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Genetic variation in *KLK2* and *KLK3* is associated with levels of hK2 and PSA in seminal plasma and in serum in young men

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Abstract

BACKGROUND—Genetic variants in *KLK2* and *KLK3* have been associated with increased serum levels of their encoded proteins human kallikrein-related peptidase 2 (hK2) and prostate-specific antigen (PSA), and with prostate cancer in older men. Catalytic PSA, possibly activated by hK2, cleaves semenogelin I and II in semen to release motile sperm; low PSA levels in seminal plasma are associated with low sperm motility. To evaluate whether common genetic variants in *KLK2* and *KLK3* affect physiological prostatic secretion, we studied the association of SNPs with hK2 and PSA levels in seminal plasma and serum of young healthy men.

METHODS—Leukocyte DNA was extracted from 303 male military conscripts (median age 18.1 years). Nine SNPs across *KLK2-KLK3* were genotyped. PSA and hK2 were measured in seminal plasma and serum with immunofluorometric assays. The association of genotype frequencies with hK2 and PSA levels was tested using the Kruskal-Wallis test.

RESULTS—Four *KLK2* SNPs (rs198972, rs198977, rs198978, and rs80050017) were strongly associated with hK2 levels in seminal plasma and serum, with individuals homozygous for the major alleles having 3- to 7-fold higher levels than the other homozygote and heterozygotes having intermediate levels (all $P<0.001$). Three of these SNPs were significantly associated with %fPSA in serum (all $P<0.007$). Three *KLK3* SNPs showed associations with PSA in seminal

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Hans Lilja holds patents for free PSA, hK2, and intact PSA assays

plasma and the rs1058205 SNP was associated with total PSA in serum (*P*=0.001), and with lower %free PSA (*P*=0.015).

CONCLUSION—In young men without prostate disease, both the seminal plasma and serum levels of hK2 and PSA are associated with several genetic variants in *KLK2* and *KLK3* that could be used to refine models of PSA cut-off values in prostate cancer testing.

Keywords

prostate cancer; prostate-specific antigen; tumor markers; seminal fluid

Introduction

Prostate-specific antigen (PSA) and a closely related protease, kallikrein-related peptidase 2 (hK2), are commonly used as markers of prostate cancer (1, 2). PSA and hK2 are members of the kallikrein gene family (3) and their expression is regulated by the ligand-dependent activation of the androgen receptor. The documented physiological roles of PSA and hK2 are in seminal plasma. PSA is responsible for the rapid degradation of the seminal vesicle-secreted proteins semenogelin I and II (4). Semenogelin I inhibits the motility of intact and demembranated spermatozoa and participates in the capacitation of sperm by blocking motility immediately after ejaculation (5). Thus, PSA is necessary for the release of progressively motile sperm. The non-catalytic proPSA-zymogen can *in vitro* be converted to catalytically active PSA by catalytic hK2 (6) which suggests that hK2 may be a physiological activator of PSA (7). Catalytic hK2 can also cleave semenogelin I and II (8).

The majority of hK2 in seminal plasma is non-catalytic and bound in complex with protein C inhibitor (encoded by *SERPINA5*), while the majority of PSA is in free, catalytically active form (fPSA), with less than 5% of PSA being complexed to *SERPIN*-type anti-proteases (cPSA) (9). The sum of fPSA and cPSA closely correspond to total PSA (tPSA). Both PSA and hK2 are non-catalytic in blood with a relationship of free to bound forms that is opposite to that in seminal fluid. hK2 in blood occurs predominantly in free unbound forms, whereas the majority of PSA is covalently bound in complexes with extracellular anti-proteases such as alpha-1-antichymotrypsin (encoded by *SERPINA3*) and only 5–35% circulates in free, unbound forms (10–12). In healthy men, the PSA concentration in seminal plasma is 0.2–5 mg/mL (13) and the retrograde release of PSA into the blood occurs with a frequency of less than one molecule per million secreted PSA molecules. hK2 is found in both seminal plasma and in serum but at approximately one percent of the concentration of PSA (7, 14). In men affected by prostate cancer, levels of PSA and hK2 in blood become elevated decades before cancer diagnosis (15, 16), and the ratio of free to total PSA (%fPSA) decreases (17). Only limited data have been reported on PSA and hK2 levels in men younger than 30 years of age. Levels of fPSA, but not cPSA, in serum correlate with levels of PSA in seminal plasma in young men (18). Low levels of PSA in seminal plasma have been shown to be associated with a reduced percentage of motile sperm (13).

The risk of prostate cancer has been associated with single-nucleotide polymorphisms (SNPs) located in the genes coding for PSA (*KLK3*) (19–24) and hK2 (*KLK2*) (25–29). A number of SNPs in these genes have been associated with serum levels of PSA and hK2 (22, 24–26, 29). The study cohorts have, however, been largely confined to older men, in whom the levels of these biomarkers may be affected by prostate cancer, benign prostatic hyperplasia, or prostatitis, all of which become more prevalent with age. Since measurement of PSA levels in serum are widely used for identifying subjects to be offered diagnostic procedures for detection of prostate cancer, it is important to know whether genetic factors may influence the non-cancer related prostatic secretion of kallikreins. To address this question, we examined the relationship between SNPs in *KLK2* and *KLK3* and levels of hK2

and PSA in seminal plasma and in serum, using a population-based cohort of young men in whom prostate conditions are very rare.

Material and Methods

Subjects

A total of 305 men under compulsory medical examination for military service in Sweden were in the year 2000 enrolled in a study of reproductive function (30). This group can be considered as representative for the Swedish general population of adolescent men since at that time more than 95% of young men in Sweden underwent examination for military service. Their median age was 18.1 years (SD, 0.4; range, 18–21 years) and median abstinence time was 85 hours (SD, 57; range, 12–504 hours). All men participated after giving written informed consent according to protocols approved by the ethical review board at Lund University. For the present study, not enough biospecimen material was available to measure all markers in all individuals; however, we expect the availability of biospecimen to be random with respect to the variable studied here.

