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Genetic variations in PI3K-AKT-mTOR pathway and bladder cancer risk

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Genetic variations in phosphoinositide-3 kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway may affect critical cellular functions and increase an individual's cancer risk. We systematically evaluate 231 single-nucleotide polymorphisms (SNPs) in 19 genes in the PI3K-AKT-mTOR signaling pathway as predictors of bladder cancer risk. In individual SNP analysis, four SNPs in regulatory associated protein of mTOR (*RAPTOR*) remained significant after correcting for multiple testing: rs11653499 [odds ratio (OR): 1.79, 95% confidence interval (CI): 1.24–2.60, $P = 0.002$], rs7211818 (OR: 2.13, 95% CI: 1.35–3.36, $P = 0.001$), rs7212142 (OR: 1.57, 95% CI: 1.19–2.07, $P = 0.002$) and rs9674559 (OR: 2.05, 95% CI: 1.31–3.21, $P = 0.002$), among which rs7211818 and rs9674559 are within the same haplotype block. In haplotype analysis, compared with the most common haplotypes, haplotype containing the rs7212142 wild-type allele showed a protective effect of bladder cancer (OR: 0.83, 95% CI: 0.70–0.97). In contrast, the haplotype containing the rs7211818 variant allele showed a 1.32-fold elevated bladder cancer risk (95% CI: 1.09–1.60). In combined analysis of three independent significant *RAPTOR* SNPs (rs11653499, rs7211818 and rs7212142), a significant trend was observed for increased risk with an increase in the number of unfavorable genotypes (P for trend <0.001). Compared with the subjects without any of the unfavorable genotypes, those carrying all three unfavorable genotypes showed a 2.22-fold (95% CI: 1.33–3.71) increased bladder cancer risk. This is the first study to evaluate the role of germ line genetic variations in PI3K-AKT-mTOR pathway as cancer susceptibility factors that will help us identify high-risk individuals for bladder cancer.

Introduction

Bladder cancer is the fourth most frequently diagnosed cancer in men with a worldwide incidence ratio of male to female of ~3.3 to 1. As estimated in 2009, there were ~70 890 new cases and 14 330 deaths from bladder cancer in the USA (1). Bladder cancer is a complex disease attributed to multiple environmental and genetic factors, of which smoking is the most important risk factor, accounting for approximately half of new cases in men and a third of new cases in women. Smokers have a 2-fold increase in the risk of developing bladder cancer compared with non-smokers. Other established risk factors include occupational exposure to aromatic amines and other chemicals, drinking water contaminated with high levels of carcinogens (e.g. arsenic, chlorinated by-products) and a family history of bladder cancer (2–4).

Recently, there has been compelling evidence that genetic factors contribute to bladder cancer etiology (2–4). The results of a large population-based twin study demonstrated the overall contributions of hereditary factors to the causation of sporadic cancers, with obvious differences among cancer types. For example, the estimated contribution of heritable factors is as high as 31% in urinary bladder cancer (5). Several epidemiological studies showed an ~2-fold increased bladder cancer risk among first-degree relatives of bladder cancer patients (6,7) and consistent associations between common genetic variations and bladder cancer risk (8). Prediction supplemented by segregation analysis in 1193 families indicated it is probably that there is no high-penetrance gene controlling the carcinogenesis of bladder cancer (9). Instead, there are probably many low-penetrance genes with a weak to moderate effects, which may interact with each other and environmental risk factors to cause cancer (10–16). Previous candidate gene studies for bladder cancer risk have identified two of the most consistent low-penetrance cancer susceptibility loci (8): *GSTM1* null genotype and *NAT2* slow acetylator genotype. Individually, these loci contributed only modestly to an elevated bladder cancer risk. More evidence was found in a recent genome-wide association study that identified two additional non-genic susceptible loci, rs9642880 on chromosome 8q24 and rs710521 on chromosome 3q28. These two loci were associated with an ~20% elevated bladder cancer risk (17). Meanwhile, numerous studies have reported that common genetic variations in critical cellular pathways may affect an individual's risk of developing bladder cancer, including polymorphisms in genes involved in carcinogen metabolism (8,18), DNA repair (19–21), cell cycle control (22,23) and inflammation (24).

