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Research Article

Cancer Prevention Research

Blood Biomarker Levels to Aid Discovery of Cancer-Related Single-Nucleotide Polymorphisms: Kallikreins and Prostate Cancer

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Abstract

Polymorphisms associated with prostate cancer include those in three genes encoding major secretory products of the prostate: *KLK2* (encoding kallikrein-related peptidase 2; hK2), *KLK3* (encoding prostate-specific antigen; PSA), and *MSMB* (encoding β-microseminoprotein). PSA and hK2, members of the kallikrein family, are elevated in sera of men with prostate cancer. In a comprehensive analysis that included sequencing of all coding, flanking, and 2 kb of putative promoter regions of all 15 kallikrein (*KLK*) genes spanning ≈280 kb on chromosome 19q, we identified novel single-nucleotide polymorphisms (SNP) and genotyped 104 SNPs in 1,419 cancer cases and 736 controls in Cancer Prostate in Sweden 1, with independent replication in 1,267 cases and 901 controls in Cancer Prostate in Sweden 2. This verified prior associations of SNPs in *KLK2* and in *MSMB* (but not in *KLK3*) with prostate cancer. Twelve SNPs in *KLK2* and *KLK3* were associated with levels of PSA forms or hK2 in plasma of control subjects. Based on our comprehensive approach, this is likely to represent all common *KLK* variants associated with these phenotypes. A *T* allele at rs198977 in *KLK2* was associated with increased cancer risk and a striking decrease of hK2 levels in blood. We also found a strong interaction between rs198977 genotype and hK2 levels in blood in predicting cancer risk. Based on this strong association, we developed a model for predicting prostate cancer risk from standard biomarkers, rs198977 genotype, and rs198977 × hK2 interaction; this model had greater accuracy than did biomarkers alone (area under the receiver operating characteristic curve, 0.874 versus 0.866), providing proof in principle to clinical application for our findings. *Cancer Prev Res; 3(5); 611–9. ©2010 AACR.*

Introduction

Prostate cancer is among the most heritable of the common cancers; one estimate of its heritability, from a twin study, was 42% (1). Based on this high heritability, several genome-wide association studies for prostate cancer susceptibility loci have been performed, and numerous polymorphisms have been associated with prostate cancer (2–7). The implicated genes include *KLK3* [encoding prostate-specific antigen (PSA); refs. 3, 8–10] and *MSMB* [encoding β-microseminoprotein (MSP or PSP_{94}); refs. 3, 7]. In addition, single-nucleotide polymorphisms (SNP) in *KLK2*, a gene that encodes kallikrein-related peptidase 2 (hK2) and a homologue of *KLK3*, have also been reported to be associated with prostate cancer (11).

All three genes (*KLK2*, *KLK3*, and *MSMB*) code for highly abundant secretory products of the prostate. PSA and hK2 are both kallikreins, a family of serine proteases that are all encoded in a gene cluster on chromosome 19q. PSA is a commonly used biomarker for detection of prostate cancer and monitoring after treatment. There is debate as to whether SNPs in *KLK3* identified by genome-wide association studies are true prostate cancer susceptibility alleles, or whether these SNPs were detected due to association with PSA levels and because an elevated PSA increases the likelihood of diagnosis of asymptomatic prostate cancer (12, 13). hK2 is also used as a biomarker for prostate cancer. Although SNPs in *KLK2* have been

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Table 1. Significant associations between plasma kallikrein values and SNPs in the kallikrein locus in the CAPS study

Measure	SNP	Location	Position on chr 19 (build 36)	CAPS1 *P**
fPSA, ng/mL	rs2271094	*KLK3* exon 2	56,051,309	<0.0001
	rs11084039	*KLK2* promoter	56,068,031	<0.0001
	rs11670728	*KLK2* promoter	56,068,301	0.0001
	rs3760728	*KLK2* promoter	56,066,404	0.0002
	rs6998	*KLK3* exon 5	56,055,473	0.0003
tPSA, ng/mL	rs2271094	*KLK3* exon 2	56,051,309	0.0003
%fPSA	rs61752561	*KLK3* exon 3	56,053,194	0.0002
hK2, ng/mL	rs198977	*KLK2* exon 5	56,073,589	<0.0001
	rs198978	*KLK2* exon 5	56,074,884	<0.0001
	rs198972	*KLK2* exon 3	56,071,705	<0.0001
	SNP11	*KLK2* exon 5	56,075,012	<0.0001
	rs1654553	*KLK4* intron 4	56,102,928	<0.0001
	rs17526278	*KLK3* promoter	56,049,699	0.0005

NOTE: Trait values (ng/mL) are given for CAPS1 + CAPS2 when available. Otherwise, trait values are for CAPS1.
Abbreviation: ND, no data.
*$P < 0.00048$ based on permutation testing; $P < 0.00012$ based on the Bonferroni correction.
†$P < 0.05$.