Semen and Blood Samples

Semen samples were obtained after masturbation and delivered between 9–11 a.m. A blood sample was subsequently drawn. All participating men were asked to abstain from sexual activities for at least 48 hours and to note the actual abstinence time. Semen volume was determined by weighing the semen sample, assuming a density of 1 g/mL. For each semen sample, 450 μl was mixed with 50 μl of 0.1 M benzamidine to inhibit liquefaction. Seminal plasma was obtained by centrifugation of the semen sample 10 000 × g for 10 minutes. Blood and seminal plasma was kept at −70°C until analysis.

hK2 and PSA Analyses

Seminal plasma samples were analyzed for hK2 using a previously reported immunofluorometric assay (12) with minor modifications. The sample volume and extent of labeling of the tracer antibody were increased and blocking of tPSA was enhanced by the use of three PSA-specific anti-PSA monoclonal antibodies (Mab) (2E9, 5F7 and 5H6) that do not cross-react with hK2. The biotinylated Mab 6H10 was used to capture hK2. Finally, hK2 was detected by use of the Mab 7G1-Eu (31). The coefficient of variation (CV) for hK2-measurement in seminal plasma was 12% at a mean concentration of 0.008 mg/mL. Measurements of free PSA (fPSA) and total PSA (tPSA) in seminal plasma and serum were performed using the commercially available assay Prostatus™ PSA Free/Total kit (Delfia® Reagents) (32). The analysis for tPSA in seminal plasma measures the sum of fPSA (> 95%), PSA in complex with protein C inhibitor (cPSA)(1–3%) and hK2 (< 1%). The analysis of fPSA in seminal plasma measures the sum of active single chain and inactive internally cleaved two-chained fPSA. The combination of Mab H117 and H50 provide equimolar detection of fPSA and cPSA but also cross-reacts with hK2, whereas fPSA is measured by the combination of Mab H117 and 5A10 with no significant cross-reactivity to cPSA or hK2. CV for PSA measurements in seminal plasma was 12% at a mean concentration of 0.66 mg/mL. The detection limit in serum was 0.05 ng/mL (CV was 5% at a mean concentration of 2.3 ng/mL) for tPSA and 0.04 ng/mL (CV was 5.9% at a mean concentration of 0.25 ng/mL) for fPSA. Serum levels of hK2, fPSA, and tPSA were analyzed in 303 men, seminal plasma tPSA in 293 men, and seminal plasma hK2 in 202 men due to missing semen and serum material. The characteristics of the study group are presented in Table 1. We note that in previous work, intra-individual variability of these measures is less than 10% and we presume the same holds here.

Genotype Analyses

Genomic DNA was prepared from peripheral leukocytes using a QIAamp DNA Maxi Kit (Qiagen). DNA concentrations were determined by PicoGreen™ DNA assay (Molecular Probes), and all samples were adjusted to the same DNA concentration. Genotypes were determined by Sequenom MassARRAY MALDI-TOF analysis and assay design was made using MassARRAY Assay Design 2.0 software (Sequenom) as previously described (33). A total of 9 SNPs were initially selected for analysis on the basis of our prior observation of SNP/biomarker correlation in older men (29), removing some redundant SNPs due to linkage disequilibrium. One SNP, rs11670728 in *KLK2*, showed a significant deviation from Hardy-Weinberg equilibrium and was excluded from further analysis. The SNPs studied are described in Table 2.

Statistical Analysis

The observed genotype distribution for each SNP was tested for consistency with Hardy-Weinberg equilibrium using Fisher's exact test. To estimate the strength of linkage disequilibrium (LD) between all possible pair-wise combinations of SNPs, we calculated D′ using Haploview 4.0 (http://www.broad.mit.edu/mpg/haploview). The group characteristics of the seminal and serum levels of hK2, fPSA and tPSA were summarized descriptively. We used the Kruskal-Wallis test to examine the associations between genotype at each SNP and kallikrein values (concentrations and absolute amounts of hK2 and PSA in seminal plasma ; and concentrations of hK2, tPSA, fPSA and the %fPSA, in serum). All statistical analyses were conducted using Stata 9.0 (Stata).

Results

Levels of hK2 and PSA in seminal plasma and serum were initially determined in a total of 303 young Swedish men. The resulting data are summarized in Table 1. A total of 9 SNPs from a 24-kbp region encompassing the *KLK2* and *KLK3* genes (Table 2) were subsequently evaluated for association with levels of hK2 and PSA in seminal plasma and in serum. LD in the region was moderate, apart from one 1-kbp haplotype block in *KLK2* extending from rs198977 through rs80050017 (Fig. 1).

SNPs associated with hK2 levels

SNP associations with levels of hK2 in seminal plasma are presented in Table 3 and Supplementary Figure 1; the associations for serum are presented in Table 4 and Supplementary Figure 2. The genotypes of all 4 *KLK2* SNPs (rs198972, rs198977, rs198978, and rs80050017), were strongly associated with hK2 amount and concentration in seminal plasma and with hK2 concentration in serum (all *P*-values < 0.001). As very few rare homozygotes were observed for rs80050017, we also performed a two-group comparison, removing the rare homozygotes, in which similar results were observed (Supplementary Table 1). In general, individuals homozygous for the major alleles showed higher hK2 values compared with individuals with the other genotypes. The effects were in all cases such that the heterozygotes had intermediate values compared with the homozygotes and the effects were more pronounced in seminal plasma compared with serum. The effects were generally quite strong; in most cases there was more than a 3-fold difference between the homozygotes. The rs198977 SNP showed the strongest effect with 4- to 7-fold differences between the homozygotes. In addition, rs61752561 in *KLK3* showed an association with hK2 amount and concentration in seminal plasma, but has a low minor allele frequency (MAF) of only 0.03. Notably, for all 4 SNPs associated with hK2 levels, *ad-hoc* two-group comparisons were consistently significant with the exception of rs80050017 for which only the comparisons between common homozygotes and heterozygotes were significant (Supplementary Table 2).

