*Am. J. Hum. Genet. 68:901–911, 2001*

# Evaluation of Linkage and Association of HPC2/ELAC2 in Patients with Familial or Sporadic Prostate Cancer

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**To investigate the relationship between HPC2/ELAC2 and prostate cancer risk, we performed the following analyses: (1) a linkage study of six markers in and around the HPC2/ELAC2 gene at 17p11 in 159 pedigrees with hereditary prostate cancer (HPC); (2) a mutation-screening analysis of all coding exons of the gene in 93 probands with HPC; (3) family-based and population-based association study of common HPC2/ELAC2 missense variants in 159 probands with HPC, 249 patients with sporadic prostate cancer, and 222 unaffected male control subjects. No evidence for linkage was found in the total sample, nor in any subset of pedigrees based on characteristics that included age at onset, number of affected members, male-to-male disease transmission, or race. Furthermore, only the two previously reported missense changes (Ser217Leu and Ala541Thr) were identified by mutational analysis of all HPC2/ELAC exons in 93 probands with HPC. In association analyses, family-based tests did not reveal excess transmission of the Leu217 and/or Thr541 alleles to affected offspring, and population-based tests failed to reveal any statistically significant difference in the allele frequencies of the two polymorphisms between patients with prostate cancer and control subjects. The results of this study lead us to reject the three alternative hypotheses of (1) a highly penetrant, major prostate cancer–susceptibility gene at 17p11, (2) the allelic variants Leu217 or Thr541 of HPC2/ELAC2 as high-penetrance mutations, and (3) the variants Leu217 or Thr541 as low-penetrance, risk-modifying alleles. However, we did observe a trend of higher Leu217 homozygous carrier rates in patients than in control subjects. Considering the impact of genetic heterogeneity, phenocopies, and incomplete penetrance on the linkage and association studies of prostate cancer and on the power to detect linkage and association in our study sample, our results cannot rule out the possibility of a highly penetrant prostate cancer gene at this locus that only segregates in a small number of pedigrees. Nor can we rule out a prostate cancer–modifier gene that confers a lower-than-reported risk. Additional larger studies are needed to more fully evaluate the role of this gene in prostate cancer risk.**

## Introduction

Using a genomewide screen together with positional cloning, Tavtigian et al. (2001) identified the HPC2/ELAC2 gene (MIM 605367) on chromosome 17 as a prostate cancer (MIM 176807) susceptibility gene in large, high-risk Utah pedigrees. A genomewide screen in eight Utah pedigrees provided suggestive evidence for linkage at 17p11 near marker D17S520, and fine-mapping studies using dense markers in the region in a larger set of pedigrees (total of 33) provided significant evidence for linkage, with a maximum two-point LOD score of 4.5 at D17S1289. The evidence for linkage in an additional 94 pedigrees was positive but much weaker, with a peak LOD of 0.44 in this region. Sequence analysis of HPC2/ELAC2 identified four sequence variants, including a rare frameshift, and three missense changes, two of which were common in the study population. These latter two polymorphisms result in a Ser-to-Leu change at amino acid 217, and an Ala-to-Thr change at amino acid 541. These two polymorphisms were reported to segregate with prostate cancer in two high-risk pedigrees. In addition, the two polymorphisms were found to be associated with the diagnosis of prostate cancer, by comparing the carrier rates of Leu217 and/or Thr541 among patients with heredi-

Received December 15, 2000; accepted for publication February 12, 2001; electronically published March 14, 2001.

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0002-9297/2001/6804-0013$02.00

tary prostate cancer (HPC), unaffected members of pedigrees with HPC, and unaffected men who had married into the pedigrees. Specifically, the overall allele frequency of Leu217 was 30%, and the frequency of Leu217 homozygotes was higher in patients with HPC (57 [13.3%]/429) than in either unaffected members of pedigrees with HPC (220 [9.3%]/2371; $P = .013$) or in unaffected men who had married into the pedigrees (9/148 [6.1%], $P = .026$). The overall allele frequency of Thr541 was 4% and its carrier rates were higher in patients with HPC (42 [9.8%]/429) than in unaffected men who had married into the pedigrees (5 [3.4%]/148; $P = .022$). There is strong linkage disequilibrium (LD) between the two polymorphisms, even though they are ~15 kb apart. All of the Thr541 variants are observed on Leu217 chromosomes (Tavtigian et al. 2001).

The finding of the association between the two polymorphisms in HPC2/ELAC2 and prostate cancer risk was examined in an independent study of 359 incident prostate cancer case subjects unselected for family history and 266 male control subjects without prostate cancer (Rebbeck et al. 2000). The frequencies of the Leu217 allele was 30.8%, 31.5%, and 31.6%, in the 359 case subjects, 258 age- and race-matched control subjects, and 383 total control subjects, respectively. There was no significant difference of the Leu217 carrier rates in the case subjects (52.1%) and in the control subjects (53.1%). The frequencies of Thr541 allele were 3.8%, 1.8%, and 2.9% in the 359 case subjects, 258 age- and race-matched control subjects, and all 383 control subjects (matched and unmatched), respectively. The Thr541 carrier rate was reported to be significantly higher in the case subjects (7.5%) than in the matched control subjects (3.5%), with an odds ratio (OR) of 2.37 (95% confidence interval [CI] 1.06–5.29), with the Leu217/Thr541 variant being estimated to account for ~5% of prostate cancer case subjects in the general population. Interestingly, the Thr541 carrier rate in case subjects was not significantly higher than the frequency observed for the complete control group (5.7%). Rebbeck et al. also reported that the Thr541 allele was only observed on the background of Leu217.

