

A Polymorphism in the *CDKN1B* Gene Is Associated with Increased Risk of Hereditary Prostate Cancer

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ABSTRACT

The loss of cell cycle control is believed to be an important mechanism in the promotion of carcinogenesis. *CDKN1B* (p27) belongs to the Cip/Kip family and functions as an important cell cycle gatekeeper. Several lines of evidence from clinical studies and laboratory experiments demonstrate that *CDKN1B* is an important tumor suppressor gene in prostate cancer etiology. In addition, a case-control study has shown that the 326T/G (V109G) polymorphism in *CDKN1B* is associated with advanced prostate cancer. In light of the evidence for linkage between the chromosomal location of the *CDKN1B* gene (12p13) and prostate cancer susceptibility in several hereditary prostate cancer (HPC) populations, we hypothesized that sequence variants of *CDKN1B* play a role in HPC. To test this hypothesis, we first resequenced this gene in 96 HPC probands to identify germ-line mutations and sequence variants. We then genotyped the identified sequence variants among all family members of 188 HPC families and tested for their cosegregation with prostate cancer. In total, 10 sequence variants were identified, including three nonsynonymous changes. A family-based test, which is free from the effects of population stratification, revealed a significant association between single nucleotide polymorphism (SNP) -79C/T and prostate cancer (with a nominal *P* of 0.0005). The C allele of -79C/T was overtransmitted from parents to their affected offspring. Evidence for this association was primarily contributed by affected offspring whose age at diagnosis was <65 years. Together with the previous association study in a sporadic prostate cancer population, our new findings additionally suggest that germ-line variants of this gene play a role in prostate cancer susceptibility.

INTRODUCTION

The loss of cell cycle control accompanied by replication of a defective genome facilitates the emergence of cancer cells. *CDKN1B* (p27) belongs to the cyclin-dependent kinase (CDK) inhibitor Cip/Kip family and functions as a cell cycle check point at G₁-S by regulating G₁ cyclin-CDK complexes (1). In prostate tumors, frequent genetic aberrations were observed at the chromosomal region 12p12-13, harboring *CDKN1B*. Indeed, a homozygous deletion region was identified in a metastatic prostate cancer xenograft (2). The critical role of *CDKN1B* in regulating cell proliferation and as a tumor suppressor was directly demonstrated in the *CDKN1B* knockout mouse, which exhibits gigantism (because of increased cell number) and increased tumorigenesis (3–5).

Recently, in a study of 96 prostate cancer cases and 106 controls, Kibel *et al.* (6) reported an association between a nonsynonymous change of *CDKN1B* and the risk of advanced prostate cancer. The single nucleotide polymorphism (SNP) 326T/G changes a valine at codon 109 to glycine. Among men with the TT genotype, an association with increased risk of advanced prostate carcinoma was reported

(odds ratio = 1.95; 95% confidence interval = 1.09–3.47), and the association was stronger in patients with androgen-independent disease (odds ratio = 2.88, 95% confidence interval = 1.19–6.97). Other results implicating *CDKN1B* and the risk for prostate cancer have come from linkage studies. The chromosomal region of *CDKN1B* (12p13) has been reported to be linked to prostate cancer susceptibility in several hereditary prostate cancer (HPC) family studies (7, 8). In our HPC study population, no evidence for linkage was observed at 12p13 in the complete set of the 188 HPC families based on the log of odds (LOD = 0); however, significant evidence for linkage at this region was found among the 34 families with the highest linkage score at 10q23 (the chromosomal region of *PTEN*), with a LOD score of 2.75 (*P* = 0.0002). This conditional linkage analysis was prompted by a mouse model, which demonstrated that the combined effects of both the *Pten* and *Cdkn1b* genes rather than a single gene cause prostate cancer in mice (9). On the basis of the critical role *CDKN1B* plays in cell cycle control, the strong functional and clinical implications of this gene in prostate carcinogenesis, and the positive findings of association and linkage studies, we hypothesized that germ-line mutations or polymorphisms in *CDKN1B* may modify an individual's risk to prostate cancer, and this may lead to familial aggregation of prostate cancer. To test this hypothesis, we first screened for germ-line mutations in 96 probands from HPC families, followed by genotyping among all 188 HPC families for the identified mutations and polymorphisms that were either rare or of potential functional importance. To evaluate the risk *CDKN1B* imparts for HPC, cosegregation of the mutations and polymorphisms in the HPC families was evaluated using family-based linkage and association tests. This study is unique in describing the *CDKN1B* mutation spectrum and its implications in HPC.