SNPs associated with PSA levels

SNP associations with levels of PSA in seminal plasma and serum are presented in Tables 3 and 4, respectively. Graphical representations of selected significant results can be found in Supplemental Tables 3 and 4. The genotypes of all 3 *KLK3* SNPs (rs2271094, rs61752561, and rs1058205), were associated with PSA amount or concentration in seminal plasma (Table 3). For rs1058205, the rare homozygote count was low; similar results are observed when the common homozygote group is compared to the heterozygote group (Supplementary Table 1). However, the effects were less strong than observed for the *KLK2* SNPs and hK2, as there were between 0.8- to 1.3-fold differences between the homozygotes. Apart from the rs61752561 SNP with a low MAF, rs1058205 was the only *KLK3* SNP showing significance for PSA in serum (Table 4). Similarly, SNP rs1058205 showed a statistically significant association with higher seminal PSA amount for the TT genotype (median tPSA amount 2.1 vs. 1.6 mg for TT vs. CC). Subjects with the TT genotype also tended to have higher median seminal plasma concentration of PSA, though the difference did not reach significance (P=0.1). The same genotype was associated with significantly higher serum concentration of tPSA (median 0.53 vs. 0.44 ng/mL for TT vs. CC genotype) and lower %fPSA (40% vs. 49% for TT vs. CC genotype). Though only the comparison between the TT and TC genotype of rs1058205 showed a significant difference in serum total PSA levels (Supplemental Table 2), a continuing trend for the C allele being associated with decreased total PSA levels in serum can be observed (Supplemental Figure 2A). In addition, the *KLK2* SNPs (rs198972, rs198977, and rs198978) showed highly significant associations with %fPSA in serum. In all three cases individuals homozygous for the minor alleles showed higher values of %fPSA (rs198972, 47% vs 38% for TT vs CC; rs198977, 44% vs 40% for TT vs CC; rs198978, 46% vs 37% for TT vs GG; all $P \leq 0.007$).

Significance of the associations

The *P*-values presented in Tables 3 and 4 are unadjusted for the multiple testing performed. A commonly used compensation for multiple testing is the Bonferroni correction, which simply divides the significance level with the number of tests. In the present study a total of 64 tests have been performed. Thus, a global significance level of 0.05 corresponds to a significance level of 0.0008 in the individual tests. Using this criterion, 14 of the 25 tests that were significant in the individual tests remain significant. It should be noted that the Bonferroni method is very strict. Another way to assess this problem is to compare the expected number of significant results given that all null hypotheses are true to the observed number. Out of the 64 tests, one expects 3.2 tests with *P*-values below 0.05 and 0.64 tests with *P*-values below 0.01. The observed numbers are 25 and 18, respectively. Thus, both the Bonferroni correction approach and the comparison of the expected and observed numbers of significant results, indicate strongly that a majority of the cases with *P*-values below 0.05 are true signals.

Correlation between the SNPs

Though linkage disequilibrium appears to be moderate in this region (Fig. 1), correlation between SNPs could still mean that some of the SNPs are tagging the same functional variant and thus are redundant. To examine this further, we calculated the pairwise correlation coefficient (r^2) between all pairs of SNPs based on the phased haplotypes and examined pairs of SNPs for which $r^2 > 0.2$. The most correlated pair of SNPs is rs3760728 and rs2271094 (r^2=0.60); these two SNPs are also both significantly associated with the concentration of PSA in seminal fluid. Additionally, numerous pairwise correlations between rs198972, rs198977, rs198978, and rs80050017 are observed; these SNPs are consistently associated with levels of hK2.

Given the strong association between several correlated SNPs and hK2 levels, we next asked which of the 4-marker haplotypes formed by rs198972, rs198977, rs198978, and rs80050017 are associated with hk2 levels. Of the five haplotypes with a frequency greater than 5%, four are strongly associated with hK2 levels in blood and semen (Table 5). Of these, two are also associated with the ratio between free and total PSA (Table 5). Notably, the three common haplotypes with a "T" allele at rs198977 are strongly associated with decreased levels of hK2, consistent with what was observed for single-marker tests.

Discussion

In the current study, we examined 9 previously reported SNPs in a 24 kbp *KLK2 - KLK3* region (28) for associations with levels of hK2 and PSA in seminal plasma and serum in young men without prostate disease. For hK2, all four *KLK2* SNPs showed association with hK2 levels both in seminal plasma and serum. Three of these SNPs (rs198977, rs198978, and rs80050017) formed a haplotype block, whereas the remaining SNP (rs198972) was in moderate LD with these SNPs. The strongest effect on hK2 level was observed for rs198977. Consistent with this observation, common haplotypes containing the "T" allele of rs198977 are associated with decreased levels of hK2. Whether the rs198977 SNP is causal or if other SNPs in LD with this SNP are causing the observed lower levels of hK2 is unknown at present.

The SNP rs198977 has previously been associated with hK2 levels among older men with no prostate cancer diagnosis (~65–70 years old) (25, 26, 29); as in this study, the T allele was consistently associated with lower levels. In all three prior studies, the T allele was also associated with slightly increased risk of prostate cancer. In contrast, a smaller study of Chinese men (27) reported increased risk of prostate cancer for men carrying the C allele. This discrepancy may possibly be explained by the different populations studied, but the differences nevertheless raise questions. The C to T substitution in rs198977 corresponds to an Arg226Trp change in hK2. An *in vitro* study suggested that the Trp^{226}-hK2 variant lacked protease activity, although the experimental system did not allow a definitive assessment (34). The observation that the T allele of rs198977 is associated with lower hK2 levels in multiple populations and among both young and old men suggests that it results from a basic aspect of hK2 biology resulting in lower hK2 expression. Another possibility is that the Trp^{226} form of hK2 (encoded by the T allele) is more rapidly degraded, or poorly detected by both of the different monoclonal antibodies each capturing and detecting hK2 through uniquely distinct epitopes in our study and that by Nam et al. (25, 26).

Several *KLK3* SNPs showed associations with levels of PSA. The rs2271094 SNP was significantly associated with PSA amount and concentration in seminal plasma. For rs61752561, the GG genotype showed an association with lower PSA concentration in seminal plasma, but an association with higher tPSA concentration in serum. Since this SNP has a MAF of only 0.03, these associations are likely due to chance. For a third *KLK3* SNP, rs1058205, the TT genotype showed a statistically significant association with higher PSA amount in seminal plasma and with higher tPSA concentration and lower %fPSA in serum. This decrease in %fPSA is a consequence of the increase in serum levels, which is due to the difference in mechanisms and rates of elimination of fPSA and complex-bound PSA. Our results with this SNP fit with a prior report in which the T variant was associated with higher serum tPSA in older men (22). This variant was also associated with higher cancer risk in one study (24), though subsequent studies failed to confirm this association (22, 29). Moreover, we and others (22) have argued that *KLK3* variants associated with higher tPSA levels may exhibit spurious associations with prostate cancer risk if PSA testing was involved in detection of the cancers.

