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Genetic variation in the prostate stem cell antigen gene *PSCA* confers susceptibility to urinary bladder cancer

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AUTHOR CONTRIBUTIONS

Texas: X.W. conceived this study and established the M.D. Anderson bladder cancer study, supervised laboratory and statistical analyses and wrote the initial draft of the manuscript. Y.Y. supervised and performed statistical analyses. C.P.D., B.C. and H.B.G. were involved in patient recruitment. J.L. was involved in epidemiologic data collection and database management. D.W.C performed *in vitro* assays. T.M., G.B.M. and K.S.H. were involved in the validation genotyping. C.I.A. provided guidance in statistical analyses, assisted in the initial development of the research and contributed in manuscript preparation. J.G. was involved in the development of the research and study design, oversaw genotyping and *in vitro* assays and wrote the initial draft of the manuscript. Other sites: L.A.K. and T.R. organized and supervised the replication efforts in European populations. P.S. performed primary statistical analysis of European populations. G.M., A.E.K., D.T.B., P.V., S. Porru, F.B., E.K., M.P.Z., R.K., P.R., E.G., K.K., J.I.M., M.S., B.S., A.L., P.d.V., G.S., S.G., S. Polidoro, S.T., U.T., K.K.H.A., J.A.W. and K.S. were involved in the subject ascertainment, DNA collection or data collection of European populations. A.S.A., A.S., Z.-f.Z. and S.-C.C. were involved in the subject ascertainment, DNA collection or data collection of US populations. D.S. was involved in the study design and data interpretation. N.S. and T.Y. provided reporter constructs of *PSCA* promoters and were instrumental in studying and discussing the function of PSCA and rs2294008. M.R.K. assisted in the initial development of the research, established the New Hampshire bladder cancer case control study and contributed to manuscript preparation. All authors contributed to the final paper.

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Abstract

We conducted a genome-wide association study on 969 bladder cancer cases and 957 controls from Texas. For fast-track validation, we evaluated 60 SNPs in three additional US populations and validated the top SNP in nine European populations. A missense variant (rs2294008) in the *PSCA* gene showed consistent association with bladder cancer in US and European populations. Combining all subjects (6,667 cases, 39,590 controls), the overall *P*-value was 2.14×10^{-10} and the allelic odds ratio was 1.15 (95% confidence interval 1.10–1.20). rs2294008 alters the start codon and is predicted to cause truncation of nine amino acids from the N-terminal signal sequence of the primary *PSCA* translation product. *In vitro* reporter gene assay showed that the variant allele significantly reduced promoter activity. Resequencing of the *PSCA* genomic region showed that rs2294008 is the only common missense SNP in *PSCA*. Our data identify rs2294008 as a new bladder cancer susceptibility locus.

Bladder cancer is the fourth most common cancer in men in the United States, with an estimated 68,810 new cases and 14,410 deaths from this disease in 2008 (ref. 1). The main environmental risk factors for bladder cancer are cigarette smoking and occupational exposure. There is also compelling evidence for a genetic component to the etiology of bladder cancer. In a large twin study, it was estimated that inherited genetic susceptibility contributes to 31% of bladder cancer risk[2]. Case reports have described familial clustering of bladder cancer[3]. Epidemiological studies showed that the risk of the disease increased by 50%–100% in first-degree relatives of individuals with bladder cancer[4–7]. However, genetic loci that account for most familial risk of bladder cancer remain elusive. A recent segregation analysis suggested a 'no major gene' model[8]. Candidate gene association studies have shown that *NAT2* slow acetylator and *GSTM1* null genotypes are associated with increased bladder cancer risks[9]. A recent genome-wide association study (GWAS) identified two bladder cancer susceptibility loci[10]. To identify additional bladder cancer susceptibility loci, we conducted a GWAS using the Illumina HumanHap610 Beadchip.

We performed the primary screen using an ongoing case-control study in Texas. We restricted study participants to self-reported Caucasians to minimize confounding by ethnicity. After applying strict quality control criteria (see Online Methods for details) to remove problematic samples and SNPs, we analyzed 556,429 SNPs in 969 cases and 957 controls. A quantile–quantile plot of observed versus expected χ^2 test statistics showed no evidence for inflation of chi-squared tests (inflation factor $\lambda = 1.002$; Supplementary Fig. 1). None of the SNPs reached genome-wide significance at this stage (Supplementary Fig. 2). After we removed highly linked SNPs, three SNPs had a *P*-value < 10^{-5} and 50 SNPs showed a *P*-value < 10^{-4} (Supplementary Table 1). We adjusted the results for population substructure using Cochran-Mantel-Haenszel tests, eigenvectors and permutation tests, and the *P*-values were consistent (Supplementary Table 1).