The phosphoinositide-3 kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway is a major pathway controlling cell growth and tumorigenesis (25). Cell growth signals are sensed by receptor tyrosine kinases, such as the insulin growth factor receptor on the cell membrane. When insulin growth factor receptors are activated through ligand binding and autophosphorylation, insulin receptor substrate is attached to the receptor and initiates a kinase cascade through activation of PI3K. PI3K then further phosphorylates a second messenger, phosphatidylinositol (3,4,5)-trisphosphate. The tumor suppressor phosphatase and tensin homolog can reverse this step and stop signaling through this pathway. Phosphatidylinositol (3,4,5)-trisphosphate then binds to the v-akt murine thymoma viral oncogene (AKT), and anchors it to cell membrane, where AKT is phosphorylated and activated by PI3K-dependent kinases 1 and 2. Activated AKT can directly or indirectly inhibit the tuberous sclerosis protein TSC2. The formation of a TSC1/2 complex inhibits Ras homolog enriched in brain activity that is required for activation of the mTOR complex 1 which includes mTOR, regulatory associated protein of mTOR (RAPTOR) and G protein beta subunit-like protein (GβL/mLST8). Activation of this complex is required for further activation of the ribosomal protein ribosomal protein S6 kinase and release of the translation initiation factor 4E to initiate messenger RNA translation of many important genes (26).

Somatic aberrations of PI3K-AKT-mTOR pathway genes have been commonly observed in a variety of malignancies; therefore, this pathway has been extensively investigated as a mechanism in tumorigenesis and as a target for cancer therapy (25). In light of the critical role of the PI3K-AKT-mTOR pathway in maintaining proper cellular function, it is probably that genetic variations in this pathway may affect bladder cancer risk. However, to date there have been no studies addressing the role of common, germ line genetic variations in this pathway as cancer susceptibility factors. Therefore, we applied a comprehensive pathway-based approach to systemically evaluate single-nucleotide polymorphisms (SNPs) in the PI3K-AKT-mTOR pathway as predictors of bladder cancer risk in a case–control study.

Abbreviations: CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; PI3K, phosphoinositide-3 kinase; RAPTOR, regulatory associated protein of mTOR; SNP, single-nucleotide polymorphism.

Materials and methods

Case–control design and population

Bladder cancer cases were recruited from The University of Texas M. D. Anderson Cancer Center and Baylor College of Medicine as a part of an ongoing case–control study since 1999. Cases were all newly diagnosed, histologically confirmed and previously untreated bladder cancer. Controls were healthy people without prior cancer history (except for non-melanoma skin cancer) who were identified through Kelsey-Seybold Clinics, the largest multispecialty physician group in the Houston metropolitan area. Controls were matched to cases on age (±5 years), gender and ethnicity. The procedure of case and control recruitment was described previously (23). Written informed consent was obtained from each participant before the collection of epidemiological data and blood samples. The response rate was 92% for cases and 75% for controls.

Epidemiology data collection

In a 45 min interview, M. D. Anderson interviewers collected data on demographics, family history and smoking status. Individuals who had smoked >100 cigarettes in their lifetime were defined as 'ever-smokers'; the remainders were considered 'never-smokers'. Smokers include current smokers and former smokers. Individuals who had quit smoking at least 1 year before diagnosis (for cases) or prior to the interview (for controls) were categorized as former smokers. Immediately after each interview, a 40 ml blood sample was collected into heparinized tubes for lymphocyte isolation and DNA extraction. All the human participation procedures were approved by The University of Texas M. D. Anderson Cancer Center, Baylor College of Medicine and the Kelsey-Seybold institutional review boards.

Selection of polymorphisms in the PI3K-AKT-mTOR pathway

We compiled our gene list together with other ongoing projects using SNPs3D bioinformatic tools (http://www.snps3d.org), which is a web-based literature mining approach to select genes according to a set of user-defined query terms of human diseases or biological processes (27). Then, we performed a literature review to refine the gene list for the PI3K-AKT-mTOR pathway. We identified tagging SNPs from the HapMap database (http://www.hapmap.org). All selected SNPs met the following criteria: $r^2 \geq 0.8$, minor allele frequency (MAF) ≥0.05 in Caucasians and within 10 kb upstream of the 5′ untranslated region and 10 kb downstream of the 3′ untranslated region of the gene. In addition, we also included potentially functional SNPs with MAF > 0.01 (e.g. coding SNPs and SNPs in untranslated region, promoter and splicing site). We also supplemented 112 SNPs in this pathway previously genotyped as part of our genome-wide association study with the same subjects in order to comprehensively screen genetic variation within this pathway. A total of 248 SNPs from 19 PI3K-AKT-mTOR pathway genes were initially identified. However, in the data analysis, 17 SNPs were removed for the following reasons: low call rate (<90%), departure from Hardy–Weinberg equilibrium ($P < 0.01$) in control subjects and MAF < 0.01 in our study population, which may differ from the population of HapMap project. Therefore, 231 SNPs of the PI3K-AKT-mTOR pathway genes were included in the statistical analysis.

