ANNIKA RÖKMAN

In Search of High-Penetrant Hereditary Prostate Cancer Susceptibility Genes in Finland

■

ACADEMIC DISSERTATION

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the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of Finn-Medi 1,
Biokatu 6, Tampere, on November 26th, 2004, at 12 o'clock.

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Perinnölliselle eturauhassyövälle voimakkaasti altistavien suomalaisten geenien tunnistus

Eturauhassyöpä on miesten yleisin syöpä Suomessa ja muissa teollisuusmaissa. Suurin osa eturauhassyövistä on satunnaisia eli sporadisia, mutta 5 – 10 %:ssa syövän taustalla on voimakas perinnöllinen alttius sairastua eturauhassyöpään. Äskettäin tehdyn kaksostutkimuksen mukaan jopa 40 % koko eturauhassyöpäriskistä voisi selittyä perinnöllisillä tekijöillä (Lichtenstein et al. 2000). Tässä tutkimuksessa selvitettiin eturauhassyövälle voimakkaasti altistavia perinnöllisiä tekijöitä suomalaisessa väestössä.

Useimmat perinnöllisistä syöpäoireyhtymistä ovat seurausta kasvurajoitegeenien geneettisistä muutoksista. Knudsonin teorian (1971) mukaan syövän synty vaatii kaksi muutosta, joista jälkimmäinen on usein kromosomin tai kromosominosan häviämä. Häviämiä voidaan siten pitää mahdollisina kasvurajoitegeenien sijaintipaikkoina. Etsiäksemme kasvurajoitegeenien sijaintipaikkoja ja määrittääksemme mitkä DNA kopioluvun muutokset ovat tyypillisiä eturauhassyöpäsukujen syöville, 21 näytettä 19 suvusta tutkittiin vertailevalla genomisella hybridisaatiolla. Yleisimmät havaitut DNA kopioluvun muutokset olivat häviämät alueilla 13q14-q22, 8p12-pter ja 6q13-q16 ja lisäykset alueilla 19p, 19q ja 7q. Ne ovat samoja, joita on havaittu satunnaisissa eturauhassyövissä. Sellaisia eturauhassyöpäsuvuille tyypillisiä geneettisiä muutoskohtia ei löytynyt, joista olisi voitu aloittaa vain perinnölliselle eturauhassyövälle altistavien geenien etsiminen. Tutkimus antoi kuitenkin viitteitä siitä, että syövän etenemiseen liittyvät mekanismit ovat periytyvissä ja satunnaisissa tapauksissa samanlaisia.

Eturauhassyöpäsukujen kytkentäanalyyseillä on löydetty useita eturauhassyöpäalttiuteen liittyviä kromosomialueita, mutta toistaiseksi niiltä on saatu tunnistettua vain kolme ehdokasgeeniä, *ELAC2* (*elaC homolog 2*), *RNASEL* (*ribonuclease L*) ja *MSR1* (*macrophage scavenger 1*). *ELAC2*:n ja *RNASEL*:n roolia eturauhassyövän synnyssä Suomessa tutkittiin seulomalla kyseisten geenien ituratamutaatiot 66 suomalaiselta eturauhassyöpäsukuun kuuluvalta potilaalta. Mielenkiintoisimpien muutosten tutkimista jatkettiin selvittämällä niiden yleisyys väestötasolla valikoimattomista eturauhassyöpäpotilaista, eturauhasen hyvänlaatuista liikakasvua sairastavista henkilöstä sekä kontrollihenkilöistä.

Mutaatioseulonnassa *ELAC2*:ssa havaittiin uusi aminohappoa vaihtava muutos Glu622Val, jolla oli yhteys valikoimattomaan eturauhassyöpään (OR= 2.94; 95 % luottamusväli 1.05-8.23). Tulokset osoittavat, että eräillä *ELAC2* -geenin muutoksilla saattaa olla merkitystä eturauhassyöpään väestötasolla. *RNASEL* geenin mutaatioseulonnassa löytyi proteiinituotetta lyhentävä mutaatio, Glu265X, jonka havaittiin olevan yleisempi eturauhassyöpäsuvuissa, joissa oli neljä tai useampia eturauhassyöpäpotilaita (9.5 %), kuin kontrolleissa (1.8 %; p=0.03). Viitteitä geenimuutosten periytymisestä taudin mukana ei kuitenkaan havaittu kuin yhdessä suvussa, mikä tukee sitä, että kytkentäanalyyseissä suomalaiset perheet eivät ole osoittaneet kytkentää *HPC1* -alueeseen 1q24-q25. Glu265X mutaatiota kantavien potilaiden keskimääräinen eturauhassyövän toteamisikä oli kuitenkin noin 11 vuotta aikaisempi kuin ei-kantajilla (p=0.07). *RNASEL* ei siten näytä selittävän eturauhassyöpäsukujen syntymistä, mutta sen tietyt muutokset voivat alentaa eturauhassyöpään sairastumisikää.

Aikaisemmin tehdyssä koko genomin laajuisessa kytkentäanalyysissä (Schleutker ym. 2003) todettiin viitteitä mahdollisista eturauhassyövän alttiusgeeneistä kahdella kromosomialueella, 3p25-p26 ja 11q14. Näiden alueiden hienokartoitus suoritettiin uudessa kytkentäanalyysissä lisäämällä sekä sukujen että geenimerkkien määrää. Genotyypitys suoritettiin 17 geenimerkillä kromosomissa 3p ja 22 geenimerkillä kromosomissa 11q. Näytteitä oli yhteensä 229 henkilöstä (46 sairaasta) 16 edustavimmasta eturauhassyöpäperheestä. Tulokset vahvistivat 3p alueen kytkentää ja paras kytkentäalue saatiin rajattua noin viidesosaan alkuperäisestä. Rajatulta alueelta valittiin 10 ehdokasgeeniä ensimmäiseen mutaatioseulontaan (8 geenin osalta julkaisematonta tietoa). Proteiinituotetta lyhentäviä mutaatioita ei löytynyt, mutta havaittiin kuusi aminohappoa vaihtavaa muutosta viidestä eri geenistä. Näiden kuuden ituratamuutoksen osuudet selvitettiin väestötasolla 200 valikoimattomasta eturauhassyöpäpotilaasta sekä 200 kontrollihenkilöstä. Yhteyttä eturauhassyöpään ei kuitenkaan havaittu yhdenkään aminohappoa vaihtavan muutoksen kohdalla tilastollisissa testeissä. Hienokartoitus vahvisti kuitenkin selkeästi 3p26 alueen yhteyden suomalaisen väestön eturauhassyöpäalttiuteen ja osoitti alueen jatkotutkimukset tarpeellisiksi.

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II Rökman A, Ikonen T, Mononen N, Autio V, Matikainen MP, Koivisto PA, Tammela TLJ, Kallioniemi O-P, Schleutker J (2001) *ELAC2/HPC2* Involvement in Hereditary and Sporadic Prostate Cancer. Cancer Res 61:6038-6041.

III Rökman A, Ikonen T, Seppälä EH, Nupponen N, Autio V, Mononen N, Bailey-Wilson J, Trent J, Carpten J, Matikainen MP, Koivisto PA, Tammela TLJ, Kallioniemi O-P, Schleutker J (2002) Germline Alterations of the *RNASEL* Gene, a Candidate *HPC1* Gene at 1q25, in Patients and Families with Prostate Cancer. Am J Hum Genet 70:1299-1304.

IV Rökman A, Baffoe-Bonnie AB, Gillanders E, Fredriksson H, Autio V, Ikonen T, Gibbs Jr KD, Jones MP, Gildea D, Freas-Lutz D, Markey C, Matikainen MP, Koivisto PA, Tammela TLJ, Kallioniemi OP, Trent J, Bailey-Wilson JE, Schleutker J (2004) Hereditary Prostate Cancer in Finland: Fine-mapping Validates 3p26 as a Major Predisposition Locus. Hum Genet, in press.

ABBREVIATIONS

2-5A	2´5´- linked oligoadenylates
AR	*Androgen receptor*
BHLHB2	*Basic helix-loop-helix domain containing, class B, 2*
bp	Base pair
BPH	Benign prostate hyperplasia
BRCA1	*Breast cancer gene 1*
BRCA2	*Breast cancer gene 2*
CAPB	*Prostate and brain cancer gene locus at 1p36*
CDC25A	*Cell division cycle 25A*
CDH1	*E-cadherin*
CDK4	*Cyclin-dependent kinase 4*
CGH	Comparative genomic hybridization
CHEK2	*CHK2 checkpoint S. pombe homolog*
CHL1	*Cell adhesion molecule with homology to L1CAM*
CI	Confidence interval
cM	CentiMorgan(~10^6 bp)
CNTN4	*Contactin 4*
CNTN6	*Contactin 6*
CSGE	Conformation-sensitive gel electrophoresis
CYP	*Cytochrome family*
DAPI	4´,6-diamidino-2-phenylindole
FHIT	*Fragile histidine triad gene*
FITC	Fluorescein isothiocyanate
*Fnu*4H I	Restriction enzyme from bacterium *F. nucleatum*
GST	*Glutathione S-transferase family*
HepG2	A hepatoma cell line
HLOD	Heterogeneity LOD
HPC1/RNASEL	*Hereditary prostate cancer gene 1, ribonuclease L*
HPC2/ELAC2	*Hereditary prostate cancer gene 2, elaC E. coli homolog 2*
HPC20	*Hereditary prostate cancer gene locus at 20q13*
HPCX	*Hereditary prostate cancer gene locus at Xq27-q28*
HSD3B	*Hydroxy-delta-5-steroid dehydrogenase, 3 beta*
IL5RA	*Interleukin 5 receptor, alpha*
LNCap	A prostate cancer cell line
LOD	Logarithm of odds
LOH	Loss of heterozygosity
MCF-7	A breast cancer cell line
MET	*Hepatocyte growth factor receptor*
MIM	Mendelian Inheritance in Man
MSR1	*Macrophage scavenger 1*
NAT	*N-acetyltransferase*
OR	Odds ratio
OXTR	*Oxytocin receptor*
p	Short arm of a chromosome
PCAP	*Hereditary prostate cancer gene locus at 1q42.2-q43*

PCR	Polymerase chain reaction
PCTA-1	*Prostate carcinoma tumor antigen 1*
PSA	Prostate specific antigen
q	Long arm of a chromosome
RB1	*Retinoblastoma gene 1*
RET	*Ret proto-oncogene*
RFLP	Restriction fragment length polymorphism (analysis)
SNP	Short nucleotide polymorphism
SRD5A2	*Steroid-5-alpha-reductase, alpha polypeptide 2*
SSCP	Single strand conformational polymorphism (analysis)
Taq$^{\alpha}$I	Restriction enzyme from bacterium *T. aquaticus*
TRNT1	*tRNA nucleotidyl transferase, CCA-adding, 1*
TURP	Transurethral resection of prostate
UTR	Untranslated region
VHL	*Von Hippel-Lindau syndrome gene*
XPD	*Xeroderma pigmentosum group D*
XRCC1	*X-ray repair complementing defective repair in Chinese hamster cells 1*

ABSTRACT

Prostate cancer is the most common malignancy among men in Finland and other industrialized countries. Most cases are sporadic but an estimated five to ten percent are due to strong hereditary predisposition. A recent twin study indicated that over 40 % of the overall prostate cancer risk may be explained by heritable factors (Lichtenstein et al. 2000). The purpose of this study was to search for susceptibility loci and genes behind prostate cancer predisposition in Finland.

Most known hereditary cancer syndromes are due to inactivating germline mutations in tumor suppressor genes. According to Knudson´s "two-hit" hypothesis (1971), inactivation of a tumor suppressor gene requires two changes, where the second hit is often a chromosomal deletion. Therefore, sites of deletion in tumor tissue of hereditary prostate cancer patients may be considered locations of possible predisposing tumor suppressor genes. Here, comparative genomic hybridization technique was used to analyze relative copy number changes in somatic tumor tissues from 21 patients from 19 prostate cancer families. The most common changes found were losses of 13q14-q22, 8p12-pter and 6q13-q16 and gains of 19p, 19q and 7q. Similar changes have previously been reported in sporadic prostate cancers, suggesting that the genetic progression of sporadic and hereditary prostate cancers may involve similar genetic steps. No copy number changes specific to familial prostate cancer were observed.

To date several susceptibility loci have been located by linkage analysis but only three genes have been implicated; *ELAC2* (*elaC homolog 2*), *RNASEL* (*ribonuclease L*) and *MSR1* (*macrophage scavenger 1*). To ascertain the roles of two prostate cancer susceptibility genes, *ELAC2* and *RNASEL*, in the causation of hereditary prostate cancer in Finland, their coding exons were screened for germline mutations in 66 hereditary prostate cancer patients. The mutations and polymorphisms found were then studied at the population level using unselected prostate cancers, benign prostate hyperplasias and healthy male blood donors as controls. A rare new *ELAC2* variant Glu622Val was associated with unselected prostate cancer (odds ratio 2.94; 95% confidence interval 1.05-8.23). The results suggest that truncating mutations in *ELAC2* are rare but some *ELAC2* variants may have a

role in prostate cancer predisposition. In the case of *RNASEL*, a truncating mutation, Glu265X, was found to be more common in hereditary prostate cancer patients from families with four or more affected members (9.5 %) than in controls (1.8 %, p= 0.03). However, only one family showed suggestive co-segregation of Glu265X with the disease. Finnish families have shown no significant linkage to *HPC1* region at 1q24-q25, making it understandable that disease-causing *RNASEL* mutations may not have a prominent role in prostate cancer in Finland. Interestingly, the median age at diagnosis of the mutation carriers was 11 years lower than in the non-carriers of the same families (p= 0.07). While, *RNASEL* does not explain prostate cancer segregation in Finnish families it could have a modifying effect on disease onset, especially in large prostate cancer families.

To determine the predisposition loci of Finnish hereditary prostate cancer a genome-wide linkage analysis was recently completed (Schleutker et al. 2003). This analysis identified two chromosomal regions, 3p25-p26 and 11q14, significant in prostate cancer causation in Finland. Here, fine-mapping of these regions was performed with additional markers and families. The results strengthened linkage to 3p and narrowed the region from 10 cM (centiMorgan) to approximately two cM. From the narrowed region 10 candidate genes were selected for initial mutation screening (data from 8 genes unpublished). No truncating mutations were found but six missense variants were identified. Frequencies of the missense variants were then determined at the population level from 200 unselected prostate cancer patients and 200 controls, but no association of any of the changes with prostate cancer was observed. The results of fine-mapping support the important role of 3p26 in prostate cancer predisposition in Finland, but further investigation is needed in order to clone the Finnish prostate cancer susceptibility gene.

INTRODUCTION

Most cancer types, both adult and pediatric, have a familial form. In hereditary cancers, the first predisposing change is present in every cell of an affected individual. Additional somatic genetic aberrations are needed for cancer development, but the inherited mutation speeds up the process. The possibility of hereditary cancer should be considered if cancer appears at an exceptionally young age, affects several close relatives or if multiple primary tumors are observed (Fearon 1997; Frank 2001). Several hereditary cancer syndromes with a mendelian inheritance pattern have been identified (Marsh and Zori 2002) (Table 1). Most of them are very rare, collectively affecting about one percent of all cancer patients (Fearon 1997).

Table 1. Hereditary cancer syndromes (modified from the review by Marsh and Zori 2002).