Three *KLK2* SNPs, rs198972, rs198977, and rs198978, were significantly associated with %fPSA in serum. In each case, the allele associated with higher %fPSA was also associated with lower hK2. How variants in *KLK2* might affect the %fPSA is not entirely clear; however, we propose two possible explanations. The first is a protein-protein interaction between hK2 and PSA, in which a variant hK2 might selectively stabilize fPSA, thereby increasing %fPSA. Second, if hK2 is responsible for PSA processing *in vivo*, then variants that reduce hK2 level or protease activity (as suggested for Trp^{226}-hK2) could result in decreased mature PSA and increased pro-PSA. Because pro-PSA is unable to form complexes with the serum anti-proteases and therefore remains free in serum, the result would be an increase in the %fPSA. Interestingly, rs198977 was also associated with both hK2 and %fPSA in our prior study of older men (29).

Other *KLK3* SNPs have also been associated with PSA levels in blood. Several SNPs in the *KLK3* promoter were reported to be associated with serum PSA levels (19, 20), and one was associated with prostate cancer risk (35), but later studies failed to confirm these associations (21, 36–39). In an earlier study of the same cohort, we found that a *KLK3* promoter SNP, rs266882, in combination with androgen receptor gene CAG repeat length, was significantly associated with serum levels of tPSA (38). In the same cohort we have also recently shown, that a *MSMB*-promoter SNP (rs10993994) at the genetic locus encoding β-microseminoprotein (*β*-MSP) is significantly associated with blood and semen levels of PSA and semen levels of hK2 (40). In the 3′ flanking region of *KLK3*, rs2735839 was found to be associated with PSA level in two studies (22, 23). In contrast, our previous study found an association for this SNP with %fPSA but not PSA level (29).

The associations others and we have detected may have implications for PSA and hK2 testing in prostate cancer screening. For men carrying genetic variants associated with altered levels of PSA or hK2, prostate cancer risk models developed on the general population may be less accurate. Conversely, the combination of biomarker levels and genotype may offer a means of increasing the accuracy of prostate cancer prediction. These considerations are particularly applicable to the *KLK2* SNP rs198977, for which the TT genotype is associated with dramatically lower hK2 levels, but with higher cancer risk. Indeed, a model incorporating rs198977 genotype and the interaction between genotype and hK2 level was suggested to have higher accuracy for predicting prostate cancer than a model with biomarker data alone (29).

In conclusion, we documented the association of SNPs in *KLK3* and *KLK2* with levels of PSA and hK2 in young men. These results demonstrate that associations exist several decades before any substantial incidence of prostate cancer or other prostate conditions were found to influence kallikrein levels in the blood (16). Moreover, most of the variants associated with altered tPSA or hK2 concentrations in serum were associated with corresponding albeit more pronounced alterations in seminal plasma. Therefore, the causative genetic variants most likely exert their effects through global changes in expression or protein stability, rather than being related to a prostate disease process. The information from analyses of variants in the *KLK2* and *KLK3* could also be used to refine models of PSA cut-off values in prostate cancer testing. In addition, because low levels of PSA are correlated with lower percentage of motile sperm in both fertile and infertile men, a better knowledge of the factors regulating the levels of hK2 and PSA is of importance to understanding the mechanisms behind male infertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

hK2	kallikrein-related peptidase 2
PSA	prostate-specific antigen
fPSA	free PSA
cPSA	complexed PSA
tPSA	total PSA
%fPSA	ratio of free to total PSA
SNP	single nucleotide polymorphism
Mab	monoclonal antibody
LD	linkage disequilibrium

Human genes

KLK2	kallikrein-related peptidase 2 encoding hK2
KLK3	kallikrein-related peptidase 3 encoding PSA
AR	androgen receptor

References

1. Darson MF, Pacelli A, Roche P, Rittenhouse HG, Wolfert RL, Young CY, et al. Human glandular kallikrein 2 (hk2) expression in prostatic intraepithelial neoplasia and adenocarcinoma: A novel prostate cancer marker. Urology. 1997; 49:857–62. [PubMed: 9187691]
2. Kuriyama M, Wang MC, Lee CI, Papsidero LD, Killian CS, Inaji H, et al. Use of human prostate-specific antigen in monitoring prostate cancer. Cancer research. 1981; 41:3874–6. [PubMed: 7284995]
3. Yousef GM, Luo LY, Diamandis EP. Identification of novel human kallikrein-like genes on chromosome 19q13.3-q13. 4. Anticancer Res. 1999; 19:2843–52. [PubMed: 10652563]
4. Lilja H. A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. The Journal of clinical investigation. 1985; 76:1899–903. [PubMed: 3902893]
5. de Lamirande E, Yoshida K, Yoshiike TM, Iwamoto T, Gagnon C. Semenogelin, the main protein of semen coagulum, inhibits human sperm capacitation by interfering with the superoxide anion generated during this process. Journal of andrology. 2001; 22:672–9. [PubMed: 11451365]
6. Lovgren J, Rajakoski K, Karp M, Lundwall a, Lilja H. Activation of the zymogen form of prostate-specific antigen by human glandular kallikrein 2. Biochemical and biophysical research communications. 1997; 238:549–55. [PubMed: 9299549]
7. Deperthes D, Chapdelaine P, Tremblay RR, Brunet C, Berton J, Hebert J, et al. Isolation of prostatic kallikrein hk2, also known as hgk-1, in human seminal plasma. Biochimica et biophysica acta. 1995; 1245:311–6. [PubMed: 8541306]