Genotyping

Genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood Maxi Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The genotyping of the above non-genome-wide association study PI3K-AKT-mTOR pathway SNPs, together with other cancer-related pathway polymorphisms, was done using Illumina's iSelect custom SNP array platform according to the manufacturer's Infinium II assay protocol (Illumina, San Diego, CA) with 750 ng of input DNA for each sample. All genotyping data were analyzed and exported using BeadStudio software (Illumina). The average call rate for the SNP array was 99.7%.

Statistical analysis

Most statistical analyses were performed using the Intercooled Stata 10 statistical software package (StataCorp College Station, TX). Pearson's χ^2 test was used to compare the difference in distribution of categorical variables (sex and smoking status) and either the Wilcoxon rank-sum test or the Student's *t*-test was used for continuous variables (age and cigarette smoking pack-years) where appropriate. Hardy–Weinberg equilibrium was tested by a goodness-of-fit χ^2 test to compare the observed genotype frequencies to the expected genotype frequencies in controls. We excluded SNPs with a departure from Hardy–Weinberg equilibrium in controls ($P < 0.01$) in the subsequent analysis.

For the main effect of SNPs, unconditional logistic regression was conducted to calculate odds ratios (ORs) and 95% confidence intervals (CIs), adjusting for potential confounders (age, gender, smoking status and pack years). For each SNP, we test its cancer association in dominant, additive and recessive models. We defined the model with most significant *P* value as best model. Only the result predicted by the best model was reported and considered in the subsequent analysis.

We performed the bootstrap resampling method to internally validate the results. Stratified analysis was used to assess the interaction between age, gender, smoking status, pack years and individual genotypes. We defined young subjects as <65 years old and old subjects ≥65 years old (the median age of controls); light smoker are subjects with <18.5 pack years and heavy smoker are subjects with ≥18.5 pack years (the median pack years in controls). All statistical analyses were two sided.

For genes with several haplotype blocks, we used the Haploview 4.1 software (Broad Institute, Cambridge, MA) to divide the genes into multiple haplotypes by importing our own genotyping data. Haplotypes and diplotypes were inferred using the expectation–maximization algorithm implemented in the HelixTree software (Golden Helix, Bozeman, MT). Haplotypes with a probability of <1% were combined together for the data analysis. The adjusted ORs and 95% CIs for haplotypes and diplotypes were assessed using multivariate logistic regression.

For the cumulative effect of multiple variants, adverse genotypes were summed and categorized by the number of adverse genotypes in subjects. Using the group of subjects without any unfavorable genotypes as the reference, the ORs and 95% CIs were calculated for the other groups using unconditional multivariate logistic regression adjusted for age, gender, smoking status and pack years. Since many SNPs and tests were incorporated in the analysis, the nonparametric multiple comparison *Q*-test (28) was used to adjust the significance level for individual polymorphisms.

Results

Characteristics of the study population

The demographic data of 803 urinary bladder cancer cases and 803 controls are presented in Table I. Due to the small size of the minority participants, our analysis was restricted to non-Hispanic Caucasians. There were no differences in the distribution of gender and age between cases and controls, with 80% of the population being male. The mean age was 64.7 (SD: 11.1) in cases and 63.8 (SD: 10.9) in controls. More ever-smokers (591 versus 448, $P < 0.001$) with longer smoking duration (median pack-years: 38 versus 22.5, $P < 0.001$) were observed in cases than controls.