MATERIALS AND METHODS

Subjects. HPC families (*n* = 188) were recruited at the Brady Urology Institute at Johns Hopkins Hospital (Baltimore, MD) through referrals, review of medical records for patients seen at Johns Hopkins Hospital for treatment of prostate cancer, and respondents to various lay publications describing our studies. The eligibility criterion for HPC was at least three first-degree relatives affected with prostate cancer. The diagnosis of prostate cancer was verified by medical records for each affected male studied. Age of diagnosis of prostate cancer was confirmed either through medical records or from two other independent sources. The mean age at diagnosis was 64.4 years, with 91 families having mean age at diagnosis < 65 years. One hundred fifty-four families are non-Ashkenazi white, 17 families are Ashkenazi, and 15 families are black. The number of families with 3, 4, and ≥5 affected individuals was 28, 47, and 113, respectively. All individuals that participated in this study gave full informed consent.

The family history for other types of cancers was also collected. For the diagnoses of other types of cancers, the majority was confirmed through interview with at least two relatives, and a small proportion (7%) was verified by medical records. Among the 188 HPC families, 68 (36.2%), 36 (19.1%), and 22 (11.7%) have at least one, two, and three or more other types of cancers in the family, respectively. Seventy-three of the prostate cancer cases were also diagnosed with another primary cancer. There were 148 other subjects diagnosed with other cancers. Colon cancer was the highest concurrent cancer (35 cases), followed by breast cancer (34 cases) and lung cancer (20 cases).

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Sequencing and Genotyping Methods. The PCR products of all three exons, exon-intron junctions, and ~800-bp promoter region of *CDKN1B* were directly sequenced in 96 HPC probands. The primers used for PCR and the components and cycling conditions for PCR are available upon request. All PCR products were purified using the QuickStep PCR purification Kit (Edge BioSystems, Gaithersburg, MD) to remove deoxynucleoside triphosphates and excess primers. All sequencing reactions were performed using dye-terminator chemistry (BigDye; ABI, Foster City, CA), and separated in an ABI 3700 DNA Analyzer. SNPs were identified using Sequencher software version 4.0.5 (Gene Codes Corporation).

SNPs were genotyped using the MassARRAY system (Sequenom, Inc., San Diego, CA). All reaction components and conditions were performed according to the manufacture's instruction (Sequenom, Inc.) and are available upon request.

Statistical Methods. Hardy-Weinberg equilibrium tests and pairwise linkage disequilibrium tests for all possible pairs of the variants were performed using the Fisher probability test statistic, as described by Weir (10). For each test, 10,000 permutations were performed, and the test statistic of each replicate was calculated. Empirical *P*s for each test were estimated as the proportion of replicates that are as probable as or less probable than the observed data, as implemented in the software package Genetic Data Analysis.

Family-based association tests were performed for the SNPs genotyped in the 188 HPC families, using the family-based association test software package (11). Family-based association test uses data from nuclear families, sibships, or a combination of the two to test for linkage and linkage disequilibrium (association) between traits and genotypes. The test for linkage is valid when multiple affected members/pedigree are used, and the power to detect linkage is increased if there is an association. The test for association is valid if one affected member/pedigree is used (the genotypes of all of the affected members can be included) or if the empirical variance is used to account for correlation between transmissions in families when linkage is present.