To examine the above findings of linkage and association between the HPC2/ELAC2 and prostate cancer risk, we performed linkage and mutational analyses in families with HPC and association studies in two data sets. We genotyped four microsatellite markers surrounding the HPC2/ELAC2 gene and the Leu217 and Thr541 polymorphisms within the gene in 159 families with hereditary prostate cancer. We have also genotyped the two polymorphisms in 249 sporadic prostate cancer case subjects, and 211 non–prostate cancer male control subjects. In addition, we performed mutational analysis on all HPC2/ELAC exons by heteroduplex analysis and direct sequencing of 93 patient DNA samples.

With these data, we can test the following alternative hypotheses. (1) If the HPC2/ELAC2 is a major, high-penetrance gene for prostate cancer, we would expect to observe significant linkage at 17p11 in the 159 total families with HPC and/or mutations in the gene segregating with disease phenotype. (2) If the variants Leu217 or Thr541 of the gene HPC2/ELAC2 were high-penetrance mutations, we would expect to observe significant linkage, in the subset of families that carry the Leu217 and/or Thr514 alleles, and over-transmission of the Leu217 and/or Thr541 alleles in these families. (3) If the variants Leu217 or Thr541 are high-prevalence but low-penetrance modifier alleles, we would expect to observe higher frequency of the Leu217 and/or Thr514 allele carrier in sporadic case subjects, compared with unaffected control subjects.

## Families and Methods

### *Ascertainment of Families*

All 159 families with HPC were ascertained and studied at the Brady Urology Institute at Johns Hopkins Hospital. Families were ascertained from three sources. Sixty-eight families were ascertained through referrals generated in response to a letter by one of us (PCW) to 8,000 urologists throughout the United States. The second source, from which 37 families were identified, was family-history records of patients seen at Johns Hopkins Hospital for treatment of prostate cancer. The remaining families (54) came from the respondents to articles, which appeared in various lay publications, describing our studies of families with HPC. Prostate cancer diagnosis was verified by medical records for each affected man studied. Age at diagnosis of prostate cancer was confirmed either through medical records or through two other independent sources. The mean age at diagnosis was 64.3 for the case subjects in these families; 84% of the families are white, and 8.8% are black.

All sporadic prostate cancer case subjects were recruited from among patients who underwent treatment for prostate cancer at the John Hopkins Hospital. The diagnosis of prostate cancer for all these subjects was confirmed by pathology reports. Preoperative prostate-specific antigen (PSA) levels, Gleason score, and pathological stages were available for 92, 244, and 245 of the 249 sporadic case subjects, respectively. Mean age at diagnosis for these case subjects was 58.6. Family histories were not available. More than 93% of the case subjects are white, and 3.2% are black.

Two hundred and twenty-two control subjects were selected from among men who participated in screening programs for prostate cancer. After excluding those who had abnormal results of a digital rectal examination (DRE) or abnormal PSA levels ($\geq$4 ng/ml), 211 were

eligible for the study. The mean age at examination was 58 years. More than 86% of the eligible control subjects are white and 7.1% are black. About 5.6% of the eligible control subjects have a brother(s) or father affected with prostate cancer. The affection status of relatives was obtained by interview of the probands.

### *Marker Genotyping*

Four microsatellite markers surrounding the HPC2/ELAC2 gene were genotyped in 159 HPC families. These markers were selected from Marshfield Comprehensive Human Genetic Maps (Broman et al. 1998) and cover ~18 cM from 17p13 to 17q1. Multiplex PCR using fluorescently labeled primers (either fam, hex, or ned) was performed, and the resulting PCR fragments were separated by means of capillary electrophoresis using an ABI 3700 DNA Analyzer (Applied Biosystems). The genotypes were scored using ABI Genotyper software. A modified version of the program Linkage Designer was used to bin the alleles and check inheritance. The output from Linkage Designer was then analyzed further for any inconsistencies by use of the program LINKAGE (Lathrop et al. 1984; Cottingham et al. 1993) without disease-phenotype information. Marker allele frequencies were estimated from the independent individuals in the data set (i.e., genetically unrelated individuals based on all the available information).

Two single-nucleotide polymorphisms (SNPs) in the HPC2/ELAC2 gene were genotyped for all subjects using PCR and restriction enzyme digestion, as described by Rebbeck et al. (2000), with the following modifications: for the region containing the Ser217Leu variant, PCR was performed in a 10-$\mu$l volume consisting of 30 ng genomic DNA, 0.2 $\mu$M each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, 50 mM KCl and 0.5 U *Taq* polymerase (Life Technologies). The primers were m5A (5′-CATTCCCATGTATGAACGTCT-3′) and m5Q (5′-AGGAAACAGCTATGACCATCTACAAGCATTA-CAAGGCAGAG-3′). These primers amplified a 276-bp fragment. PCR cycling conditions were as follows: 95°C for 3 min, followed by 28 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 20 s, with a final extension of 72°C for 2 min; 5 $\mu$l of PCR products were digested with *Taq*$\alpha$I (New England Biolabs) at 65°C for 2 hours. Genotypes were read on 2% agarose gels. Among Ser/Ser individuals, 172- and 104-bp products were observed, whereas Leu/Leu produced an uncut 276-bp band. For the region containing the Ala541Thr variant, PCR was performed as with the Ser217Leu variant. The primers were m15A (5′-CCAGCCTTTGTGTAAGTCTAC-3′) and m15P (5′-TCTGGGCAAGTTTGGAAGC-3′). A 495-bp fragment was amplified. PCR cycling conditions were the same as for Ser217Leu, except that the annealing temperature was 57°C; 5 $\mu$l of PCR products were then digested with *Fnu*4HI (New England Biolabs) at 37°C for 2 h, and the fragments were separated on 2% agarose gels. Among Thr/Thr individuals, 162-bp products were observed; for Ala/Ala, 110-bp products were observed. Genotyping of the two SNPs in 159 HPC probands was performed in three independent laboratories (Wake Forest, National Human Genome Research Institute, and Johns Hopkins University) as a quality-control measure. All the genotyping results were identical.