Risks associated with the individual variant genotype

A total of 231 SNPs within 19 genes in the PI3K-AKT-mTOR pathway were analyzed. We assessed the association of each individual SNP with bladder cancer risk under dominant, recessive and additive models. We defined the model with most significant *P* value as best model. Only the result predicted by the best model reported (Table II) and considered in the subsequent analysis. Twenty-four potential significant associations were identified based on the *P* value <0.05

Table I. Characteristics of bladder cancer cases and controls

	Case	Control	P^a
Gender			
Male	639 (79.6%)	639 (79.6%)	1
Female	164 (20.4%)	164 (20.4%)	
Age			
Mean (SD)	64.7 (11.1)	63.8 (10.9)	0.1
Smoking status			
Never	212 (26.4%)	355 (44.2%)	**<0.001**
Former	404 (50.3%)	381 (47.5%)	
Current	187 (23.3%)	67 (8.3%)	
Ever	591 (73.6%)	448 (55.8%)	**<0.001**
Pack year[b], *N*	590	447	
Median (range)	38 (0.05–176)	22.5 (0.05–165)	**<0.001**

Significant *P* values <0.05 were in bold.
[a]*P* values were derived from the χ^2 test for categorical variables, such as gender and smoking status; rank-sum test for pack year and *t*-test for age.
[b]Among ever-smokers.

(Table II). Of particular interest, 17 significant associations were observed for SNPs in the gene encoding RAPTOR. In addition, two SNPs in *AKT3*, one SNP in *IRS2*, one SNP in *RHEB*, one in *RPS6KA5* and two linked SNPs in *TSC2* also reached significance. After multiple comparison adjustment by the *Q*-test, four variants in *RAPTOR*, rs11653499 (OR: 1.79, 95% CI: 1.24–2.60, $P = 0.002$), rs7211818 (OR: 2.13, 95% CI: 1.35–3.36, $P = 0.001$), rs7212142 (OR: 1.57, 95% CI: 1.19–2.07, $P = 0.002$) and rs9674559 (OR: 2.05, 95% CI: 1.31–3.21, $P = 0.002$) remained significant at a false discovery rate of 5%. To internally validate the associations, we performed bootstrap sampling. The overall ORs and 95% CIs generated by bootstrapping were consistent with our initial results. Table II lists the number of times that the bootstrap-generated *P* value was <0.05, 0.01 or 0.005 for each SNP. For the four *RAPTOR* SNPs in 100 bootstrap samplings, each of the SNPs reached significance at the $P < 0.01$ level in >75% of the samplings. This indicates that the results for these SNPs are highly unlikely to be due to chance alone.

To explore potential interactions between genetic variants in *RAPTOR* and age, gender, smoking status and pack years, we performed stratified analysis for the four polymorphisms of interest (Supplementary Table 1 is available at *Carcinogenesis* Online). The results were more evident in the subgroup of old subjects, male subjects, never-smokers and light smokers, compared with young subjects, female subjects, ever-smokers and heavy smokers. We also assessed the interaction using the likelihood-ratio test. The gene-environment interaction between four *RAPTOR* SNPs and light smokers were significant (*P* for interaction <0.05).

Haplotype and diplotype analysis

We performed haplotype and diplotype analysis for all *RAPTOR* SNPs genotyped in this study (Tables III and IV). As shown in Table III, three haplotype blocks were identified in *RAPTOR*. Two haplotypes were significantly associated with bladder cancer risk compared with the most common haplotypes in our population. Block 2 is composed of SNPs rs7212142-rs12939076 and the haplotype H2 of this block, containing the wild-type G allele of rs7212142 in combination with the C allele for rs12939076, showed a significant protective effect (OR: 0.83, 95% CI: 0.70–0.97, $P = 0.022$) compared with the most common haplotype with variant A allele of rs7212142. In the diplotype analysis, compared with diplotypes without the protective haplotype H2, an increase in the number of H2 haplotypes resulted in a stronger protective effect (*P* for trend = 0.039, Table IV). Block 3 contains 15 SNPs (rs1485330-rs7217223-rs4889875-rs35544492-rs9901366-rs7501659-rs9906827-rs7208502-rs12948054-rs4062178-rs7211818-rs6565478-rs12603074-rs9915378-rs9674559). Compared with the most frequent haplotype, haplotype H2 of this block showed a 1.32-fold (95% CI: 1.09–1.60, $P = 0.004$) increase in bladder cancer risk. This haplotype contains two of the potential risk variant alleles identified in the single SNP analysis: rs7211818 and rs9674559. Compared with the diplotypes without haplotype H2 of block 3, the diplotype with one and two H2 haplotypes showed 1.16-fold (95% CI: 0.93–1.46, $P = 0.182$) and 2.23-fold (95% CI: 1.38–3.60, $P = 0.001$) increased bladder cancer risk with a significant *P* for trend of 0.003.

