBCS and SBC/EPICURO study

	rs11685068*			rs1233560		
	Genotype count	HR (95% CI)	*P*	Genotype count	HR (95% CI)	*P*
Gene		*GLI2*			*SHH*	
Allele		G>A			A>G	
Best model†		DOM			ADD	
TXBCS	124/15/2	2.19 (1.22-3.93)	0.01	47/70/24	1.49 (1.07-2.07)	0.02
SBC/EPICURO study	335/21/0	1.91 (0.97-3.76)	0.06	105/175/76	1.34 (1.05-1.71)	0.02
Combined	459/36/2	2.07 (1.33-3.21)	1.3×10^{-3}	152/245/100	1.39 (1.14-1.70)	1.0×10^{-3}

Abbreviations: DOM, dominant; ADD, additive.

**GLI2* rs11685068 is not genotyped in the Spanish study, but in strong linkage with rs11677381 with $R^2 = 1.0$. The validation result of rs11685068 was derived from the result of rs11677381.

†Best model: the model with smallest *P* value.

Progression in NMIBC patients

We also assessed the association of SNPs with progression of NMIBC; however, we did not observe any SNPs significantly associated with bladder cancer progression (data not shown). Compared with recurrence, the progression rate in NMIBC patients is relatively low; therefore, we did not have adequate sample size to do the stratification analysis by BCG treatment status.

Overall survival in MiMBC patients

Two SNPs on *GLI2*, rs735557 and rs4848632, and the *SHH* SNP rs208684 showed individual associations with overall survival in MiMBC patients ($P < 0.05$; Supplementary Table S7). All three associations became nonsignificant, however, after adjusting for multiple comparisons (data not shown).

Discussion

The present results have important implications for the clinical management of NMIBC. Nine SNPs were significantly associated with recurrence of NMIBC patients in the TXBCS who received TUR treatment only; two of these SNPs, *SHH* rs1233560 and *GLI2* rs11685068, were validated independently in the SBC/EPICURO cohort. With initial TXBCS and replicated SBC/EPICURO results, *SHH* rs1233560 and *GLI2* rs11685068 have potential real-time clinical utility for predicting recurrence in NMIBC patients receiving TUR only. In NMIBC patients who received BCG, two variant genotypes of *GLI3* (rs6463089 and rs3801192) were significantly associated with an increased risk of recurrence and a shorter recurrence-free survival (~2.5-fold changes in each) after adjusting for multiple comparisons, and the associations were internally validated by bootstrap analysis. These results suggest that NMIBC patients with wild-type genotypes of these two *GLI3* SNPs are good candidates for BCG therapy, whereas those with the variant genotypes of these two SNPs should be spared BCG therapy.

The homozygous variant genotypes of three SNPs in *GLI3* showed significant associations with increased overall bladder cancer risk in our 803 bladder cancer cases and 803 controls. These associations remained at the top of the list of risk-associated SNPs in the case-control study restricted to the 419 NMIBC cases or 318 MiMBC cases (versus 803 controls). These associations became nonsignificant, however, after adjusting for multiple comparisons in the overall analysis and the analysis stratified for NMIBC or MiMBC. Further validation studies in independent populations are warranted to clarify the associations of these SNPs with bladder cancer risk. Two *GLI2* SNPs and an *SHH* SNP were significantly associated with overall survival in MiMBC patients, but these associations became nonsignificant after adjusting for multiple comparisons.

It is suggested that Shh signaling plays an important role in the development and prognosis of bladder cancer. For example, the most frequent loss-of-heterozygosity region in NMIBC is the locus of the Shh pathway gene *PTCH* on 9q22 (42). Changes in the level of Shh pathway gene expressions also might predict overall survival in bladder cancer patients (43). All previous studies were conducted in tumor tissues, where they showed that somatic changes in Shh genes (such as gene expression level and loss of heterozygosity) may be involved in cancer development. Our study was the first, however, to examine germ-line genetic variations in Shh signaling as cancer susceptibility factors and predictors of outcome.