### *Genomic Mutational Analysis*

For HPC2/ELAC2 exons, PCR was performed in 50-$\mu$l reactions consisting of 20 ng genomic DNA, 10 mM dNTPs, 10 × PCR Buffer (Gibco BRL), 4.5 mM $MgCl_2$, 0.5 U Platinum™ *Taq* DNA polymerase (Gibco BRL), 0.5 U Ampli*Taq* Gold™ (Applied Biosystems), and 10 pmol of each forward and reverse primer (Gibco BRL). PCR cycles consisted of 95°C for 14 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 5 min. PCR products were denatured for 3 min at 95°C and then were reannealed gradually over 30 min using a 95°C to 65°C temperature gradient. The optimal melting temperature for each PCR amplicon was obtained by analysis of wild-type sequence, using an algorithm at the Stanford Denaturing High-Performance Liquid Chromatography (DHPLC) Web site.

### *DHPLC*

DHPLC heteroduplex analysis was performed using automated HPLC instrumentation equipped with an analytical 2.1 × 75–mm Eclipse dsDNA column (Agilent Technologies). The analytical gradient was composed of Buffer A (100 mM triethylammonium acetate and 0.10 mM EDTA) and Buffer B (100 mM triethylammonium acetate, 0.10 mM EDTA, and 25% acetonitrile) with a flow rate of 0.4 ml/min. The injection volume of each PCR sample was 4 $\mu$l. The analysis time for each sample was 10 min, including a column wash and an equilibration step.

### *Sequencing Analysis*

PCR products were purified using the Qiagen PCR purification kit (Qiagen) with the Qiagen BIOROBOT 9600 dual vacuum system. One-half–volume sequencing reactions were prepared in a 96-well format using the 3700 Big Dye™ Terminator Chemistry (Applied Biosystems) as follows: 6 $\mu$l of purified PCR product, 4 $\mu$l Big Dye Terminator reagent, and 1 $\mu$l of 5 pmol/$\mu$l of primer. Exons 7, 11, and 17 were sequenced using M13 forward and reverse primers. All remaining exons were sequenced using corresponding primers (table 1). Sequencing reactions were performed using the following conditions:

**Table 1**

**Primers Used for Mutation Analysis**

| Exon | Primer Sequence | | Size (bp) |
|---|---|---|---|
| | Forward | Reverse | |
| 1 | CCGCTTGAGACGCTCTAGTAT | CTGTCAGCACTTTCGGAGC | 735 |
| 2 | AATGGTGTCAGAGAGTTTACAG | ATAGCAAAAGTGGTCCTTGTTC | 214 |
| 3 | TTTATAGCAAAAGTGGTCCTTG | GAGGCTGGTGGGAAGTCTC | 178 |
| 4 | CCTTGCTGCTTCACCCTAG | CGTAGCAGCACATATAAAGCA | 578 |
| 5 | CTACATTTGTTCAACCATAACTG | CATCAACATCAAACCTCAAAATC | 208 |
| 6 | TCGTGTCAGATTCCCACCATA | ACGGATGTCTGACTTATGCCT | 388 |
| 7 | CATTCCCATGTATGAACGTCT | TCCTTCTTCCTGGGCTTACTAT | 335 |
| 8 | AGTGTCTTCAGCCTTTGTATTG | TGAGACAAGAAGAGATAGCAGAT | 325 |
| 9 | TAAAACCAACCTTCTTCATTAG | CATCGCTCCCATCATTGCT | 245 |
| 10 | GGCTTCTGGGGACTCACTG | CTACAGACACCACTTTTGAAGG | 251 |
| 11 | GTATCCACAAAGAGACCAGAAG | TAAGTCACTGTTGGTAGTTGGTG | 448 |
| 12 | GCTTGCCAGATACAGGAATC | CACCTGCCTAAACTTTCTGT | 433 |
| 13 | GAACACCTCATCCTCATTACCA | CCATGAATGTGTTTTGTCTCTT | 316 |
| 14 | GTTTCCGCTGTAAGGTAGTGT | CCACATAGTAAATGTTCCAG | 266 |
| 15 | TGCTAGTGGGTAGAGGTCAG | CATTCTAACCTGGCTTTCAGT | 528 |
| 16 | TGTGAAGACGGGATAACCTGA | TGCGGTATCAAGCCCTGTC | 534 |
| 17 | CCAGCCTTTGTGTAAGTCTAC | CTTCCAAACTTGCCCAGA | 392 |
| 18 | CGCTTTCTGCCTGTGACAT | GCATTGGCTGAAGGACAGAA | 634 |
| 19 | CACTTGATGGGCGTTCTGAG | GCATTGGCTGAAGGACAGAA | 394 |
| 20 | GGGTTCTCCAGCCAAAGACT | CAGAGCCTTCCAGCCCCACA | 256 |
| 21 | AAGAGGTAAGGGGCACAGC | GCAGAGGCAGGAGACTCAGA | 313 |
| 22 | GCTGAGTGTTGAGACCAGGA | GAGCAGCCGTCGTTTGTCT | 252 |
| 23 | GGGAGATGGTGCTGGCTAC | ATCTACCCATCACTAACCAGG | 439 |
| 24 | TTGATTTTGAGAGCATCTGGAC | CAGTGGGTCTAAGTGTCCGAG | 860 |

95°C for 3 min, 98°C for 45 s, 50°C for 10 s, 60°C for 4 min, followed by 25 cycles of 98°C for 15 s, 50°C for 10 s, 60°C for 4 min.

Sequencing reactions were subsequently purified using a 96-well Sephadex plate (preparation of Sephadex G-50 containing microtiter filter plates from the University of Oklahoma Advanced Center for Genome Technology) and were dried in a Speedvac (Savant). Samples were rehydrated and denatured in 10 $\mu$l Hi-Di formamide loading buffer at 95°C for 3 min. Samples were electrophoresed on a 3700 DNA Analyzer (Applied Biosystems), according to the manufacturer's protocols.