Combined effect of unfavorable genotypes

To evaluate the combined effect of multiple SNPs associated with risk, we summed the unfavorable genotypes of each individual and analyzed the resulting bladder cancer risk. Only one SNP with the most significant association, identified in the initial main effect assessment, was selected as a representative if there were several SNPs within the same haplotype block. Therefore, three significant SNPs of *RAPTOR*

Table II. Significant SNPs associated with bladder cancer risk

SNP	Gene	Genotype (Number of case/control)				HWE[a]	OR (95%CI)[d]	*P*	Best Model[b]	Bootstrap *P* Counts[c]		
		ww	wv	vv	MAF	*P*				<0.05	<0.01	<0.005
rs12045585	*AKT3* (G > A)	632/592	152/198	18/13	0.12/0.14	0.44	0.75 (0.59–0.95)	0.019	DOM	68	46	29
rs2994329	*AKT3* (G > A)	514/509	246/267	41/27	0.20/0.20	0.26	1.72 (1.03–2.87)	0.039	REC	49	31	19
rs717775	*RHEB* (A > C)	406/415	315/331	82/56	0.30/0.28	0.37	1.55 (1.06–2.25)	0.022	REC	65	36	28
rs8018757	*RPS6KA5* (G > A)	225/245	368/390	210/168	0.49/0.45	0.58	1.29 (1.02–1.65)	0.037	REC	56	35	26
rs2073636	*TSC2* (G > A)	275/315	397/379	130/108	0.41/0.37	0.74	1.22 (1.05–1.42)	0.01	ADD	72	54	48
rs8063461	*TSC2* (G > A)	272/313	393/360	123/108	0.41/0.37	0.47	1.31 (1.05–1.62)	0.014	DOM	67	50	37
rs9515120	*IRS2* (G > A)	699/721	100/80	4/2	0.07/0.05	0.89	1.46 (1.07–1.98)	0.016	ADD	66	41	30
rs1062935	*RAPTOR* (A > G)	210/203	369/407	210/171	0.50/0.48	0.60	1.28 (1.01–1.63)	0.045	REC	61	30	22
rs11653499	***RAPTOR* (G > A)**	**390/434**	**312/294**	**87/54**	**0.31/0.26**	0.49	**1.79 (1.24–2.60)**	**0.002**[e]	REC	**95**	**86**	**80**
rs2589143	*RAPTOR* (C >A)	472/505	263/232	42/36	0.22/0.20	0.10	1.26 (1.02–1.56)	0.035	DOM	65	36	26
rs2672890	*RAPTOR* (G > A)	539/491	216/260	34/30	0.18/0.20	0.68	0.78 (0.62–0.97)	0.023	DOM	69	44	34
rs4889875	*RAPTOR* (G > A)	346/340	355/334	88/108	0.34/0.35	0.03	0.73 (0.53–1.00)	0.048	REC	50	24	16
rs4969230	*RAPTOR* (A > G)	306/277	369/374	114/131	0.38/0.41	0.45	0.86 (0.74–0.99)	0.042	ADD	61	43	25
rs4969444	*RAPTOR* (G > A)	518/534	228/226	43/22	0.20/0.17	0.86	1.94 (1.13–3.34)	0.017	REC	65	43	36
rs7208536	*RAPTOR* (G > A)	420/441	302/300	67/41	0.28/0.24	0.39	1.70 (1.12–2.58)	0.012	REC	85	60	48
rs7211818	***RAPTOR* (A > G)**	**462/487**	**267/263**	**60/32**	**0.25/0.21**	0.78	**2.13 (1.35–3.36)**	**0.001**[e]	REC	**98**	**84**	**74**
rs7212142	***RAPTOR* (G > A)**	**270/291**	**364/380**	**155/111**	**0.43/0.39**	0.78	**1.57 (1.19–2.07)**	**0.002**[e]	REC	**95**	**76**	**67**
rs7219896	*RAPTOR* (A > G)	518/533	228/226	43/23	0.20/0.17	0.99	1.85 (1.09–3.16)	0.024	REC	62	36	31
rs8071015	*RAPTOR* (A > C)	390/432	311/292	88/58	0.31/0.26	0.25	1.25 (1.07–1.47)	0.005	ADD	91	76	67
rs9674559	***RAPTOR* (A > G)**	**462/485**	**266/262**	**60/33**	**0.25/0.21**	0.91	**2.05 (1.31–3.21)**	**0.002**[e]	REC	**97**	**76**	**65**
rs9890502	*RAPTOR* (G > A)	395/435	307/290	87/57	0.30/0.26	0.25	1.69 (1.17–2.43)	0.005	REC	91	77	63
rs9894401	*RAPTOR* (G > A)	410/433	309/299	70/50	0.28/0.26	0.93	1.48 (1.01–2.19)	0.047	REC	73	41	32
rs9897968	*RAPTOR* (G > A)	502/459	243/278	44/44	0.21/0.23	0.65	0.79 (0.64–0.98)	0.033	DOM	51	39	35
rs9915378	*RAPTOR* (G > A)	350/345	352/329	87/108	0.33/0.35	0.01	0.72 (0.52–0.98)	0.038	REC	58	27	19