We used three more US sets to perform a fast-track replication of the top 50 SNPs ($P < 10^{-4}$) and the top 10 additional SNPs in 8q24 ($P < 5 \times 10^{-3}$), a region associated with genetic susceptibility to several cancers[10–14]. One SNP (rs2294008) was consistent across the US discovery and the replication sets ($P = 7.34 \times 10^{-4}$ and 3.53×10^{-5}, respectively; Table 1). The *P*-value was 1.07×10^{-7} in the combined US populations (Supplementary Table 2). We next used nine European populations to replicate this SNP. The overall *P*-value for the combined European populations was 9.83×10^{-5}. Combining all US and European subjects (6,667 cases, 39,590 controls), the *P*-value was 2.14×10^{-10} (Table 1). The allelic odds ratio (OR) was 1.15 (95% confidence interval (CI), 1.10–1.20). There was no significant heterogeneity among the ORs of all the populations (*P* for heterogeneity = 0.423). We also performed a multivariable logistic regression analysis, adjusting for age, gender and smoking status (5,038 cases and 9,363 controls, excluding three European populations with missing individual age or smoking data for controls; Table 2). The OR for individuals carrying one copy of the variant allele (T) was 1.30 (95% CI, 1.18–1.42) and for those carrying two copies was 1.40 (95% CI, 1.25–1.56). We tested the equality of the ORs for heterozygous and homozygous carriers of the variant allele and observed a nonsignificant *P*-value of 0.14, suggesting a dominant model. The OR for individuals carrying at least one variant allele was 1.33 (95% CI, 1.22–1.45). We also performed stratified analyses for age, gender and smoking status and observed similar associations across different strata (Table 2). The association was also similar in superficial and invasive bladder cancer (for US populations only; data not shown). The risk allele (T) frequency is 46%, and individuals carrying the homozygous risk genotype (TT) account for about 22% of the control population.

rs2294008 is a missense SNP located in exon 1 of the *PSCA* gene. Linkage disequilibrium (LD) analysis of all HapMap SNPs in the vicinity of rs2294008 showed that it maps to an 11-kb LD block on chromosome 8q24 (Fig. 1). We imputed genotypes within 1 Mb of rs2294008 for SNPs in the HapMap database but not on the HumanHap610 chip. rs2294008 was among the top SNPs showing the strongest association (Fig. 1a). To identify unknown variants, we resequenced the genomic region of *PSCA* in 106 individuals of European ancestry. We found 27 SNPs, 23 of which had a minor allele frequency (MAF) > 5% (Supplementary Table 3). Several of these SNPs are listed in the dbSNP database, but this study is the first to validate them (SNPs no. 4, no. 5, no. 9 and no. 16). We also validated an insertion/deletion polymorphism (no. 7) and identified two new low-frequency SNPs (no. 1 and no. 2). All of the high frequency SNPs are in strong LD ($D' > 0.9$) with rs2294008. We genotyped seven of these SNPs in our discovery set and observed nearly identical ORs compared to rs2294008 (Supplementary Table 4).

A recent study[15] found that the T allele of rs2294008 resulted in a significant reduction in transcriptional activity of the *PSCA* promoter in gastric cell lines. We obtained four constructs representing the top four 5′ upstream haplotypes (including promoter and exon 1