associated with prostate cancer risk (11), this association has not been consistently replicated (14). Also, interpretation of prior associations between SNPs in *KLK2* and hK2 levels in blood is complicated by both lack of replication and that all study subjects had elevated PSA and/or abnormal findings on digital rectal examination in these studies. Taken together, prior data suggest that the *KLK* locus may influence prostate cancer risk (15). We therefore undertook a detailed analysis of association across the entire kallikrein locus. We performed comprehensive resequencing of all *KLK* genes and genotyped known and newly discovered kallikrein SNPs, as well as an *MSMB* SNP, in a large prostate cancer case-control cohort from Sweden. By examining the relationship between SNP genotype, serum levels of PSA and hK2, and disease status, we found that several SNPs at this locus are associated with PSA and hK2 levels in the blood and that an interaction between one SNP in *KLK2* and hK2 levels in plasma is associated with prostate cancer.

Materials and Methods

SNP discovery

Ninety-four men referred for prostate biopsy at the University Hospital, Malmö, Sweden and 47 male patients at the University Hospital with no signs of prostate cancer were resequenced for the *KLK2* and *KLK3* genes. A subset of the men referred for prostate biopsy were sequenced for the other genes (Supplementary Table S2). Genomic DNA was isolated from whole blood, PCR amplified, and sequenced using Big Dye Terminator chemistry (Applied Biosystems 3730). For each gene, all coding regions, flanking intronic sequences, and 2 kb of the putative promoter were sequenced. The generally bidirectional sequence data were assembled and compared using SeqScape v2.5 (Applied Biosystems) with manual confirmation of candidate heterozygotes followed by independent genotyping to confirm all detected polymorphisms. This sequencing strategy is expected to detect 95% of SNPs with an allele frequency ≥1% given a sample size of 48 individuals (16).

Case-control study population

Subjects were recruited for Cancer Prostate in Sweden (CAPS), a population-based case-control study (17, 18), in two stages (Supplementary Table S1). Due to the setup of Swedish regional oncology centers, the individual cohorts of patients contributed by them are genuinely population-based. Patients ages 35 to 65 years were enrolled in the southern regions, compared with ages 35 to 79 years in the northern regions. Concurrent with recruitment of case subjects, control subjects were randomly selected from the Swedish Population Registry, matched by geographic region and the expected age distribution of cases (within 5 years). Clinical tumor characteristics and treatment information were collected from the National Prostate Cancer Register (19).

Immunodetection of biomarkers

Measurement of levels of hK2, free PSA (fPSA), and total PSA (tPSA) in EDTA-anti-coagulated blood plasma from cases and controls was performed in Dr. Lilja's laboratory at Department of Laboratory Medicine, Lund University, University Hospital UMAS during 2005-2006. For most

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Table 1. Significant associations between plasma kallikrein values and SNPs in the kallikrein locus in the CAPS study (Cont'd)

CAPS2 $P^{\dagger}$	CAPS1 + CAPS2 P	Mean trait value, ng/mL (95% CI)		
		Common homozygote	Heterozygote	Rare homozygote
0.002	<0.0001	0.62 (0.081-2.1)	0.69 (0.086-2.5)	0.98 (0.094-3.6)
ND	ND	0.075 (0.069-2.9)	0.075 (0.10-2.7)	0.96 (0.13-3.5)
0.024	<0.0001	0.66 (0.084-2.3)	0.67 (0.085-2.5)	1.0 (0.092-3.9)
0.17	<0.0001	0.66 (0.083-2.3)	0.68 (0.085-2.5)	1.0 (0.094-3.8)
0.4	<0.0001	0.66 (0.083-2.3)	0.74 (0.084-2.6)	0.87 (0.094-3.5)
0.031	<0.0001	2.6 (0.20-10)	2.8 (0.21-12)	4.00 (2.4-16)
0.0001	<0.0001	32 (8.1-56)	36 (9.5-63)	48 (27-69)
<0.0001	<0.0001	0.069 (0.013-0.21)	0.050 (0.0086-0.14)	0.032 (0.0016-0.071)
<0.0001	<0.0001	0.068 (0.014-0.20)	0.062 (0.0091-0.18)	0.036 (0.0023-0.11)
<0.0001	<0.0001	0.065 (0.012-0.20)	0.060 (0.0078-0.19)	0.049 (0.0024-0.14)
<0.0001	<0.0001	0.062 (0.0096-0.20)	0.060 (0.0038-0.19)	0.014 (0.0043-0.033)
<0.0001	<0.0001	0.065 (0.012-0.20)	0.062 (0.0086-0.21)	0.057 (0.0052-0.19)
ND	ND	0.068 (0.0097-0.22)	0.056 (0.0047-0.22)	0.038 (0.0050-0.15)

cases, blood samples were collected after initiation of treatment for prostate cancer; hence, these plasma levels generally reflect treatment effects. Free PSA and tPSA were simultaneously measured with a dual-label assay (DELFIA Prostatus PSA F/T, Perkin-Elmer Life Sciences; ref. 20). Total hK2 was measured according to a three-step in-house assay (21, 22).