8. Deperthes D, Frenette G, Brillard-Bourdet M, Bourgeois L, Gauthier F, Tremblay RR, Dube JY. Potential involvement of kallikrein hk2 in the hydrolysis of the human seminal vesicle proteins after ejaculation. Journal of andrology. 1996; 17:659–65. [PubMed: 9016396]
9. Christensson A, Lilja H. Complex formation between protein c inhibitor and prostate-specific antigen in vitro and in human semen. European journal of biochemistry/FEBS. 1994; 220:45–53. [PubMed: 7509746]
10. Lilja H, Christensson A, Dahlen U, Matikainen MT, Nilsson O, Pettersson K, Lovgren T. Prostate-specific antigen in serum occurs predominantly in complex with alpha 1-antichymotrypsin. Clinical chemistry. 1991; 37:1618–25. [PubMed: 1716536]
11. Grauer LS, Finlay JA, Mikolajczyk SD, Pusateri KD, Wolfert RL. Detection of human glandular kallikrein, hk2, as its precursor form and in complex with protease inhibitors in prostate carcinoma serum. Journal of andrology. 1998; 19:407–11. [PubMed: 9733142]
12. Becker C, Piironen T, Kiviniemi J, Lilja H, Pettersson K. Sensitive and specific immunodetection of human glandular kallikrein 2 in serum. Clin Chem. 2000; 46:198–206. [PubMed: 10657376]
13. Ahlgren G, Rannevik G, Lilja H. Impaired secretory function of the prostate in men with oligo-asthenozoospermia. Journal of andrology. 1995; 16:491–8. [PubMed: 8867597]
14. Piironen T, Lovgren J, Karp M, Eerola R, Lundwall A, Dowell B, et al. Immunofluorometric assay for sensitive and specific measurement of human prostatic glandular kallikrein (hk2) in serum. Clinical chemistry. 1996; 42:1034–41. [PubMed: 8674186]
15. Nam RK, Diamandis EP, Toi A, Trachtenberg J, Magklara A, Scorilas A, et al. Serum human glandular kallikrein-2 protease levels predict the presence of prostate cancer among men with elevated prostate-specific antigen. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2000; 18:1036–42. [PubMed: 10694554]
16. Lilja H, Cronin AM, Dahlin A, Manjer J, Nilsson PM, Eastham JA, et al. Prediction of significant prostate cancer diagnosed 20 to 30 years later with a single measure of prostate-specific antigen at or before age 50. Cancer. 2011; 117:1210–9. [PubMed: 20960520]
17. Christensson A, Bjork T, Nilsson O, Dahlen U, Matikainen MT, Cockett AT, et al. Serum prostate specific antigen complexed to alpha 1-antichymotrypsin as an indicator of prostate cancer. The Journal of urology. 1993; 150:100–5. [PubMed: 7685416]
18. Savblom C, Malm J, Giwercman A, Nilsson JA, Berglund G, Lilja H. Blood levels of free-psa but not complex-psa significantly correlates to prostate release of psa in semen in young men, while blood levels of complex-psa, but not free-psa increase with age. Prostate. 2005; 65:66–72. [PubMed: 15880475]
19. Xue WM, Coetzee GA, Ross RK, Irvine R, Kolonel L, Henderson BE, Ingles SA. Genetic determinants of serum prostate-specific antigen levels in healthy men from a multiethnic cohort. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2001; 10:575–9.
20. Cramer SD, Chang BL, Rao A, Hawkins GA, Zheng SL, Wade WN, et al. Association between genetic polymorphisms in the prostate-specific antigen gene promoter and serum prostate-specific antigen levels. Journal of the National Cancer Institute. 2003; 95:1044–53. [PubMed: 12865450]
21. Xu J, Meyers DA, Sterling DA, Zheng SL, Catalona WJ, Cramer SD, et al. Association studies of serum prostate-specific antigen levels and the genetic polymorphisms at the androgen receptor and prostate-specific antigen genes. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2002; 11:664–9.
22. Ahn J, Berndt SI, Wacholder S, Kraft P, Kibel AS, Yeager M, et al. Variation in klk genes, prostate-specific antigen and risk of prostate cancer. Nat Genet. 2008; 40:1032–4. author reply 5–6. [PubMed: 19165914]
23. Eeles RA, Kote-Jarai Z, Giles GG, Olama AA, Guy M, Jugurnauth SK, et al. Multiple newly identified loci associated with prostate cancer susceptibility. Nat Genet. 2008; 40:316–21. [PubMed: 18264097]
24. Pal P, Xi H, Sun G, Kaushal R, Meeks JJ, Thaxton CS, et al. Tagging snps in the kallikrein genes 3 and 2 on 19q13 and their associations with prostate cancer in men of european origin. Hum Genet. 2007; 122:251–9. [PubMed: 17593395]