Mode of inheritance	**Syndrome**	**Chromosomal location(s)**	**Gene name**	**References**
Dominant	Carney complex (type 1)	17q23-q24	*PRKAR1A*	Kirschner et al. 2000
	Beckwith-Wiedemann syndrome	11p15.5	*CDKN1C*	Lam et al. 1999
	Cowden syndrome	10q23.31	*PTEN*	Liaw et al. 1997; Steck et al. 1997
	Familial adenomatous polyposis	5q21	*APC*	Bodmer et al. 1987; Groden et al. 1991
	Familial melanoma	9p21	*CDKN2A*	Cannon-Albright et al. 1992; Kamb et al. 1994
		12q14	*CDK4*	Zuo et al. 1996
	Hereditary breast and ovarian cancer	17q21	*BRCA1*	Miki et al. 1994
	Hereditary breast cancer	13q12.3	*BRCA2*	Wooster et al. 1995; Tavtigian et al. 1996
	Hereditary nonpolyposis colorectal cancer	3p21	*MLH1*	Bronner et al. 1994; Papadopoulos et al. 1994
		2p16	*MSH2*	Leach et al. 1993 Fishel et al. 1993
			MSH6	Miyaki et al. 1997; Akiyama et al. 1997
		2q31-q33	*PMS1*	Nicolaides et al. 1994
		7p22	*PMS2*	Nicolaides et al. 1994
	Hereditary papillary renal cell carcinoma	7q31	*MET*	Schmidt et al. 1997 Fischer et al. 1998
	Hereditary paraganglioma and phaeochromosocytoma	1p35-p36.1	*SDHB*	Astuti et al. 2001
		1q21	*SDHC*	Niemann and Muller 2000

		11q23	*SDHD*	Baysal et al. 2000
	Hereditary prostate cancer	1q25	*RNASEL*	Carpten et al. 2002
		17p11	*ELAC2*	Tavtigian et al. 2001
		8p22	*MSR1*	Xu et al. 2002
	Juvenile polyposis	18q21.1	*SMAD4*	Friedl et al. 1999
		10q22.3	*BMPR1A*	Howe et al. 2001; Zhou et al. 2001
	Li-Fraumeni syndrome	17p13	*TP53*	Malkin et al. 1990; Srivastava et al. 1990
	Multiple endocrine neoplasia type 1	11q13	*MEN1*	Lemmens et al. 1997, Chandrasekharappa et al. 1997
	Multiple endocrine neoplasia type 2A and 2B	10q11.2	*RET*	Mulligan et al. 1993; Hofstra et al. 1994
	Multiple exostoses	8q24.11-q24.13	*EXT1*	Ahn et al. 1995
		11p11-p12	*EXT2*	Stickens et al. 1996
		19p	*EXT3*	Le Merrer et al. 1994
	Neurofibromatosis type 1	17q11.2	*NF1*	Wallace et al. 1990; Viskochil et al. 1990
	Neurofibromatosis type 2	22q12	*NF2*	Trofatter et al. 1993; Rouleau et al. 1993
	Nevoid basal cell carcinoma	9q22.3	*PTCH*	Hahn et al. 1996; Johnson et al. 1996
	Peutz-Jegher´s syndrome	19p13.3	*STK11*	Hemminki et al. 1997, 1998
	Familial retinoblastoma	13q14	*RB1*	Sparkes et al. 1980; Friend et al. 1986; Dunn et al. 1988
	Tuberous sclerosis	9q34	*TSC1*	van Slegtenhorst et al. 1997
		16p13	*TSC2*	The European Chr 16 Tuberous Sclerosis Consortium 1993
	Von Hippel-Lindau syndrome	3p25	*VHL*	Hosoe et al. 1990; Latif et al. 1993
	Wilms´ tumor	11p13	*WT1*	Riccardi et al. 1978; Call et al. 1990
Recessive	Ataxia-telangiectasia	11q22.3	*ATM*	Savitsky et al. 1995
	Bloom´s syndrome	15q26.1	*RECQL3*	Ellis et al. 1995
	Fanconi´s anemia	16q24.3	*FANCA*	Lo Ten Foe et al. 1996
		9q22	*FANCC*	Youssoufian et al. 1994
		3p25.3	*FANCD2*	Timmers et al. 2001
		6p22	*FANCE*	de Winter et al. 2000
		11p15	*FANCF*	de Winter et al. 2000
		9p13	*FANCG*	Yamada et al. 2000
		13q12.3	*BRCA2*	Howlett et al. 2002
	Rothmund-Thomson syndrome	8q24.3	*RECQL4*	Kitao et al. 1999
	Werner syndrome	8p12	*RECQL2*	Yu et al. 1996
	Xeroderma pigmentosum	9q22.3	*XPA*	reviewed by Boulikas 1996
		2q21	*XPB*	
		3p25	*XPC*	
		19q13	*XPD*	
		11p12	*XPE*	
		16p13	*XPF*	
		13q33	*XPG*	

In inherited cancer syndromes the risk of disease varies depending on the mutation, other genes, dietary, lifestyle and other environmental factors. However, in most of them the likelihood of developing cancer is so high that it leads to a dominant pattern of inheritance. Yet at the cellular level, inactivating mutations in recessive tumor suppressor genes rather than activating mutations in dominant oncogenes predominate (Fearon 1997). Only three oncogenes, *RET* (*ret proto-oncogene*) at 10q11.2 in multiple endocrine neoplasia type 2A and 2B (Mulligan et al. 1993; Hofstra et al. 1994), *MET* (*hepatocyte growth factor receptor*) at 7q31 in hereditary papillary renal cell carcinoma (Schmidt et al. 1997) and *CDK4* (*cyclin-dependent kinase 4*) at 12q14 in familial melanoma (Zuo et al. 1996), have been found to be implicated in hereditary cancer syndromes. The proteins encoded by inherited cancer genes function in a diverse array of cellular processes including proliferation, differentiation, apoptosis, and the maintenance of genomic integrity.

Most prostate cancers are sporadic, but in five to ten percent of all prostate cancers familial aggregation of the disease is seen, suggesting the existence of a hereditary component. A twin study of 44,788 pairs of twins from Sweden, Denmark and Finland indicated that over 40 % of the overall prostate cancer risk may be explained by heritable factors (Lichtenstein et al. 2000), which was higher than that observed in colorectal (35 %) and breast cancers (27 %). The purpose of this study was to identify high-penetrant susceptibility loci for hereditary prostate cancer in Finland.

REVIEW OF THE LITERATURE

1. Knudson´s theory and tumor suppressor genes

Knudson´s "two-hit" hypothesis (Knudson 1971; 1986) was based on childhood retinoblastoma, which is a rare eye cancer affecting one out of 20 000 children. About 40 % of all retinoblastoma cases are caused by hereditary mutations in *RB1* (*retinoblastoma 1*) located on chromosome 13q. Knudson´s model suggests that two events are needed to inactivate both alleles of a particular tumor suppressor gene (Figure 1).

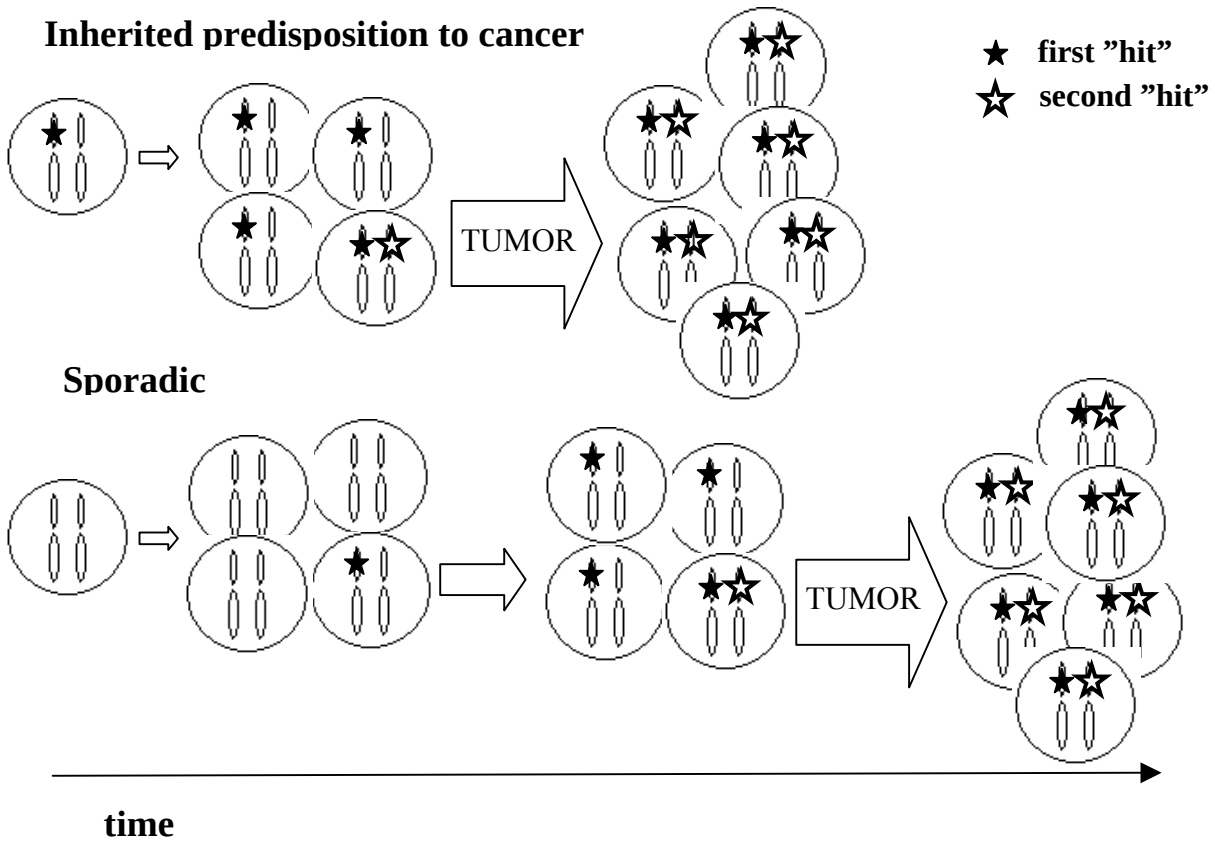

Figure 1. Schematic presentation of Knudson´s "two-hit" hypothesis (1971): In sporadic cases, both events occur somatically during the lifetime of a person. However, in hereditary cases, the first mutation is already present in all cells of the body and, therefore, only one extra mutation is required for cancer development.

The first inactivating "hit" may be sporadic or inherited point mutation, but the second is often a larger chromosomal deletion. Therefore, sites of deletion in the tumor tissue of hereditary prostate cancer patients can be considered locations of possible predisposing tumor suppressor genes. After the initiating event, a set of additional somatic genetic changes, estimated to involve a minimum of four to eight genes for typical solid tumors (Renan 1993; Hahn et al. 1999), takes place before tumor can progress.

Tumor suppressor genes can be divided, based on their roles, into gatekeepers and caretakers (Kinzler and Vogelstein 1997; 1998). Gatekeepers regulate cell proliferation either by controlling the cell division or by promoting programmed cell death. Over 30 gatekeeper genes have been identified so far, and inactivating mutations in them are responsible for most of the known hereditary cancer syndromes (Frank 2001). Caretakers consist of genes that are not directly responsible for the regulation of growth and differentiation in the cell. Instead, they affect other cancer genes by influencing DNA repair and genomic integrity. More than 130 genes have been identified with roles in DNA damage surveillance and repair (Wood et al. 2001). Recent evidence of haploinsufficiency in a growing number of tumor suppressor genes suggests that the inactivation of only one allele may be sufficient for tumor initiation (Kwabi-Addo et al. 2001; Buchholz et al. 2002; Chen et al. 2003a; Magee et al. 2003). This group of tumor suppressor genes may eventually form a group of its own apart from the classic tumor suppressor genes (Balmain 2002).

2. Epidemiology of prostate cancer

2.1 Incidence

Prostate cancer constitutes a major health problem for many countries. In Finland, as well as in other industrialized countries, it is the most common male malignancy, and the second leading cause of cancer deaths after lung cancer (Figure 2, Finnish Cancer Registry 2004 at http://www.cancerregistry.fi).

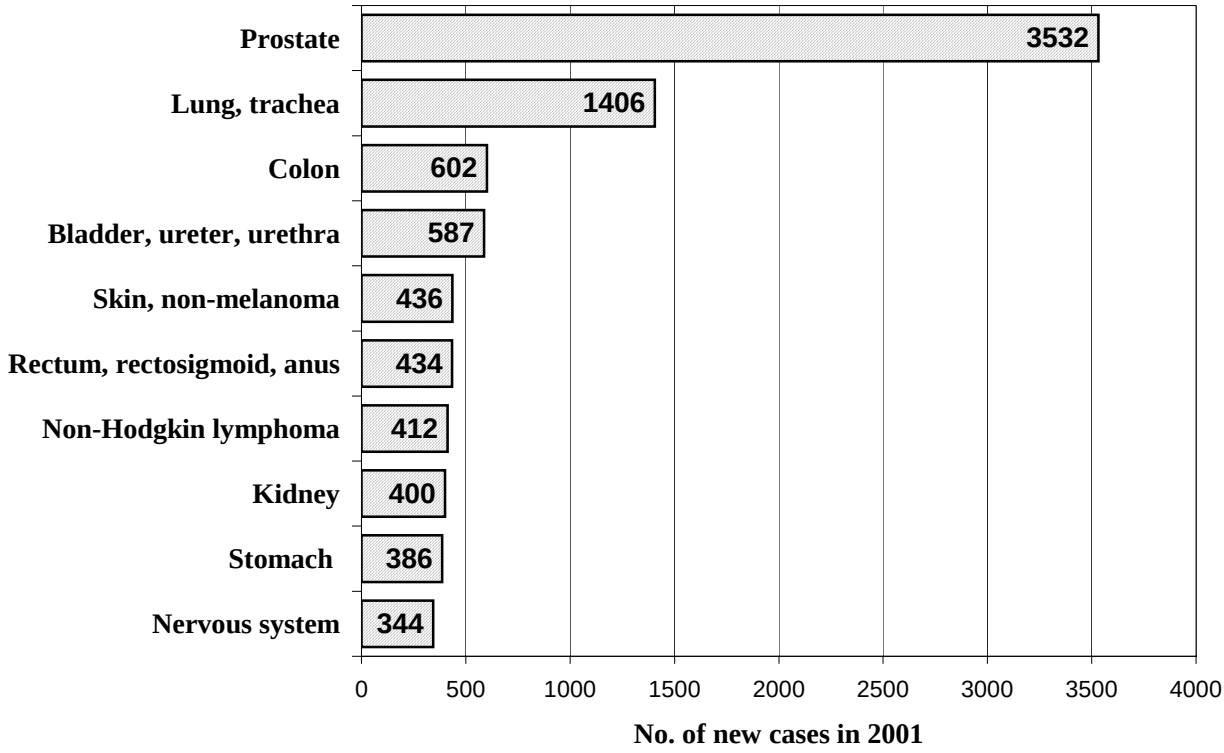

Figure 2. Ten leading primary sites of male cancers in Finland (2001) (Finnish Cancer Registry, 2004 at http://www.cancerregistry.fi).

In 2001, the incidence of prostate cancer in Finland was 82.5/100 000 men, with over 3500 new cases annually (Finnish Cancer Registry 2004). A rapid increase in the incidence has been observed since 1985 (Figure 3). In many countries, the increase in incidence may to a large extent be due to the use of prostate specific antigen (PSA) in the screening of prostate cancers. It has been estimated that up to 75 % of new prostate cancer cases detected by PSA screening would not have been clinically detected (Etzioni et al. 1998). One of the main questions in prostate cancer research is, how to separate the incidental prostate cancers from the clinically relevant ones.

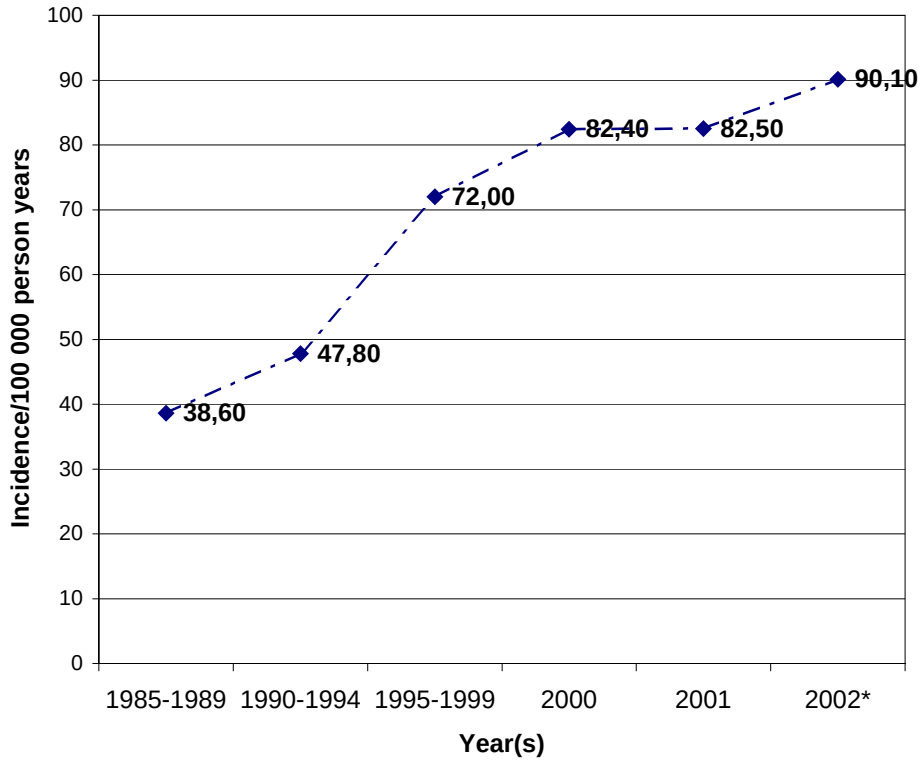

Figure 3. Age-adjusted incidence rates of prostate cancer in Finland 1985–2002. *Rate for 2002 is an estimate (Finnish Cancer Registry 2004 at http://www.cancerregistry.fi).