vv, homozygous variant genotype; ww, homozygous wild-type genotype; wv, heterozygous variant genotype. SNPs remain significant after multiple comparison by *Q*-test were in bold.

[a]HWE: Hardy–Weinberg Equilibrium was tested by a goodness-of-fit χ^2 test to compare the observed genotype frequencies to the expected genotype frequencies in controls.

[b]DOM (dominant model): ww versus (wv and vv); REC (recessive model): (ww and wv) versus vv; ADD (additive model): P for the trend with increasing variant alleles (v). We defined the model with the most significant *P* value as the best model for each SNP.

[c]Bootstrapping was conducted 100 times for each SNP.

[d]Adjusted by age, gender, smoking status and pack year using unconditional logistic regression.

[e]Remain significant after multiple comparison adjustment by *Q*-test.

Table III. Haplotype analysis of significant *RAPTOR* SNPs

RAPTOR		Haplotype	Number of haplotype		OR (95% CI)[a]	*P* value
			Case (%)	Control (%)		
Block 1	H1	AGGGAGAGACA	390 (24.78)	409 (26.29)	Ref.	
	H2	AAAGAAAAACC	471 (29.92)	392 (25.19)	1.10 (0.92–1.31)	0.311
	H3	GGAAGGAGCAA	299 (19.00)	308 (19.79)	0.99 (0.80–1.23)	0.925
	H4	AAAGGGCGCAA	181 (11.50)	181 (11.63)	1.03 (0.80–1.33)	0.814
	H5	AAAGGGAGCCA	127 (8.07)	146 (9.38)	0.86 (0.65–1.13)	0.280
	H6	AGAAGGAGCAA	43 (2.73)	49 (3.15)	0.95 (0.61–1.50)	0.829
	H7	AGAGAGAGACA	12 (0.76)	20 (1.29)	0.63 (0.30–1.34)	0.234
	H8	GGAAGGAGCCA	16 (1.02)	9 (0.58)	1.71 (0.73–4.03)	0.220
	H9	Others	35 (2.22)	42 (2.70)	0.88 (0.55–1.42)	0.608
Block 2	H1	AC	674 (42.71)	602 (38.49)	Ref.	
	H2	GC	549 (34.79)	595 (38.04)	**0.83 (0.70–0.97)**	**0.022**
	H3	GA	355 (22.50)	367 (23.47)	0.91 (0.75–1.09)	0.307
Block 3	H1	GGAAGGGGGAAAGAA	523 (33.23)	537 (34.42)	Ref.	
	H2	GAGAGGAAGAGGGGG	371 (23.57)	302 (19.36)	**1.32 (1.09–1.60)**	**0.004**
	H3	AGGAGGAAGAAGAGA	222 (14.10)	207 (13.27)	1.13 (0.90–1.42)	0.290
	H4	GGGTGAGGGGAGGGA	172 (10.93)	168 (10.77)	1.13 (0.88–1.46)	0.333
	H5	AGGAGGAAAAAGAGA	138 (8.77)	168 (10.77)	0.94 (0.72–1.22)	0.647
	H6	GAGAAGGGGGAGGGA	116 (7.37)	134 (8.59)	0.90 (0.68–1.19)	0.463
	H7	GGGAGGAAGAGGGGG	11 (0.70)	19 (1.22)	0.65 (0.30–1.40)	0.272
	H8	Others	21 (1.33)	25 (1.60)	0.85 (0.46–1.59)	0.613

Block 1: rs12937147-rs7209040-rs4889863-rs12951309-rs12949279-**rs11653499**-rs4890055-rs9890502-rs7503807-rs901065-rs8071015. Block 2: **rs7212142**-rs12939076. Block 3: rs1485330-rs7217223-rs4889875-rs35544492-rs9901366-rs7501659-rs9906827-rs7208502-rs12948054-rs4062178-**rs7211818**-rs6565478-rs12603074-rs9915378-**rs9674559**. Significant associations and significant SNPs found were in bold.
[a]Adjusted by age, gender, smoking status and pack year.

