Germline mutations in the ribonuclease L gene in families showing linkage with *HPC1*

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Although prostate cancer is the most common non-cutaneous malignancy diagnosed in men in the United States[1,2], little is known about inherited factors that influence its genetic predisposition[3–5]. Here we report that germline mutations in the gene encoding 2′-5′-oligoadenylate(2-5A)–dependent RNase L (*RNASEL*)[6–8] segregate in prostate cancer families that show linkage to the HPC1 (hereditary prostate cancer 1) region at 1q24–25 (ref. 9). We identified *RNASEL* by a positional cloning/candidate gene method, and show that a nonsense mutation and a mutation in an initiation codon of *RNASEL* segregate independently in two HPC1-linked families. Inactive *RNASEL* alleles are present at a low frequency in the general population. *RNASEL* regulates cell proliferation and apoptosis through the interferon-regulated 2-5A pathway and has been suggested to be a candidate tumor suppressor gene[10–12]. We found that microdissected tumors with a germline mutation showed loss of heterozygosity and loss of RNase L protein, and that *RNASEL* activity was reduced in lymphoblasts from heterozyogous individuals compared with family members who were homozygous with respect to the wildtype allele. Thus, germline mutations in *RNASEL* may be of diagnostic value, and the 2-5A pathway might provide opportunities for developing therapies for those with prostate cancer.

On the basis of reports of familial clustering of prostate cancer[3] and segregation analyses that support the existence of dominantly acting alleles, which confer high risk for prostate cancer[4], we carried out a genetic linkage study using families affected with hereditary prostate cancer (HPC). Results implicated several prostate susceptibility loci, including one on the long arm of chromosome 1, at 1q24–25 (termed HPC1)[9]. We used recombination mapping and candidate gene analysis to map several genes, including *RNASEL*, to the critical region[13,14] of HPC1 (Fig. 1*a*). RNase L is a constitutively expressed latent endoribonuclease that mediates the antiviral and proapoptotic activities of the interferon-inducible 2-5A system[10,11,15]. The gene consists of eight exons. Northern-blot analysis shows that there are two mRNA species of 5 kb and 9.5 kb in the spleen, thymus, prostate, testis, uterus, small intestine, colon and peripheral blood leukocytes (data not shown). Expression varies according to the tissue, with the highest expression in the spleen and thymus.

We initially screened a set of DNA samples representing one affected individual from each of 26 families at high risk for prostate cancer, including 8 families that showed linkage to the HPC1 region and that had at least four affected individuals sharing an HPC1 haplotype. We identified a mutation (Glu265X) in the proband from family 065 (Fig. 1*b*). Four affected brothers had the base substitution 795G→T in exon 2 (starting from the initiating methionine) of *RNASEL*, which is predicted to result in the conversion of a glutamic acid codon to a termination codon at amino-acid position 265 and can lead to the loss of function of that allele[8]. These brothers were heterozygous with respect to the mutation. Three of the four affected brothers had prostate cancers with clinical features that are associated with poor prognosis (that is, Gleason score greater than or equal to 7, stage greater than or equal to T2B, and/or evidence of disseminated disease); this information was not available for the fourth affected brother.

We identified a second mutation in the proband of family 097, a family of African-American descent (Fig. 2*c*). On initial evaluation, five of the six brothers in this family had been diagnosed with prostate cancer; the sixth brother (097-016) was diagnosed subsequently. The average age of diagnosis in this family was 59. The mutation in this family is characterized by the base substitution 3G→A in the codon that corresponds to the initiating methionine (AUG) of the RNase L transcript. This guanine is conserved 100% in the initiation codons of all eukaryotes and in most prokaryotes[16]. This mutation was inherited heterozygously by four of the six affected brothers in family 097. The two affected brothers that do not carry the mutation possibly represent phenocopies (Fig. 2*c*). Whereas three of the four mutation carriers had cancers with poor prognostic indicators, as described above for family 065, the two affected non-mutation carriers had cancers with clinical features that are associated with more favorable disease outcomes (that is, a lower tumor grade and stage, Gleason score 5, and clinically non-palpable, T1C stage).

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To assess the frequency of the Glu265X and the Met1Ile mutations, we analyzed the DNA from control populations and from individuals with non-familial prostate cancer (Table 1). We found one Glu265X heterozygote in 144 normal control individuals and two Glu265X heterozygotes in 186 participants with no family history of prostate cancer and normal serum concentrations of prostate-specific antigen. Analysis of germline DNA from 258 men with non-familial prostate cancer revealed two Glu265X heterozygotes. Thus, this nonsense variant is found in the control population at an estimated allele frequency of 0.5%; as yet we cannot identify a difference in allele frequency between affected individuals and controls.