2.2 Risk factors

Prostate cancer is a very heterogeneous disease, where definitive risk factors include age, ethnic origin and family history (Pentyala et al. 2000; Crawford 2003). In addition, many other endogenous and exogenous risk factors have been suggested, such as hormones, diet and physical activity.

2.2.1 Age

Prostate cancer is a disease of older men with about 80 % of cancers occurring in men over the age of 65 (Carter and Coffey 1990). Of all cancers, the prevalence of prostate cancer increases most rapidly with age (Carter and Coffey 1990). In 2002, the mean age at diagnosis of prostate cancer in Finland was 72 years (Finnish Cancer Registry 2003), while in the US the mean age at diagnosis is little lower, 70 years (National Cancer Institute 2004 at http://www.cancer.gov). The initiation of prostate cancer i.e. the formation of a histologically detectable lesion is a very frequent event, present in nearly 50 % of cases in

an autopsy series of men of 70 – 80 years who had died of other causes than cancer (Breslow et al. 1977; Sheldon et al. 1980; Sakr et al. 1993). The majority, perhaps 75 %, of such lesions does not progress to clinically detectable tumors (Etzioni et al. 1998).

2.2.2 Ethnic origin

There is a striking difference in prostate cancer risk between different ethnic groups, with African-American men having reported incidence rates that are 40 to 60 fold higher than those reported for Asian men (Hsing et al. 2000). In Scandinavia, too, the incidence has been quite high (Zaridze et al. 1984). Interestingly, incidental or latent prostate cancer cases are found equally commonly in all these populations (Yatani et al. 1982; Sakr et al. 1993). It is therefore the rate at which prostate cancer becomes clinically manifest that varies markedly in different areas (Akazaki and Stemmerman 1973; Carter and Coffey 1990). The risk for prostate cancer among Asians increases when they immigrate to North America, implicating the environment and lifestyle-related factors such as diet in prostate cancer causation (Shimizu et al. 1991; Whittemore et al. 1995). While most international differences in cancer rates seem to be due to environmental or lifestyle rather than genetic effects, some, such as the higher prostate cancer risk among African-Americans than among Caucasian Americans, could to some extent be due to racial differences in the allelic spectrum of a particular gene (Shibata and Whittemore 1997).

2.2.3 Family history

Most prostate cancers are sporadic but in a subset of cases, about ten percent, there is a positive family history of the disease. In early onset cases the proportion of familial clusters is much higher, up to 40 – 50 % (Carter et al. 1992). Familial clustering may be due to a variety of mechanisms, such as familial exposure to environmental or dietary risk factors, contribution of several predisposing genes or the contribution of a single gene with reduced penetrance. Hereditary prostate cancer is a more specific term referring to a subset of these familial cases, approximately five percent of all prostate cancers in which a pattern of mendelian inheritance consistent with the presence of a rare susceptibility gene is seen (Carter et al. 1993; Keetch et al. 1995; Bratt et al. 1999).

In case-control studies the relative risks of a first-degree relative developing prostate cancer range from 1.7 – 3.7 (Stanford and Ostrander 2001). The closer relative a man is to an affected person and the greater the number of persons affected in a family, the greater is the

risk for prostate cancer. For example, men with three or more first-degree relatives with prostate cancer have an almost 11-fold elevated risk for prostate cancer in comparison with men with no family history (Steinberg et al. 1990). Early age at diagnosis of prostate cancer also increases the risk of prostate cancer in relatives (Carter et al. 1993; Keetch et al. 1995).

2.3 Epidemiology of prostate cancer in Finland

A Finnish population-based registry study indicated over two-fold risk for prostate cancer for the relatives of prostate cancer patients with early age at onset (<70 years) (Matikainen et al. 2001). No significantly increased risk for prostate cancer was seen in the relatives with median age at onset (70-79 years) patients. However, among relatives with the old age at onset (>80 years) again almost two-fold risk was seen, suggesting a contribution of inherited factors also in the late onset disease. The only other cancer for which the risk was significantly elevated in relatives of Finnish prostate cancer patients was gastric cancer (Matikainen et al. 2001). A potential candidate gene for the connection between prostate and gastric cancer was *CDH1* (*e-cadherin*), which has been observed to be mutated in familial gastric cancer (Guilford et al. 1998; 1999). However, additional analyses suggested that mutations in *CDH1* do not explain the association between prostate and gastric cancer in Finnish population, although its variants such as Ser270Ala may contribute to prostate cancer onset (Ikonen et al. 2001). Elevated risk for gastric cancer in the relatives of prostate cancer patients has also been detected in Sweden (Grönberg et al. 2000) but not elsewhere, suggesting possible differences between Nordic and other populations in the etiology of prostate cancer.

3. Hereditary prostate cancer

3.1 Mode of inheritance of hereditary prostate cancer

Familial aggregation of prostate cancer was first reported by Morganti et al. in 1956 and by Woolf et al. in 1960, but the concept of hereditary prostate cancer was not established until the first segregation analysis was published in 1992 (Carter et al. 1992). The analysis of 691 men with prostate cancer from prostate cancer families indicated a rare high-risk allele with a penetrance of 88 % by the age of 85 years, causing 9 % of all prostate cancers and 43 % of early onset prostate cancers (diagnosed before 55 years of age). To date several analyses of the mode of inheritance of prostate cancer have been performed; most of them support the autosomal dominant mode of inheritance (Grönberg et al. 1997a; Schaid et al. 1998; Verhage et al. 2001; Gong et al. 2002; Valeri et al. 2003), although autosomal recessive as well as X-linked mode have also been proposed (Monroe et al. 1995; Narod et al. 1995). X-linked mode is supported by the fact that men with an affected brother are more likely to develop the disease than are men with an affected father (Monroe et al. 1995). It has also been suggested that dominantly inherited genes increase the risk, especially at younger ages, whereas a recessive or X-linked risk may especially affect older men (Cui et al. 2001). Also synergistic action of two or more genes has also been proposed (Page et al. 1997; Conlon et al. 2003). In a recent study, a multifactorial model, where the risk of prostate cancer in families is determined by both environmental and genetic factors, explained the data better than did the pure mendelian models (Gong et al. 2002). It should be noted that segregation models cannot deal optimally with complex diseases with multiple etiological factors and multiple genetic loci, and such estimates are mainly needed to set up parameters for linkage analyses.

3.2 General features of hereditary prostate cancer

In 1993, Carter et al. suggested criteria for the classification of hereditary prostate cancer cases. These are still the most commonly used in the field, especially in selecting families for linkage analysis. According to Carter et al. (1993) hereditary cases should meet one of the following criteria: 1) three or more affected relatives in the nuclear family, 2)

occurrence of prostate cancer in three successive generations in either maternal or paternal lineage, or 3) two affected relatives with an early age at diagnosis (55 years or less). The definition is somewhat biased towards autosomal dominant transmission, and is likely to miss some families with autosomal recessive or X-linked transmission. Familial cases are those that do not fullfill the criteria but where a familial aggregation is detected. The etiology of hereditary prostate cancer is thought to result from a single (or few) genes passed along in families and conferring a greatly increased risk for the development of prostate cancer. As no strongly associated susceptibility genes have yet been identified, hereditary prostate cancer is presently defined only by the pedigree. An example of a Finnish hereditary prostate cancer family is presented in Figure 4.

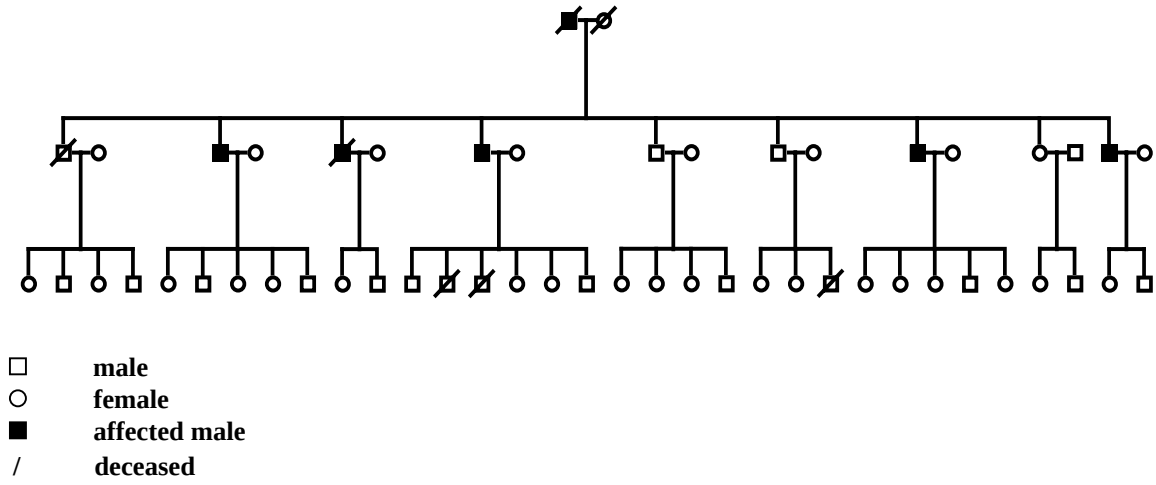

Figure 4. An example of a Finnish hereditary prostate cancer family.

3.3 High-risk predisposing loci and genes identified by positional cloning

According to the majority of segregation analyses, familial clustering of prostate cancer is most likely due to rare high-penetrant genes that can be found by positional cloning methods. Many genes that are implicated in the causation of other cancers have been identified by linkage analysis (Futreal et al. 2004). Several linkage analyses (Table 2) of hereditary prostate cancer families have been performed, implicating high-risk loci in many different chromosomes.

Table 2. Linkage analyses of hereditary prostate cancer. Only logarithm of odds (LOD) scores of original findings are indicated and only those with LOD score equal to or greater than 1.5 have been included.

Chr	Location	Locus/gene	References	No. of families	2-point LOD
1	1p36	*CAPB*	Gibbs et al. 1999b	12	3.22
			Badzioch et al. 2000	207	
			Gibbs et al. 2000	94	
			Suarez et al. 2000a	230	
			Goode et al. 2001	149	
			Xu et al. 2001b	159	
			Matsui et al. 2004	44	
	1q21-q22		Gibbs et al. 2000	94	1.99
			Suarez et al. 2000a	230	
	1q24-q25	*HPC1/ RNASEL*	Smith et al. 1996	66	3.65
			Cooney et al. 1997	59	
			Grönberg et al. 1997c	13	
			Hsieh et al. 1997	92	
			Grönberg et al. 1999	40	
			Neuhausen et al. 1999	41	
			Berry et al. 2000a	144	
			Xu 2000	772	
			Goddard et al. 2001	254	
			Goode et al. 2001	149	
			Powell et al. 2001	43	
			Xu et al. 2001b	159	
			Janer et al. 2003	254	
			Xu et al. 2003a	188	
			Brown et al. 2004	33	
	1q42.2-q43	*PCAP*	Berthon et al. 1998	47	2.70
			Gibbs et al. 1999a	152	
			Suarez et al. 2000b	49	
			Cancel-Tassin et al. 2001a	64	
			Goddard et al. 2001	254	
			Goode et al. 2001	149	
			Paiss et al. 2001	189	
			Xu et al. 2001b	159	
			Brown et al. 2004	33	
2	2p11		Gibbs et al. 2000	94	1.58
	2q23		Xu et al. 2003a	188	2.03
	2q32-q36		Suarez et al. 2000a	230	2.22
			Witte et al. 2003	259	
3	3p25-p26		Schleutker et al. 2003	13	2.57
4	4q21		Xu et al. 2003a	188	2.80
	4q23		Suarez et al. 2000a	230	2.72
			Goddard et al. 2001	254	
	4q27		Smith et al. 1996	66	1.80
5	5p13		Hsieh et al. 2001	98	1.98
	5q11		Wiklund et al. 2003a	50	2.24
6	6p21		Gibbs et al. 2000	94	1.62
8	8p22-p23	*MSR1*	Xu et al. 2001c	254	1.84

			Goddard et al. 2001	159	
			Wiklund et al. 2003b	57	
			Xu et al. 2003a	188	
	8q21		Gibbs et al. 2000	94	2.17
			Matsui et al. 2004	44	
9	9p22-p23		Gibbs et al. 2000	94	1.60
10	10q25-q26		Gibbs et al. 2000	94	1.68
11	11p13-p15		Gibbs et al. 2000	94	3.02
	11q14		Schleutker et al. 2003	13	2.97
12	12p13-p14		Suarez et al. 2000a	230	1.85
			Hsieh et al. 2001	98	
	12p11-q13		Gibbs et al. 2000	94	1.76
14	14q24		Gibbs et al. 2000	94	1.74
15	15q12-q13		Suarez et al. 2000a	230	3.01
	15q25-q26		Gibbs et al. 2000	94	1.65
16	16p13-p14		Gibbs et al. 2000	94	1.58
			Suarez et al. 2000a	230	
	16q22-q23		Suarez et al. 2000a	230	3.15
			Goddard et al. 2001	254	
			Witte et al. 2003	259	
17	17p11	*HPC2 / ELAC2*	Tavtigian et al. 2001	33	4.50
	17q		Lange et al. 2003	175	2.36
19	19p13		Hsieh et al. 2001	98	2.87
			Wiklund et al. 2003a	50	
20	20q11-q13	*HPC20*	Berry et al. 2000b	162	2.69
			Bock et al. 2001	172	
			Zheng et al. 2001	159	
			Cunningham et al. 2003	160	
			Brown et al. 2004	33	
22	22q		Lange et al. 2003	175	2.35
X	Xq27-q28	*HPCX*	Xu et al. 1998	360	4.60
			Lange et al. 1999	153	
			Schleutker et al. 2000	57	
			Paiss et al. 2001	189	
			Peters et al. 2001	186	
			Bochum et al. 2002	104	
			Xu et al. 2003a	188	
			Brown et al. 2004	33	

A large number of proposed loci indicates that genetics of hereditary prostate cancer is more complex than other cancers. Of these, seven loci, *HPC1* (*Hereditary prostate cancer gene 1;* Smith et al. 1996), *PCAP* (*Hereditary prostate cancer gene locus at 1q42.2-q43;* Berthon et al. 1998), *HPCX* (*Hereditary prostate cancer gene locus at Xq27-q28;* Xu et al. 1998), *CAPB* (*Prostate and brain cancer gene locus*; Gibbs et al. 1999b), *HPC20* (*Hereditary prostate cancer gene locus at 20q13;* Berry et al. 2000b), *HPC2* (*Hereditary prostate cancer gene 2*; Tavtigian et al. 2001) and locus at chromosome 8p (Xu et al.

2001c), have been most widely investigated in different populations (Figure 5). To date, only three candidate susceptibility genes have been cloned within the loci implicated in the linkage analyses. These include *ELAC2* (*elaC E-coli homolog* 2; Tavtigian et al. 2001), *RNASEL* (*ribonuclease L 2',5'-oligoisoadenylate synthetase-dependent*; Carpten et al. 2002) and *MSR1* (*macrophage scavenger receptor*; Xu et al. 2002) genes (Figure 5). However, none of these genes shows consistent patterns of linkage or explains more than a small fraction of the cases suggested by linkage analysis.

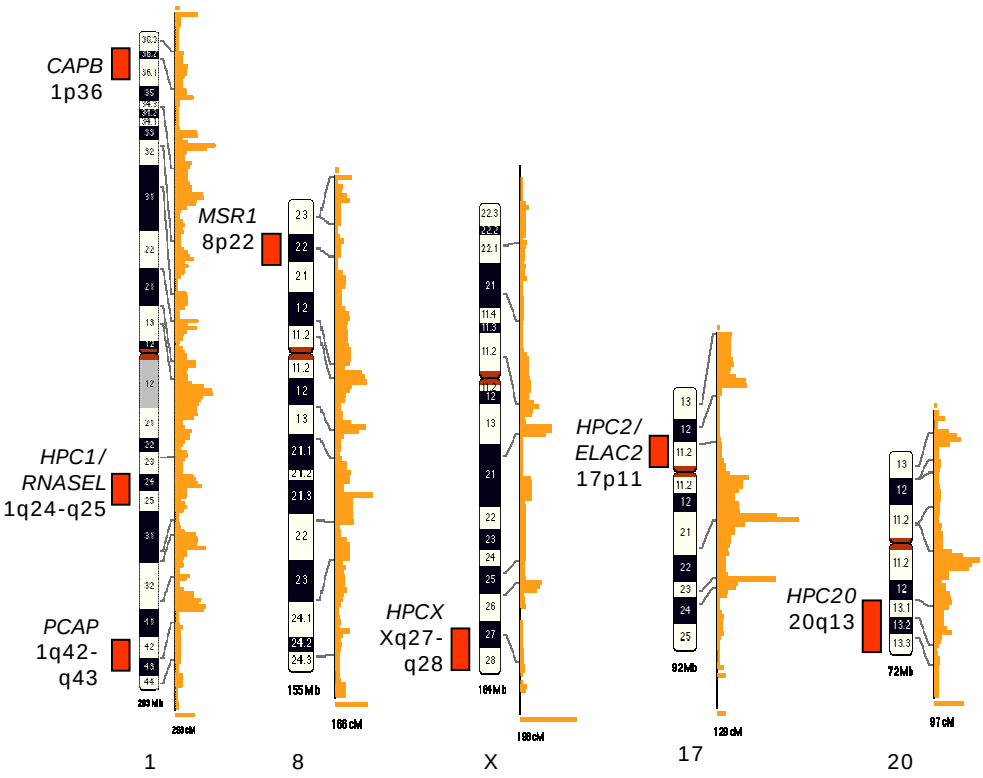

Figure 5. Predisposing loci and candidate genes for hereditary prostate cancer.