Table IV. Diplotype analysis of significant haplotypes *RAPTOR* SNPs

RAPTOR	Haplotype H2	Number of subject		OR (95% CI)[a]	*P* value
	No.	Case (%)	Control (%)		
Block 2	0	358 (44.58)	324 (40.35)	Ref.	
	1	341 (42.47)	363 (45.21)	**0.80 (0.65–1.00)**	**0.054**
	2	104 (12.95)	116 (14.45)	0.76 (0.56–1.05)	0.100
				***P* for trend**	**0.039**
Block 3	0	487 (60.65)	530 (66.00)	Ref.	
	1	261 (32.50)	244 (30.39)	1.16 (0.93–1.46)	0.182
	2	55 (6.85)	29 (3.61)	**2.23 (1.38–3.60)**	**0.001**
				***P* for trend**	**0.003**

Block 2: **rs7212142**-rs12939076. Block 3: rs1485330-rs7217223-rs4889875-rs35544492-rs9901366-rs7501659-rs9906827-rs7208502-rs12948054-rs4062178-**rs7211818**-rs6565478-rs12603074-rs9915378-**rs9674559.** Significant associations and significant SNPs found were in bold.
[a]Adjusted by age, gender, smoking status and pack year

Table V. Cumulative effect of unfavorable genotypes

Number of unfavorable SNPs[a]	Case (%)	Control (%)	OR (95% CI)[b]	*P*
0	641 (79.83)	689 (85.80)	Ref.	
1	68 (8.47)	56 (6.97)	1.34 (0.91–1.97)	0.137
2	48 (5.98)	33 (4.11)	1.63 (1.01–2.64)	0.045
3	46 (5.73)	25 (3.11)	2.22 (1.33–3.71)	0.002
For each unfavorable SNP	*P* for trend			<0.001

[a]Unfavorable genotypes were potentially hazard genotypes. We used the genotyping data and haploview software to determine the haplotype block structure of the RAPTOR gene. The three SNPs included in this combined analysis were in different haplotype blocks. For SNPs in the same haplotype block, we just selected one with the most significant elevated bladder cancer risk. Unfavorable genotypes: *RAPTOR: rs11653499 (vv)*, *rs7212142 (vv)*, *rs7211818 (vv).*
[b]Adjusted by age, gender, smoking status and pack year.

[rs11653499 (vv), rs7212142 (vv) and rs7211818 (vv)], all of which had a false discovery rate < 0.05 and were located in different haplotype blocks defined in our haplotype analysis. For *RAPTOR* rs7211818 (vv) and rs9674559 (vv), which are within same haplotype block, we only selected one [rs7211818 (vv)] as a representative for this analysis. As shown in Table V, we divided the study into four subgroups based in the number of unfavorable genotypes. We found a significant gene-dosage effect for increasing bladder cancer risk, with an increasing number of unfavorable genotypes (*P* for trend <0.001). Compared with Group 1, Groups 2 through 4 exhibited a progressively increased bladder cancer risk with ORs of 1.34 (95% CI: 0.97–1.97), 1.63 (95% CI: 1.01–2.64) and 2.22(95% CI: 1.33–3.71), respectively.

Discussion

This study systematically evaluated the association between a comprehensive set of polymorphisms in the PI3K-AKT-mTOR pathway genes and bladder cancer. The major finding was the significant association of four polymorphisms in *RAPTOR* with elevated bladder cancer risk, especially among male and older populations. Moreover, the combined effect of multiple potential SNPs showed a gene-dosage trend in a pathway-based polygenic approach.