By contrast, we did not observe the Met1Ile mutation in 698 control individuals, 284 of whom were African American (Table 1). We note that in rare, highly penetrant disorders with a young age of onset, it is unusual to find mutant alleles in unaffected individuals. For low penetrance disorders with a late age of onset and extremely high prevalence (for example, prostate cancer), however, it is common to identify individuals in a general control population who may be carriers of a mutant allele[17,18]. In addition to these two mutations, we also identified a series of missense mutations in the probands of HPC-affected individuals (Web Fig. A). Studies are underway to determine the frequency of these changes in affected individuals and controls.

Single-strand conformation polymorphism (SSCP) analysis of microdissected tumor DNA from individual 065-009 showed that, compared with the heterozygosity of their normal lymphocyte DNA, there was clear loss of the wildtype allele in tumor cells from this individual (Fig. 2*a*). We used the same tumor sample from individual 065-009 in an immunohistochemical analysis of RNase L protein expression using a monospecific RNase L monoclonal antibody[19]. Although we observed cytoplasmic staining in non-cancerous prostate epithelial cells, there was a consistent absence of staining in cancer cells throughout the tumor, consistent with inactivation of both *RNASEL* alleles in tumor tissue from this individual (Fig. 2*b*).

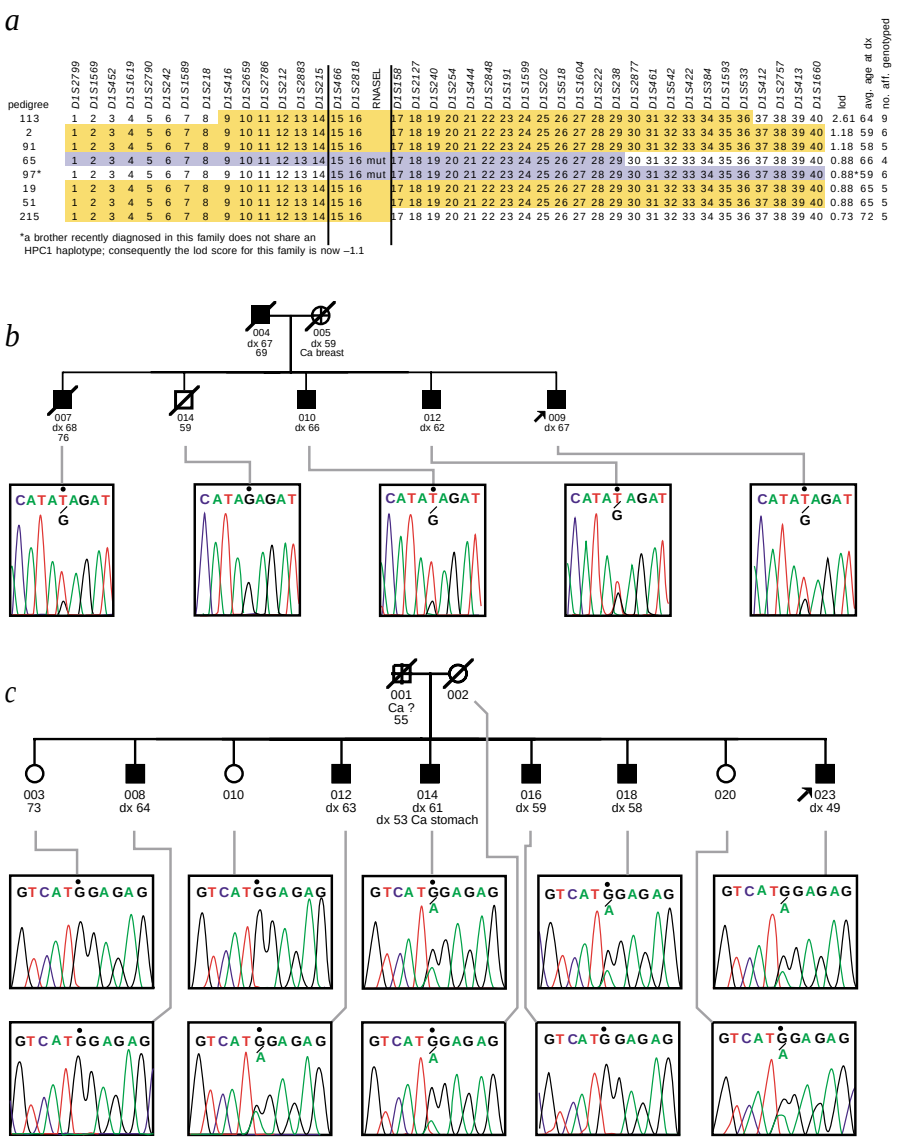

Fig. 1 Multipoint linkage analysis assuming heterogeneity on 91 high-risk prostate cancer families suggests *HPC1* maps to *D1S2883–D1S158–D1S422*. ***a***, Informative affected recombinants, prioritized on the basis of the individual lod scores of families, led to the identification of a critical interval for *HPC1*. ***b***,***c***, Detailed family structure and sequence chromatograms showing transmission of the Glu265X mutation in family 065 and the Met1Ile mutation in family 097. The variable nucleotide is marked by a dot in each representative chromatogram.

