EIJA SEPPÄLÄ

Heterogeneity in Genetic Susceptibility to Prostate Cancer

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ACADEMIC DISSERTATION
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YHTEENVETO

Eturauhassyövän geneettinen monimuotoisuus

Eturauhassyöpä on nykyisin miesten yleisin syöpä. Lisäksi eturauhassyöpä on toiseksi yleisin syöpäkuolemien syy keuhkosyövän jälkeen. Taudin syyt ja riskitekijät tunnetaan kuitenkin huonosti. Mono- ja ditsygoottisten kaksosten syöpäriskitutkimus osoittaa, että perimä saattaa selittää eturauhassyövän synnystä suuremman osan (42 %) kuin minkään muun yleisen syövän etiologiasta. Aluksi eturauhassyövälle altistavan geneettisen riskin arveltiin johtuvan siitä, että mies on perinyt harvinaisen, mutta voimakkaasti altistavan geenimutaation. Myöhemmät tutkimukset ovat kuitenkin osoittaneet, että ainakin osa perheisiin kasaantuneista eturauhassyöpätapauksista selittyy todennäköisesti useiden geenien polymorfioiden yhteisvaikutuksella. Näissä tapauksissa yhden mutaation aiheuttama tautiriski on pienempi, ja vasta useiden, kenties kymmenien polymorfismien yhteisvaikutus viimekädessä johtaa syövän syntyyn. Tämän työn kokonaistavoitteena oli tunnistaa eturauhassyövän riskitekijöitä, ja lisätä tietoamme syövän synnystä ja kehityksestä. Tavoitteena oli myös tuoda esiin uutta tietoa, joka tulevaisuudessa voi ehkä mahdollistaa nykyistä paremman syövän diagnostiikan, hoidon ja ehkäisyn; esim. auttamalla seulomaan riskihenkilöt ajoissa seurannan pariin.

Kirjallisuudessa kuvatut eturauhassyöpään assosioituvat lokukset ovat *HPC1* (1q24-q25), *PCAP* (1q42-q43), *HPCX* (Xq27-q28), *CAPB* (1p36), *HPC20* (20q13), *HPC2* (17p11) ja 16q23. Eturauhassyöpään kytkeytyviltä kromosomialueilta on tunnistettu toistaiseksi vain *ELAC2*-geeni *HPC2*-lokuksessa, *RNASEL*-geeni *HPC1*-lokuksessa sekä *MSR1*-geeni uudesta eturauhassyöpään kytkeytyvästä lokuksesta 8p22-23. *RNASEL*- ja *ELAC2*-geenien merkitys eturauhassyövän synnyssä on jatkotutkimuksissa osoittautunut luultua vähäisemmäksi; myöskään Suomessa ne eivät selitä eturauhassyövän kasautumista perheisiin. Tässä tutkimuksessa selvitettiin *MSR1*-geenin osuutta eturauhassyövän synnyssä. Suomalainen aineisto koostui eturauhassyöpäperheisiin kuuluvista henkilöistä, valikoimattomista eturauhassyöpäpotilaista sekä terveistä verrokeista. Tutkimustulokset viittaavat siihen, että myöskään *MSR1*-geenin mutaatiot eivät ole voimakkaasti syövälle altistavia geenimuutoksia. Havaittiin kuitenkin, että Arg293X-mutaatio voi vaikuttaa sairauden kulkuun alentamalla sairastumisikää.

Ns. alhaisen penetranssin geeneistä eniten on tutkittu androgeenireseptoria ja muita hormonaalisen solukasvuun liittyviä geenejä. Androgeenit säätelevät eturauhassolujen kasvua, erilaistumista ja solukuolemaa. Geneettiset tekijät, jotka muuttavat solujen hormonaalista ympäristöä ja järkyttävät siten solusäätelyn tasapainoa, voivat lisätä eturauhassolujen mahdollisuutta muuttua syöpäsoluiksi. Useiden androgeenin signaalinvälitysreitin geenien polymorfioiden onkin osoitettu assosioituvan eturauhassyöpään. Näitä ovat mm. androgeenireseptorin, 5α-reduktaasin ja *CYP17A1*-geenien polymorfiat. Kyseiset tutkimukset on usein suoritettu maissa, joiden etninen tausta on hyvin kirjava, jolloin saattaa syntyä valikoitumisvirheitä. Lisäksi tutkitut potilasryhmät ovat

usein olleet hyvin pieniä. Tässä työssä tutkittiin androgeenien tuotantoon ja metaboliaan liittyvien geenien roolia eturauhassyövässä analysoimalla 10 geeniä (*SRD5A2*, *HSD3B1*, *HSD17B2*, *HSD17B3*, *AKR1C3*, *CYP19A1*, *CYP17A1*, *KLK3*, *HSD3B2* and *CYP11A1*) suuresta näytejoukosta (yhteensä 1891 näytettä). Havaittiin, että *CYP19A1* Thr201Met muutos on yhteydessä ns. kliinisesti merkityksettömään syöpään eli syöpään, joka on yhä eturauhaskapselin sisäpuolella ja jonka solut ovat säilyttäneet erilaistumisasteensa. *CYP17A1* -34T>C-polymorfia puolestaan assosioitui astetta vakavampaan syöpään, eli syöpään joka on myös kapselin sisällä, mutta jonka solut ovat menettäneet erilaistuneen ulkomuotonsa. Lisäksi havaittiin, että *KLK3* -252A>G polymorfia ei yksistään aiheuta syöpää, mutta kun miehellä on samanaikaisesti *CYP19A1* Thr201Met-mutaatio, on hänen eturauhassyöpäriskinsä kohonnut. Samassa työssä tutkittiin myös kahden ennalta tunnetun geenivariantin osuutta eturauhassyövässä. Kumpikaan näistä, androgeenireseptorin Arg726Leu-mutaatio tai luteinisoivan hormonin Ile15Thr-polymorfia, ei lisännyt suomalaisen miehen riskiä sairastua eturauhassyöpään.

Androgeenien toimintaan liittyvien geenien lisäksi on nykyvuosina tutkittu muitakin eturauhassyövän ehdokasgeenejä. Tällaiset geenit koodaavat proteiineja, jotka osallistuvat mm. DNA:n korjaukseen, solusyklin säätelyyn, tulehdusreaktioihin, verisuonten muodostumiseen ja lääkeaineiden metaboliaan. Tässä työssä tutkittiin *CHEK2*-geenin variaatioita, joiden on aikaisemmin osoitettu altistavan rintasyövälle. Normaalilla CHEK2-proteiinilla on tärkeä rooli solussa DNA:n vaurioituessa. Se osallistuu solusyklin tarkastuspisteiden toimintaan, jotka takaavat sen, että vaurioittunut solu ei pääse jakaantumaan ennen kuin DNA on korjattu; ja tarvittaessa säädeltyyn solukuolemaan. Havaittiin, että sekä 1100delC- että Ile157Thr-variaatiot assosioituvat eturauhassyöpäperheisiin, joissa on vain kaksi sairastunutta miestä. Tällaiset perheet ovat hyvin yleisiä, joten *CHEK2*-geenin muutokset voivat olla merkittäviä populaatiotasolla. Tulokset tukevat väitettä, että *CHEK2* on alhaisen penetranssin geeni, jonka muutokset altistavat rintasyövän lisäksi eturauhassyövälle. Yhdessä osatyössä tutkittiin *KLF6*-geenissä sijaitsevan intronialueen polymorfian osuutta eturauhassyövän synnyssä. Kyseisen polymorfian on osoitettu lisäävän vaihtoehtoisen silmukoinnin määrää ja näin syntyvä proteiinimuoto toimii päinvastoin kuin normaali KLF6-proteiini kiihdyttäen solukasvua. Normaalia *KLF6*-geeniä pidetään ns. syövänestäjägeeninä, joka säätelee solun kasvua, jakaantumista ja erilaistumista. Päinvastoin kuin aikaisemmin on julkaistu, tässä työssä ei havaittu minkäänlaista yhteyttä *KLF6*-geenivariantin ja eturauhassyövän välillä. Syöpänäytteiden lisäksi analysoitiin DNA-näytteitä potilailta, joilla esiintyy eturauhasen hyvälaatuista liikakasvua. Havaittiin, että tässä potilasjoukossa *KLF6*-geenivariantin osuus oli kaikista suurin, mutta ero verrokkinäytteisiin ei ollut tilastollisesti merkitsevä.

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List of original communications

This thesis is based on the following communications, which are referred to in the text by their Roman numerals:

I. **Seppälä E.H.**, Ikonen T., Autio V., Rökman A., Mononen N., Matikainen M.P., Tammela T.L.J. and Schleutker J., Germ-line alterations in *MSR1* gene and prostate cancer risk.
Clinical Cancer Research, 2003; 9:5252-6.

II. **Seppälä E.H.**, Ikonen T., Mononen N., Autio V., Rökman A., Matikainen M.P., Tammela T.L.J., Schleutker J., *CHEK2* variants associate with hereditary prostate cancer.
British Journal of Cancer, 2003; 89:1966-70.

III. **Seppälä E.H.**, Ikonen T., Autio V., Tammela T.L.J., Schleutker J., *KLF6* variant IVS1 -27G>A and the risk of prostate cancer in Finland.
European Urology, in press

IV. Mononen N.*, **Seppälä E.H.***, Duggal P., Autio V., Ikonen T., Ellonen P., Saharinen J., Saarela J., Vihinen M., Tammela T.L., Kallioniemi O., Bailey-Wilson J.E., Schleutker J., Profiling genetic variation along the androgen biosynthesis and metabolism pathways implicates several single nucleotide polymorphisms and their combinations as prostate cancer risk factors.
Cancer Research, 2006; 66:743-7.
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Publication IV is also included in the thesis of Nina Mononen (Polymorphisms in genes associated with androgen biosynthesis and metabolism as risk factors for human prostate cancer, Acta Universitatis Tamperensis 1108, Tampere University Press 2005)

Abbreviations

AKR	Aldo-keto reductase protein family
AKR1C3	3α-hydroxysteroid dehydrogenase type 2
Ala	Alanine
APC	Annual percent change
APC	Adenomatosis polyposis coli
AR	Androgen receptor
ARE	Androgen responsive element
Arg	Arginine
Asn	Asparagine
ASO	Allele specific nucleotide
Asp	Aspartic acid
ATM	Ataxia telangiectasia mutated
BPH	Benign prostate hyperplasia
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
CAPB	Prostate cancer/brain cancer susceptibility locus
CASP8	Caspase 8
CDH1	E-cadherin
CHEK2	Checkpoint kinase 2
CI	Confidence interval
Cy5-dCTP	Cyanine-5-labeled deoxycytidine tripfosphate
Cy5-dUTP	Cyanine-5-labeled deoxyuridine tripfosphate
CYP11A	Cytochrome P450, family 11, subfamily A (cholesterol desmolase)
CYP17A1	Cytochrome P450, family 17, subfamily A1 (Steroid 17α-hydroxylase/17,20 lyase)
CYP19A1	Aromatase
Cys	Cysteine
dATP	Deoxyadenosine triphosphate
ddATP	Dideoxyadenosine triphosphate
ddGTP	Dideoxyguanosine triphosphate
del	Deletion
dGTP	Deoxyguanosine triphosphate
DHT	Dihydrotestosterone
DNA	Deoxyribonuclease acid
dNTP	Deoxyribonucleotide
EDTA	Ethylenediaminetetraacetic acid

ELAC2	elaC (E. coli) homolog 2 (tRNase Z 2)
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FAP	Familial adenomatous polyposis
FHA	Forkhead-associated domain
FRR	Familial relative risk
FSH	Follicle stimulating hormone
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GSTM1	Glutathione S-transferase M1
GSTP1	Glutathione S-transferase P1
GSTT1	Glutathione S-transferase T1
H^3	Tritium
HCl	Hydrochloric acid
HGPIN	High grade prostatic intraepithelial neoplasia
His	Histidine
HLOD	Heterogeneity logarithm of odds
HNPCC	Hereditary nonpolyposis colorectal cancer
hOGG1	8-oxoguanine DNA glycosylase
HPC	Hereditary prostate cancer
HPC1	Hereditary prostate cancer 1
HPC2	Hereditary prostate cancer 2
HPC20	Hereditary prostate cancer 20
HPCX	Hereditary prostate cancer X
HRAS1	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
HSD17B2	17b-hydroxysteroid dehydrogenase type 2
HSD17B3	17b-hydroxysteroid dehydrogenase type 3
HSD3B1	3β-hydroxysteroid dehydrogenase type 1
HSD3B2	3β-hydroxysteroid dehydrogenase type 2
ICPCG	International consortium for prostate cancer genetics
IL-10	Interleukin 10
IL-1RN	Interleukin 1 receptor antagonist
Ile	Isoleusine
ins	Insertion
IRAK4	Interleukin-1 receptor-associated kinase 4
IVS	Intron
k_{cat}	Turnover number of the enzyme
KLF6	Krüppel-like factor 6
KLK3	Kallikrein 3 (prostate specific antigen)
K_m	The concentration of substrate that leads to half-maximal velocity
LDOC1	Leucine zipper, down-regulated in cancer 1
Leu	Leusine

LGPIN	Low grade prostatic intraepithelial neoplasia
LH	Luteinizing hormone
LHB	Luteinizing hormone beta polypeptide
LHR	Luteinizing hormone releasing hormone
LOD	Logarithm of odds
LOH	Loss of heterozygosity
Lys	Lysine
Met	Methionine
MLH1	mutL (E. coli) homolog 1
MMLV	Moloney Murine Leukemia Virus
MnSOD	Manganese superoxide dismutase
mRNA	Messenger RNA
MSH2	mutS (E. coli) homolog 2
MSH6	mutS homolog 6 (E. coli)
MSR1	Macrophage scavenger receptor 1
MTA3	Metastasis associated 3
MTHFR	5,10-methylenetetrahydrofolate reductase (NADPH)
NaCl	Natrium Cloride
NaOH	Natrium hydroxide
OR	Odds ratio
p	Short arm of the chromosome
PCAP	Predisposing for prostate cancer
PCR	Polymerase chain reaction
Phe	Phenylalanine
PIA	Proliferative inflammatory athropy
PIN	Prostatic intraepithelial neoplasia
PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)
Pro	Proline
PSA	Prostate specific antigen
PTGS2	Prostaglandin-endoperoxide synthase 2
q	Long arm of the chromosome
RNA	Ribonuclease acid
RNASEL	Ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)
RR	Relative risk
Ser	Serine
SHBG	Sex hormone binding globulin
SIFT	Sorting intolerant from tolerant -program
SNP	Single nucleotide polymorphism
SPANX	Sperm protein associated with the nucleus, X-linked
SR-A	Class A machrophage scavenger receptor
SRD5A2	Steroid 5α-reductase type 2
SSCP	Single-strand conformation polymorphism
SV1	Splice variant 1
TGFβ	Transforming growth factor β
Thr	Threonine

TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR1	Toll-like receptor 1
TLR10	Toll-like receptor 10
TLR5	Toll-like receptor 5
TLR6	Toll-like receptor 6
TNM-stage	Tumor, Node, Metastasis -stage
TP53	Tumor protein p53
tRNA	Transfer RNA
Trp	Tryptophan
Tyr	Tyrosine
UTR	Untranslated region
Val	Valine
VDR	Vitamin D receptor
V_{max}	The maximum enzyme velocity
X	Stop codon
XRMV	Gammaretrovirus related to xenotropic murine leukemia viruses

Abstract

The Finnish Cancer Registry estimates that there will be about 5485 new cases of prostate cancer in Finland in 2006. Over 800 men will die of the disease. Even though most men die with prostate cancer rather than from it, yet physicians are unable to determine which men are at greatest risk of developing clinically apparent prostate cancer. Older age, African ancestry and a positive family history of prostate cancer have long been recognized as important risk factors, yet we are only at the early stage of unravelling the complex genetic and environmental influences on this disease. The characterization of genetic alterations which are in the germline and predispose to prostate cancer will enable the identification of individuals at elevated risk, and may provide an insight into the pathogenesis of the disease. Several putative loci identified by genetic linkage have been reported to exist on chromosomes 1 (*HPC1*, *PCAP*, and *CAPB*), X (*HPCX*), 17 (*HPC2*), 20 (*HPC20*) and 8, with genes *RNASEL* (*HPC1*), *ELAC2* (*HPC2*) and *MSR1* (8p22-23) tentatively defined. In this dissertation, to further evaluate the role of *MSR1* in prostate cancer susceptibility in Finland, sequence variants of *MSR1* were studied in familial and unselected prostate cancer patients. In addition to family-based approaches to identify the rare high penetrant susceptibility genes, studies of common polymorphisms of genes related to the metabolism and biosynthesis of androgens and other steroids have suggested that variation in these genes may affect an individual's risk for prostate cancer. Here the findings of a large association study that included ten androgen pathway genes and over 1800 samples are reported. In addition to genes encoding products that play a role in androgen stimulation of the prostate, the role of *CHEK2* and *KLF6*, putative tumor suppressor genes in prostate cancer were studied. The *CHEK2* gene, recently identified as a breast cancer susceptibility gene, could also be a candidate gene for prostate cancer susceptibility. *KLF6* IVS1 –27G>A variant was reported to enhance alternative splicing of the *KLF6* tumor suppressor gene and to be associated with increased prostate cancer risk. An attempt was made to confirm these findings in Finnish population.

Introduction

The incidence of prostate cancer has increased markedly in recent decades. It is now the most prevalent noncutaneus cancer in males in developed regions of the world. The two strongest predictors of increased risk for prostate cancer, apart from age and ethnicity, are the presence of several affected first-degree relatives and an affected brother who had an unusually early age at onset (Keetch et al. 1995). There is also excellent evidence from twin studies that this familial risk has an inherited basis (Page et al. 1997). In 1993, it was suggested that 5% to 10% of incident cases are attributable to rare, highly penetrant dominant alleles in single gene forms of the disease (Carter et al. 1993). Based on this assumption several linkage analyzes were performed during the following decade. Researchers found evidence of linkage of prostate cancer to several loci including 1q24-25, 1q42.2-43, 1p36, 8p22-23, 17p11, 20q13, and Xq27-28. However, most of these findings could not be confirmed in other studies. At the identified loci, some putative prostate cancer susceptibility genes have been studied: *RNASEL* for 1q24-25, *ELAC2* for 17p11 and *MSR1* for 8p22-23 (Schaid 2004). The few mutations found in the families analyzed have not allowed a clear definition of the involvement of mutations of these genes in susceptibility to hereditary prostate cancer.

In 2001, it was suggested that the genetic basis of prostate cancer is not explained by independent, rare autosomal dominant mutations but rather by recessive and/or multiple interacting loci (Risch 2001). Further evidence supporting multiple interacting genes was provided by a new segregation analysis (Conlon et al. 2003). Therefore the most recent analyzes, both in the field of linkage and association studies, have considered gene-gene interactions in addition to assessing the main effects on prostate cancer risk for each sequence variant or loci.

In parallel with linkage analyzes, many candidate gene studies have been performed. At first the association studies concentrated in the genes involved in the metabolism of testosterone and other androgens such as the *AR* gene encoding the androgen receptor that is involved in androgen binding and transport, the *SRD5A2* gene encoding steroid 5α-reductase type 2 that converts testosterone into the more potent androgen dihydrotestoserone, the *CYP17A1* gene, whose enzyme product regulates steps in testosterone biosynthesis, and the two genes of the *HSD3B* gene family which encode 3β-hydroxysteroid dehydrogenases that are involved in the metabolism of dihydrotestoserone in the prostate, as well as the catalysis of testosterone biosynthesis (Schaid 2004). Nowadays the candidate gene studies have expanded to cell-cycle control-related

genes, as well as genes involved in immune response, inflammatory change, and the extracellular matrix, genes involved in drug and xenobiotic metabolism and genes involved in DNA repair and genome stability. These genes most likely involve more common, low- to moderate-penetrance alleles. In addition, environmental exposures are important in prostate cancer etiology and may interact with underlying genetic susceptibility to determine both the risk of developing prostate cancer and clinical features of the disease.

The purpose of this study was to confirm the role of *MSR1* as a prostate cancer susceptibility gene and to investigate whether genetic variation in several candidate genes affects prostate cancer risk in Finland.

Review of the literature

1. Inheritable factors in common cancers

Most genetic alterations that lead to cancer are somatic and are found only in an individual's cancer cells. However, a small fraction of all cancers is associated with inherited predisposition to cancer (Ponder 2001). The association of cancer risk with genetic status can result basically from two kinds of mechanisms (Table 1.). First of all, genetic predisposition associated with a very high risk can explain inherited cancer syndromes. The second genetic mechanism associated with familial cancers may result from genetic susceptibility via individual or ethnic polymorphisms. The effect on individual risk is then moderate to weak.

Table 1. *Inherited predisposition to cancer. Data modified from Ponder 2001.*

	Contribution to overall cancer incidence	Clinical features	Frequency of predisposing alleles	Effect on individual risk
Inherited cancer syndromes	1-2%	Rare cancers or combination of cancers. Mendelian dominant inheritance	Rare (1:1000 or less)	Strong: lifetime risk up to 50-80%
Familial cancers	Up to 10% depending on definition	Families with several cases of common cancers. Generally dominant inheritance	Uncommon to common	Moderate to weak
Predisposition without evident family clustering	No precise figure possible; substantial fraction of cancer incidence within predisposed population	Single cases of cancer at any site, some with one or two affected relatives.	Multiple common alleles	Weak

Table 2. shows the estimated values for the heritability of four common cancers obtained from cohort or twin studies. In the first study, Goldgar et al. (1994) estimated familial relative risks (FRR) from the Utah Population Database by identifying all cases of cancer in first-degree relatives of 35,228 cancer probands. In the second study, Dong and Hemminki (2001) used the Swedish Family Cancer Database to estimate FRR. The study population included 4,225,232 parents and 5,520,756 offspring. 435,000 (10.3%) of the

parents and 71,424 (1.3%) of the offspring had cancer. In both studies, the most common cancers are characterized by moderate risk ratios. Lichtenstein et al. (2000) combined data on 44,788 pairs of twins listed in the Swedish, Danish, and Finnish twin registries in order to assess the risks of cancer at 28 anatomical sites for the twins of individuals with cancer. In terms of heritability, prostate cancer was placed first among the common cancers, colorectal cancer was second and breast cancer third (Table 2.).

Table 2. *Heritability of four common cancers*

Cancer type	Study 1[a] Family risk ratio	Study 2[b] Family risk ratio	Proportion of variance due to heritable factors[c]
Lung	2.55	1.68	0.26
Colorectal	2.54	1.86	0.35
Prostate	2.21	2.82	0.42
Breast	1.83	1.86	0.27

[a]Goldgar et al. (1994), the ratios shown here were in part recalculated by Risch (2001)

[b]Dong and Hemminki (2001) , the ratios shown here were recalculated by Risch (2001)

[c]Lichtenstein et al. (2000)

1.1. Colorectal cancer

Of all common cancers, colorectal cancer is probably best characterized in terms of genes affected by cancer-causing mutations, their normal functions and their carcinogenic effects when mutated (de la Chapelle 2004). High-penetrance mutations confer predisposition to hereditary nonpolyposis colorectal cancer (HNPCC, also known as Lynch syndrome) and in familial adenomatous polyposis (FAP). Together these conditions account for 5% or less of all cases of colorectal cancer. Lynch syndrome involves mutations in mismatch-repair genes *MLH1*, *MSH2*, *MSH6* and *PMS2* with a penetrance of approximately 80% for colorectal cancer, 60% for endometrial cancer, and below 20% for other cancers (Lynch, de la Chapelle 2003). Using family history and age at onset as a criteria for Lynch syndrome, the proportion of all colorectal cancer caused by Lynch syndrome is 5% (Abdel-Rahman, Mecklin & Peltomäki 2006). However, only half of traditionally defined Lynch syndrome cases carry a mutation in the mismatch-repair gene. Therefore, 2.5% of all colorectal cancers are calculated to be caused by Lynch syndrome (Abdel-Rahman, Mecklin & Peltomäki 2006). In FAP, the mutations are located in the *APC* tumor suppressor gene. The penetrance of this syndrome is 100%, but the proportion of all colorectal cancer cases is just 0.2%, assuming an incidence of 1:10,000 for FAP and a lifetime risk of 1:20 for colorectal cancer (Potter 1999, de la Chapelle 2004).

Low penetrance susceptibility genes, such as tumor suppressor *TGFβ*, account for a high proportion of all attributable risks of colorectal cancer in both

familial and sporadic cases (de la Chapelle 2004). Carriers of six alanines (*6Ala) in the polyalanine repeat of exon 1 of *TGFβ* gene have a 20% increased risk (OR=1.20, 95% CI 1.01-1.43) of colorectal cancer (Pasche et al. 2004). The most common genotype contains nine alanines (*9Ala). The *6Ala-allele is so common in Caucasian population (with a carrier frequency of 14%) that its contribution to the total burden of colorectal cancer in the population is relatively high, in spite of its markedly modest relative risk (Pasche et al. 2004). Several other common, low-penetrance alleles affecting colorectal cancer have been proposed. To assess the evidence that any of these confers a risk, Houlston and Tomlinson (2001) performed a systematic review and meta-analysis of 50 published studies concerning 13 genes. Significant associations were seen for only three polymorphisms – *APC* Ile130Lys, *HRAS1* variable number tandem repeat polymorphism and *MTHFR* Val677Val.

1.2. Breast cancer

Breast cancer is the most frequent carcinoma in women. Linkage studies and positional cloning led to the identification of breast-ovarian cancer susceptibility genes *BRCA1* and *BRCA2* (Miki et al. 1994, Wooster et al. 1995). Based on early linkage analyzes, *BRCA1* and *BRCA2* germline mutations were estimated together to account for the great majority of breast-ovarian cancer families (Easton et al. 1993). However, germline mutations of *BRCA1* and *BRCA2* have been detected in 20% of breast cancer families (Wooster, Weber 2003), implying that the contribution of germline mutations to hereditary breast cancer predisposition is not as high as originally estimated. In Finland, *BRCA1* and *BRCA2* germline mutations have been detected in 11% and 9% of families with three or more cases of breast or ovarian cancer in first- or second degree relatives (Vehmanen et al. 1997, Vahteristo et al. 2001a). Mutations in *BRCA1/BRCA2* account for only 2-3% of all breast cancers (Ford, Easton & Peto 1995, Newman, Millikan & King 1997).

The recent discovery of 1100delC mutation in *CHEK2* gene validated the idea that there are common variants that confer an appreciably enhanced risk of breast cancer (Vahteristo et al. 2002). Segregation analysis estimated that *CHEK2* 1100delC conferred an increased risk of breast cancer of approximately two-fold in noncarriers of *BRCA1/2* mutations (Meijers-Heijboer et al. 2002). This risk has been confirmed in the collaborative analysis of over 10,000 breast cancer cases and matched controls (CHEK2 Breast Cancer Case-Control Consortium. 2004). *CHEK2* 1100delC is, therefore, not a high penetrance mutation, but rather a relatively common variant conferring a more moderate risk of breast cancer. The *CHEK2* 1100delC variant does not appear to increase the risk of breast cancer in carriers of *BRCA1* or *BRCA2* mutations, possibly reflecting functional interactions between the three genes (Meijers-Heijboer et al. 2002, CHEK2 Breast Cancer Case-Control Consortium. 2004).

The residual inherited susceptibility to breast cancer may be partly due to rare mutations in one or few additional major breast cancer-susceptibility genes with either dominant or recessive mode of inheritance (Antoniou et al. 2001, Cui et al. 2001). In addition, several common, low-penetrance alleles with multiplicative effects on breast cancer risk must exist, and they have been proposed to be responsible for a large fraction of hereditary breast cancers (Antoniou et al. 2002). *TGFβ* might be an example of such a gene. Pasche et al. (2004) reported that *6Ala carriers have a 38% increased risk of breast cancer and 41% increased risk of ovarian cancer. Therefore, its contribution to the total burden of breast and ovarian cancers in the Caucasian population seems to be even higher than to colorectal cancer.

1.3. Prostate cancer

Results from segregation analyzes suggest that familial clustering of prostate cancer can be best explained by transmission of a rare hereditary factor accounting for 5-10% of total prostate cancer cases (Carter et al. 1993). In addition, two large twin studies reported higher prostate cancer concordance rates for monozygotic twins versus dizygotic twins, suggesting a strong genetic influence on risk (Page et al. 1997, Lichtenstein et al. 2000). Therefore, the search for prostate cancer susceptibility genes by linkage studies offered early hope that finding genes would be as easy as it was for breast and colorectal cancer. However, this hope has been diminished by the difficulty of replicating promising regions of linkage (Nupponen, Carpten 2001, Schaid 2004). A major dilemma in prostate cancer genetics is the assignment of the correct modes of inheritance for familial prostate cancer. Some cases of prostate cancer are due to an autosomal susceptibility locus with an allele or alleles that collectively behave in a dominant and age-dependent fashion (Carter et al. 1992, Grönberg et al. 1997a, Schaid et al. 1998). Other investigators have argued either for recessive or X-linked mode of inheritance (Monroe et al. 1995, Pakkanen et al. submitted). One reason for unsuccessful linkage studies is the high prevalence of phenocopies. When the sporadic cases are analyzed as affected individuals, but they do not share the disease locus with the hereditary cases in the family, linkage results are substantially diminished. However, the evidence also points toward a much more complex genetic basis of prostate cancer than initially anticipated. A segregation study in 263 prostate cancer families found that the disease is more likely due to the contributions of two to four prostate cancer susceptibility genes than one gene (Conlon et al. 2003). Risch (2001) applied a new analysis method to twin study data provided by Lichtenstein et al. (2000). Similarly, Schaid et al. (2004) reanalyzed the results of Page et al. (1997). The new results suggest that the genetic basis of prostate cancer is not explained by independent, rare, autosomal dominant mutations but rather by recessive and/or multiple interacting loci (Schaid 2004). Furthermore, the modifier genes and environmental factors can influence the phenotype of both high and low penetrance genes (de la Chapelle 2004). Our current understanding of gene-gene

and gene-environment interactions is limited and should be improved before a full understanding of predisposition to common cancers is can be achieved.

2. Pathological findings of the prostate

The healthy adult prostate is nearly the same size and shape as a chestnut. It is located in front of the rectum, just below the bladder, and wraps around the urethra. The organ is made up of four regions: three glandular zones (transition, central and peripheral zone) and fibromuscular stroma (McNeal, 1981). Though its function is not fully understood, the prostate produces 20% of the seminal fluid as well as other substances that may facilitate sperm motility and penetration.

2.1 Benign prostate hyperplasia

Histologically, benign prostate hyperplasia (BPH) is characterized by overgrowth of the epithelium and fibromuscular tissue of the transition zone and periurethral area. At the cellular level, BPH contains alterations including basal cell hyperplasia, increased stromal mass, enhanced extracellular matrix deposition, reduced elastic tissue, more infiltrating lymphocytes around ducts, and acinar hypertrophy (Bostwick et al. 1992). The prevalence of histological BPH increases rapidly with age. More than half of men in their sixties and as many as 90% in their seventies and eighties have some symptoms of BPH. The age specific prevalence is remarkably similar in populations throughout the world (Bostwick et al. 1992). The development of BPH requires the production of testosterone. In men, testosterone is synthesized in large amounts, primarily by the Leydig cells of the testes. Testosterone is transported to the prostate where it is irreversibly metabolised to dihydrotestosterone (DHT). This reaction is catalysed by an enzyme called 5α-reductase. Elevated prostate dihydrotestosterone concentrations, increased 5α-reductase activity in the hypertrophic prostate, and prostate atrophy following castration all suggest a significant role for testosterone and dihydrotestosterone in the pathogenesis of BPH (Geller 1989). BPH usually responds to androgen-deprivation treatment (Bostwick et al. 2004). Based on histologic evidence and anatomic locations, BPH is currently not considered a precursor lesion for prostate cancer (Miller & Torkko, 2001).

2.2 Prostatic intraepithelial neoplasia

Prostatic intraepithelial neoplasia (PIN) is defined as cellular proliferations within the epithelium of the prostatic ducts, ductules, and acini. PIN was

originally graded from 1 to 3, but current recommendations recognize two grades (low grade and high grade). High grade PIN (HGPIN) refers to architecturally benign prostatic acini and ducts lined by atypical cells (Epstein, Herawi 2006). These atypical cells share morphological, histochemical, immunohistochemical and genetic changes with cancer. In addition, HGPIN is often multifocal (Qian, Wollan & Bostwick 1997). However, unlike cancer, HGPIN lacks invasion of the basement membrane of the prostatic glands. Most patients with HGPIN will develop carcinoma within ten years (Bostwick, Qian 2004). HGPIN does not need to be present, however, for carcinoma to develop. Low grade PIN (LGPIN) has milder deviations from the normal cells and it is often difficult to distinguish histologically from BPH. LGPIN is not always documented in pathology reports (Epstein, Herawi 2006).

2.3 Prostate cancer

Ninety-five per cent of prostate cancers are adenocarcinomas originating from the epithelial cells of the glandular tissue. Seventy per cent of the adenocarcinomas originate from the peripheral zone of the prostate, whereas 20% of the cancers arise from the transition zone and the rest 5-10% rise from central zone (McNeal 1981). The prostatic epithelium is composed of three distinct cell populations: secretory luminal, basal, and neuroendocrine cells. Bonkhoff and Remberger (1996) proposed a stem cell model for the prostate, suggesting that a small population in the basal cell layer gives rise to all epithelial cell lineages encountered in normal, hyperplastic and neoplastic prostate. More recent evidence supports the hypothesis that prostate cancer arises from malignant transformation of transiently proliferating/amplifying cell population, which serves as an intermediate between the undifferentiated stem cells of the basal layer and the highly differentiated secretory cells of the lumen (Shalken, van Leenders 2003).

Prostate cancer can be present as an asymptomatic latent entity that is diagnosed only on histologic examination. A latent form can be identified in approximately 30% of men over the age of 50 and 60% to 70% of men over the age of 80 (Pienta, Goodson & Esper 1996). Alternatively, prostate cancer may present clinically with an elevated serum prostate specific antigen (PSA) or a palpable nodule on digital rectal examination without any other symptoms. This disease may also present symptomatically with complaints ranging from low urinary track symptoms to severe bone pain as a result of metastasis. With increasing awareness and routine PSA testing, a remarkable migration in the clinical presentation of the disease has occurred in the past 20 years (Mannuel, Hussain 2005). An increasingly greater proportion of men are diagnosed with clinically organ-confined disease. In parallel, the incidence of men presenting with clinically metastatic disease has decreased. However, as yet there is no conclusive data to confirm that early detection will decrease disease-specific morbidity and mortality. The Laval University/Quebec Screening Trial conducted from 1988 to 1998 reported a 69% reduction in prostate cancer

mortality in screened men (Labrie et al. 1999). This trial was criticized for low statistical power, possible selection bias, and a short observation period of four to seven years. Two large-scale, randomized prostate cancer screening trials, The European Randomised Study of Screening for Prostate Cancer and the Prostate, Lung, Colorectal, and Ovary Cancer Screening Trial in the US are in progress, but conclusive mortality analyzes are not expected until 2008-2010 (Auvinen et al. 1996, Prorok et al. 2000, Mäkinen et al. 2004).

