ELECTRONIC LETTER

No fumarate hydratase (*FH*) mutations in hereditary prostate cancer

R Lehtonen, M Kiuru, A Rökman, T Ikonen, J M Cunningham, D J Schaid, M Matikainen, N N Nupponen, A Karhu, O-P Kallioniemi, S N Thibodeau, J Schleutker, L A Aaltonen

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Mutations in fumarate hydratase (fumarase, *FH*), a nuclear gene encoding a mitochondrial tricarboxylic acid cycle or Krebs cycle protein also present in the cytosol, have recently been shown to predispose to hereditary leiomyomatosis and renal cell cancer (HLRCC, OMIM 605839)[1] or multiple cutaneous and uterine leiomyomatosis (MCL, OMIM 150800).[2] In addition to leiomyomas of the skin and uterus, some subjects also develop papillary renal cell carcinoma of rare type II histology.[3 4] Germline *FH* mutations were found in approximately 60% (24/42) of families segregating HLRCC.[1] Mutations in *FH* have been recently observed in apparently sporadic tumours. In a series of lesions studied, one uterine leiomyosarcoma and one cutaneous leiomyoma harboured a germline mutation while one soft tissue sarcoma represented the first known purely somatic case.[5] Homozygous germline *FH* mutations have been found to cause recessive fumarate hydratase deficiency (OMIM 136850).[6–8] FH has been mapped to chromosome 1q43, between markers D1S2785 and D1S2842, within 500 kb of D1S2785.

The first observation that a Krebs cycle component is involved in tumorigenesis was made by Baysal *et al*.[9] Germline mutations in subunits of succinate ubiquinone oxidoreductase (mitochondrial complex II), succinate dehydrogenase subunit D (*SDHD*) and C (*SDHC*) and B (*SDHB*), have been observed in patients with hereditary paraganglioma (PGL) and subsequently in sporadic phaeochromocytomas.[9–13] Homozygous succinate dehydrogenase subunit A (*SDHA*) mutations display a phenotype resembling more classical mitochondrial gene defects, such as Leight syndrome.[14] Succinate dehydrogenase oxidises succinate to fumarate in the Krebs cycle, and complex II is involved in a mitochondrial electron transport chain.

Hereditary prostate cancer (HPRCA) is a genetically complex disease involving multiple susceptibility genes and a strong environmental component. Segregation analyses in high risk prostate cancer families support a model of autosomal dominant inheritance with multiple rare high penetrance genes, particularly in early onset subgroups.[15 16] One of the potential HPRCA loci has been mapped to chromosome 1q42.2-43, putative predisposing gene for cancer of prostate (*PCAP*), spanning a 20 cM region.[17 18] A maximum two point logarithm of odds (lod) score was obtained with a microsatellite marker D1S2842 in a selection of French and German families. Furthermore, the subset of early onset families gave the highest multipoint lod score of 3.31 at the *PCAP* locus at marker D1S2785.[17] Loss of heterozygosity (LOH) in prostate tumours at 1q42.2-43[17] further supported the linkage evidence and indicated that the causative gene may act as a tumour suppressor. Suggestive evidence for linkage, maximum multipoint non-parametric lod (NPL) score 1.45 at D1S2785, was observed by Berry *et al*[18] for a subset of US families fulfilling the criteria of male to male transmission, age under 66 years at the time of diagnosis, and more than four affected subjects within the families.[18] Linkage to the *PCAP* locus has remained unconfirmed in some data sets.[19 20] These and other studies propose a heterogeneous background for HPRCA. Linkage analyses have indicated that several other chromosomal regions may be involved in inherited prostate cancer: first putative hereditary prostate cancer locus (*HPC1*) at 1q24-25,[21] prostate and brain cancer susceptibility locus (*CAPB*) at 1p36,[22] hereditary prostate cancer X linked locus (*HPCX*) at Xq27-28,[23 24] hereditary prostate cancer locus at chromosome 20q13 (*HPC20*),[25] and a putative prostate cancer gene *HPC2/ELAC2* at 17p.[26] A few genes have been reported to be mutated in hereditary prostate cancer, none of them representing a high penetrance major susceptibility gene: *ELAC2*,[27 28] ribonuclease L (*RNASEL*) at *HPC1* locus,[29–32] 5α reductase type II gene (*SRD5A2*),[33 34] and cytochrome P450c17 (*CYP17*).[35 36] The two latter genes are involved in androgen synthesis and action.

