RNASEL mutations in hereditary prostate cancer

H Chen, A R Griffin, Y-Q Wu, L P Tomsho, K A Zuhlke, E M Lange, S B Gruber, K A Cooney

MATERIAL AND METHODS

Patient selection

Men with prostate cancer selected for this study are participants in the University of Michigan PCGP. Current enrolment criteria for this family based study of inherited prostate cancer susceptibility include families with two or more living members with prostate cancer. Confirmation of the diagnosis is obtained via review of medical records and/or death certificates. In some cases, the diagnosis of prostate cancer in dead subjects is achieved through verbal confirmation by two independent family members. Genomic DNA was isolated from whole blood using a commercial kit (Puregene, Gentra Systems, Inc, Plymouth, MN).

Ninety-five men with prostate cancer were selected from 75 PCGP families for direct sequence analysis of the RNASEL gene. Genotype data using two markers that surround the gene, D1S4466 and D1S158, were available from affected men in 39 of the 75 families. Nineteen of the 39 families with genotype data were considered to have high evidence of linkage because the family non-parametric linkage (NPL) score was >1 (table 1). The 11 families with moderate evidence of linkage consisted of families with NPL scores 1 but 1. The remaining nine families with low evidence of linkage consisted of families who had NPL scores <1. In the 11 families with moderate evidence for linkage to 1q24-25 markers, genomic DNA samples from all affected men in these families were evaluated by direct sequencing (28 subjects). DNA samples from all affected men from three additional families without available linkage data were also sequenced (six men). In the remaining 61 families, which included 19 families with strong evidence and nine families with little evidence for linkage using markers D1S466 and D1S158, genomic DNA from only one affected man from each family was sequenced.

Control samples

To estimate allele frequencies in both African Americans and whites, control samples were obtained from two sources. The Flint Men’s Health Study (FMHS) is a continuing population based case-control study of prostate cancer in African American men conducted in Genesee County, Michigan. For this report, DNA samples from 103 men with prostate cancer and 323 male controls were used. The average age at diagnosis for cases was 62.8 ± 8.3 years. All control men were screened for prostate cancer with serum PSA and digital rectal exam per protocol. The average age of the controls at the time of consent was 56.0 ± 10.2 years. Genomic DNA samples from 27 men and 48 women were also obtained from a continuing study called Genes, Environment and Melanoma (GEM) at the University of Michigan. The 75 DNA samples were obtained from spouses of study participants with melanoma. These subjects are white and range between 20 and 85 years of age.

For participants in all three studies (PCGP, FMHS, and GEM), written consent was obtained and all protocols were

Key points

- Mutations in the RNASEL gene have been identified in some prostate cancer families leading to the hypothesis that RNASEL is the prostate cancer susceptibility gene HPC1 on chromosome 1q.
- To test this hypothesis, we tested genomic DNA from 95 affected men in 75 multiplex prostate cancer families, including 19 families with evidence of linkage to 1q24-25 markers, from the University of Michigan Prostate Cancer Genetics Project for RNASEL mutations.
- Direct sequence analysis of the coding region showed four rare missense/nonsense mutations (G59S, I97L, V247M, and E265X) occurring in four unrelated families. None of four reported rare mutations (M1L, G59S, V247M, and E265X) were observed in a panel of 75 control DNA samples or in any of the 103 cases and 323 control samples from a population based study of prostate cancer in African Americans.
- Additional studies are needed to elucidate more clearly the role of RNASEL mutations in hereditary prostate cancer.
performed by the University of Michigan DNA Sequencing Corporation, Bedford, MA). Sequencing of PCR products was using Microcon® PCR centrifugal filter devices (Millipore Corporation, Bedford, MA). Amplification was carried out as follows: 95°C/1 minute, 56°C/30 seconds, and 72°C/45 seconds, and final denaturing (95°C/14 minutes), annealing (35 cycles of 95°C/30 seconds, 56°C/30 seconds, and 72°C/45 seconds), and final extension (72°C/10 minutes). PCR products were purified of AmpliGold® (Applied Biosystems, Foster City, CA) DNA polymerases. PCR primers were M13 forward and reverse primers, 0.55 µmol/l for both forward and reverse primers, 1 unit of Platinum® dNTPs, 0.2 mmol/l primer sequences from Carpten et al.3 PCR primers were M13ailed and purchased from GIBCO BRL (Invitrogen, Carlsbad, CA). Each reaction contained 2.5 mmol/l MgCl₂, 0.2 mmol/l dNTPs, 0.55 µmol/l for both forward and reverse primers, 1 unit of Platinum® Taq (Invitrogen, Carlsbad, CA), and 1 unit of AmpliGold® (Applied Biosystems, Foster City, CA) DNA polymerases. Amplification was carried out as follows: denaturing (95°C/14 minutes), annealing (35 cycles of 95°C/30 seconds, 56°C/30 seconds, and 72°C/45 seconds), and final extension (72°C/10 minutes). PCR products were purified using Microcon® PCR centrifugal filter devices (Millipore Corporation, Bedford, MA). Sequencing of PCR products was performed by the University of Michigan DNA Sequencing Core using ABI PRISM® 3700 Genetic Analyzers (Applied Biosystems, Foster City, CA). M13 forward and reverse primers were used to obtain both strand sequences. Sample sequences were aligned with RNASEL DNA sequence using the NCBI BLAST program to identify changes. Only changes clearly present in both strands were further evaluated.