We found that certain Shh pathway SNPs affected recurrence in patients receiving BCG. Although its antitumor mechanism is not fully understood, BCG can trigger a strong local immune response that leads to the expression of many cytokines at the tumor site and to an influx of granulocytes and mononuclear cells into the bladder wall (44, 45). It is hypothesized that intravesical BCG treatment can directly inhibit urothelial tumor cell growth

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

Table 5. Recurrence in NMIBC patients receiving BCG treatment versus those receiving TUR only and internal bootstrap validation

BCG vs TUR only

SNP	Gene	Genotype	Best model*	BCG subgroup recurrence				TUR-only subgroup recurrence			
				Yes/No		HR (95% CI)†	*P*	Yes/No		HR (95% CI)†	*P*
				ww	wv + vv			ww	wv + vv		
rs6463089	***GLI3***	G>A	**DOM**	**92/78**	**26/5**	**2.40 (1.50-3.84)**	**2×10^{-4}**	78/38	14/11	0.74 (0.42-1.33)	0.32
rs3801192	***GLI3***	G>A	**DOM**	**100/77**	**17/6**	**2.54 (1.47-4.39)**	**9×10^{-4}**	83/37	9/12	0.43 (0.21-0.88)	0.02
rs277534	*GLI2*	A>G	DOM	69/46	49/37	0.60 (0.40-0.90)	0.01	63/31	29/18	1.02 (0.64-1.61)	0.95
rs3801210	*GLI3*	G>A	ADD	52/37	65/46	1.43 (1.08-1.89)	0.01	38/14	54/35	0.86 (0.62-1.20)	0.39
rs6974655	*GLI3*	C>A	DOM	67/42	51/41	0.62 (0.42-0.93)	0.02	50/21	42/28	0.72 (0.47-1.10)	0.13
rs2237425	*GLI3*	G>C	ADD	76/60	42/20	1.45 (1.04-2.03)	0.03	70/38	22/11	1.08 (0.71-1.63)	0.73
rs2286294	*GLI3*	A>G	DOM	28/29	89/51	1.63 (1.04-2.55)	0.03	31/11	61/37	0.97 (0.62-1.52)	0.90
rs2306924	*HHIP*	A>G	DOM	25/15	93/68	0.61 (0.38-0.99)	0.04	18/14	74/35	1.44 (0.83-2.50)	0.19
rs7785287	*GLI3*	G>A	ADD	73/58	44/25	1.39 (1.00-1.93)	0.05	56/36	36/13	1.30 (0.89-1.89)	0.18

Internal bootstrap validation of significant results in patients receiving BCG treatment

SNP	Gene	Genotype	Best model*	Bootstrap‡ BCG subgroup recurrence			
				HR (95% CI)†	*P* < 0.0001	*P* < 0.001	*P* < 0.05
rs6463089	***GLI3***	G>A	**DOM**	**2.40 (1.55-3.71)**	**46**	**68**	**92**
rs3801192	***GLI3***	G>A	**DOM**	**2.54 (1.46-4.40)**	**34**	**56**	**93**
rs277534	*GLI2*	A>G	DOM	0.60 (0.38-0.94)	8	18	66
rs3801210	*GLI3*	G>A	ADD	1.43 (1.06-1.91)	9	23	69
rs6974655	*GLI3*	C>A	DOM	0.62 (0.38-1.02)	3	16	58
rs2237425	*GLI3*	G>C	ADD	1.45 (1.11-1.91)	3	6	55
rs2286294	*GLI3*	A>G	DOM	1.63 (1.01-2.62)	3	11	53
rs2306924	*HHIP*	A>G	DOM	0.61 (0.36-1.02)	4	16	61
rs7785287	*GLI3*	G>A	ADD	1.39 (1.02-1.89)	1	10	46

NOTE: Significant SNPs after correcting for multiple comparisons by *Q* value with a false discovery rate of ≤10% are in boldface.
Abbreviations: w-wild type allele; v-variant allele; ww, homozygous wildtype genotype; wv, heterozygous variant genotype; vv, homozygous variant genotype.
*Best model: the model with smallest *P* value.
†Adjusted by age, sex, smoking status, tumor stage, and tumor grade using Cox proportional hazard regression where appropriate.
‡We did internal validation of the results choosing from the best genetic model using bootstrap 100 times.