Key points

- Mutations in nuclear genes encoding tricarboxylic acid (Krebs) cycle proteins fumarate hydratase (FH) and some subunits of succinate dehydrogenase complex have been associated with familial tumour predisposition and certain sporadic tumour types. The mechanisms by which *FH* defects promote tumorigenesis are unknown, but may involve activation of hypoxia pathways or alterations in citrate production through Krebs cycle.
- *FH* is located in the same chromosomal region as one of the putative familial prostate cancer loci, *PCAP* at 1q42.2-43. Thus it is a positional candidate gene for PCAP.
- We performed *FH* mutation analysis in a series of 89 HPRCA families. Our data set consisted of 19 US families showing suggestive linkage to the *PCAP* locus and 70 Finnish families in which *PCAP* linkage information was not available.
- No mutations were found. The absence of mutations in this large series strongly suggests that *FH* is not a predisposing gene for familial prostate cancer, but additional candidates at 1q42.2-43 should be screened.

Abbreviations: FH, fumarate hydratase; HLRCC, hereditary leiomyomatosis and renal cell cancer; MCL, multiple cutaneous and uterine leiomyomatosis; HPRCA, hereditary prostate cancer; DHPLC, denaturing high performance liquid chromatography; LOH, loss of heterozygosity

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The occurrence of prostate cancer in a *FH* mutation carrier in one HLRCC family and a frequent occurrence of kidney cancer (nine cases in 19 families) among US HPRCA families showing linkage to 1q42.2-43 supported the possible role of FH defects in prostate cancer predisposition.[3] To test this hypothesis we performed extensive *FH* mutation analyses in two series of prostate cancer families. We analysed two subjects per family from 19 US families with suggestive evidence for linkage at the *PCAP*/*FH* locus, and one affected family member from each of 70 Finnish prostate cancer families. In addition, the *PCAP* region was scrutinised for other candidate prostate cancer predisposition genes, to be analysed in subsequent studies.

MATERIALS AND METHODS

Finnish families

The Finnish family collection has been described previously.[25] DNA samples from the youngest affected subjects (when available) of each of the 70 Finnish families were collected for mutation analysis. At least one inclusion criterion had to be fulfilled: (1) prostate cancer in three generations, (2) three or more first degree relatives with prostate cancer, or (3) two subjects with prostate cancer diagnosed under the age of 60. The average age of diagnosis was 62.2 years (range 44-79) and the number of affected subjects per family was 3.2 (range 2-6). Two of the patients had kidney cancer, but their leiomyoma status was unknown. No 1q42.2-43 linkage data were available.

US families

US families were ascertained through the Mayo Clinic radical prostatectomy database. All men who had undergone radical prostatectomy for clinically localised prostate cancer, in the Department of Urology, or who received radiation therapy, in the Division of Radiation Oncology at the Mayo Clinic (Rochester, MN), were subjected to a family cancer history survey.[19] A total of 162 families with a minimum of three men affected with prostate cancer were collected for linkage studies. All men who contributed a blood specimen and who had prostate cancer had their cancers verified by review of medical records. The average age of diagnosis per pedigree was 66.5 years (range 47-77 years), with 73 pedigrees having an average age of diagnosis <66 years. The average number of affected men per pedigree was 4.4 (range 3-11), the average number of affected men with blood specimens per pedigree was 2.8 (range 2-7), and the average number of total blood specimens per pedigree was 3.7 (range 2-12). The research protocol and informed consent forms were approved by the Mayo Clinic Institutional Review Board. The 19 US families for *FH* mutation screening were selected on the basis of having a maximum lod score >0.5 (range 0.52-1.31) and maximum NPL >0.9 (range 0.94-2.75) over the *PCAP* locus. The average age of onset in the families was 64.89 years (range 54.75-69.67 years) and the average number of affected subjects in each family was 3.36 (range 2-6). Within these 19 families, kidney cancer was observed in six different kindreds in nine subjects altogether. Four of these cases also had prostate cancer. The average age at diagnosis of the kidney cancer cases was 58.55 years.