region) in our populations (Fig. 2). Except for the wild-type haplotype, the other three haplotypes all contained the variant T allele at rs2294008. We found that in three different bladder cancer cell lines (UC1, UC3 and UC13) the T allele-containing haplotypes showed significantly lower promoter activity ($P < 0.001$ for all comparisons between wild-type and the other three constructs; Fig. 2b). Furthermore, substitution of a single nucleotide in the wild-type haplotype (rs2294008 C to T) significantly reduced promoter activity, whereas a single substitution of rs2294008 T to C in UP-H1 increased promoter activity (Fig. 2c). These results are in complete concordance with data in gastric cancer[15], providing compelling evidence that rs2294008 is a functional variant *in vitro*. We then used real-time PCR to detect *PSCA* mRNA expression in nine bladder cancer cell lines. We found that UC9 (TT genotype) had the highest expression, whereas three CT-genotype cell lines (UC1, UC5 and UC7) showed intermediate expression, and three CC genotype cell lines (UC3, UC13 and UC17) and two TT cell lines (UC12 and UC18) showed very low expression (data not shown). This lack of genotype–expression correlation in cancer cell lines is likely due to somatic changes because PSCA is upregulated in most bladder tumors[16]. By contrast, expression of PSCA in most normal tissues is very low, except for prostate, esophagus and stomach[17,18]. We did not have normal bladder tissues and thus could not measure PSCA expression in them. We used 135 lymphoblastoid cell lines with different genotypes of rs2294008 (TT, CT and CC) and attempted to determine *PSCA* mRNA expression, but *PSCA* mRNA was not detectable (data not shown). Finding sufficient numbers of normal tissue samples, particularly of tissues abundantly expressing PSCA (prostate, esophagus and stomach), is warranted in order to compare endogenous *PSCA* expression for the different rs2294008 genotypes and determine whether the T allele reduces *PSCA* gene expression *in vivo*.

Previous GWASs have identified several independent susceptibility alleles at 8q24 for prostate, breast and colorectal cancers[11–14]. None of these SNPs showed significant associations with bladder cancer risk in our discovery set (Table 3). The power calculation showed that we had adequate power (>80%) to detect an OR of 1.33 for an additive model and 1.37 for a dominant model when the MAF was greater than 0.10 in our discovery set. These 8q24 SNPs have also been examined in the nine European studies previously[10], and the results were consistent with ours. The 8q24 SNP (rs9642880) identified from the previous bladder cancer GWAS[10] was validated in our US populations (OR = 1.14; 95% CI, 1.06–1.22; $P = 2.1 \times 10^{-4}$; Table 3). When we performed a meta-analysis of all the US and European populations, the OR was 1.19 (95% CI, 1.14–1.24; $P = 1.35 \times 10^{-14}$; Supplementary Fig. 3a). We also validated a second SNP (rs710521) at 3q28 (OR = 1.16, 95% CI, 1.10–1.22; $P = 1.09 \times 10^{-8}$; Supplementary Fig. 3b).

PSCA was initially identified as a prostate-specific cell-surface marker[17]. PSCA is overexpressed in prostate cancer, and the level of expression increases with tumor grade and stage[17,19]. It may be involved in cell proliferation, and migration as monoclonal antibodies to PSCA could inhibit tumor growth and metastasis formation in animal models[20,21]. PSCA is expressed at low levels in the transitional epithelium of normal bladder but is overexpressed in the majority of bladder cancers[16]. Immunocytochemical analysis of PSCA in voided urine was shown to be a complementary marker for cytological diagnosis of bladder cancer[22]. The expression level of PSCA was an independent predictor of recurrence in superficial bladder cancer[23]. These observations provide biological plausibility for the association between *PSCA* and bladder cancer risk.

rs2294008 is a missense variation that alters the start codon of *PSCA*. The first report on PSCA protein[17] was based on the cDNA sequence carrying the T allele (the longer, 123-amino-acid protein). In the cDNA sequence carrying the C allele, the translation is predicted to start from the next ATG codon, resulting in a nine-amino-acid truncation. *In vitro*

translation showed that cDNA sequences containing these two alleles produced proteins of almost the same size, compatible with a difference of only nine amino acids[15]. PSCA is a member of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored cell surface proteins. All GPI-anchored proteins undergo complex cellular processing before becoming mature protein[24]. The 123-amino-acid primary PSCA translation product, like other GPI-anchored proteins, has two signal sequences: an N-terminal signal sequence (20 amino acids) for endoplasmic reticulum targeting and a C-terminal sequence that directs the GPI-anchoring[15]. Both these signal sequences are removed in the endoplasmic reticulum, and the GPI-anchored form is then carried through a secretory pathway to the cell surface. Therefore, the mature proteins are the same for the two alleles of rs2294008. However, because rs2294008 changes the length of the N-terminal signal peptide, it may change protein folding, intracellular modifications and/or trafficking of PSCA proteins. An *in vitro* assay using EYFP (enhanced yellow fluorescent protein)-fused PSCA expression vectors transfected into HSC60 cells found no detectable difference in the amount or distribution of the EYFP-fused short and long forms of PSCA protein[15]. *In vivo* measurements of PSCA protein have been challenging owing to the low expression of PSCA in most normal tissues and the uncertainty of the sensitivity and specificity of the available PSCA antibody. Furthermore, if the truncated N-terminal signal peptide affects protein folding and/or intracellular modifications, then an antibody could recognize mature PSCA protein processed from the long and short signal sequences differently even though the mature protein sequence is the same. Future efforts are needed to determine the protein expression and physiological function of PSCA and the functional consequence of rs2294008 *in vivo*.