RESULTS AND DISCUSSION

A total of ten sequence variants were identified when we screened *CDKN1B* for germ-line mutations in the ~800 bp promoter region, all three exons, and the exon-intron junctions among 96 HPC probands (Table 1). Among these variants, three are in the promoter region, one is in the 5'-untranslated region, four are in the coding region of exon 1, one is in intron 2, and one is in the 3'-untranslated region. Three of the four SNPs in the coding sequence cause amino acid changes: SNP 258G/C changes a glutamic acid at codon 86 to aspartic acid (E86D); SNP 326T/G changes a valine at codon 109 to glycine (V109G); and SNP 356T/C changes an isoleucine at codon 119 to threonine (I119T). The two nonsynonymous changes 258G/C (E86D) and 356T/C (I119T) are rare, and each occurred only once among the probands. The nonsynonymous change 326T/G (V109G) is frequent and has been reported to be associated with increased sporadic prostate cancer risk (6). Interestingly, only 4 of these 10 SNPs were previously reported in the public database.

To preliminarily evaluate the functional impact of these identified

Table 2 Family based association test for *CDKN1B* sequence variants among 188 hereditary prostate cancer families

SNPs ^a	No. of informative families	χ^2 (df = 1)	<i>P</i>
p27_A(-1220)C	32	0.000	0.998
p27_(-79)C/T	80	11.971	0.0005
p27_T326G	70	0.482	0.487
p27_G2460C	78	0.098	0.754
p27_A4149C	79	0.572	0.449

^a Single nucleotide polymorphisms.

SNPs, we performed bioinformatic analyses using various computational methods. In a search of the TRANSFAC database, SNPs -1220T/G, -987C/T, and -838C/A were found to lie in potential binding sites for transcription factors Pbx-1a, SP1, and E2F, respectively. The SNP -79C/T was found to eliminate one CpG site in a CpG island. All three nonsynonymous changes were found to be located in the cyclin dependent kinase inhibitor (CDI) domain (pfam02234), which is the CDK-CDKN interaction motif. In addition, missense changes V109G and I119T might alter the interaction between *CDKN1B* and its negative regulator p38^α because they are located in the interaction surface that spans amino acid residues 97 to 151 (6, 12). When the exonic SNPs were evaluated for changes in exonic splice enhancer elements, SNPs -79C/T and 258G/C were found to be in these potentially important areas.

To assess whether the identified nonsynonymous changes and other sequence variants are associated with prostate cancer risk, we genotyped 9 of these 10 variants among all 188 HPC families. The SNP -987C/T was excluded because a reliable assay could not be developed. Hardy-Weinberg equilibrium and pairwise linkage disequilibrium tests were performed among unrelated probands of non-Ashkenazi whites. Eight of the 9 remaining SNPs were in Hardy-Weinberg equilibrium (*P* > 0.05). The SNP -838C/A significantly deviated from Hardy-Weinberg equilibrium (*P* < 0.005) and therefore was dropped from additional analysis. The family-based association test was used to test whether the sequence variants in *CDKN1B* are associated with prostate cancer risk. Three SNPs were rare (114C/T, 258G/C, and 356T/C) and were uninformative for this analysis. Among the five remaining SNPs, -79C/T in the 5'-untranslated region was significantly associated with prostate cancer, and the C allele was found to be overtransmitted from parents to affected offspring (nominal *P* = 0.0005; Table 2). Eighty HPC families were informative for the test of this SNP. We further explored this result by performing a stratified analysis based on age of diagnosis. Interestingly, the association was primarily observed among affected offspring with age at diagnosis < 65 years (*P* = 0.0015). There was no significant association among affected offspring with age at diagnosis ≥ 65 years (*P* = 0.34). It is worth noting that age of diagnosis is a crude indicator

Table 1 Sequence variants identified in *CDKN1B* among 96 Caucasian hereditary prostate cancer probands

SNP ^a	Location	Variant	Amino acid change	Variant frequency	SNP identity
T(-1220)G	Promoter	G		0.08	
C(-987)T	Promoter	T		0.04	
C(-838)A	Promoter	A		0.49	
-79C/T	5'-untranslated region	T		0.27	rs34330
C(114)T	Exon1	T		0.006	
G(258)C	Exon1	C	E86D	0.006	
T(326)G	Exon1	G	V109G	0.19	rs2066827
T(356)C	Exon1	C	I119T	0.006	
C(2460)G	Intron2	G		0.37	rs34329
A(4149)C	3'-untranslated region	C		0.48	rs7330

^a The positions of single nucleotide polymorphisms (SNPs) are corresponding to the translation start site ATG (+1), which is based on the reference sequences NT009714 and NM_004064.

for age of onset and is not a good surrogate for clinically aggressive disease. Because the empirical variance, which accounts for correlation between transmission in families when linkage is present (13), was used in the statistical test, this significant result reflects an association between SNP -79C/T and prostate cancer. The significant association remained when the analysis was limited to non-Ashkenazi white families only ($P = 0.02$). None of the other SNPs was significantly linked or associated with prostate cancer.