3.3.1 HPC1 region at 1q24-q25 and RNASEL gene

In 1996, the first high-risk loci for hereditary prostate cancer, named *HPC1* (MIM 601518), was mapped to 1q24-q25, by carrying out the first genome-wide linkage analysis (Smith et al. 1996). Altogether 66 American high-risk prostate cancer families were genotyped with a marker density of 10 cM. All families had at least three first-degree relatives with prostate cancer, the average age at diagnosis being 65 years. The number of families was then increased to 91, and additional markers from the region were typed, providing the

maximum two-point logarithm of odds (LOD) score 3.65 at θ = 0.18 with marker D1S2883. Under the assumption of heterogeneity a maximum multipoint LOD score of 5.43 was obtained, with the postulated susceptibility locus mapping close to D1S422 and one third of the families linking to it.

Several subsequent analyses confirmed the existence of a susceptibility gene at this locus (Cooney et al. 1997; Grönberg et al. 1997c; Hsieh et al. 1997; Grönberg et al. 1999; Neuhausen et al. 1999; Berry et al. 2000a; Xu 2000; Goddard et al. 2001; Goode et al. 2001; Powell et al. 2001; Xu et al. 2001b; Janer et al. 2003; Xu et al. 2003a; Brown et al. 2004), while others failed to confirm it (McIndoe et al. 1997; Berthon et al. 1998; Eeles et al. 1998; Bergthorsson et al. 2000; Gibbs et al. 2000; Goode et al. 2000; Schleutker et al. 2000; Suarez et al. 2000b; Cancel-Tassin et al. 2001a; Hsieh et al. 2001; Paiss et al. 2001). These negative reports on *HPC1* provided the first indications of the genetic heterogeneity of prostate cancer, as well as the fact that the cloning of prostate cancer susceptibility genes might prove more complex than originally anticipated. While the first data suggested a linked fraction of 36 % (Smith et al. 1996), a subsequent analysis of 772 families implied that the proportion of *HPC1*-linked families was only 6 % (Xu 2000). Characteristic features of the *HPC1*-linked families include a male-to-male transmission, early-onset disease (< 66 years), and at least 5 affected family members (Grönberg et al. 1997b).

In 2002, six years after the initial linkage report of *HPC1*, Carpten et al. reported the finding of a possible candidate gene for *HPC1*. Mutation screening was performed from the germline DNA of an index case from 26 pedigrees, including eight pedigrees showing linkage to *HPC1* locus with at least four affected individuals sharing an *HPC1* haplotype. Two germline mutations, a nonsense mutation Glu265X and an initiation codon mutation Met1Ile which co-segregated with prostate cancer in two high-risk families were found in a gene called *RNASEL* (MIM 180435) at 1q25. Glu265X was found in a family with five prostate cancer cases, where DNA was available from four affected members all of whom carried the mutation. Met1Ile was found in a family with six prostate cancer cases, four of whom carried the mutation. Two patients that did not carry the mutation had an older average age at diagnosis than the carriers, suggesting that they represent sporadic cases. Two affected non-carriers also had cancers with clinical features associated with more favorable outcomes (lower tumor grade and stage) than the affected carriers. Further supportive evidence was obtained from the observation of loss of heterozygosity (LOH) in

the tumor tissue as well as reduced enzymatic activity in heterozygotes of Glu265X in comparison to homozygotes for the normal allele. In addition to the two deleterious segregating mutations, several missense mutations of *RNASEL* were found.

RNASEL is a constitutively expressed latent endoribonuclease involved in the mediation of the antiviral and proapoptotic activities of the interferon 2-5A system (Zhou et al. 1993). *RNASEL* has been suggested to be a tumor suppressor gene, based on its putative function (Lengyel 1993), and it has previously been demonstrated to be homozygously lost in a hepatoma cell line, HepG2 (Tnani and Bayard 1998). However, allelic imbalance at 1q24-q25 has not been frequently found in hereditary prostate cancers (Dunsmuir et al. 1998; Åhman et al. 2000). A mouse model of *RNASEL* function demonstrates that null mice have defects in both interferon-induced apoptosis and antiviral response, although they do not develop tumors (Zhou et al. 1997).

3.3.2 PCAP region at 1q42.2-q43

The second putative susceptibility locus for prostate cancer, *PCAP* (MIM 602759), was mapped to 1q42.2-q43 (Berthon et al. 1998). A linkage analysis using 47 French and German families with three or more prostate cancer patients per family was performed. A maximum two-point LOD score of 2.7 at $\theta = 0.1$ with marker D1S2785, distal to *HPC1*, was observed, with the estimated proportion of linked families 50 %. LOH at 1q42.2-q43 was also observed in 11 out of 55 cases of sporadic prostate cancer further supporting the existence of a potential tumor suppressor gene at this locus (Berthon et al. 1998). There are several confirmatory studies, but the evidence from most of them is weak (Gibbs et al. 1999a; Suarez et al. 2000b; Cancel-Tassin et al. 2001a; Goddard et al. 2001; Goode et al. 2001; Paiss et al. 2001; Xu et al. 2001b; Brown et al. 2004), and some studies, especially with North American families, have failed completely in confirmation (Whittemore et al. 1999; Berry et al. 2000a). This may reflect the greater genetic diversity of the North American families in comparison to the families in the original study. However, negative results have also been obtained from other populations such as Iceland (Bergthorsson et al. 2000). A likely candidate at 1q42.2-q43 is *PCTA-1* (*prostate carcinoma tumor antigen 1*). However, a study of 77 familial cases in Germany and France did not identify any functional *PCTA-1* sequence variants that would associate with prostate cancer (Maier et al. 2002).

3.3.3 HPCX region at Xq27-q28

Evidence for a susceptibility locus at chromosome Xq27-q28 has been suggested by linkage analysis (Xu et al. 1998), and the locus was named *HPCX* (MIM 300147). The findings of a segregation analysis suggested X-chromosomal inheritance model for prostate cancer (Monroe et al. 1995). Linkage to *HPCX* locus was observed in a multi-center study of 360 prostate cancer pedigrees from North America, Sweden and Finland on average in 16 % of the families, with a maximum two point LOD score 4.60 at $\theta = 0.26$ for marker DXS1113. The proportion of linked families ranged from 15 % in American families to over 40 % in Finnish families. The strongest results came from 129 families without evidence of paternal transmission and with late age (> 65 years) at diagnosis. The finding has been confirmed in 57 Finnish prostate cancer families, where over 40 % of the families seem to be linked to *HPCX* (Schleutker et al. 2000). The same fraction of German prostate cancer families also seem to be linked to *HPCX* (Paiss et al. 2001; Bochum et al. 2002). Studies in populations other than Finnish and German, have only been able to give little (Lange et al. 1999; Peters et al. 2001; Xu et al. 2003a; Brown et al. 2004) or no confirmation for *HPCX* linkage (Bergthorsson et al. 2000; Cancel-Tassin et al. 2001a; Goode et al. 2001). While *AR* (*androgen receptor)* gene may play a role in the causation of prostate cancer, it is not likely to be the target gene for *HPCX*, as it is located more than 50 cM from the region of linkage. Recently, deletions of Xq27-q28 were found in two out of 19 somatic tumor tissues of sporadic prostate cancer patients, suggesting that the mutations of putative *HPCX* gene, although quite rare, might play also a role also in sporadic disease (Kibel et al. 2003).

3.3.4 CAPB region at 1p36

From 12 prostate cancer families with a confirmed family history of primary brain cancer, a prostate cancer susceptibility locus *CAPB* (MIM 603688) at 1p36 was identified with an overall maximum two-point LOD score of 3.22 $\theta = 0.06$ with marker D1S507 (Gibbs et al. 1999b). In the younger age group (mean age at diagnosis < 66 years), a maximum two-point LOD score of 3.65 at $\theta = 0.0$ with marker D1S407 was observed. After the exclusion of three families showing linkage to either *HPC1* or *PCAP*, a maximum two-point LOD score increased to 4.74 at $\theta = 0.0$ with marker D1S407. The finding of *CAPB* was of particular interest because it could be the locus behind the excess of brain and central nervous system tumors that has been previously reported in prostate cancer families (Goldgar et al. 1994; Isaacs et al. 1995). In addition, 1p36 region has shown frequent LOH in brain tumors (Bello et al. 1995; Kaghad et al. 1997). Several confirmatory studies were

published later (Badzioch et al. 2000; Gibbs et al. 2000; Suarez et al. 2000a; Goode et al. 2001; Xu et al. 2001b; Matsui et al. 2004), but again there are also negative findings (Berry et al. 2000a; Cancel-Tassin et al. 2001a; Goddard et al. 2001; Hsieh et al. 2001; Brown et al. 2004) with no meta-analyses published to date.

3.3.5 HPC20 region at 20q13

HPC20 (MIM 176807) was mapped to 20q13 in a genome-wide linkage of 162 North American families (Berry et al. 2000b). The highest two point LOD score was 2.69 with a maximum multipoint non-parametric LOD score of 3.02 (p= 0.002). Families with fewer than five affected members, a late age at diagnosis and no male-to-male transmission gave the strongest evidence for linkage with a multipoint non-parametric LOD score of 3.69 with a p-value of 0.0001. Interestingly these results are consistent with the segregation results of Cui et al. (2001), where a recessive model is more likely for older-onset disease. The findings have been confirmed in a few independent studies (Bock et al. 2001; Zheng et al. 2001; Cunningham et al. 2003; Brown et al. 2004), but negative findings have also been published (Cancel-Tassin et al. 2001b). The International Collaboration of Prostate Cancer Genetics (ICPCG) failed to replicate linkage to *HPC20* in a study of 1,234 prostate cancer pedigrees (unpublished data).

3.3.6 HPC2 region at 17p11 and ELAC2 gene

A genome-wide linkage analysis of eight large Utah prostate cancer families with 300 polymorphic markers provided indicative evidence for linkage on chromosome 17 short arm (Tavtigian et al. 2001). Fine-mapping with a denser set of markers in a total of 33 prostate cancer families gave a maximum two-point LOD score of 4.53 at $\theta = 0.07$ with marker D17S1289 at 17p11. However, when the series was expanded to 127 families, the results were no longer significant. The analysis of a common haplotype highlighted a 1.5 Mb region, from which eventually the first candidate prostate cancer susceptibility gene, *ELAC2* at 17p11 (MIM 605367) or *HPC2*, was identified. ELAC2 is an 826 amino acid long protein present in most human tissues. It encodes a novel protein whose function is still mainly unclear. First it was thought to serve as metal-dependent hydrolase and to be potentially linked to interstrand-crosslink repair functions. However, it was later purified and shown to be a binuclear zinc phosphodiesterase (Vogel et al. 2002). Knockouts of the yeast ortholog are lethal. It has been shown that overexpression of ELAC2 in tumor cells results in increase in G2 phase cells, suggesting that overexpression of the protein may alter

mitotic entry (Korver et al. 2003). ELAC2 seems to physically interact with the γ-tubulin complex, possibly promoting tumorigenesis through irregular cell division.

In the original study by Tavtigian et al. (2001), one kindred contained eight prostate cancer cases, from which six shared an *ELAC2* haplotype. Germline mutation screening of the youngest patient, diagnosed at the age of 46, revealed a protein truncation mutation; a single insertion at codon 547 (1641insG), leading to miscorporation of 67 amino acids followed by a stop codon. The frameshift occurred within the most highly conserved segment of the protein and eliminated one-third of the protein including several other conserved segments. However, the mutation was not completely co-inherited with the disease; three out of four prostate cancer patients carried the mutation. In addition, it has not been found in other studies. As the 1641insG mutation was found in an individual with early onset prostate cancer, 45 prostate cancer cases with early age at diagnosis (≤ 55 years) were also screened. A new missense mutation Arg781His was found in an individual diagnosed at the age of 50. The missense change occurs in a very highly charged stretch of amino acid residues near the C-terminus of the protein. In that family, the mutation was found in six out of 14 cases, six were non-carriers and two unknown. In addition, two common missense changes, Ser217Leu and Ala541Thr, were found. Initial results indicated that individuals homozygous for Leu217 and individuals carrying Thr541 allele were at significantly increased risk for prostate cancer, and that the combination of the genotypes was the most significant, with an odds ratio (OR) of 2.94 (95 % confidence interval, CI 1.52-5.69).

3.3.7 Region at 8p22 and MSR1 gene

The first genome-wide linkage analysis (Smith et al. 1996) also suggested other regions of interest, in addition to *HPC1* at 1q24-q25. One of them was 8p22-p23, where LOH suggesting the location of a tumor suppressor gene has frequently been observed in sporadic prostate cancers (Cunningham et al. 1996). Xu et al. (2001c), therefore, performed linkage analysis in 159 prostate cancer families using 21 microsatellite markers over 35 cM area at 8p22-p23. Evidence for new susceptibility loci was obtained with a peak multipoint heterogeneity LOD (HLOD) score of 1.84 (p= 0.004). The estimated proportion of linked families was 14 %. Especially families with patients having late average age at diagnosis (> 65 years) seemed to be linked to this new locus. Evidence for linkage to 8p22-p23 has been confirmed by several studies (Goddard et al. 2001; Wiklund et al. 2003b; Xu et al. 2003a).

After the linkage analysis (Xu et al. 2001c), 159 prostate cancer patients were screened for germline mutations, and disease-segregating mutations in *MSR1* gene (MIM 176807) at 8p22 were found (Xu et al. 2002). MSR1 is a transmembrane protein that functions as a homotrimeric receptor for a number of polyanionic ligands including a variety of bacteria. *MSR1* is not expressed in normal prostate, rather, it is only found in macrophages. Thus, the identification of a second prostate cancer susceptibility gene connected with the immune responses suggests that inflammation might contribute in some way to the development of prostate cancer. In the mutation screening one nonsense and six rare missense mutations were found. The nonsense mutation Arg293X removes most of the extracellular ligand binding domain as well as the conserved extracellular scavenger receptor cystein-rich domain, suggesting gross interference with its function. A further 31 prostate cancer families were added to the screening and altogether 13 pedigrees were found to harbor at least one of the rare sequence variants. A family based linkage/segregation test provided evidence that there was disproportionate segregation of these variants with disease (p= 0.0007). Six of these 13 pedigrees, all of European descent, harbored the nonsense mutation Arg293X. In addition, the prevalence of Arg293X was studied among 317 prostate cancer patients of European descent. The frequency among patients was 4.4 % in comparison with 0.8 % in unaffected men. The same values in African-Americans were 12.5 % and 1.8 % respectively. These results suggested that *MSR1* may be important in susceptibility to prostate cancer in men of both African-American and European descent.

More recently, Xu et al. (2003b) studied five common polymorphisms of *MSR1* among 301 sporadic prostate cancer patients and 250 control subjects. Haplotype analysis revealed a significant difference in haplotype frequencies in a global score test (p=0.011). In a confirmatory study by Miller et al. (2003) 134 African-American men and 340 unaffected controls were screened and an association of a common polymorphism as well as a rare missense change with prostate cancer were found. A subsequent study of the role of *MSR1* in causation of prostate cancer in Finland (Seppälä et al. 2003a) did not find any statistically significant association of *MSR1* variants with prostate cancer. However, the mean age at diagnosis of the carriers of Arg293X was significantly lower than that of non-carriers (55.4 versus 65.4; p= 0.04). Consistent with these negative results, the genome-wide linkage analysis in Finnish hereditary prostate cancer families found no linkage to chromosome 8p (Schleutker et al. 2003). A study by Wang et al. (2003) also failed to find

an association of *MSR1* variants with prostate cancer. A Swedish study by Lindmark et al. (2004) found Arg293X to be more common in unselected prostate cancer cases than in controls, although the results were not statistically significant. Nupponen et al. (2004) investigated somatic mutations of *MSR1* in 39 clinical sporadic prostate cancer specimens, 10 prostate cancer xenografts and 4 prostate cancer cell lines. Truncating mutation Arg293X was found in one sample and it was later proven to be a germline mutation, indicating that somatic *MSR1* mutations in sporadic prostate cancer are rare events.