rs2294008 was recently identified as a susceptibility allele for diffuse-type gastric cancer in Japan[15]. Notably, the T allele has higher frequency in individuals of European ancestry (MAF = 0.46) and Koreans (MAF = 0.46) than in Chinese (MAF = 0.26) and Africans (MAF = 0.25), based on HapMap data; however, it is a major allele in Japanese (frequency = 0.62). The population genetic history of this SNP and why only Japanese possess a different minor allele remain to be explained. Whether rs2294008 is associated with gastric cancer or any other cancers in individuals of European descent warrants further investigation.

The T allele reduced the transcriptional activity of the *PSCA* promoter *in vitro*. It seems paradoxical that the T risk allele reduces transcription, whereas PSCA has been shown to be overexpressed in bladder tumors. Because the physiological function of PSCA and the functional impact of different N-terminal signal lengths on protein function are still unknown the functional consequence of the risk T allele *in vivo* is unclear, but it would be the cumulative result of transcriptional, translational and post-translational effects. All the previously identified cancer susceptibility variants in 8q24 in individuals of European descent are located near the *MYC* gene, and the causal variants and biologic mechanisms remain elusive. rs2294008 (position 143758933) is 15 Mb distal from the previous bladder cancer susceptibility locus (rs9642880, position 128787250) on 8q24, and these two SNPs are not in LD ($D' = 0.01$, $r^2 = 0.00$), suggesting that rs2294008 is an independent bladder cancer susceptibility locus on 8q24. Future functional studies are warranted to delineate the physiological role of PSCA and the biological mechanisms underlying the association of rs2294008 in *PSCA* with bladder carcinogenesis.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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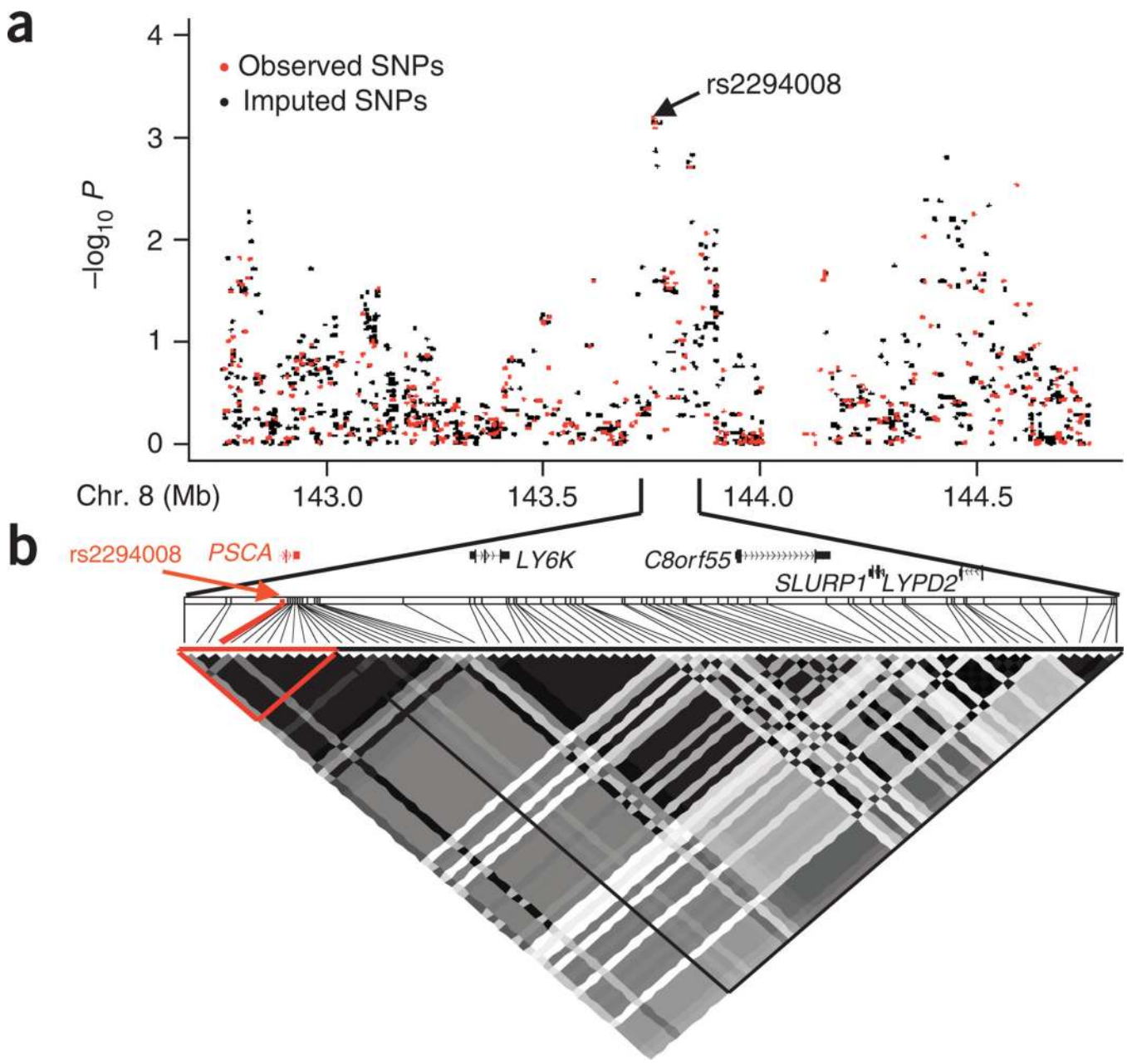