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

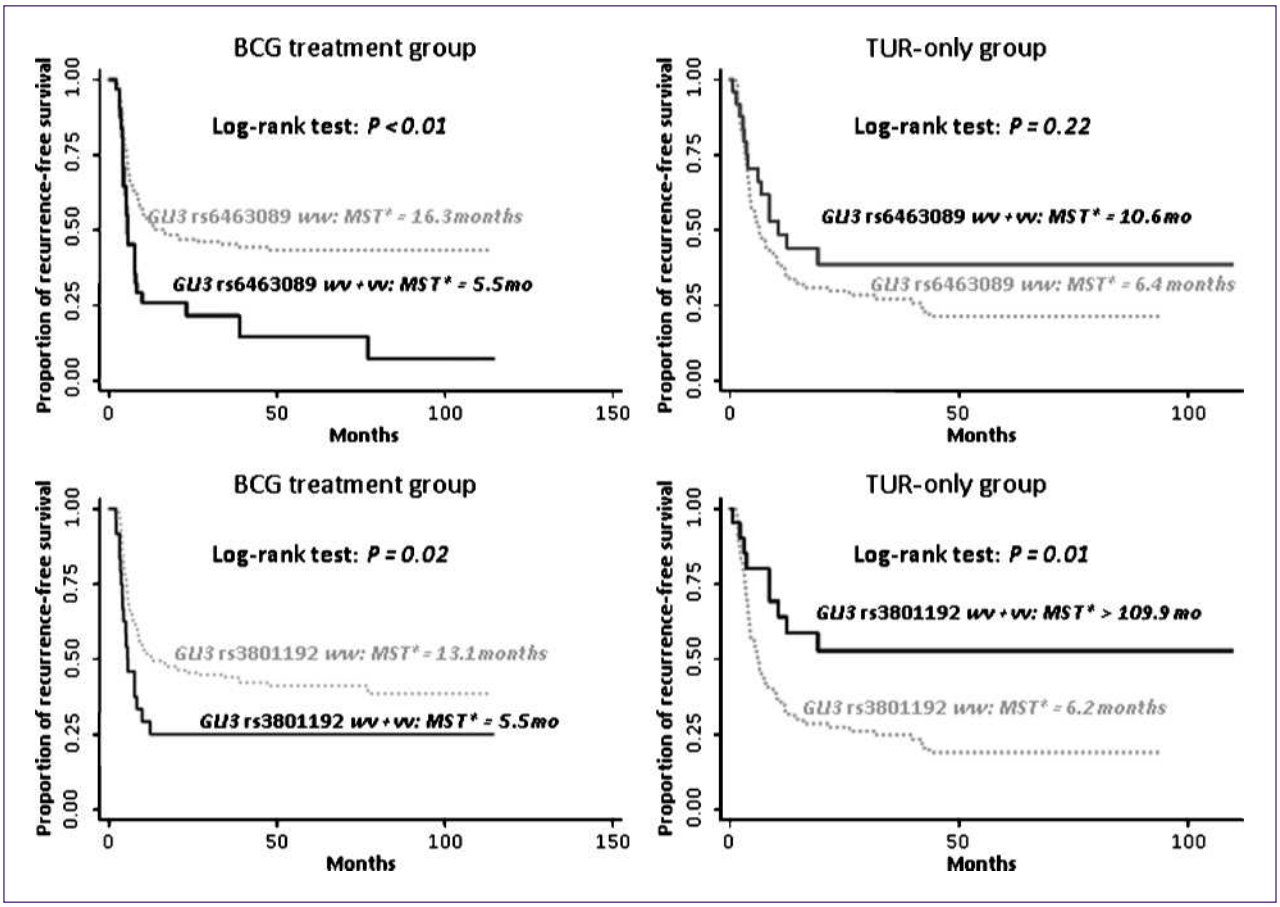

Fig. 2. Kaplan-Meier curve of recurrence-free survival in NMIBC patients receiving BCG treatment versus those receiving TUR only. w-wild type allele; v-variant allele; ww, homozygous wildtype genotype; wv, heterozygous variant genotype; vv, homozygous variant genotype.