Genotyping

All SNPs discovered in our resequencing and selected *KLK* locus SNPs identified in dbSNP (http://www.ncbi.nlm.nih.gov/SNP) were genotyped on parent-child trios to check data quality, confirm Mendelian segregation, and check for segregation patterns consistent with deletions. For the current study, DNA suitable for genotyping was available from 2,686 cases and 1,637 controls in CAPS1 + CAPS2. Genotypes were determined using the MassARRAY matrix-assisted laser desorption/ionization time-of-flight system (Sequenom), using the Homogenous MassEXTEND protocol. We initially assessed 137 probes corresponding to 112 SNPs on the CAPS1 samples, using the Sequenom system; 124 probes corresponding to 104 SNPs passed quality control. Where one SNP had multiple probes, only the probe with the fewest missing observations was used for statistical analysis. Based on results from CAPS1, 36 probes corresponding to 34 SNPs were chosen for replication in CAPS2. After quality control, 33 probes remained, each corresponding to a unique SNP. Technical sample replicates showed >99.9% genotype concordance, and multiple probes for the same SNP showed >99% concordance. The final sets of SNPs for CAPS1 and CAPS2 had, on average, 1.9% and 2.8% missing observations (maximum, 6.7% and 7.8%).

For confirming the genotype of the SNP marker rs198977, we used a predesigned TaqMan assay (C_736084, Applied Biosystems). The genotypes were called automatically using an ABI 7900HT SDS and verified manually. Agreement between Sequenom and TaqMan assays for non-missing measurements was 99.93% across CAPS1 and CAPS2.

Statistical analysis

Case-control study. Statistical analyses were generally performed using R version 2.3.1 (http://www.r-project.org). Association with case-control status was done using Pearson's χ^2 or Fisher's exact test on a 3 × 2 contingency table. Stratified unconditional logistic regression was used to test for association between genotype and disease status, controlling for age and geographic area; recessive, dominant, codominant, and additive models were tested. Haploview version 3.3.2 with default parameters was used for evaluation of multimarker haplotypes and for permutation testing. For tests of Hardy-Weinberg equilibrium, a χ^2 test with 1 degree of freedom was used to assess deviation of observed from expected allele frequencies.

Quantitative variables. Kallikrein levels in plasma were analyzed for association with SNP genotypes in controls. Free fPSA, tPSA, and hK2 levels were normalized by taking the log. Association was tested using linear regression with an additive model (23). Ten thousand permutations of the quantitative variables were used to assess the cutoff for an empirical $P < 0.05$ across all quantitative association tests.

Conditional haplotype-based testing was done for all pairs of significant SNPs at the *KLK* locus. For each pair of SNPs, independent association was tested for each condition on the other using *whap*, version 2.09 (24).

Interaction analysis. To test for interaction between hK2 levels and rs198977, we removed all case individuals for whom hK2 was measured in a blood sample obtained after any type of treatment and individuals with missing rs198977 or hK2 data, resulting in 189 CAPS1 cases, 143 CAPS2 cases, and 1,600 controls. Using logistic regression, we tested for association of any *T* allele at rs198977 with

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prostate cancer in a univariate manner both solely and after controlling for hK2 and also in interaction with hK2 levels. To prevent bias due to an age difference between treated and untreated cases, sensitivity analyses were conducted adjusting for age, which confirmed that our results were not affected by the exclusion of treated cases (data not shown).

Predictive models. Log-transformed values for all plasma biomarkers were used to model their nonlinear association with outcome. These analyses included those participants with complete data for all biomarkers, rs198977, rs10993994, and rs2271094. The models were constructed using 178 cases and 661 controls from CAPS1 and independently validated by calculating the area under the receiver operating characteristic curve (AUC) among 136 cases and 819 controls from CAPS2.

Results

SNP discovery

Sequencing in all 15 kallikrein genes identified 140 polymorphisms (Supplementary Tables S2 and S3), including 38 novel SNPs. We designed and successfully validated genotyping assays for 13 novel SNPs and 89 previously described SNPs in this region. These assays capture most of the common variations in the entire region (r^2 > 0.5 for more than half of HapMap tag SNPs).

SNP association analysis

The 102 SNPs were genotyped in 1,419 prostate cancer cases (Supplementary Table S1) and 736 controls in the CAPS1 study (17). We did not find any significant association between SNP and prostate cancer risk under a variety of genetic models after correcting for the number of SNPs tested. Therefore, no novel cancer susceptibility associations were revealed.