Denaturing high performance liquid chromatography (DHPLC) analysis

Mutation screening of all 38 US samples (two per family) was performed by DHPLC. DNA samples were extracted from blood by standard procedures. The exons were amplified for the DHPLC analysis in 50 µl PCR reactions consisting of 50 ng genomic DNA, 0.7 × Platinum PCR Buffer (Invitrogen, Carlsbad, CA), 200 µmol/l each dNTP (Finnzymes, Espoo, Finland), 0.3 µmol/l both primers, and DNA polymerase Platinum *Taq* (Invitrogen) 1.25 units, Titanium *Taq* (Clontech, Palo Alto, CA) 0.60 units, and Ampli*Taq* Gold (Applied Biosystems, Foster City, CA) 0.60 units. The hot start PCR cycling conditions were as follows: 94°C for 12 minutes, 94°C for 12 minutes, followed by 35 cycles of denaturing for 30 seconds, varying annealing temperatures for 30 seconds, elongation at 72°C for 45 seconds, and final extension at 72°C for 10 minutes. Denaturing temperature was lowered from 94°C to 89°C after 10 cycles. $MgCl_2$ concentrations, annealing temperatures, and primer sequences are provided in table 1. Samples from two subjects were pooled to enable the detection of putative homozygous variation before they were denatured at 95°C for three minutes and reannealed by cooling down 0.5°C/30 seconds for 40 minutes. DHPLC heteroduplex analyses were performed using automated HPLC instrumentation with Agilent 2G experimental dsDNA 2.1 × 75 mm 3.5 micron column (Agilent Technologies, Palo Alto, CA). The optimal melting

Table 1 Oligonucleotide primers, $MgCl_2$ concentrations, and annealing temperatures for *FH* PCR. DHPLC melting temperatures correspond to column temperatures used in heteroduplex analysis. Mitochondrial targeting signal is defined as exon 0 (FH0)

Exon	Primer	Primer sequence (5′ > 3′)	Product size (bp)	$MgCl_2$ (mmol/l)	Annealing temp (°C)	DHPLC melting temp (°C)
0	FH0F	TGT GAG GCT GTT GAT TGG AT	299	0	59	66
	FH0R	GGA GGG CTG AAG GTC ACT G				
1	FH1F	AAG ATG CGA TTA CTT TTG ATC C	256	2.8	58	58
	FH1R	TGA ATA CAG CCT ACT TCA TCCA A				
2	FH2F	CTG CCA AAA TAA TAA ACT TCC ATG C	254	1.4	58	53/56
	FH2R	GCC AGA GCA TAT CGT CAT CC				
3	FH3F	TGT GGG TCA ACT GTA TTC AAA C	321	1.4	58	57
	FH3R	CAA TCT CAG GTA TGC TTT TCA A				
4	FH4F	GCT GGG TTT TGA GTA GTT AGT TGG	349	1.4	58	56/57
	FH4R	GGC CAT TTG TAC CAA GCT CT				
5	FH5F	TTTGCTCATCATAAGATTTGAAGT	381	2.8	60	59
	FH5R	CAGACCACGTATAATGAGAAATGAA				
6	FH6F	GTT CAC CCA TCT AGG ATA TTT TTC T	340	2.8	60	61
	FH6R	GCT TCG GGA TAA CTT TAA ACA AA				
7	FH7F	ATGGTTGGGCCTTGCTTTAT	300	2.8	58	54/57
	FH7R	CCAAGATAATAAGCCTTTGGTCA				
8	FH8F	CATGTTGCCTTAGTAATGTCTCTCTC	317	1.4	58	56
	FH8R	TGCTGTTCTCAAACACTGATCC				
9	FH9F	CAATTATGTCACCTTTTGCTTTAGG	312	2.8	58	53/56
	FH9R	GCAGTTTCCTTTCAAACTTATCC				