Prostate adenocarcinoma is characterized by four distinctive features. First, prostate cancer tends to be slow growing, with a typical doubling time of three to four years (Friberg, Mattson 1997). This slow growth rate most likely accounts for the extended latency of the prostate cancer. Second, prostate cancer is remarkably age-related, rarely appearing before age 40 years and typically identified in men around 70 years (Pienta, Goodson & Esper 1996). Prostatic carcinogenesis starts in the second to third decade of life and may require over 50 years for progression to pathologically detectable metastatic disease (Berges et al. 1995). This suggests that prostate cancer results from an accumulation of genetic damage, perhaps due to oxidative stress or other endogenous or exogenous factors (Bostwick et al. 2004). Third, prostate cancer usually is multifocal (Arora et al. 2004), so that most men have prostate cancers instead of a just one cancer. Similarly, the likely precursor HGPIN is usually multifocal and often in intimate spatial association with cancer (Qian, Wollan & Bostwick 1997). Finally, prostate cancer is heterogeneous in its morphology and genotype (Nwosu et al. 2001, Arora et al. 2004). This remarkable heterogeneity suggests that multiple pathways and perhaps multiple mechanisms lead to prostate cancer.

3. Epidemiology of prostate cancer

3.1 Trends in incidence and mortality

Excluding basal and squamous cell cancers of the skin, prostate cancer is the most common malignancy diagnosed in the USA and most western countries, and its incidence is rising rapidly in most countries, including low risk populations (Hsing, Tsao & Devesa 2000). An estimated 234,460 new cases will occur in the USA during 2006 (American Cancer Society 2006). As shown in Figure 1., in the USA the incidence of prostate cancer had been increasing for some time; however, from 1989 to 1992 it increased, on average, 16.4% per year, reaching the peak incidence of 237.7 per 100,000 men in whites in 1992 and 342.4 per 100,000 in blacks in 1993 (Ries et al. 2005). Since 1993 a decreasing incidence trend, at a rate of 11.2% a year, has been observed, and in 1995, the incidence was 163.4 per 100,000 among whites and 278.5 among blacks. Since 1995 the incidence of prostate cancer has been modestly increasing among whites and slowly decreasing among blacks with the annual percent

change (APC) from 1995 to 2002 being 1.5 and -0.7, respectively (Ries et al. 2005). The sharp rise in incidence and the subsequent decline observed in the USA are consistent with the effects of introducing PSA screening into the population (Hankey et al. 1999). Widespread screening via PSA measurement became available in 1986, and an increasing number of men are subsequently being diagnosed at earlier stages, when the cancer is still clinically organ confined.

In Finland the incidence of prostate cancer increased slowly from the 1960s to the beginning of the 1990s with an age-adjusted incidence per 100,000 person years increasing from 18.4 to 40.0 (Finnish Cancer Registry 2006). A rapid increase in prostate cancer incidence has been observed since 1991 with age-adjusted incidence per 100,000 men increasing from 43.2 in 1991 to 91.2 in 2002 (Figure 1.). The annual number of prostate cancer cases is still increasing in Finland; in 2004 the age-adjusted incidence was 115.3 per 100,000 men.

With gradual Westernisation, the incidence of prostate cancer has risen by 5-118% in Asian countries during 1978-1982 to 1993-1997 (Sim, Cheng 2005). The increase in incidence was highest among Singaporean Chinese, where the incidence rose from 6.6 per 100,000 person-years to 14.4 per 100,000 person-years.

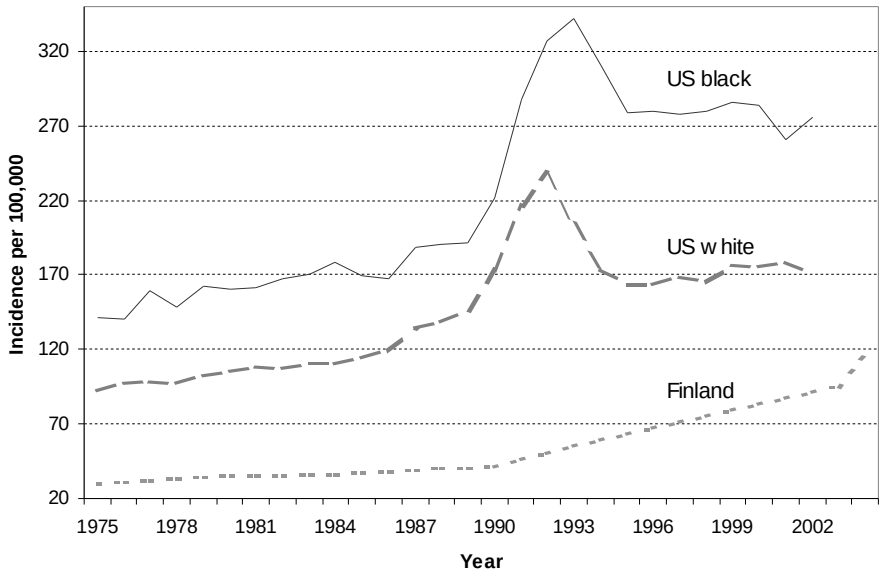

Figure 1. *Trends in prostate cancer incidence in the USA and Finland 1975-2004. Data modified from Finnish Cancer Registry 2006 and Ries et al. 2005.*

With an estimated 27,350 deaths in 2006, prostate cancer is the second leading cause of cancer death in men in the USA, second only to lung cancer. From 1975 until 1987, the rate of increase in prostate cancer mortality in African-Americans (APC 1.9) was roughly twice that for whites (APC 0.8; Ries et al. 2005). There was acceleration in the trend of mortality rates in both whites and blacks in the late 1980s with the APC being 3.1 for whites and 3.4 for

blacks. Prostate cancer mortality in the USA has been declining since 1993, but in relation to the changes in incidence, the magnitude of the mortality decline has been small, from 36.3 deaths per 100,000 men in 1993 to 25.8 deaths per 100,000 men in 2002 among whites and from 81.9 deaths per 100,000 men in 1993 to 63.0 deaths per 100,000 men in 2002 among blacks. Death rates in African-American men remain more than twice as high as death rates in white men.

In Finland prostate cancer mortality has been slowly increasing, reaching its peak of 18.4 deaths per 100,000 men in 1992-1996 (Finnish Cancer Registry 2006). Since then the mortality has declined. In 2004, 5252 men were diagnosed with prostate cancer and 806 died of it. Mortality was 16.0 deaths per 100,000 men.

3.2. Risk factors

In addition to age, the only well-established risk factors for prostate cancer are ethnicity and family history of the disease (Schaid 2004). The evidence for hormones and diet acting as a risk factors is more contradictory (Bostwick et al. 2004). A new hypothesis for the etiology of prostate cancer is that prostate inflammation may initiate and promote prostate cancer development (Nelson et al. 2004).

3.2.1 Family history

Based on family history, one can identify three prostate cancer patient groups: hereditary, familial, and sporadic. Hereditary prostate cancer (HPC) was first described by Carter et al. (1993), who suggested that it accounts for 5% to 10% of all cases of prostate cancer. According to Carter et al. (1993), men with HPC represent families that meet at least one of the following criteria: 1) three or more affected first-degree relatives, 2) prostate cancer occurring in three generations through the paternal or maternal lineage, and/or 3) two first-degree relatives diagnosed at an early age (≤55 years). Until the genes for HPC are cloned, the definition of HPC is based on the pedigree only. Familial prostate cancer does not meet these strict criteria, but it represents families in which there are two first-degree or one first-degree and two or more second-degree relatives with prostate cancer. Familial prostate cancer is estimated to account for 10% to 20% of all cases of prostate cancer (Carter et al. 1993, Stanford, Ostrander 2001). Sporadic prostate cancer signifies that only one man in a family has been diagnosed with prostate cancer.

Men with HPC are diagnosed an average of five to six years earlier than sporadic prostate cancer cases but they do not otherwise differ clinically from the sporadic form (Bratt et al. 2002). Tumor grade and pathological stage at diagnosis do not differ between patients with HPC and those with sporadic

prostate cancer (Bastacky et al. 1995, Valeri et al. 2000, Bratt et al. 2002). HPC is multifocal, but the same is true also for familial and sporadic prostate cancers (Bastacky et al. 1995).

In terms of defined risk factors for prostate cancer, epidemiological studies show that a family history of the disease is strongly and consistently associated with an elevated relative risk (RR). A meta analysis of 11 case-control studies and two cohort studies that reported the risk of prostate cancer according to family history among first degree relatives estimated a pooled RR of 2.5 (95% confidence interval [CI] 2.2-2.8; Johns, Houlston 2003). RR was greater if a brother was affected than if a father was affected. This is consistent with the hypothesis of an X-linked or recessive model of inheritance (Monroe et al. 1995). However, this could be explained by the screening effect leading to over-diagnosis in brothers of cases. The risk for prostate cancer increases as the age of probands decreases, as the closeness and number of affected members in the family increases, or when both factors are considered together (Eeles 1999).

3.2.2 Ethnic origin

The prevalence of small latent cancer at autopsy is constant across countries and ethnic groups (Breslow et al. 1977, Bostwick et al. 2004), but there exists considerable ethnic variation in the incidence of clinically detected prostate cancer. The highest rates are in the USA, Canada, Sweden, Australia and France (48.1-137.0 cases per 100,000 person-years 1988-1992); European countries (Spain, Italy, England, Denmark) have intermediate rates (27.2-31.0 cases per 100,000 person-years), and Asian countries the lowest rates (2.3-9.8 cases per 100 000 person-years; Hsing, Tsao & Devesa 2000). The incidence in Finland is a little higher than in Central-Europe (40.0 per 100,000 person-years in 1987-91; Finnish Cancer Registry). As shown in Figure 2., there are substantial racial differences both in prostate cancer incidence and mortality in the USA. African-Americans have the highest incidence, next come whites, then Hispanic, and Asian/Pacific Islanders (Weir et al. 2003). The lowest incidence in the USA is among natives of Alaska. Correct incidence rates from Africa have been difficult to obtain, but recent studies show that incidence rates are comparable to those of African-American men (127 per 100,000 in Nigeria and 304 per 100,000 in Jamaica; Osegbe 1997, Glover et al. 1998). Differences in prostate cancer risk by ethnic origin may reflect three factors: differences in exposure, such as dietary differences; differences in detection (including clinical practice patterns and screening methods); and genetic differences. The observations that prostate cancer risk increases when Japanese migrate to Hawaii (Maskarinec, Noh 2004) or to Los Angeles (Shimizu et al. 1991) suggests that diet and environmental differences play a major role. There is, however, consistent evidence across different racial and ethnic groups that a family history increases the risk of prostate cancer (Monroe et al. 1995, Whittemore et al. 1995, Cunningham et al. 2003a). Therefore, genetics is likely to play an important role at least in some forms of prostate cancer.

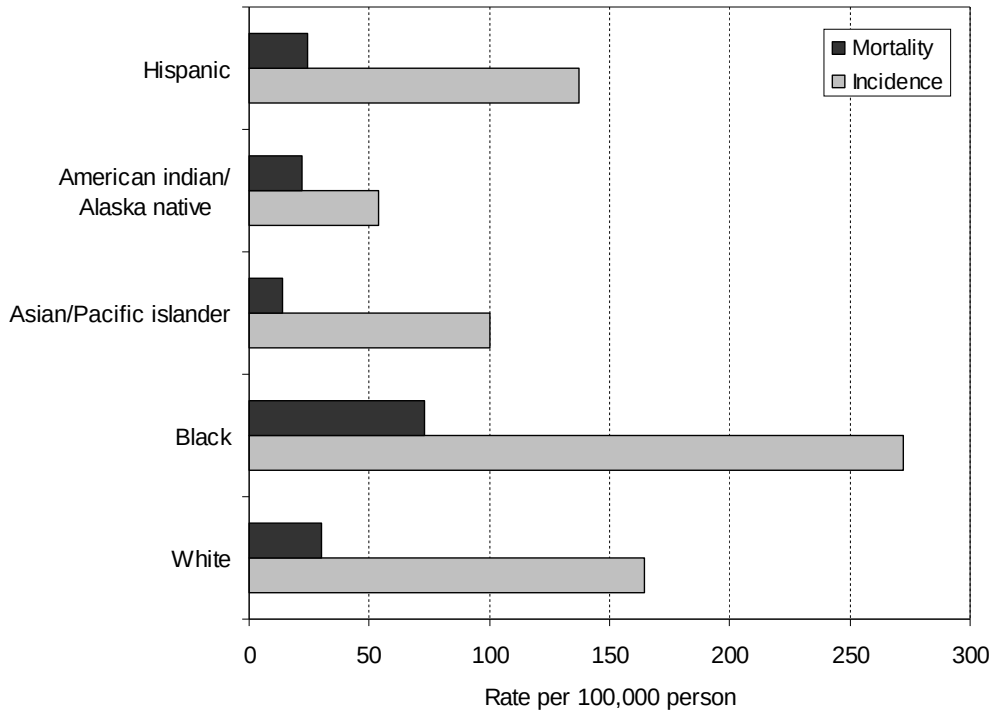

Figure 2. *Prostate cancer incidence and death rates in different US populations 1996-2000. Rates are per 100,000 persons and are age-adjusted to the 2000 US standard population. Data modified from Weir et al. 2003.*

3.2.3 Hormones

Endogenous hormones, especially androgens are required for the growth, maintenance, and function of the prostate, affecting both the proliferation and the differentiation status of the luminal epithelium (Kellokumpu-Lehtinen 1985, Naslund, Coffey 1986). The effect of steroid hormones is mediated through nuclear receptors that bind to DNA sequences named hormone response elements in a ligand-dependent manner. Nuclear receptors repress or stimulate transcription by recruiting corepressor or coactivator proteins in addition to directly contacting the basal transcription machinery (Lee et al. 2001).

Studies of androgens and prostate cancer go back over 60 years, for which Charles Huggins won the Nobel prize for his discoveries concerning the hormonal treatment of prostate cancer in 1966 (Huggins, Hodges 1941). Castration results in the involution of the prostate gland as a result of diffuse atrophy of the luminal epithelial cells, but not the stromal cells (English, Santen & Isaacs 1987). The replacement of androgen results in the proliferation of the epithelial cells, but once normal volume is attained additional androgenic stimulation does not further increase the size of the gland as a result of balance between proliferation and apoptosis (Bruchovsky et al. 1975, Arnold, Isaacs 2002). Withdrawal of testosterone by surgical or medical castration is a well

known treatment for extracapsular prostate cancer in humans (Tammela 2004). This treatment is often successful in reducing the size of metastases and bone pain until androgen independent growth is acquired. Furthermore, there are case reports of prostate cancer in men who used androgenic steroids as anabolic agents or therapy for pituitary dysfunction, suggestive of causal relationship between androgens and prostate cancer (Roberts, Essenhigh 1986, Ebling et al. 1997).

Epidemiologic studies of androgen levels and prostate cancer risk have been inconsistent (Meikle, Smith & West 1985, Nomura et al. 1996). Eaton et al. (1999) performed a meta-analysis from the data of eight prospective studies published during 1966-1998 in order to compare mean serum concentrations of sex hormones in men who subsequently developed prostate cancer with those men who remained cancer-free. There was no evidence that the serum concentrations of testosterone or DHT were different between cases and controls. However, all five studies (Gann et al. 1996, Nomura et al. 1996, Guess et al. 1997, Vatten et al. 1997, Dorgan et al. 1998) that measured the DHT metabolite androstanediol glucuronide reported a higher concentration among cases relative to controls with a pooled ratio of 1.05 (95% CI 1.00-1.11). This may reflect an increased conversion of testosterone to DHT within prostatic tissue, resulting in increased cell growth and progression from subclinical tumor foci into a clinically manifest form. More recent studies did not detect an association between serum testosterone, sex hormone binding globulin (SHBG), or androstenedione concentrations and the occurrence of subsequent cancer (Heikkilä et al. 1999, Chen et al. 2003). Chen et al. (2003) also measured the levels of 3α-androstanediol glucuronide, but the concentration did not differ significantly between cases and controls (15.08 nmol/l vs. 13.80 nmol/l; P=0.06).

Meta-analysis of prospective epidemiologic studies tentatively suggest that men who would be predicted to have higher intraprostatic levels of DHT based on higher serum levels of androstanediol glucuronide appear to have a higher risk of prostate cancer (Eaton et al. 1999). This hint of a link is now supported by recent findings from the Prostate Cancer Prevention Trial (Thompson et al. 2003). In that trial, 18,882 healthy men with median age of 63 years were randomised to take finasteride, an inhibitor of 5α-reductase type 2, or placebo for seven years. At the time when the trial was stopped, the period prevalence of prostate cancer was 24% lower in the finasteride group than in the placebo group (Thompson et al. 2003). Because the trial period was so short (slightly less than seven years on average), it is likely that many of the men diagnosed with prostate cancer already had one or more foci at the start of the trial. Thus, the trial indirectly suggests that DHT is at least important in the promotion of the growth of existing small prostate tumors. Interestingly, the period prevalence of high-grade cases (Gleason score 7-10) was greater in the finasteride group than in the placebo group, indicating that low intraprostatic DHT due to finasteride treatment may lead to the loss of differentiation of the prostatic tissue (Thompson et al. 2003). However, it is also possible that finasteride merely altered the visual appearance of the epithelium such that pathologists perceived worse histological patterns (Scardino 2003).

Although the development of prostate cancer is dependent upon androgens, animal studies have suggested that androgens alone are insufficient to induce tumorigenesis. Aromatase knockout mouse, deficient in estrogens and elevated in androgens due to a non-functional aromatase enzyme, developed prostatic hyperplasia, but no malignant changes were detected (McPherson et al. 2001). Excessive exposure to estrogens during critical stages of development or long-term treatment of adult animals with estrogens and androgens leads to prostatic neoplasia (Leav et al. 1988, Bosland, Ford & Horton 1995, Bosland 2000).

Estrogens regulate the development and function of prostate by indirect and direct mechanisms (Härkönen, Mäkelä 2004). The direct effect of estrogen treatment on adult prostate has been best described in rodents. A specific direct response to estrogens is the induction of epithelial squamous metaplasia and it requires estrogen receptor ERα in the prostate (Cunha et al. 2001). Squamous epithelial metaplasia has also been observed in human prostate, detected often after hormonal therapy for prostatic adenocarcinoma (Das et al. 1991, Parwani et al. 2004). Risbridger et al. (2001) showed that transformation of the epithelium involved proliferation of cells with a basal cell phenotype. Aside from direct signaling by estrogen through its steroid receptor, estrogen may influence prostate cancer risk via its mutagenic metabolites. Certain catechol metabolites of estrogen, including 2-hydroxyestradiol and 4-hydroxyestradiol, may be converted in situ into DNA damaging agents (Yager 2000). Estrogens are also believed to have beneficial effects in the prostate. Phytoestrogens, and isoflavones in particular show structural similarities to estradiol and demonstrate a number of anti-carcinogenic properties, including the inhibition of angiogenesis (Fotsis et al. 1993), and tumor cell growth (Geller et al. 1998) although the mechanisms behind these actions are still poorly understood. Furthermore, oral estrogen treatment with diethylstilbestrol used to be the most common hormonal treatment for prostate cancer. However, it was largely abandoned in the 1970s due to its significant thromboembolic and cardiovascular toxicity (Cox, Crawford 1995). The therapeutic effect of estrogen in preventing prostate cancer was mainly obtained indirectly by feedback inhibition of the hypothalamic release of luteinizing hormone (LH)/follicle stimulating hormone (FSH)-releasing hormone (LRH) leading to lowered serum androgen levels (Härkönen, Mäkelä 2004).

The incidence of prostate cancer rises exponentially in elderly men, in whom the ratio of estrogen to androgen increase due to a decline in testicular function and increase in aromatization of adrenal androgens by peripheral adipose tissue during aging (Gray et al. 1991, Griffiths 2000). However, there is no conclusive clinical evidence of a strong correlation between estrogen/androgen ratio and increase in prostate cancer incidence (Gann et al. 1996, Eaton et al. 1999). Eaton et al. (1999) compared the levels of estrogens, luteinizing hormone and prolactin among human prostate cancer cases and healthy controls in their meta-analysis of eight prospective epidemiological studies. No statistically significant differences were seen.

Differences in endogenous sex hormone levels have been hypothesized to explain ethnic differences in prostate cancer risk. According to de Jong et al. (1991), plasma levels of testosterone and estradiol were significantly lower in 258 Japanese men, when compared to 368 Dutch men. Probably as a result of this difference in testosterone levels, the testosterone:SHBG ratio was lower among Japanese men, while DHT:testosterone ratio was higher. In contrast, Wu et al. (1995) showed that the DHT:testosterone ratio was highest in African-Americans, intermediate in whites, and lowest in Asian-Americans, corresponding to the respective incidence rates in these groups. Platz et al. (2000) measured the concentrations of testosterone, DHT, androstanediol glucuronide, estradiol and SHBG in a sample of 43 African-American, 52 Asian and 55 white US male health professionals. In their study steroid hormone levels did not vary appreciably by race. Similarly, Cheng et al. (2005) did not detect any correlation between ethnic background and androgen levels when they examined testosterone and 3α-androstanediol glucuronide levels among Singapore Chinese, African-American, US white, US Latino and Japanese-American men.

3.2.4 Diet and nutrition

A wide variety of dietary factors have been implicated in the development of prostate cancer in prospective intervention, cohort, and case-control studies (Bostwick et al. 2004, Dagnelie et al. 2004). Unfortunately, most of the results are contradictory or inconclusive.

Overall fruit consumption was not associated with prostate cancer risk in several studies (Mills et al. 1989, Hsing et al. 1990, Shibata et al. 1992, Giovannucci et al. 1995), but with an increased risk in some (Schuurman et al. 1998, Chan et al. 2000) and reduced risk in one study (Giovannucci et al. 1998). For individual fruits there was no association except for raisins, dates and other dried fruits, which showed a decreased prostate cancer risk (Mills et al. 1989). The consumption of vegetables is associated with a decreased risk of many cancers (Verhoeven et al. 1996), but for prostate cancer inverse (Hsing et al. 1990) and null (Shibata et al. 1992, Giovannucci et al. 1995, Schuurman et al. 1998, Chan et al. 2000) associations were observed.

Tomatoes and tomato-based products are the main source of lycopene in most of the Western populations. Lycopene is a carotenoid with antioxidant properties. Therefore, its relation to prostate cancer has been widely studied (Giovannucci 2002). A large prospective study in male health professionals found that high intake of tomatoes and tomato products was associated with a 35% lower risk of total prostate cancer, and a 53% lower risk of advanced prostate cancer (Giovannucci et al. 1995). In a large plasma-based study very similar risk reductions were observed (Gann et al. 1999). However, several other studies, mostly dietary case-control studies, do not support the protective effect of lycopene (Key et al. 1997, Cohen, Kristal & Stanford 2000, Kolonel et al. 2000).

For fat and fatty acids, most cohort studies suggest either an increased risk or no relation with prostate cancer (Gann et al. 1994, Harvei et al. 1997). A questionnaire-based study showed an increased risk for intake of alpha-linolenic acid (Giovannucci et al. 1993). In the study by Giovanucci et al. (1993) red meat represented the food group with the strongest positive association with advanced cancer (RR = 2.64; 95% CI = 1.21-5.77), whereas another questionnaire-based study found no association between energy-adjusted intake of total fat, saturated fat, mono-unsaturated fat or poly-unsaturated fat and the incidence of prostate cancer (Veierod, Laake & Thelle 1997).

Retinoids, including vitamin A, help regulate epithelial cell differentiation and proliferation (Sporn, Roberts 1984). β-Carotene and few other carotenoids can be converted to vitamin A. Paganini-Hill et al. (1987) reported a slightly positive association between vitamin A intake and prostate cancer. Some other studies report null association (Shibata et al. 1992, Giovannucci et al. 1995). In one intervention study β-carotene supplementation seemed to reduce prostate cancer incidence in subjects with low baseline plasma β-carotene levels (RR = 0.68, 95% CI 0.46-0.99), but to increase prostate cancer incidence in subjects with high baseline levels (RR=1.33, 95% CI 0.91-1.96; Cook et al. 1999).

Other vitamins investigated include C, D and E. Vitamin C is a scavenger of reactive oxygen species and free radicals (Yu et al. 1994). Maramag et al. (1997) showed that vitamin C inhibits cell proliferation in prostate cancer cell lines. However, data from prospective cohort studies show no consistent effect (Shibata et al. 1992, Giovannucci et al. 1995). Vitamin E (α-tocopherol) is an antioxidant that inhibits prostate cancer cell growth in vitro through apoptosis (Sigounas, Anagnostou & Steiner 1997). The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study reported a 32% decrease (95% CI -47%- -12%) in the incidence of prostate cancer among the subjects receiving 50 mg α-tocopherol daily for 5-8 years (n = 14564) compared with those not receiving it (n = 14569) (Heinonen et al. 1998). Similarly, mortality decreased by 41% (95% CI -65% - -1%). This is supported by a serum-based cohort study (Helzlsouer et al. 2000). In contrast, vitamin E intake from food (Giovannucci et al. 1995, Schuurman et al. 2002) and supplements (Shibata et al. 1992, Chan et al. 1999) showed no association with prostate cancer risk.

The hypothesis that vitamin D protects against the risk of prostate cancer is based on evidence that the vitamin D endocrine system regulates prostate growth and differentiation, that black skin colour and residence in northern latitudes, risk factors for prostate cancer, are potentially associated with low circulating levels of vitamin D (Schwartz, Hulka 1990). In two studies, the lowest risk of prostate cancer occurred in men with high 1-25-dihydroxyvitamin D and low 25-hydroxyvitamin D (Corder et al. 1993, Gann et al. 1996). It has been hypothesized that high intakes of calcium and dairy products may increase the risk of prostate cancer by suppressing production of 1-25-dihydroxyvitamin D, the biologically active form of vitamin D (Giovannucci 1998). In the Physicians' Health Study, 1012 patients with prostate cancer had been prospectively assessed for dietary calcium intake before they were diagnosed with prostate cancer (Chan

et al. 2001). Men who had more than 600 mg Ca per day from dairy products were 1.32 (95% CI 1.08–1.63) times more likely to develop prostate cancer than were those who consumed 150 mg Ca per day or less, and the risk was highest in patients with advanced disease. This finding was confirmed in a large meta-analysis of 12 prospective studies (Gao, LaValley & Tucker 2005). Men with the highest intake of dairy products (RR=1.11, 95% CI 1.00-1.22) and calcium (RR=1.39 95% CI 1.09-1.77) were more likely to develop prostate cancer than men with the lowest intake.

One explanation for the low incidence of prostate cancer in Asia might be high consumption of dietary phytoestrogens. Phytoestrogens are natural plant substances which can be classified to isoflavones, flavonoids, coumestans and lignans (Ganry 2005). A recent study demonstrated that a long-term administration of dietary 7-hydroxymatairesinol, a plant lignan, inhibits the growth of LNCaP human prostate cancer xenografts in athymic nude mice (Bylund et al. 2005). Soybeans have one of the highest contents of phytoestrogens, especially isoflavones, which seem to have a prophylactic effect on prostate cancer (Severson et al. 1989, Jacobsen, Knutsen & Fraser 1998, Ström et al. 1999, Kolonel et al. 2000, Lee et al. 2003). However, it should be noted that in most of the studies the sample groups are rather small and the results are not always statistically significant. Two Nordic studies measured the serum concentrations of enterolactone, a phytoestrogene belonging to the class of lignans (Stattin et al. 2002, Hedelin et al. 2006). A Nordic nested case-control study did not observe a protective effect of enterolactone on prostate cancer risk (Stattin et al. 2002), whereas the Swedish population-based case-control study reported that intermediate serum levels of enterolactone were associated with a decreased risk of prostate cancer (Hedelin et al. 2006).

Selenium is an essential trace element and a versatile anticarcinogenic agent (Schrauzer 1992). Criqui et al. (1991) reported that plasma selenium levels were lower in patients with prostate cancer compared to matched controls. However, the difference was not statistically significant. Another serum-based study showed no effect (Hartman et al. 1998). In a randomized intervention trial, the risk of prostate cancer for men receiving a daily supplement of 200 μg selenium was one third of that for men receiving placebo (Clark et al. 1998). In addition, two studies reported a significant inverse association between toenail selenium level and prostate cancer risk (Yoshizawa et al. 1998, van den Brandt et al. 2003).

In conclusion, epidemiological studies are most consistent for selenium, and possibly calcium, vitamin E and tomatoes/lycopene. Selenium, vitamin E and lycopene seem to have a protective effect against prostate cancer, whereas high calcium intake may be associated with increased risk of prostate cancer.

3.2.5 Inflammation

Increased attention has recently been directed at the role of prostatic infection and/or inflammation in the pathogenesis of prostate cancer (Nelson et al. 2004).

Prostate inflammation is very common; although the precise prevalence is uncertain, available epidemiological data suggest that 2-10% of adult men suffer symptoms from prostatitis at some point in life (Krieger 2004). Chronic inflammation may lead to tumorigenesis by damaging DNA through radical oxygen and nitrogen species, enhancing cell proliferation, and stimulating angiogenesis (Coussens, Werb 2002). An increase in prostate cancer risk has been correlated with symptoms of prostatitis and with sexually transmitted infections independent of the specific pathogen (Dennis, Dawson 2002, Dennis, Lynch & Torner 2002). Furthermore, young men with sexually transmitted infections have been shown to have higher PSA values than healthy controls (Sutcliffe et al. 2006). A link between prostate inflammation and prostate cancer is supported by three recent findings: 1) the identification of *RNASEL* and *MSR1*, encoding proteins with critical functions in host responses to infections, as prostate cancer susceptibility genes (Carpten et al. 2002, Xu et al. 2002a); 2) the likelihood that an inflammatory proliferative lesion in the prostate, proliferative inflammatory atrophy (PIA), is a precursor to prostate cancer (De Marzo et al. 1999); and 3) the increased sensitivity of neoplastic prostate cells to damage by reactive oxygen and nitrogen species as a result of somatic crippling of glutathione S-transferase P1 (*GSTP1*; De Marzo et al. 1999).

PIA stands for focal atrophic lesions that are associated with chronic inflammation and are often directly adjacent to PIN lesions and to prostatic carcinomas, or both (De Marzo et al. 1999). The frequent association of PIA lesions with chronic inflammation suggests that these lesions arise as a consequence of the regenerative proliferation of prostate epithelial cells in response to injury caused by inflammatory oxidants. The intermediate cell population is greatly enriched in PIA lesions and might thus be susceptible to genetic damage (van Leenders et al. 2003). Several somatic alterations such as *TP53* mutations and gain at the chromosome 8 centromere, which have also been detected in prostate cancers, have been found in some PIA lesions (Nelson et al. 2004). *GSTP1* CpG island hypermethylation, which is present in 90% of prostate cancer cases, has been found in 6.3% of PIA lesions (Nakayama et al. 2003). *GSTP1* encodes for a detoxification enzyme that helps to catalyse conjugation reactions between potentially damaging oxidants and electrophiles and gluthatione. Thus inactivation of *GSTP1* may leave cells vulnerable to oxidative DNA damage and/or tolerant to accumulation of oxidised DNA base adducts. *GSTP1* CpG island hypermethylation is present in at least 70% of PIN lesions (Brooks et al. 1998). Therefore, loss of *GSTP1* may define the transition between PIA and PIN or prostate cancer (Nelson, De Marzo & Isaacs 2003).

Interestingly, Urisman et al. (2006) identified a novel virus, named XRMV, in a subset of prostate tumor samples. A strong association between the presence of the virus and being homozygous for the *RNASEL* Arg462Gln variant was observed. The Arg462Gln variant has been shown to have 3-fold decrease in catalytic activity compared with the wild type (Casey et al. 2002, Xiang et al. 2003). As Rnase L has a role in viral defence, this data suggests that *RNASEL*

Arg462Gln homozygotes are more sensitive to the acquisition of infection or are simply less likely to clear infection once acquired.

In addition to variants in *RNASEL* and *MSR1* genes, other polymorphisms in genes encoding oxidant defence enzymes and factors involved in response to infection have been implicated in prostate cancer risk. These genes include *MnSOD* encoding a mitochondria enzyme that protects cells against oxidative damage (Woodson et al. 2003), *hOGG1* encoding an enzyme that repairs oxidative genome damage (Xu et al. 2002b), *PTGS2* encoding a key enzyme in the production of prostaglandins (Shahedi et al. 2006) and genes encoding Toll-like receptors important in the innate immune response to pathogens (Sun et al. 2005). These studies support the hypothesis that inflammation is involved in prostate carcinogenesis.

4. Genetic susceptibility to prostate cancer

4.1 Linkage studies for prostate cancer susceptibility loci

Since 1996, research groups worldwide have recruited families with multiple prostate cancer cases and have performed linkage analyses to search for prostate cancer susceptibility genes. Numerous regions have been suggested to harbor hereditary prostate cancer genes. Table 3 summarizes the most significant initial linkage reports.

Table 3. *Putative hereditary prostate cancer susceptibility loci*

Locus/Gene	Location	Reference
HPC1/RNASEL	1q24-25	(Smith et al. 1996)
PCAP	1q42.2-43	(Berthon et al. 1998)
HPCX	Xq27-28	(Xu et al. 1998)
CAPB	1p36	(Gibbs et al. 1999b)
HPC20	20q13	(Berry et al. 2000)
MSR1	8p22-23	(Xu et al. 2001c)
HPC2/ELAC2	17p11	(Tavtigian et al. 2001)

The first genomic scan reported chromosome 1q24-25 as containing prostate cancer loci with a maximum HLOD of 5.43 (Smith et al. 1996). The initial report did not suggest that any subgroup of families was more or less likely to be linked, but the subsequent analysis of the same set of families reported strongest evidence for linkage to *HPC1* among men with an early age of diagnosis (age <65 years), and the evidence increased if in addition there were at least five men affected (Grönberg et al. 1997b). Given the strength of the initial results, it was surprising that subsequent reports attempting to replicate the linkage for *HPC1* were inconclusive. One of the strongest confirmations came from a study using

Gleason score as a covariate in the analysis (Goddard et al. 2001). Men with a higher grade provided stronger evidence of linkage for *HPC1* (LOD score 3.25). At the same time, other studies failed to confirm linkage (Eeles et al. 1998, Goode et al. 2000). Therefore, the International Consortium for Prostate Cancer Genetics (ICPCG) performed a meta-analysis in 772 families affected by hereditary prostate cancer from North America, Australia, Finland, Norway, Sweden, and the United Kingdom (Xu 2000). Confirmatory linkage evidence to *HPC1* locus with a HLOD of 1.4 was found. Furthermore, the evidence was greater among 491 pedigrees with male-to-male transmission, with an HLOD of 2.6.

Other findings on chromosome one were reported in a genome-wide scan of 47 French and German families, in which a locus *PCAP* located in 1q42.2-43 was detected with a maximum two-point LOD score of 2.7 (Berthon et al. 1998). The replication of this finding has been difficult (Gibbs et al. 1999a, Whittemore et al. 1999). Xu et al. (2001b) performed multipoint linkage analyzes with 50 microsatellite markers spanning chromosome 1 in 159 hereditary prostate cancer families. The highest LOD scores were observed at 1q24-25 at which the parametric HLOD was 2.54.