Previous studies have noted associations between *KLK* locus SNPs and PSA or hK2 levels, which could confound the clinical use of PSA or hK2 measurements. Therefore, we next asked if any of the 102 SNPs were associated with plasma levels of hK2 and PSA [fPSA, tPSA, and the ratio of fPSA to tPSA (%fPSA)]. We restricted this analysis to controls to avoid any bias from measuring posttreatment PSA and hK2 levels. We identified 13 associations with $P < 0.00048$, a threshold determined through permutation testing (Table 1). One associated SNP was in *KLK4*; all others were in the *KLK2-KLK3* region (Fig. 1; Supplementary Table S4). Levels of hK2 were clearly associated with several SNPs in the *KLK2* region. The most striking association involved rs198977 ($P < 0.0001$; Fig. 1A; Table 1). Several other SNPs were associated with fPSA, tPSA, or %fPSA (Table 1); most SNPs were in or adjacent to a haplotype block that includes *KLK3* and the *KLK3-KLK2* intergenic region (Fig. 2). On retesting in CAPS2, almost all significant associations were replicated with $P < 0.05$ (Table 1).

Associations with previously reported prostate cancer susceptibility alleles

We next considered three SNPs previously associated with prostate cancer susceptibility: rs198977 in *KLK2* (11), which

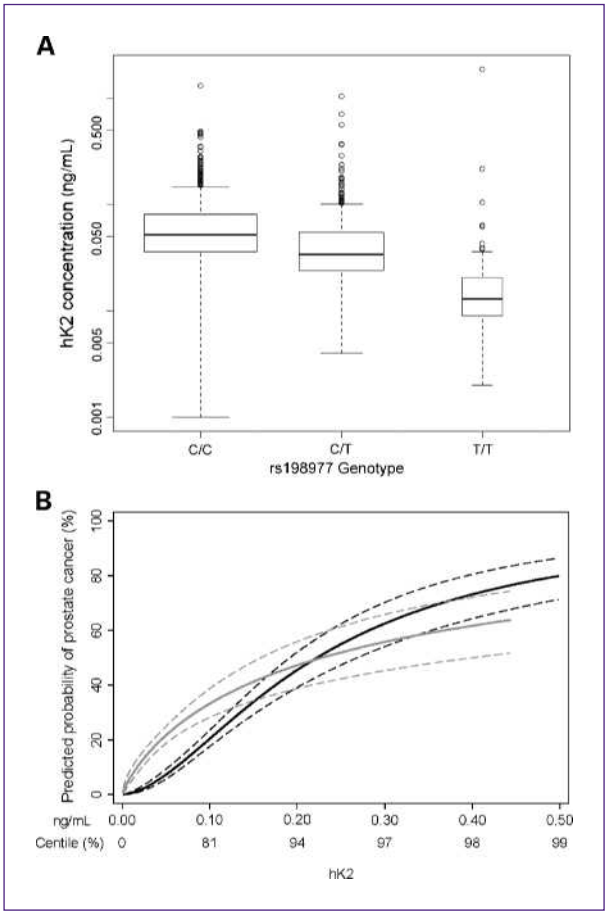

Fig. 1. A, association of rs198977 with hK2 levels. Box plots show hK2 levels in controls from both CAPS1 and CAPS2 stratified by rs198977 genotype. Each box covers the middle two quartiles, with the bar representing the median. Plot whiskers extend out to 1.5 times the interquartile range from the 25th and 75th percentiles. Widths of boxes are proportional to the square root of the number of observations. B, interaction plot. Association between hK2 level in plasma and prostate cancer risk in CAPS is shown for men with *C/C* genotype at rs198977 (black curve) and for men with at least one *T* allele (gray curve). Dashed lines, 95% CIs.

was one of the 102 SNPs described above, rs2735839 in the *KLK2-KLK3* intergenic region (3, 12, 13), and rs10993994 in *MSMB* (3, 7). *MSMB*, like *KLK2* and *KLK3*, encodes a protein for which serum levels are associated prostate cancer (25, 26). The three SNPs were genotyped in the combined CAPS (17, 18). rs2735839 was not associated with case status ($P = 0.82$). Both rs198977 and rs10993994, however, were significantly associated [rs198977 $P = 0.029$; odds ratio (OR), 1.08; 95% confidence interval (CI), 0.97–1.19; rs10993994 $P = 0.0020$; OR, 1.17; 95% CI, 1.07–1.28], thus replicating the previous findings. Although rs198977 showed deviation from Hardy-Weinberg equilibrium in controls ($P = 0.00013$), genotype calls were confirmed with a second genotyping method, ruling out genotyping error. To enhance the weak association of rs198977 with prostate cancer, we conducted a meta-analysis of this SNP using four cohorts [Nam et al. (11),

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CAPS1, CAPS2, and CGEMS (27)]. Using Fisher's exact test, rs198977 was significantly associated with prostate cancer risk ($P = 0.011$), even when the Nam et al. replication cohort was excluded ($P = 0.039$; Table 2). Therefore, rs198977 seems to be weakly associated with prostate cancer susceptibility.