### *Statistical Analyses*

Hardy-Weinberg equilibrium (HWE) tests for all markers and LD tests between all pairs of markers were performed using independent individuals of HPC families and all sporadic case subjects and non-prostate cancer control subjects (GDA software, Weir et al. 1996). The HWE tests were based on exact tests, in which a large number of the possible arrays are generated by permuting the alleles among genotypes, and the proportion of these permuted genotypic arrays that have a smaller conditional probability than the original data is calculated. The LD tests were based on an exact test, assuming multinominal probability of the multilocus genotype, conditional on the single-locus genotype (Zaykin et al. 1995). A Monte Carlo simulation was used to assess the significance, by permuting the single-locus genotypes among individuals in the sample to simulate the null distribution. The empirical *P* values of both HWE and LD tests were based on 10,000 replicate samples. All six markers are in HWE in 159 probands with HPC. Both SNPs are in HWE in both case and control subjects.

Multipoint linkage analyses were performed using both parametric and nonparametric methods, implemented by GENEHUNTER-PLUS (Kruglyak et al. 1996; Kong and Cox 1997). Two genetic models were used for the parametric analyses. The autosomal dominant model was the same as that used by Smith et al. (1996). Under this model, the disease gene frequency of .003, incomplete penetrance, and phenocopies were assumed. Specifically, affected men were assumed to be disease-gene carriers with a fixed 15% phenocopy rate, and all unaffected men $\leqslant$75 years old and all women were assumed to be of unknown phenotype. In men >75 years old, the lifetime penetrance of gene carriers was estimated to be 63%, and the lifetime risk of prostate cancer for noncarriers was 16% in this age class. A similar autosomal recessive model was also used, except that the disease-gene frequency was set at .077, giving the same population disease frequency (Berry et al. 2000*b*). Linkage in the presence of heterogeneity was assessed by use of Smith's admixture test for heterogeneity (Ott 1998). In this test, two types of families are assumed: one type linked to the disease locus with a proportion

**Table 2**

**Multipoint Linkage Results in 159 Families with HPC**

| Marker | Distance (cM)[a] | HLOD[b] | | HLOD[b] (Recessive Model) | | Allele-Sharing LOD | |
|---|---|---|---|---|---|---|---|
| | | Two Point | Multipoint | Two Point | Multipoint | Two Point | Multipoint |
| D17S786 | 10.9 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ser217Leu | 15.5 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ala541Thr | 15.5 | .14 | 0 | .13 | 0 | .03 | 0 |
| D17S799 | 15.9 | 0 | 0 | 0 | 0 | .07 | 0 |
| D17S1843 | 22.1 | 0 | 0 | 0 | 0 | .05 | 0 |
| D17S783 | 28 | 0 | 0 | 0 | 0 | .01 | 0 |

[a] Distance from 17 pter.
[b] HLOD = LOD score, assuming locus heterogeneity.

of $\alpha$ and the other type not linked, with the proportion $1 - \alpha$. A maximum-likelihood approach was used to estimate the proportion of linked families ($\alpha$), by maximization of the admixed LOD score.

For the nonparametric analysis, the estimated marker identical by descent (IBD) sharing of alleles for the various affected relative pairs was compared with its expected values under the null hypothesis of no linkage. A statistic $Z_{all}$ in the program was used (Whittemore and Halpern 1994). Allele-sharing LOD scores were then calculated, using the computer program ASM (Kong and Cox 1997), on the basis of the statistic $Z_{all}$ with equal weight assigned to all families.

Family-based association tests were performed for all six markers in the 159 families with HPC, using the software package FBAT (Laird et al. 2000). Unlike the classic transmission/disequilibrium test (TDT), which is limited to a specific pedigree structure (one genotyped proband and two genotyped parents per pedigree), the FBAT uses data from nuclear families, sibships, or a combination of the two to test for linkage and LD between traits and genotypes. The test for linkage is valid when multiple affected members in each pedigree are used, and the power to detect linkage is increased if there is an association. The test for association is valid if the empirical variance is used to account for correlation between transmissions in families when linkage is present. In brief, the FBAT determines an $S$ statistic from the data, which is the linear combination of offspring genotypes and phenotypes. The distribution of the $S$ statistic is generated by treating the offspring genotype data as random and conditioning on the phenotypes and parental genotypes. When the marker is biallelic, a $Z$ statistic (together with its corresponding $P$ value) is calculated. When the marker is multiallelic, a $\chi^2$ test is performed, with the number of df being equal to the number of alleles.

Population-based association tests were performed for the two polymorphisms in patients with prostate cancer and in control subjects without prostate cancer. An unconditional logistic regression is used to test for association between genotypes and affection status, adjusting for potential confounding variables such as age. The association tests were also performed only for white subjects to decrease the potential confounding effects of population stratification.

## Results

### *Hardy-Weinberg Equilibrium and LD Tests*

All four microsatellite markers and two SNPs are in HWE in this study population. The empirical $P$ values for the HWE tests were .51, .48, .08, .52, .40, and .60 for D17S786, Ser217Leu, Ala541Thr, D17S799, D17S1843, and D17S783, respectively. There was a strong LD between the two SNPs ($P < 10^{-6}$), and almost all Thr541 allele carriers also carried Leu217. We observed three white patients with sporadic prostate cancer who had the haplotype Thr541 and Ser217.