A combined study of 360 families from four groups representing North America, Finland and Sweden identified a locus at Xp27-28 (Xu et al. 1998). There was a maximum two-point LOD score of 4.60 and *HPCX* was estimated to account for 16% of HPC overall. Schleutker et al. (2000) performed a subgroup analyzis among 57 Finnish HPC families indicating that families with no male-to-male transmission and a late age at diagnosis (>65 years) accounted for most of the *HPCX*-linked cases. Using the linkage disequilibrium approach in Finnish *HPCX*-linked families, Baffoe-Bonnie et al. (2005) were able to reduce the *HPCX* critical locus to approximately 750 kb region containing the cluster of five *SPANX* genes and *LDOC1*. A comprehensive analysis of *SPANX-C, -B,* and *-D* revealed an extensively complex and dynamic organization of the *SPANX* genes (Kouprina et al. 2005). The authors suggest that the X-linked susceptibility to prostate cancer is a genomic disorder caused by a specific architecture of the *SPANX* gene cluster. A few studies have provided some supporting evidence for linkage to *HPCX*, although, contrary to expectation, the evidence was strongest among families with male-to-male transmission (Lange et al. 1999, Bochum et al. 2002).

Gibbs et al. (1999b) screened 12 families with a history of both prostate and primary brain cancers for linkage. They observed a maximum two-point LOD score of 3.22 on chromosome 1p36 and the locus was termed *CAPB* for Cancer of the Prostate and Brain. An excess of brain and central nervous system cancers had been previously reported in high-risk prostate cancer families (Goldgar et al. 1994, Isaacs et al. 1995). In addition, 1p36 is a region of frequent loss of heterozygosity (LOH) in brain tumors (Takayama et al. 1992, Bello et al. 2000). The original linkage result has been difficult to replicate. Badzioch et al. (2000) report a maximum HLOD of 0.07 for nine prostate-brain cancer families from the UK. Partitioning the families by mean age at diagnosis of prostate cancer

resulted in suggestive, but not significant differential linkage with five early onset families (mean age at diagnosis <66 years) providing a maximum LOD score of 0.48. In another study, six hereditary prostate cancer families with a history of primary brain cancer showed HLOD of 0.39 (Xu et al. 2001b).

In 2000, Berry et al. (2000) reported linkage to a locus on 20q13 with two-point LOD score of 2.69 for the dominant model and 3.11 for the recessive model. The strongest evidence of linkage was evident with the pedigrees having <5 family members affected with prostate cancer, a later average age at diagnosis, and no male-to-male transmission. Two studies confirm the finding (Bock et al. 2001, Zheng et al. 2001), whereas one study reported no evidence for linkage (Cancel-Tassin et al. 2001). Furthermore, a large study performed by the ICPCG among 1234 pedigrees failed to replicate linkage to *HPC20* (Schaid, Chang & ICPCG. 2005).

Xu et al. (2001c) found evidence for prostate cancer linkage at 8p22-23, with a peak HLOD of 1.84 in 159 pedigrees affected by HPC. This was a very interesting finding because in prostate cancer, LOH on 8p was reported to be one of the most frequent somatic alterations, occurring in >60% of cancers (Cunningham et al. 1996). Wiklund et al. (2003) were able to confirm the linkage among 57 families from Sweden. In their study, evidence of linkage was seen in families with early-onset prostate cancer with a peak multipoint non-parametric linkage score of 2.01 (P=0.03) and in families with a small number of affected individuals with a linkage score of 2.25 (P=0.01). Furthermore, a recent genome-wide linkage analysis from Germany observed linkage at 8p22 in the family collection of 139 prostate cancer families (Maier et al. 2005b).

Tavtigian et al. (2001) originally carried out a genome-wide search for prostate cancer predisposition loci using a small set of high-risk prostate cancer pedigrees from Utah. The first 8 pedigrees analyzed gave suggestive evidence of linkage on chromosome 17p11 and finally, the analysis was expanded to 33 pedigrees which gave a maximum multipoint LOD score of 4.3 (Tavtigian et al. 2001). In contrast, Xu et al. (2001a) found no evidence for linkage for the *HPC2* locus in a total sample of 159 families, nor in any subset of pedigrees based on characteristics that included age at onset, number of affected members, male-to-male disease transmission, or race.

In December 2003, eight genome-wide scans for prostate cancer susceptibility (Cunningham et al. 2003b, Edwards et al. 2003a, Janer et al. 2003, Lange et al. 2003, Schleutker et al. 2003, Wiklund et al. 2003, Witte et al. 2003, Xu et al. 2003) were published in *Prostate* together with a summary written by Easton et al. (2003). Cunningham et al. (2003b) reported the only LOD score >3 when they identified a linkage to chromosome 20 with HLOD score of 4.77. Thus they confirmed their initial finding on chromosome 20 (Berry et al. 2000). Ten other linkage regions with LOD scores >2 were reported, on chromosomes 2, 3, 4, 5, 6, 7, 9, 16, 17, and 19. Remarkably, none of these peaks coincides precisely with any of the previously reported linkage peaks. The peak on chromosome 19p13.3 reported by Wiklund et al. (2003) is close to the peak reported by Hsieh et al. (2001). A Finnish genome-wide linkage analysis indicated two chromosomal regions, 3p25-26 with two-point LOD score of 2.57

and 11q14 with two-point LOD score of 2.97 (Schleutker et al. 2003). Fine-mapping with 39 microsatellite markers in 16 families validated 3p26 as a prostate cancer susceptibility locus in Finland (Rökman et al. 2005). The maximum multipoint HLOD was 3.39 at 3p26 and 1.42 at 11q14.

Gillanders et al. (2004) performed a combined genome-wide linkage analysis among 188 families recruited at the Johns Hopkins Hospital, 175 families recruited at the University of Michigan, 50 families recruited at the University of Umeå and 13 families recruited at the University of Tampere and Tampere University Hospital (total of 426 families). The strongest evidence for linkage was obtained at 17q22, which had a LOD score of 3.16 in the total sample set. LOD scores greater than 2 were also obtained for chromosomal regions 2q32, 15q11, Xq25 and 6q22. Furthermore, stratified analysis revealed the 15q11 region among families with late-onset prostate cancer and the 4q35 region among families with four or more affected family members (Gillanders et al. 2004). Then Chang et al. (2006) analyzed the same linkage data from 426 families by modeling two-locus gene-gene interactions for all possible pairs of loci across the genome. Suggestive evidence for an epistatic interaction for six pairs of loci was found. The possible interacting pairs of loci were 12q24-16p13, 11q13-13q12, 22q13-21q22, 8q24-7q21, 20p13-16q21 and 5p13-16p12. Considering that the 17q22 region was implicated by a single gene approach (Gillanders et al. 2004), the various interactions involving this locus were also examined. Suggestive interactions between chromosome 17q22 loci and loci on chromosomes 4q35 and 11q14 respectively, were found (Chang et al. 2006).

In 2005, an even larger combined genome-wide linkage analysis was performed by ICPCG when they analyzed data from 1233 families with prostate cancer (Xu et al. 2005). The highest overall LOD score was 2.28 from the nonparametric analysis, found on chromosome 5q12. The LOD score was higher in the combined analysis than those observed in any of the ten individual groups. In the subset analysis, for 269 families with at least five affected members, significant evidence of linkage with the LOD score of 3.57 at 22q12 was found. Suggestive evidence for linkage at five other regions (1q25, 8q13, 13q14, 16p13, and 17q21) was also observed in this subset of families. For 606 families with family mean age at diagnosis of ≤65 years, suggestive evidence of linkage at 3p24, 5q35, 11q22, and Xq12 was found. Many of the regions identified in this study may represent false positive finding due to multiple tests. However, the significant linkage at 22q12 is probably due to prostate cancer susceptibility gene(s) at this region (Xu et al. 2005). The chance of observing such a high LOD score under a null hypothesis of no linkage is <0.25 times in the study. In addition, the linkage was identified in a subset of families with several affected individuals that are more likely to segregate mutations in genes conferring a strong prostate cancer risk. Furthermore, the evidence of linkage was supported by multiple individual groups.

Effort has been invested in overcoming the problems of disease and locus heterogeneity, which reduce the power to find significant linkage signals by focusing on men with aggressive disease. Gleason score was first analyzed as

quantitative trait in a sib-pairs study by Witte et al. (2000) with evidence for linkage at chromosomes 5q31-33 (P=0.0002), 7q32.3 (P=0.0007), and 19q12 (P=0.0004). Subsequent studies provided support for the results on chromosomes 7 and 19 (Neville et al. 2002, Neville et al. 2003, Paiss et al. 2003, Slager et al. 2003), and in addition reported linkage signals for more aggressive disease based on Gleason score on chromosome 4q (Goddard et al. 2001, Slager et al. 2003) and on chromosomes 6q23, 1p13-q21, and 5p13-q11 (Slager et al. 2006). Recently, the first linkage analysis of HPC families recording only men with "clinically significant disease" as affected revealed HLOD score above 2.0 on chromosomes Xq27-28 and 22q13 (Chang et al. 2005). Subsequently, two other genome-wide linkage scans coding only men with aggressive disease as affected observed suggestive linkage at chromosomes 22q11.1, 22q12.3-q13.1 and 15q12 (Lange et al. 2006, Stanford et al. 2006). ICPCG performed a genome-wide scan on pedigrees that had at least three men with clinically aggressive prostate cancer (unpublished data). Among 166 pedigrees, the strongest linkage signals were on 6p22.3 (LOD=3.0), 11q14.1-14.3 (LOD=2.4) and 20p11.21-q11.21 (LOD=2.5). These findings suggest that focusing on aggressive prostate cancer may reveal linkage that is not apparent among all types of prostate cancer.

4.2 Variation in genes suggested by linkage studies

4.2.1 RNASEL/HPC1

In 2002, six years after the initial linkage report of *HPC1* locus, Carpten et al. (2002) identified *RNASEL* gene by a positional cloning/candidate gene method. 2',5'-Oligoadenylate-dependent RNase L functions in the interferon-inducible RNA decay pathway known as the 2-5A system that mediates cell proliferation and apoptosis (Zhou et al. 1997, Castelli et al. 1998). Its tumor suppressor potential has been postulated since the introduction of truncated RNase L protein in murine cells abolished the antiproliferative effect of interferon (Hassel et al. 1993, Lengyel 1993). RNase L is a 741-amino acid protein with a bipartite domain structure in which the N terminal half represses the RNase domain in the COOH-terminal region (Figure 3., Dong, Silverman 1997). The repressor half consists of nine ankyrin repeats. Binding of 2',5'-oligoadenylate to the repressor region of RNase L relieves the inhibition caused by ankyrin repeats, presumably as a result of conformational change that unmasks the dimerization and RNase domains (Dong et al. 2001).

In the initial report by Carpten et al. (2002), two deleterious mutations, Glu265X and Met1Ile, were reported to co-segregate with prostate cancer in hereditary prostate cancer families linked to *HPC1*. In Finnish families with prostate cancer, Glu265X was associated with prostate cancer risk; especially in families with four or more affected relatives (OR=5.85, 95% CI 1.20-28.87, Rökman et al. 2002). However, the mutation did not segregate with the disease.

Wiklund et al. (2004) identified the Glu265X mutation among 5 out of 350 (1.4%) familial and HPC patients. None of these families provided evidence for co-segregation and the carrier frequency was similar among unselected cases and controls. A third deleterious mutation, 471delAAAG, was more common among unselected prostate cancer patients compared with controls in Ashkenazi Jews (Rennert et al. 2002). This deletion causes a frameshift at codon 157 and a translation stop after seven additional codons. Subsequent study identified only one 471delAAAG carrier among 111 Ashkenazi Jewish probands, whereas in 105 controls, two carriers were identified (OR=0.47, 95% CI 0.08-9.2, Kotar et al. 2003). Loss of the wild type *RNASEL* allele in prostate tumor tissue has been reported in cases with either Glu265X or 471delAAAG mutation (Carpten et al. 2002, Rennert et al. 2002).

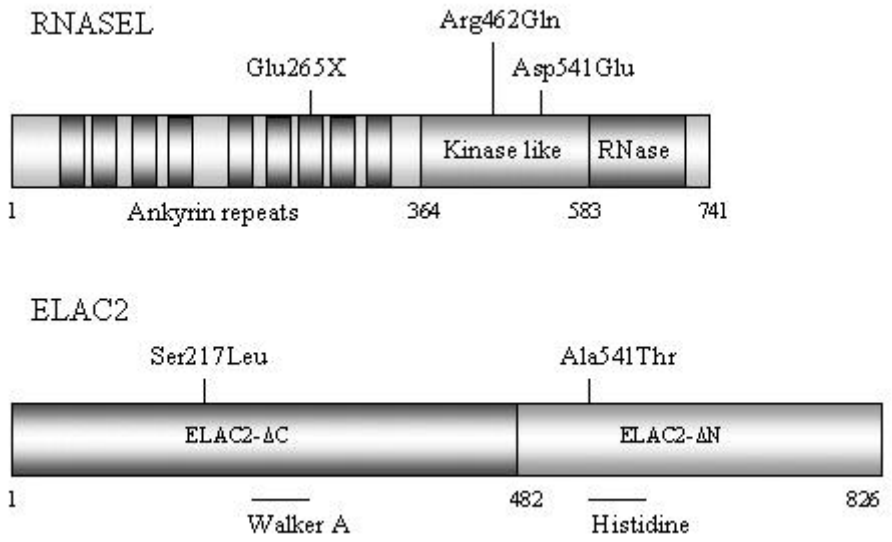

Figure 3. *Ribonuclease L* (RNASEL) *and elaC homolog 2 (*ELAC2) *proteins. Structural domains are indicated with boxes. For* ELAC2, *regions of the Walker A motif and the histidine motif are denoted by solid lines. The main germline coding region mutations studied in prostate cancer are listed above the protein.*

In addition to rare mutations, several polymorphisms in *RNASEL* have been reported (Figure 3.). Arg462Gln, a common missense variant of *RNASEL*, has been associated with both increased (Casey et al. 2002, Rökman et al. 2002) and decreased risk of prostate cancer (Wang et al. 2002, Nakazato et al. 2003). Other studies found no association (Wiklund et al. 2004, Maier et al. 2005a). Recently, Xiang et al. (2003) showed that Arg462Gln significantly decreased RNase L activity and reduced the ability of RNase L to cause apoptosis in response to activation by 2',5'-oligoadenylates. Another missense variant, Asp541Glu, was associated with an increased risk for prostate cancer in a study from Japan (Nakazato et al. 2003). By contrast, a Swedish study found an inverse association between the Asp541Glu and sporadic prostate cancer risk (Wiklund et al. 2004).

Several other studies found no association (Casey et al. 2002, Rökman et al. 2002, Wang et al. 2002, Maier et al. 2005a). According to Xiang et al. (2003) RNase L containing the Asp541Glu variant produced a similar level of activity as wild type enzyme.

Taken together, these results indicate that *RNASEL* could only account for a minor portion of familial prostate cancer. Glu265X has so far been observed as the only truncating *RNASEL* mutation in Caucasian population and it co-segregates with the disease in only few studied families. The results for common variants are inconsistent and further work will be required to ascertain the roles of the various variants of the *RNASEL* gene. Although the precise mechanisms by which defects in an interferon inducible RNA degradation pathway might lead to prostate cancer have not been established, studies on *RNASEL* -/- mice have revealed decreased interferon-α antiviral activity and deficiencies in apoptosis induction (Zhou et al. 1997).

4.2.2 ELAC2/HPC2

The *ELAC2* gene on chromosome 17p11.2 was the first candidate gene for prostate cancer susceptibility to be identified from a linkage analysis and positional cloning project (Tavtigian et al. 2001). At that time, the function of *ELAC2* product was totally unknown. Sequence analysis suggested that *ELAC2* encodes an evolutionary conserved metal-dependent hydrolase (Melino et al. 1998, Tavtigian et al. 2001). In addition, the gene product showed a similarity to DNA interstrand crosslink proteins (Niegemann, Brendel 1994, Tavtigian et al. 2001). Finally, Takaku et al. (2003) demonstrated that *ELAC2* encodes a tRNA 3' processing nuclease (3' tRNase), which is responsible for the removal of a 3' trailer from precursor tRNA. The C-terminal half of *ELAC2* (ELAC2-ΔN in Figure 3.) containing the histidine motif is essential for the 3'-tRNase activity (Takaku et al. 2003). In contrast, the Walker A motif that exists in the N-terminal half region (ELAC2-ΔC) is not essential for activity. This ATP/GTP-binding motif may be involved in regulation of the catalysis by means of ATP or GTP. In addition, it has been reported that ELAC2 interacts with the γ-tubulin complex (Korver et al. 2003). This interaction suggests an additional function of ELAC2, such as cell cycle regulation.

In the original report, two segregating mutations (1641insG, Arg781His) were found in two Utah pedigrees (Tavtigian et al. 2001). In addition, two common missense variants, Ser217Leu and Ala541Thr, were also found to be associated with familial prostate cancer. Moreover, a combination of Leu217 and Thr541 genotypes gave the most significant association with OR of 2.94 (95% CI 1.52-5.69). At about the same time, Rebbeck et al. (2000) found that men who carried both Leu217 and Thr541 variants were at an increased risk of prostate cancer with an OR of 2.37 (95% CI 1.06-5.29). Several groups have attempted to confirm these findings, with varying levels of success. In a Finnish study, no truncating mutations were found and neither Leu217 nor Thr541 was associated with prostate cancer (Rökman et al. 2001). A meta-analysis of six studies

(Rebbeck et al. 2000, Tavtigian et al. 2001, Vesprini et al. 2001, Wang et al. 2001, Xu et al. 2001a) published by July 2002 showed that Thr541 allele, either alone or in combination with Leu217, is significantly associated with prostate cancer (Camp, Tavtigian 2002). In contrast, Leu217 did not increase the risk for prostate cancer. In a large population-based study from Australia, no evidence that either *ELAC2* polymorphism is associated with prostate cancer was found (Severi et al. 2003). Their meta-analysis, which included their data, the data from Rökman et al. (2001) and the six studies from meta-analysis performed by Camp and Tavtigian (2002), estimated summary ORs of 1.04 (95% CI 0.85-1.26) for Leu217 homozygotes and 1.18 (95% CI 0.98-1.42) for carriers of Thr541. Due to the fact that the selection criteria for controls differed across studies, PSA level might differ by genotype and hence explain the discrepancies between study findings. However, the meta-analysis showed that excluding control subjects with elevated plasma PSA levels did not change the ORs (Severi et al. 2003).

Other association studies published after the meta-analyzes have been inconsistent. A population based studies from UK and Trinidad and Tobago showed no significant associations between Leu217 or Thr541 and prostate cancer (Meitz et al. 2002, Shea et al. 2002). Similarly, Stanford et al. (2003) found no overall significant associations when these genotypes were examined separately among residents of King County, Washington. When considering the joint effect of these polymorphisms, however, there was evidence of an increased relative risk in the subset of men who were homozygous for both the Leu217 allele and Thr541 allele (OR=1.84, 95% CI 1.11-3.06). A study of 199 cases and 525 controls from Canada found that carriers of Leu217 were at an elevated risk, with OR of 1.6 (95% CI 1.15-2.23; but not when considering only homozygotes), and that Thr541carrier frequency increased with age in the cases, but not in the controls (P=0.028, Adler et al. 2003). In a Japanese ethnic group, one study observed an association between Leu217 and prostate cancer but not between Thr541 and cancer (Takahashi et al. 2003), one study found no associations at all (Suzuki et al. 2002); one study reported that both Ser217Leu and Thr541 were significantly associated with prostate cancer (Fujiwara et al. 2002) and one study reported that Thr541 but not Leu217 is a significant risk allele for prostate cancer (Yokomizo et al. 2004).

After the function of *ELAC2* product was identified, the effect of these polymorphisms on enzyme activity could also be studied. Takaku et al. (2003) analyzed eight *ELAC2* variants (1641insG, Ser217Leu, Ala541Thr, Arg781His, Ser217Leu/Ala541Thr, Ala541Thr/ Arg781His, Arg781His/ Ser217Leu, Ser217Leu/ Ala541Thr/ Arg781His). There was no significant difference in the tRNase activity between the wild type and seven missense variants; only 1641insG, a truncating variant, which lacks a C-terminal half region, had no activity. Furthermore, Minagawa et al. (2005) showed that there is no significant difference in the K_m and k_{cat} values between wild type tRNase and these variants. Additional rare sequence variants that alter the protein coding sequence have been found in familial prostate cancer patients, but has not been well studied whether these variants are more common in patients than controls and the effect

of these variants on enzyme function is not known (Rökman et al. 2001, Wang et al. 2001).

Camp et al. (2005) performed a haplotype block analysis of *ELAC2*. None of the eight tagging SNPs was found to be significantly associated with familial early-onset prostate cancer. Similarly, no individual haplotype reached the level of significant association. However, three haplotypes analyzed together showed significant evidence for association when compared to disease free individuals over the age of 70 years (OR=2.23, 95% CI 1.28-2.57), indicating that there is allelic heterogeneity in the association between *ELAC2* and prostate cancer. All three haplotypes contain the minor allele at Ser217Leu. Based on all of these studies, it appears that if *ELAC2* plays a role in prostate cancer, it is a minor role for most forms. The genetic architecture of *ELAC2* seems to be more complex than initially thought and therefore, Ser217Leu and Ala541Thr, either alone or in combination, are not sufficient to "tag" the genetic variance in *ELAC2*.

4.2.3 MSR1

After identifying a linkage at 8p22-23 (Xu et al. 2001c), the same group detected seven rare mutations in *MSR1* gene among 13 of 190 HPC families recruited at Johns Hopkins Hospital (Xu et al. 2002a). The *MSR1* gene encodes the class A macrophage scavenger receptor (SR-A; Emi et al. 1993). The functional SR-A protein is a homotrimeric molecule consisting of six domains: the amino-terminal cytoplasmic domain, transmembrane domain, spacer domain, α-helical coiled-coil domain, collagen-like domain, and the scavenger receptor cysteine-rich carboxy-terminal domain (Figure 4.; Matsumoto et al. 1990). Functional studies have shown that the receptor can bind to a wide range of ligands (Krieger, Herz 1994). These polyanionic ligands include modified lipoproteins, Gram-positive and Gram-negative bacteria, certain polynucleotides, and some sulfated polysaccharides. SR-A proteins are believed to play a role in multiple pathophysiological processes, including atherosclerosis and host defence through ligand internalisation and promotion of cell adhesion (Peiser, Gordon 2001).

The only nonsense mutation among the seven rare mutations identified by Xu et al. (2002a) was Arg293X. This mutation results in the deletion of the ligand-binding region and of the highly conserved cysteine-rich domain. Similar synthetic mutations of *MSR1* have been found to have a dominant-negative phenotype when expressed in vitro (Dejager et al. 1993). A family-based linkage and association test indicated that the rare mutations, including Arg293X, co-segregated with prostate cancer among families affected with HPC (Xu et al. 2002a). In contrast, the common variant Pro275Ala found in 30/190 affected families did not show co-segregation with the disease. After re-analysis of the linkage data using the same markers to compare families with and without *MSR1* mutations from the original study, the 13 families with *MSR1* mutations had significantly higher LOD scores (HLOD=1.40, P=0.01) in this region compared to families without mutations (HLOD=0.05; Xu et al. 2002a). Furthermore, the association analysis revealed that Arg293X was found more often in individuals

with non-HPC (2.52%) than in unaffected men (0.39%, P=0.047; Xu et al. 2002a). Asp174Tyr was observed only in African-American subjects, where it occurred more often in individuals with non-HPC (12.5%) than in controls (1.82%, P=0.01). Subsequently, the same authors genotyped five common variants in 301 patients with prostate cancer and in 250 unaffected controls, both of European descent (Xu et al. 2003). The genotyped variants were an SNP in the promoter region (PRO3), a 15-base pair insertion/deletion in intron 1 (INDEL1), an SNP in intron 5 (IVS5-59), a missense change in exon 6 (Pro274Ala), and a 3-base pair insertion/deletion in intron 7 (INDEL7). The allele frequencies of these variants were all significantly different between cases and controls. In addition, when haplotypes were examined, a specific haplotype of these five variants was identified with a significantly higher frequency in prostate cancer patients (6.6%) than in control subjects (2.6%, P=0.004). Interestingly, this haplotype did not harbor any of the rare mutations identified in the earlier study (Xu et al. 2002a), indicating that the association of the five common variants with prostate cancer risk was independent of the presence of rare mutations.

Miller et al. (2003) performed a case control study of *MSR1* variants among 474 African-American men. In their study, the Asp174Tyr variant was identified twice as frequently in men with prostate cancer than in control subjects, but the difference was not statistically significant (P=0.14). In contrast, significantly different allele frequencies were observed for one of the common sequence variants, IVS5-59 (P=0.02). However, after adjustment for age, neither this nor any other common variant was associated with a significantly increased risk for prostate cancer. Contrary to the findings of Xu et al. (2003), haplotype analyzes revealed no statistically significant findings. Similarly, Wang et al. (2003) did not detect any associations or co-segregation of *MSR1* variants with prostate cancer in their study of 483 individuals with familial prostate cancer, 499 with sporadic prostate cancer and 493 population based-controls.

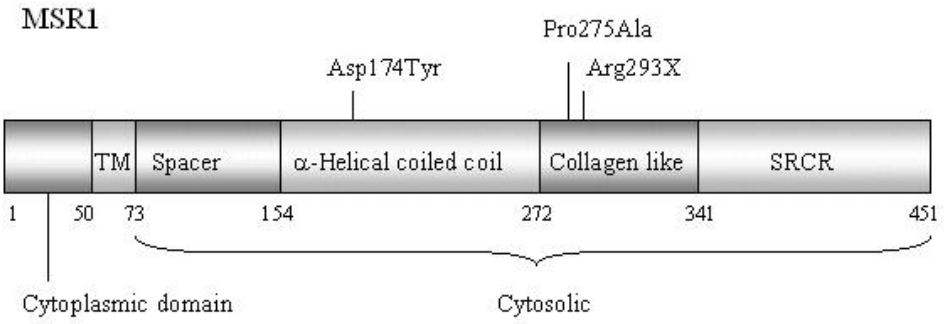

Figure 4. *Macrophage scavenger receptor 1 (*MSR1*) protein. Structural domains and functional motifs are indicated with boxes. The main germline coding region mutations studied in prostate cancer are listed above the protein. TM, transmembrane domain; SRCR, scavenger receptor cysteine-rich domain.*

4.2.4 BRCA1 and BRCA2

Different epidemiological studies have observed coaggregation of prostate and breast cancers in families (Anderson, Badzioch 1992, Tulinius et al. 1992). Familial clustering of these two cancers could be due to the same inherited susceptibility. Breast cancer predisposing genes *BRCA1* and *BRCA2* are involved in repair of DNA damage and transcriptional regulation (Welcsh, King 2001). The evidence that *BRCA1* is important in prostate cancer is controversial. An estimated relative risk of prostate cancer of 3.3 (1.8-6.2) was calculated for obligate male carriers of a deleterious *BRCA1* mutation when compared with general population in a co-operative study using 33 *BRCA1*-linked breast/ovarian cancer families (Ford et al. 1994). However, several negative studies have been reported (Gayther et al. 2000, Sinclair et al. 2000, Ikonen et al. 2003). By contrast, the evidence in support of a link between *BRCA2* and prostate cancer is much stronger. In a study by the Breast Cancer Linkage Consortium (1999) including 173 breast/ovarian cancer families with a deleterious *BRCA2* mutation, a significantly increased risk (RR=4.6, 95% CI 3.5-6.2) for prostate cancer was observed. The RR for prostate cancer for men below the age of 65 years was 7.3 (95% CI = 4.7-11.5). According to Sigurdsson et al. (1997) the founder Icelandic *BRCA2* mutation (999del5) was associated with increased risk of prostate cancer. Furthermore, Gayther et al. (2000) found two novel *BRCA2* deletions among affected individuals from 38 prostate cancer familial clusters. In a study of 263 men with prostate cancer who were aged 55 years or younger, protein truncating mutations in *BRCA2* were present in six men (2%), four of whom did not have a family history of breast or ovarian cancer (Edwards et al. 2003b). However, it should be noted that the strongest support for *BRCA2* involvement in prostate cancer is obtained in populations in which the prevalence of *BRCA2* mutations is high, such as the United Kingdom and Iceland. Therefore, the estimate that germline mutations in *BRCA2* account for 5% of all prostate cancer families

worldwide (Gayther et al. 2000) is probably excessive, since other studies of families with prostate cancer have been negative (Sinclair et al. 2000, Ikonen et al. 2003).

4.3 Candidate genes for prostate cancer

Although a number of putative high penetrant prostate cancer loci have been detected as described above, the majority of cases are unlikely to be due to major susceptibility genes and genetic polymorphisms are likely to be more important from a public health perspective. The most likely candidate genes are those involved in the synthesis and metabolism of androgens, because the growth of prostatic cells depends on testosterone (Platz et al. 2004). Other well studied genes include vitamin D receptor (*VDR*), due to the fact that vitamin D may influence prostate cancer risk; phase I enzymes of the cytochrome P450 (*CYP*) family, that activate carcinogens to reactive forms to produce DNA adducts; and glutathione S-transferases (*GSTs*) that catalyze the conjugation of glutathione to numerous potentially genotoxic compounds (Coughlin, Hall 2002). As shown in Table 4., several studies have evaluated genes involved in DNA repair and cell-cell interactions, as well as genes involved in angiogenesis, immune response to infection and cell cycle regulation as possible low penetrant susceptibility genes.

Table 4. *Examples of low penetrant candidate genes studied in prostate cancer*

Gene	Gene product	Reference
Sex steroid hormone receptors, synthesis and metabolism		
AR	Androgen receptor	(Elo et al. 1995)
ERα	Estrogen receptor *α*	(Modugno et al. 2001)
ERβ	Estrogen receptor *β*	(Thellenberg-Karlsson et al. 2006)
SRD5A2	Steroid 5α-reductase type 2	(Makridakis et al. 1997)
CYP17A1	Steroid 17α -hydroxylase/17,20 lyase	(Lunn et al. 1999)
HSD17B3	17 β-hydroxysteroid dehydrogenase type 3	(Margiotti et al. 2002)
HSD3B1	3β-hydroxysteroid dehydrogenase type 1	(Chang et al. 2002a)
HSD3B2	3β-hydroxysteroid dehydrogenase type 2	(Chang et al. 2002a)
CYP3A4	Nifedipide oxidase	(Rebbeck et al. 1998)
CYP19A1	Aromatase	(Modugno et al. 2001)
*CYP1A1**	Cytochrome P450 1A1 (Aryl hydrocarbon hydroxylase)	(Murata et al. 1998)
*CYP1B1**	Cytochrome P450 1B1 (Aryl hydrocarbon hydroxylase)	(Tang et al. 2000)
LHB	Luteinizing hormone β subunit	(Elkins et al. 2003)
COMT	Catechol O-methyltransferase	(Suzuki et al. 2003a)

Drug and carcinogen metabolising enzymes		
CYP2D6	Debrisoquine hydroxylase	(Febbo et al. 1998)
NAT1	N-acetyltransferase 1	(Fukutome et al. 1999)
NAT2	N-acetyltransferase 2	(Agundez et al. 1998)
GSTM1	Glutathione S-transferase M1 (μ)	(Murata et al. 1998)
GSTT1	Glutathione S-transferase T1 (θ)	(Autrup et al. 1999)
GSTP1	Glutathione S-transferase P1 (π)	(Autrup et al. 1999)
Enzymes involved in oxidative DNA damage		
hOGG1	8-oxoguanine DNA glycosylase	(Xu et al. 2002b)
MnSOD	Manganese superoxide dismutase	(Woodson et al. 2003)
PON1	Paraoxonase 1	(Marchesani et al. 2003)
Infection, inflammation and immune response		
TLR4	Toll-like receptor 4	(Zheng et al. 2004)
TLR6-TLR1-TLR10 gene cluster	Toll-like receptors 6, 1, and 10	(Sun et al. 2005)
PTGS2 (*COX-2)*	Prostaglandin-endoperoxide synthase 2 (Cyclooxygenase-2)	(Shahedi et al. 2006)
IL8	Interleukin 8	(McCarron et al. 2002)
IL10	Interleukin 10	(McCarron et al. 2002)
MIC-1	Macrophage-inhibitory cytokine 1	(Lindmark et al. 2004b)
Proteins involved in cell adhesion and cell-cell interactions		
CDH1	E-cadherin	(Ikonen et al. 2001)
ICAM1-ICAM4-ICAM5 gene cluster	Intercellular adhesion molecules 1, 4 and 5	(Kammerer et al. 2004)
Regulators of angiogenesis		
LEP	Leptin	(Ribeiro et al. 2004)
VEGF	Vascular endothelial growth factor	(McCarron et al. 2002)
COL18A1	Endostatin	(Iughetti et al. 2001)
Regulators of cell proliferation, differentiation and apoptosis		
BCL2	B-cell CLL/lymphoma 2	(Kidd et al. 2006)
IGF1	Insulin-like growth factor 1	(Schildkraut et al. 2005)
KLF6	Krüppel-like factor 6	(Narla et al. 2005a)
NKX3.1	Homeobox protein Nkx-3.1.	(Gelmann et al. 2002)
TGFB1	Transforming growth factor β1	(Li et al. 2004)
Enzymes involved in DNA repair and genome stability		
CHEK2	Cell cycle checkpoint kinase 2	(Dong et al. 2003)
ATM	Ataxia telangiectasia mutated	(Angele et al. 2004)
NBS1	Nijmegen breakage syndrome 1	(Cybulski et al. 2004a)
Others		
KLK3	Kallikrein 3, (prostate specific antigen)	(Gsur et al. 2002)
VDR	Vitamin D receptor	(Taylor et al. 1996)

*Gene products are also drug and carcinogen metabolising enzymes

4.3.1 Sex steroid hormone receptors

Androgen receptor

The actions of testosterone and DHT in androgen-responsive tissues are mediated by the androgen receptor (Jänne et al. 1993). This receptor is sometimes mutated in prostate cancer cells with various predicted effects on its functionality and possibly a wider array of activating steroid and non-steroid ligands (Shi et al. 2002). The *AR* gene encoding this receptor, located on the long arm of the X chromosome, contains a variable length CAG repeat (encodes polyglutamine) in exon 1. In experimental constructs the fewer the number of CAG repeats, the greater the transactivational activity of the receptor (Chamberlain, Driver & Miesfeld 1994, Kazemi-Esfarjani, Trifiro & Pinsky 1995). Expansion of the CAG microsatellite to ~40 or more repeats causes a rare, adult-onset, neurodegenerative disorder called spinal and bulbar muscular atrophy or Kennedy's disease (Igarashi et al. 1992, La Spada et al. 1992). Men with this disease frequently develop partial androgen insensitivity and this has been correlated with decreased transactivation activity of the mutant AR protein (Mhatre et al. 1993, Chamberlain, Driver & Miesfeld 1994). Several (Irvine et al. 1995, Giovannucci et al. 1997, Stanford et al. 1997, Hsing et al. 2000, Modugno et al. 2001), but not all (Miller et al. 2001, Chang et al. 2002b, Chen et al. 2002, Santos et al. 2003), epidemiologic studies support that shorter *AR* gene CAG repeats are associated with a higher risk of prostate cancer. In Finland, a tendency for an association with prostate cancer was seen (OR=1.47, P=0.05, Mononen et al. 2002). Interestingly, Swedish prostate cancer patients had shorter *AR* CAG repeats than Swedish controls, but Japanese prostate cancer patients had longer repeats than controls (Li et al. 2003). These differences between the populations may suggest that the *AR* CAG repeats are in linkage disequilibrium with a prostate cancer susceptibility locus localized in Xq12.1.