We next examined the three SNPs for association with plasma hK2, fPSA, tPSA, and %fPSA in CAPS controls. Both rs2735839 in the *KLK2-KLK3* region and rs10993994 in *MSMB* were previously associated with tPSA levels among controls (3, 12). The association of rs2735839 with tPSA was not replicated in our study, although this SNP was associated with %fPSA (Table 3). We did, however, replicate the association of the *MSMB* SNP rs10993994 with tPSA, and also found stronger associations with fPSA and with hK2. As noted above, the *T* allele of rs198977 in *KLK2* was strikingly associated with lower hK2 level, and it was also associated with higher %fPSA.

Independence of the discovered associations

To remove redundancies in the SNP/kallikrein associations due to linkage disequilibrium between SNPs, we tested for the independence of each SNP association relative to the other SNPs (ref. 24; Supplementary Table S5). Overall, our data suggest that the observed association with

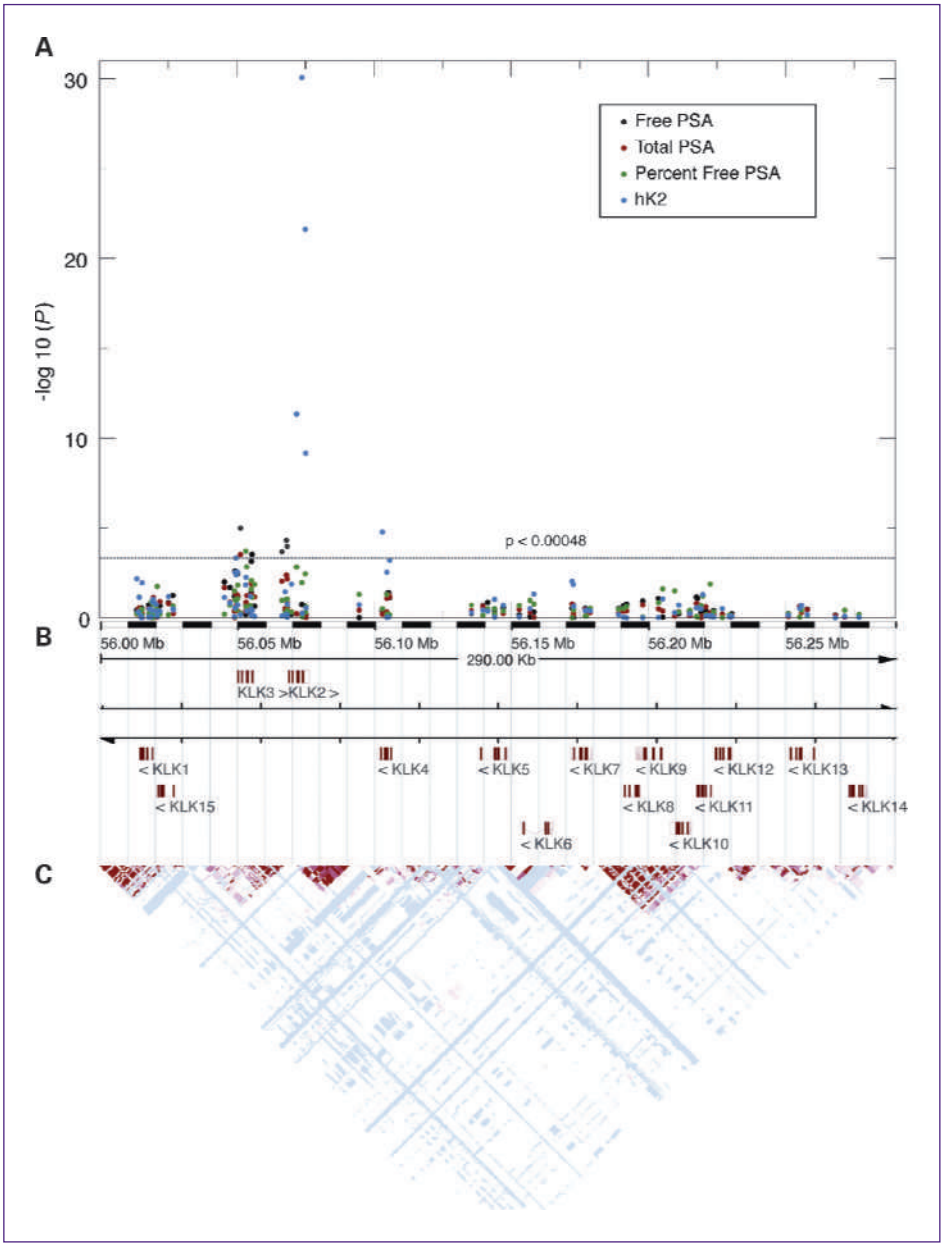

Fig. 2. Association of polymorphisms at the kallikrein locus with PSA forms and hK2 in plasma. A, plot showing strength of association between SNPs and levels of fPSA and tPSA, %fPSA, and hK2. B, schematic of the kallikrein locus, showing the position and orientation of all 15 genes. C, linkage disequilibrium plot from the CEU population in HapMap. Red, high linkage disequilibrium as measured by D′.