Figure 1.

The 8q24 locus encompassing rs2294008. (**a**) Results of SNP association from the genome-wide screen. Observed results from genotyped SNPs are in red and imputed results are in black. All known genes in this region are also shown. (**b**) LD structure of this region. Red arrow, position of rs2294008. Represented in each location is the square of the correlation coefficient (r^2) derived from phase 2 genotypes in Haploview software (version 4.1), with darker shading indicating greater extent of LD between two SNPs. Triangles represent LD blocks identified by Haploview software.

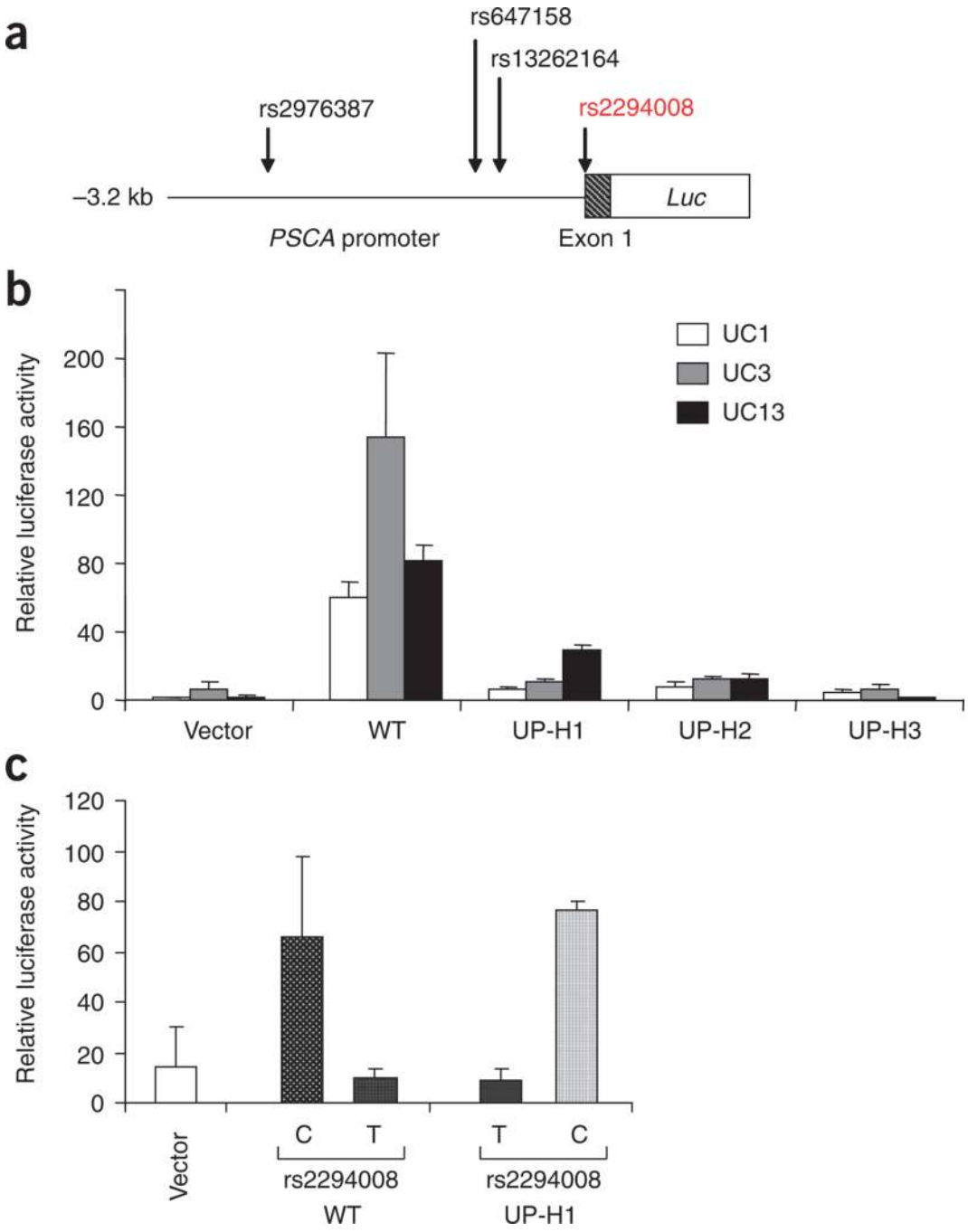

Figure 2.

In vitro reporter assay of the four most frequent haplotypes of the *PSCA* 5′ upstream region (nucleotides −3236 to +28). (**a**) The four SNPs that comprise these haplotypes are rs2976387, rs6471587, rs13262164 and rs2294008. The frequencies of top four haplotypes in our population were wild-type (G-C-C-C), 58%; UP-H1 (A-C-T-T), 25%; UP-H2 (A-C-C-T), 16%; and UP-H3 (G-G-C-T), 1%, respectively. (**b**) Three bladder cell lines were transfected with a luciferase (*Luc*) reporter gene driven by *PSCA* upstream sequences containing these four haplotypes. In all three cell lines, the wild-type sequence (containing C at rs2294008) showed significantly higher promoter activity than the other three (all containing T at rs2294008) ($P < 0.001$). (**c**) Substitution of a single nucleotide (rs2294008 C