A second polymorphic *AR* gene trinucleotide repeat has also been described in exon 1 consisting of GGN repeats encoding polyglycine. Recently, Hillmer et al. (2005) reported that the GGN repeat was strongly associated with androgenetic alopecia, or male-pattern baldness, whose pathogenesis is androgen dependent. Previously obtained functional data, in which shorter repeat alleles of the GGN repeat were associated with higher protein levels and thereby higher *AR* activity (Ding et al. 2005), support the possibility of a causal role for the repeat in androgenetic alopecia. The association between GGN repeat and prostate cancer has not been consistent among studies (Irvine et al. 1995, Stanford et al. 1997, Hsing et al. 2000, Miller et al. 2001, Chang et al. 2002b, Chen et al. 2002).

Zeegers et al. (2004) performed a meta-analysis of 19 case-control studies comprising a total of 4,274 cases and 5,275 controls in order to provide summary estimates of CAG and GGN repeats with sufficient power. Men with shorter CAG and GGN repeats experienced a 1.19- (95% CI 1.07-1.31) and 1.31-fold (95% CI 1.06-1.61) increased risk of prostate cancer. However, the absolute

difference in number of repeats between cases and controls was <1 repeat for both CAG and GGN repeats. It remains to be resolved whether such a small difference is enough to yield a measurable biological impact in prostate carcinogenesis.

In addition to trinucleotide repeat polymorphisms, only six germline alterations have been reported in prostate cancer patients so far: Arg726Leu in exon 5 (Elo et al. 1995), Gln798Glu in exon 6 (Evans et al. 1996), Glu213Glu (also known as E211 G>A or StuI polymorphism) in exon 1 (Hayes et al. 2005), G2T and C214A within the 5'-UTR (untranslated region; Crocitto, Henderson & Coetzee 1997), and Gln211Gln in exon 1 (Koivisto et al. 2004a). The functional consequences of Arg726Leu mutation have been studied. Elo et al. (1995) showed that Arg726Leu, although located in the ligand binding domain of androgen receptor, does not alter the ligand binding specificity, but the mutated receptor can be activated by estradiol to a significantly greater extent than the wild-type receptor. However, it should be noted that in their experiments high doses of estradiol were used (10 and 100 mmol/l). A similar study by Shi et al. (2002) demonstrated that the mutated or the wild type receptor did not respond to physiological dose of estradiol. However, the combination of estradiol and progesterone at physiological concentrations weakly activated the Arg726Leu mutant receptor. Thompson et al. (2001) reported that the Arg726Leu mutation did not affect the stability of receptor-DNA complexes, and the mutant showed amino/carboxyl-terminal interactions similar to those of wild-type AR. The transcriptional activity of Arg726Leu, however, was slightly reduced.

Estrogen receptor

In the prostate, estrogens interact with two forms of estrogen receptor, ERα and ERβ (Linja et al. 2003). ERα is expressed only at low levels and is confined to the stroma (Leav et al. 2001, Royuela et al. 2001). In contrast, ERβ is highly expressed in prostatic epithelium and levels seem to decrease in prostate cancer (Leav et al. 2001). ERα, but not ERβ, is essential for prostate development (Prins et al. 2001). ERβ appears to be a physiological regulator of prostatic epithelial growth and differentiation (Weihua et al. 2001). Loss of ERβ expression has been reported to be associated with progression from normal prostate epithelium to prostate cancer, and those cancers that retained ERβ expression had a higher rate of relapse (Horvath et al. 2001). *ERα* knockout mice develop enlarged prostates and seminal vesicles upon aging without any pathological changes in the prostatic histology (Prins et al. 2001). Aged *ERβ* knockout mice have been reported to display hyperplastic foci and increased proliferation in the ventral prostate (Weihua et al. 2002). In contrast, in another *ERβ* deficient mouse line no prostatic abnormalities were observed (Dupont et al. 2000).

Unlike the *AR* gene, only a handful of case-control studies have evaluated polymorphisms in *ERα* gene, located in the long arm of chromosome 6 (Modugno et al. 2001, Cancel-Tassin et al. 2003, Suzuki et al. 2003a, Tanaka et al. 2003, Hernandez et al. 2006). Most of these studies are small and several

polymorphisms have been evaluated only in couple of the studies. Therefore, no conclusive evidence for the role of *ERα* variants in prostate cancer etiology could be drawn. *ERβ* gene, cloned in 1996, is located in chromosome 14 (Mosselman, Polman & Dijkema 1996). So far only one study has been conducted for polymorphisms in *ERβ* and risk of prostate cancer (Thellenberg-Karlsson et al. 2006). One single nucleotide polymorphism (SNP) in the promoter region of *ERβ* was shown to associate with prostate cancer (OR 1.22, 95% CI 1.02-1.46).

4.3.2 Sex steroid hormone synthesis and metabolism

Figure 5. presents the simplified androgen and estrogen metabolic pathway in the testes, adrenal cortex, prostate and liver. Many of the genes in this pathway are polymorphic. However, only *SDR5A2* and *CYP17A1* encoding steroid 17α-hydroxylase/17,20 lyase have been well studied for their variation and relation to prostate cancer.

Luteinizing hormone secreted from the anterior pituitary signals testicular Leydig cells to secrete testosterone. Luteinizing hormone is an α:β heterodimer with a common α subunit and a unique β subunit that determines specificity (Pierce, Parsons 1981). The gene encoding β subunit, *LHB*, is located on chromosome 19 and harbours two linked SNPs, Trp8Arg and Ile15Thr (Haavisto et al. 1995). This genetic variant of the LH-β protein (LH-βV) has higher bioactivity and a shorter half-life than that of the wild-type protein (Suganuma et al. 1996). Elkins et al. (2003) hypothesized that genetically determined variation in luteinizing hormone function might affect susceptibility to prostate cancer via altered testosterone secretion. In their study, familial cases were more likely to carry the variant allele (18.6%) compared to controls (13.7%). However, the association was not statistically significant (OR=1.29; 95% CI 0.96–1.75). The same was true for the sporadic case group (15.2%, OR=1.33; 95% CI 0.86–2.07).

SRD5A2 is located on chromosome 2p23 and encodes the steroid 5α-reductase type 2 enzyme expressed primarily in the androgen-sensitive cells of genital skin and the prostate gland (Thigpen et al. 1993). This enzyme irreversibly converts testosterone into DHT, which has a greater affinity for androgen receptor, resulting in greater transactivation of androgen responsive genes (Wilbert, Griffin & Wilson 1983). Several polymorphic regions in *SRD5A2* have been identified. A TA dinucleotide repeat in the 3'-UTR (Davis, Russell 1993), a missense variant Val89Leu, which reduces enzyme activity both in vitro and in vivo (Makridakis et al. 1997), and another missense variant Ala49Thr, which results in higher enzymatic activity in vitro (Makridakis et al. 1999), have been evaluated in relation to prostate cancer risk. The results of these studies have been contradictory (reviewed in Platz, Giovannucci 2004). Consequently, Ntais et al. (2003b) performed a meta-analysis of nine studies with Val89Leu genotyping, seven studies with Ala49Thr genotyping, and four studies with TA repeat genotyping. There was no evidence that the Leu89 allele modified the risk of prostate cancer. The summary OR was 1.02 by both random

(P=0.61) and fixed effects (P=0.64). In contrast, based on the meta-analysis, the Thr49 allele might modify the risk of prostate cancer. The summary OR was 1.56 by random effects (P=0.089) and 1.44 by fixed effects (P=0.029). However, no clear effect was seen in subjects of European descent. Excluding the first study in the field (Makridakis et al. 1999) that may be considered to provide the hypothesis-generating data, the remaining studies did not suggest any strong association between the Thr49 allele and prostate cancer (random effects OR=1.08; 95% CI 0.72–1.61). In addition, the meta-analysis suggested that homozygotes for longer *TA* repeats may have a smaller risk for prostate cancer. However, this association was not statistically significant *(P*=0.07 by both random and fixed effects).

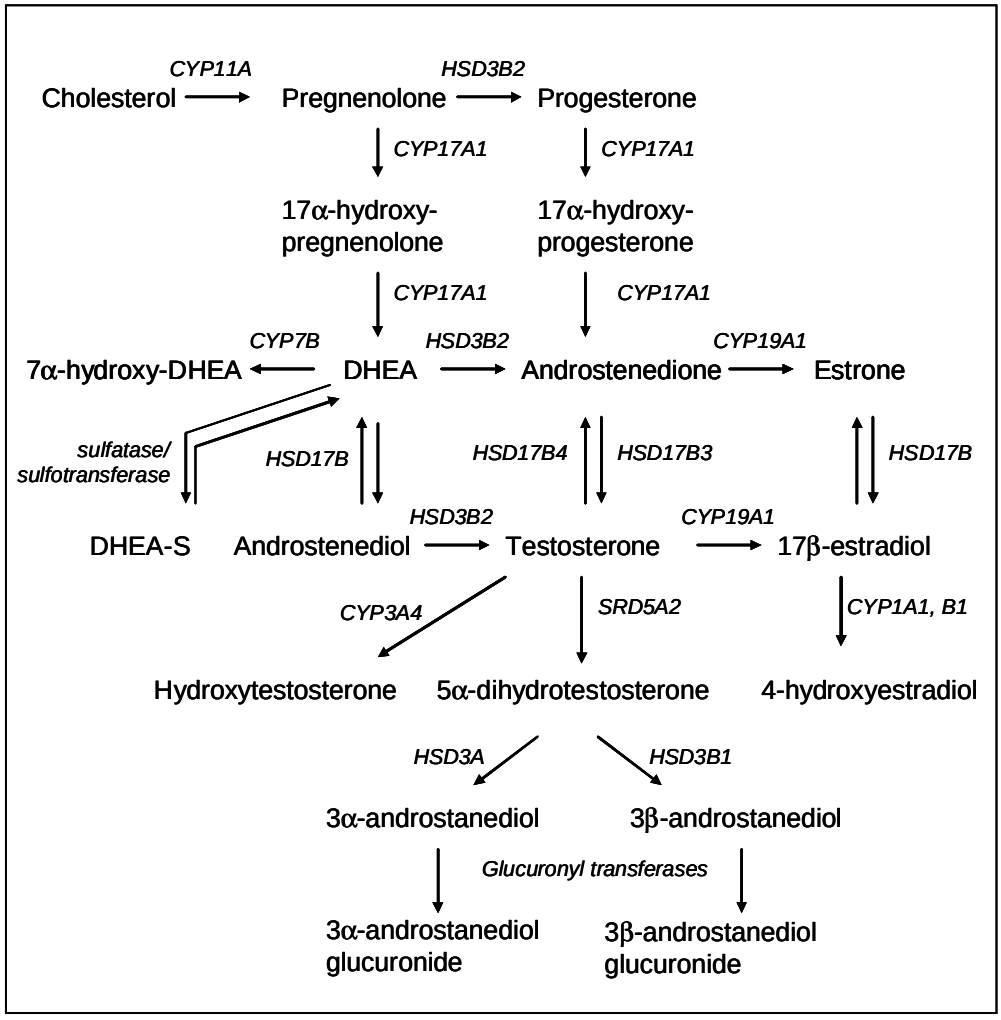

Figure 5. *Simplified version of sex hormone biosynthesis and metabolism pathway. Most of the testosterone is produced in the testes. In addition, steroid precursors DHEA-S, DHEA and androstenedione are produced in adrenal cortex. In the prostate, testosterone is converted into dihydrotestosterone and androgens can be aromatized into estrogens. Testosterone, dihydrotestosterone and estradiol are metabolised in the liver. DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate.*

The enzyme encoded by *CYP17A1* catalyzes two reactions in testosterone synthesis pathway, pregnenolone to 17α-hydroxypregnenolone via its 17α-hydroxylase activity and the latter to dehydroepiandrosterone via its 17,20-lyase activity in the testes (Lunn et al. 1999). *CYP17A1* is located in the long arm of chromosome 10. The 5'-untranslated promoter region of the *CYP17A1* gene contains a polymorphic -34T>C substitution that gives rise to A1 (T) and A2 (C) alleles (Carey et al. 1994). This base pair substitution in *CYP17A1* was originally hypothesized to create an additional binding site for the transcription factor Sp-1, which may lead to increased transcription of the enzyme and enhanced steroid hormone production. However, in an in vitro assay, Nedelcheva Kristensen et al. (1999) did not observe Sp-1 binding at this polymorphic site or within the promoter sequenc of *CYP17A1*. Whether other regulatory factors differentially bind to this polymorphic motif remains unknown. In a meta-analysis of 10 case-control studies published before the end of 2002, there was no overall association between a C>T substitution and prostate cancer (Ntais, Polycarpou & Ioannidis 2003a). No effect of C-allele was seen in subjects of European descent (OR=1.04; 95% CI 0.92-1.18) or Asian descent (OR=1.06; 95% CI 0.66-1.71), whereas C-allele increased susceptibility to prostate cancer in subjects of African descent (OR=1.56; 95% CI 1.07-2.28). However, the data available on men of African descent was very limited and should thus be interpreted with caution.

4.3.3 CHEK2

Cell cycle checkpoint kinase 2 (CHEK2) has an important role together with checkpoint kinase 1 (CHEK1) in various DNA-damage responses, including cell cycle checkpoints, genome maintenance, DNA repair and apoptosis (Zhou, Bartek 2004). The *CHEK2* gene is located on chromosome region 22q12, and its 14 exons encode a protein of 543 amino acids. The protein has three functional domains: an N-terminal regulatory region with several serine-glutamine and threonine-glutamine (SerGln/ThrGln) pairs, a forkhead-associated (FHA) domain that mediates multiple protein-protein interactions, and a large C-terminal kinase domain (Figure 6.; Matsuoka, Huang & Elledge 1998). CHEK2 is mainly activated by DNA strand-breaking agents such as ionising radiation through the ATM-dependent pathway, but it can also be activated in an ATM-independent manner (Matsuoka et al. 2000). Activated CHEK2 induces rapid G1/S cell cycle arrest and/or S phase delay by phosphorylating Cdc25A (Falck et al. 2001b). In addition, activated CHEK2 participates in maintaining the G2/M block by phosphorylating the mitosis promoting Cdc25C phosphatase (Matsuoka, Huang & Elledge 1998). CHEK2 can also phosphorylate p53 tumor suppressor, which results in stabilization of p53 and transactivation of its target genes involved in controlling G1/S and G2/M checkpoints, DNA repair and apoptosis (Chehab et al. 2000, Hirao et al. 2000). Furthermore, CHEK2 has been shown to phosphorylate BRCA1 tumor suppressor releasing it from the complex with CHEK2 itself (Lee et al. 2000). Prevention of CHEK2-mediated

phosphorylation via mutation of the Ser988 residue of *BRCA1* disrupted both the BRCA1-dependent promotion of homologous recombination and the suppression of non-homologous recombination (Zhang et al. 2004).

Somatic mutations in *CHEK2* have been found in small subsets of diverse types of sporadic human malignancies, including carcinomas of the breast (Sullivan et al. 2002), lung (Haruki et al. 2000), vulva (Reddy et al. 2002), and ovary (Miller et al. 2002), osteosarcomas (Miller et al. 2002) and lymphomas (Hofmann et al. 2001, Tavor et al. 2001). Significant subsets of human tumors with low or undetectable CHEK2 protein in the apparent absence of any mutations in the CHEK2 gene have also been reported (Bartkova et al. 2001, Sullivan et al. 2002, Tort et al. 2002, Vahteristo et al. 2002). Epigenetic silencing of gene expression through promoter methylation has been excluded as a cause of this phenotype (Sullivan et al. 2002, Tort et al. 2002), and the finding of normal mRNA levels on CHEK2 in such cases suggested potential posttranscriptional aberrations of CHEK2 as a plausible way to downregulate CHEK2 levels during oncogenesis.

The role of CHEK2 in controlling the cell cycle after DNA damages, a position in the middle of a pathway that is frequently targeted in cancer, and regulation of many tumor suppressors makes it a good candidate for being a tumor suppressor itself (Bartek, Falck & Lukas 2001). Therefore, it was not a surprise when Bell et al. (1999) reported on germline *CHEK2* mutations in patients with Li-Fraumeni syndrome. The frameshift 1100delC mutation found in a classic Li-Fraumeni family with rhabdomyosarcoma, brain tumors and multiple cases of breast cancer, results in premature termination at codon 381. A second variant, Ile157Thr, was detected in a Li-Fraumeni syndrome variant family in which a proband had three primary tumors: breast cancer, melanoma and lung cancer. In addition, Arg145Trp missense mutation was detected in colon cancer cell line (Bell et al. 1999). Afterwards, it was shown that the 1100delC mutation results in the loss of kinase activity (Wu, Webster & Chen 2001), Ile157Thr variant is defective in its ability to bind and phosphorylate Cdc25A, and Arg145Trp variant, although retaining some basal kinase activity, cannot be phosphorylated at an ATM-dependent phosphorylation site (Thr68) and cannot be activated following gamma radiation. (Falck et al. 2001b).

In 2001, Vahteristo et al. (2001b) detected the 1100delC mutation in two of the 44 Finnish families only suggestive of Li-Fraumeni syndrome. This observation together with the fact that 1100delC mutation was identified in breast cancer patients in two different populations (Bell et al. 1999, Vahteristo et al. 2001b) led to the idea that *CHEK2* might be a breast cancer susceptibility gene in general. Indeed, it was shown that *CHEK2* 1100delC mutation was significantly associated with positive family history of breast cancer (Vahteristo et al. 2002). The same mutation was subsequently found in seven breast cancer cases in a family showing linkage to chromosome 22 close to *CHEK2* (Meijers-Heijboer et al. 2002). Further screening revealed that this mutation was present in over 5% of breast cancer cases with a family history of breast cancer and no *BRCA1/2* mutation, compared with a frequency of 1% in controls.

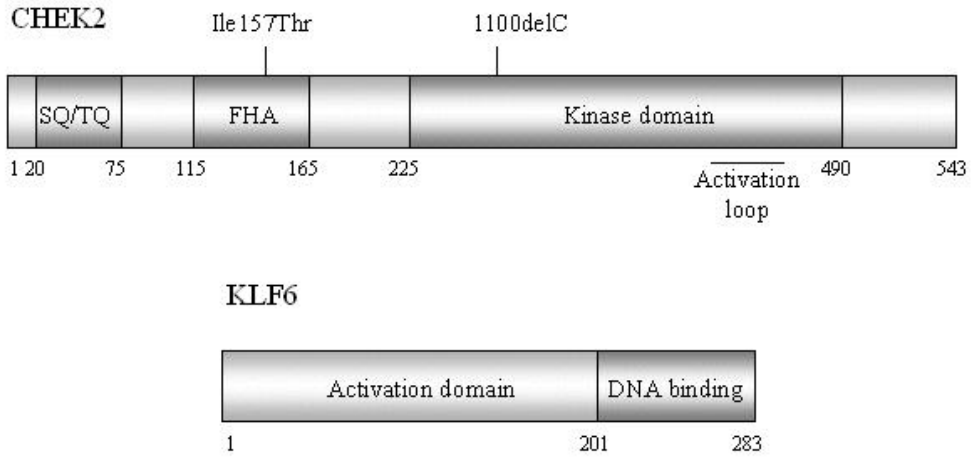

Figure 6. *Cell cycle checkpoint kinsase 2 (*CHEK2*) and Krüppel-like factor 6 (*KLF6*) proteins. Structural domains and functional motifs are indicated with boxes. The CHEK2 germline mutations studied in Li-Fraumeni syndrome and breast cancer are listed above the protein. SQ/TQ, regulatory region with several serine-glutamine and threonine-glutamine pairs; FHA, forkhead-associated domain.*

4.3.4 KLF6

Krüppel-like factor 6 (KLF6) is a ubiquitously expressed zinc-finger transcription factor that is part of a growing KLF family. The KLF family is broadly involved in differentiation and development, growth-related signal transduction, cell proliferation, apoptosis and angiogenesis (Bieker 2001, Black, Black & Azizkhan-Clifford 2001). Transcriptional targets of KLF6 include a placental glycoprotein (Koritschoner et al. 1997), human immunodeficiency virus (HIV-1; Suzuki et al. 1998), collagen α1 (I) (Ratziu et al. 1998), transforming growth factor β1 (TGFβ1), types I and II TGFβ1 receptors (Kim et al. 1998), nitric oxide synthase (Warke et al. 2003), urokinase type plasminogen activator (Kojima et al. 2000), insulin-like growth factor I receptor (IGF-IR; Rubinstein et al. 2004), and ceramidase (Park et al. 2005). *KLF6* gene maps to chromosome 10p, which is frequently deleted in prostate carcinomas (Ittmann 1996a, Ittmann 1996b) and codes for a 283 amino acid protein consisting of 201 amino acid activation domain and an 82 amino acid zinc finger DNA binding domain (Ratziu et al. 1998).

Mutations in *KLF6* have been reported in several cancers including colorectal cancer (Reeves et al. 2004), malignant glioma (Jeng, Hsu 2003), hepatocellular carcinoma (Kremer-Tal et al. 2004), and nasopharyngeal carcinoma (Chen et al. 2002), indicating its possible generalized role in cancer pathogenesis, whereas, in other studies, *KLF6* mutations or chromosome 10p loss have not been found in hepatocellular carcinoma, glioblastomas, meningiomas or astrocytomas (Kohler et al. 2004, Koivisto et al. 2004, Montanini, Bissola & Finocchiaro 2004, Boyault et al. 2005).

In 2001, Narla et al. reported that one *KLF6* allele is deleted in 77% (17 of 22) of primary prostate tumors. Twelve of the 17 (71%) tumors showing LOH had somatic mutations in the retained *KLF6* allele. Subsequently, Chen et al. (2003) showed that LOH was present in four (19%) and point mutations in three (14%) of the 21 xenografts and cell lines. Among the three xenografts/cell lines with mutations, one also had LOH. Furthermore, expression levels of *KLF6* were significantly reduced in four out of the 20 (20%) xenografts/cell lines. In the same study, LOH was detected in eight out of 29 (28%) informative prostate cancer tumors. In addition, 12 mutations in 11 out of the 75 (15%) tumors were identified. The allelic status was available for only three out of the 11 tumors with mutations; one of these three tumors could have LOH at *KLF6* locus. None of the mutations detected in xenografts/cell lines resulted in a change in amino acid sequence of the *KLF6* gene, whereas seven of the tumor-derived mutations changed amino acid codons, but none of them resulted in a stop codon. Furthermore, none of the mutations found by Chen et al. (2003) occurred in the zinc-finger domains of *KLF6*, and none was identical to the mutations discovered by Narla et al. (2001). Mülbauer et al. (2003) examined 32 primary prostate tumors and three prostate tumor cell lines for *KLF6* exon 2 mutations, because earlier studies report clustering of mutations in exon 2 (Narla et al. 2001, Chen et al. 2003). No mutations were found, suggesting that *KLF6* mutations in tumors of the prostate are uncommon.

A wild type KLF6 upregulates the cell cycle inhibitor p21 in a p53-independent manner and suppresses growth, whereas tumor-derived KLF6 mutants fail to upregulate p21 or suppress proliferation in prostatic and non-small cell lung cancer cell (Narla et al. 2001, Ito et al. 2004). In addition, KLF6 can mediate growth suppression by disrupting the interaction between cyclin D1 and cyclin-dependent kinase 4 and thus forcing the redistribution of p21 onto cyclin-dependent kinase 2, which promotes G1 cell cycle arrest (Benzeno et al. 2004). This evidence indicates that *KLF6* is a tumor suppressor gene and a good candidate for prostate cancer predisposition. To test this hypothesis, Koivisto et al. (2004b) screened genomic DNA from probands of 69 Finnish prostate cancer families using single strand conformational polymorphism (SSCP) analysis. Sequencing revealed six 603G>A (Arg201Arg) variants as well as one –4C>A and one 956T>C alteration in the 5'- and 3'-UTRs respectively. DNA samples of two additional patients with prostate cancer and two healthy males aged over 70 years were screened from three families with the -4C>A, Arg201Arg and 956T>C variants. All subjects studied were found to be carriers of the variant in question, suggesting that these variants are not disease related.

Narla et al. (2005a) identified one very frequent polymorphism (IVS1 –27G>A) by direct sequencing. They also found a significant association between this variant and both sporadic and familial prostate cancer when they genotyped a total of 1,253 sporadic prostate cancer patients, 882 familial cancer patients and 1,276 control men from three independent institutions in the United States. Furthermore, Narla et al. (2005a) demonstrated that *KLF6* is alternatively spliced, that there is an overabundance of *KLF6* splice variants to wild type 1n tumor tissue, and that IVS1 –27G>A is consistently associated with enhanced

KLF6 alternative splicing and variant protein expression. A splicing enhancer motif prediction program showed that the A-allele generates a functional SRp40 binding sequence, and studies with minigene constructs showed that spliced forms of KLF6 antagonize wild type KLF6 effects on p21 and cell growth.

Aims of the study

The aim of the study was to provide essential information on the genetic risk factors leading to prostate cancer, which may help to understand molecular changes and cancer development at the cellular level. Specific aims were:

1. to study the role of *MSR1*, a known prostate cancer predisposition gene in prostate cancer in Finland (I).

2. to study the role of *CHEK2* in prostate cancer (II).

3. to study the role of intronic variant of *KLF6* gene in prostate cancer in Finland (III).

4. to study the variation in some of the major genes acting along the sex steroid hormone synthesis and metabolism pathway in Finnish prostate cancer patients (IV).

5. to investigate possible interactions of sex steroid hormone pathway genes in relation to prostate cancer (IV).

Materials and methods

1. Human subjects

1.1 Families with prostate cancer (I-IV)

Finnish prostate cancer families with two or more affected relatives have been collected in the Laboratory of Cancer Genetics at Tampere University Hospital and the University of Tampere since 1995. Families were identified by several methods, including a questionnaire-based approach, a nation-wide cancer registry-based search, advertisements in newspapers, television and radio, and referrals from urologists in Finland (Schleutker et al. 2000). Diagnoses were confirmed from the Finnish Cancer Registry or individual patient records from regional hospitals.

The youngest affected patient from each of 120 prostate cancer families was initially used for screening for *MSR1* and *CHEK2* mutations. *KLF6* IVS1 -27G>A variant was first genotyped among probands of 164 families. *MSR1* Arg293X, Arg26His, and Pro275Ala; *CHEK2* 1100delC and Ile157Thr; and *KLF6* IVS1 -27G>A variants were then assayed in all other available affected and unaffected members of the mutation–positive families to ascertain the possible segregation of these variants with the disease.

The initial screening of genetic variation in 10 genes belonging to androgen pathway was performed among 32 men with familial prostate cancer and 32 men with unselected prostate cancer. The selected 18 variants were then assayed among 121 prostate cancer families. Again, the youngest affected member with available sample was genotyped from each family. A description of the families used in these studies is presented in Table 5.

1.2 Unselected prostate cancer patients (I-IV)

The unselected prostate cancer patients were consecutive patients diagnosed in the Department of Urology at Tampere University Hospital or Tampere City Hospital or Valkeakoski District Hospital. After informed consent had been given, blood samples were taken and DNA extracted. Patients also filled in a family questionnaire. Clinical data was collected from hospital records. The coverage was 85% of all prostate cancer patients in Pirkanmaa Hospital District since 1996. Due to the structure of the Finnish health care system, the material is

truly population based. The unselected prostate cancer patients used in Studies I-IV are summarised in Table 5.

1.3 Patients with benign prostate hyperplasia (III)

The men with BPH were also collected from the Pirkanmaa Hospital District. The diagnosis of BPH 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 the possibility of prostate cancer. The indication for biopsy was total PSA of ≥ 4 ng/ml or total PSA of 3.0-3.9 ng/ml with the proportion of free PSA < 16%. The diagnoses of BPH were made between 06/1992 - 04/2003. Eighty-eight of these BPH cases are now deceased and none of them had a prostate cancer diagnosis during their lifetime. The mean age of the deceased men was 75.8 years (range 48-94) at the time of death, whereas the mean age of the remaining 371 men with BPH is now 72.1 years (range 49-95).

Table 5. *The DNA samples used in studies I-IV*

		Study I	Study II	Stydy III	Study IV
Familial cases	No. of families	120	120	164	121
	Median no. of affected subjects	3 (2-7)	3 (2-7)	2 (2-7)	3 (2-7)
	Mean age at diagnosis	65.1 (44-86)	65.1 (44-86)	63.0 (43-86)	64.8 (44-86)
Unselected cases	No. of samples	537	537	852	847
	Year of diagnosis	1999-2000	1999-2000	1999-2001	1999-2001
	Mean age at diagnosis	68.6 (47-90)	68.6 (47-90)	69.0 (45-93)	68.9 (45-93)
BPH	No. of samples	-	-	459	-
Population controls	No. of samples	480	480	923	923
PSA controls	No. of samples	-	-	950	-
	Mean age	-	-	67.5 (64-74)	-

1.4 Healthy control individuals (I-IV)

The population controls consisted of DNA samples from anonymous male blood donors obtained from the Finnish Red Cross in the cities of Tampere, Turku and

Kuopio. The blood donors are 18 to 65 year-old healthy men providing an unbiased survey of population genotype frequencies. In Study III, an additional 950 men who had PSA less than 1.0 ng/ml in the third round of the Finnish population-based prostate cancer screening trial were genotyped. A detailed description of the PSA screening trial has been published elsewhere (Määttänen et al. 1999, Mäkinen et al. 2004).

1.5 Ethical considerations (1-IV)

Permission for the collection of the families throughout Finland as well as the use of the Finnish Cancer Registry data was granted on 20th July 1995 by the Ministry of Social Affairs and Health (licence 859/08/95). Permission to collect and use blood samples and clinical data from the prostate cancer patients in the Tampere University Hospital District was granted on 8th March 1995 (latest extension 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 samples and clinical data from prostate cancer patients treated in Hatanpää City Hospital was granted on 1st July 1996 (latest extensions 30th January 2001) by the Institutional Review Board of the City of Tampere. Written informed consent for use of their samples as well as medical records was obtained from all living individuals participating in the study.

2. Methods

2.1 DNA extraction (I-IV)

Genomic DNA was extracted from peripheral blood samples using a commercially available kit (Puregene, Gentra systems, Minneapolis, MN, USA).

2.2 Mutation Screening with SSCP Analysis (I, II, IV)

Single-strand conformation polymorphism (SSCP) is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair), which results in a different secondary structure, and a measurable difference in mobility through a gel. SSCP analysis of the entire coding sequence of the *MSR1, CHEK2, SRD5A2, HSD17B2, HSD17B3, HSD3B1, HSD3B2, CYP11A, CYP17A1, CYP19A1, KLK3* and *AKR1C3* genes was performed using primer sequences designed to include all intron-exon boundaries. The sequences of the primers are listed in Table 6. and

at http://uta.fi/sgy/schleutker/indexb.html. *CHEK2* exons 10-14 have copies on several other chromosomes. Therefore, primers used for amplification of these exons were designed so that both primers for each primer pair had a base mismatch in the most 3' nucleotide, compared with sequences from nonfunctional copies. In addition, a large-scale population screening of the *MSR1* -14743A>G and *CHEK2* Ile157Thr variants was performed by SSCP analysis. The *CYP19A1* exons were numbered 1-9 starting from the first coding exon.

The 15 µl reaction mixture contained 1.5 mM $MgCl_2$; 20 µM each of dATP, dCTP, dGTP, and dTTP; 0.5 µCi of α(^{33}P)-dCTP (Amersham Pharmacia, Uppsala, Sweden); 0.6 µM of each primer; 1.0 U Ampli*Taq*Gold; the reaction buffer provided by the supplier (PE Biosystems, Foster City, CA); and 25 ng of the genomic DNA. Radiolabeled PCR products were mixed with 95% formamide dye, denatured at 95°C for 5 min, and chilled on ice. The (^{33}P)-labeled PCR products were electrophoresed at 800 V for 12 h at room temperature, in 0.5×MDE (mutation-detection–enhancement) gel (FMC BioProducts, Rockland, ME) with 1% glycerol in 0.5× Tris-borate EDTA. After electrophoresis, gels were dried and exposed to Kodak BioMax MR (maximum-resolution) films for 6 h. All samples in which variant bands were detected as well as two to three normal bands per exon, were analyzed by sequencing using an automated ABI Prism 310 Genetic Analyzer (PE Biosystems, Foster City, CA). Variants were identified using Sequencher software version 3.0 or 4.5 (Gene Codes Corporation, Ann Arbor, MI).

2.3 Sequencing (I-IV)

In Study III, the specific region of the *KLF6* gene was initially sequenced from the 78 unselected prostate cancer cases using the previously published PCR primers (Narla et al. 2005a) in order to identify some mutation carriers among Finnish population. Sequencing was also used to identify the variants found by SSCP analysis in Studies I, II and IV; in these cases the same primers were used in SSCP and sequencing analyzes.

PCR products were purified in 96 format Acro Prep Filter Plates (Pall Life Sciences, Ann Arbor, MI) using Perfect Vac Manifold vacuum machine (Eppendorf AG, Hamburg, Germany). Sequencing was performed using the ABI PRISM ®BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with the ABI 310 and ABI3100 sequencers (Applied Biosystems, Foster City, CA). Sequence analysis was performed with Sequencher 4.5 software (Gene Codes Corporation, Ann Arbor, MI).