Although the relationship between the PI3K-AKT-mTOR pathway and cancer has been established by numerous previous studies, the function of RAPTOR within the tumorigenesis process remains unclear. RAPTOR is a scaffold protein interacting with mTOR, eukaryotic translation initiation factor 4E binding protein 1 and ribosomal protein S6 kinase. In response to nutrient and growth factor signals, RAPTOR catalyzes mTOR phosphorylation required for ribosomal protein S6 kinase activation (29–31). However, a potential disease-related polymorphism, rs2019154, in intron 3 has been reported to be associated with psoriasis (32). In this study, we interrogated SNPs within a 440 kb region that included and surrounded *RAPTOR*. Four intronic polymorphisms (rs11653499, rs7211818, rs7212142 and rs9674559) were significantly associated with increased bladder cancer risk, among which the polymorphisms rs7211818 and rs9674559 were located within the same haplotype block (Tables II–IV). The

observed robust associations remained significant after adjustment for multiple comparisons, suggesting that the finding was unlikely due to chance alone. The MAFs of these four SNPs ranges from 21 to 42% in both cases and controls, which indicates this effect will probably influence bladder cancer risk for a large fraction of population.

The SNP rs11653499 is located within intron 1 of *RAPTOR*, a region with a high regulatory potential based on sequence alignments among different species identified using the UCSC Genome Browser (http://genome.ucsc.edu) (33). This indicates that this SNP may disrupt a potential *cis*-regulatory module affecting gene transcription and translation. Further study is needed to address the function of rs11653499. It is also probable that this SNP is merely a tagging SNP that is in linkage disequilibrium with the real causal SNP. The real causal SNP may be located in the coding region and affect the protein function at the posttranslational level. Previous studies suggested that the activity of RAPTOR is modified through posttranslational modifications such as phosphorylation events (34) and through its interaction with various other proteins in this pathway (29). Therefore, fine mapping will be necessary to identify the causal variant.

Cancer, as a multifactorial disease, requires the interaction of many genetic and environmental factors. This has also been observed in bladder cancer. For example, there is a significant interaction between *NAT2* genotype and smoking in elevating bladder cancer risk (8). Lin *et al.* (35) showed that familial history of cancer and smoking jointly contributed to a 5.3-fold increased bladder cancer risk . Other studies have shown that the carcinogen in tobacco can potently activate the PI3K-AKT-mTOR pathway (36).

Interestingly, a significant interaction was detected between the four significant SNPs of *RAPTOR* and light smokers when we dichotomized the smokers based on the distribution of the pack years in controls (Supplementary Table 1 is available at *Carcinogenesis* Online). Although the significance may be influenced by the small sample size in this subgroup of light smokers, the finding is consistent with previous reports (37,38). We hypothesize that the strong environmental exposure of increased pack years smoking may be overpowering the contributions of genetic variation to bladder cancer susceptibility.

Given the large number and close physical proximity of tagging SNPs in *RAPTOR*, we did haplotype and diplotype analysis for the four potential risk polymorphisms. The results of the haplotype analysis were consistent with the individual effect of the potential risk conferring polymorphisms and confirmed our findings in individual SNP analysis that variants of rs7212142, rs9915378 and rs9674559 are associated with increased bladder cancer risk.

Although the individual SNPs analyzed in this pathway exhibited only moderate bladder cancer risk, we found a strong cumulative effect of multiple SNPs (Table V). The pathway-based cumulative effect analysis is able to amplify the modest effect of each individual SNP and enhance the predictive power. The identification of multiple risk variants may therefore improve risk prediction and could conceivably be applied to assess an individual's bladder cancer risk.

This is a hospital-based case–control study and selection bias may exist since the controls from a clinic may not be ideal representatives of the geographically matched population with similar environmental exposure. We compared our control with local population where cases were from and found no difference in the social economic status. Recall bias is always a concern for retrospective study. But genetic factors are the major focus instead of environment factor in this association study and the effect of recall bias on genetic susceptibility is limited. Patients with muscle-invasive disease are overpresented in this study since M. D. Anderson is a tertiary referral center; however, there were no evidence to suggest that there is different etiology between muscle invasive and superficial bladder cancer patients. Finally, although we adjusted for multiple testing, it is still probable that our findings are false positives. Validation in independent population is necessary to confirm these associations.

In conclusion, this is the first study to evaluate germ line genetic variations in the PI3K-AKT-mTOR pathway and cancer risk. We identified four SNPs in *RAPTOR* significantly associated with increased bladder cancer risk, found a significant gene-dosage effect, and detected potential gene-environment interaction. The identification of novel genetic susceptibility markers for bladder cancer etiology will not only help us understand the biology of bladder carcinogenesis but may also be integrated with known clinical, epidemiological and genetic risk factors to help us identify individuals at high risk for developing bladder cancer.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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