### *Parametric and Nonparametric Linkage Analyses*

There was no evidence for linkage between prostate cancer–susceptibility genes and markers at 17p13-17q11 in the 159 pedigrees with HPC (table 2). The total inheritance information in the 159 pedigrees using these six markers is intermediate (0.58–0.67). Parametric and nonparametric linkage analyses provided similar results. The multipoint LOD scores, under the assumption of heterogeneity and under either the dominant or the recessive model, were 0 across the region. Sixteen pedigrees had LOD scores (under the dominant model) $\geq$0.3, and three pedigrees had LOD scores $\geq$0.6. The highest LOD score, 0.97, occurred in a pedigree with six affected members (two affected siblings, an affected father, and three affected paternal uncles [father and one uncle without genotype]). The nonparametric allele-sharing LOD scores were 0 across the region.

The stratified linkage analyses did not provide evidence for linkage in any subsets of pedigrees (table 3). The allele-sharing LOD scores were 0 across the region regardless of age at diagnosis. In addition, allele-sharing LOD scores were 0 in pedigrees with three, four, and more than four affected members; in white and black

**Table 3**

**Allele-Sharing LOD Scores in Subsets of Families with HPC**

| Family Characteristic | No. of Pedigrees | LOD Scores with | | | | | |
|---|---|---|---|---|---|---|---|
| | | D17S786 | Ser217Leu | Ala541Thr | D17S799 | D17S1843 | D17S783 |
| Proband age at diagnosis: | | | | | | | |
| <65 | 79 | .11 | .02 | .02 | .03 | .16 | .19 |
| ⩾65 | 80 | 0 | 0 | 0 | 0 | 0 | 0 |
| No. of affected members: | | | | | | | |
| 3 | 29 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 40 | 0 | 0 | 0 | 0 | 0 | 0 |
| ⩾5 | 90 | .03 | .18 | .14 | .19 | .21 | .1 |
| Race: | | | | | | | |
| White | 133 | 0 | 0 | 0 | 0 | .02 | 0 |
| Black | 14 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other | 12 | 0 | 0 | 0 | 0 | 0 | 0 |
| Male-to-male disease transmission: | | | | | | | |
| Yes | 98 | 0 | .01 | 0 | .01 | .02 | .1 |
| No | 60 | 0 | 0 | 0 | 0 | 0 | 0 |
| Probands with Leu217 | 89 | 0 | 0 | 0 | 0 | 0 | 0 |
| Probands with Thr541 | 17 | 0 | 0 | 0 | 0 | 0 | 0 |

subjects; and in pedigrees with and without male-to-male disease transmission. When the linkage analyses were limited to the 89 pedigrees in which probands are Leu217 carriers or to the 17 pedigrees in which probands are Thr541 carriers, no evidence for linkage was found.

### *Mutational Screens for All Exons*

To directly assess the HPC2/ELAC2 gene for mutations that may be segregating in the families with HPC reported here, the complete coding region of the gene was screened for sequence variants in 93 probands with HPC. Although the two previously reported missense changes were readily observed, no other DNA sequence variants were found that altered the amino acid sequence of HPC2/ELAC2.

### *Family and Population-Based Association Tests*

Family-based linkage and association tests using a multiallelic method did not provide evidence for either linkage or LD between the markers and prostate cancer–susceptibility genes (table 4). The association tests using the biallelic method were also performed for the two single-nucleotide polymorphisms (SNPs). For the SNP Ser217Leu, 84 nuclear pedigrees were informative for the analysis. The observed *S* score was 187.0, and the expected *S* score was 194.3 (empirical variance 40.3; $Z = -1.15$; $P = .25$). For the SNP Ala541Thr, 24 nuclear pedigrees were informative for the analysis. The observed *S* score was 33.0, and the expected *S* score was 30.8 (variance 6.40; $Z = 0.87$; $P = .38$). The results were similar when the analyses were limited to white subjects.

Allele frequencies of the two SNPs were compared between patients with prostate cancer and control subjects. To decrease the confounding effect of racial differences, the comparison was limited to white subjects. The allele frequencies for Leu217 were 34.0%, 29.2%, and 27.2%, in the 134 probands with HPC, 228 patients with sporadic disease, and 182 unaffected control subjects, respectively. There was no statistically significant difference in the frequencies between the HPC case subjects and control subjects (Fisher's exact test [FET] $P = .08$), between the sporadic case subjects and control subjects (FET $P = .58$), and between all case subjects and control subjects (FET $P = .21$). The allele frequencies for Thr541 were 6.1%, 4.8%, and 4.4% in the probands with HPC, the patients with sporadic disease, and the unaffected control subjects, respectively. No significant difference was observed in the allele frequencies between the probands and control subjects (FET $P = .45$), between the patients with sporadic disease and the control subjects (FET $P = .87$), or between all patients and control subjects (FET $P = .65$).

Genotype frequencies of the two SNPs were also compared in an analysis restricted to white subjects (table 5). There was a trend toward higher Leu217 homozygous rates in the patients with HPC (11.2%) and in the patients with sporadic disease (8.3%) than in the control subjects (7.7%); however, the difference was not statistically significant. There was no statistical difference in the Thr541 carrier rates in the patients with HPC (10.5%), in the patients with sporadic disease (9.0%), or in the unaffected control subjects (9.0%). When the two SNPs are considered together, no significant difference in the frequencies was found. The frequencies of individuals carrying both Leu217 and Thr541 were 10.4% in the HPC case subjects, 8% in the sporadic case subjects, and 8.8% in the unaffected control subjects.

**Table 4**

**Results of Family-Based Association Test in 159 Families with HPC**

| Marker | No. of Carriers | df[a] | $\chi^2$ | *P* |
|---|---|---|---|---|
| D17S786 | 11 | 6 | 2.4 | .88 |
| Ser217Leu | 2 | 1 | 1.42 | .23 |
| Ala541Thr | 2 | 1 | .61 | .44 |
| D17S799 | 10 | 6 | 2.89 | .82 |
| D17S1843 | 14 | 6 | 4.91 | .55 |
| D17S783 | 10 | 7 | 6.11 | .53 |

NOTE.—Families comprised 653 and 97 affected and unaffected subjects, respectively.