Table 6. *Sequences of the primers used in the SSCP analyzes*[a]

Gene	Exon	Forward (5'->3')	Reverse (5'->3')
MSR1	1	TTGATGAGAGTGCTATTGAAAC	ACAATCAATAGAAACAATTCTGG
MSR1	2	AGCCCTAGCCATTTCATGTG	ACTCTAAGAACAACCTCCATG
MSR1	3	CAACACTTTCTAACAGTAGGC	ATAATTCACGGGACGAGTTAC
MSR1	4A	CATTCAAGGATCAGGCCATG	CTTTGATCAGTTGTCATGCTG
MSR1	4B	TAGACATGGAAGCCAACCTC	TGGATGGATTCAGTTCCAGC
MSR1	5	GCTGTGAATGCTCACTTATG	TTTCTGGAGAAATGACAAGAC
MSR1	6	ATAAGTACCTTGACAGATGAC	TGTATATCATCTATCTTCACAG
MSR1	7	CTAGATGTTTTCATTATACTCTC	CTGGTGTTTCTTCTACATATTC
MSR1	8	TTGTGATGATCTGTCTCCAG	AAGTTGCGAACATTTGCCTC
MSR1	9[b]	TTGTAAGGCTTTCTGAAAATATG	AAAGTCATTTGGAGGAGTCAC
MSR1	10	TACATAATTAGTCCTTGCTTGC	TGAGACTCTGTCTGAAACAAC
MSR1	11	AAACTCTAAACTTACTAATGCAG	ATTTGGGCAATATTCTTAATCTC
CHEK2	1A	AAGTCTTGTGCCTTGAAACTC	TAGAGTTCCTGAGTGGACAC
CHEK2	1B	TGGGACACTGAGCTCCTTAG	CCTTCCACCTGGTAATACAAC
CHEK2	2	ATTCAACAGCCCTCTGATGC	CCAGCTCTCCTAGATACATG
CHEK2	3	AATCTCTGCTATTCAAAGTCTG	TTCCTCCTATGAGAGAGTGG
CHEK2	4	ATCAGTGATCGCCTCTTGTG	CACCAATCACAAATGTATAGTG
CHEK2	5	TAATACTTGAAGTGGACCCAG	TTGGGAAGTTATGAAGACGTG
CHEK2	6	TTGAGTCAACTGAGTTTAACTG	TGAAAGGCTTTATACTCTTCTC
CHEK2	7	TGTGGTTTTCCTCTTGGGAG	GATGAGAAAGGCAAGCCTAC
CHEK2	8	TTGTCTTCTGTCCAAGTGCG	AGGCAGCTGTCAAAAGAATTG
CHEK2	9	AAGTATCTACTGCATGAATCTG	AGAATCTACAGGAATAGCCAC
CHEK2	10	TGTTAATCTTTTTATTTTATGG	CTCCTACCAGTCTGTGC
CHEK2	11	CTGGGATTACAAGCCTAAGG	GAAGAAACTCCCACCACAGC
CHEK2	12	CCTGTTAATTCTGGCATACTC	ATCTAAAACAATTAGATTAAACAC
CHEK2	13	GATGATTCTTGGACGGACATT	AAATCTAAGGGTGCTGGAGC
CHEK2	14	CCCCCACTTTACTGGAAGC	GAAAGAAGGTACATTTCTTTCG

[a]The primers used in Study IV are listed in http://uta.fi/sgy/schleutker/indexb.html

[b]Specific to *MSR1* type 2

2.4 Minisequencing (I, II, IV)

Minisequencing (Syvänen 1998) was used to determine the frequencies of *MSR1* Arg26His, *CHEK2* 1100delC and *CYP19A1* Thr201Met variants at the population level. PCR primers were designed to amplify 100-200 bp regions 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 designated to anneal with its 3' end immediately adjacent to the variant nucleotide to be analyzed (Table 7.). A DNA fragment was first amplified as follows: 100 ng of DNA, 200 nM of both primers, 200 µM of each deoxy-NTP, 1.5 mM $MgCl_2$, and 1.5 U AmpliTaqGold™ DNA Polymerase (Applied Biosystems, Foster City, CA) in a final volume of 50 µl. Fifteen µl of the amplified biotinylated fragment was then captured on streptavidin-coated microtiter plates (Scintiplates

Streptavidin covalent, Wallac, Turku, Finland) by incubation in 0.1% Tween 20 (VWR International, Espoo, Finland) in phosphate buffered saline at 37ºC 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 treatment with 100 µl 50 mM NaOH for 5 min at room temperature. 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 H^3-labeled dNTP and 0.5 U Dynazyme^{TM}II polymerase (FinnZymes, Espoo, Finland) in 1xPCR buffer by incubating at 50ºC for 20 min. Every sample had two wells for two different labelled dNTPs, one for the detection of the normal allele and the other for the variant allele. After washing, the plates were scanned with a liquid scintillator counter (1450 Microbeta, Wallac, Turku, Finland). The results were obtained as counts per minute, which directly expressed the amount of incorporated H^3-labeled dNTPs.

2.5 5' -nuclease assay (III, IV)

Two *KLK3* alterations, -252A>G and Ile179Thr, and *KLF6* IVS1 -27G>A variant were genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions in 96 or 384-well format. The *KLK3* -252A>G genotypes were determined using the TaqMan®Pre-Designed Assay, product number C_1531052_10. For *KLK3* Ile179Thr and *KLF6* IVS1 -27G>A genotyping a Custom TaqMan®SNP Genotyping Assays was ordered.

The nucleotide sequences of the primers and probes used in the PCR of custom assays were deduced from publicly available sequences deposited in the GeneBank database and the UCSC Genome Browser and were chosen and synthesized by Applied Biosystems using the Assay-by-Design service (Table 7.). DNA samples were genotyped by means of 5'-nuclease assay for allelic discrimination using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). A PCR containing genomic DNA (20 ng), 1×Universal PCR Master Mix, 1×Assay Mix containing the unlabeled PCR primers (900 nM) and TaqMan probes with a conjugated minor groove binder group (200 nM) was performed in 96- or 384-plates using the standard protocol in a total volume of 25 or 5 µl respectively. The DNA and the PCR master mix were pipetted to the 384-plates using a Tecan Freedom EVO 100 instrument and Instrument Software V4.8 (Tecan Schweiz AG, Switzerland). To monitor genotyping errors, known control samples previously genotyped by sequencing were run in parallel with unknown samples. After PCR, end-point fluorescence was measured and genotype calling carried out using the allelic discrimination analysis module.

Table 7. *Primers used in the minisequencing (MSR1 Arg26His, CHEK2 1100delC and CYP19A1 Thr201Met) and in the Custom TaqMan SNP Genotyping Assays (KLK3 Ile179Thr and KLF6 IVS1 -27G>A).*

Gene	Variant	Forward (5'->3')	Reverse (5'->3')	Detection (5'->3')
MSR1	Arg26His	AGCCCTAGCCATTTCATGTG	Biotin-ACTCTAAGAACAACCTCCATG	ATCTGTGAAATTTGATGCTC
CHEK2	1100delC	Biotin-TGTTAATCTTTTTATTTTATGG	CTCCTACCAGTCTGTGC	TCTTGGAGTGCCCAAAATCA
CYP19A1	Thr201Met	AATCGGGCTATGTGGACGTG	Biotin-GATGGTCAAGATGTGAGAGTG	ATGCTGGACACCTCTAACA
KLK3[a]	Ile179Thr	CCCGTAGTCTTGACCCCAAAG	CTTGCGCACACACGTCAT	CCTCCATGTTA[T/C]TTCC[b]
KLF6	IVS1-27G>A	CTTTTGCTTTTACTTTTGGTCGTCATG	CAGGTCTGTGCAAAATGAACCA	CAATCAC[G/A]TGCCTTC[b]

[a]*KLK3* -252A>G genotypes were determined using the TaqMan Pre-Designed Assay, product number C_1531052_10 with no primer information available

[b][VIC labeled oligo/FAM labeled oligo]

2.6 Allele-specific primer extension on microarrays (IV)

A system for specific, high-throughput genotyping by allele-specific primer extension on microarrays was first described by Pastinen et al. (2000). Afterwards, the method was slightly modified by Riise Stensland et al. (2005). We adapted the method with some further modifications to genotype the following variants: *LHB* Ile15Thr, *SRD5A2* Ala49Thr, *SRD5A2* Val89Leu, *HSD3B1* Asn367Thr, *HSD3B1* Arg71Ile, *HSD17B2* Ala111Thr, *HSD17B3* Gly289Ser, *HSD17B3* 729_735 delGATAACC, *AKR1C3* Gln5His, *AKR1C3* Pro180Ser, *CYP19A1* Arg264Cys, *CYP17A1* -34T>C, *AR* Arg726Leu, *KLK3* Asp102Asn, *KLK3* Leu132Ile.

Briefly, the detection is based on hybridisation on microarray glass slides. First the corresponding genomic fragments of the individual studied are amplified by multiplex PCR followed by *in vitro* transcription to RNA. The RNA fragments are then allowed to hybridise with the allele specific oligonucleotides (ASOs) bound to a glass plate. Next a sequence specific extension of two immobilized ASOs, differing at their 3' ends, is performed by a reverse trasncriptase involving fluorescent labeled nucleotides. Finally the glass plates are washed and the amount of fluorescence is measured by a microarray scanner.

2.6.1 Primers

The allele-specific oligonucleotides (ASOs) contained a 5' NH_2 group, a spacer sequence of 9 T residues 5' of the actual gene specific sequence of 18-21 nucleotides in length and a 3' nucleotide complementary either to the normal or mutant nucleotide. One PCR primer of each pair contained a 5' RNA polymerase promoter sequence (TAATACGACTCACTATAGGGAGA) and the other primer for each pair had a 5' tail of random sequence (GCG GTC CCA AAA GGG TCA GT). The polarity of the latter primer was the same as that for the corresponding detection primer sequence. All primer pairs are available at http://www.uta.fi/imt/sgy/schleutker/indexb.html.

2.6.2 Preparation of Microarrays

Aminosilane microarray slides were manufactured as described by Guo et al. (1994) in the National Institute of Health, Biomedicum, Helsinki. The ASOs were printed onto aminosilane coated microarray slides from 20µM solution containing 0.4 M Na-carbonate (pH 9.0) forming a covalent bonding with coated slide surface. Printing was carried out with Telechem SMP5 contact printing pins (ArrayIt) using an OmniGrid arraying instrument (GeneMachines/A1 Biotech). Each microarray slide consisted of 80 identical subarrays containing ASOs for

all multiplexed SNPs. Each subarray consisted of two copies of each ASO spot to ensure quality of printing.

2.6.3 Multiplex PCR amplification and RNA transcription

The PCR primer pairs were grouped into multiplex PCR reactions with 4, 4, 4 and 3 primer pairs per reaction for 15 variants. The amplifications were carried out using 50 ng of DNA, 200 μM dNTPs, and 1.0 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) in 25 μl of DNA polymerase buffer supplied with the enzyme. The primer concentration varied from 0.12 μM to 0.68 μM and had been adjusted to give similar signal intensities in the reactions on the arrays. After initial activation of the polymerase at 95°C for 10 minutes, the thermocycling parameters were as follows: 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds for 30 cycles; and finally 72°C for 5 min. Two of the PCR products were then pooled so that in the next step there were 4, 4 and 7 variants in the same reaction. The PCR products carrying a 5' T7-RNA polymerase promoter sequence were transcribed to RNA using the T7 AmpliScribe Kit (Epicentre Technologies, Madison, WI). The 4μl reaction contained 0.86 × T7 reaction buffer, 6.17 mM NTPs, 8.64 mM dithiothreitol, 0.35 μl AmpliScribe T7 enzyme solution and 2.0 μl of the PCR product. The reaction was carried out in 37°C for two hours. After transcription, the DNA templates were degraded using 0.1 MBU of DNase I enzyme (provided in the T7 Ampliscribe Kit) in 37°C for 15 min. Finally, the DNase I was denaturated in 65°C for 5 min.

2.6.4 Hybridization and allele-specific extension

Before hybridization, 2 μl of 5 M NaCl was added to 5 μl of DNase I treated RNA. Then the RNA was allowed to anneal to ASOs on the arrays at 42°C for 20 minutes, followed by a brief rinse in washing buffer containing 5 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.3 M NaCl and 0.1% Triton X-100 at RT. Immediately after hybridization the allele-specific extension reaction was carried out using 0.04 U MMLV RT enzyme (Epicentre Technologies, Madison, WI), 10 mM dithiothreitol, 0.5 μM dATP, dGTP, ddATP and ddGTP, 1.0 μM Cy5-dCTP and Cy5-dUTP, 0.5 M trehalose and 8% glycerol. The reaction temperature was 52°C for 20 min. The slides were washed with washing buffer, briefly dipped in 50 mM NaOH and once again rinsed with washing buffer. Finally, the slides were rinsed with water and dried under compressed air.

2.6.5 Array scanning and signal quantitation

The microscope glass slides were scanned using the confocal ScanArray 4000 (GSI Lumonics, Watertown, MA), with excitation at 630 nm and emission at 670 nm. Ten µm resolution 16-bit TIFF images were analyzed using QuantArray software (GSI Lumonics, Watertown, MA). Accurate allele calling and genotyping were produced by SNPSnapper 3.88b software developed by Juha Saharinen, National Public Health Institute, Helsinki, Finland (http://www.bioinfo.helsinki.fi/SNPSnapper/).

2.7 Statistical Analysis (1-IV)

Odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) were calculated using logistic regression to estimate prostate cancer risk. Association with demographic, clinical, and pathological features of the disease was tested by the Mann-Whitney test (continuous variables), the Pearson χ^2 test (dichotomous variables with expected count more than five), and Fisher's exact test (dichotomous variables with expected count less than five). These analyzes were performed with SPSS 11.0 statistical software package. The magnitude of the association between the *CYP19A1*, *CYP17A1* and *KLF6* SNPs and the occurrence of prostate cancer and other related outcomes was measured with OR using polytomous logistic regression. Outcome definitions included WHO grade (I-III), Gleason score (2-6, 7-10), PSA at diagnosis (<20 ng/ml, >20 ng/ml) and T stage (T1-T2, T3-T4). Polytomous logistic regression analyzes were performed using STATA v8.0. In Study III, power was calculated using the Power and Sample Size Program by Dupont and Plummer (Dupont, Plummer 1990).

2.8 Bioinformatics (IV)

The effect of the Thr201Met mutation on the structure and function of aromatase was investigated with several bioinformatics methods and tools including PHD and PROF (Rost, Sander 1993, Rost, Sander 1994), Jpred (Cuff et al. 1998), SADM (Chen, Zhou 2005) and SIFT (Ng, Henikoff 2001, Saunders, Baker 2002). The effect of point mutation had to be evaluated sequence-based, because the known structures are only for the core domain of P450.

Results

1. *MSR1*, a known prostate cancer susceptibility gene (I)

MSR1 is the third prostate cancer susceptibility gene identified first by linkage and then by genetic analyzes that indicated that mutations in *MSR1* may be associated with risk of prostate cancer (Xu et al. 2001c, Xu et al. 2002a, Xu et al. 2003). To determine the role of *MSR1* in prostate cancer susceptibility in Finland, we performed a mutation screening analysis of the entire coding region of the *MSR1* gene in 120 probands from prostate cancer families. Five sequence variants were identified, including one nonsense mutation at codon 293 (Arg293X), two missense mutations (Arg26His and Pro275Ala), and two sequence variants (A>G in promoter region and variation in number of T nucleotides in intron 9). Arg293X, Pro275Ala, and SNP in the promoter region (PRO3 = -14743A>G) were also reported by Xu et al. (2002a).

The frequencies of Pro275Ala and Arg293X variants were then determined in 537 unselected prostate cancer patients and 480 population controls. No significantly elevated or lowered risks for prostate cancer among these three variants were detected (Table 8.). Arg26His mutation was screened in 239 patients with unselected prostate cancer and in 240 healthy male blood donors. No mutation carriers were found. Thus Arg26His is a very rare novel mutation. The variants Arg293X, Arg26His, and Pro275Ala were then sequenced among all available affected and unaffected male relatives from the mutation positive families to investigate the possible co-segregation with the disease. There was no clear evidence for segregation. However, the mean age at diagnosis of the Arg293X mutation carriers among probands from families was significantly lower compared to non-carriers (55.3 vs. 65.4 years, P=0.04). The same trend was observed among unselected prostate cancer cases, but the difference was not statistically significant (65.7 vs. 68.7 years, P=0.37). No other statistically significant associations of the Arg293X, Pro275Ala, or -14743A>G variant with demographic, clinical, or pathological features of the disease were observed.

Table 8. *Association of MSR1, CHEK2 and KLF6 variants with prostate cancer*

Gene variant and sample	No. of carriers/total	OR	95% CI	P
MSR1 Arg293X				
Controls[b]	5/480 (1.0%)	1.00		
Unselected PRCA	6/537 (1.1%)	1.07	0.33-3.54	0.91
Familial PRCA	3/120 (2.5%)	2.44	0.57-10.34	0.23
MSR1 Pro275Ala				
Controls[b]	20/480 (4.2%)	1.00		
Unselected PRCA	21/537 (3.9%)	0.94	0.50-1.75	0.84
Familial PRCA	3/120 (2.5%)	0.59	0.17-2.02	0.40
MSR1 -14743A>G				
Controls[b]	14/192 (7.3%)	1.00		
Unselected PRCA	24/239 (10.0%)	1.42	0.71-2.83	0.32
Familial PRCA	15/120 (12.5%)	1.82	0.84-3.91	0.13
CHEK2 1100delC:				
Controls[b]	2/480 (0.4%)	1.00		
Unselected PRCA	7/537 (1.3%)	3.14	0.65-15.16	0.15
Familial PRCA	4/120 (3.3%)	8.24	1.49-45.54	0.02[a]
CHEK2 Ile157Thr:				
Controls[b]	26/480 (5.4%)	1.00		
Unselected PRCA	42/537 (7.8%)	1.48	0.89-2.46	0.13
Familial PRCA	13/120 (10.8%)	2.12	1.06-4.27	0.04[a]
KLF6 IVS1-27G>A				
Controls[b]	206/923 (22.3%)	1.00		
Unselected PRCA	182/852 (21.4%)	0.95	0.76-1.19	0.63
Familial PRCA	32/164 (19.5%)	0.84	0.56-1.28	0.42
BPH	112/459 (24.4%)	1.12	0.86-1.46	0.39
PSA Controls[c]	188/950 (19.8%)	1.00		
Unselected PRCA	182/852 (21.4%)	1.10	0.87-1.38	0.41
Familial PRCA	32/164 (19.5%)	0.98	0.65-1.49	0.93
BPH	112/459 (24.4%)	1.30	1.00-1.71	0.05

[a] Statistically significant

[b] Anonymous male blood donors

[c] PSA<1 ng/ml in the third round of Finnish PSA screening trial

PRCA, prostate cancer; BPH, benign prostate hyperplasia

2. *CHEK2* and *KLF6*, candidate genes for prostate cancer

2.1 CHEK2 (II)

To explore the significance of *CHEK2*, a potential tumor suppressor gene, in prostate cancer causation in Finland, 120 genomic DNA samples from probands of 120 families with prostate cancer were screened for mutations in the coding exons and exon/intron boundaries. Five sequence variants were detected by SSCP analysis. Two of the variants, a missense variant Ile157Thr and a frameshift mutation 1100delC, were the same as previously reported in Li-Fraumeni syndrome and breast cancer (Bell et al. 1999, Allinen et al. 2001, Meijers-Heijboer et al. 2002, Vahteristo et al. 2002). In addition, a silent change in exon 1 (also reported by Bell et al. 1999), insertion of A-nucleotide in intron 1, and a novel mutation 1312G>T leading to a missense change Asp438Tyr in exon 11 were observed. Asp438Tyr mutation was observed in only one family with two affected persons, of whom only one was available for this study.

To determine the frequencies of *CHEK2* Ile157Thr and 1100delC variants, they were genotyped from altogether 1137 samples, including the 120 familial prostate cancer cases, 537 unselected prostate cancer patients and 480 population controls. The frameshift mutation 1100delC was detected in 3.3% of the familial cases compared with 0.4% of the controls (OR=8.24; 95% CI 1.49-45.54). In addition, the mutation was identified in 1.3% of the unselected prostate cancer patients (OR=3.14; 95% CI 0.65-15.16, compared to controls). The missense variant Ile157Thr was also more frequent in men with familial prostate cancer (10.8%) than in controls (5.4%; OR=2.12; 95% CI 1.06-4.27). Among the unselected prostate cancer cases, the difference was not statistically significant (7.8%, OR=1.48; 95% CI 0.89-2.46). Both of these mutations were genotyped from other family members in mutation positive families. 1100delC mutation showed a suggestive co-segregation with the disease in all four families, whereas Ile157Thr variant did not segregate with prostate cancer.

The association between the frequency of the two variants and disease phenotype, including age at diagnosis, tumor WHO-grade, Gleason score, T-, N- and M-stage and PSA-value at diagnosis, was also analyzed among unselected prostate cancer cases. No significant associations emerged from these analyzes. The mean ages at diagnosis of the *CHEK2* variant carriers in patients with familial prostate cancer were 62.7 years for 1100delC carriers and 64.0 years for Ile157Thr carriers. These ages were only marginally different from the mean age of familial patients with no mutations (65.2 years for both variants; P=0.57 for 1100delC and P=0.62 for Ile157Thr).

2.2 KLF6 IVS1 -27G>A (III)

The intronic variant IVS1 -27G>A of the *KLF6* gene was recently reported to associate with prostate cancer regardless of a family history of the disease (Narla et al. 2005a). To confirm these findings, we first sequenced 78 unselected prostate cancer cases to identify some variant carriers in Finland. Subsequent genotyping and case control association analysis was carried out in series of 164 familial prostate cancer cases, 852 unselected prostate cancer cases, 459 BPH cases, 923 population controls and 950 men from a PSA screening trial with PSA < 1 ng/ml. Carrier frequencies for the A allele, previously reported as the risk allele in prostate cancer, were 19.5% in patients with familial prostate cancer, 21.4% in the patients with unselected prostate cancer, 24.4% in the patients with BPH, 22.3% in the population controls and 19.8% in PSA screened controls. No statistically significant association between the *KLF6* IVS1 -27G>A and prostate cancer (either familial or unselected) or BPH was observed when these groups were compared with population controls or with PSA controls (Table 8.). We also calculated the ORs by comparing the prostate cancer cases with the combined set of controls. Neither these logistic regression analyzes revealed any significant associations.

Association tests were performed between *KLF6* IVS1 –27G>A genotype and tumor T stage, Gleason score and WHO grade; no significant association was observed. The mean age at diagnosis was almost equal for wild type genotype and for A-allele carriers (P=0.82 for unselected prostate cancer cases and P=0.77 for familial prostate cancer cases). Furthermore, the median number of affected family members was similar among wild type and variant carriers (P=0.89). In addition, segregation of the SNP with the disease was incomplete, in that both unaffected variant carriers and variant-negative patients with prostate cancer were observed.

The unselected prostate cancer cases were further subclassified according to T stage and WHO grade so that patients had severe or aggressive cancer (T3-T4 and WHO II-III), moderate cancer (T1-T2 and WHO II-III) or clinically less significant cancer (T1-T2 and WHO I). When these prostate cancer subgroups were compared with PSA screened controls, no associations were observed.

3. Variation along the androgen biosynthesis pathway (IV)

In order to establish whether the genetic variants in the androgen pathway are predictive of prostate cancer, we screened 10 genes (*SRD5A2*, *HSD3B1*, *HSD17B2*, *HSD17B3*, *AKR1C3*, *CYP19A1*, *CYP17A1*, *KLK3*, *HSD3B2* and *CYP11A1*) for possible disease-associated variations. A total of 50 variants was identified by SSCP among the 10 genes studied. Of the variants found 15 were located in the introns, three in the 3'-UTR areas, five in the 5'-UTR areas and 27

in the coding exons. Sixteen of the variants identified in the screening as well as two other variants (*AR* Arg726Leu and *LHB* Ile15Thr) previously suggested to be risk factors for prostate cancer (Mononen et al. 2000, Elkins et al. 2003) were then selected for large-scale genotyping in 1891 Finnish men.

3.1 CYP19A1

Two variants of the *CYP19A1* gene were analyzed in a large sample set. The C to T polymorphism results in a non-conservative replacement of threonine for methionine at codon 201 in exon 4. At codon 264 in exon 6 the C to T polymorphism results in a non-conservative arginine to cysteine amino acid change.

A statistically significant association with the *CYP19A1* Thr201Met mutation and unselected prostate cancer was identified (OR=2.04, 95% CI 1.03-4.03, P=0.04; Table 9.). However, no association was seen with the *CYP19A1* Arg264Cys variant (OR=1.08, 95% CI 0.77-1.51, P=0.65). To determine if the apparent association with Thr201Met was to severe prostate cancer, the categories were narrowed according to stage and grade classifications. Interestingly, for both T stage and PSA value at diagnosis classifications, individuals with moderate disease were more likely to carry the Thr201Met mutation than controls (P=0.006 and P=0.03 respectively). Individuals with severe stage classification showed no association with the T-allele. Similar results were seen for the tumor grade classifications, WHO and Gleason score, in which individuals with less aggressive prostate cancer defined by a low grade tumor (WHO grade I) were 4.5 times more likely to carry the T-allele than population based controls (OR=4.51, 95% CI 1.94-10.5, P<0.0001). More severe cases (WHO II and III or Gleason >7) did not have any association with the *CYP19A1* Thr201Met T allele.

To further refine the risk categories, a risk score was created in which an individual had severe or aggressive prostate cancer (T3-T4 and WHO grade II-II), moderate cancer (T1-T2 and WHO grade II-III) or clinically less significant cancer (T1-T2 and WHO grade I). Individuals with clinically less significant cancer were more than five times more likely to carry the *CYP19A1* Thr201Met T-allele (OR=5.42, 95% CI 2.33-12.6, P<0.0001) than population based controls. This association persisted after accounting for multiple testing.

The effect of the Thr201Met mutation on the structure and function of aromatase was investigated with numerous bioinformatics methods and tools. According to sequence database searches, position 201 is not highly conserved in aromatases and in P450 family. The residue most common in this position in the aromatase family is arginine. Thr201 was predicted to be close to the C-terminal end of a long α-helix by several methods including PHD (Rost, Sander 1993), PROF (Rost, Sander 1993) and Jpred (Cuff et al. 1998). The mutation was predicted not to increase disorder or aggregation tendency of the protein. The predictions of surface accessibility of Thr201 with programs PHD (Rost, Sander

1994), PROF (Rost, Sander 1994) and SADM (Chen, Zhou 2005) are somewhat contradictory. It is possible that the residue is on the buried surface of the helix. The program SIFT (sorting intolerant from tolerant) uses multiple sequence information to predict whether an amino acid substitution affects protein function (Ng, Henikoff 2001). According to SIFT prediction Thr201Met mutation is not tolerated.

3.2. Other candidate genes along the androgen biosynthesis pathway

Association studies showed no increased risk for prostate cancer among the carriers of *AR* Arg726Leu, *SRD5A2* Ala49Thr, *SRD5A2* Val89Leu, *HSD17B3* Gly289Ser, *AKR1C3* Gln5His, *AKR1C3* Pro180Ser, *CYP17A1* -34T>C, *KLK3* Asp102Asn, *KLK3* -252A>G, *KLK3* Ile179Thr and *LHB* Ile15Thr (Table 9.). For the very rare mutations (*HSD3B1* Arg71Ile, *HSD17B2* Ala111Thr, *HSD17B3* 729_735delGATAACC, *KLK3* Leu132Ile) no ORs were calculated because of the small number of carriers.

However, some of the variants were associated with the clinicopathological features of the unselected prostate cancer cases. *KLK3* -252A>G carriers had a low Gleason score (2-6) more often than non-carriers (P=0.05). *LHB* Ile15Thr showed a borderline association with organ-confined tumor (P=0.07). In contrast, carriers of the *KLK3* Ile179Thr alteration were more likely to have metastases than non-carriers (P=0.009). The same cancer categories in the further analysis of *CYP17A1* –34T>C were used as in the case of *CYP19A1* variants (aggressive prostate cancer = [T3-T4 and WHO grade II-III], moderate cancer = [T1-T2 and WHO grade II-III] and clinically less significant cancer = [T1-T2 and WHO grade I]). No association was seen to clinically less significant prostate cancer (OR=1.09, 95% CI 0.75-1.59, P=0.65). However, this alteration increased the risk for moderate cancer (OR=1.42, 95% CI 1.09-1.83, P=0.007). The association was marginally significant after the conservative Bonferroni correction (n=8).

Table 9. *Association of genotypes studied with prostate cancer among unselected and familial cancer cases. Table modified from Supplementary Table 1 in Study IV.*

	Unselected Cases (%)	Familial Cases (%)	Controls (%)	Unselected Cases		Familial Cases	
				OR	(95% CI)	OR	(95% CI)
LHB Ile15Thr							
CC/TC	240/846 (28)	34/121 (28)	236/923 (26)	1.15	0.93-1.42	1.14	0.75-1.74
SRD5A2 Ala49Thr							
AA/GA	44/845 (5.3)	10/121 (8.3)	46/923 (4.9)	1.05	0.69-1.60	1.72	0.84-3.50
SRD5A2 Val89Leu							
CC/GC	448/847 (53)	65/121 (54)	477/923 (52)	1.05	0.87-1.27	1.09	0.74-1.59
GC	366/847 (43)	55/121 (45)	394/923 (43)	1.04	0.85-1.26	1.11	0.75-1.65
CC	82/847 (10)	10/121 (8)	82/923 (9)	1.10	0.79-1.54	0.96	0.47-1.96
HSD3B1 Asn367Thr							
CC/AC	384/847 (45)	60/121(50)	414/923 (45)	1.02	0.85-1.23	1.21	0.83-1.77
AC	317/847 (37)	53/121 (44)	334/923 (36)	1.04	0.86-1.27	1.32	0.89-1.96
CC	67/847 (8)	7/121 (6)	80/923 (9)	0.92	0.65-1.30	0.73	0.32-1.65
HSD17B3 Gly289Ser							
AA/GA	51/847 (6.0)	6/121 (5.0)	58/923 (6.3)	0.95	0.64-1.40	0.78	0.33-1.84
AKR1C3 Gln5His							
GG/CG	493/847 (58)	73/120 (61)	544/923 (59)	0.97	0.80-1.17	1.08	0.73-1.60
GC	394/847 (47)	56/120 (47)	441/923 (48)	0.96	0.78-1.17	1.02	0.68-1.55
GG	99/847 (12)	17/120 (14)	103/923 (11)	1.03	0.75-1.41	1.33	0.73-2.42

	Unselected Cases (%)	Familial Cases (%)	Controls (%)	Unselected Cases		Familial Cases	
				OR	(95% CI)	OR	(95% CI)
AKR1C3 Pro180Ser							
TT/CT	40/847 (4.7)	10/120 (8.3)	63/922 (6.8)	0.68	0.45-1.02	1.24	0.62-2.49
CYP19A1 Thr201Met							
TT/TC	24/847 (2.8)	4/121 (3.3)	13/923 (1.4)	2.04	1.03-4.03	2.39	0.77-7.46
CYP19A1 Arg264Cys							
TT/CT	74/846 (8.7)	14/121 (12)	75/921 (8.1)	1.08	0.77-1.51	1.48	0.81-2.70
CYP17A1 -34T>C							
CC/TC	568/845 (67)	73/120 (61)	597/923 (65)	1.12	0.92-1.36	0.85	0.55-1.25
TC	426/845 (50)	55/120 (46)	453/923 (49)	1.11	0.90-1.36	0.84	0.56-1.28
CC	142/845 (17)	18/120 (15)	144/923 (16)	1.16	0.88-1.54	0.87	0.49-1.55
AR Arg726Leu							
T	11/847 (1.3)	3/121 (2.5)	8/923 (0.9)	1.04	0.96-1.13	1.10	0.98-1.25
KLK3 Asp102Asn							
AA/GA	68/847 (8.0)	13/121 (11)	66/923 (7.2)	1.13	0.80-1.61	1.56	0.84-2.93
KLK3 -252A>G							
GG/AG	480/842 (57)	66/121 (55)	510/923 (55)	1.08	0.90-1.30	0.97	0.66-1.42
AG	385/842 (46)	49/121 (40)	417/923 (45)	1.06	0.87-1.29	0.88	0.59-1.33
GG	95/842 (11)	17/121 (14)	93/923 (10)	1.19	0.86-1.63	1.37	0.76-2.47
KLK3 Ile179Thr							
CC/TC	115/846 (14)	15/120 (13)	116/923 (13)	1.11	0.84-1.46	0.99	0.56-1.77

3.3 Joint effect analysis

Combined genetic variations in the androgen biosynthesis pathway genes may alter an individual's risk of prostate cancer (Modugno et al. 2001). We further tested a hypothesis of a joint impact of *CYP19A1* Thr201Met with five common polymorphisms (*LHB* Ile15Thr, *CYP17A1* -34T>C, *KLK3* -252A>G, *AKR1C3* Gln5His and *SRD5A2* Val89Leu) in prostate cancer risk. Increased risk was noted in individuals carrying both *CYP19A1* Thr201Met and *KLK3* -252A>G (OR=2.87, 95% CI 1.10-7.49, Table 10.).

Table 10. *Joint effect of CYP19A1 Thr201Met and five common polymorphisms in prostate cancer risk. Table modified from Supplementary Table 2 in Study IV.*

Genotypes (Interaction)		Cases	Controls	OR	95% CI	P
CYP19A1 T201M[b]	*LHB* I15T					
CC	TT	587	676	1.00		
CC	TC/CC	235	234	1.15	(0.93-1.43)	0.18
CT/TT	TT	19	11	1.99	(0.94-4.21)	0.07
CT/TT	TC/CC	5	2	2.88	(0.56-14.9)	0.21
CYP19A1 T201M	*CYP17A1* –34T>C					
CC	TT	271	325	1.00		
CC	TC/CC	550	585	1.12	(0.92-1.37)	0.24
CT/TT	TT	6	1	7.19	(0.86-60.1)	0.07
CT/TT	TC/CC	18	12	1.79	(0.85-3.81)	0.12
CYP19A1 T201M	*KLK3* –252A>G					
CC	AA	353	406	1.00		
CC	AG/GG	465	504	1.06	(0.88-1.28)	0.54
CT/TT	AA	9	7	1.47	(0.54-4.01)	0.44
CT/TT	AG/GG	15	6	2.87	(1.10-7.49)	0.03
CYP19A1 T201M	*AKR1C3* Q5H[a]					
CC	CC	383	372	1.00		
CC	CG/GG	480	538	0.97	(0.79-1.17)	0.74
CT/TT	CC	11	7	1.70	(0.65-4.45)	0.28
CT/TT	CG/GG	13	6	2.34	(0.88-6.25)	0.09
CYP19A1 T201M	*SRD5A2* V89L					
CC	GG	388	441	1.00		
CC	GC/CC	435	469	1.05	(0.87-1.27)	0.58
CT/TT	GG	11	5	2.50	(0.86-7.26)	0.09
CT/TT	GC/CC	13	8	1.86	(0.76-4.50)	0.18

[a]All genetic variants were coded as dominant with the exception of *AKR1C3* Q5H since the minor allele was not present as a homozygote.

[b]T, Thr; M, Met; I, Ile; Q, Gln; H, His; V, Val; L, Leu

Discussion

1. Methodological considerations

SSCP is widely used in mutation detection in association with inherited diseases, genetic polymorphisms, and also for somatic mutations in tumor tissue. SSCP is based on conformational changes in DNA due to sequence alterations (Orita et al. 1989, Sekiya 1993). It is estimated to yield over 90% efficiency in detecting single base substitutions in sequences of 300 bp or less in length (Fan et al. 1993, Sekiya 1993). There are two major disadvantages of SSCP. First, the amounts of mobility differences have little if any correlation to the amount of sequence differences. Second, the optimal amplicon size for detection of most point mutations is rather small, around 200-300 bp.

For detection of known mutations in certain sites of a gene, several different methods were used. Direct sequencing of PCR amplified products was one method of choice for mutation analysis; it is perhaps more labor intensive compared to some other methods such as TaqMan assay, but has the advantage of giving precise information on the sequence alterations unlike some other methods like SSCP. For some variants we used an application called solid-phase minisequencing (Syvänen 1998). It is a good choice when the variant is rare, so that four to six samples can be pooled together. In addition to minisequencing, we used TaqMan 5' nuclease assay and microarray analysis developed in the National Institute of Health. TaqMan 5' nuclease assay is very suitable for the detection of one variant in a large sample set, whereas the allele-specific primer extension on microarray method is designed to genotype large amounts of SNPs simultaneously from a large sample set.

2. Contribution of *MSR1* to prostate cancer in Finland

Despite reports that *MSR1* is involved in prostate cancer etiology (Xu et al. 2002a, Xu et al. 2003), other studies could not always replicate the association between cancer and this gene (Miller et al. 2003, Wang et al. 2003). Our results are consistent with other studies that have not detected any associations of *MSR1* variants with prostate cancer risk. In our study, the rare Arg293X mutation, which was previously reported in prostate cancer families from North America (Xu et al. 2002a, Miller et al. 2003, Wang et al. 2003), was found in three of the 120 families (2.5%). Similarly, the subsequent studies detected the Arg293X

mutation in two out of the 83 (2.4%) Swedish families affected with prostate cancer and in an affected sib pair and a single prostate cancer case from another family from Germany (Lindmark et al. 2004a, Maier et al. 2006). In contrast, Bar-Shira et al. (2006) did not detect any sequence alterations in *MSR1* gene among 300 prostate cancer cases or 50 control individuals (mostly Jewish Ashkenazi). In our study, the frequency of Arg293X mutation was highest among familial prostate cancer cases (2.5%), whereas in the Swedish study, the highest frequency was observed among incident prostate cancer cases (4.9%). However, in both studies the difference between cases and controls was not statistically significant. In Germany, carriers of Arg293X were equally frequent in sporadic prostate cancer cases (1.9%) and in controls (2.0%).