3.4 Low-penetrant predisposing genes associated with prostate cancer

Family-based linkage analysis will identify rare moderate- to high-penetrant susceptibility genes, which are likely to account for only a small proportion of all prostate cancers. Yet, part of the prostate cancer susceptibility is probably due to common polymorphisms, which can be found by association studies. Such polymorphisms are likely to cause a small relative risk of disease, but account for a larger proportion of cancers due to the high frequency of the risk alleles in the population. These include genes capable of modifying the disease manifestation without having a direct predisposing role. In prostate cancer, such candidate gene approaches have mainly been focused on genes involved in the metabolism of testosterone and other androgens (e.g. *AR*, *CYP*, *SRD5A2*, *HSD3B*), because the growth of prostate cells is heavily dependent on testosterone (Rebbeck et al. 2002; Gsur et al. 2004). Besides genes involved in androgen metabolism, a number of studies have evaluated genes involved in environmental carcinogen metabolism (e.g. *CYP*, *GST*, *NAT*), and DNA repair (e.g. *CHEK1*, *XRCC1*, *XPD*) pathways (Rebbeck et al. 2002; Gsur et al. 2004). However, studies mainly focused on sporadic rather than familial prostate cancer cases. Our group has studied *AR* (*androgen receptor*), *BRCA1* and *2* (*breast cancer genes 1 and 2*), *CDH1* (*e-cadherin*), *CHEK2* (*CHK2 checkpoint homolog, S. pombe)* and *SRD5A2* (*steroid-5-alpha-reductase*) with familial or hereditary prostate cancer samples (Mononen et al. 2000; 2001; 2002; Ikonen et al. 2001; 2003; Seppälä et al. 2003b). Association with familial or hereditary prostate cancer has been seen in Arg726Leu variant of *AR* (Mononen et al. 2000), 1100delC and Ile157Thr variants of *CHEK2* (Seppälä et al. 2003b) as well as *CDH1* variant Ser270Ala (Ikonen et al. 2001). Some prostate cancer susceptibility genes, such as *ELAC2* or *MSR1*, that have been found by linkage analyses, may in fact prove to be low-penetrant rather than high-penetrant genes.

3.5 Allelic imbalance and relative DNA copy number changes in the search for hereditary prostate cancer susceptibility genes

Besides linkage and association analyses, molecular studies provide insight into the location of putative disease genes. Most familial cancer syndromes result in inactivating mutations in tumor suppressor genes. The first mutation, usually a point mutation, is inherited, but it is the loss of the remaining wild-type allele later in life that initiates the carcinogenesis. The involvement of tumor suppressor genes is indicated by allelic loss or imbalance in tumor tissue.

3.5.1 Allelic imbalance in hereditary prostate cancer

So far only four studies of allelic imbalance on tumor tissue from hereditary prostate cancer patients have been published (Dunsmuir et al. 1998; Bergthorsson et al. 2000; Åhman et al. 2000; Verhage et al. 2003a). Studies have been made in three countries focusing mainly on *HPC1*, *PCAP*, *CAPB* and *HPC20* regions, and results differ from the lower allelic imbalance found in the United Kingdom, Iceland and Sweden (*HPC1*: 7-11 %; *PCAP*: 20 %; *CAPB*: 11 %) to higher frequencies found in the Netherlands (*HPC1*: 38 %; *PCAP*: 26 %; *HPC20*: 36 %). In the latter study several other regions were also studied, including the 8p22-p23 region, where *MSR1* is located, in which over 30 % of the tumors had allelic imbalance. The results from allelic imbalance studies reflect the enormous heterogeneity among hereditary prostate cancer cases.

3.5.2 Relative DNA copy number changes in hereditary prostate cancer

Comparative genomic hybridization (CGH) allows the whole genome to be scanned for relative DNA copy number changes in a single hybridization (Kallioniemi et al. 1992). So far, one published and one unpublished study have been conducted with CGH on tumor tissue from familial prostate cancer patients (Verhagen et al. 2000; Herman et al. unpublished, in Verhage et al. 2003b). The most commonly seen changes in hereditary prostate cancer specimens have been losses of 6q, 8p, 13q and 16q as well as gains of 8q. All of these changes have also been detected in primary sporadic prostate cancers (Bova and Isaacs 1996), loss of 8p and 13q as well as gain of 8q being the most common changes. In addition, losses have been more common in hereditary prostate tumors than gains, as is the case in sporadic cases (Visakorpi et al. 1995). All in all, studies on hereditary prostate cancer specimens by CGH have not been able to demonstrate systematic differences

between hereditary and sporadic prostate cancers, nor have they pinpointed the locations of tumor suppressor genes specific to only hereditary cases.

3.6 Clinical and pathological features of hereditary prostate cancer

3.6.1 Age at diagnosis

The most prominent feature of hereditary prostate cancer is the comparatively early age at diagnosis. On average, hereditary prostate cancer is diagnosed seven years earlier than sporadic prostate cancer (Smith et al. 1996; Bratt et al. 1999). This is a small difference in comparison to data from analogous studies of breast (Marcus et al. 1996), ovarian (Rubin et al. 1996) and colorectal cancers (Watson et al. 1998) that have reported up to 20 years of difference in the age at diagnosis between hereditary and sporadic cases.

3.6.2 Prognosis and clinical characteristics

As a consequence of the earlier onset, prostate cancer could be the cause of death for a larger proportion of men with hereditary than for men with sporadic prostate cancer. Comparison of survival between sporadic and hereditary cases is difficult, because an increased awareness in prostate cancer families may result in an earlier diagnosis and longer survival. Most of the studies have indicated similar outcomes for sporadic and hereditary prostate cancers (Carter et al. 1993; Keetch et al. 1996; Bauer et al. 1998; Bova et al. 1998; Grönberg et al. 1998; Hanlon and Hanks 1998; Hanus et al. 1999; Valeri et al. 2000). However, there are a few studies suggesting poorer (Kupelian et al. 1997a; 1997b; Rodriguez et al. 1997) or on the contrary better prognosis for men with a family history of prostate cancer (Norrish et al. 1999). In most studies no differences in tumor grade and pathological stage at diagnosis between hereditary and sporadic prostate cancers have been reported (Bastacky et al. 1995; Valeri et al. 2000).

The tumors of carriers of certain predisposing genes may have specific biological characteristics. The small amount of data about cancers in *HPC1* linked families suggests that they are more likely to be of high grade and to have disease which has spread beyond the prostatic capsule in comparison with cases from unlinked families (Grönberg et al. 1997b; Goode et al. 2001). Goddard et al. (2001) observed that detection of linkage to *HPC1* was enhanced when the Gleason score was considered in the linkage model.

Therefore, *HPC1* may confer not only the susceptibility to develop prostate cancer but could also influence the development of aggressive disease (Laniado 1998; Walther 1998). In a genome-wide analysis, Witte et al. (2000; 2003) mapped prostate cancer aggressiveness loci to 5q31-q33, 7q32, and 19q12 by taking the Gleason score as a quantitative measure in linkage analysis. The two last regions have been confirmed in independent studies (Neville et al. 2002; 2003; Paiss et al. 2003; Slager et al. 2003). These regions may contain genes that influence the progression of prostate cancer from histological to invasive disease.

Overall, no substantial clinical or pathological differences seem to exist between hereditary, familial or sporadic prostate cancer cases. However, until the genes involved are identified, gene-specific effects on tumor phenotypes cannot be excluded.

AIMS OF THE STUDY

The purpose of this study was to identify high-risk predisposition loci and genes in Finnish hereditary prostate cancer families. The specific aims were:

1. To explore somatic genetic changes in tumor tissue of patients from prostate cancer families to identify prostate cancer predisposition loci (I).

2. To determine the role of known prostate cancer predisposition genes in prostate cancer in Finland (II, III).

3. To search for novel susceptibility loci by linkage analysis and candidate gene search (IV).

MATERIALS AND METHODS

1. Human subjects

1.1 Finnish prostate cancer families (I – IV)

1.1.1 Collection (I – IV)

Since 1996 Finnish prostate cancer families have been collected by the Hereditary Prostate Cancer Study Group in the Laboratory of Cancer Genetics at the University of Tampere and Tampere University Hospital. Identification of the families has been accomplished through nation-wide registry based searches, referrals from physicians and newspaper, television, and radio advertisements. From the families gathered, only families having at least two affected first or second degree relatives were accepted for the study. Diagnoses and the family histories were initially obtained by questionnaire and subsequently confirmed using the Finnish Cancer Registry or individual patient records from regional hospitals and parish records.

1.1.2 Tumor samples (I)

To study relative somatic DNA copy number alterations in patients with a positive family history of prostate cancer by comparative genomic hybridization (CGH; Study I), all available formalin-fixed paraffin-embedded primary prostate carcinoma specimens from patients belonging to prostate cancer families were obtained from the pathology archives of regional hospitals throughout Finland. Those patients who had received hormonal therapy before sampling, and who had recurrent cancer were excluded. Tumor samples from altogether 21 persons belonging to 19 families were obtained (Table 3). Most of the samples were from prostatectomies (n=13), the rest were from transurethral resection of prostate (TURP; n=6) or needle biopsies (n=2). Due to the rareness of the hereditary tumor samples only one family was among the families used in Studies II – IV.

Table 3. DNA sample numbers used in different studies.

DNA source			Study I	Study I I	Study III	Study IV
Tumor	PRCA[a] families	No. of families	19			
		Mean no. of aff.[c] (range)	2.7 (2-4)			
		Mean age at diagn.[d] (range)	68 (55-86)			
		No. of aff. samples	21			
Genomic	PRCA families	No. of families		107	116	16
		Mean no. of aff. (range)		2.8 (2-5)	2.6 (2-6)	4.3 (3-6)
		Mean age at diagn. (range)		65 (45-86)	66 (44-86)	69 (44-98)
		No. of aff. samples		107 (1/ family)	116 (1/family)	49 (all aff.)
		No. of unaff.[e] samples				180
	Unselected PRCA	No. of samples		467	492	200
		Mean age at diagn. (range)		68 (48-92)	68 (52-88)	67 (47-88)
	BPH[b]	No. of samples		223	223	
	Controls	No. of samples		568	566	200

[a]PRCA= prostate cancer
[b]BPH= benign prostate hyperplasia
[c]aff. = affected persons
[d]diagn. = diagnosis
[e]unaff. = unaffected persons

1.1.3 Blood samples (II-IV)

In order to study germline mutations in prostate cancer families, blood samples were collected from patients from prostate cancer families as well as their healthy relatives. At the moment, there are 121 prostate cancer families having at least one blood sample from an affected person. In addition lymphoblastoid cell lines were established from 186 blood samples of affected family members. The blood samples used in different studies are presented in Table 3.

In Study II initial mutation screening was performed with 66 families with three or more affected first or second degree relatives or two with the ages at diagnosis under 65 years. An additional 41 families that had only two affected members diagnosed over 65 years of age were used to determine the frequencies of the known mutations in a larger sample size making the total number of families analyzed 107 (Table 3).

In Study III initial mutation screening was performed with 66 families having three or more affected first or second degree relatives or two with age at diagnosis under 60 years. An additional set of 50 families with two affected members with age at diagnosis over 60 years

of age were used to determine the frequencies of the known mutations making the total number of families analyzed 116 (Table 3).

In Study IV, sixteen multiple case and most informative prostate cancer families were selected for the fine-mapping of the chromosomal regions at 3p25-p26 and 11q14 (Table 3). These families had at least three affected first or second degree relatives with at least two samples from affected members as well as several samples from unaffected members. Ten of these families were also used in the initial genome-wide linkage analysis (Schleutker et al. 2003). All available samples from these 16 families (229 samples, 46 from affected members) were genotyped in Study IV.

1.2 Unselected prostate cancer patients and patients with benign prostate hyperplasia (II – IV)

Since 1995 blood samples have been collected from consecutive unselected prostate cancer patients and benign prostate hyperplasia patients diagnosed in Tampere University Hospital. Tampere University Hospital is a regional referral center in the area for all patients with prostate cancer, which results in unselected, population-based collection of the patients. The diagnosis of benign prostate hyperplasia was based on lower-urinary tract symptoms, free uroflowmetry, and evidence, by palpation or transrectal ultrasound, of increased prostate size. If PSA was elevated then the patients underwent biopsies to exclude prostate cancer. The indication for biopsy was total PSA of ≥ 4 ng/ml or total PSA of 3.0 – 3.9 µg/l with the proportion of free PSA < 16 %. Most patients with benign prostate hyperplasia were followed up for 3 - 5 years and did not develop prostate cancer during that time. At the moment there are blood and DNA samples from 2312 unselected prostate cancer patients and 680 benign prostate hyperplasia (BPH) patients in this collection. For approximately 90 % of the unselected prostate cancer patients information is available on tumor grade, T-stage (tumor) and M-stage (metastasis, ascertained by bone scan). The numbers of unselected prostate cancer and benign prostate hyperplasia samples used in different studies are presented in Table 3.

1.3 Healthy control individuals (I – IV)

DNA extracted from the blood samples of healthy male blood donors of the Blood Center of the Finnish Red Cross in Tampere was used for control purposes. The number of controls used in different studies is shown in Table 3.

2. Methods

2.1 DNA extraction (I-IV)

2.1.1 Tumors (I)

Paraffin blocks were initially analyzed by a pathologist who selected representative parts from the tumors in order to increase the proportion of tumor cells in each sample. After this, the number of tumor cells in samples was estimated to be well over 50 %, which is the minimum requirement for comparative genomic hybridization. DNA was extracted from the selected parts of the paraffin block using standard methods for paraffin-embedded tissue (Isola et al. 1994) using Qiagen kit (Qiagen, Valencia, CA, USA) for paraffin-embedded tissue according to the instructions provided by the manufacturer.

2.1.2 Blood (I-IV)

Genomic DNA was extracted from blood lymphocytes using the Puregene kit (Gentra Systems, Inc., Minneapolis, MN, USA) according to the instructions of the manufacturer.

2.2 Relative DNA copy number analysis by comparative genomic hybridization (I)

With comparative genomic hybridization (CGH) relative DNA copy number changes of the tumor tissue from hereditary prostate cancer patient could be characterized in a single hybridization (Kallioniemi et al. 1992). Genomic DNA from tumor was labeled with directly fluorochrome-conjugated FITC-12-dUTP (DuPont, Boston, MA, USA), and the normal reference DNA from healthy male blood donors with TexasRed-5-dUTP (DuPont, Boston, MA, USA) by nick-translation. In the reaction, the amount of DNAase was adjusted to produce double stranded DNA fragments between the length of 600 - 2000 bp. About 400 ng of each labeled DNA sample together with 10 μg of Cot-1 DNA (Invitrogen, Carlsbad, CA, USA) was ethanol precipitated and dissolved in 10 μl of hybridization buffer (50 % formamide, 10 % dextran sulfate, 2 x SSC [standard saline citrate], pH 7.0). The hybridization mixture was denatured at 72^{o}C for 5 min and applied to normal lymphocyte metaphase spreads (Vysis Inc., Downers Grove, IL, USA). Before hybridization the spreads were denaturated in a formamide solution (70 % formamide, 2 x SSC, pH 7.0) at 73^{o}C for 3

min, followed by dehydration in ethanol series (70 %, 85 % and 100 % ethanol). Hybridization was performed in a moist chamber at 37°C for two days. In each hybridization batch a negative control (normal male against normal female) and a positive control (MCF-7 breast cancer cell line against normal female) were used.

After hybridization the slides were washed for two minutes at 74°C in wash solution I (0.4 x SSC, 0.3 % NP-40 [VWR International, Espoo, Finland]) and for one minute in wash solution II (2 x SSC, 0.1 % NP-40) at room temperature. Finally the slides were left in distilled water for 10 minutes at room temperature. All the washes were made in the dark, to stop the fluorochromes from fading. After air-drying, the slides were counterstained with a 0.5 μM DAPI (4′,6-diamidino-2-phenylindole) in an antifade solution (Vectashield, Vector, Burlingame, CA, USA).