### Table 1: Characteristics of the 75 PCGP families analysed for RNASEL mutations

<table>
<thead>
<tr>
<th>No of families</th>
<th>No of family members with prostate cancer</th>
<th>No of African American families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Families with high evidence for linkage to 1q24-25</td>
<td>19</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>Families with moderate evidence for linkage to 1q24-25 markers</td>
<td>11</td>
<td>5.6 ± 3.8</td>
</tr>
<tr>
<td>Families with low evidence for linkage to 1q24-25 markers</td>
<td>9</td>
<td>4.4 ± 1.6</td>
</tr>
<tr>
<td>Families with no linkage data</td>
<td>36</td>
<td>5.3 ± 2.3</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>4.7 ± 2.4</td>
</tr>
</tbody>
</table>

*All affected family members from three of these families were sequenced for RNASEL mutations.

### Table 2: Clinical features of 95 men with prostate cancer from 75 PCGP families

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average age at diagnosis</td>
<td>58.3 ± 8.1 years</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td>56.4</td>
</tr>
<tr>
<td>White</td>
<td>69</td>
</tr>
<tr>
<td>African American</td>
<td>21</td>
</tr>
<tr>
<td>Median serum PSA at diagnosis</td>
<td>6.3 ng/ml</td>
</tr>
<tr>
<td>Histological grade</td>
<td>35.1</td>
</tr>
<tr>
<td>Well differentiated (Gleason grade ≤6)</td>
<td>53</td>
</tr>
<tr>
<td>Moderately differentiated (Gleason 7)</td>
<td>33</td>
</tr>
<tr>
<td>Poorly differentiated (Gleason 8-10)</td>
<td>8</td>
</tr>
<tr>
<td>Treatment</td>
<td>8.5</td>
</tr>
<tr>
<td>Radical prostatectomy</td>
<td>72</td>
</tr>
<tr>
<td>External beam radiotherapy</td>
<td>7.4</td>
</tr>
<tr>
<td>Hormones</td>
<td>8</td>
</tr>
<tr>
<td>Other</td>
<td>8.4</td>
</tr>
</tbody>
</table>

*Histological grade was not available from one participant.

DNA sequencing

PCRs were used to amplify 20-40 ng of genomic DNA using primer sequences from Carpten et al.3 PCR primers were M13ailed and purchased from GIBCO BRL (Invitrogen, Carlsbad, CA). Each reaction contained 2.5 mmol/l MgCl₂, 0.2 mmol/l dNTPs, 0.55 µmol/l for both forward and reverse primers, 1 unit of Platinum® Taq (Invitrogen, Carlsbad, CA), and 1 unit of AmpliGold® (Applied Biosystems, Foster City, CA) DNA polymerases. Amplification was carried out as follows: denaturing (95°C/14 minutes), annealing (35 cycles of 95°C/30 seconds, 56°C/30 seconds, and 72°C/45 seconds), and final extension (72°C/10 minutes). PCR products were purified using Microcon® PCR centrifugal filter devices (Millipore Corporation, Bedford, MA). Sequencing of PCR products was performed by the University of Michigan DNA Sequencing Core using ABI PRISM® 3700 Genetic Analyzers (Applied Biosystems, Foster City, CA). M13 forward and reverse primers were used to obtain both strand sequences. Sample sequences were aligned with RNASEL DNA sequence using the NCBI BLAST program to identify changes. Only changes clearly present in both strands were further evaluated.