Hope et al. (2005) tested for the Arg293X mutation in 2,943 men with invasive carcinoma and in 2,870 controls from case-control, cohort and prostate cancer family studies conducted in several Western countries. The overall prevalence of mutation carriers was 2.7% in cases and 2.2% in controls and did not differ by country, ethnicity, or source of subjects. The country-adjusted risk ratio from the logistic regression analysis was 1.31 (95% CI 0.93-1.84). To adjust the bias that is due to the fact that some cases were ascertained on the basis of having a family history, the data were also analyzed by a modified segregation analysis. This way the risk ratio was estimated to be 1.20 (95% CI 0.87-1.66). Hope et al. (2005) also performed a meta-analysis that combined their data and data from Xu et al. (2002a), Miller et al. (2003), Wang et al. (2003) and our study (Study I). With the pooled OR of 1.34 (95% CI 0.94-1.89) the meta-analysis showed that if the mutation is associated with an increased risk of prostate cancer, its effect on risk is not large and is unlikely to be more than 2-fold.

Rennert et al. (2005) genotyped 888 prostate cancer cases and 473 controls of European American descent, and 131 cases and 163 controls of African-American descent. Similar to our result, no overall associations between *MSR1* variants and prostate cancer were seen. However, after stratification by race, family history and disease severity, some associations were detected. IVS7delTTA (INDEL7) homozygous genotype was significantly associated with family-history negative cancer and lower-grade disease (OR=3.0, 95% CI 1.3-6.9 for European Americans and OR=2.9, 95% CI 1.2-7.2 for African-Americans). In contrast, Arg293X was associated with high-grade disease in a subset of European Americans with no family history of prostate cancer (OR=4.0, 95% CI 1.1-14.1). Rennert et al. (2005) also performed a meta-analysis using their data and data from four published studies including our study (Study I, Miller et al. 2003, Xu et al. 2003, Lindmark et al. 2004a). They identified significant associations of any INDEL7, any PRO3, and any Arg293X with prostate cancer in Caucasians with or without a family history of cancer. Unfortunately there was not enough data to replicate this result in strata defined by family history.

Very recently, the third meta-analysis of eight published studies (Study I, Xu et al. 2002a, Miller et al. 2003, Wang et al. 2003, Xu et al. 2003, Lindmark et al. 2004a, Hope et al. 2005, Rennert et al. 2005) was performed including three rare

mutations and five common polymorphisms of the *MSR1* gene (Sun et al. 2006a). Overall, there was evidence for an association between sporadic prostate cancer risk and Arg293X among white men (OR=1.35; 95% CI 1.01-1.79, based on fixed effects model analysis assuming common effects for all studies), and for Asp174Tyr among black men (OR=2.39; 95% CI 1.23-4.63, based on fixed effects model analysis). However, if the random effects model was used, which assumes that effect estimates may vary across studies, the results were not statistically significant. Likewise, results were not statistically significant when the initial report of Xu et al. (2002a) was excluded. Furthermore, there was no evidence supporting an association between Arg293X and hereditary/familial prostate cancer. Thus Arg293X is unlikely to be a highly penetrant variant that leads to hereditary prostate cancer. Additional studies to investigate the Asp174Tyr variant, which is only observed among black men, are warranted, since so far it has been genotyped only among 273 cases and 576 controls.

In addition to previously reported variants, studies from both Sweden and Germany detected several novel variants. In the study by Lindmark et al. (2004a) two variants were potential splice site mutations, others were intronic or in 5'- or 3'-UTR and therefore considered not to be deleterious. Maier et al. (2006) identified a second nonsense allele (Ser84X) in one family with two affected and one unaffected carrier, a splice site mutation c.818-1G>A that leads to an unstable transcript in one affected sib pair and several missense mutations, most in one single proband. Unfortunately, the newly identified nonsense, splice-site and missense variants were not found in additional samples and therefore their role in prostate cancer risk could not be evaluated. Homozygosity for rare *MSR1* variants has been observed for both Arg293X (Rennert et al. 2005) and Asp174Tyr (Miller et al. 2003). However, whereas Asp174Tyr homozygous carriers were affected with the disease, the Arg293X homozygous carrier was a 77-year-old control individual.

According to our study, the mean age at diagnosis of the Arg293X mutation carriers among probands from prostate cancer families was significantly lower compared to non-carriers (P=0.04), indicating that *MSR1* might modify the age at diagnosis. Likewise, Maier et al. (2006) report that the subgroup of carriers appeared to be diagnosed 3.5 years earlier than patients with wild-type *MSR1* genotypes (P=0.09). In contrast, in the Swedish study, the mean age at diagnosis was almost identical among Arg293X carriers and non-carriers (P=0.65, Lindmark et al. 2004a). Although the exact role of *MSR1* in prostate carcinogenesis is unknown, some processes involving macrophages have been implicated in the development of prostate cancer (De Marzo et al. 1999). In the prostate MSR1 expression is restricted to macrophages, particularly those present at sites of inflammation. In addition, the degree of macrophage infiltration has been shown to associate with prostate cancer prognosis (Shimura et al. 2000). These reports support our finding that MSR1 might modify age at diagnosis. However, the mechanisms by which the defects in macrophage function might lead to prostate cancer or influence prostate cancer severity or progression have not been elucidated. Studies on knockout mice have shown that *Msr-A* -/- mice appear vulnerable to infection by Listeria monocytogenes,

Staphylococcus aureus, Estercia coli and Herpes simplex virus type 1 (Suzuki et al. 1997, Thomas et al. 2000, Platt, Gordon 2001). Taken together, *MSR1* is not a high penetrant prostate cancer susceptibility gene, but rather a modifier gene that can influence the age at onset.

3. *CHEK2* in prostate cancer predisposition

Defects in *CHEK2* contribute to the development of both hereditary and sporadic human cancers (Bartek, Falck & Lukas 2001). Therefore it is a potential tumor suppressor gene that could also have a role in prostate cancer. Our results suggest that 1100delC, a protein-truncating variant of *CHEK2*, is associated with a positive family history of prostate cancer (OR=8.24, P=0.02). Notably, the families that carried the mutation had only few affected members. At about the same time as us, Dong et al. (2003) published a study suggesting that mutations in *CHEK2* may contribute to prostate cancer risk. Previously, the 1100delC allele has been found to confer an elevated risk of breast cancer, especially in families with only two affected relatives (Vahteristo et al. 2002). It has also been reported to be in excess among Dutch families with aggregates of both breast and colon cancer (Meijers-Heijboer et al. 2003). So far, only few studies of the role of *CHEK2* 1100delC variant in colorectal cancer have been published (Kilpivaara et al. 2003, Lipton et al. 2003, de Jong et al. 2005). The results are consistent with a low-penetrance effect of the mutation on colorectal cancer risk (de Jong et al. 2005). The only homozygous 1100delC carrier reported so far was affected with colon cancer, indicating that the mutation appears not to be lethal in humans (van Puijenbroek et al. 2005).

Recently another truncating variant (IVS2 +1G>A) was shown to associate with familial prostate cancer in Poland and, similar to our results, only two men with prostate cancer were observed in mutation-positive Polish prostate cancer families (Cybulski et al. 2004c). We did not detect IVS2 +1G>A variant in SSCP analysis of 120 probands, but it has been detected previously in a single family with prostate cancer in the United States (Dong et al. 2003). In the Polish study, IVS2 +1G>A variant was the dominant truncating mutation with frequencies of 3.1% in familial prostate cancer patients, 1.2% in unselected prostate cancer cases and 0.3% in controls (Cybulski et al. 2004c). These frequencies are almost identical to the frequencies of 1100delC in our study. In Poland, the 1100delC mutation was found in 0.4% of unselected prostate cancer cases and in 1.0% of familial cases, compared to 0.2% of controls. In Sweden, the 1100delC mutation was detected in 1.2% of prostate cancer cases (sporadic: 0.7%; familial: 1.6%; hereditary: 1.4%) and in 1.0% of the controls (Wagenius et al. 2006). Dong et al. (2003) found the mutation only in 1/298 patient with familial prostate cancer and in 1/400 individual with sporadic prostate cancer. In contrast to our study, no statistically significant associations were detected in any of these three studies between the 1100delC mutation and prostate cancer (Dong et al. 2003, Cybulski

et al. 2004c, Wagenius et al. 2006). However, due to the limited size of the study (Wagenius et al. 2006) or the rarity of the mutation (Dong et al. 2003, Cybulski et al. 2004c) they cannot rule out the fact that 1100delC may moderately affect prostate cancer risk. Indeed, when both truncating mutations (IVS2 +1G>A and 1100delC) were analyzed together in the Polish study, significant associations were seen with OR of 3.4 (95% CI 1.4-8.3) for unselected cases and OR of 9.0 (95% CI 2.7-29.9) for familial cases (Cybulski et al. 2004c). Furthermore, the failure to detect a significant association between *CHEK2* variants and familial cases in the United States could be attributable to the fact that the families included a relatively high number of affected men (Dong et al. 2003).

Loss of heterozygosity analyzes have shown that in tumor tissue, loss of either the wild-type or 1100delC variant allele can occur (Vahteristo et al. 2002, Jekimovs et al. 2005). Jekimovs et al. (2005) performed functional analysis of *CHEK2* 1100delC using heterozygous lymphoblastoid cell lines. Mutant mRNA represented about 20% of the total CHEK2 transcript, demonstrating that variant message is present but may undergo nonsense-mediated decay. In contrast, 1100delC protein was absent in heterozygous lymphoblastoid cell lines. In these cell lines, the amount of total CHEK2 protein and phosphorylation of CHEK2 on Thr68 was reduced to half compared to wild type lymphoblastoid cell lines, suggesting that 1100delC variant may act simply by haploinsufficiency (Jekimovs et al. 2005). The increased breast and prostate cancer risk associated with *CHEK2* 1100delC may therefore be attributed to a threshold level of phosphorylation of *CHEK2* below which cells become more susceptible to other genetic and environmental factors promoting tumorigenesis. Furthermore, compensation of the 1100delC defect by *CHEK1* might explain the rather low cancer risks associated with *CHEK2* variant.

In our study, the missense variant Ile157Thr was also more frequent in men with familial prostate cancer (10.8%) than in controls (5.4%, OR=2.12, P=0.04). Similarly, in the Polish study, Ile157Thr was detected in 16% of men with familial prostate cancer and in 4.8% of the controls (OR=3.8, P=0.00002, Cybulski et al. 2004c). In the same study, Ile157Thr was identified in 7.8% of consecutive patients with prostate cancer (OR=1.7, P=0.03). Our results together with the results of Cybulski et al. (2004c) support the hypothesis that the Ile157Thr allele confers increased susceptibility to prostate cancer. In contrast, Dong et al. (2003) did not detect any association between Ile157Thr and prostate cancer. Interestingly, Kilpivaara et al. (2004), in a case-control study from Finland, found some evidence that the variant Ile157Thr may also be associated with breast cancer risk (OR=1.43, 95% CI 1.06-1.95). While Bogdanova et al. (2005) were able to confirm this result both in German and Byelorussian populations, Schutte et al. (2003) produced contradictory results. Based on these results, Ile157Thr variant seems to be more common in Eastern Europe (Cybulski et al. 2004c, Kilpivaara et al. 2004, Bogdanova et al. 2005) than elsewhere (Dong et al. 2003, Schutte et al. 2003). The reasons for the discrepancies in both prostate and breast cancer studies may be heterogeneous and could be due to a smaller cohort size, a very low frequency of the Ile157Thr allele or a sampling bias toward multiple-case families.

The CHEK2 Ile157Thr protein is stable and itself deficient in recognizing its physiological substrates p53 (Falck et al. 2001a), Cdc25A (Falck et al. 2001b), and BRCA1 (Li et al. 2002), and it undermines the function of wild-type CHEK2 when expressed in the same cells, resulting in checkpoint defects in response to ionising radiation (Falck et al. 2001b). Thus, the Ile157Thr may contribute to tumorigenesis by a dominant negative effect on the remaining wild-type CHEK2.

Recently, another study identified *CHEK2* as a multiorgan cancer susceptibility gene in Polish cancer patients (Cybulski et al. 2004b). Interestingly, both truncating mutations (1100delC and IVS2 +1G>A) and Ile157Thr were found at higher frequencies in a series of 690 Polish prostate cancer patients than in 4,000 population controls (OR=2.2, P=0.04 for any truncating mutation and OR=1.7, P=0.002 for Ile157Thr). These results are in line with our data and support the view that *CHEK2* variants are common cancer predisposing alleles.

4. *KLF6* IVS1 –27G>A – a disease causing variant or a neutral polymorphism?

KLF6, an ubiquitously expressed transcription factor, has extensive activity in regulating cell growth, tissue injury, and differentiation. The researchers from Mount Sinai School of Medicine, New York, claim that *KLF6* is a tumor suppressor gene inactivated by allelic loss and somatic mutation in sporadic prostate cancers that can mediate growth suppression both by a p53-independent up-regulation of p21 (Narla et al. 2001) and by disrupting the interaction between cyclin D1 and cyclin dependent kinase 4 (Benzeno et al. 2004). Based on the study by Narla et al. (2001), tumor-specific *KLF6* mutations would be expected overall in up to half of sporadic prostate tumors with Gleason scores in the range of 3-8, and thus would constitute the most frequent gene mutation event identified to date in prostate carcinogenesis. In contrast, Chen et al. (2003) report a low prevalence of miscoding *KLF6* mutations in tumors of patients with high-grade prostate cancer (9%, average Gleason score >8; 75 tumors analyzed) and Mühlbauer et al. (2003) did not detect any mutation among 32 tumors (average Gleason score 7.3).

The results of Narla et al. (2005a) suggested that *KLF6* IVS1 –27G>A effectively disrupts a regulated pattern of KLF6 splicing and, through overexpression of splice variants, could lead to an increased relative risk of prostate cancer. Subsequently they demonstrated that the KLF6 splice variant 1 (SV1), whose expression is increased in cells carrying IVS1 –27A-allele, antagonizes the ability of wild type KLF6 to suppress cell proliferation and tumorigenity in vivo (Narla et al. 2005b). We missed the IVS1 –27G>A variant in our earlier study, probably because the polymorphism is located at the first nucleotide contiguous to the PCR primer used then (Koivisto et al. 2004b). Now we detected the variant first by direct sequencing in a small sample set, and then

genotyped it among 3,348 Finnish men. Surprisingly, the carrier frequency of the A-allele turned out to be considerably higher in the Finnish population (21% in combined set of controls) compared to a tri-institutional study from the United States (13% in combined controls, Narla et al. 2005a). In contrast to Narla et al. (2005a), we did not detect any association between *KLF6* IVS1 –27G>A variant and prostate cancer when cases were compared to population controls or to PSA tested controls. The combined data from John Hopkins University and Mayo Clinic studies showed a significant association with both sporadic (OR=1.47; 95% CI 1.08-2.00) and familial prostate cancer (OR=1.61; 95% CI 1.20-2.16). However, no association between genotype and sporadic cancer was observed in the third centre from USA, namely the Fred Hutchinson Cancer Research Center (OR=0.86; 95% CI 0.62-1.20). Interestingly, the frequency of the heterozygotes was higher among controls from the Fred Hutchinson Cancer Research Center (15.6%) compared to Johns Hopkins University (10.2%) and Mayo Clinic (11.4%). This might indicate that the sample groups from three different US centers are genetically heterogeneous.

Bar-Shira et al. (2006) detected five different germline sequence variations in *KLF6* among 300 (188 Ashkenazi and 112 non-Ashkenazi) Jewish prostate cancer patients. Two alterations (-4C>A and –80C>T) were found in the 5'-UTR. The carrier frequencies of these variants did not differ between 300 cases and 200 controls. Three variants were novel: 366G>C (Thr122Thr), 515C>T (Pro172Leu) and 478C>T (Gln160X). These mutations were so rare that association analyzes could not be performed. *KLF6* IVS1 –27G>A polymorphism was detected in 48 of 402 (11.9%) Jewish prostate cancer patients and in 52 out of 300 (17.3%) controls. Two hundred and one and 100 of patients and controls respectively were Ashkenazi. The A-allele was significantly less frequent in the total prostate cancer population compared to controls (P=0.030). When Ashkenazi and non-Ashkenazi patients were analyzed separately, significantly lower frequency of A-allele was detected only in Ashkenazi population (P=0.047). These results are in line with our study suggesting that *KLF6* IVS1 –27G>A polymorphism is not a disease causing variant at least in the Finnish and Ashkenazi Jewish populations.

To our knowledge, our study was the first report on the role of IVS1 –27G>A in BPH. Given the growth promoting effects of the SNP reported by Narla et al. (2005a), its role in BPH could be possible. However, we found no evidence that A-allele is associated with BPH.

5. Variation along the androgen biosynthesis pathway in relation to prostate cancer

LHB, *AR*, *SRD5A2* and *CYP17A1*

In our study, no statistically significant association was observed between the two previously reported polymorphisms, *AR* Arg726Leu and *LHB* Ile15Thr, and prostate cancer. In the initial study of the *LHB*, a trend towards positive association between carriers of the LH-βV variant allele and familial prostate cancer was seen (OR= 1.29, 95% CI 0.96-1.75, Elkins et al. 2003). As far as we know, this is the only replication study of the role of LH-βV protein in prostate cancer. Douglas et al. (2005) studied the role of *LHB* –1864C>T polymorphism in prostate cancer using family-based association tests. No significant associations were found.

AR Arg726Leu mutation was initially identified in a patient with prostate cancer and his offspring (Elo et al. 1995). Afterwards it was detected in another Finnish patient whose cancer appeared during finasteride treatment for BPH (Koivisto et al. 1999). In both cases, the mutation was in the germline. Since then, three other studies have looked for this variant in prostate cancer patients from the United States, in male breast cancer patients from Finland and in patients with psychiatric disorders from the United States and Finland (Gruber et al. 2003, Syrjäkoski et al. 2003, Yan et al. 2004). Only one carrier was found – a Finnish man suffering from alcoholism (Yan et al. 2004). In 2000, Mononen et al. (2000) found the mutation in eight out of 418 (1.9%) sporadic prostate cancer patients, in two out of the 106 (1.9%) familial prostate cancer patients and in nine out of 900 (0.3%) population controls from Finland. Accordingly, *AR* Arg726Leu seemed to be 6-fold more frequent among prostate cancer patients compared to controls. We were able to detect the mutation in additional Finnish cases but the statistically significant association did not persist. In the study by Mononen et al. (2000) the sporadic cases were diagnosed 1996-1999; whereas in our study the unselected cases were diagnosed 1999-2001. Therefore, the discrepancy between studies may reflect the different disease spectrum before and after the increase use of PSA testing in Tampere region. Also, in the first study this mutation was found in two families, where it segregated with the disease. In our new study, we identified a new mutation-positive family with two prostate cancer cases. Unfortunately there was no sample available from the second prostate cancer patient and therefore the segregation could not be studied.

SRD5A2 Ala49Thr missense substitution has been reported to increase the apparent maximal steroid 5α-reductase activity (V_{max}) 5-fold in vitro (Makridakis et al. 1999). Therefore, it is a good candidate to increase the risk of prostate cancer and some studies seemed to prove this hypothesis (Makridakis et al. 1999, Margiotti et al. 2000). Other, more recent epidemiological studies,

however, have reported a non-significant association between the Ala49Thr variant and prostate cancer predisposition (Chang et al. 2003, Loukola et al. 2004). Mononen et al. (2001) likewise detected no association between Ala49Thr and prostate cancer. Our present study extends and supplements the work of Mononen et al. (2001) showing that Ala49Thr is not a risk allele for prostate cancer in the Finnish population. The results are in line with the meta-analysis, which did not detect an association in subjects of European descent (Ntais, Polycarpou & Ioannidis 2003b). The Ala49Thr mutation seems to be absent or very rare in Asian population (Hsing et al. 2001, Li et al. 2003).

Another *SRD5A2* SNP, the Val89Leu missense substitution has also been associated with an increased prostate cancer risk (Cicek et al. 2004, Loukola et al. 2004, Lindström et al. 2006). However, in a study by Loukola et al. (2004), the Val89Leu variant was reported to be in extremely high linkage disequilibrium with another SNP (-3001G>A), and therefore they could notdistinguish which of the SNPs is causal for disease. Our results and those from a meta-analysis of nine studies do not show any increased risk conferred by the Val89Leu polymorphism (Ntais, Polycarpou & Ioannidis 2003b).

It should be noted that the meta-analysis performed by Ntais et al. (2003b) did not address whether these *SRD5A2* polymorphisms may have an effect on the clinical behavior of prostate cancer or other clinicopathological attributes. Nam et al. (2001) reported a 3.3-fold significantly increased risk of disease progression in patients with at least one Val-allele at codon 89. Conversely, Söderström et al. (2002) found an association with homozygous Leu89 genotype and metastases at the time of diagnosis, and Shibata et al. (2002) observed poorer prognosis among men homozygous for Leu89 genotype. Jaffe et al. (2000) claimed that the presence of the Ala49Thr variant is associated with characteristics suggesting poor prognosis. Söderström et al. (2002) observed that cases heterozygous for Ala49Thr variant were significantly younger than cases that were homozygous for Ala49 genotype. We also wanted to study whether the carrier status of Ala49Thr and Val89Leu was associated with clinicopathological features, but no associations emerged from these analyzes. Similarly, Latil et al. (2001) found no significant associations for ether of the *SRD5A2* variants with pathological or clinical manifestations of tumors. Definitions of clinicopathological correlates varied considerably across different studies, thus making it difficult to compare the results.

CYP17A1 is also a fairly well studied gene acting in the sex steroid hormone synthesis pathway. In a meta-analysis conducted by Ntais et al. (2003a) there was no overall association between a substitution of C to T in *CYP17A1* existing 34 basepairs upstream of the translation start site, but downstream of the transcription start site and prostate cancer. Similar results were obtained in the meta-analysis of breast cancer studies (Ye, Parry 2002). Neither did several subsequent case-control studies observe an association with prostate cancer (Lin et al. 2003, Madigan et al. 2003, Cicek et al. 2004, Loukola et al. 2004, Vesovic et al. 2005). Therefore, the lack of overall association we observed for *CYP17A1* -34T>C was not surprising. In contrast, however, a very recent Swedish population-based study reports an inverse association with prostate cancer risk

(Lindström et al. 2006). Interestingly, when stratifying by T-stage and WHO grade, a significantly increased risk of moderate prostate cancer (T1-T2 and WHO grade II-III) was seen in our study.

HSD3B1, *HSD3B2*, *HSD17B2*, *HSD17B3*, *AKR1C3* and *KLK3*

Human 3β-hydroxysteroid dehydrogenase may regulate DHT levels by initiating the inactivation of this potent androgen in the target tissue. In addition, it is required for the biosynthesis of androgens. The enzyme is encoded by two homologous and closely linked loci: the *HSD3B1* and *HSD3B2* genes, which are both located on chromosome 1p13 (Berube et al. 1989, Labrie et al. 1992). The *HSD3B1* gene encodes the type 1 enzyme, which is exclusively expressed in the placenta and peripheral tissues, such as prostate, breast, and skin. The *HSD3B2* gene encodes the type 2 enzyme, which is predominantly expressed in classical steroidogenic tissues, namely the adrenals, testis, and ovary (Simard et al. 1996). One missense *HSD3B1* variant, Asn367Thr associated with prostate cancer has been reported to date (Chang et al. 2002b). In addition, this SNP and 7519C>G polymorphism in *HSD3B2* seemed to have an additive effect on risk. We also detected the Asn367Thr polymorphism, but could not replicate the association either in familial or unselected cases. We were, however, able to find another, very rare missense variant Arg71Ile from *HSD3B1* gene. The function of these variants is unknown.

Other androgen metabolic loci that may play a role in prostate cancer predisposition are the *HSD17B* genes. 17β-hydroxysteroid dehydrogenases are of crucial importance in the regulation of the intracellular levels of biologically active steroid hormones in a variety of tissues. Generally, the reduction step is essential for the formation of active estrogens as well as active androgens, whereas the oxidative reaction is required for the inactivation of potent sex steroids into compounds having only low biological activity or no activity at all (Peltoketo, Vihko & Vihko 1999).

HSD17B3 gene encodes 17β-hydroxysteroid dehydrogenase type 3 and catalyses testosterone biosynthesis in the testes (Geissler et al. 1994). The *HSD17B3* gene is located in chromosomal band 9q22 and contains 11 exons. Several mutations that cause male pseudohermaphrodism have been functionally characterized in this gene (Geissler et al. 1994). These mutations, however, do not appear to be involved in prostate diseases in adults. Moghrabi et al. (1998) reported a missense substitution, Gly289Ser, in exon 11 of the *HSD17B3* gene. Our results contradict the claim of Margiotti et al. (2002) that Gly289Ser variant increases the risk for sporadic cancer. Enzymes bearing either glycine or serine at this position have similar substrate specificities and kinetic constants (Moghrabi et al. 1998). We also identified a novel deletion of 7 nucleotides in exon 10, which was found to be a very rare mutation. There were only four heterozygous carriers among the 1,457 samples genotyped. This novel deletion leads to a truncated protein so that the first affected amino acid is at codon 201.

This is followed by 10 novel amino acids and a stop codon, whereas the wild-type protein is 310 amino acids long.

HSD17B2 encoding 17β-hydroxysteroid dehydrogenase type 2 is another member of the large *HSD17B* gene family. This enzyme is widely expressed in different tissues such as placenta, breast, uterus, testis, liver and prostate (Moghrabi, Head & Andersson 1997, Peltoketo, Vihko & Vihko 1999). Härkönen et al. (2003) reported a remarkable decrease in the expression of the *HSD17B2* gene during the transition of cultured prostate cancer LNCaP cells to an androgen-independent stage. This observation of a decrease in the oxidative 17β-hydroxysteroid dehydrogenase type 2 activity during the cellular transformation is in line with previous results suggesting an association between a chromosomal deletion at 16q24.1-q24.2, including the *HSD17B2* gene, and clinically aggressive features of prostate cancer (Elo et al. 1999). In earlier studies, reduced expression of 17β-hydroxysteroid dehydrogenase type 2 mRNA has also been detected in prostate cancer specimens (Elo et al. 1996). No *HSD17B2* mutations associated with either predisposition to or progression of prostate cancer have been reported to date. We detected an Ala111Thr missense mutation in four unselected prostate cancer cases and in one familial prostate cancer patient. None of the 878 controls carried the mutation.

Type 2 3α-hydroxysteroid dehydrogenase encoded by *AKR1C3* gene is a multi-functional enzyme that possesses 3α-, 17β- and 20α- hydroxysteroid dehydrogenase as well as prostaglandin F synthase activities and catalyzes androgen, estrogen, progestin and prostaglandin metabolism (Penning et al. 2000, Komoto et al. 2004). *AKR1C3* was cloned from human prostate cDNA library, and is a member of the aldo-keto reductase (AKR) superfamily (Lin et al. 1997). In androgen target tissues such as the prostate, AKR1C3 catalyzes the conversion of Δ^4-androstene-3,17-dione to testosterone, 5α-DHT to 5α-androstane-3α,17β-diol, and 3alpha-diol to androsterone (Penning et al. 2000). Thus AKR1C3 may regulate the balance of androgens and hence the transactivation of the androgen receptor in these tissues. According to Fung et al. (2006) elevated expression of *AKR1C3* is strongly associated with prostate carcinoma. We identified two *AKR1C3* missense variants (Gln5His and Pro180Ser), on which no information could be found in the literature. Neither of the variants affected prostate cancer risk.

KLK3 is not actually an androgen pathway gene, but rather a target gene for androgen receptor. Transcriptional regulation of the *KLK3* gene is mediated through binding of the androgen receptor to regions of the promoter containing androgen response elements (AREs; Kim, Coetzee 2004). *KLK3* encodes for PSA, which is a serine protease that is expressed in both normal prostate epithelium as well as prostate cancer. PSA expressed by malignant cells, however, are released into the serum at an increased level, which can be detected to diagnose and monitor prostate cancer. *KLK3* is a member of a family of 15 kallikrein genes clustered on chromosome 19q13.3-13.4 (Diamandis, Yousef 2001). A number of studies have examined associations between genetic polymorphisms in *KLK3* gene, PSA levels, and prostate cancer risk. Rao et al. (2003) initially identified a single SNP within the ARE I region, a -158G>A

substitution. The associations between the -158G>A polymorphism and PSA levels have not been consistent (Medeiros et al. 2002, Xu et al. 2002, Nam et al. 2003, Wang et al. 2003). Similarly, the A allele has also been associated with prostate cancer risk in some studies (Gsur et al. 2002, Medeiros et al. 2002, Chiang et al. 2004), but not in all (Wang et al. 2003). Cramer et al. (2003) reported that specific germline genetic polymorphisms (-4643G<A, -5412C<T,-5429T<G) in the promoter region of the *KLK3* gene were associated with higher serum PSA levels among Caucasian males. Beebe-Dimmer et al. (2006) genotyped the same SNPs from 475 African-American men; no association was observed with either serum PSA level or prostate cancer diagnosis. Yang et al. (2001) identified two polymorphisms at positions -252 (G>A) and -205 (A>AA). According to this study with a small number of Japanese subjects, -252 polymorphism was suggested to be associated with prostate cancer progression. In contrast, Wang et al. (2003) did not find any significant association between the polymorphism at position -252 and the risk of prostate cancer and its progression. According to our data, there was no overall association between -252 polymorphism and prostate cancer, but the G-allele carriers had more often well-differentiated tumor compared to non-carriers (P=0.045). In addition to -252G>A variant, we genotyped three missense variants (Asp102Asn, Leu132Ile, Ile179Thr) in a large sample set, but no significant associations were seen. However, carriers of Ile179Thr were more likely to have metastases than non-carriers (P=0.009).

CYP19A1

The most interesting finding of our study was the *CYP19A1* Thr201Met variant that seemed to be associated with clinically less significant cancer. *CYP19A1* located on chromosome 15q21.2 encodes for aromatase that catalyses the formation of aromatic C18 estrogens from C19 androgens (Sebastian, Bulun 2001). The entire gene spans more than 123 kb of DNA. Only the 30 kb 3' region encodes aromatase, whereas a large 93 kb 5' flanking region serves as the regulatory unit of the gene (Sebastian, Bulun 2001). The complex expression and regulation of aromatase are achieved through the use of multiple exon 1 that encodes the 5'-UTR. Each exon 1 is used in a tissue-specific fashion by alternative splicing and is flanked by its own unique promoter region. However, the aromatase protein is the same irrespective of the site of expression and promoter used. In breast cancer, aromatase and its aberrant expression are significant (Lu et al. 1996) and therefore aromatase inhibitors have been used successfully to treat breast cancer (Brodie, Njar 2000). In normal prostate tissue, aromatase expression seems to be absent from epithelium, but localises to stroma (Ellem et al. 2004). In contrast, in malignant prostate tissue, aromatase expression was detected in epithelium and a different promoter use compared to stroma was observed. Significantly, the levels of activity measured in the prostate cell lines were within, but at the lower end of, the range of activity reported to be present in breast tumors (Ellem et al. 2004).

The *CYP19A1* has a tetranucleotide TTTA repeat polymorphism in intron 4 and the polymorphism has been reported to associate with a risk of breast cancer, prostate cancer and postmenopausal bone metabolism (Haiman et al. 2000, Masi et al. 2001, Suzuki et al. 2003b). Haiman et al. (2000) demonstrated that the presence of at least one allele more than seven TTTA repeats was associated with a lower level of plasma androstenedione, a higher level of estrogens, and a higher estrone to androstenedione ratio. A recent study did not observe an association between the tetranucleotide repeat and susceptibility to or aggressiveness of prostate cancer (Li et al. 2004). In contrast, Tsuchiya et al. (2006) reported that long allele (over seven TTTA repeats) was associated with poorer survival of prostate cancer patients with distant metastasis.

Other germline *CYP19A1* variants suggested to be associated with breast cancer risk include a SNP in 3'-UTR in exon 10 (Kristensen et al. 2000) and Trp39Arg localized in N-terminal region (Miyoshi et al. 2000). In addition, Arg264Cys has also been studied in relation to breast cancer, but appears to have no effect in this context (Probst-Hensch et al. 1999, Miyoshi et al. 2000). Modugno et al. (2001) identified the Arg264Cys variant in 9 out of 88 (10%) Caucasian prostate cancer patients and in 6 out of 241 (6%) controls from Pittsburg (all carriers were heterozygous). After adjusting for age and body mass index, this association was of borderline significance (OR=2.50; 95% CI 0.99-6.28). Suzuki et al. (2003a) genotyped 101 prostate cancer patients with at least one affected first-degree relative and 114 controls from Japan. The C/C, C/T and T/T genotypes of Arg264Cys variant were observed in 59%, 33%, and 9% of control patients and in 45% 42% and 14% of cases respectively. Thus the high prevalence of T allele of Arg264Cys polymorphism seems to be a characteristic in Asian populations (Suzuki et al. 2003a, Ma et al. 2005). According to Suzuki et al. (2003a), the presence of at least one T allele was associated with prostate cancer risk (OR=1.77; 95% CI 1.02-3.09), especially with high-grade carcinoma (OR=2.56; 95% CI 1.47-4.46). In contrast, we did not detect any association between Arg264Cys polymorphism and prostate cancer risk. The combined frequencies of C/T and T/T genotypes were 9%, 12% and 8% among unselected prostate cancer cases, familial prostate cancer cases and population controls respectively. Two studies report that the Cys246 variant was similar to the wild type enzyme with regard to substrate and inhibitor kinetics (Watanabe et al. 1997, Ma et al. 2005). Watanabe et al. (1997) claimed that aromatase activity was not affected by Cys264, whereas Ma et al. (2005) observed a slight decrease in the enzyme activity of this variant. The studies were otherwise identical, but Watanabe et al. (1997) used cytosolic marker enzyme to correct for transfection efficiency, whereas in the study by Ma et al. (2005), a fusion protein targeted at the endoplasmic reticulum was used. In addition to Arg264Cys, Trp39Arg mutation also seems to be specific for Asian populations (Miyoshi et al. 2000, Ma et al. 2005). Therefore, as expected, we did not detect any Trp39Arg mutation carrier in our study.