The hybridized slides were examined with an Olympus BX 50 (Tokyo, Japan) epifluorescence microscope equipped with suitable filters for the fluorochromes used (DAPI, FITC and Texas Red). From every analyzed metaphase a digital image was captured with a CCD-camera (charge-coupled device, Photometrics Image Point, Photometrics, Tucson, AZ, USA) attached to the microscope. Five to ten best metaphases were captured from each hybridization and edited using Karyotyper and Analyzer programs of the CGH software (Vysis, Downers Grove, IL, USA) to compose CGH karyograms.

2.3 Mutation detection (II-IV)

2.3.1 Restriction fragment length polymorphism analysis (II)

Restriction fragment length polymorphism analysis (RFLP) was used for the genotyping of Ser217Leu and Ala541Thr variants of *ELAC2* gene. RFLP analysis exploits the fact that a mutation may alter the recognition sites of restriction enzymes. The target sequences were amplified with specific primers for each variant using 100 ng DNA in 25 μl reaction mixture (containing 2mM $MgCl_2$, 0.2 μM each primer, 0.2 mM each dNTP, 1.25 U Ampli*Taq*Gold in 1 x polymerase chain reaction buffer).

Ser217Leu variant was analyzed after an overnight digestion with *Taq*$^{\alpha}$I (NEB, Beverly, MA, USA) at 65^{o}C in a reaction mixture of 25 µl (containing 12 µl of the PCR product, 1 x bovine serum albumine, 6U *Taq*$^{\alpha}$I, in 1 x buffer). The recognition site for *Taq*$^{\alpha}$I is 5´…T↓CGA…3´. Ala541Thr variant was analyzed after three-hour digestion with *Fnu*4H I (NEB, Beverly, MA, USA) at 37^{o}C in a reaction mixture of 25 µl (containing 20 µl of the PCR product, 3U *Fnu*4H I, in 1 x NEBuffer 4). The recognition site for *Fnu*4H I is 5´…GC↓NGC…3´. After digestion the fragments were separated using 2.5 % agarose gel electrophoresis containing ethidium bromide. Confirmation of the results was performed for altogether 172 samples from the original 1365 samples genotyped using an automated ABI PRISM 310 Genetic Analyzer (PE Biosystems, Foster City, CA, USA) according to the instructions of the manufacturer. Analysis of the sequences was conducted with Sequencher 3.0 software (Gene Codes Corporation, Ann Arbor, MI, USA). In sequencing a complete concordance with the RFLP analysis was observed i.e. no false positives or false negatives were detected that could result from incomplete digestion.

2.3.2 Single strand conformational polymorphism analysis (II, III)

Single strand conformational polymorphism analysis (SSCP; Orita et al. 1989) was used for screening for new mutations in the coding sequences of *ELAC2* (II) and *RNASEL* (III). In both studies the primer sequences were designed to include all intron-exon boundaries, in Study III primer sequences were kindly provided by Dr. John Carpten (Translational Genomics, Phoenix, USA). 100 ng genomic DNA was used in a 15 µl reaction mixture, which contained 1.5 mM $MgCl_2$, 0.6 µM each primer, 20 µM each dNTP, 0.5 µCi of α(^{33}P)-dCTP (Amersham Pharmacia, Piscataway, NJ, USA), 1.5U Ampli*Taq*Gold polymerase and the reaction buffer provided by the supplier (PE Biosystems, Foster City, CA, USA). In order to create single stranded DNA, the radiolabeled PCR products were mixed with 95 % formamide dye, denaturated for 5 min at 95^{o}C and chilled on ice. Electrophoresis of the [^{33}P] labeled products was performed at 800 V for 13 hours at room temperature using a commercial polyacrylamide gel (0.5 x MDE; mutation detection enhancement, FMC Bioproducts, Rockland, ME, USA) with and without 1 % glycerol in 0.5 x TBE (Tris-borate EDTA) buffer. After electrophoresis the dried gels were exposed to Kodak BioMax MR (maximum resolution) films for 4 hours. Samples which created aberrantly moving bands as well as two to three normally moving bands per run were analyzed by sequencing, using the original PCR primers.

2.3.3 Conformation-sensitive gel electrophoresis (III)

Conformation-sensitive gel electrophoresis (CSGE; Ganguly et al. 1993) was used to screen for mutations in the coding sequence of *RNASEL*. Moreover, the purpose was also to compare the effectiveness of single strand conformational polymorphisms analysis and conformational sensitive gel electrophoresis using the same primers and same PCR conditions in both methods. The forward primer was labeled by 5´-labelling in 37°C for 60 min, in a 30 µl reaction volume, which contained 45 pmol forward primer, 45 µCi of $\gamma(^{33}P)$-dATP (Amersham Biosciences, Piscataway, NJ, USA), and 15 U T4 polynucleotide kinase. A PCR, which contained 2.25 mM $MgCl_2$, 200 µM each dNTP, 5.4 pmol of the labeled forward primer, 6 pmol of the reverse primer and 1.25 U of Ampli*Taq*Gold, was performed. Afterwards the PCR product was heated to 95°C for 10 min and then allowed to slowly cool to room temperature allowing the formation of the heteroduplexes. Before loading the samples, 7.5 µl sample buffer containing 30 % glycerol containing 0.25 % xylene cyanol and 0.25 % bromophenol blue was added to 5 µl of each sample. Electrophoresis was performed with a 10 % polyacrylamide gel (1:75 ratio of acrylamide:bisacrylamide, 10 % ethylene glycol, 15 % formamide in 0.5 x TTE [Tris-taurine EDTA] buffer), at 5 W (for two gels) overnight. Analysis of the aberrant bands was performed by sequencing.

2.3.4 Solid phase minisequencing (II – IV)

Solid phase minisequencing (Syvänen 1998) was used to determine the frequencies of the variants found in the SSCP and CSGE screening (II and III) and direct sequencing (IV) at the population level. PCR primers were designed to amplify 100 – 200 bp region containing the predetermined mutation. One PCR primer was biotinylated from the 5´ end resulting in a PCR product with one biotinylated strand. In addition, a detection primer, complementary to the biotinylated strand was designed to anneal with its 3´ end immediately adjacent to the variant nucleotide to be analyzed. The target sequences were amplified by PCR using 100 ng genomic DNA at a 50 µl reaction mixture (containing 1.5 mM $MgCl_2$, 0.2 µM each primer, 0.2 mM each dNTP, and 1.0 U of Ampli*Taq*Gold). Fifteen µl of the amplified biotinylated fragment was then captured on a streptavidin-coated solid support on microtiter plates (Scintiplates Streptavidin covalent, Wallac, Turku, Finland) by incubation in 0.1 % Tween 20 (VWR International, Espoo, Finland) in PBS (phosphate buffered saline) at 37°C for 1.5 hours. The excess PCR reagents were removed by washing with automated microtitration plate washer (Delfia Platewash, Wallac, Turku, Finland). The

captured biotinylated DNA strand was rendered single stranded by alkaline treatment with 100 µl 50mM NaOH for 5 min at room temperature followed by washing and drying of the plate. The detection primer was then allowed to anneal to the biotinylated strand and to be extended with a single labeled nucleoside triphosphate complementary to the nucleoside at the polymorphic site. This was performed in a 100 µl reaction mixture (containing 20 pmol detection primer, 2 pmol [^{3}H]-labeled dNTP and 0.5 U Dynazyme^{TM}II polymerase [Finnzymes, Espoo, Finland] in 1 x PCR buffer), in incubation at 50^{o}C for 20 min. Every sample had two wells for two different labeled dNTPs, one for the detection of the normal allele and the other for the variant allele. In addition, on each plate a negative and a positive control were included on each plate. After the plates had been washed, 200 µl of washing solution was added to each well and the amount of radioactivity matching the amount of incorporated label, was measured with a liquid scintillator counter (1450 Microbeta, Wallac, Turku, Finland). The results were obtained as counts per minute (cpm) values which directly expressed the amount of incorporated [^{3}H] -dNTP. The ratio between cpm values (R-value) for the two nucleotides (normal and variant) for each sample reflected the ratio between the two sequences in the original sample e.g. for homozygous subjects an allele ratio of ≥ 2:0 was accepted and for heterozygous subjects ratios close to 1:1. All borderline results were verified by sequencing.

2.3.5 Direct sequencing (IV)

Ten candidate genes were selected for mutation screening of the coding exons and exon / intron boundaries from the chromosomal area of 3p26 giving the best LOD scores in the fine-mapping by direct sequencing. All available affected samples (n=18) of the six families linked to 3p26 (family LOD score > 0.5) were screened with an ABI PRISM 3100 Genetic Analyzer (PE Biosystems, Foster City, CA, USA) which is a multi-color fluorescence-based DNA analysis system using capillary electrophoresis with 16 capillaries operating in parallel. It is fully automated from sample loading to data analysis. PCR products were purified in 96-format Acro Prep Filter Plates (Pall Life Sciences, Ann Arbor, MI, USA) using Perfect Vac Manifold vacuum machine (Eppendorf AG, Hamburg, Germany). Sequencing was performed according to the instructions of the manufacturer using BigDye Terminator v.3.1 Cycle Sequencing Kit (PE Biosystems, Foster City, CA, USA). Sequence analysis was performed with Sequencher 4.1 software (Gene Codes Corporation, Ann Arbor, MI, USA).

2.3.6 Statistical analysis of the association (II – IV)

The association of different genotypes with hereditary prostate cancer, unselected prostate cancer and benign prostatic hyperplasia was tested with logistic regression analysis, using SPSS statistical software package (SPSS 9.0 for Windows, SPSS Inc. 1999). Association with demographic, clinical, and pathological features of the disease was tested by the Mann-Whitney or Chi´s square test from the SPSS software package or Fisher´s Exact Test with StatXact (Cytel Software 1999; SPSS Inc. 1999). In this study the focus was on the search for high-penetrant susceptibility genes, the effect of which would be seen in prostate cancer families. According to the power calculations the effect of rare low-penetrant mutations at the population level would be detected with 4-fold sample sizes than used here.

2.4 Fine-mapping (IV)

2.4.1 Selection of microsatellite markers

In order to narrow down the region obtained from the genome-wide linkage analysis of 10 Finnish prostate cancer families (Schleutker et al. 2003), a fine-mapping of the candidate regions on 3p25-p26 and 11q14 was performed. Seventeen microsatellite markers were selected for chromosome 3p and 22 for 11q using the Marshfield genetic map (http://www.marshfieldclinic.org). The regions were selected so that they included markers from the genome-wide linkage analysis that gave the maximum multipoint LOD scores as well as their flanking markers. New markers were selected in between in order to narrow the spacing of the markers from 10 cM (centiMorgans) used in the genome-wide linkage analysis to 0.1-1 cM. Markers also existing on the DeCode genetic map (http://www.decodegenetics.com; according to Unigene in National Center for Biotechnology Information (NCBI) STS-UNIsts– database at http://www.ncbi.nlm.nih.gov) were preferred. The map positions of the markers were determined as a combination of the DeCode, Marshfield and Généthon (http://www.genethon.fr) genetic maps.

2.4.2 Selection of families

Sixteen most informative and multiplex hereditary prostate cancer families were chosen for the fine-mapping. Ten of these families were also used in the original genome-wide analysis (Schleutker et al. 2003). Meanwhile the continuous collection of the families and

the verification of the diagnoses had produced six families that also fulfilled the selection criteria of the linkage analysis. From the 16 families altogether 229 DNA samples were available for genotyping, forty-six of these were from affected individuals. The criteria for the families were three or more affected first- or second-degree relatives and at least two sampled affected members. The mean number of affected individuals genotyped per family was 2.9 and the mean number of all genotyped individuals per family was 14.4.

2.4.3 Genotyping

A total of 5 µl of 10 ng/µl DNA was used per PCR reaction with fluorescently labeled primers. The sizes of the fragments were analyzed with a 96-capillary MegaBace 1000 – machine (Amersham Biosciences, Piscataway, NJ, USA), which is a fluorescence-based DNA system utilizing capillary electrophoresis with up to 96 capillaries operating in parallel. Genotyping with the MegaBace 1000 was performed in the Finnish Genome Center in Helsinki according to the instructions of the manufacturer, producing altogether over 8900 genotypes.

2.4.4 Linkage analysis and statistics

Linkage is the tendency for genes and other genetic markers to be inherited together because of their location close to one another on the same chromosome. Linkage analysis is a gene-hunting technique that traces patterns of heredity in large, high-risk families, in an attempt to locate a disease-causing gene mutation by identifying traits that are co-inherited with it. The statistical estimate of whether two loci are likely to lie near each other on a chromosome and are therefore likely to be inherited together is called a LOD score, logarithm of the odds (to the base of 10). In the search for a simple mendelian disease a LOD score of 3 or more is generally taken to indicate that the two loci are linked and are close to one another and a LOD score of –2 is taken as evidence against linkage. However, this threshold may not be appropriate for a disease like prostate cancer (Lander and Kruglyak 1995). Recombinations may occur between the linked loci and the recombination fraction (θ), the proportion of recombinants out of all opportunities for recombination can be taken as a measure of genetic linkage. In two-point linkage analysis the segregation of a marker locus is compared to the inheritance of a linkage locus, whereas in multipoint analysis more than two loci are analyzed simultaneously. Parametric tests take into account the mode of inheritance, whereas non-parametric methods are model free. Heterogeneity

LOD scores, i.e. HLODs, allow the possibility that linkage is only present in a proportion of families.

Mendelian inconsistencies of the genotypes were checked using SIBPAIR and RELCHECK programs. Finnish marker allele frequencies were estimated from the data using imputation in the SIBPAIR program. First, all genotype data available for the family members were used to infer the genotypes for untyped founders. Then allele frequencies were estimated from the typed and untyped but imputed founder genotypes. The standard two-point and multipoint parametric likelihood analyses were performed using the computer program FASTLINK. The GENEHUNTER and GENEHUNTER PLUS programs were used for additional parametric and nonparametric analyses and a biallelic major locus was assumed. In the parametric analyses, a dominant affected-only model (Smith et al. 1996) was used. The frequency of the disease allele was set at 0.003 in the parametric analyses. All unaffected men and all women were treated as unknown. Only individuals with verified diagnoses of prostate cancer were considered affected and one liability class was specified for the affected men (penetrance was specified to be 1.0 and 0.001 for genotypes DD/Dd, and dd).

2.5 Ethical considerations (I-IV)

Permission for the collection of the families throughout Finland as well as the use of the Finnish Cancer Registry data was granted 20th July 1995 by the Ministry of Social Affairs and Health (license 859/08/95). Permission to collect and use blood samples, tissue samples as well as clinical data from the prostate cancer patients in the Tampere University Hospital District was granted 8th March 1995 (extensions: 17th June 1997, 23rd April 1999, 31st October 2000, 22nd May 2001, 1st November 1999 and 30th December 2003) by the Institutional Review Board of Tampere University Hospital (assurance numbers 95062, and 99228, valid to 31st December 2010). The use of blood and tissue samples as well as clinical data from prostate cancer patients treated in Hatanpää City Hospital was granted 1st July 1996 (extensions: 7th October 1999 and 30th January 2001) by the Institutional Review Board of the City of Tampere. This study was also approved by the National Human Genome Research Institute (HG-0158). Written informed consent for use of their samples as well as medical records was obtained from all living individuals participating in the study.

2.6 Electronic databases (I-IV)

The following sources of information were most often used during this study:

Finnish Cancer Registry, Finland

http://www.cancerregistry.fi

National Cancer Institute (NCI), USA

http://www.cancer.gov

Marshfield map

http://research.marshfieldclinic.org/genetics

DeCode Genetics

http://www.decodegenetics.com

Généthon

http://www.genethon.fr

National Center for Biotechnology Information (NCBI), USA

http://www.ncbi.nlm.nih.gov (PubMed, Locus Link, Map Viewer, UniSTS, Unigene, DbSNP)

University of California Santa Cruz Genome Bioinformatics (UCSC), USA

http://genome.ucsc.edu

Online Mendelian Inheritance in Man, USA (OMIM)

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

RESULTS

1. Characterization of somatic genetic changes in tumor tissue of familial prostate cancer patients (I)

1.1 Relative DNA copy number changes by comparative genomic hybridization (I)

In order to search for the locations of predisposing genes, relative DNA copy number changes were characterized from 21 tumor tissue specimens from 19 Finnish prostate cancer families by comparative genomic hybridization (CGH). Families had at least two first- or second-degree affected relatives. With a cut-off of 1.15 for gains and 0.85 for losses, relative DNA copy number changes were found in 17 out of 21 samples studied (81 %). The mean number of alterations per tumor was 4.0 (± 1.9). Four tumors did not have any DNA copy number changes detectable by CGH. Gains were found in half of the cases with an average number per tumor 2.0 ± 2.1, but no high level amplification (cut-off point 1.5) was detected. Losses were found in 75 % of the cases with the average number per tumor 2.0 ± 2.2. The most common relative DNA copy number alterations, present in at least three cases, were losses of 6q13-q16 (14 %), 8p12-pter (24 %) and 13q14-q22 (29 %) and gains of 19p (25 %), 19q (14 %) and 7q (14 %). Overall the changes in tumors were quite heterogenous, the only chromosome that did not show any copy number changes was chromosome 15.