To determine whether the Glu265X mutation affected enzyme activity, we measured rRNA cleavage in lymphoblasts after transfections with the biostable 2-5A analog psA(2′ps5′A)$_3$ (Fig. 2*c* and Table 2). The prostate cancer cell line PC3, which originated from a bone metastasis of a grade IV prostatic adenocarcinoma in a 62-year-old male who lacked the described mutations in *RNASEL*, showed prominent products specific to RNase L cleavage[20] of 28S and 18S rRNA (Fig. 2*c*, lanes 1–3). The lymphoblasts had less activity, partly owing to lower uptake of 2-5A, as determined by fluorescein-tagged 2-5A and confocal microscopy (data not shown). Lymphoblasts from heterozygous individuals from family 065, including 065-012, showed decreased *RNASEL* activity compared with lymphoblasts from individuals homozygous with respect to the wildtype allele, for example 065-016 (Fig. 2*c*, lanes 4–9, and Table 2). Three separate experiments on the lymphoblasts from family 065 resulted in homozygous/heterozygous ratios of *RNASEL* activity towards 18S and 28S rRNAs of 2.2 and 1.9, respectively (Table 2). Similarly, several experiments with lymphoblasts from family 097 produced homozygous/heterozygous ratios of *RNASEL* activity towards 18S and 28S rRNA of 2.4 and 1.5, respectively (Table 2). The average of the ratios from both families (2.0) suggests that, as predicted, homozygous cells contain twice the amount of RNase L as do heterozygous cells. These findings also indicate that both the Glu265X and Met1Ile mutations prevent synthesis of a functional RNase L.