and exerts its antitumor activity through cell immunity mediated by the T-helper type 1 cytokines (46). Previous studies have suggested that genetic variations in inflammation genes and DNA repair genes have regulatory effects on these cytokines and, hence, on BCG response (47, 48). Recent data indicate that the activation of Shh signaling is mediated by NF-κB, which is the hallmark of the inflammatory response (49, 50). Shh signaling also may influence T-cell activation (51). Therefore, it is plausible that genetic variations in the Shh pathway may affect response to BCG through their effects in regulating the inflammatory response to BCG treatment. Alternatively to this direct mediation, these genetic variations may function as a surrogate biomarker of response to BCG.

Our present findings that the SNPs most significantly associated with recurrence in NMIBC patients were *GLI2* rs11685068 (Table 4) in TUR-only patients and the independent (i.e., not or only weakly linked to one another) *GLI3* SNPs rs6463089 and rs3801192 in BCG (Table 5), induction plus or minus maintenance (Supplementary Table S8), patients showed the importance of *GLI*-family genes in bladder cancer. The *GLI* family encodes zinc-finger proteins that are amplified in malignant glioma (52). These GLI proteins are transcription factors with distinct functions in a context-dependent manner (53). The effect of Shh signaling finally depends on the flipping of GLI in a combinatorial and cooperative manner. In the absence of Shh, GLI1 generally is transcriptionally repressed, whereas GLI2 and GLI3 are cleaved by the proteasome and act as repressors of transcription. On activation by Shh, the full-length GLI becomes a transcriptional activator. GLI2 and GLI3 are weaker than GLI1 in activating transcription (54).

Strong evidence suggests that altered *GLI2* and *GLI3* play a role in cancer development and progression. *GLI2* is less studied in human diseases, but several studies in mice indicate that GLI2 overexpression or mutation is associated with basal cell carcinomas and skeletal defects and disorders (55). *GLI3* translocation, deletion, and mutations have been implicated in several types of birth defects (55). It is interesting to note that genetic variations in *GLI3* seem to modulate both bladder cancer recurrence in patients treated with BCG and bladder cancer risk. A recent *in vitro* study showed that arsenic treatment activated Shh signaling in bladder cancer cells by decreasing the stability of the repressor form of GLI3; furthermore, high levels of arsenic exposure were associated with high levels of SHH activity in

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

tumor samples from a cohort of bladder cancer patients (56). These results suggest that GLI3-mediated activation of the Shh pathway plays an important role at least in bladder cancer induced by the environmental toxin arsenic. It is likely that smoking, BCG, and other exposures also may affect GLI3 and lead to the activation of the Shh pathway, which may explain the risk and recurrence associations in our study.

SHH is the secreted ligand that activates the PTCH receptor and thus initiates the Shh signaling pathway. Endogenous overexpression of SHH can drive abnormal activation of the Shh signaling pathway and cell growth in solid tumors (57). SHH has been reported to be an early and late mediator of pancreatic tumorigenesis (58). We found that the SNP *SHH* rs1233560 was associated with increased recurrence in TUR-only NMIBC patients in both the TXBCS and SBC/EPICURO cohorts. Because this SNP is located in the flanking 3′ untranslated region, we hypothesized that it may affect SHH expression. Because all of the SNPs in *SHH*, *GLI2*, and *GLI3* with significant associations are haplotype-tagging SNPs located either in intergenic regions or introns without a clear functional indication, they likely are not the causal variants but are in strong linkage disequilibrium with the causal SNPs proximate to these tagging SNPs. Future studies to accurately map the causal SNPs and identify the biological mechanisms underlying the associations of *SHH*, *GLI2*, and *GLI3* with BCG-associated recurrence are needed.