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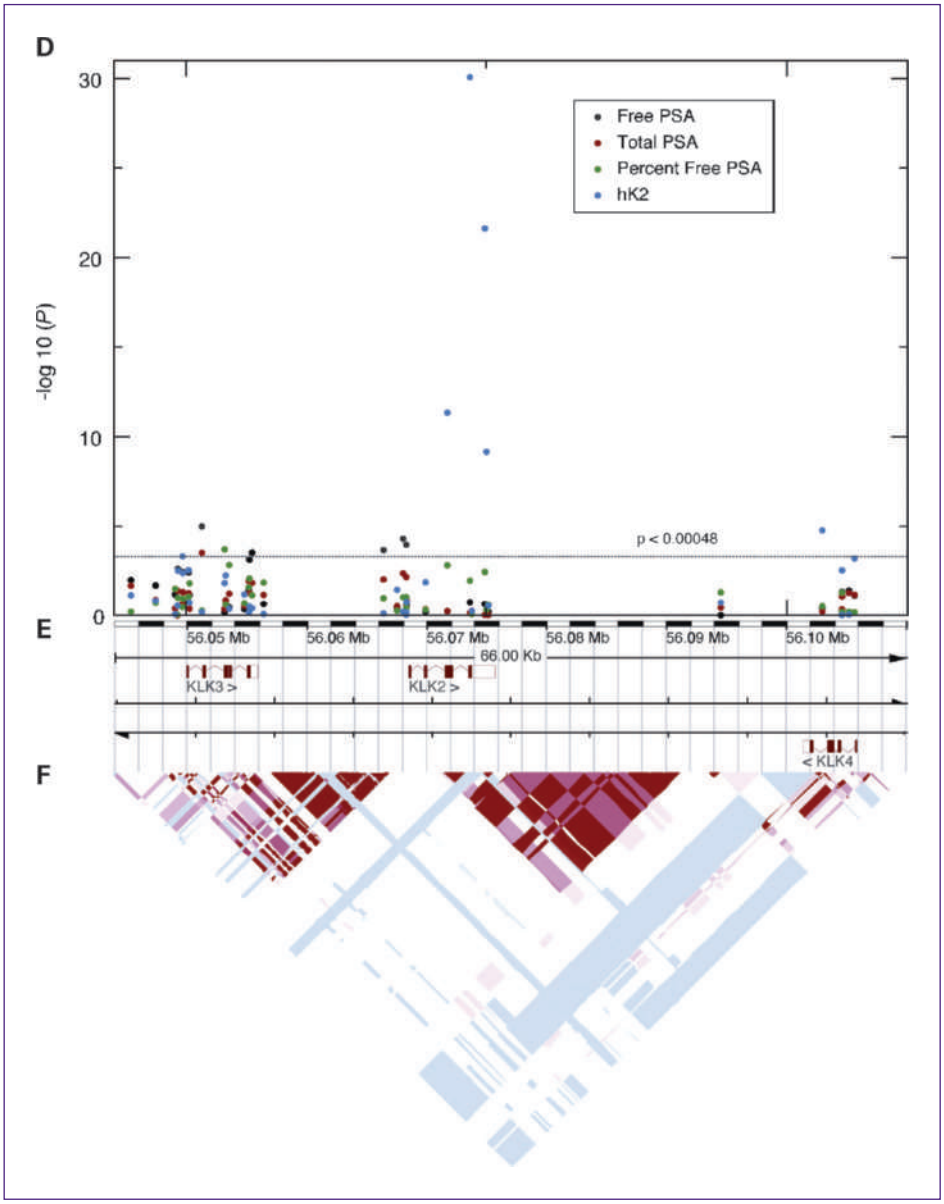

Fig. 2. *Continued.* D, E, and F, similar plots as in A, B, and C, but restricted to the *KLK2-KLK3* region.

fPSA is explained by rs2271094 and that the observed association with hK2 is explained by rs198977. For the three SNPs associated with %fPSA, conflicting results were obtained from CAPS1, CAPS2, and the combined data set, and therefore we cannot determine their interdependence in influencing %fPSA.