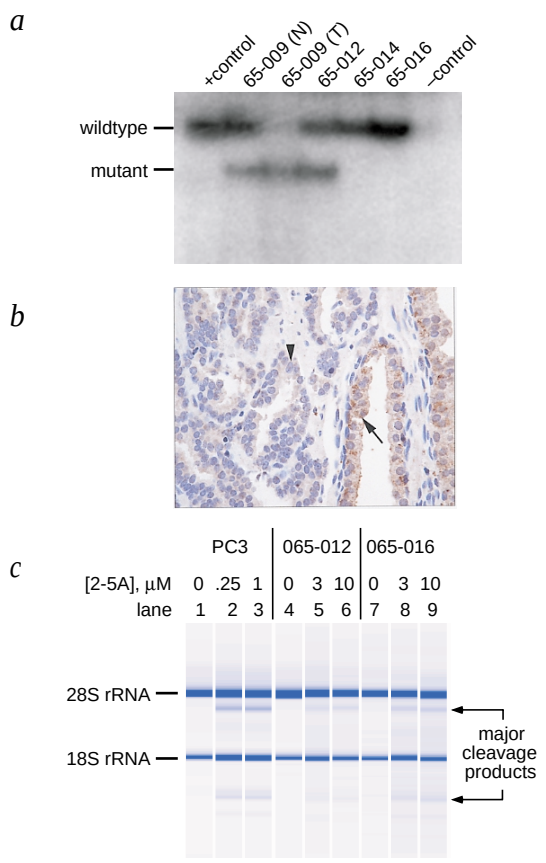

Fig. 2 Deficiences in RNase L in tumor tissue and lymphoblasts from HPC1-affected individuals. ***a***, Loss of heterozygosity using SSCP analysis was carried out on lymphocyte DNA from individual 065-009 and controls, and on tumor DNA from individual 065-009 to determine loss of the wildtype allele in tumors. Wildtype and mutant alleles are indicated. ***b***, Immunohistochemical analysis of the expression of RNAse L protein[19] in a tumor specimen from an Glu265X mutation carrier in family 065. The cytoplasm of normal prostate epithelium stains positively (brownish red stain in cells marked by the arrow on the right of the section), whereas tumor cells are negative (arrowhead). ***c***, Activity of *RNASEL* in intact PC3 cells and lymphoblast cell lines from individual 065-012 (heterozygous with respect to the Glu265X mutation in *RNASEL*) and individual 065-016 (homozygous, lacking this mutation). The positions of the 28S and 18S rRNA and their main cleavage products are indicated.

It has been proposed that *RNASEL* is a candidate tumor-suppressor gene on the basis of its known function[12]. RNase L has been shown to be lost completely in the hepatoma cell line HepG2 (ref. 21). An animal model of RNase L function shows that mice devoid of RNase L have defects in both interferon-induced apoptosis and antiviral response[11]; however, these animals do not develop tumors. Although the function of the 2-5A pathway has not yet been explored in prostate tissue, the balance between hormonally regulated growth and cell death is crucial in this organ[22]. It is possible that reduced 2-5A function shifts this balance toward cell growth, creating a favorable environment for the development of prostate cancer.

We propose that *RNASEL* is a candidate cancer-susceptibility gene for HPC1. We have identified two mutations that are potentially responsible for prostate cancer cases in families showing linkage to the HPC1 locus. We have also shown that loss of the wildtype allele occurs in tumor DNA from a mutation carrier, suggesting that there is complete loss of function of this protein. The low frequency of mutations suggests that there is likely to be a high rate of heterogeneity in prostate cancer. It is also possible that we did not identify mutations in other potential families with HPC1 that we screened owing to either the location of the mutations in regulatory elements or technical limitations. The identification of other functionally significant mutations in *RNASEL* using an independent data set will be necessary to confirm this gene as the prostate cancer–susceptibility gene in families showing linkage to HPC1. These findings could be significant, as the identification of germline mutations in this gene could lead to early diagnosis and therapeutic approaches for prostate cancer cases linked to HPC1.

Methods

Subjects. We obtained informed consent from each participant in this study.

PCR analysis. The primers for PCR are available upon request. We carried out PCR of *RNASEL* exons in a volume of 50 µl containing 20 ng of genomic DNA, PCR buffer (Gibco BRL), 2.25 mM Mg^{2+}, 250 nM dNTPs, 10 pmol of each forward/reverse primer mix, 0.06 U Platinum Taq DNA polymerase (Gibco BRL) and 0.06 U AmpliTaq Gold (PE Biosystems).

The PCR protocol was 95 °C for 14 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. We analyzed PCR products by 2% agarose gel electrophoresis.

DNA sequencing and sequence analysis. We amplified the exons for a given gene using M13-tailed primers. The subsequent PCR products were cleaned up using a PCR purification kit (Qiagen) and the BIOROBOT 9600 dual vacuum system (Qiagen). We prepared half-volume cycle sequencing reactions in 96-well plates using standard M13 forward and reverse primers and 3700 Big Dye Terminator Chemistry (PE/Applied Biosystems). After purification, sequencing reactions were run on a 3700

Table 1 • Frequency of Glu265X and Met1Ile mutations in familial prostate cancer cases, nonfamilial cases and controls

Mutation	Study groups	Number screened	Number of mutants	Frequency (%)	Sample type	Method
Glu265→X						
	unaffected white men*	186	2	0.54	blood DNA	direct sequencing and SSCP
	CEPH parents	96	1	0.52	blood DNA	direct sequencing
	US population controls	48	0	0.00	blood DNA	
		330	3	0.45		
	prostate cancer cases (non-HPC)	258	2	0.39	normal tissue DNA	SSCP
Met1→Ile	unaffected African American men*	92	0	0.00	blood DNA	*Nla*III digest
	unaffected white men*	186	0	0.00	blood DNA	direct sequencing
	African American population control	192	0	0.00	blood DNA	direct sequencing
	US population controls	48	0	0.00	blood DNA	direct sequencing
	total number of samples screened	240	0	0.00		
	prostate cancer cases (non-HPC)	180	0	0.00	normal tissue DNA	*Nla*III digest

*From prostate cancer screenings (PSA<4.0, ages 35–70, men were excluded if they had an abnormal digital rectal exam). CEPH, Centre d'Etude du Polymorphisme Humain.