25. Nam RK, Zhang WW, Trachtenberg J, Diamandis E, Toi A, Emami M, et al. Single nucleotide polymorphism of the human kallikrein-2 gene highly correlates with serum human kallikrein-2 levels and in combination enhances prostate cancer detection. J Clin Oncol. 2003; 21:2312–9. [PubMed: 12805332]
26. Nam RK, Zhang WW, Klotz LH, Trachtenberg J, Jewett MA, Sweet J, et al. Variants of the hk2 protein gene (klk2) are associated with serum hk2 levels and predict the presence of prostate cancer at biopsy. Clin Cancer Res. 2006; 12:6452–8. [PubMed: 17085659]
27. Chiang CH, Hong CJ, Chang YH, Chang LS, Chen KK. Human kallikrein-2 gene polymorphism is associated with the occurrence of prostate cancer. The Journal of urology. 2005; 173:429–32. [PubMed: 15643194]
28. Mittal RD, Mishra DK, Thangaraj K, Singh R, Mandhani A. Is there an inter-relationship between prostate specific antigen, kallikrein-2 and androgen receptor gene polymorphisms with risk of prostate cancer in north indian population? Steroids. 2007; 72:335–41. [PubMed: 17257635]
29. Klein RJ, Hallden C, Cronin AM, Ploner A, Wiklund F, Bjartell AS, et al. Blood biomarker levels to aid discovery of cancer-related single nucleotide polymorphisms: Kallikreins and prostate cancer. Cancer Prev Res (Phila Pa). 2010; 3:611–9.
30. Richthoff J, Rylander L, Hagmar L, Malm J, Giwercman A. Higher sperm counts in southern sweden compared with denmark. Hum Reprod. 2002; 17:2468–73. [PubMed: 12202443]
31. Vaisanen V, Eriksson S, Ivaska KK, Lilja H, Nurmi M, Pettersson K. Development of sensitive immunoassays for free and total human glandular kallikrein 2. Clinical chemistry. 2004; 50:1607–17. [PubMed: 15247158]
32. Mitrunen K, Pettersson K, Piironen T, Bjork T, Lilja H, Lovgren T. Dual-label one-step immunoassay for simultaneous measurement of free and total prostate-specific antigen concentrations and ratios in serum. Clin Chem. 1995; 41:1115–20. [PubMed: 7543033]
33. Klein RJ, Hallden C, Gupta A, Savage CJ, Dahlin A, Bjartell A, et al. Evaluation of multiple risk-associated single nucleotide polymorphisms versus prostate-specific antigen at baseline to predict prostate cancer in unscreened men. European urology. 2012; 61:471–7. [PubMed: 22101116]
34. Herrala A, Kurkela R, Porvari K, Isomaki R, Henttu P, Vihko P. Human prostate-specific glandular kallikrein is expressed as an active and an inactive protein. Clinical chemistry. 1997; 43:279–84. [PubMed: 9023130]
35. Medeiros R, Morais A, Vasconcelos A, Costa S, Pinto D, Oliveira J, et al. Linkage between polymorphisms in the prostate specific antigen are1 gene region, prostate cancer risk, and circulating tumor cells. The Prostate. 2002; 53:88–94. [PubMed: 12210484]
36. Beebe-Dimmer JL, Lange LA, Cain JE, Lewis RC, Ray AM, Sarma AV, et al. Polymorphisms in the prostate-specific antigen gene promoter do not predict serum prostate-specific antigen levels in african-american men. Prostate cancer and prostatic diseases. 2006; 9:50–5. [PubMed: 16247489]
37. Rao A, Chang BL, Hawkins G, Hu JJ, Rosser CJ, Hall MC, et al. Analysis of g/a polymorphism in the androgen response element i of the psa gene and its interactions with the androgen receptor polymorphisms. Urology. 2003; 61:864–9. [PubMed: 12670590]
38. Savblom C, Giwercman A, Malm J, Hallden C, Lundin K, Lilja H, Giwercman Y. Association between polymorphisms in the prostate-specific antigen (psa) promoter and release of psa. International journal of andrology. 2009; 32:479–85. [PubMed: 18336535]
39. Wang LZ, Sato K, Tsuchiya N, Yu JG, Ohyama C, Satoh S, et al. Polymorphisms in prostate-specific antigen (psa) gene, risk of prostate cancer, and serum psa levels in japanese population. Cancer Lett. 2003; 202:53–9. [PubMed: 14643026]
40. Xu X, Valtonen-Andre C, Savblom C, Hallden C, Lilja H, Klein RJ. Polymorphisms at the microseminoprotein-beta locus associated with physiologic variation in beta-microseminoprotein and prostate-specific antigen levels. Cancer Epidemiol Biomarkers Prev. 2010; 19:2035–42. [PubMed: 20696662]

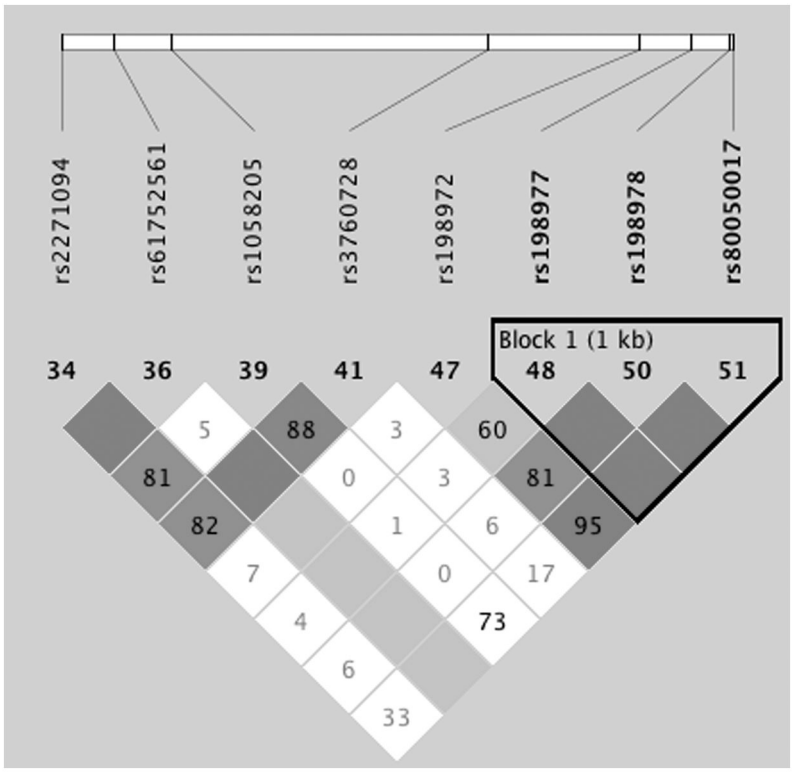

Figure 1.
Linkage disequilibrium plot of the SNPs studied.

Table 1

hK2 and PSA levels in seminal plasma and serum.

Characteristic	No.	Mean	Median	IQR	SD
Semen volume (mL)	303	3.2	3.2	2.3–4.0	1.3
Seminal plasma hK2 (mg)	202	0.02	0.02	0.01–0.03	0.02
Seminal plasma hK2 (mg/L)	202	7.2	6.1	4.0–9.2	4.6
Seminal plasma PSA (mg)	293	2.2	2.0	1.3–2.9	1.3
Seminal plasma PSA (mg/mL)	293	0.70	0.64	0.46–0.88	0.34
Serum hK2 (ng/mL)	303	0.04	0.04	0.03–0.05	0.02
Serum fPSA (ng/mL)	303	0.29	0.19	0.13–0.27	0.72
Serum tPSA (ng/mL)	303	0.64	0.50	0.35–0.67	0.84

IQR, inter-quartile range.

Table 2

SNP descriptions and allele frequencies in the study cohort.