[a] Alleles observed in <10 subjects were not included in the analysis.

We also examined the relationships of Leu217 and Thr541 frequencies and Gleason scores and pathological stages in sporadic prostate cancer case subjects. There was no statistically significant difference in the genotypic frequencies of the two SNPs between the groups with low (≤6) and high (≥7) Gleason scores or between the groups with disease confined to the prostate and the group with non–organ-confined disease (table 6).

## Discussion

We tested several alternative hypotheses in the current study. The first hypothesis—that HPC2/ELAC2 is a high-prevalence, high-penetrance major gene for prostate cancer—was rejected, because linkage results using both parametric and nonparametric methods in the 159 pedigrees with HPC did not provide any evidence for linkage. The finding of no novel mutations in the coding region of HPC2/ELAC2 in 93 probands with HPC is consistent with this conclusion. The lack of evidence for linkage from the parametric analyses (under either a dominant or recessive model) is unlikely to have resulted solely from the misspecification of the parameters in the genetic model. The impact of misspecification of penetrance estimates on the linkage results is small, as long as a dominant or recessive model is correctly specified (Clerget-Darpoux et al. 1986).

The second hypothesis—that the HPC2/ELAC2 was a less prevalent, high-penetrance major gene—was also rejected, because linkage evidence was not found when heterogeneity was assumed, which tested for a subset of pedigrees linked to this gene or region. No evidence for linkage was found in predefined subsets of families based on the pedigree characteristics, such as age at diagnosis, number of affected members per pedigree, male-to-male disease transmission, and race. Finally, no evidence for linkage was observed in subsets of pedigrees in which probands carried the Leu217 and/or Thr541 alleles.

The third alternative hypothesis—that the HPC2/ELAC2 is a common, low-penetrance modifier gene—was rejected, because neither family-based nor population-based tests found evidence for association between the genotypes at Ser217Leu and/or Ala541Thr and prostate cancer risk. Leu217 and/or Thr541 carrier rates in probands with HPC or in the patients with sporadic disease were not significantly increased, compared with unaffected control subjects.

Although these alternative hypotheses were rejected because of the absence of statistically significant differences, the results should be interpreted cautiously, be-

**Table 5**

**Genotypes of Ser217Leu and Ala541Thr in Patients with HPC, Patients with Sporadic Disease, and Unaffected Control Subjects (White Only)**

| Ser217Leu | Ala541Thr | No. of Control Subjects | No. of Patients with SPC[b] | No. of Patients with HPC | OR[a] (95% CI) of SPC vs. Control Subjects | OR[a] (95% CI) of HPC vs. Control Subjects | OR[a] (95% CI) of All Patients vs. Control Subjects |
|---|---|---|---|---|---|---|---|
| Ser/Ser | | 97 | 114 | 58 | 1 | 1 | 1 |
| Ser/Leu | | 71 | 95 | 61 | 1.14 (.75–1.71) | 1.45 (.90–2.35) | 1.39 (.98–1.97) |
| Leu/Leu | | 14 | 19 | 15 | 1.14 (.54–2.40) | 1.63 (.71–3.73) | 1.34 (.68–2.63) |
| Any Leu | | 85 | 114 | 76 | 1.14 (.77–1.68) | 1.49 (.94–2.35) | 1.26 (.87–1.84) |
| | Ala/Ala | 166 | 211 | 111 | 1 | 1 | 1 |
| | Ala/Thr | 16 | 20 | 11 | .98 (.49–1.96) | .95 (.42–2.15) | .99 (.52–1.87) |
| | Thr/Thr | 0 | 1 | 2 | | | |
| | Any Thr | 16 | 20 | 13 | 1.03 (.52–2.04) | 1.16 (.53–2.55) | 1.09 (.58–2.05) |
| Ser/Ser | Ala/Ala | 97 | 110 | 55 | 1 | 1 | 1 |
| Any Leu | Ala/Ala | 69 | 97 | 56 | 1.25 (.82–1.89) | 1.43 (.87–2.35) | 1.33 (.90–1.95) |
| Ser/Ser | Any Thr | 0 | 3 | 0 | | | |
| Any Leu | Any Thr | 16 | 17 | 13 | .94 (.45–1.95) | 1.37 (.61–3.11) | 1.10 (.57–2.14) |

[a] All ORs were adjusted for age.

[b] SPC = sporadic prostate cancer.

cause various forms of genetic heterogeneity, high phenocopy rates, and incomplete penetrance in prostate cancer can significantly decrease the power to detect linkage and association of a true susceptibility gene. Different modes of inheritance have been reported for the transmission of prostate cancer in families, including autosomal dominant and X-linkage modes (Woolf 1960; Carter et al. 1992; Hayes et al. 1995; Monroe et al. 1995; Narod et al. 1995; Grönberg et al. 1997; Schaid et al. 1998; Cerhan et al. 1999; Schuurman et al. 1999), and various loci have been reported as prostate cancer–susceptibility genes, including HPC1 (MIM 601518; Smith et al. 1996; Cooney et al. 1997; Hsieh et al. 1997; McIndoe et al. 1997; Eeles et al. 1998; Neuhausen et al. 1999; Xu 2000), PCAP (MIM 602759; Berthon et al. 1998; Gibbs et al. 1999*a*; Whittemore et al. 1999; Berry et al. 2000*a*), HPCX (MIM 300147; Xu et al. 1998; Lange et al. 1999; Peters et al. 2001), CAPB (MIM 603688; Gibbs et al. 1999*b*; Berry et al. 2000*b*), and HPC20 (Berry et al. 2000*b*). With these various forms of genetic heterogeneity, it would not be surprising that only a small proportion of pedigrees and patients had prostate cancer that was attributable to the HPC2/ECLA2 gene. Furthermore, the high phenocopy rate caused by high prevalence of the disease can prevent the detection of linkage even in the pedigrees where the HPC2/ECLA2 gene segregates (e.g., some affected individuals in these pedigrees with HPC could be nongenetic case subjects) which lead to false recombinants in the linkage analysis and to misclassification in the association study. These problems could be compounded by incomplete and age-dependent penetrance of HPC2/ECLA2. Finally, some of the unaffected men could be HPC2/ECLA2 gene carriers but remain unaffected because of lack of background genes (modifier genes) and/or lack of environmental risk factors.