Table 2 • RNase L activity in intact lymphoblasts

Cell line	18S rRNA cleavage (%)	28S rRNA cleavage (%)
RNase $L^{+/+}$		
065-016 (*n*=3)	15.3 ± 1.5	11.2 ± 0.8
065-061 (*n*=3)	12.3 ± 2.7	11.3 ± 2.2
Average	13.8	11.3
RNase $L^{+/-}$		
065-007 (*n*=3)	5.7 ± 4	6.8 ± 3.6
065-009 (*n*=3)	8.2 ± 2	6.1 ± 1.5
065-012 (*n*=4)	5.0 ± 2.1	4.8 ± 1.7
Average	6.3	5.9
RNase $L^{+/+}$/ RNase $L^{+/-}$	2.2-fold	1.9-fold
RNase $L^{+/+}$		
097-008 (*n*=6)	33.1 ± 4.4	15.7 ± 3.1
097-016 (*n*=6)	26.7 ± 5.6	14.2 ± 2.1
Average	29.9	15.0
RNase $L^{+/-}$		
097-012 (*n*=3)	18.5 ± 6.6	15.3 ± 7.1
097-014 (*n*=5)	7.7 ± 1.6	9.8 ± 5.1
097-018 (*n*=5)	11.5 ± 3.9	8.3 ± 3.2
097-023 (*n*=5)	12.0 ± 4.3	6.0 ± 2.2
Average	12.4	9.9
RNase $L^{+/+}$/ RNase $L^{+/-}$	2.4-fold	1.5-fold

DNA Analyzer (PE/Applied Biosystems) according to the manufacturer's protocols. We aligned and analyzed sequence chromatograms using Sequencher version 4.1 (Gene Codes).

Laser capture microdissection. Glass slides containing sections 8 mm thick cut from paraffin-embedded tissue blocks were deparaffinized and stained with hematoxylin and eosin. We dehydrated slides in xylene and used them immediately for laser capture microdissection of tumor cells using the PixCell II LCM system (Arcturus). A polymer cap was placed on the slide, and all available tumor cells (~2,000) were transferred to the cap using a laser beam. We then placed the cap on an Eppendorf tube containing 50 ml of digestion buffer (1 mg ml^{-1} proteinase K, 10 mM Tris-HCl, pH 8, 1 mM EDTA and 1% Tween-20). We incubated the solution at 52 °C overnight with the tube in an inverted position so that digestion buffer was in contact with the tissue on the cap. The cap was removed after centrifugation for 5 min, and proteinase K was inactivated by incubation at 95 °C for 10 min.

Loss of heterozygosity. We determined loss of heterozygosity using SSCP analysis for the Glu265X mutation. An expected 166-bp product spanning the Glu265X mutation was identified by PCR. We modified the PCR protocol such that the volume was reduced to 15 ml, [a-^{32}P]dCTP was added, and 40 cycles were carried out. The PCR products were mixed with formamide loading buffer, denatured and separated by electrophoresis on a Hydrolink MDE gel (BioWhittaker Molecular Applications) with 5% glycerol for 16 h. We dried the gels in a vacuum and subjected them to autoradiography.

***RNASEL* activity assay.** Lymphoblastoid cell lines were cultured in RPMI 1640 medium supplemented with glutamine and 15% fetal bovine serum. The PC3 prostate cancer cell line[23] was grown in the same medium, except that 10% fetal bovine serum was used. The biostable, all phosphorothioate mixed isomer analog of tetramer 2-5A, psA(2′ps5′A)$_3$ was synthesized chemically using an ABI 380B DNA synthesizer, purified by high-performance liquid chromatography and desalted (Z. Wang and R.H.S., unpublished data). We tranfected cells with 3.0 μM psA(2′ps5′A)$_3$ or at the indicated concentrations for 4–5 h using lipofectamine (Gibco-BRL). We isolated total RNA from transfected cells using Trizol reagent (Gibco-BRL) and quantified it by measuring absorbance at 260 nm. We separated RNA molecules on RNA chips and analyzed them with an Agilent Bioanalyzer 2100 (Agilent Technologies). We determined peak areas of 28S and 18S rRNA and their main cleavage products using the Bio Sizing (version A.01.30 S1220) program (Agilent Technologies).

Note: Supplementary information is available on the Nature Genetics web site (http://genetics.nature.com/supplementary_info/).

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