In addition to common Arg264Cys polymorphism, we identified a novel Thr201Met mutation. The overall association among the unselected prostate cancer cases was weak (OR=2.04; P=0.04), but the stratified analysis revealed a

strong association with clinically less significant cancer (OR=5.42; P<0.0001). According to SIFT prediction Thr201Met mutation is not tolerated. SIFT predictions have been shown to be very accurate in detecting deleterious mutations (Saunders, Baker 2002). Met201 could affect testosterone levels in the prostate through altered enzyme function. Based on the bioinformatics analyzes we can assume that Thr201 is in structurally important α-helix, most likely at least partly buried to the core of the protein. Substitution by methionine might affect the packing of the helix and consequently the local and global folding of the enzyme. The effect may also rise from losing stabilizing polar interactions formed by the hydroxyl group of threonine. Apparently, the change does not completely alter the function of the protein, but could lead to slightly modified activity and phenotype. A higher activity of the variant enzyme could result in lower levels of androgens in carriers, as androgens are more efficiently converted into estrogens. Thus lower androgen levels would explain why the cancer does not progress to aggressive state. Alternatively, the lower activity of the enzyme could lead to increased androgen levels, and androgens would then maintain the well-differentiated phenotype of the tumors. This hypothesis is in line with the finding of the Prostate Cancer Prevention Trial showing that low intraprostatic DHT levels due to finasteride treatment may lead to the loss of differentiation of the prostatic tissue (Thompson et al. 2003). However, the results of the Prostate Cancer Prevention Trial need further evaluation, since 5α-reductase inhibitors decrease the risk of prostate cancer but also alter the detection of disease through effects on PSA, and prostate volume and histology (Andriole et al. 2005). It should be noted that the hypothalamic-pituitary-gonadal axis regulates the levels of hormones in a complex way. It has been shown that testosterone concentration does not change even though the levels of estrogens are significantly decreased (Lew et al. 2003). What happens on the cellular level in the prostate is totally unknown. Interestingly, while our study was under review, Ma et al. (2005) published a study showing that Thr201Met variant does not have an effect on enzyme activity, protein quantity or substrate kinetics. However, as mentioned earlier, their enzyme activity measurements of Arg264Cys variant differed from earlier results (Watanabe et al. 1997). An even more striking difference occurred with the Trp39Arg variant, which had previously been reported to be inactive (Nativelle-Serpentini et al. 2002) and Ma et al. (2005) observed only slight decrease in enzyme activity.

To date, no information is available regarding prostate phenotypes in humans with aberrant aromatase expression (Ellem, Risbridger 2006). Therefore, transgenic and knockout mouse models are the only means to study the impact of aberrant aromatase expression in the prostate. In the aromatase overexpressing mice, squamous epithelial metaplasia is evident throughout the prostate, consistent with exposure to high levels of endogenous estrogens, while seminal vesicles and testis show atrophy and Leydig cell hyperplasia respectively, consistent with dramatically reduced peripheral androgens (Li et al. 2001). In aromatase knockout mice, weight gain of seminal vesicles and prostate is observed because of increased content of secreted material (Fisher et al. 1998).

This overall hyperplastic response is attributed to the elevation in androgens, prolactin and androgen receptor expression resulting from the estrogen deficiency. The primary influences driving the observed phenotypic changes in both of these mouse models are most likely due to the significant changes in the systemic hormone profiles of these animals. The importance of local hormone metabolism, however, remains unknown.

6. Multigenic model on cancer susceptibility

It has been widely hypothesized that the interactions of multiple genes influence individual risk for common cancers. Fu et al. (2003) demonstrated a trend toward increased risk of developing breast cancer in women harboring a greater number of putative high-risk genotypes of non-homologous end-joining pathway. Yu et al. (2006) observed that specific combination of variant genotypes in *ERα-MTA3-CDH1* pathway were more common in breast cancer patients than controls. Furthermore, Shen et al. (2006) observed a joint effect on breast cancer risk between *CYP1A1* and *ERα* polymorphisms. Goodman et al. (2006) explored the association between colon cancer and 94 SNPs in 63 genes. They identified variants in *TP53* and *CASP8* that may interact with *GSTT1* polymorphism, which was the best predictor of colon cancer among the studied SNPs.

Few studies have investigated the combined effect of polymorphic alleles in different genes on prostate cancer risk and not all studies have had a sufficient sample size or statistical power to adequately examine gene-gene interactions. Stanford et al. (1997) analyzed the polymorphic CAG and GGN repeats within the *AR* gene among 301 cases and 277 controls. When both repeat lengths were considered jointly, the subgroup with two short repeats had a 2-fold elevation in risk (OR=2.05; 955 CI 1.09-3.84) relative to those with two long repeats. Murata et al. (1998) analyzed the combined effect of the *CYP1A1* and *GSTM1* polymorphisms in 115 prostate cancer patients and 204 control individuals. They found that the combination of the *CYP1A1* Val allele and *GSTM1* null (0/0) genotype was associated with a higher risk (OR=2.3, 95% CI 1.18-4.48) than the *CYP1A1* Val allele alone. Autrup et al. (1999) reported that homozygote deletion of either *GSTM1* or *GSTT1* was not associated with increased risk of prostate cancer (OR=1.3; 95% CI 0.9-1.9 and 1.3; 0.8-2.2, respectively), but deletion of both *GSTM1* and *GSTT1* gave a near-significant increased risk (OR=1.7; 95% CI 0.9-3.4). In contrast, by studying 237 cases and 239 controls Rebbeck et al. (1999) observed no interaction of these genes in prostate cancer etiology. Chang et al. (2002a) studied the association between prostate cancer and *HSD3B* genes. Men with variant allele at *HSD3B1* Asn367Thr or *HSD3B2* c7519g were at increased risk for prostate cancer compared with men who were homozygotes for the wild-type allele at both genes (RR=2.17; 95% CI 1.29-3.65 for hereditary prostate cancer and RR=1.61; 95% CI 1.07-2.42 for sporadic prostate cancer). Unfortunately, they did not have adequate power to test if men with mutations in both genes had even higher risk.

Xue et al. (2000) genotyped 57 prostate cancer cases and 156 controls to study whether allelic differences in AR-driven PSA expression may affect prostate cancer risk. Subjects with both short *AR* CAG allele and *KLK3* genotype –158G/G were shown to have more than 5-fold increase in prostate cancer risk (OR=5.08; 955 CI 1.59-16.25). At first glance, our data do not seem to support the idea that variants in *KLK3* gene increase the risk of prostate cancer, since the genotype distribution was not significantly different between our prostate cancer patients and controls. However, our observation that men carrying both *KLK3* -252A>G and *CYP19A1* variants were more common among patients than controls suggested that the SNP in the *KLK3* is associated with prostate cancer, but this can only be seen in the subgroup of men harboring high risk variant genotype of *CYP19A1*. Because of the small number of people with variant genotype at *CYP19A1* Thr201Met, we did not have adequate power to test for all possible interactions of this SNP. Of the five common polymorphisms tested, only *KLK3* –252A>G showed significant interaction with *CYP19A1* Thr201Met. Interestingly, Modugno et al. (2001) studied the joint effects of *CYP19A1* Arg264Cys variant, *AR* CAG repeat and two intronic restriction sites in *ERα*. Homozygosity for the *ERα* XbaI restriction site together with a longer CAG repeat was more frequent among controls than cases (OR=0.39, 95% CI 0.19-0.78). The *CYP19A1* Arg/Arg genotype together with longer CAG repeat was also more frequent among controls than cases (OR=0.51, 95% CI 0.30-0.89). However, care must be taken when interpreting these data because this study included only 88 prostate cancer patients and 241 controls and therefore certain genotype subgroups were very small.

The most recent interaction studies are large, with over 1,300 cases and involve the genes in the inflammatory pathway. Xu et al. (2005) found that the interaction of *IL-10*, *IL-1RN*, *TIRAP* and *TLR5* genes significantly predicts prostate cancer risk. Sun et al. (2006b) observed a gene-gene interaction between sequence variants in *IRAK4* and *TLR1*. Furthermore, when combined with the risk genotype at *TLR-6-1-10*, one SNP in *IRAK4* conferred a multiplicative risk of prostate cancer. In addition, very recently Chang et al. (2006) performed linkage scan by modeling two-locus gene-gene interactions for all possible pairs of loci across the genome. Evidence for linkage in the six sets of loci was found, suggesting an epistatic interaction of two prostate cancer susceptibility genes; i.e., mutations in two genes are needed to increase prostate cancer risk. It is also possible that one of the two loci is a major gene while the other is a modifying gene.

7. Genetic aspects

It is clear from the linkage data to date that prostate cancer is genetically complex (Easton et al. 2003). One possible type of genetic complexity is locus heterogeneity, whereby mutations in different genes can give rise to a high risk

of the disease. Another possible complexity is that much of the genetic variation may not be due to individual major genes but rather to the combined effect of a larger number of genes associated with smaller risk. The risks associated with individual genes under such a polygenic model may be small; therefore, detecting them by linkage may be difficult or even impossible. We used case-control studies to directly examine associations between gene variants and prostate cancer risk. This approach has greater power than linkage studies to detect low penetrance genes.

We were interested in estimating the magnitude of the genetic risks of the certain gene variants in Finnish population. In addition, we wanted to find out how different genetic variants act together and form the basis for disease progression. Therefore, we mainly used population-based selection of cases and controls instead of extreme groups. Our controls were blood donors, who are free of the disease, but who represent the population at risk of becoming cases. However, a small fraction of controls may be misclassified due to the fact that the blood donors are relatively young compared to prostate cancer cases and therefore, some of them might be affected with prostate cancer at a later age. In Study III, older men with PSA levels below 1.0 ng/ml were used as additional controls. When such a sample set with a very low risk is used as a control group, it might be difficult to distinguish whether a gene with a protective effect is enriched in controls; meaning that a gene variant that is more common in cases might actually be a normal and not a high risk gene variant.

Population stratification can be a confounding factor in genetic case-control or association studies (Freedman et al. 2004). The confounding occurs when individuals are selected from two genetically different populations in different proportions in cases and controls. Thus, the cases and controls are not matched for their genetic background. This may cause spurious associations, or it may mask true associations, like any other unknown confounder. In our studies, all samples were of Finnish origin, which is known to be an isolated population that has experienced marked recent population growth, starting from a small founding population (Peltonen, Palotie & Lange 2000). Isolated populations tend to be more homogeneous than the general human population and thus have the advantage of exhibiting reduced etiological, phenotypic, and genetic heterogeneity (Ellsworth, Manolio 1999). A very recent study reported that Finnish population shows particularly extensive genome-wide linkage disequilibrium with very few holes (Service et al. 2006). Furthermore, earlier studies have successfully utilized Finnish population to examine common variants of genes implicated as risk factors for complex diseases such as coronary heart disease or myocardial infection (Stengard et al. 1995, Pastinen et al. 1998).

With the exception of *MSR1* in Study I, we have used the candidate gene approach in the search for genetic markers of prostate cancer susceptibility. Despite its widespread use, the candidate gene approach is currently limited, because many important genes influencing complex diseases such as prostate cancer are unknown, or the functions of their protein products have not been well characterized (Ellsworth, Manolio 1999). A further complication is that disease

may not be associated with the candidate gene but, rather, the candidate gene may be in linkage disequilibrium with a gene that is the actual causative agent. Furthermore, all changes in DNA sequence of the candidate genes do not result in differences in protein function. Luckily for us, in many cases such as *CHEK2* 1100delC and *KLF6* IVS1 –27G>A, the function of the studied variant was already known.

8. Future prospects

In the future, the major goal will be to set up an SNP combination map for Finnish population, which will result in better prostate cancer diagnostics, improved outcome of treatments including possible new clues for therapy, and tools for prevention of the disease. So far, no genetic tests are available for prostate cancer patients. If high-risk prostate cancer predisposition mutations can be identified, genetic testing would mostly benefit families in which mutation has been discovered. In addition, by identifying several low penetrant genetic variants, risk assessment for prostate cancer in the population might be possible. Treatment of prostate cancer could also be improved by identifying genetic risk factors. Molecular changes and cancer development at the cellular level are largely unknown and therefore prostate cancer specific drugs do not exist. A major challenge is to identify aggressive cancers. Also of concern is the mounting evidence that there is a significant proportion of prostate cancers for which treatment may not be necessary. If these subclasses could be identified by genetic testing, expression analysis or proteomics assays, it would be possible to offer aggressive treatment only to men who benefit from it and avoid treatment-related side effects and complications for those who do not need treatment. Ideally, cancer specific gene-therapy could be offered to prostate cancer patients.

Screening of candidate genes and pathways will continue in the future. The details of androgen pathway, the whole network of genome-surveillance pathways and genes involved in inflammation will be under active investigation. In addition, new candidates will certainly emerge. Then, after identifying a cancer causing gene with a known function, it will be possible to exploit this information in the search for further susceptibility genes. One possibility is that cancer is caused by several genes with different functions, acting, however, in the same pathway. Alternatively, various genes with the same function may underlie the predisposition to certain cancers. This approach has been proven to be very efficient in the case of Lynch syndrome which is caused by mutations in mismatch repair genes (Plotz, Zeuzem and Raedle, 2006). Mismatch repair is performed by two protein dimers, MutS and MutL. The majority of the mutations are in *MLH1* and *MSH2* genes which are obligate factors for the mismatch repair system, since they are required for formation of all heterodimeric MutS and MutL complexes. In addition, the involvement of

mutations in *MSH6* and *PMS2* has been proven. However, patients with *PMS2* mutation have been found to show a milder phenotype of Lynch syndrome. This is explained by a partial compensation of the function of *PMS2* by another mismatch repair gene, *MLH3*. Likewise, the identification of *CHEK2-BRCA1* pathway and functional resemblance of *BRCA1* and *BRCA2* genes have helped to understand the tumorigenesis of breast cancer (Bartek, Falck and Lukas, 2001; Wooster, Weber 2003).

So far association studies have used the candidate gene approach in the search for prostate cancer susceptibility alleles. However, in the next few years, association studies will be performed on a genome-wide scale. The challenge has been due to large sample size and large number of genetic markers required for genome wide association scans. However, recent developments give great hope for future work. One solution will be to combine microarrays with hundreds of thousands SNPs and DNA pooling (Meaburn et al. 2006).

It is now clear that several major genes and many modifier genes underlie genetic susceptibility to prostate cancer. Therefore the aim will be to discover the interactions of these genes to determine the individual risk for prostate cancer. There are currently some algorithms designed to examine complex gene-gene interactions and disease risk. These methods, including multifactor dimensionality reduction and polymorphism interaction analysis have already been used successfully to explore the joint effects of multiple sequence variants in common cancers (Xu et al. 2005, Goodman et al. 2006). Future studies that include additional genes and environmental factors will likely improve our understanding of the high order gene-gene and gene-environment interactions in prostate cancer etiology. Furthermore, current linkage studies to identify the major prostate cancer susceptibility genes rely primarily on single gene approaches. Linkage analysis methods that model interactions may increase the statistical power to detect linkage when interactions among genes exist. In the future, the development of new analytical methods will make it feasible to systemically explore genome-wide interactions, also in linkage analyzes.

Resources beyond those available to any single institution will most likely be required to obtain the power necessary to map and identify prostate cancer susceptibility genes. In addition, both genome-wide association studies and gene-gene interaction studies require large sample size to achieve reasonable power. International collaboration is therefore needed. An attempt to bridge this gap is the formation of the International Consortium for Prostate Cancer Genetics (ICPCG). The ICPCG consists of 19 independent laboratories in North America, Europe and Australia, who share a common interest in genetic susceptibility for prostate cancer, and all of which have major ongoing, individual research efforts in this area.

Conclusions

To date, no high penetrant prostate cancer gene has been identified. The etiology of prostate cancer is unlikely to be explained by allelic variability at a single locus. Instead, the prevalence of prostate cancer in the population probably results from complex interactions among many genetic and environmental factors over time. The interaction of genes leading to disease may be described with either a heterogeneity model, in which alterations in any of the several genes are sufficient, or an epistatic model in which several simultaneous genetic alterations are required.

Neither our study nor any of the meta-analyzes support a major role for *MSR1* in the causation of prostate cancer (Hope et al. 2005, Rennert et al. 2005, Sun et al. 2006a). However, certain *MSR1* sequence variants may influence age at onset, prostate cancer severity or progression. Our recent genome-wide linkage study in Finnish prostate cancer families found no evidence for linkage on chromosome 8p (Schleutker et al. 2003). Therefore, it is not surprising that we did not detect any significant association between *MSR1* and prostate cancer. Regarding the fact that three research groups identified a linkage to 8p22 (Xu et al. 2001c, Wiklund et al. 2003, Maier et al. 2005b), but only one of these was able to show a significant association between *MSR1* variants and prostate cancer risk (Xu et al. 2002a, Lindmark et al. 2004a, Maier et al. 2006), this coincidence is unlikely due to *MSR1* as a major causative gene in these prostate cancer families. The number of pedigrees that segregate *MSR1* mutations is low overall, and thus cannot explain the observed linkage signal. However, the possible epistatic interactions between *MSR1* and other prostate cancer susceptibility genes, such as *RNASEL* or *ELAC2* have not been studied.

Polymorphisms in genes that code for sex steroid hormones and their signaling and metabolic pathways are compelling biological candidates for prostate cancer. At present, there is no convincing evidence for a strong effect of polymorphisms in *AR*, *LHB*, *SRD5A2* and *CYP17A1* on risk of prostate cancer (Ntais, Polycarpou & Ioannidis 2003a, Ntais, Polycarpou & Ioannidis 2003b, Zeegers et al. 2004). However, more work is needed to define the influence of polymorphisms in relation to early versus advanced prostate cancer, grade of disease, and survival with prostate cancer. Many other genes in this pathway such as those encoding for 3β- and 17β-hydroxysteroid dehydrogenases have been understudied for their relation to prostate cancer. We found an SNP in the *CYP19A1* gene that seems to be associated with less aggressive prostate cancer. Due to the extensive use of PSA screening, the most typical prostate cancers today are small organ-confined cancers. Therefore, *CYP19A1* Thr201Met variant

may serve as a marker for individuals with clinically less significant disease. Further studies, including functional analyzes, will be required to fully understand the role of this gene in prostate cancer.

In addition to sex hormone signaling pathway, other prostate cancer candidate genes exist, including genes involved in DNA repair and cell cycle control. Finding of *CHEK2* 1100delC and Ile157Thr variants in families with small numbers of affected relatives support the idea that *CHEK2* variants are low-penetrance prostate cancer predisposition alleles that contribute significantly to familial clustering of prostate cancer at the population level (Study II, Cybulski et al. 2004c). Given their low penetrance, testing for these *CHEK2* variants alone would likely have little predictive value for individual patients. However, it may eventually allow risk assessment for prostate cancer on population, or even individual level as part of a broader panel of analogous variants suitable for profiling and screening, and thereby prove useful for chemoprevention or lifestyle counselling.

In contrast to Narla et al. (2005a), in our study the *KLF6* IVS1 –27G>A variant was not associated with increased risk of prostate cancer either among familial or unselected patients. The results from our study suggest that *KLF6* IVS1 -27G>A variant is a neutral polymorphism frequently found in the Finnish population. If *KLF6* IVS1 -27G>A had an effect on cancer risk, it could act in concert with some other, so far unknown factors that could be population-specific and not prevalent in our population.

Because we tested only one or a few SNPs in every gene, we cannot exclude the role of other polymorphisms in these genes or other functionally relevant sequences in linkage disequilibrium with these SNPs. Fully deciphering the impact of these genes on prostate cancer may require the evaluation of multiple variants or haplotypes.

The highly penetrant causative mutations in prostate cancer that have a strong hereditary component might exist but have not yet been identified. In addition, there must be several low-penetrance variants that contribute to prostate cancer susceptibility in an additive way, involving interactions between genes and with environmental factors. As well as accounting for cases of hereditary or familial prostate cancer, these variants are also likely to contribute to cases of prostate cancer that are classified as sporadic. Furthermore, modifier genes are also likely to influence the effects of genetic and environmental factors that contribute to prostate cancer. Therefore, the concepts of hereditary, familial and sporadic cases are perhaps no longer applicable.

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Germ-Line Alterations in *MSR1* Gene and Prostate Cancer Risk

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ABSTRACT

Purpose: **The *MSR1* gene maps to 8p22–23, a novel susceptibility locus for hereditary prostate cancer (HPC). Mutations in *MSR1* have been reported to associate with prostate cancer (PRCA) risk. Here we report a follow-up study from Finland to evaluate the association between PRCA and *MSR1* gene.**

Experimental Design: **The youngest affected patient from each of 120 HPC families was initially used for the screening of *MSR1* mutations by single-strand conformational polymorphism analysis. Selected variants of *MSR1* gene were then screened in 537 unselected PRCA cases and in 480 controls.**

Results: **Among 120 HPC families, five *MSR1* sequence variants were identified. The carrier frequencies of the R293X, P275A, and −14743A>G variants were compared between the probands with HPC, unselected PRCA cases, and healthy male blood donors. No significant differences were observed. The odds ratios for R293X, P275A, and −14743A>G mutations were also calculated to estimate the PRCA risk. No significantly elevated or lowered risks for PRCA among these three variants were detected. However, the mean age at diagnosis of the R293X mutation carriers among HPC probands was significantly lower compared with noncarriers (55.4 *versus* 65.4 years; *t* test, $P = 0.04$). The same trend was observed among unselected PRCA cases (65.7 *versus* 68.7 years; *t* test, $P = 0.37$).**

Conclusions: **Our results do not support a major role for the *MSR1* gene in the causation of hereditary or unselected PRCAs but suggest a possible modifying role in cancer predisposition.**

INTRODUCTION

A positive family history is among the strongest epidemiological risk factors for PRCA.[2] Although an increasing number of PRCA susceptibility loci have been reported, including *HPC1* (1q24–25), *PCAP* (1q42–43), *HPCX* (Xq27–28), *CAPB* (1p36), *HPC20* (20q13), *HPC2* (17p11), and *16q23* (1), only two genes have been identified from these loci: *ELAC2* from HPC2-locus (2) and *RNASEL* from HPC1-locus (3). Some of the subsequent studies have been able to confirm the role of these genes (4, 5), whereas others have not (6, 7). Therefore, additional studies using larger cohorts are needed to fully evaluate the role of these two susceptibility genes in PRCA risk. In addition, more statistical power can also be generated with meta-analyses, as has been done recently with *ELAC2* (8). Xu *et al.* (9) reported recently evidence of linkage to a new locus at 8p22–23 in 159 pedigrees affected with HPC. The same group identified both germ-line mutations and common sequence variants from *MSR1* gene locating in 8p22 (10, 11). Wiklund *et al.* (12) confirmed the linkage to 8p22–23 in Swedish HPC material. The *MSR1* gene encodes the SR-A, which include three different isoforms (I, II, and III) generated by alternative splicing (13, 14). The functional SR-A protein is a trimeric molecule composed of three identical protein chains. SR-AI and SR-AII consists of six domains: cytoplasmic, membrane spanning, spacer, α-helical coiled-coil, collagen-like, and isoform-specific COOH-terminal domain (15). SR-A types I and II are functional integral membrane glycoproteins that bind diverse array of macromolecules (16, 17). Type III protein also contains six domains, but it is a nonfunctional protein acting as a dominant-negative isoform by blocking modified low-density lipoprotein uptake (14). Putative biological roles of SR-As include macrophage-host cell interactions, macrophage adhesion to substratum, endocytosis of ligands, phagocytosis of apoptotic cells and microbes, and clearance and detoxification of microbial products (17). Here, we report a study from Finland to additionally evaluate the association between PRCA and *MSR1* gene.

MATERIALS AND METHODS

Families with HPC. A detailed description of the original collection of families, confirmation of diagnosis, and prostate specific antigen testing for unaffected males in the HPC families is described elsewhere (18). In this study we used two different cohorts of families: the first cohort included 68 fami-

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[2] The abbreviations used are: PRCA, prostate cancer; HPC, hereditary prostate cancer; SSCP, single-strand conformation polymorphism; SR-A, class A macrophage scavenger receptor.

Table 1 Summary of *MSR1* germ-line variants found in 120 patients with HPC in the SSCP analysis

Mutation[a]	Amino acid change	Exon/intron	Domain	Number of families
-14743A>G	–	–	–	15
77G>A	R26H	Exon 2	Cytoplasmic	1
823C>G	P275A	Exon 6	Collagen like	3
877C>T	R293X	Exon 6	Collagen like	3
1034-10 variation in n. of T nucleotides[b]	–	Intron 9	–	17

[a] Numbering is according to the cDNA (NM_138715) starting at the A in the start codon.
[b] Min. number of T nucleotides = 18; max = 29; most frequently 21 T nucleotides.

lies and the second included 52 families. Families in the first cohort had either three or more affected members or two affected members with at least the index patient diagnosed with PRCA ≤60 years of age. The mean age at diagnosis for the index patients was 62.2 years (range, 44–81 years), and the mean number of affected family members was 3.2 (range, 2–6). The second cohort of families had only two affected members with ages at diagnosis >60 years. The mean age at diagnosis for the index patients in this cohort was 69.0 years (range, 61–86). In both family cohorts the affected persons were first- or second-degree relatives. The youngest affected patient from each of 120 HPC families was initially used for the screening of *MSR1* mutations by SSCP analysis. Selected variants of *MSR1* gene were assayed in all of the other available affected and unaffected members of the mutation-positive families.

Patients with PRCA and Controls. There were 634 consecutive patients diagnosed with PRCA in the Pirkanmaa Hospital District with a population of ~450 000 during 1999–2000. We had samples from 85% of these patients, which results in an unselected, population-based collection of patients. The mean age at diagnosis for the patients with unselected PRCA was 68.6 years (range, 47–90). Of the patients with unselected PRCA, 12% (66 of 537) reported a positive family history of PRCA. The controls consisted of DNA samples from anonymous male blood donors obtained from the Blood Center of the Finnish Red Cross in Tampere.

Written informed consent was obtained from all of the living patients and also, for families with HPC, from the unaffected members. The research protocols were approved by the Ethical Committee of the Tampere University Hospital (93175, 95062, and 99228), and the National Human Genome Research Institute (HG-0158). Permission for collection of families, in the entirety of Finland, was granted by the Ministry of Social Affairs and Health (59/08/95).

Mutation Screening with SSCP Analysis. SSCP analysis of the entire coding sequence of the *MSR1* gene was performed using primer sequences that were designed to include all of the intron-exon boundaries (GenBank accession nos. NM_138715, NM_002445, and NM_138716[3]). On request, all of the primers are available from the authors. The 15-μl reaction mixture contained 1.5 mM $MgCl_2$; 20 μM each of dATP, dCTP, dGTP, and dTTP; 0.5 μCi of α [^{33}P]-dCTP (Amersham Pharmacia, Uppsala, Sweden); 0.6 μM of each primer; 1.0 unit AmpliTaqGold; the reaction buffer provided by the supplier (PE Biosystems, Foster City, CA); and 25 ng of the genomic DNA. For exon 10 the PCR reaction mixture contained 5% DMSO. Annealing temperature of 55°C was used for exons 1–4, 9, and 11; temperature of 51°C was used for exons 5–8, and exon 10 needed temperature of 53°C. Radiolabeled PCR products were mixed with 95% formamide dye, denatured at 95°C for 5 min, and chilled on ice. The ^{33}P-labeled PCR products were electrophoresed at 800 V for 12 h at room temperature, in 0.5× mutation-detection-enhancement gel (FMC BioProducts, Rockland, ME) with 1% glycerol in 0.5× Tris-borate EDTA. After electrophoresis, gels were dried and exposed to Kodak BioMax maximum resolution films for 6 h. All of the samples in which variant bands were detected, as well as two to three normal bands per exon, were analyzed by sequencing using an automated ABI Prism 310 Genetic Analyzer (PE Biosystems, Foster City, CA). Variants were identified using Sequencher software version 3.0 (Gene Codes Corporation, Ann Arbor, MI).

Minisequencing and SSCP for Large-Scale Population Screening of Identified Variants. The frequencies of P275A and R293X variants were determined in the entire set of 1137 samples by SSCP analysis as described above. R26H mutation was screened in 239 patients with unselected PRCA and in 240 controls by minisequencing (19). PCR for minisequencing was performed with 100 ng of DNA, 0.2 μM each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, and 1.0 unit of AmpliTaqGold (PE Biosystems), in a final volume of 50 μl. The single nucleotide polymorphism in the promoter region was screened in 239 patients with unselected PRCA and in 192 controls by SSCP as described above.

Statistical Analyses. Association of the *MSR1* genotypes with HPC and unselected PRCA was tested by logistic-regression analysis, by use of the SPSS statistical software package (SPSS 11.0). Association with demographic, clinical, and pathological features of the disease was tested by the Mann-Whitney test, Pearson χ^2 test, and Fisher's exact test by use of the SPSS statistical software package (SPSS 11.0).

RESULTS AND DISCUSSION

Among 120 HPC families we identified five sequence variants, including one nonsense mutation at codon 293 (R293X), two missense mutations (R26H and P275A), and two sequence variants (A>G in promoter region and variation in number of T nucleotides in intron 9; Table 1). R293X, P275A, and SNP in the promoter region were also reported by Xu *et al.*

[3] Internet address, GenBank: http://www.ncbi.nlm.nih.gov/Genbank/index.html.

Table 2 Association of the variants of the *MSR1* gene with patients with unselected PRCA or HPC

Sample and mutation	No. of carriers/total (frequency)	Odds ratio	95% confidence interval	*P*
R293X				
Controls	5/480 (1.0%)	1.00		
Patients with unselected PRCA	6/537 (1.1%)	1.07	0.33–3.54	0.91
Patients with HPC	3/120 (2.5%)	2.44	0.57–10.34	0.23
P275A				
Controls	20/480 (4.2%)	1.00		
Patients with unselected PRCA	21/537 (3.9%)	0.94	0.50–1.75	0.84
Patients with HPC	3/120 (2.5%)	0.59	0.17–2.02	0.40
14743 A>G				
Controls	14/192 (7.3%)	1.00		
Patients with unselected PRCA	24/239 (10.0%)	1.42	0.71–2.83	0.32
Patients with HPC	15/120 (12.5%)	1.82	0.84–3.91	0.13

(10, 11). R293X mutation deletes most of the ligand-binding domain and the entire COOH-terminal cysteine-rich domain. P275A changes the first Gly-Xaa-Yaa repeat in the collagen-like domain.

To investigate the possible segregation of the variants R293X, R26H, and P275A, we sequenced all of the available affected and unaffected male relatives from the mutation-positive families. R26H was found only in 1 family including 3 affected men. The proband and his 50-year-old unaffected brother carried the mutation. The affected father could not be genotyped, but his affected brother did not carry the variant. In addition, R26H mutation was screened in 239 patients with unselected PRCA and in 240 healthy male blood donors. No mutation carriers were found. Thus, R26H is a very rare novel mutation. The SNP in promoter region (−14743A>G) was found in 12.5% (15 of 120) of the HPC families. We screened the variant from all of the other available affected male relatives from these families. There was no clear evidence of cosegregation; 19 of 32 of the screened affecteds carried the variant. In addition, 10.0% (24 of 239) of the unselected PRCA patients and 7.3% (14 of 192) of the control individuals carried the variant. The carrier frequencies did not differ significantly between the three different sample groups (Pearson χ^2 test, $P = 0.30$).

R293X and P275A were both found in three probands from different families. One family having 3 affected members carried both mutations but in different individuals. In this family, there was 1 affected (the proband) and 2 unaffected R293X carriers, and 1 unaffected P275A carrier. The second affected did not carry either of the variants, and the third affected could not be genotyped. The 2 other R293X mutation-positive families had only 2 affecteds; in both families they both carried the mutation. Two of the P275A-positive families had 2 affected relatives, and 1 had 3 affected relatives. P275A did not segregate with the disease in these families.

Carrier frequencies of the R293X and P275A variants were compared between patients and control subjects. The carrier frequencies for R293X were 2.5%, 1.1%, and 1.0%, in the 120 probands with HPC, 537 unselected PRCA cases, and 480 healthy blood donors, respectively. There was no statistically significant difference in the carrier frequencies between the different sample groups (Pearson χ^2 test, $P = 0.41$), although the percentage was highest among HPC patients. The carrier frequencies for P275A were 2.5%, 3.9%, and 4.2% in the probands with HPC, unselected PRCA cases, and healthy blood donors, respectively. No significant difference was observed in the carrier frequencies between the different sample groups (Pearson χ^2 test, $P = 0.70$). We also calculated the odds ratios for R293X, P275A, and −14743A>G mutations to estimate the PRCA risk (Table 2.). There were no significant elevated or lowered risks for PRCA among these three variants.

The mean age at diagnosis of the R293X mutation carriers among HPC probands was significantly lower compared with noncarriers (55.3 *versus* 65.4 years; *t* test, $P = 0.04$; Table 3). Difference in ages at diagnosis in these groups can be because of small number of mutation carriers ($n = 3$). However, the same trend was observed among unselected PRCA cases (65.7 *versus* 68.7 years; *t* test, $P = 0.37$). Although the exact role of *MSR1* in prostate carcinogenesis is unknown, some processes involving macrophages have been implicated in the development of PRCA (20). In addition, the degree of macrophage infiltration has been shown to associate with PRCA prognosis (21). These reports support our finding that MSR1 might modify age at diagnosis. No other statistically significant associations of the R293X, P275A, or −14743A>G variant with demographic, clinical, or pathological features of the disease were observed (Tables 3 and 4.).

In the study from the United States, R293X was observed in 3.2% (6 of 190) of families (all 6 were of European descent; Ref. 10). Each family had at least three first-degree relatives affected with PRCA. In the same study, the carrier frequency of R293X was 2.5% (8 of 317) among individuals with non-HPC (affected men either without a family history of PRCA or with one affected first degree relative). In the present study, the detected R293X frequency of 2.5% among our HPC probands is the same as the frequency in the non-HPC cases in the United States study. It should be noticed that the sample grouping criteria and definition were different in these two studies, because 54% of our HPC families had only two affected relatives. In addition, R293X was observed in 1.5% of men exposed to asbestos in the study of Xu *et al.* (10). This group was analyzed to determine the frequency in the general United States population. Interestingly, we detected similar frequency of 1.0% among our population controls, which were anonymous male blood donors.

Table 3 Association of *MSR1* genotype with age and PSA value at diagnosis in PRCA patients

	R293X			P275A			nt-14743		
	C/C	C/T[a]	P^b	C/C	C/G[a]	P^b	A/A	A/G + G/G[c]	P^b
Mean age at diagnosis									
HPC probands	65.4	55.3	0.04	65.1	66.3	0.80	65.1	65.3	0.95
Unselected PRCA cases	68.7	65.7	0.37	68.6	68.0	0.70	67.7	68.0	0.87
Median PSA-value at diagnosis[d]									
Unselected PRCA cases	10.7	5.2	0.10	10.4	10.9	0.88	11.0	10.8	0.39

[a] All mutation carriers were heterozygous.
[b] Analyses were carried out using the *t* test for equality of means and Mann-Withney U-test for equality of medians.
[c] One HPC proband was homozygous, all other mutation carriers were heterozygous.
[d] Information was not available from HPC probands.

Table 4 Association of *MSR1* genotype with clinicopathological characteristics of the unselected PRCA patients

	R293X			P275A			nt-14743		
	C/C $n = 531$	C/T[a] $n = 6$	P^b	C/C $n = 516$	C/G[a] $n = 21$	P^b	A/A $n = 215$	A/G[a] $n = 24$	P^b
T stage			1.00			0.26			0.83
T1–T2	339	4		327	16		127	15	
T3–T4	192	2		189	5		88	9	
N stage			0.40			0.16			0.64
N0	155	3		153	5		71	10	
N1	4	0		3	1		3	0	
Nx	372	3		360	15		141	14	
M stage			0.71			0.77			0.63
M0	212	2		205	9		98	10	
M1	58	1		58	1		21	1	
Mx	261	3		253	11		96	13	
Gleason score			0.50			0.95			0.10
2–6	216	4		212	8		77	14	
7–10	251	2		242	11		102	7	
Unknown	64	0		62	2		36	3	
WHO grade			0.50			0.08			0.37
I	124	3		126	1		58	5	
II	305	3		293	15		118	14	
III	78	0		75	3		34	3	
Unknown	24	0		22	2		5	2	

[a] All mutation carriers were heterozygous.
[b] Analyses were carried out using the Fisher's exact test.