Because *CDKN1B* has been implicated in other types of cancers, we investigated this possibility in the HPC families by testing the hypothesis that *CDKN1B* functions as a general tumor suppressor gene for all types of cancers. A family-based association test was used to examine the presence of an association between the SNP -79C/T and the risk to all types of cancers, including prostate cancer. A significant association remained when including other cancers in the test ($P = 0.0009$), implicating the SNP -79C/T as contributing not only to the risk of prostate cancer but also to other cancers among HPC families. To explore the possibility that the association between SNP -79C/T and the risk to other cancers is independent of prostate cancer, we included all cancers except for prostate cancer in an additional analysis. After excluding prostate cancer cases, no association was observed between SNP -79C/T and the risk to other cancers ($P = 0.61$). These results suggest *CDKN1B* plays a role as a general tumor suppressor gene, and its tumor suppressor function is particularly profound for prostate cancer.

The SNP -79C/T is located in the 5'-regulatory CpG island. Previous studies indicate that methylation of the *CDKN1B* promoter, although occurring in some cancer cells (14), is rare in prostate carcinomas (15, 16), and the methylation that does occur in prostate cancer typically does not affect this particular CpG dinucleotide. Although a primary focus of *CDKN1B* gene regulation studies has been the increase in protein degradation rates mediated by SKP2 ubiquitin ligase complex, ultimately decreasing CDKN1B protein in prostate cancer (17), CDKN1B protein levels can also be affected by the rates of gene transcription (18) as well as mRNA translation rates. Interestingly, this latter mechanism has been shown to be affected by sequences in the 5'-untranslated region of the *CDKN1B* transcript (19–21). Whether the basal transcription or transcript translation rates are affected by the -79C/T polymorphism is unknown at present.

The evidence for cosegregation of the two rare nonsynonymous mutations with prostate cancer in our HPC families is inconclusive. For the SNP 258G/C (E86D), the variant allele C was only identified in a proband but not in other family members available for study. For the SNP 356T/C (I119T), all four affected siblings were found to be carriers of the variant allele C. In addition, one of the sisters of the proband was diagnosed with lung cancer and found to be a C allele carrier. However, the unaffected brother and three unaffected nephews (ages 42, 60, and 65 years) of the proband also carried the C allele. Additional follow-up studies on these two rare variants may clarify their roles in HPC.

Recently, in a study by Kibel *et al.* (6), which included 96 cases and 106 controls from a European-American population, the SNP 326T/G (V109G) was found to be associated with the risk of advanced prostate cancer, with the genotype TT significantly increasing the risk of advanced disease (odds ratio = 1.95; 95% confidence interval = 1.09–3.47). In their study, the variant T allele frequency was found to be 72.4% in controls and 82.3% in cases. We observed a similar frequency for the variant T allele in our collection of unrelated probands of non-Ashkenazi white families with HPC (81%; Table 1). Furthermore, the T allele at SNP 326T/G was found to be overtransmitted, although not significantly, to affected offspring (observed S

statistics = 334, expected statistics = 331.9, $P = 0.74$) in our family based association test. It is also possible that the SNP 326T/G may play an important role in sporadic cases but less so in the etiology of high-risk hereditary cases, where -79C/T may be more relevant.

Given the biological relevance of cell cycle controls to cancer etiology, as well as the recent findings of association between prostate cancer and cell cycle regulation candidate genes (*CHEK2* and *CDKN1B*), additional studies are warranted. Specifically, follow-up studies should either evaluate the existing results in other populations or expand the analyses to other candidate genes in the same pathway. For *CDKN1B*, additional studies that evaluate interaction effects with *PTEN* among human populations will be interesting because these may build upon the biological mechanism already described in prostate cancer mouse models.

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