### Table 3: Summary of missense and nonsense mutations identified through sequence analysis of 95 men with prostate cancer from 75 PCGP families

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer pair</th>
<th>Amino acid change</th>
<th>Nucleotide substitution</th>
<th>No of affecteds identified with mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>RNASEL2</td>
<td>G59S</td>
<td>G175A</td>
<td>1 [PCGP 1115-02]</td>
</tr>
<tr>
<td>2</td>
<td>RNASEL2</td>
<td>I97L</td>
<td>A289C</td>
<td>2 [PCGP 345-02 and 392-02]</td>
</tr>
<tr>
<td>2</td>
<td>RNASEL3</td>
<td>V247N</td>
<td>G739A</td>
<td>1 [PCGP 168-01]*</td>
</tr>
<tr>
<td>2</td>
<td>RNASEL4</td>
<td>E265X</td>
<td>G799T</td>
<td>1 [PCGP 1115-02]</td>
</tr>
<tr>
<td>2</td>
<td>RNASEL5</td>
<td>R462Q</td>
<td>G1385A</td>
<td>Common</td>
</tr>
<tr>
<td>4</td>
<td>RNASEL7</td>
<td>D541E</td>
<td>T1623G</td>
<td>Common</td>
</tr>
</tbody>
</table>

*Novel mutation in this report.

Allele specific oligonucleotide (ASO) hybridisation and restriction fragment length polymorphism (RFLP) analysis

ASO experiments were performed as previously described.6 Control DNA samples for the M11 substitution were kindly provided by W Issacs. For RFLP analysis, PCR products were generated using the appropriate RNASEL primers, incubated at 36°C in the presence of BseRI or 65°C in the presence of BsrDI for one hour, and analysed on 2% agarose gels.

**RESULTS**

Sixteen of the 75 families (17%) included in this report were African American and the remainder of the families were white (table 1). The average number of confirmed affected men in the families was 4.7 ± 2.4. The clinical features of the 95 men with prostate cancer who were sequenced for RNASEL mutations are shown in table 2.

Sequence analysis of the RNASEL gene in 95 men with prostate cancer selected from 75 PCGP families showed three rare missense substitutions and one nonsense mutation (table 3). One of the missense substitutions, 197L, was identified in two unrelated affected men. Sequence analysis of the other affected men in each family from which DNA was available (family 345: brother and maternal cousin; family 392: second cousin) failed to identify this mutation. These results were also confirmed using RFLP analysis, since this mutation creates a novel BseRI site in the PCR product created using primer RNASEL2 (data not shown). This 197L substitution has been identified in men with prostate cancer in two separate reports with a frequency of ≤1%.1,7

A novel missense mutation was identified in subject 168-01, which results in the substitution of methionine for valine at
amino acid position 247 (table 3). The presence of this mutation in the proband was confirmed by RFLP analysis (data not shown). We also identified the E265X nonsense mutation previously described by Carpten et al in one of the 95 men with prostate cancer who were selected for the initial mutation screen (table 3). Sequence analysis confirmed the presence of the stop codon in one of two affected brothers but also in one of two unaffected brothers in this same family (fig 1C). All three men who were heterozygous for the E265X substitution were also heterozygous for the G59S substitution.

In the report of Carpten et al the E265X mutation was identified in 0.54% of unaffected white men (n=186) from a prostate cancer screening study and 0.52% of CEPH parents (n=96). Over 50% of the participants in the PCGP receive their care at the University of Michigan Medical Center. To determine the frequency of this mutation in the patient population at our medical centre, we analysed DNA from 75 spouses of patients with melanoma (GEM study) for the E265X mutation as well as the G59S and V247M substitutions (table 4). No heterozygous or homozygous carriers of any of these mutations were identified in this panel of controls. We also tested genomic DNA samples from a population based prostate cancer case-control study that focuses solely on African American men for the M1I mutation. This substitution of isoleucine for methionine at codon 1 was one of the two detrimental mutations described in the report of Carpten et al and it was identified in a single African American family. None of the case or control men was shown to be a carrier of the M1I mutation or the G59S, V247M, or E265X mutations.

**DISCUSSION**

There are several factors that make RNASEL a plausible candidate for the prostate cancer susceptibility gene HPC1. First, although the candidate region remains large, linkage peaks and recombinant data often indicate that the most likely region for HPC1 is near marker D1S158. The gene encodes an endoribonuclease which is a member of the interferon regulated 2-5A system. In vitro experiments suggest that the RNASEL gene may function as a tumour suppressor gene. RNase L -/- murine thymocytes have suppressed apoptosis, although similar studies have not been completed with prostate cells in which the RNASEL gene has been inactivated. Finally, the identification of deleterious mutations in two of eight multiplex prostate cancer families linked to 1q24-25 suggest that RNASEL requires further study as a prostate cancer susceptibility gene.