Prostate cancer risk models

We next tested for a statistical interaction in which the association between hK2 level and disease status (case or control) depends on rs198977. We compared three logistic regression models (Table 4). The association between rs198977 and prostate cancer risk was strengthened by adding hK2 level as an independent factor. When the interaction between rs198977 and hK2 was added, rs198977, hK2, and the interaction term were all strongly associated with disease (Fig. 1B). Among men with low hK2 levels, those with a *T* allele at rs198977 had a greatly elevated probability of prostate cancer, whereas among men with higher hK2 levels, those with and without any *T* allele had little difference in probability of prostate cancer.

Various prediction models for prostate cancer use tPSA, fPSA, %fPSA, and hK2 in plasma (28). We tested whether such a prediction model could be further improved by incorporating rs198977, rs10993994, rs2271094, and the SNP × kallikrein interaction. We evaluated the predictive accuracy for a base model (tPSA, fPSA, %fPSA, and hK2), full model (base plus rs198977, rs198977 × %fPSA, and rs198977 × hK2), and two exploratory models (model 1: full plus rs10993994, rs10993994 × tPSA, rs10993994 × fPSA, and rs10993994 × hK2; model 2: full plus rs2271094, rs2271094 × tPSA, and rs2271094 × fPSA). The AUC was

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Table 2. Case-control analysis of rs198977 in the *KLK2* gene in three studies of prostate cancer

	Nam et al. (11)	CGEMS (27)	CAPS1	CAPS2	CAPS1 + CAPS2
Cases, *n* (%)					
C/C	335 (52)	618 (53)	788 (56)	670 (55)	1,458 (56)
C/T	260 (40)	472 (40)	515 (37)	460 (38)	975 (37)
T/T	50 (8)	80 (7)	94 (7)	89 (7)	183 (7)
Controls, *n* (%)					
C/C	356 (59)	630 (57)	443 (61)	483 (57)	926 (59)
C/T	216 (36)	404 (37)	233 (32)	287 (34)	520 (33)
T/T	34 (6)	63 (6)	48 (7)	72 (9)	120 (8)
P (3 × 2 table)	0.038	0.079	0.088	0.19	0.029
Heterozygote OR (95% CI)	1.3 (1.0-1.6)	1.2 (1.0-1.4)	1.2 (1.0-1.5)	1.2 (0.96-1.4)	1.2 (1.0-1.4)
Homozygote OR (95% CI)	1.6 (1.0-2.5)	1.3 (0.91-1.8)	1.1 (0.76-1.6)	0.89 (0.64-1.2)	1.0 (0.76-1.2)
Hardy-Weinberg *P* (controls)	0.9	0.9	0.025	0.002	0.0001

NOTE: Data for the combined cohort of CAPS1 and CAPS2 are included for informational purposes in the last column; these two cohorts were considered separately in the meta-analysis.

0.866 for the base model, slightly increasing to 0.874 for the full model. A small enhancement was observed with the rs10993994 exploratory model (AUC, 0.877), but not for the rs2271094 exploratory model (AUC, 0.872).

Discussion

Here we analyzed common genetic variation in the kallikrein genes in association with kallikrein levels in plasma and risk of developing prostate cancer. To our knowledge, this represents the first comprehensive analysis of this locus, and we believe that it has identified all common variants in the Swedish population associated with prostate cancer risk and kallikrein levels.

This analysis identified a novel association of rs2271094 with fPSA and tPSA levels. Although rs2271094 is not associated with prostate cancer risk, it still may have clinical importance: Because it is associated with tPSA, this SNP may influence whether a patient's early-stage prostate cancer gets detected.

We were unable to replicate previously reported associations of rs2735839 with prostate cancer risk and tPSA (3, 12). However, we identified an association between rs2735839 and %fPSA, suggesting that rs2735839 influences kallikrein levels in blood. Although the nearby SNP rs2271094 was associated with tPSA, linkage disequilibrium between this SNP and rs2735839 is low (r^2 = 0.08). Although we cannot explain the discrepancy with previous association results with tPSA, our denser genotyping and measures of different PSA isoforms provide finer-grained information on the relationship of *KLK3* SNPs to PSA levels. The reported association of rs2735839 with prostate cancer could be confounded if this SNP affects PSA levels, as previously noted (12). Specifically, the association with PSA could introduce selection bias in which individuals with the risk allele tend to have higher PSA levels and, therefore, are more likely to have asymptomatic prostate cancer diagnosed. Supporting this notion, we found no significant difference in genotype frequencies of rs2735839 in a cohort of cases not

Table 3. Associations of three selected SNPs (rs198977, rs2735839, and rs10993994) with prostate cancer risk and plasma kallikrein values in the CAPS study