2. Characterization of the role of the two prostate cancer susceptibility genes, *ELAC2* and *RNASEL*, in Finland (II, III)

2.1 *ELAC2* (II)

ELAC2 is the first prostate cancer susceptibility gene identified by linkage analysis and subsequent positional cloning (Tavtigian et al. 2001). To study the role of *ELAC2* in

predisposition to prostate cancer in Finland 66 genomic DNA samples from the probands of Finnish hereditary prostate cancer families were screened for mutations in the coding exons and exon/intron boundaries. Seventeen sequence variants, four exonic and 12 intronic and one from 3´-UTR, were found. Of the exonic variants three were missense mutations, Ser217Leu, Ala541Thr and a novel Glu622Val, and one was silent. No truncating mutations were found. To determine the frequencies of the three missense variants they were genotyped from altogether 1365 individuals, including probands of 41 additional prostate cancer families, 467 unselected prostate cancer cases, 223 benign prostate hyperplasias and 568 male blood donors as controls.

The frequencies of Ser217Leu and Ala541Thr did not differ significantly between hereditary prostate cancer cases (42.1 %, 7.5 % respectively), unselected prostate cancer cases (47.8 %, 7.5 %), and controls (54.6 %, 7.4 %). No association was found for the combination of the two variants. However, the novel variant Glu622Val was found to be significantly more frequent (OR= 2.94; 95 % CI 1.05-8.23) in unselected prostate cancers (3.0 %) than in controls (1.0 %), and the trend became even stronger when only cases without positive family history were examined (3.5 %; OR= 3.45; 95 % CI 1.23- 9.66). There were no differences between the ages at diagnosis of the carriers or non-carriers in patients with unselected prostate cancer. Interestingly, the Ala541Thr variant was slightly more frequent in the cases of benign prostate hyperplasia (BPH) samples than in controls (OR= 1.73; 95 % CI 1.04-2.87). Three missense variants were also analyzed for the disease phenotype, including tumor grade, Gleason score, T-stage and M-stage in unselected prostate cancer cases. No effect of clinical or pathological features on the prostate cancer risk by *ELAC2* was found.

2.2 *RNASEL* (III)

RNASEL has been proposed as a second potential predisposition gene for prostate cancer found by linkage analysis (Carpten et al. 2002). To determine the significance of *RNASEL* in prostate cancer susceptibility in Finland, mutations of *RNASEL* coding exons and exon/intron boundaries were screened in 66 probands from hereditary prostate cancer families. Seven sequence variants were found in the SSCP analysis, including a previously found nonsense mutation, Glu265X, and four missense mutations, Gly59Ser, Ser406Phe,

Arg462Gln and Asp541Glu and two silent variants. No additional changes were found in conformational sensitive gel electrophoresis analysis.

The most interesting variant, Glu265X, was then genotyped in probands of an additional 50 prostate cancer families, 492 unselected prostate cancer patients, 223 benign prostate hyperplasia patients and 566 healthy male blood donors for the association analysis. All Glu265X carriers found were heterozygotes. There was no association for either unselected prostate cancers or benign prostate hyperplasias. However, in the stratified analysis of hereditary prostate cancer families, the frequency of Glu265X was significantly higher (OR= 5.85; 95 % CI 1.20– 28.87) in patients from prostate cancer families having four or more affected members (9.5%) than in controls (1.8 %), whereas no statistically significant association was observed in families with fewer affected members. Indicative evidence for co-segregation with the disease was seen only in a single family with three affected members, where two affected brothers were carriers, an unaffected brother was a non-carrier, with no sample available from the affected father. In other families having Glu265X mutation, only one affected member carried the mutation. However, in all Glu265X families the median age at diagnosis for mutation carriers was 11 years lower than in patients from the same families who were not carriers (p= 0.07). Of the four missense variants, Gly59Ser was found to be in strong linkage disequilibrium with Glu265X.

In addition to the 66 patients from hereditary prostate cancer families the frequencies of the Ser406Phe, Arg462Gln and Asp541Glu were determined in 167 unselected prostate cancer patients and 176 healthy male blood donors for the association analysis. Gln462 homozygotes were found to be associated with prostate cancer families (OR= 1.96; 95 % CI 0.9 – 4.0). Again, the strongest association was found in families with at least four affected members but no segregation was observed. The other missense variations did not associate with any of the groups studied. In addition, no significant results were obtained in stratification by T-stage, M-stage, and tumor grade among patients with unselected prostate cancer.

3. Search for new Finnish prostate cancer predisposition loci (IV)

3.1 Fine-mapping of 3p25-p26 and 11q14 regions

A Finnish genome-wide linkage analysis (Schleutker et al. 2003) indicated two chromosomal regions, 3p25-p26 and 11q14, as candidates for further analysis. For the fine-mapping of these two regions, a set of 17 markers for chromosome 3p25-p26 and 22 for chromosome 11q14 were genotyped from 229 persons belonging to 16 multiplex prostate cancer families.

For the best marker in the previous genome-wide linkage analysis (D11S901 at 11q14; Schleutker et al. 2003), FASTLINK two point LOD score in the fine-mapping was 3.21 (θ = 0.0). However, the flanking markers were only slightly positive. The maximum GENEHUNTER multipoint HLOD was 1.42 near marker D11S4950. The estimated proportion of the linked families (α) was 0.50. GENEHUNTER multipoint NPL score was 2.25 at the same position (p= 0.0170).

At 3p25-p26 the best FASTLINK two-point LOD score was 2.26 (θ = 0.0) at marker D3S2426. Two proximal markers (D3S1297 and D3S3525) and two distal markers (D3S1307 and D3S1270) of D3S2426 gave also positive results. The best GENEHUNTER multipoint HLOD score was 3.39 at position 2.53 cM proximal from D3S4559 (α=0.89). The GENEHUNTER multipoint non-parametric linkage (NPL) score was 2.92 (p=0.0035). Four of the six families with the best HLOD scores at 3p26 tended to aggregate on the west coastal area of Finland.

3.2 Mutation screening of potential candidate genes of 3p26

The fine-mapping of 3p25-p26 pinpointed an approximately 2 cM area covering the most positive LOD score region around marker D3S4559 for the location of the possible predisposing gene. Known genes located near this area are presented in Figure 6.

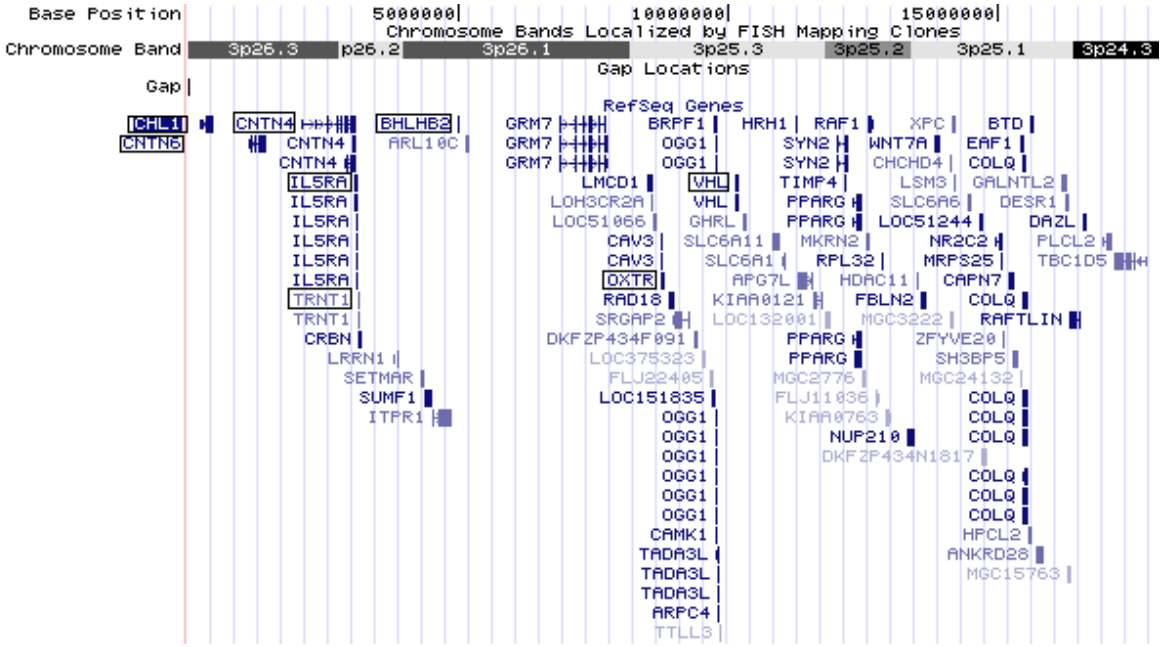

Figure 6. Known genes at 3p24.3-p26.3 (University of California Santa Cruz Genome Bioinformatics at http://genome.ucsc.edu/). The eight out of the ten genes selected for the mutation screening are marked with rectangles. Two genes, *FHIT* and *CDC25A*, not presented here locate proximal to 3p24.3.

From these genes, ten were selected (unpublished data for 8 genes) for mutation screening (Table 4). Seven genes, *CHL1* (*cell adhesion molecule close homolog to LICAM1*), *CNTN6 (contactin 6*), *CNTN4 (contactin 4)*, *IL5RA (interleukin 5 receptor alpha)*, *TRNT1 (tRNA nucleotidyl transferase, CCA-adding, 1)*, *BHLHB2 (basic helix-loop-helix domain containing class B 2*) and *OXTR (oxytocin receptor*), were selected because of their location in the area of best linkage. *CHL1* and *CNTN6* were scored highest based on their location exactly at the peak of multipoint HLOD scores (Study IV). Two genes, *FHIT (fragile histidine triad gene)* and *CDC25A (cell division cycle 25A)*, were selected because they had been suggested in a recent abstract of a linkage study (Ding et al. 2003) as candidate genes for prostate cancer predisposition at 3p. The Von Hippel-Lindau gene (*VHL*) at 3p25 was chosen to be tested because it is the target gene behind the Von Hippel-Lindau hereditary cancer syndrome, which has been associated with several cancers, including renal cell carcinoma, pheo and extra-adrenal paragangliomas (Lonser et al. 2003).

Table 4. Ten genes selected for the mutation screening in the six families showing best multipoint linkage to 3p26.

Gene	No coding exons	Alias	Location	OMIM
CHL1	26	*cell adhesion molecule with homology to L1CAM*	3p26.1	607416
CNTN6	22	*contactin 6*	3p25-p26	607220
CNTN4	22	*contactin 4*	3p25-p26	607280
IL5RA	10	*interleukin 5 receptor, alpha*	3p25-p26	147851
TRNT1	7	*tRNA nucleotidyl transferase, CCA-adding, 1*	3	-
BHLHB2	5	*basic helix-loop-helix domain containing, class B, 2*	3p26	604256
OXTR	2	*oxytocin receptor*	3p25	167055
VHL	3	*von Hippel-Lindau syndrome gene*	3p25-p26	193300
CDC25A	15	*cell division cycle 25A; protein-tyrosine-phosphatase*	3p21	116947
FHIT	5	*fragile histidine triad gene*	3p14.2	601153

Genomic DNA samples from altogether 18 persons were selected to be screened, representing the six families with the best family multipoint HLOD scores (> 0.5) at 3p26 (Table 5). Coding exons as well as exon/intron boundaries of 10 genes were screened with direct sequencing. No truncating mutations but six missense variations from five genes were found; one from *CHL1 (*Leu17Phe), *CNTN6* (Thr867Ala), *IL5RA* (Ile129Val) and *BHLHB2* (Gln113His), and two from *OXTR* (Ala218Thr and Ala238Thr).

Table 5. Family multipoint heterogeneity LOD (HLOD) scores from the peak region on chromosome 3p26.

Family	HLOD	3p26 linked families (HLOD >0.5)
2001	0.555	X
2015	0.244	
2045	0.299	
2062	0.589	X
2102	0.301	
2109	0.594	X
2176	0.299	
2215	0.294	
2232	0.300	
2236	0.327	
2238	0.589	X
2264	0.293	
2279	0.589	X
2291	-0.561	
2292	0.592	X
2308	-0.397	

To determine the frequencies of these six missense variations in unselected prostate cancers and controls, the variations were genotyped by minisequencing from 200 unselected prostate cancers and 200 healthy male blood donors. The frequencies of the missense variants in hereditary prostate cancer cases (n=18), and unselected prostate cancer cases (n=200) and controls (n=200) as well as the results from the association tests between unselected prostate cancer cases and controls are shown in Table 6. No significant associations were observed with any of the missense variants and prostate cancer.

Table 6. Frequencies of the six missense mutations in hereditary prostate cancer cases, unselected prostate cancer cases and controls (unpublished data).

Gene	**Nucleotide**	**Genotype**	**Controls (%) n=200**	**HPC[a] (%) n=18**	**Unselected PRCA [b](%) n=200**	**OR**	**95% CI**	**p-value**
CHL1	49	C	111 (55.5)	13 (72.2)	108 (54.0)	1.00	...	...
		CT	79 (39.5)	3 (16.7)	76 (38.0)	0.99	0.66 - 1.49	0.957
		T	10 (5.0)	2 (11.1)	16 (8.0)	1.64	0.72 - 3.78	0.242
		CT+T	89 (44.5)	5 (2.8)	92 (46.0)	1.06	0.72 - 1.58	0.763
CNTN6	2599	A	200 (100)	17 (94.4)	200 (100)	-		
		AG	-	1 (5.6)	-	-		
IL5RA	385	A	112 (56.0)	11 (64.7)	126 (63.0)	1.00	...	...
		AG	73 (36.5)	6 (33.3)	63 (31.5)	0.77	0.50 - 1.17	0.219
		G	15 (7.5)	1 (5.6)	11 (5.5)	0.65	0.29 - 1.48	0.306
		AG+G	88 (44.0)	7 (38.9)	74 (37.0)	0.75	0.50 - 1.12	0.154
BHLHB2	339	G	200 (100)	16 (88.9)	200 (100)	-		
		GT	-	2 (11.1)	-	-		
OXTR	652	G	137 (68.5)	12 (66.7)	136 (68.0)	1.00	...	...
		GA	58 (29.0)	5 (27.8)	60 (30.0)	1.04	0.68 - 1.61	0.852
		A	5 (2.5)	1 (5.6)	4 (2.0)	0.81	0.21 - 3.07	0.752
		GA+A	63 (31.5 %)	6 (33.3)	64 (32.0 %)	1.02	0.67 - 1.60	0.914
	712	G	196 (98.0)	16 (88.9)	191 (95.5)	1.00	…	…
		GA	4 (2.0)	2 (11.1)	9 (4.5)	2.31	0.70 - 7.62	0.170

[a]HPC= hereditary prostate cancer

[b]PRCA= prostate cancer

DISCUSSION

1. Relative DNA copy number changes in familial prostate cancer (I)

A comparative genomic hybridization (CGH) analysis of 21 paraffin embedded primary prostate cancer samples was performed to characterize somatic genetic changes of patients from prostate cancer families. Most of the cases (81 %) showed relative DNA copy number changes. Losses were more common (75 %) than gains (50 %), which has also been the tendency among sporadic primary prostate tumors (Visakorpi et al. 1995). At the time of the study no prostate cancer susceptibility genes had been cloned from the linked areas. Most of the samples showed no changes at the seven confirmed susceptibility loci. Yet, in a few tumors changes were seen at the 1p region including the *CAPB* locus (gains in 2 out of 19 samples) and at Xq region including the *HPCX* (losses in 2/19 samples). Changes were also seen at 8p region including the *MSR1* gene (losses in 5/19 samples) and at 17p region including the *ELAC2* gene (losses in one sample and gains in two out of 19 samples).

Several CGH studies on sporadic primary prostate tumors (Joos et al. 1995; Visakorpi et al. 1995; Sattler et al. 1999; Wolter et al. 2002a; 2002b) have been conducted. In the largest study of 50 primary prostate adenocarcinomas (Wolter et al. 2002a) the most common losses were at 13q, 8p, and 6q and gains at 17q, 20q and 9q. The most common losses found in that study are the very same as those observed in our analysis. Gains of 17q and 9q were detected as well but they were somewhat less common in our study. The second largest study contains the results of 31 Finnish sporadic primary prostate cancers (Visakorpi et al. 1995). The results were very similar to those in our study of familial cancers, with most common losses of 6q, 8p and 13q as well as the most common gain at chromosome 19.