Our data provide limited support for the hypothesis that the RNASEL gene is HPC1. Analysis of one or more probands from 75 large (average >4 affected men/family) prostate cancer families has shown four rare missense/nonsense mutations among four unrelated families. Only two of 19 families with strong evidence of HPC1 linkage were found to have RNASEL mutations (families 168 and 392). In family 168, DNA samples from two affected brothers are not available for RNASEL mutation testing. In family 392, however, only one of two affected family members harboured the 197L mutation (see below). The other RNASEL mutations detected in our study (G59S, 197L, and E265X) did not completely cosegregate with affected status in three families.

Prostate cancer genetic studies are complicated by several factors including the variable participation in early detection or screening tests and the occasional diagnosis of latent or clinically insignificant prostate cancer within a family. The E265X nonsense mutation was first identified in a family with four affected brothers and each brother was heterozygous for the allele with the stop codon. Clinical information was available from three of the four brothers; all three men had clinically significant prostate cancer with Gleason scores in each case >7. However, in our report, only two of three affected brothers in family 1115 were discovered to be heterozygous carriers of the E265X mutation (fig 1). Review of clinical records shows no significant differences in clinical stage at presentation between the brothers with and the one without the E265X mutation. There are also two unaffected brothers in family 1115 who are participating in the PCGP research project; both brothers have had serum PSA tests with values...
<2.5 ng/ml within the last 12 months. Thus, it is unlikely that the unaffected brother harbouring the E265X mutation (1113-07), who was 37 at the time of study enrolment, has undetected prostate cancer. Rokman et al. recently reported the identification of the RNASEL E265X mutation in five index patients from 166 Finnish hereditary prostate cancer families. Similar to our results, segregation of the E265X mutation with disease status was observed in only one of the five families. In our study, the E265X mutation was found to be in complete linkage disequilibrium with the G595 mutation. This observation is consistent with the findings of others and suggests the occurrence of a rare (allele frequency <1%) founder allele in the US white population.

Previous studies by us and others have shown rare cases of allelic loss in prostate cancer tumour tissue using markers that map to the HPC1 candidate interval. The frequency of 1q24-25 LOH appears to ~10% in both sporadic (C Bettis, L Huang, K A Cooney, unpublished data) and hereditary21-22 prostate cancer. Previous investigations in our laboratory using tumour tissue from subject 392-01 showed evidence of allelic loss using two chromosome 1q markers (D1S158 and D1S518, data not shown). Examination of the linkage data from subject 392-01 and his affected second cousin (392-02) showed that the non-shared, presumably normal, allele was deleted consistent with the hypothesis that HPC1 may be acting as a tumour suppressor gene in this family. This observation led us to include both 392-01 and his affected second cousin 392-02 in our sequence analysis of the RNASEL gene. However, the 197L RNASEL missense mutation was identified only in subject 392-02 and presumably on the non-shared allele. Taken together, the findings do not provide evidence to support the hypothesis that the RNASEL gene is a tumour suppressor gene causing hereditary prostate cancer in this family.

Review of published linkage studies provides reasonable evidence to support the existence of a prostate cancer susceptibility gene at 1q24-25. Mutations in this HPC1 gene are likely to be relatively rare and may preferentially lead to aggressive prostate cancer.23 RNASEL mutations may contribute to prostate cancer in some families, as suggested by our results, as well as those contained in two recent publications.22,24 Wang et al. screened 326 men with prostate cancer from 167 unrelated families for RNASEL mutations. These investigators failed to identify any clearly deleterious mutations (including E265X); however, a common polymorphism, namely Arg462Gln, was associated with familial prostate cancer compared to either sporadic prostate cancer or compared to control men. Rennert et al.25 identified a founder frameshift mutation in RNASEL (471delAAAG) in Ashkenazi Jews that was found in higher frequency among men with prostate cancer (6.9%) compared to either elderly male or young women controls (2.4% and 4.0%, respectively). Additional studies, including a meta-analysis, are needed to elucidate more clearly the role of RNASEL mutations in hereditary prostate cancer.

ACKNOWLEDGEMENTS

This work was supported by USPHS Grants P50-CA69568 (SPORE) and R01-CA79956. We gratefully acknowledge Caroline Mohai, Gina Claeyts, Kay Doerr, and the Flint Men’s Health Study Research Team for assistance with patient recruitment and data collection.

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J Med Genet 2003 40: e21
doi: 10.1136/jmg.40.3.e21

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