The major strength of our present study is that our recurrence results in TUR-only NMIBC patients of the TXBCS were externally validated in the SBC/EPICURO cohort, which reduced the possibility of chance findings. Although it was not feasible to externally validate recurrence results in BCG-treated patients in the SBC/EPICURO population (because of substantial differences in BCG regimens between the TXBCS and SBC/EPICURO study, as mentioned earlier), we controlled for the false discovery rate of these results by adjusting for multiple testing, and we performed an internal validation bootstrap analysis. Another study strength is that the TXBCS population of NMIBC patients was large and ethnically homogeneous, which limited potential confounding by population substructure. Furthermore, all NMIBC patients in the TXBCS had complete clinical data and a long median follow-up of 48.2 months (59), which allowed for a more accurate assessment of recurrence outcomes in TUR-only and BCG patients.

In conclusion, this study indicates that Shh signaling may predict the outcome of TUR only or BCG in NMIBC patients and thus could help in future approaches to the clinical management of these patients and reducing the burden of this morbid disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

NIH grants U01 CA 127615, R01 CA 74880, P50 CA 91846, and R01 CA 131335.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 02/16/2010; revised 08/12/2010; accepted 08/13/2010; published OnlineFirst 09/21/2010.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225–49.
2. Johansson SL, Cohen SM. Epidemiology and etiology of bladder cancer. Semin Surg Oncol 1997;13:291–8.
3. Wu X, Gu J, Grossman HB, et al. Bladder cancer predisposition: a multigenic approach to DNA-repair and cell-cycle-control genes. Am J Hum Genet 2006;78:464–79.
4. Yang H, Gu J, Lin X, et al. Profiling of genetic variations in inflammation pathway genes in relation to bladder cancer predisposition. Clin Cancer Res 2008;14:2236–44.
5. Yang H, Dinney CP, Ye Y, Zhu Y, Grossman HB, Wu X. Evaluation of genetic variants in microRNA-related genes and risk of bladder cancer. Cancer Res 2008;68:2530–7.
6. Dalbagni G. The management of superficial bladder cancer. Nat Clin Pract Urol 2007;4:254–60.
7. Sylvester RJ. The use of intravesical chemotherapy and possibilities for improving its efficacy. Eur Urol 2006;50:233.
8. Babjuk M, Oosterlinck W, Sylvester R, Kaasinen E, Bohle A, Palou-Redorta J. EAU guidelines on non-muscle-carcinoma of the bladder. Actas Urol Esp 2009;33:361–71.
9. Lamm DL, Blumenstein BA, Crissman JD, et al. Maintenance bacillus Calmette-Guerin immunotherapy for recurrent TA, T1 and carcinoma *in situ* transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study. J Urol 2000;163:1124–9.
10. Merz VW, Marth D, Kraft R, Ackermann DK, Zingg EJ, Studer UE. Analysis of early failures after intravesical instillation therapy with bacille Calmette-Guerin for carcinoma *in situ* of the bladder. Br J Urol 1995;75:180–4.
11. Brandau S, Suttmann H. Thirty years of BCG immunotherapy for non-muscle invasive bladder cancer: a success story with room for improvement. Biomed Pharmacother 2007;61:299–305.
12. Schrier BP, Hollander MP, van Rhijn BW, Kiemeney LA, Witjes JA. Prognosis of muscle-invasive bladder cancer: difference between primary and progressive tumours and implications for therapy. Eur Urol 2004;45:292–6.
13. Saint F, Salomon L, Quintela R, et al. Do prognostic parameters of remission versus relapse after Bacillus Calmette-Guerin (BCG) immunotherapy exist? Analysis of a quarter century of literature. Eur Urol 2003;43:351–60, discussion 60–1.
14. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–11.
15. Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 2003;3:895–902.
16. Dittmar T, Nagler C, Schwitalla S, Reith G, Niggemann B, Zanker KS. Recurrence cancer stem cells—made by cell fusion? Med Hypotheses 2009;73:542–7.
17. Salama P, Platell C. Colorectal cancer stem cells. ANZ J Surg 2009;79:697–702.
18. Varnat F, Duquet A, Malerba M, et al. Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. EMBO Mol Med 2009;1:338–51.