To investigate the power to detect linkage in the 159 pedigrees with HPC in the presence of genetic heterogeneity, high phenocopy rate, and incomplete penetrance, we performed a computer simulation study using FASTLINK. The dominant model, as described in the Families and Methods section, which incorporates a 15% phenocopy rate and 63% penetrance by age 75 years, was used to simulate a disease gene that segregates in the 159 pedigrees with HPC. A marker with six equally frequent alleles was simulated to be linked to the disease gene at a recombination fraction ($\theta$) of .025, using these exact pedigree structures, affection status, and availability of DNA. When 20% of the 159 pedigrees were linked to the disease gene, 46%, 17%, and 6% of the 1,000 replicates reached allele-sharing LOD scores of 1, 2, and 3, respectively. When one-third of the 159 pedigrees were linked to the disease gene, 89%, 65%, and 40% times among the 1,000 replicates reached allele-sharing LOD scores of 1, 2, or 3, respectively. The simulation results suggested that we had reasonable power to reach suggestive evidence for linkage in our study sample only if one-third of the pedigrees segregate the gene. When the proportion of pedigrees that segregate the gene is below that level, the power is very limited. Clearly, a much larger collection of pedigrees with HPC is needed to detect linkage of genes that segregate in a small proportion of pedigrees.

**Table 6**

**Genotypes of Ser217Leu and Ala541Thr in White Men with Sporadic Prostate Cancer**

| | No. (%) of Patients with | | | |
|---|---|---|---|---|
| | Gleason Score | | Pathological Stage[a] | |
| Allele | ≤6 | ≥7 | OC | NOC |
| Ser217Leu: | | | | |
| Ser/Ser | 42 | 72 | 32 (45.07) | 82 (52.23) |
| Ser/Leu | 38 | 57 | 33 (46.48) | 62 (39.49) |
| Leu/Leu | 6 (6.98) | 13 (9.15) | 6 (8.45) | 13 (8.28) |
| Ala541Thr: | | | | |
| Ala/Ala | 83 | 128 | 62 (87.30) | 149 (92.55) |
| Ala/Thr | 6 (6.74) | 14 (9.79) | 9 (12.68) | 11 (6.83) |
| Thr/Thr | 0 (0) | 1 (.7) | 0 (0) | 1 (.62) |

[a] OC = organ-confined disease; NOC = non–organ-confined disease.

Similarly, we estimated the power to detect an association in our study sample. When the point estimates of ORs and frequencies from Tavtigian et al. (2001) are used, the power to detect an OR of 2.4 at the significance level of .05, with a frequency of Leu217 homozygous carrier rate of 6.1% in control subjects, is 87% in our combined 364 patients and 182 control subjects. The power to detect an OR of 2.9 at the significance level of .05, with a frequency of any Thr541 carrier rate of 3.4% in control subjects, is 69% in our combined patient and control sample. However, if we consider the lower estimates of the 95% CI of the reported ORs, our study sample has very low power to detect this level of effect. For example, if the Leu217 and Thr541 have an OR of 1.3 each, our sample has only 18% and 13% power, respectively.

Considering the difficulties in the linkage and association studies of complex diseases and the lack of power to detect linkage and association of genes with relatively small effects, our negative linkage and association results are not surprising. On the basis of our linkage results, we probably can rule out any major gene that segregates in a large number of pedigrees, but we cannot rule out the possibility that a small proportion of our pedigrees segregate a major gene in the region. However, two pieces of evidence suggested that, even if there is a major gene in the region that segregates in a small number of pedigrees, it is unlikely that they are the Leu217 and/or Thr541 variants of the HPC2/

ECLA2 gene. The first piece of evidence comes from the negative linkage results in pedigrees whose probands carried the Leu217 and/or Thr541 alleles. If the variants of the Leu217 and/or Thr541 were high-penetrance mutations, we would expect to observe linkage in these pedigrees. Although substantial phenocopies in these pedigrees could disguise the linkage even if the variants were high-penetrance mutations, it is difficult to use this argument to explain the second piece of evidence that the Leu217 and/or Thr541 alleles are not overtransmitted to affected individuals in family-based association tests.

Because our case-control sample has a reasonable power to detect association when Leu217 homozygotes have an OR of 2.4 or when Thr541 carriers have an OR of 2.9, our negative association results suggested that the Leu217 and Thr541, separately or together, are not the modifier mutations that increase the prostate cancer risk at the previously reported magnitude in our study population. However, our results cannot rule out the association between these variants and prostate cancer, if these variants confer lower risks than the point estimates (in the lower ranges of the reported 95% CI). In fact, although the differences were not statistically significant, we observed higher homozygous Leu217 carrier rates in the patients (9.4%) than in the control subjects (7.7%) (OR = 1.3). It is interesting that these rates were highest in the patients with HPC (11.2%) (OR = 1.6), intermediate in the patients with sporadic disease (8.3%) (OR = 1.1), and lowest in the control subjects (7.7%).