Measure	CAPS1 *P*	CAPS2 *P*	Combined CAPS *P*	CAPS1 *P*	CAPS2 *P*	Combined CAPS *P*	CAPS1 *P*	CAPS2 *P*	Combined CAPS *P*
	rs198977 (*KLK2*)			rs2735839 (*KLK2-KLK3* intergenic region)			rs10993994 (*MSMB*)		
Cancer risk	0.09	0.19	**0.029**	0.7	0.2	0.8	0.3	**0.003**	**0.002**
tPSA	0.7	0.3	0.5	0.07	0.5	0.08	0.09	0.055	**0.006**
fPSA	0.18	0.3	0.13	0.2	0.7	0.5	**0.011**	**0.024**	**0.0006**
%fPSA	**0.011**	**<0.0001**	**<0.0001**	0.014	**0.006**	**0.0002**	0.18	0.86	0.5
hK2	**<0.0001**	**<0.0001**	**<0.0001**	0.8	0.16	0.3	**0.015**	**0.006**	**0.0002**

NOTE: Boldface indicates statistically significant results ($P \leq 0.05$).

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Table 4. Prediction of prostate cancer: univariate and multivariable analyses of rs198977, hK2 level, and the interaction between rs198977 and hK2 level

	CAPS1 (707 controls, 189 cases)		CAPS2 (893 controls, 143 cases)		CAPS1 + CAPS2 (1,600 controls, 332 cases)	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Univariate						
Any *T* allele*	1.48 (1.07-2.04)	0.018	1.15 (0.81-1.64)	0.4	1.29 (1.02-1.64)	0.034
Controlling for hK2						
Any *T* allele*	3.10 (2.11-4.55)	<0.0005	2.07 (1.38-3.09)	<0.0005	2.55 (1.94-3.37)	<0.0005
Log hK2	3.24 (2.57-4.08)	<0.0005	3.63 (2.82-4.67)	<0.0005	3.43 (2.90-4.07)	<0.0005
Interaction analysis						
Any *T* allele*	0.34 (0.10-1.19)	0.091	0.20 (0.05-0.82)	0.025	0.27 (0.11-0.68)	0.005
Interaction	0.43 (0.27-0.69)	<0.0005	0.41 (0.24-0.69)	0.0008	0.42 (0.30-0.60)	<0.0005

*Heterozygous genotypes were encoded as intermediate between *C/C* and *T/T* genotypes.

ascertained through PSA screening (3) versus a comparable population sample (ref. 29; nominal $P = 0.2$; see Supplementary Table S6).

We replicated previously observed associations of rs10993994 in the *MSMB* promoter with prostate cancer risk and with tPSA. We extended these observations by noting that rs10993994 shows even stronger evidence for association with fPSA and hK2 levels in plasma. These data could suggest that MSP, the *MSMB* gene product, plays a role in the etiology of prostate cancer. Alternatively, the association between rs10993994 and cancer could result from its association with tPSA, causing a selection bias. Arguing against this selection bias, the genotype frequency of rs10993994 does significantly differ between prostate cancer cases ascertained without PSA screening and a comparable population sample ($P < 0.0005$ after correcting for multiple tests; see Supplementary Table S6).

Similarly, we replicated observed associations of rs198977 (in *KLK2*) with prostate cancer risk and hK2 level. Although association between rs198977 and hK2 level was previously observed in a mixed group of cases and controls (11, 30), our results are first to show an association among controls, with remarkably strong effect. We also identified an association between rs198977 and %fPSA. These data again suggest a role of hK2 and/or PSA in the etiology of prostate cancer. The potential selection bias described for SNPs affecting tPSA is implausible for rs198977, as neither hK2 nor %fPSA is routinely used to screen for prostate cancer. Moreover, the allele associated with cancer risk is also associated with lower hK2 and higher %fPSA, both of which are associated with lower tPSA (absolute value of Spearman's $\rho > 0.5$ for both markers in cases and controls separately). Therefore, any selection bias from tPSA would make diagnosis of asymptomatic cancer less, not more, likely in men with the risk allele. We also showed an interaction between rs198977 genotype and hK2 levels in predicting prostate cancer risk. This suggests that it is important to consider SNP-biomarker level interactions in genetic association studies.

Our data suggest that measuring a blood marker can help identify cancer-related SNPs. Assessed in isolation, the association between rs198977 and prostate cancer was small (OR of 1.29 for combined cohorts) and significant in only one cohort. In contrast, after controlling for hK2 levels in plasma, the association was much stronger (OR of 2.55) and highly significant in both cohorts ($P < 0.0005$). Our data suggest that the ability of a marker to predict cancer is importantly enhanced if SNP status is known and vice versa ($P < 0.0005$ for the interaction term). This suggests a potential role for incorporating both genotype and marker levels in predictive models. Further studies should examine the degree to which evaluation of genotype at these SNPs increases the predictive accuracy of PSA or other kallikrein measures in blood.

Disclosure of Potential Conflicts of Interest

H. Lilja holds patents for free PSA and hK2 assays. The other authors disclosed no potential conflicts of interest.

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