SNP	Location	Genomic Position[a]	Amino Acid Change	Major/Minor Allele[b]	MAF[b]	HWE *P*-value
rs2271094[c]	*KLK3* exon 2	51359497	Gly16 syn	A/G	0.39	0.04
rs61752561	*KLK3* exon 3	51361382	Asp-Asn 102	G/A	0.03	1.0
rs1058205	*KLK3* exon 5	51363398	3′UTR	T/C	0.09	0.84
rs3760728	Intergene	51374592	-	C/G	0.36	0.10
rs11670728[d]	*KLK2* upstream	51376489	promoter	G/A	0.39	<0.0001
rs198972	*KLK2* exon 3	51379893	Leu124 syn	C/T	0.31	0.24
rs198977	*KLK2* exon 5	51381777	Arg-Trp 250	C/T	0.23	0.49
rs198978	*KLK2* exon 5	51383072	3′UTR	G/T	0.36	0.88
rs80050017	*KLK2* exon 5	51383200	3′UTR	G/T	0.08	0.14

[a] Position on chromosome 19 according to NCBI dbSNP build 137.

[b] Based on the study cohort.

[c] This SNP (rs2271094) is represented by rs11573 in build 137 of NCBI dbSNP. We provide the build 137 coordinates for the SNP here, but use the older nomenclature of rs2271094 to be consistent with our prior publications.

[d] This SNP was excluded from further analysis due to deviation from Hardy-Weinberg equilibrium (HWE).

Table 3

Association between SNPs in *KLK2* and *KLK3* and levels of hK2 and PSA in seminal plasma.

		hK2					PSA				
SNP	**Genotype**	**No.**	**Amount, mg Median (IQR)**	***P*-value**	**Conc., mg/L Median (IQR)**	***P*-value**	**No.**	**Amount, mg Median (IQR)**	***P*-value**	**Conc., mg/mL Median (IQR)**	***P*-value**
rs2271094	AA	85	0.020 (0.011, 0.034)	0.8	6.8 (3.7, 10.1)	0.8	117	1.8 (1.1, 2.6)	**0.01**	0.57 (0.42, 0.80)	**0.01**
	GA	85	0.019 (0.011, 0.029)		5.8 (4.3, 8.6)		118	2.1 (1.5, 3.1)		0.65 (0.52, 0.90)	
	GG	30	0.019 (0.011, 0.027)		5.8 (4.0, 9.1)		53	2.1 (1.2, 3.3)		0.69 (0.49, 0.98)	
	Total	200					288				
rs61752561	GG	188	0.018 (0.010, 0.030)	**0.01**	5.9 (3.8, 9.1)	**0.004**	273	2.0 (1.3, 2.9)	0.3	0.63 (0.45, 0.88)	**0.03**
	GA	13	0.030 (0.020, 0.040)		10.0 (7.7, 11.4)		18	2.7 (1.4, 3.3)		0.80 (0.58, 1.07)	
	Total	201					291				
rs1058205	TT	123	0.018 (0.011, 0.029)	0.7	5.9 (4.0, 9.1)	0.2	189	2.1 (1.4, 3.1)	**0.02**	0.67 (0.47, 0.92)	0.1
	CT	70	0.018 (0.010, 0.034)		6.2 (3.7, 10.1)		92	1.8 (1.1, 2.6)		0.57 (0.44, 0.84)	
	CC	7	0.029 (0.014, 0.032)		9.0 (5.5, 13.9)		9	1.6 (0.94, 2.2)		0.49 (0.34, 0.63)	
	Total	200					290				
rs3760728	CC	90	0.018 (0.010, 0.034)	0.6	6.4 (3.5, 10.0)	0.6	125	1.8 (1.2, 2.6)	0.1	0.57 (0.42, 0.78)	**0.01**
	CG	87	0.020 (0.011, 0.030)		5.9 (4.3, 9.1)		121	2.1 (1.4, 3.1)		0.66 (0.52, 0.90)	
	GG	24	0.018 (0.012, 0.024)		4.7 (3.7, 8.0)		44	2.1 (1.5, 3.0)		0.69 (0.51, 1.0)	
	Total	201					290				
rs198972	CC	99	0.024 (0.012, 0.034)	**0.0007**	7.1 (5.0, 11.3)	**<0.0005**	136	1.9 (1.1, 2.9)	0.7	0.63 (0.48, 0.86)	0.8
	CT	89	0.017 (0.010, 0.030)		5.6 (3.4, 8.2)		133	2.0 (1.3, 3.1)		0.65 (0.45, 0.90)	
	TT	14	0.008 (0.004, 0.016)		2.9 (1.0, 4.9)		24	2.0 (1.3, 2.4)		0.69 (0.42, 0.86)	
	Total	202					293				
rs198977	CC	118	0.027 (0.016, 0.038)	**<0.0005**	7.7 (5.2, 11.2)	**<0.0005**	173	2.0 (1.4, 2.9)	1	0.61 (0.47, 0.87)	0.5
	CT	69	0.014 (0.010, 0.022)		4.8 (3.2, 6.8)		96	2.0 (1.3, 2.9)		0.65 (0.45, 0.95)	
	TT	12	0.004 (0.002, 0.005)		1.1 (0.8, 1.5)		18	2.0 (1.1, 3.3)		0.67 (0.32, 0.82)	
	Total	199					287				
rs198978	GG	83	0.026 (0.014, 0.038)	**<0.0005**	7.7 (5.2, 11.7)	**<0.0005**	120	1.9 (1.2, 2.9)	0.9	0.63 (0.48, 0.87)	1

		hK2					PSA				
SNP	**Genotype**	**No.**	**Amount, mg Median (IQR)**	***P*-value**	**Conc., mg/L Median (IQR)**	***P*-value**	**No.**	**Amount, mg Median (IQR)**	***P*-value**	**Conc., mg/mL Median (IQR)**	***P*-value**
	TG	95	0.017 (0.011, 0.029)		5.8 (3.7, 8.4)		132	2.0 (1.3, 3.0)		0.62 (0.45, 0.91)	
	TT	22	0.008 (0.004, 0.015)		2.0 (1.0, 4.1)		36	2.0 (1.3, 2.6)		0.68 (0.41, 0.83)	
	Total	200					288				
rs80050017	GG	169	0.021 (0.012, 0.032)	**<0.0005**	6.8 (4.6, 10.0)	**<0.0005**	245	1.9 (1.3, 2.9)	0.7	0.64 (0.47, 0.88)	0.4
	TG	27	0.008 (0.006, 0.021)		3.0 (1.4, 5.9)		37	2.2 (1.3, 3.1)		0.69 (0.43, 0.99)	
	TT	1	0.004 (0.004, 0.004)		1.0 (1.0, 1.0)		3	0.8 (0.8, 4.4)		0.25 (0.23, 0.87)	
	Total	197					285				

Bold type indicates statistical significance.