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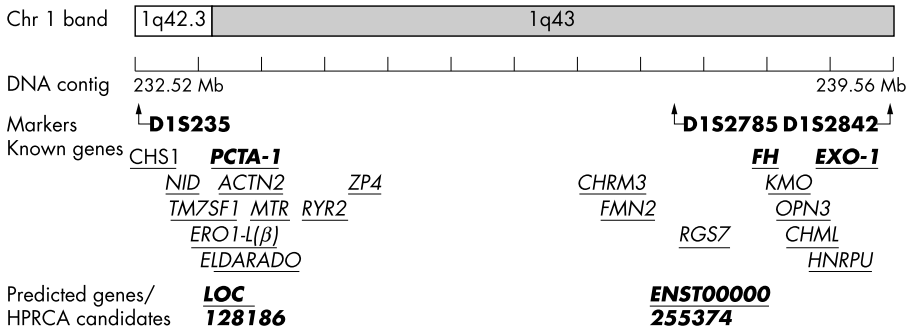

Figure 1 Map of the *PCAP* locus defined by markers D1S235 and D1S2842. All known genes and the most interesting predicted genes are included in the map. Genes/transcripts discussed in the text are printed in bold.

temperatures for each amplicon (table 1) was obtained using algorithm at the Stanford DHPLC Melt program web page (http://insertion.stanford.edu/melt1.html). The analytical acetonitrile gradient was composed by mixing Helix BufferPak A for dHPLC and 55-75% B (Varian Analytical Instruments, Walnut Creek, CA) at flow rate of 0.4 ml/minute.

Sequencing

The Finnish sample set was analysed by genomic sequencing. The PCR reactions, conditions, and oligonucleotide primers used were identical to the preceding study of Kiuru *et al*.[5] Any samples with heteroduplex DHPLC peaks were also reanalysed by genomic sequencing. PCR products were purified using NucleoSpin PCR purification kit (Matcherey-Nagel, Duren, Germany). Direct sequencing of PCR products was performed using the BigDye3 termination chemistry (Applied Biosystems) with ABI 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Bioinformatics

To evaluate candidate genes, the *PCAP* locus between markers D1S235 and D1S2842[19] spanning the 7 Mb region of the chromosome 1 physical map (23 250 000-23 980 000 bp) was browsed through Ensemble Human Map View www server (http://www.ensembl.org/perl/mapview?chr=1). Translated products of known genes and predicted transcripts were subjected to homology and conserved domain search at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and EBI (http://www.ebi.ac.uk/interpro/) web sites respectively.

RESULTS

We could not detect any germline mutations in the coding sequence or within the conserved splice site regions of *FH* in the series of 108 prostate cancer patients from 89 families. We succeeded in obtaining results from 1073 out of 1080 (99.4%) *FH* fragments analysed. One unique base pair substitution was observed in the 3' untranslated region (UTR) of the gene at position IVS9+9 T>C. A silent polymorphism 798G>A was observed in five samples in exon 7. The only heteroduplex peak displayed in the DHPLC analysis resulted from the heterozygous substitution of C to T at position –11 upstream from the translation initiation codon. Loss of a wild type allele was not present in the prostate cancer tissue of the *FH* mutation carrier in HLRCC family (data not shown).

Through the computational analyses, we localised 19 known genes and almost 100 predicted transcripts to the *PCAP* locus (fig 1).

DISCUSSION

Recent studies on SDH and FH suggest that some proteins predominantly known as Krebs cycle enzymes are involved in the development of multiple human tumour types. We hypothesised that FH defects may be associated not only with HLRCC, but also with prostate cancer predisposition. A *FH* mutation carrier in one HLRCC family had had prostate cancer. Kidney cancer cases were unexpectedly frequent among US HPRCA families showing linkage to 1q42.2-43. The fact that the genetic locus harbouring *FH* had been associated with hereditary prostate cancer made *FH* an attractive positional candidate for a prostate cancer predisposition gene.

The product of the *FH* gene acts as a key enzyme in mitochondrial energy metabolism in the citric acid cycle. In addition to energy metabolism, the intermediates of Krebs cycle are the precursors of amino acid metabolism and biosynthesis including the formation of steroids from cholesterol through lipid metabolism. In normal prostate cells, the oxidation of citrate is inhibited by aconitase, which leads to the accumulation of citrate. Transformation from citrate producing benign cells to citrate oxidising malignant cells appears to be a key event in prostate carcinogenesis.[37] Succinate, the Krebs cycle intermediate and respiratory chain substrate, is postulated to restore HIF-1α hypoxic induction through electron transport chain or through some metabolic pathway.[38 39] This indicates that Krebs cycle could be linked to tumorigenesis through regulation of hypoxia inducible genes.