to T) in the wild-type haplotype significantly reduced promoter activity, whereas a single substitution (rs2294008 T to C) in UP-H1 increased promoter activity to a level comparable to that in wild-type in UC1 cells.

Table 1

Summary results of rs2294008 in discovery and replication populations

Study group	Cases n = 6,667	Controls n = 39,590	MAF Cases	MAF Controls	Allelic OR (95% CI)	P value	P for heterogeneity
Discovery (Texas)	969	957	0.501	0.446	1.24 (1.10–1.41)	7.34×10^{-4}	
US validation							
New Hampshire	800	912	0.499	0.448	1.23 (1.07–1.41)	2.87×10^{-3}	
Texas	764	2,807	0.485	0.450	1.15 (1.02–1.29)	0.018	
MSKCC	149	152	0.527	0.443	1.40 (1.02–1.94)	0.04	
European validation							
Iceland	551	28,757	0.485	0.469	1.07 (0.95–1.20)	0.278	
Netherlands	1,276	1,831	0.471	0.449	1.09 (0.98–1.20)	0.1	
UK	706	514	0.458	0.426	1.14 (0.97–1.34)	0.123	
Italy-Torino	357	310	0.458	0.439	1.08 (0.86–1.34)	0.506	
Italy-Brescia	182	190	0.448	0.442	1.02 (0.77–1.37)	0.876	
Sweden	335	909	0.513	0.450	1.29 (1.08–1.54)	5.25×10^{-3}	
Belgium	192	380	0.500	0.471	1.12 (0.88–1.44)	0.355	
Spain	172	1,362	0.439	0.435	1.01 (0.81–1.27)	0.9	
Eastern Europe (Hungary, Romania, Slovakia)	214	509	0.521	0.444	1.36 (1.09–1.71)	7.37×10^{-3}	
Summary							
Discovery	969	957	0.501	0.446	1.24 (1.10–1.41)	7.34×10^{-4}	
US validation	1,713	3,871	0.495	0.449	1.19 (1.10–1.30)	3.53×10^{-5}	0.456
European validation	3,985	34,762	0.475	0.465	1.12 (1.06–1.18)	9.83×10^{-5}	0.498
All combined	6,667	39,590	0.484	0.463	1.15 (1.10–1.20)	2.14×10^{-10}	0.423