In addition to our study, only one CGH study on familial prostate cancer cases has been published (Verhagen et al. 2000). In both studies the number of cases per analysis has been relatively small, reflecting the difficulties in obtaining tissue samples of familial cancers. In the study by Verhagen et al. (2000) 19 paraffin embedded tissue samples from familial prostate carcinoma and seven sporadic prostate carcinoma specimens were analyzed. The most commonly observed changes were losses of portions of 3p, 7q, 10q, 11q and 16q and

gains of 4q, 8q, 21q and portions of X chromosome. While the most common changes seem different compared to our study, the overall picture is similar. The only changes not detected at all in our analysis were losses of 3p and 7q and gains of X chromosome. Verhagen et al. (2000) reported three novel changes, loss of 3p12-p22 and 6p21.1-p22 and gain of 6q11-q21, which seemed to be restricted to *HPC1* or *HPCX* –linked cases or both. None of these changes was observed in our patient set.

No differences specific to familial prostate cancer cases were found in our study, suggesting that the genetic progression events in familial and sporadic prostate cancer may be similar. The finding is different from those published on other hormone-dependent cancers, such as breast cancer, where prominent differences between sporadic and hereditary cases were revealed, both for *BRCA1* and *BRCA2* (*breast cancer genes 1 and 2*) carriers (Tirkkonen et al. 1997) as well as non-carriers (Kainu et al. 2000). Similar studies will be possible in prostate cancer only after prevalent disease-causing mutations have been identified.

2. Contribution of *ELAC2* and *RNASEL* in prostate cancer predisposition in Finland (II, III)

The original *ELAC2* study by Tavtigian et al (2001) was followed by altogether 14 studies on *ELAC2* variants and two meta-analyses (Camp and Tavtigian 2002; Severi et al. 2003). In five studies, including ours (Study II), a mutational screening of *ELAC2* was performed (Wang et al. 2001; Xu et al. 2001a; Shea et al. 2002; Takahashi et al. 2003), but in nine studies only the two common polymorphisms were analyzed (Rebbeck et al. 2000; Suarez et al. 2001; Vesprini et al. 2001; Fujiwara et al. 2002; Meitz et al. 2002; Suzuki et al. 2002; Adler et al. 2003; Severi et al. 2003; Stanford et al. 2003). Only one study was able to find an additional deleterious mutation, Glu216X, of *ELAC2* (Wang et al. 2001) and none were able to detect 1641insG reported in the original study (Tavtigian et al. 2001).

In six studies, evidence in favor of an association of the Ser217Leu and Ala541Thr or their joint effect (Rebbeck et al. 2000; Suarez et al. 2001; Fujiwara et al. 2002; Adler et al. 2003; Stanford et al. 2003; Takahashi et al. 2003), with sporadic prostate cancer has been proposed. Eight studies including our study (Study II), were negative. In our study, Thr541

was found to be associated with benign prostate hyperplasia, which has also been studied by Takahashi et al. (2003), however, with negative results. One of the supportive studies associates Leu217 with less aggressive prostate cancer (Stanford et al. 2003) and another Thr541 with late-age at onset disease (Adler et al. 2003). A meta-analysis of six studies (Camp and Tavtigian 2002) indicated that Thr541 either alone or with Leu217 allele is significantly associated with prostate cancer, while another meta-analysis of eight studies obtained negative results (Severi et al. 2003). All studies found Thr541, the less common allele, to be in very strong linkage disequilibrium with Leu217, which makes it essentially impossible to distinguish the effects of Thr541 alone from the joint effect of the two missense changes Leu217 and Thr541 allele. In our study the novel missense mutation of *ELAC2*, Glu622Val, was associated with unselected prostate cancer. The variant seems to be specific to Finnish population since it has not been observed in other populations. Because of the low frequency of the variant there is not enough power to detect its true effect within this material.

Based on the present evidence, it seems that *ELAC2* is not a high-penetrant susceptibility gene for prostate cancer, as it was first classified. In fact, given the LOD scores and the chromosomal area it is quite surprising that it was even found by linkage analysis and positional cloning and it may also be possible that it is not the target gene for linkage in this region. It has been suggested to be able to promote tumorigenesis through irregular cell division (Korver et al. 2003) but the possible role of *ELAC2* in the causation of prostate cancer seems to be restricted to specific populations and its deleterious mutations are very rare.

In the original study of *RNASEL* two segregating truncating mutations, Met1Ile and Glu265X, were found (Carpten et al. 2002). Met1Ile has not been observed in subsequent studies, but Glu265X has been found in one study besides ours (Study III; Chen et al. 2003b). In our study an association of Glu265X with hereditary prostate cancer but no segregation were observed (Study III). A novel founder mutation 471delAAAG was detected in unselected Ashkenazi prostate cancer patients (Rennert et al. 2002; Kotar et al. 2003), but no significant difference between unselected cases and controls was seen. Yet, carriers of 471delAAAG tended to be diagnosed at a younger age than non-carriers, just as Glu265X in our study (Rennert et al. 2002). 471delAAAG has not been found in other populations but was recently observed in LNCap cell line (Nupponen et al. 2004).

Confirmatory studies have focused on the common variants of *RNASEL*, and most of them are in favor of an association with prostate cancer (Casey et al. 2002; Wang et al. 2002; Chen et al. 2003b; Nakazato et al. 2003; Xiang et al. 2003) but negative reports also exist (Rennert et al. 2002; Kotar et al. 2003). An association of Arg462Gln with prostate cancer has been observed in three studies (Casey et al. 2002; Wang et al. 2002; Nakazato et al. 2003). In our study Gln462 homozygotes tended to be associated with prostate cancer (Study III), suggesting a recessive effect. Gln462 has been implicated to be involved in up to 13 % of all prostate cancers (Casey et al. 2002) and to have only one third of the enzymatic activity of the normal allele as well as inability to cause apoptosis (Casey et al. 2002; Xiang et al. 2003). Men who are heterozygous for it have been suggested to have 1.5 times greater risk than non-carriers and for homozygotes the risk is more than two fold. Recently, inactivating mutations of *RNASEL* in sporadic prostate tumors were reported to be exceedingly rare (Nupponen et al. 2004). In summary, although some studies suggest only a minor role, a greater number of studies provide functional and epidemiological support for the claim that *RNASEL* plays a role in hereditary prostate cancer predisposition.

RNASEL is a uniquely regulated endoribonuclease that requires 5´-triphosphorylated, 2´5´-linked oligoadenylates (2-5A) for its activity. It functions in the molecular pathways of interferon action against viral infections. The cell with a defective *RNASEL* allele loses its ability to break down RNA, and may avoid apoptosis, which is triggered by RNA degradation. Carpten et al. (2002) speculate that this pathway may be particularly responsive to androgen. Studies on the role of viruses in the etiology of prostate cancer are few and the results inconclusive. Association of human herpesvirus 8 (Hoffman et al. 2004), human papilloma virus 33 (Adami et al. 2003), and human cytomegalovirus (Sanford et al. 1977; Boldogh et al. 1983) with prostate cancer have been suggested. Several viruses have been found on the same tissue sample, suggesting a possible joint effect (Zambrano et al. 2002). Support for an infectious etiology comes from the association of macrophage scavenger 1 (*MSR1*) with prostate cancer (Xu et al. 2002). Null mice for *MSR1* have shown enhanced susceptibility to bacterial and viral pathogens (Thomas et al. 2000).

Our results for *ELAC2* and *RNASEL* suggest that their variants associate with unselected prostate cancer and hereditary prostate cancer respectively. However, neither alone is

sufficient for the formation of prostate cancer families. Co-segregation of *ELAC2* or *RNASEL* variants with prostate cancer has been detected world-wide in only few families. Results from the Finnish population suggest that they may possibly act as modifier genes, or if independent effect exists it would be low-penetrant. In the Finnish population *ELAC2* seems to increase the risk for prostate cancer at the population level and *RNASEL* acts in prostate cancer families by lowering the age at onset. The results of the Finnish genome-wide linkage analysis support this minor role of *ELAC2* and *RNASEL* (Schleutker et al. 2003), because no significant LOD score peak was obtained at 17p11 or at 1q25. In addition, in earlier linkage analyses, *HPC1* linkage seemed not to be important in Finland (Schleutker et al. 2000; Xu et al. 2001b). However, the number of affected members in the Finnish prostate cancer families is much smaller than in the North American *HPC1* study (Smith et al. 1996), which may also indicate different etiology behind smaller and larger families.

3. Fine-mapping of 3p25-p26 and 11q14 in hereditary prostate cancer families and mutation screening of specific candidate genes at 3p26 (IV)

The difficulties in cloning the hereditary prostate cancer genes, together with the large number of proposed loci, indicate that the genetics of hereditary prostate cancer is more complex than the genetics of hereditary cancers at many other sites. Confounding factors are frequency of sporadic cases and late age at onset. There are particular difficulties in pursuing candidate loci for prostate cancer through linkage studies. These are the gender specificity of the disease, which limits information within a family, possible locus heterogenity within and between different populations, making the joint analysis of many kindreds or many populations potentially confusing. As evidence accumulates in favor of a genetic heterogeneous etiology of prostate cancer, the power of subset identification becomes acknowledged. Also, population isolates with a limited number of founders tend to have less mutational heterogeneity and an increased frequency of founder effects, which makes them particularly useful in positional cloning studies.

The motivation for the first genome-wide linkage analysis using only Finnish families (Schleutker et al. 2003) was the negative results of other previously reported linkage loci other than *HPCX* in Finnish families (Schleutker et al. 2000). About 60 % of Finnish

families were not linked to any of the loci studied (Schleutker et al. 2000). From the Finnish genome-wide linkage analysis of 10 multiplex hereditary prostate cancer families two previously unreported regions, 3p25-p26 (LOD score 2.57, $\theta = 0.01$ at D3S1297) and 11q14 (LOD score 2.97, $\theta = 0.0$ at D11S901), were the most promising (Schleutker et al. 2003). Therefore, fine-mapping with additional markers and families of these regions was performed. Meanwhile the collection of prostate cancer families had succeeded in composing six new multiplex prostate cancer families, and the number of families in the fine-mapping was increased to 16. The number of markers with approximately 0.1 – 1.0 cM spacing was increased to 17 at 3p25-p26 and 22 at 11q14.

Region 11q14, which gave the best LOD score in the original genome-wide analysis, obtained lower multipoint HLOD scores than in the original analysis (1.42 vs. 2.08). This may be due to a false positive result in the original analysis. It has been estimated that peaks with LOD score over two should occur at a rate of approximately 0.8 per genome scan, assuming an infinitely dense marker map (Lander and Kruglyak 1995). Additional explanations are that there are inconsistencies in the order of the markers in the 11q map. Two-point analysis is not sensitive to map locations of markers like multipoint analysis. This is because in two-point analysis the disease locus is tested with one marker at a time, whereas in multipoint analysis the disease locus is slid along the fixed marker map. A strong two-point LOD score 3.21 at D11S901 with no support from the flanking markers can be seen as supportive of the inconsistencies of the map, although it can also be seen as indicating false positive results. In the fine-mapping the distance between markers is very narrow and determining the order of the markers is not unambiguous. The order of the markers was based on three different genetic maps, which on some occasions gave different locations for the same markers, therefore the re-analysis of the region continues. In CGH analysis (Study I) two out of 19 samples had a loss of 11q, but the minimal region of involvement was located at 11q22-q23, distal from the best region of the linkage analysis. Loss of 11q was also a common change in another CGH study of familial prostate tumors (Verhagen et al. 2000), but the minimal region of involvement was not reported.

On the contrary to 11q14 case, the addition of families and markers strengthened the evidence for linkage to chromosome 3p25-p26 (multipoint HLOD score 3.39 vs. 2.35). The families with best multipoint LOD scores tended to aggregate on the west coastal area of Finland, suggesting a possible founder effect, although no clear shared haplotypes between

linked families were detected at this marker density (approximately 1.0 cM). Previously, Finnish founder effects have helped in the cloning of many single gene disorders (Peltonen 1997; Kere 2001). Most recently, Finnish founder effect proved its power in the cloning of the first asthma gene (Laitinen et al. 2004), for which patients from a restricted area of Kainuu in Finland were analyzed demonstrating the usefulness of this phenomenon, also in complex diseases. Chromosome 3p loss has frequently been associated with various epithelial cancers, such as breast cancer (Yang et al. 2002), ovarian cancer (Leary et al. 1993), primary lung cancer (Whang-Peng et al. 1982), non-small cell lung carcinomas (Thiberville et al. 1995) and head and neck tumors (Li et al. 1994). Allelic imbalance at 3p (Cunningham et al. 1996) and LOH at 3p24-p26 have been reported in prostate cancer (Dahiya et al. 1997). The 3p loss was also reported as a specific somatic change for familial prostate cancers by CGH analysis (Verhagen et al. 2000), although the minimal region of involvement was located at 3p12-p22, proximal from our region of interest. In addition, there is some linkage evidence of susceptibility gene at 3p in the North American population (Ding et al. 2003; Xu et al. 2003a). These facts supported the results obtained from our linkage and fine-mapping analysis and suggested that either a single or a few tumor suppressor genes reside at 3p. Therefore, it was reasonable to continue with the mutation screening of the positional candidate genes of the narrowed region of 3p.

From the refined area of about two cM (equal to about two million base pairs), which still contained dozens of genes, ten genes (data from 8 genes unpublished) were selected for mutation screening. Six missense mutations from five genes were found, but no statistically significant associations of the variants with prostate cancer were observed. As there are no obvious candidate genes in the region, all genes near the best LOD score peak need to be screened for mutations. In addition to coding exons and exon/intron boundaries, the regulatory elements need to be carefully investigated in order to rule out a gene with a high probability. Sometimes also intronic areas may also prove to be important, which was the case in the Finnish study of adult-type hypolactasia, where the associated change was located in the intron region (Enattah et al. 2002).

The mutation screening of additional candidate genes at 3p26 is in progress. Most importantly, an SNP (short nucleotide polymorphism) mapping of the best region of linkage at 3p, with over 200 SNPs at approximately 5 kb intervals, is in progress and will be followed by association analyses. Material for an LOH study on tumors of patients from

3p -linked families is also being collected. In addition, a Finnish segregation analysis will be performed in the near future and, if recessive mode is predicted, the linkage results of the fine-mapping will be re-analyzed with a recessive model in addition to the dominant model used in this study.

This is the first attempt to exploit the homogenous Finnish population in the search for high-penetrant genes responsible for prostate cancer susceptibility. Genes identified in the Finnish population would most likely also have a role in other populations, although their effect may vary in more mixed genetic pools. Genetic information on prostate cancer susceptibility and development would enhance cancer prevention, diagnosis and possibly cure in the future.

SUMMARY AND CONCLUSIONS

Family history is one of the most prominent risk factors for prostate cancer. Inherited genetic defects may exert a major influence in five to ten percent of prostate cancers. The aim of this study was to identify high-penetrant susceptibility loci for hereditary prostate cancer in Finland. Identification of the genes responsible for the increased cancer risk in inherited prostate cancer may also elucidate the development as well as the mechanisms of sporadic prostate cancer.

In a CGH study of 21 familial prostate tumors no DNA copy number changes specific to familial cases were detected. The results suggest that the progression of familial and sporadic cases proceeds in a similar manner and may involve the activation and inactivation of the same genes.

Based on the studies of two putative susceptibility genes, *ELAC2* and *RNASEL*, it seems that neither alone is sufficient for the formation of prostate cancer families. However, a novel mutation Glu622Val of *ELAC2* was associated with unselected prostate cancer, indicating a role of *ELAC2* variants at the population level. A truncating variant Glu265X of *RNASEL* was associated with large prostate cancer families. No segregation with prostate cancer in hereditary prostate cancer families was observed, but it was demonstrated to lower the age at onset of the mutation carriers by approximately 11 years. All in all the role of *ELAC2* and *RNASEL* in hereditary prostate cancer in Finland seems quite small. Their effect may be direct but low-penetrant or they may act as modifier genes for some other predisposing loci.

A recently completed genome-wide linkage analysis pinpointed two novel chromosomal regions, 3p25-p26 and 11q14, that may contain predisposing genes for hereditary prostate cancer in Finland (Schleutker et al. 2003). Fine-mapping of 3p25-p26 indicated it to be the most valuable region for follow-up in Finland. Ten genes from 3p26 were screened for mutations, however, no statistically significant associations of the variants with prostate cancer emerged. The results indicate that although no deleterious mutations were found in coding exons and exon/intron boundaries of a set of ten candidate genes, the fine-mapping succeeded in considerably narrowing down the most positive linkage region at 3p. There is a strong possibility that 3p26 region contains predisposing gene or genes important for Finnish hereditary prostate cancer, and this genetic region deserves further study.

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ORIGINAL COMMUNICATIONS