IQR, inter-quartile range.

Table 4

Association between SNPs in *KLK2* and *KLK3* and concentrations of hK2 and PSA in serum.

			hK2, ng/mL		tPSA, ng/mL		fPSA, ng/mL		Percent fPSA	
SNP	**Genotype**	**No.**	**Median (IQR)**	***P*-value**	**Median (IQR)**	***P*-value**	**Median (IQR)**	***P*-value**	**Median (IQR)**	***P*-value**
rs2271094	AA	121	0.037 (0.026, 0.052)	0.4	0.50 (0.33, 0.67)	0.8	0.18 (0.13, 0.26)	0.3	40 (32, 50)	0.8
	GA	122	0.036 (0.028, 0.051)		0.50 (0.37, 0.67)		0.21 (0.15, 0.27)		43 (35, 49)	
	GG	54	0.035 (0.022, 0.045)		0.52 (0.32, 0.67)		0.21 (0.13, 0.29)		43 (35, 52)	
	Total	297								
rs61752561	GG	282	0.035 (0.025, 0.051)	0.3	0.50 (0.35, 0.68)	**0.02**	0.19 (0.14, 0.27)	0.05	42 (34, 50)	0.6
	GA	19	0.039 (0.035, 0.046)		0.42 (0.29, 0.51)		0.15 (0.12, 0.21)		44 (36, 51)	
	Total									
rs1058205	TT	195	0.036 (0.025, 0.050)	0.8	0.53 (0.37, 0.76)	**0.001**	0.20 (0.15, 0.28)	0.06	40 (34, 48)	**0.01**
	CT	94	0.036 (0.026, 0.050)		0.41 (0.32, 0.58)		0.17 (0.12, 0.24)		44 (34, 51)	
	CC	10	0.034 (0.025, 0.040)		0.44 (0.30, 0.53)		0.19 (0.18, 0.27)		49 (41, 61)	
	Total	299								
rs3760728	CC	130	0.036 (0.025, 0.051)	0.2	0.48 (0.33, 0.67)	0.9	0.18 (0.13, 0.24)	0.3	40 (32, 49)	0.3
	CG	123	0.036 (0.027, 0.051)		0.50 (0.36, 0.65)		0.21 (0.14, 0.27)		44 (35, 49)	
	GG	46	0.034 (0.019, 0.042)		0.50 (0.32, 0.70)		0.20 (0.13, 0.30)		44 (37, 53)	
	Total	299								
rs198972	CC	138	0.040 (0.030, 0.052)	**<0.0005**	0.52 (0.39, 0.68)	0.2	0.19 (0.13, 0.27)	0.6	38 (31, 47)	**<0.0005**
	CT	140	0.035 (0.026, 0.050)		0.45 (0.32, 0.67)		0.20 (0.15, 0.27)		44 (37, 52)	
	TT	25	0.017 (0.008, 0.023)		0.52 (0.32, 0.60)		0.18 (0.13, 0.24)		47 (27, 56)	
	Total	303								
rs198977	CC	178	0.044 (0.033, 0.054)	**<0.0005**	0.51 (0.36, 0.68)	0.5	0.19 (0.13, 0.26)	0.9	40 (32, 48)	**0.007**
	CT	100	0.029 (0.021, 0.038)		0.45 (0.34, 0.66)		0.19 (0.14, 0.28)		45 (37, 53)	
	TT	18	0.008 (0.005, 0.014)		0.52 (0.30, 0.60)		0.18 (0.15, 0.27)		44 (32, 55)	
	Total	296								
rs198978	GG	121	0.043 (0.033, 0.054)	**<0.0005**	0.52 (0.36, 0.68)	0.5	0.18 (0.13, 0.26)	0.3	37 (31, 46)	**<0.0005**

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			hK2, ng/mL		tPSA, ng/mL		fPSA, ng/mL		Percent fPSA	
SNP	**Genotype**	**No.**	**Median (IQR)**	***P*-value**	**Median (IQR)**	***P*-value**	**Median (IQR)**	***P*-value**	**Median (IQR)**	***P*-value**
	TG	139	0.034 (0.026, 0.050)		0.47 (0.35, 0.67)		0.21 (0.15, 0.27)		45 (38, 52)	
	TT	38	0.015 (0.008, 0.023)		0.48 (0.31, 0.61)		0.18 (0.13, 0.27)		46 (32, 55)	
	Total	298								
rs80050017	GG	253	0.039 (0.028, 0.051)	**<0.0005**	0.50 (0.35, 0.67)	0.8	0.19 (0.14, 0.27)	0.9	41 (35, 49)	0.6
	TG	37	0.023 (0.017, 0.029)		0.47 (0.35, 0.66)		0.18 (0.15, 0.25)		43 (32, 53)	
	TT	4	0.011 (0.007, 0.015)		0.48 (0.33, 0.56)		0.20 (0.11, 0.31)		52 (37, 55)	
	Total	294								

Bold type indicates statistical significance.

IQR, inter-quartile range.

Table 5

Haplotype association with hK2 levels and percent fPSA

		Serum				Seminal plasma			
		Percent fPSA		log10 hK2, ng/mL		log10 hK2, mg		log10 hK2, mg/L	
Haplotype (rs198972, rs198977, rs198978, rs80050017)	**Frequency**	**Beta**	***P*-value**	**Beta**	***P*-value**	**Beta**	***P*-value**	**Beta**	***P*-value**
CCGG	0.61	−5.0	<0.0005	0.48	<0.0005	0.19	<0.0005	0.21	<0.0005
TCTG	0.1	2.8	0.1	−0.048	0.6	0.082	0.1	0.057	0.3
TTTG	0.098	5.3	0.007	−0.38	<0.0005	−0.27	<0.0005	−0.23	<0.0005
TTTT	0.074	0.62	0.8	−0.60	<0.0005	−0.30	<0.0005	−0.34	<0.0005
CTTG	0.056	1.4	0.6	−0.71	<0.0005	−0.25	<0.0005	−0.24	<0.0005
All other haplotypes (frequency <5% each)	0.061	4.2	0.08	0.26	0.02	0.22	0.002	0.15	0.02