Table 2

Multivariate logistic regression analysis of rs2294008

			Genotype					
		All	**CC**	**CT**	**TT**	**Dominant**	***P*-value**	***P* for heterogeneity**
All[a]	Case/Control	5,038 / 9,363	1,288 / 2,842	2,613 / 4,668	1,137 / 1,853			
	OR[c] (95% CI)		1	1.30 (1.18–1.42)	1.40 (1.25–1.56)	1.33 (1.22–1.45)	1.30×10^{-10}	0.58
Male	Case/Control	4,159 / 5,104	1,080 / 1,576	2,147 / 2,500	932 / 1,028			
	OR[d] (95% CI)		1	1.30 (1.17–1.44)	1.38 (1.21–1.56)	1.32 (1.20–1.45)	2.05×10^{-8}	0.22
Female	Case/Control	871 / 3,558	205 / 1,037	464 / 1,827	202 / 694			
	OR[d] (95% CI)		1	1.29 (1.06–1.58)	1.48 (1.16–1.88)	1.34 (1.11–1.62)	2.47×10^{-3}	0.57
Never smoker	Case/Control	983 / 2,871	233 / 866	511 / 1,431	239 / 574			
	OR[e] (95% CI)		1	1.41 (1.17–1.70)	1.54 (1.23–1.93)	1.45 (1.21–1.73)	5.50×10^{-5}	0.30
Ever smoker	Case/Control	3,727 / 5,660	955 / 1719	1,938 / 2,825	834 / 1,116			
	OR[e] (95% CI)		1	1.26 (1.13–1.39)	1.34 (1.18–1.52)	1.28 (1.16–1.41)	1.20×10^{-6}	0.53
Age ≤ 62[b]	Case/Control	1,996 / 4,561	511 / 1,353	1,054 / 2,318	431 / 890			
	OR[c] (95% CI)		1	1.24 (1.08–1.43)	1.35 (1.14–1.61)	1.27 (1.11–1.45)	4.20×10^{-4}	0.87
Age > 62[b]	Case/Control	3,042 / 4,802	777 / 1,489	1,559 / 2,350	706 / 963			
	OR[c] (95% CI)		1	1.33 (1.17–1.50)	1.44 (1.24–1.66)	1.36 (1.21–1.52)	1.41×10^{-7}	0.52

[a] Excludes Iceland, UK and Sweden populations owing to lack of individual epidemiologic data in controls.

[b] Age cutoff is the median age in controls.

[c] Adjusted for age, gender, smoking status and study site.

[d] Adjusted for age, smoking status and study site.

[e] Adjusted for age, gender and study site.

Table 3

Previously reported cancer susceptibility alleles on 8q24 and bladder cancer risk[a]

		Allele		MAF				LD with rs2294008	
SNP	**Position**	**Major**	**Minor**	**Case**	**Control**	**OR (95% CI)**	***P*-value**	***D′***	**Reported cancer association**
rs10505483[b]	128194377	G	A	0.031	0.029	1.08 (0.75–1.56)	0.688	0.02	Prostate
rs13281615	128424800	A	G	0.414	0.412	1.01 (0.89–1.15)	0.868	0.03	Breast
rs6983267	128482487	C	A	0.491	0.487	1.02 (0.89–1.15)	0.815	0.01	Prostate, colorectal
rs1447295	128554220	C	A	0.102	0.086	1.21 (0.97–1.50)	0.090	0.07	Prostate
rs9642880	128787250	G	T	0.488	0.457	1.14 (1.06–1.22)	2.1×10^{-4}	0.01	Bladder

[a]Results for rs9642880 were from all US populations; results of other SNPs were from discovery phase population.

[b]rs10505483 is in complete LD with rs16901979.