Published OnlineFirst September 21, 2010; DOI: 10.1158/1940-6207.CAPR-10-0035

19. Xu X, Xing B, Han H, et al. The properties of tumor-initiating cells from a hepatocellular carcinoma patient's primary and recurrent tumor. Carcinogenesis 2009;31:167–74.
20. Liu G, Yuan X, Zeng Z, et al. Analysis of gene expression and chemoresistance of CD133$^+$ cancer stem cells in glioblastoma. Mol Cancer 2006;5:67.
21. Rich JN. Cancer stem cells in radiation resistance. Cancer Res 2007; 67:8980–4.
22. Ma S, Lee TK, Zheng BJ, Chan KW, Guan XY. CD133$^+$ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene 2008;27:1749–58.
23. Shafee N, Smith CR, Wei S, et al. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. Cancer Res 2008;68:3243–50.
24. Shervington A, Lu C. Expression of multidrug resistance genes in normal and cancer stem cells. Cancer Invest 2008;26:535–42.
25. Scales SJ, de Sauvage FJ. Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. Trends Pharmacol Sci 2009;30:303–12.
26. Kelleher FC, Fennelly D, Rafferty M. Common critical pathways in embryogenesis and cancer. Acta Oncol 2006;45:375–88.
27. Rubin LL, de Sauvage FJ. Targeting the Hedgehog pathway in cancer. Nat Rev Drug Discov 2006;5:1026–33.
28. Yang L, Xie G, Fan Q, Xie J. Activation of the hedgehog-signaling pathway in human cancer and the clinical implications. Oncogene 2010;29:469–81.
29. Pasca di Magliano M, Hebrok M. Hedgehog signalling in cancer formation and maintenance. Nat Rev Cancer 2003;3:903–11.
30. Habuchi T, Devlin J, Elder PA, Knowles MA. Detailed deletion mapping of chromosome 9q in bladder cancer: evidence for two tumour suppressor loci. Oncogene 1995;11:1671–4.
31. Linnenbach AJ, Pressler LB, Seng BA, Kimmel BS, Tomaszewski JE, Malkowicz SB. Characterization of chromosome 9 deletions in transitional cell carcinoma by microsatellite assay. Hum Mol Genet 1993; 2:1407–11.
32. Spruck CH III, Ohneseit PF, Gonzalez-Zulueta M, et al. Two molecular pathways to transitional cell carcinoma of the bladder. Cancer Res 1994;54:784–8.
33. LaRue H, Simoneau M, Aboulkassim TO, et al. The PATCHED/Sonic Hedgehog signalling pathway in superficial bladder cancer. Med Sci (Paris) 2003;19:920–5.
34. McGarvey TW, Maruta Y, Tomaszewski JE, Linnenbach AJ, Malkowicz SB. PTCH gene mutations in invasive transitional cell carcinoma of the bladder. Oncogene 1998;17:1167–72.
35. Hudmon KS, Honn SE, Jiang H, et al. Identifying and recruiting healthy control subjects from a managed care organization: a methodology for molecular epidemiological case-control studies of cancer. Cancer Epidemiol Biomarkers Prev 1997;6:565–71.
36. Puente D, Malats N, Cecchini L, et al. Gender-related differences in clinical and pathological characteristics and therapy of bladder cancer. Eur Urol 2003;43:53–62.
37. Wu X, Ye Y, Kiemeney LA, et al. Genetic variation in the prostate stem cell antigen gene PSCA confers susceptibility to urinary bladder cancer. Nat Genet 2009;41:991–5.
38. Rothman N, Garcia-Closas M, Chatterjee N, et al. A multi-stage genome-wide association study of bladder cancer identifies multiple susceptibility loci. Nat Genet. In revision.
39. Storey JD. A direct approach to false discovery rates. J R Stat Soc Ser B-Stat Method 2002;64:479–98.