The combination of functional and linkage data indicates that mutations in *FH* might underlie HPRCA in a subset of families. An extensive effort to screen for FH defects in HPRCA was performed to test the hypothesis that FH mutations may predispose to prostate cancer. An almost complete data set (99.4% success rate in mutation analyses by DHPLC and/or sequencing) from 89 families was obtained, but no pathogenic variants of FH were detected. The negative result is unlikely to be because of technical reasons. The sensitivity of DHPLC has been shown to vary between 93 and 100%, consistently exceeding 96% in many data sets.[40] Considering that a large number of families representing two different populations were scrutinised, in addition to the fact that some families showed evidence for *PCAP* linkage, and no mutations were found, other 1q42.2-43 candidate genes should be considered.

A small number of known genes map to the *PCAP* locus, some of them being candidate genes of interest. The region restricted by the markers giving the highest lod scores, D1S2785 and D1S2842, and the flanking sequence include only seven genes (fig 1). Exonuclease 1 (*EXO1*) interacts with *MSH2*,[41] the mismatch repair gene mutated in hereditary nonpolyposis colorectal cancer (HNPCC).[42] Another promising candidate gene at the *PCAP* locus is prostate cancer antigen 1 (*PCTA-1*, *LGALS8*, galectin 8). Putative functional association of galectins have been reported in cell adhesion and apoptosis.[43 44] Some interesting predicted genes (transcripts with multiple ESTs and conserved domains) could be functionally linked to tumorigenesis. Ensemble transcript ENST00000255379, a putative member of the DAN domain family, is very similar to Gremlin protein which is an antagonist of the bone morphogenetic protein (BMPR) signalling pathway.[45] BMPR receptor 1A (*BMPR1A/ALK3*) mutations segregate in juvenile polyposis (JPS) families.[46] Additionally, *DAN*

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gene product has been shown to have tumour suppressive activity.[47] NCBI transcript LOC128186 (similar to aconitase 2, *ACO-2*) includes two conserved aconitase domains, the aconitase C-terminal domain and the aconitate hydratase domain. Mitochondrial aconitase (m-aconitase, *ACO-2*) catalyses the first step leading to the oxidation of citrate via the Krebs cycle. In malignant prostate cells, the citrate production, a characteristic phenomenon of normal prostate epithelial cells, is shifted towards citrate oxidation which is activated by m-aconitase overexpression regulated by testosterone and prolactin.[48] Reaction between m-aconitase and superoxide generates free hydroxyl radicals that may enhance mitochondrial oxidative damage.[49] Citrate serves as an oxidisable intermediate in the Krebs cycle and as a precursor for lipogenesis, which is accelerated in malignant cells possibly owing to increased ATP production.[37]

Efforts to identify predisposing genes for complex diseases like HPRCA have been difficult. We have analysed 89 high risk HPRCA families, of which 19 showed suggestive evidence of linkage to 1q42.2-43, for *FH* mutations. The absence of coding region or splice site aberrations in this data set strongly suggests that *FH* is not a predisposing gene for hereditary prostate cancer. Future work will focus on the other positional candidates.

Authors' affiliations

R Lehtonen, M Kiuru, N N Nupponen, A Karhu, L A Aaltonen , Departments of Medical Genetics, Biomedicum Helsinki or Haartman Institute, FIN-00014 University of Helsinki, Finland
A Rökman, T Ikonen, M Matikainen, O-P Kallioniemi, J Schleutker, Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, 33521 Tampere, Finland
J M Cunningham, D J Schaid, S N Thibodeau, Departments of Laboratory Medicine and Pathology, Health Sciences Research, Mayo Clinic/Foundation, Rochester, MN, and Prostate Cancer Investigation Group, National Human Genome Research Institute, National Institutes of Health, Bethesda, MA, USA

Correspondence to: Dr L A Aaltonen, Department of Medical Genetics, Biomedicum Helsinki, PO Box 63, FIN-00014 University of Helsinki, Finland; lauri.aaltonen@helsinki.fi

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