Our study is the first reported replication study to investigate the linkage results at 17p11. The initial report by Tavtigian et al. (2001) found a maximum two-point LOD of 4.5 and a maximum three-point LOD of 4.3 in the 17p11 region in the first 33 pedigrees. They found a much weaker linkage in the additional 94 pedigrees. Several factors may explain the difference between their study and ours. Most of their pedigrees are large. The mean numbers of affected and genotyped affected members were 18.9 and 5.5 per pedigree, respectively. The mean numbers of affected and genotyped affected members were only 5.1 and 3.3, respectively, in our study. Interestingly, the most notable positive LOD scores in our study came from the 90 pedigrees with five or more affected members. The Utah pedigrees may be more homogeneous in both genetic and environmental background than our study pedigrees. The study by Tavtigian et al. mainly used two-point or three-point methods because of the large size of the pedigrees. These linkage methods are sensitive to allele frequencies, and false-positive linkage can arise when marker allele frequencies are wrongly assumed (Ott 1998). This is especially critical in the study of prostate cancer, because most parental genotype data are missing. Our linkage analyses were based on both two-point and multipoint analyses and thus were robust to the incorrect estimates of marker allele frequencies.

Our study is the second reported replication study to investigate the association between the two common HPC2/ELAC2 sequence variants and prostate cancer risk. For the Ser217Leu missense change, Tavtigian et al. (2001) found significantly higher homozygous Leu217 carriers in the related patients (13.3%) than in the unaffected related pedigree members (9.3%) or in the unrelated married-in unaffected males (6.1%). Rebbeck et al. (2000) did not report the homozygous Leu217 carrier rate in their study but found lower Leu217 carrier rates in the patients (30.8%) than in the control subjects (31.5%). We report here a higher Leu217 homozygous carrier rate in probands with HPC (11.2%) and in the patients with sporadic disease (8.3%) than in the control subjects (7.7%), although this difference is not statistically significant. For the Ala541Thr variant, Tavtigian et al. (2001) found a significantly higher Thr541 carrier rate in the related patients (9.8%) than in the unrelated married-in unaffected men (3.4%). Rebbeck et al. (2000) reported a marginally significant, higher Thr541 carrier rate in the patients (7.5%) than in the 266 age- and race-matched control subjects (3.5%). However, the Thr541 carrier rate was 5.7% in their 383 control subjects. We found no difference in the Thr541 carrier rates in the patients with HPC (10.5%), in patients with sporadic disease (9.0%), or in unaffected control subjects (9.0%). As an additional control population, we genotyped 90 independent subjects (all whites) from one of our nonprostate cancer study populations (ages 45–65 years). Although the prostate cancer status was unknown for this population, it represents general population control subjects. In this population, we found a similar frequency for Thr541 carrier rate (11.1%) (J.X. and L.Z., unpublished data).

Although the differences between studies are unexplained, several of the following factors may contribute: First, the point estimates of the ORs in the study by Tavitigian et al. (2001) could be overestimated, because the case subjects were not independent; most of their study pedigrees are large, and if some of the pedigrees were linked to this chromosomal region and the affected individuals carried the variants, they could inflate the frequency of the variants in the case subjects. Second, the young age of some of the control men may lead to potential misclassification, thereby decreasing the power to detect association. Even though the age differences between case and control subjects were not statistically significant and the ORs were adjusted for age, some of the younger control subjects in the study reported here (40–50 years of age) may develop prostate cancer later. Third, population stratification may lead

to false-positive findings. Although this is unlikely, because race was matched in the two positive association studies, it is still possible that there are different genetic backgrounds between case and control subjects within the whites. The present study employed a family-based association test, which is robust to population stratification. Fourth, genotyping error is a potential problem in case-control studies. Although caution has been exercised and some genotypes were confirmed by multiple methods (Rebbeck et al. 2000), genotyping error in other subjects cannot be ruled out. It is worth noting that all the significant findings were marginal and that one misclassified genotype may change the results. To address this issue in our study, genotypes of the 159 HPC probands were confirmed by three independent laboratories.

Caution is warranted when interpreting and generalizing from the results of the present population-based association study. The case subjects collected in our study had early mean age of onset and thus may represent more hereditary case subjects. The potential bias could be two ways, either bias toward a significant finding if the HPC2/ELAC2 contributing to the susceptibility or bias against a significant finding if other competing major locus contributing to the susceptibility in these subjects. The control subjects in our study came from a prostate cancer screen population; the group therefore is likely to be at high risk (because of self selection). Although this could partially account for the higher frequency (compared with the studies of Tavtigian et al. [2001] and Rebbeck et al. [2000]) of the suspected alleles observed in our control subjects, we think the impact is limited for the following three reasons. First, all the control subjects were carefully examined and had normal DRE and PSA results. Thus, they are unlikely to be case subjects, at least at the time of examination. Second, very few of the control subjects have a positive family history. We collected extensive information on family history of the control subjects, and only six control subjects reported positive family history (defined as affected father and/or brothers) among 182 white control subjects. Furthermore, when we performed additional analysis with the six individuals excluded, the results were similar. Third, the frequency of the suspect alleles in 90 additional control subjects was similar to the screen control subjects.

In summary, the results of the study reported here are not consistent with a major role for HPC2/ELAC2 as a prostate cancer susceptibility gene. In addition, we find no significant evidence that the Leu217 or Thr541 variants of the HPC2/ELAC2 increase prostate cancer risk in our study population.

## Acknowledgments

The authors thank all the men who participated in this study. This work was partially supported by PHS SPORE grant CA58236, by two grants from the Department of Defense (to W.B.I. and J.X.), and by the Ella and Georg Ehrnrooth Foundation, the Maud Kuistila Foundation, the Paulo Foundation (N.N.N.), and the GE Fund (S.D.I.).

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Linkage Designer, http://dnalab-www.uia.ac.be/dnalab/ld.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for prostate cancer [MIM 176807], HPC2 [MIM 605367], HPC1 [MIM 601518], PCaP [MIM 602759], PCBP/CAPB [MIM 603688], and HPCX [MIM 300147])
Stanford Denaturing High-Performance Liquid Chromatography, http://insertion.stanford.edu/melt.html
University of Oklahoma Advanced Center for Genome Technology, http://www.genome.ou.edu/

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