40. Storey JD, Taylor JE, Siegmund D. Strong control, conservative point estimation and simultaneous conservative consistency of false discovery rates: a unified approach. J R Stat Soc Ser B-Stat Method 2004;66:187–205.
41. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 2003;100:9440–5.
42. Aboulkassim TO, LaRue H, Lemieux P, Rousseau F, Fradet Y. Alteration of the PATCHED locus in superficial bladder cancer. Oncogene 2003;22:2967–71.
43. Quint K, Stintzing S, Alinger B, et al. The expression pattern of PDX-1, SHH, Patched and Gli-1 is associated with pathological and clinical features in human pancreatic cancer. Pancreatology 2009;9: 116–26.
44. Alexandroff AB, Jackson AM, O'Donnell MA, James K. BCG immunotherapy of bladder cancer: 20 years on. Lancet 1999;353: 1689–94.
45. Bohle A, Brandau S. Immune mechanisms in bacillus Calmette-Guerin immunotherapy for superficial bladder cancer. J Urol 2003; 170:964–9.
46. Luo Y, Chen X, O'Donnell MA. Role of Th1 and Th2 cytokines in BCG-induced IFN-γ production: cytokine promotion and simulation of BCG effect. Cytokine 2003;21:17–26.
47. Gu J, Zhao H, Dinney CP, et al. Nucleotide excision repair gene polymorphisms and recurrence after treatment for superficial bladder cancer. Clin Cancer Res 2005;11:1408–15.
48. Leibovici D, Grossman HB, Dinney CP, et al. Polymorphisms in inflammation genes and bladder cancer: from initiation to recurrence, progression, and survival. J Clin Oncol 2005;23:5746–56.
49. Nakashima H, Nakamura M, Yamaguchi H, et al. Nuclear factor-κB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. Cancer Res 2006;66: 7041–9.
50. Yamasaki A, Kameda C, Xu R, et al. Nuclear factor κB-activated monocytes contribute to pancreatic cancer progression through the production of Shh. Cancer Immunol Immunother 2010;59: 675–86.
51. Crompton T, Outram SV, Hager-Theodorides AL. Sonic hedgehog signalling in T-cell development and activation. Nat Rev Immunol 2007;7:726–35.
52. Kinzler KW, Bigner SH, Bigner DD, et al. Identification of an amplified, highly expressed gene in a human glioma. Science 1987;236:70–3.
53. Ruiz i Altaba A, Sanchez P, Dahmane N. Gli and hedgehog in cancer: tumours, embryos and stem cells. Nat Rev Cancer 2002; 2:361–72.
54. Stecca B, Ruiz IAA. Context-dependent regulation of the GLI code in cancer by HEDGEHOG and non-HEDGEHOG signals. J Mol Cell Biol 2010;2:84–95.
55. Villavicencio EH, Walterhouse DO, Iannaccone PM. The sonic hedgehog-patched-gli pathway in human development and disease. Am J Hum Genet 2000;67:1047–54.
56. Fei DL, Li H, Kozul CD, et al. Activation of Hedgehog Signaling by the Environmental Toxicant Arsenic May Contribute to the Etiology of Arsenic-Induced Tumors. Cancer Res 70:1981–8.
57. Berman DM, Karhadkar SS, Maitra A, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature 2003;425:846–51.
58. Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature 2003; 425:851–6.
59. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. Control Clin Trials 1996;17:343–6.

AACR American Association for Cancer Research

Cancer Prevention Research

Genetic Variations in the Sonic Hedgehog Pathway Affect Clinical Outcomes in Non–Muscle-Invasive Bladder Cancer

Meng Chen, Michelle A.T. Hildebrandt, Jessica Clague, et al.

Cancer Prev Res 2010;3:1235-1245. Published OnlineFirst September 21, 2010.