The carrier frequencies for P275A in the United States studies were 15.8% (30 of 190), 9.6% (29 of 301), and 16.4% (41 of 250) in the HPC families, non-HPC patients, and unaffected controls, respectively (10, 11). These frequencies were much higher compared with our results. This suggests that P275A is a more common variant in the heterogeneous United States population compared with the Finnish population, which is known to be historically isolated and genetically homogeneous (22). P275A did not show cosegregation in the United States families either.

Our study is the first reported follow-up study to investigate the role of *MSR1* in PRCA causation. The initial reports by Xu *et al.* (10, 11) found that germ-line mutations and sequence variants of the *MSR1* gene are associated with PRCA risk. Our results do not support a major role for the *MSR1* gene in the causation of hereditary or unselected PRCAs. However, R293X mutation might influence disease onset by lowering the age at diagnosis. Consistent with the results, our recent genome wide linkage study in Finnish HPC families found no evidence for linkage on chromosome 8p (23). Therefore, it is not surprising that we did not detect any significant association between *MSR1* and PRCA. The present results warrant additional studies of the role that MSR1 variants have as risk factors for HPC and unselected PRCA in other populations.

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CHEK2 variants associate with hereditary prostate cancer

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Recently, variants in *CHEK2* gene were shown to associate with sporadic prostate cancer in the USA. In the present study from Finland, we found that the frequency of 1100delC, a truncating variant that abrogates the kinase activity, was significantly elevated among 120 patients with hereditary prostate cancer (HPC) (four out of 120 (3.3%); odds ratio 8.24; 95% confidence interval 1.49–45.54; $P = 0.02$) compared to 480 population controls. Suggestive evidence of segregation between the 1100delC mutation and prostate cancer was seen in all positive families. In addition, I157T variant had significantly higher frequency among HPC patients (13 out of 120 (10.8%); odds ratio 2.12; 95% confidence interval 1.06–4.27; $P = 0.04$) than the frequency 5.4% seen in the population controls. The results suggest that *CHEK2* variants are low-penetrance prostate cancer predisposition alleles that contribute significantly to familial clustering of prostate cancer at the population level.
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Analyses using families with hereditary prostate cancer (HPC) have suggested that multiple genetic loci may harbour prostate cancer susceptibility genes, including HPC1 (MIM 601518) at 1q24–q25, HPC2 (MIM 605367) at 17p11, PCAP (MIM 602759) at 1q42–q43, HPCX (MIM 300147) at Xq27–q28, CAPB (MIM 603688) at 1p36, and HPC20 (MIM 176807) at 20q13 (Nwosu *et al*, 2001). So far, only two genes have been identified from these chromosomal regions: *ELAC2* from HPC2 –locus (Tavtigian *et al*, 2001) and *RNASEL* (MIM 180435) from HPC1 –locus (Carpten *et al*, 2002). In Finland neither *ELAC2* nor *RNASEL* did explain the disease segregation in HPC families, but seemed to have some kind of modifying role in prostate carcinogenesis (Rokman *et al*, 2001; Rokman *et al*, 2002). Xu *et al* (2001) identified a new locus at 8p22–23, and mutations in *MSR1* gene (MIM 153622) were reported to associate with prostate cancer (Xu *et al*, 2002). However, recent results do not support a major role for the *MSR1* gene in the causation of prostate cancer (Seppala *et al*, in press). Definitive confirmations of the role of *ELAC2*, *RNASEL*, or *MSR1* in prostate cancer predisposition are still warranted.

Recently, mutations in *CHEK2* (MIM 604373) were identified in patients with prostate cancer (Dong *et al*, 2003). The *CHEK2* gene localises to chromosome 22q12.1 and contains 14 exons. Originally, germline mutations in *CHEK2* gene were reported in Li–Fraumeni syndrome and breast cancer (Bell *et al*, 1999; Allinen *et al*, 2001). Rare somatic mutations in *CHEK2* have also been identified in a number of cancer types, including lung and ovarian cancers and osteosarcomas (Miller *et al*, 2002). These results together with the normal function of *CHEK2* in DNA damage checkpoints are consistent with the idea that *CHEK2* might act as a tumour suppressor gene. Here, we explored the significance of *CHEK2* gene in prostate cancer causation in Finland. The Finnish population is known to be historically isolated and genetically homogeneous (Peltonen *et al*, 2000). Therefore, there may be a limited number of prostate cancer causing mutations, and the effect of individual risk genes could be identified more readily than in more heterogeneous populations.

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MATERIALS AND METHODS

Families with HPC

Collection of Finnish families with HPC has been described elsewhere (Schleutker *et al*, 2000). For single-strand conformation polymorphism (SSCP) analysis, youngest affected patient from each of the 120 HPC families was initially used for the screening of *CHEK2* mutations. The HPC families consisted of two cohorts. The families in the first cohort ($n = 68$) had either three or more first- or second-degree- affected members or two affected members with at least the index patient diagnosed with prostate cancer $\leqslant 60$ years of age. The mean age at diagnosis for the index patients was 62.2 years (range 44–81 years), and the mean number of affected family members was 3.2 (range 2–6). The second cohort of families ($n = 52$) had only two first- or second-degree affected members with ages at diagnosis >60 years. The mean age at diagnosis for the index patients was 69.0 years (range 61–86).

Patients with prostate cancer and controls

The 1100delC and I157T variants were analysed in 537 patients with unselected prostate cancer, and in 480 healthy male blood donors. There were altogether 634 consecutive patients diagnosed with prostate cancer in the Pirkanmaa Hospital District with a population of around 450 000 during 1999–2000. We had samples from 85% of these patients, which results in an unselected,

population-based collection of patients. The mean age at diagnosis for the patients with unselected prostate cancer was 68.6 years (range 47–90). Information was available on the tumour WHO-grade in 96%, on Gleason score in 88%, on T-stage in 100%, and on N-stage in 30% of the patients. M-stage was ascertained by bone scan in 73% of the patients with PSA $\geqslant 10\,\mu g\,l^{-1}$ and in 26% of the patients with PSA $< 10\,\mu g\,l^{-1}$. In all, 12% (66 out of 537) of the patients reported a positive family history of prostate cancer. The controls consisted of DNA samples from anonymous male blood donors obtained from the Blood Center of the Finnish Red Cross in Tampere.

Written informed consent was obtained from all living patients and also, for families with HPC, from the unaffected members. The research protocols were approved by the Ethical Committee of the Tampere University Hospital (93175, 95062, and 99228), and the National Human Genome Research Institute (HG-0158). Permission for collection of families, in the entirety of Finland, was granted by the Ministry of Health and Social Affairs (59/08/95).

Mutation screening with SSCP analysis

Single-strand conformation polymorphism analysis of the entire coding sequence of the *CHEK2* gene was designed to include all intron–exon boundaries (GenBank accession number AF086904). Primers used for amplification of exons 10–14, which are known to be repeated on several other chromosomes (Sodha *et al*, 2000), were designed so that both primers for each primer pair had a base mismatch in the most 3′ nucleotide, compared with sequences from nonfunctional copies of *CHEK2*. Genomic DNA was used at 25 ng per 15 μl reaction mixture containing 1.5 mM $MgCl_2$; 20 μM each of dNTP; 0.5 μCi of $\alpha(^{33}P)$-dCTP (Amersham Pharmacia, Uppsala, Sweden); 0.6 μM of each primer; 1.0 U Ampli*Taq*Gold; and the reaction buffer provided by the supplier (PE Biosystems, Foster City, CA, USA). Annealing temperature of 50°C (for exons 10 and 12) or 55°C (for all other exons) was used. After denaturation, the (^{33}P)-labeled PCR products were electrophoresed at 800 V for 12 h at room temperature, in 0.5 × mutation-detection-enhancement gel (FMC BioProducts, Rockland, ME, USA) with 1% glycerol in 0.5 × Tris-borate EDTA. For exon 11, the electrophoresis was also performed without glycerol in the gel. After electrophoresis, gels were dried and exposed to Kodak BioMax maximum-resolution films for 6 h. All samples, in which variant bands were detected, as well as two normal bands per exon, were sequenced using the same PCR primers and ABI Prism 310 Genetic Analyzer (PE Biosystems, Foster City, CA, USA). Also, the genotypes of the available family members of the mutation carriers were determined by sequencing.

Minisequencing and SSCP for large-scale population screening of identified variants

The frequencies of the two *CHEK2* variants were determined in the entire sample of patients described above. 1100delC variant was screened by minisequencing (Syvanen, 1998). PCR was performed with 100 ng of DNA, 0.2 μM each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, and 1.0 U of Ampli*Taq*Gold (PE Biosystems, Foster City, CA, USA), in a final volume of 50 μl. I157T variant was screened by SSCP analysis as described above. Positive results from both mutation analyses were confirmed by sequencing.

Statistical analyses

Association of the CHEK2 genotypes with HPC and unselected prostate cancer was tested by logistic-regression analysis, by use of the SPSS statistical software package (SPSS 11.0). Association with demographic, clinical, and pathological features of the disease was tested by the Mann–Whitney test, Pearson χ^2 test, and Fisher's exact test by use of the SPSS statistical software package (SPSS 11.0).

RESULTS

Five sequence variants were identified in the SSCP analysis of the *CHEK2* gene in 120 index patients from Finnish families with HPC (Table 1). Two of the variants, a missense variant 470T > C (I157T) in exon 3 and a frameshift mutation 1100delC in exon 10, were the same as previously reported in patients with Li–Fraumeni syndrome (Bell *et al*, 1999), breast (Allinen *et al*, 2001; Vahteristo *et al*, 2002), and prostate cancer (Dong *et al*, 2003). The frameshift 1100delC mutation has been proven to result in the loss of kinase activity (Wu *et al*, 2001), and I157T variant has been shown to be defective in its ability to bind and phosphorylate Cdc25A, one of its normal substrates (Falck *et al*, 2001). These variants were further studied in a set of 1137 samples. In addition, a silent exonic change also reported by Bell *et al* (1999), an intronic change (not affecting splice site), and a novel missense mutation 1312G > T (D438Y) were observed. D438Y mutation was found only in one proband. In this family there were two prostate cancer patients. Unfortunately, we did not have a sample from the second affected person (deceased).

The 120 patients with HPC included 3.3% (four out of 120) patients who carried the 1100delC mutation (Table 2). This was significantly higher (odds ratio (OR) = 8.24; 95% confidence interval (CI) 1.49–45.54; $P = 0.02$) than the frequency 0.4% seen in population sample of 480 blood donors. Among the unselected patients with prostate cancer, the frequency of 1100delC variant was 1.3% (seven out of 537). All 1100delC carriers were heterozygous. All other affected and unaffected male relatives were also genotyped from 1100delC-positive families. Suggestive evidence of segregation between the 1100delC mutation and prostate cancer was seen in all four families (Figure 1). Since 1100delC mutation has been reported in Li–Fraumeni syndrome and was previously called a mutation hot spot (*CHEK2* [MIM 604373]), we looked other cancers in these four 1100delC-positive families. In family 001, there was one second-degree relative diagnosed with lung cancer, but no other cancers were found among first- or second-degree relatives.

Table 1 Summary of *CHEK2* germline variants found in 120 patients with HPC in the SSCP analysis

Mutation	Amino-acid change	Exon/intron	Domain
252A > G	Silent (E84)	Exon 1	Unknown
319+(43–44)insA	—	Intron1	—
470T > C	I157T	Exon 3	FHA[a]
1100delC	Frameshift	Exon 10	Kinase
1312G > T	D438Y	Exon 11	Kinase

[a]FHA = forkhead-associated domain.

Table 2 Association of the 1100delC and I157T variants of the *CHEK2* gene with patients with unselected prostate cancer or HPC

Mutation and sample	No. of carriers/total (frequency)	OR	95% CI	P
1100delC				
Controls	2/480 (0.4%)	1.00		
Patients with unselected Prostate cancer	7/537 (1.3%)	3.14	0.65–15.16	0.15
Patients with HPC	4/120 (3.3%)	8.24	1.49–45.54	0.02[a]
I157T				
Controls	26/480 (5.4%)	1.00		
Patients with unselected Prostate cancer	42/537 (7.8%)	1.48	0.89–2.46	0.13
Patients with HPC	13/120 (10.8%)	2.12	1.06–4.27	0.04[a]

[a]Statistically significant.

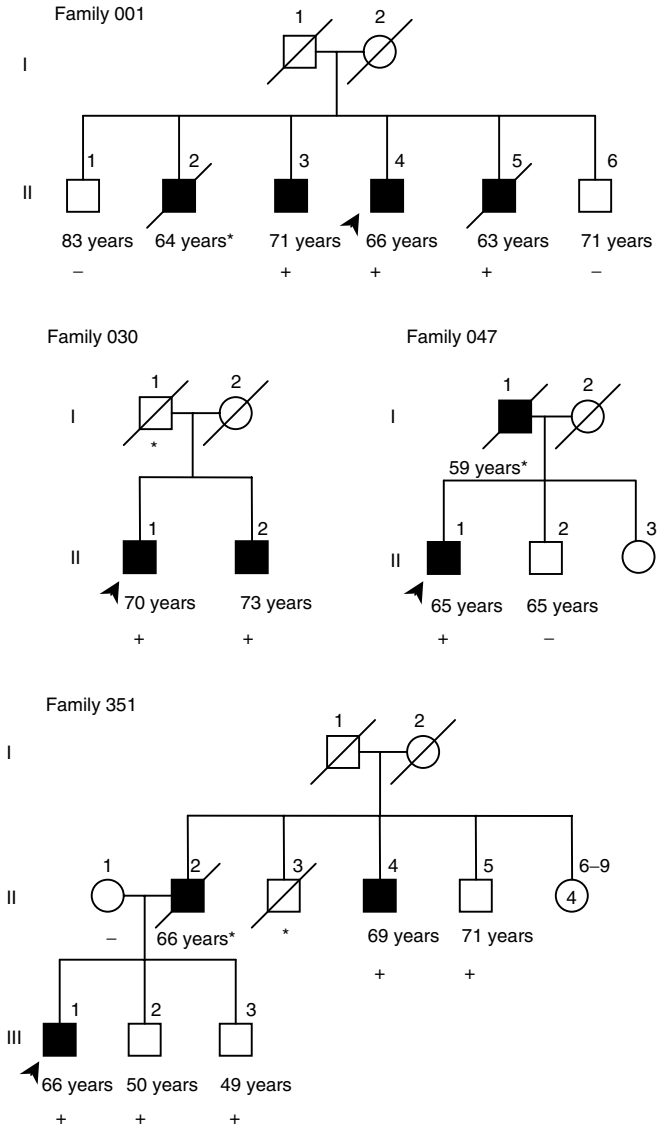

Figure 1 Segregation of *CHEK2* 1100delC mutation in four families with HPC. 1100delC variant carriers are denoted by a plus sign (+), and noncarriers by a minus sign (−). An asterisk (*) denotes the persons with no sample available. No sample was available from affected father (II-2) in family 351, but because the mother (II-1) did not carry the mutation, the father is a likely 1100delC mutation carrier. Current age of the unaffected members or age at diagnosis for prostate cancer patients (in years) is indicated below the symbol for each family member. In each family, the index patient is marked with an arrow. Squares denote male subjects, and circles denote female subjects; black symbols denote patients with prostate cancer.

The I157T variant was seen in 10.8% (13 out of 120) of patients with HPC (Table 2). This was also significantly higher (OR = 2.12; 95% CI 1.06–4.27; $P = 0.04$) than the frequency of 5.4% seen in the population controls. Nine of the I157T-positive families had only two affecteds, three families had three affecteds, and one family had four affecteds. Segregation of the variant with the disease was incomplete, in that both unaffected mutation carriers and mutation-negative patients with prostate cancer were observed. In addition, the I157T variant was found in 7.8% (42 out of 537) of unselected prostate cancer patients. One of these carriers was homozygous; all other I157T variant carriers were heterozygous. The homozygous carrier did not have family history of cancer and there was nothing unusual in his phenotype. None of the patients or controls carried both 1100delC and I157T variant.

The mean ages at diagnosis of the *CHEK2* variant carriers in patients with HPC were 62.7 years for 1100delC carriers and 64.0 years for I157T carriers. These ages were only marginally different from the mean age of HPC patients with no mutations (65.2 years for both variants; $P = 0.57$ for 1100delC and $P = 0.62$ for I157T). A similar trend was observed in the cohort of unselected prostate cancer patients (64.6 *vs* 68.7 years; $P = 0.18$ for 1100delC and 68.4 *vs* 68.6 years; $P = 0.86$ for I157T). The association between the frequency of the two variants and disease phenotype, including tumour WHO-grade, Gleason score, T-, N- and M-stage and PSA value at diagnosis, were also analysed among unselected prostate cancer cases. No significant associations emerged from these analyses (data not shown).

DISCUSSION

CHEK2 has been suggested to be a candidate tumour suppressor gene on the basis of the findings that normal function of CHEK2 is involved in DNA-damage respond and some of the mutations identified in Li–Fraumeni families were expected to result in a truncated protein (Bell *et al*, 1999). Subsequently, these findings were supported by the reports concerning identical and additional mutations in patients with Li–Fraumeni syndrome (Lee *et al*, 2001) and breast cancer (Meijers-Heijboer *et al*, 2002; Vahteristo *et al*, 2002). While this manuscript was in preparation, another study was published showing that mutations in *CHEK2* were associated also with prostate cancer risk (Dong *et al*, 2003).

Our results suggest that *CHEK2* 1100delC mutation is associated with positive family history of prostate cancer. The mutation segregated almost completely in all mutation-positive families (Figure 1). In family 351, there were three unaffected men, who carried the variant. Two of them were rather young, about 50 years old (III-2 and III-3), and the third unaffected carrier was 71 years old (II-5). The total PSA values of the unaffected mutation carriers of this family were measured in July 2000. The values were <0.5, 2.2, and 2.4 $\mu g\,l^{-1}$ for III-2, III-3 and II-5, respectively. The mean age at diagnosis of prostate cancer in Finland was 71.1 years in 1999 (Finnish Cancer Registry; cancer statistics at http://www.cancerregistry.fi/) and all three affecteds of the family 351 were over 66 years old when diagnosed for prostate cancer, thus the future diagnosis of prostate cancer cannot be ruled out for the healthy carriers of this family. On the other hand, in the four 1100delC-positive families, there were no mutation-negative prostate cancer patients. The association of 1100delC mutation with families that include small number of affected relatives, the most common types of prostate cancer families, implies that the mutation is likely to have a significant contribution to familial prostate cancer at the population level. In addition, I157T seems to be a disease-associated polymorphism at least in the Finnish population. It has a slightly higher frequency among patients with unselected prostate cancer than among control individuals and it is strongly associated with family history of the disease. However, according to the previous reports, the I157T allele does not make a significant contribution to breast cancer susceptibility (Allinen *et al*, 2001; Schutte *et al*, 2003). Therefore, the association with this allele is less conclusive.

Previously, Vahteristo *et al* (2002) reported the strong association of the *CHEK2* 1100delC with breast cancer families that included only two affected patients, suggesting that 1100delC is a low-penetrance genetic alteration. In contrast to our results, Dong *et al* (2003) reported the association of the *CHEK2* mutations (all mutations pooled together) only with sporadic prostate cancer. In addition, they did not observe any association between prostate cancer and I157T variant. The reason why Dong *et al* (2003) did not detect any association with HPC could be due to different

sample settings: the families from the USA represent more extreme HPC families than the Finnish families in the present study. In their study two affected members from 149 HPC families with at minimum of three affected men over at least two generations were used. In our recent genome-wide linkage analysis, no positive signals were seen on chromosome 22 (Schleutker *et al*, in press). This is probably due to the selected study material, as only the most extreme families were genotyped, possibly reflecting the same phenomenon as seen in the study of Dong *et al* (2003). Also, the low allele frequencies of *CHEK2* variants (<10%) make this kind of an association almost impossible to detect by linkage analysis.

Dong *et al* (2003) reported a total of 13 different *CHEK2* germline mutations among 400 sporadic prostate cancer patients and 298 individuals with familial prostate cancer. Most of these mutations occurred only once in their study population. The reason why fewer variants were found in our study can possibly be due to the limited sensitivity of the SSCP analysis and the number of screened patients. Most likely, however, the reason is the study population itself. The Finnish population is genetically much more homogeneous than the US population, and therefore it is not surprising that fewer variants were detected.

Taken together, finding of the 1100delC and I157T variants in families with small numbers of affected relatives support the idea that *CHEK2* variants are low-penetrance prostate cancer predisposition alleles that contribute significantly to familial clustering of prostate cancer at the population level, especially in families with small number of affected relatives. However, variants in *CHEK2* gene alone do not explain the familial clustering of prostate cancer in Finland as the majority of families did not have any *CHEK2* alterations. The present results warrant further studies of the role of *CHEK2* variants as a risk factor for prostate cancer in other populations.

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Profiling Genetic Variation along the Androgen Biosynthesis and Metabolism Pathways Implicates Several Single Nucleotide Polymorphisms and Their Combinations as Prostate Cancer Risk Factors

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Abstract

Several candidate genes along androgen pathway have been suggested to affect prostate cancer risk but no single gene seems to be overwhelmingly important for a large fraction of the patients. In this study, we first screened for variants in candidate genes and then chose to explore the association between 18 variants and prostate cancer risk by genotyping DNA samples from unselected (n = 847) and familial (n = 121) prostate cancer patients and population controls (n = 923). We identified a novel single nucleotide polymorphism (SNP) in the *CYP19A1* gene, T201M, with a mild significant association with prostate cancer [odds ratio (OR), 2.04; 95% confidence interval (95% CI), 1.03-4.03; P = 0.04]. Stratified analysis revealed that this risk was most apparent in patients with organ-confined (T_1-T_2) and low-grade (WHO grade 1) tumors (OR, 5.42; 95% CI, 2.33-12.6; P < 0.0001). In contrast, *CYP17A1* −34T>C alteration was associated with moderate to poorly differentiated (WHO grade 2-3) organ-confined disease (OR, 1.42; 95% CI, 1.09-1.83; P = 0.007). We also tested a multigenic model of prostate cancer risk by calculating the joint effect of *CYP19A1* T201M with five other common SNPs. Individuals carrying both the *CYP19A1* and *KLK3* −252A>G variant alleles had a significantly increased risk for prostate cancer (OR, 2.87; 95% CI, 1.10-7.49; P = 0.03). In conclusion, our results suggest that several SNPs along the androgen pathway, especially in *CYP19A1* and *CYP17A1*, may influence prostate cancer development and progression. These genes may have different contributions to distinct clinical subsets as well as combinatorial effects in others illustrating that profiling and joint analysis of several genes along each pathway may be needed to understand genetic contributions to prostate cancer etiology. (Cancer Res 2006; 66(2): 743-7)

Introduction

Prostate cancer is the most common male malignancy and the second leading cause of cancer deaths in many western countries. There is large variation in the risk to prostate cancer among different racial/ethnic groups (1). Although the reason for this is mostly unknown, differences in diet and hormonal levels may be involved (2). Androgens are essential for both the normal and malignant growth and differentiation of the prostate. Castrated men never develop prostate cancer and androgen ablation is widely used as the primary treatment for extracapsular prostate cancer (3, 4). Further evidence of the role of the androgens in prostate cancer etiology comes from rat experiments wherein testosterone and dihydrotestosterone have been used to induce prostate cancer (5, 6).

Because of the importance of androgens to prostate cancer development, genes involved in the biosynthesis and metabolism of androgens have been under intensive study. Already several genes have been identified along the androgen pathway, such as *SRD5A2, CYP19A1, CYP17A1, HSD3B1*, and *AR* (7–11), of which genetic variation is suggested to be associated with an increased risk of prostate cancer. However, many of the effects observed have been rather modest. Furthermore, studies have often been done using rather small sample sets and replication of the data in independent clinical cohorts has not been possible. To establish whether the genetic variants in the androgen pathway are predictive of prostate cancer, we screened 10 genes for possible disease-associated variations and then genotyped 18 selected alterations among a large sample set, including a total of 1,891 samples from unselected and familial prostate cancer patients and controls.

Materials and Methods

Samples used in the screening of genetic variation. All samples collected and used are of Finnish origin. The initial screening by single-strand conformational polymorphism (SSCP) of genetic variation in 10 genes was done among 32 men with familial prostate cancer and 32 men with unselected prostate cancer (family history for prostate cancer unknown). Collection of the Finnish families with prostate cancer has been previously reported (12). For SSCP, we randomly picked 32 samples from families that had either at least three affected members or two affected with at least one affected diagnosed <60 years of age. The mean number of affected family members in these 32 families was 3.6 (range, 2-7) and the mean age at diagnosis was 64.8 years (range, 50-76 years). The samples from

Note: N. Mononen and E.H. Seppälä contributed equally to this work.

Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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the unselected prostate cancer that were used in SSCP analysis were randomly picked among cases that had been diagnosed <60 years of age. The mean age at diagnosis of the 32 men with unselected prostate cancer was 54.9 years (range, 49-58 years). The patients were diagnosed during 1990 to 1999.

Variants and samples used in the large-scale population study. From the detected variants, 14 changed the protein sequence (*SRD5A2* A49T, *SRD5A2* V89L, *HSD3B1* N367T, *HSD3B1* R71I, *HSD17B2* A111T, *HSD17B3* G289S, *AKR1C3* Q5H, *AKR1C3* P180S, *CYP19A1* R264C, *KLK3* D102N, *KLK3* L132I, *KLK3* I179T, *CYP19A1* T201M, *HSD17B3* 729_735 delGATAACC). These and two found noncoding variants (*CYP17A1* −34T>C and *KLK3* −252A>G), previously reported to be associated with prostate cancer risk, were selected for large-scale analysis in 1,891 Finnish men (9, 13). In addition, based on previous reports of positive association with prostate cancer, variants *AR* R726L and *LHB* I15T were included in the study (14, 15). Patients with familial prostate cancer (n = 121) used in the genotyping of 18 selected variants had two or more first- or second-degree affected members. The youngest affected member with available sample was genotyped from each family. All 32 familial SSCP samples described above were part of the large-scale genotyping set. The unselected cases (n = 847) were the consecutive patients diagnosed with prostate cancer during 1999 to 2001 in the Pirkanmaa Hospital District with a population of ~450,000. Because the SSCP sample set contained nine patients diagnosed during 1999, those nine patients were also part of the large-scale population study. The allele frequencies for the nine individuals were similar to the entire population. The mean age at diagnosis of the unselected prostate cancer cases was 68.9 years with a range of 45 to 93 years. There was a total of 998 diagnoses of prostate cancer in Pirkanmaa during the 3 years, indicating that we obtained DNA samples and clinical data from 85% of all cancers diagnosed in the area during these years. Table 1 shows the clinicopathologic characteristics of the unselected cases. The population controls consisted of 923 DNA samples from anonymous male blood donors obtained from the Finnish Red Cross in Tampere, Kuopio, and Turku. The blood donors are 18- to 65-year-old healthy men providing an unbiased survey of population genotype frequencies.

Written informed consent was obtained from all living patients and their family members and research protocols were approved by the Ethical Committee of the Tampere University Hospital. All prostate cancer diagnoses were confirmed through medical records or from the Finnish Cancer Registry.

Mutation screening with SSCP analysis. SSCP analysis (16) of the entire coding sequence of the genes *SRD5A2, HSD17B2, HSD17B3, HSD3B1, HSD3B2, CYP11A, CYP17A1, CYP19A1, KLK3*, and *AKR1C3* was done using primer sequences that were designed to include all intron-exon boundaries. All primers are available in http://www.uta.fi/imt/sgy/schleutker/indexb.html.

Table 1. Clinical and pathologic findings at diagnosis of the unselected prostate cancer patients

Clinical/pathologic category	*n* (%)
Stage	
T stage	
T_1-T_2 (organ confined)	542 (64)
T_3-T_4 (extracapsular)	305 (36)
Prostate-specific antigen value at diagnosis	
<20 ng/mL	625 (74)
≥20 ng/mL	217 (25)
Grade	
WHO grade	
I (low)	165 (20)
II (mid)	526 (64)
III (high)	132 (16)
Gleason score	
2-6	349 (45)
7-10	432 (55)

Genotyping by allele-specific primer extension on microarrays. The following variants were genotyped using allele-specific primer extension assay: *LHB* I15T, *SRD5A2* A49T, *SRD5A2* V89L, *HSD3B1* N367T, *HSD3B1* R71I, *HSD17B2* A111T, *HSD17B3* G289S, *HSD17B3* 729_735 delGATAACC, *AKR1C3* Q5H, *AKR1C3* P180S, *CYP19A1* R264C, *CYP17A1* −34T>C, *AR* R726L, *KLK3* D102N, and *KLK3* L132I. Some modifications were made to the method described by Riise Stensland et al. (17). The allele-specific oligonucleotides and multiplex PCR primers are available at http://www.uta.fi/imt/sgy/schleutker/indexb.html. Arrays were spotted with allele-specific oligonucleotides from forward or reverse orientations. The PCR primer pairs were grouped into multiplex PCR reactions with four, four, four, and three primer pairs per reaction for 15 variants. Specific PCR conditions can be obtained from the corresponding author. Products of two multiplex PCRs were then pooled so that in the RNA transcription step using the T7 Ampliscribe Kit (Epicentre Technologies, Madison, WI), there were four, four, and seven variants in the same reaction. A 5′ Cy3-(A)9 3′ blocked probe was not used in the hybridization reaction. The microscope glass slides were scanned using the confocal ScanArray 4000 (GSI Lumonics, Watertown, MA). Ten-micron resolution, 16-bit TIFF images were analyzed using the QuantArray software (GSI Lumonics). Accurate allele calling and genotyping were produced by SNPSnapper 3.88b software developed by Juha Saharinen, National Public Health Institute, Helsinki, Finland (http://www.bioinfo.helsinki.fi/SNPSnapper/).

5′ Nuclease assay. Two *KLK3* alterations, −252A>G and I179T, were genotyped by using the TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer in 96-well format. The *KLK3* −252A>G genotypes were determined by using the TaqMan Pre-Designed Assay. For *KLK3* I179T genotyping, a Custom TaqMan SNP Genotyping Assay was ordered. Briefly, the DNA was amplified for I179T analysis using the following *KLK3*-specific primers: forward 5′-CCCGTAGTCTTGACCCCAAAG-3′ and reverse 5′-CTTGCGCACACACGTCAT-3′. The *KLK3* I179T genotypes were determined using the following fluorogenic allele-specific probes with a conjugated minor groove binder group: VIC-labeled 5′-CCTCCATGTTATTTCC-3′ for T allele and FAM-labeled 5′-CCTCCATGTTACTTCC-3′ for C allele. The nucleotide sequences of the primers and probes used in the PCR were deduced from publicly available sequences deposited in the GeneBank database and were chosen and synthesized by Applied Biosystems using the Assay-by-Design service. DNA samples were genotyped by means of 5′ nuclease assay for allelic discrimination using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Known control samples previously genotyped by sequencing were run in parallel with unknown samples. After PCR, end-point fluorescence was measured and genotype calling was carried out using the allelic discrimination analysis module.

Sequencing. Sequencing was done using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with the ABI 310 and ABI3100 sequencers (Applied Biosystems).

Minisequencing. The genotypes for *CYP19* T201M alteration were determined by minisequencing. A 121-bp fragment was first amplified as follows: 100 ng of DNA, 200 nmol/L of both primers, 200 μmol/L of each deoxynucleotide triphosphate, 1.5 mmol/L $MgCl_2$, and 1.5 units of AmpliTaqGold DNA Polymerase (Applied Biosystems) in a final volume of 50 μL; at 95°C for 10 minutes, followed by 35 cycles of 95°C 30 seconds, 62°C for 30 seconds, and 72°C for 45 seconds, with a 5-minute extension at 72°C after the last cycle. Primers for PCR were 5′-AATCGGGCTATGTGGACGTG-3′ and 5′biotin-GATGGTCAAGATGTGAGAGTG-3′. Minisequencing was done as described by Syvanen (18) with detection primer 5′-ATGCTGGACACCTCTAACA-3′. Minisequencing results were confirmed by sequencing with ABI PRISM 310 Genetic Analyzer (Applied Biosystems) as recommended by the manufacturer. Primers used in sequencing were the same as those used in PCR.

Statistics. Odds ratios (OR) and corresponding 95% confidence intervals (95% CI) were calculated using logistic regression to estimate prostate cancer risk. Categorical variables were compared with the

Fisher's exact test and Pearson χ^2 test for independence. These analyses were done with SPSS 11.0 statistical software package. The magnitude of the association between the *CYP19A1* and *CYP17A1* single nucleotide polymorphisms (SNP) and the occurrence of prostate cancer and other related outcomes was measured with the OR using polytomous logistic regression. Outcome definitions included WHO grade (2-3), Gleason score (2–10), prostate-specific antigen at diagnosis (<20 ng/mL, ≥20 ng/mL), and T stage (T_1-T_2, T_3-T_4). Polytomous regression analyses were done using STATA v8.0.

Bioinformatics. The effect of the T201M mutation on the structure and function of aromatase was investigated with several bioinformatic methods and tools including PHD (19, 20), PROF (19, 20), Jpred (21), SADM (22), and SIFT (23). The effect of point mutation had to be evaluated sequence based because the known structures are for the core domain of P450.

Results

Table 2 shows the genetic alterations found in the screening of 64 samples from prostate cancer patients in 10 target genes by SSCP analysis. A subset of the variants identified in the screening as well as two other variants (*AR* R726L, *LHB* I15T) previously suggested to be associated with prostate cancer (14, 15) were then selected for large-scale genotyping in 1,891 Finnish men (Supplementary Table S1).

We identified an association of a previously unpublished SNP, *CYP19A1* T201M (602C>T), with unselected prostate cancer (OR, 2.04; 95% CI, 1.03-4.03; P = 0.04, Supplementary Table S1). Other variants showed no statistically significant association either with unselected or familial prostate cancer. For the rare mutations, no ORs were calculated because of a small number of carriers.

We also wanted to study whether the carrier status of the studied genotypes was associated with the clinicopathologic features (T stage, M stage, WHO grade, Gleason score, prostate-specific antigen at diagnosis, and age at diagnosis) of the unselected prostate cancer cases. *KLK3* −252A>G carriers had more often a lower Gleason score (2–6) than noncarriers (P = 0.045, Pearson χ^2 test). *LHB* I15T showed a borderline association with organ-confined tumor (P = 0.074, Pearson χ^2 test). In contrast, carriers of the *KLK3* I179T alteration were more likely to have metastases than noncarriers (P = 0.009, Pearson χ^2 test). To determine the nature of disease association with *CYP19A1* T201M, a polytomous logistic regression analysis was done (Table 3). Interestingly, the T201M association was only seen in patients with organ-confined disease as well as in those with a low prostate-specific antigen value at diagnosis. In contrast, individuals with severe stage classification showed no association with the *CYP19A1* T allele. We saw similar results for the histologic classifications, WHO grade, and Gleason score, in which individuals with less aggressive prostate cancer defined by a low-grade tumor (WHO grade I) were 4.5 times more likely to carry the *CYP19A1* T allele than population controls (OR, 4.5; 95% CI, 1.94-10.5; P < 0.0001). More severe cases (WHO II and III or Gleason >7) did not show an overrepresentation of the *CYP19A1* T allele.

To further refine our risk categories, we created a risk score in which an individual had severe or aggressive prostate cancer (T_3-T_4 and WHO grade II-III), moderate cancer (T_1-T_2 and WHO grade II-III), or clinically less significant cancer (T_1-T_2 and WHO grade I). Individuals with clinically less significant prostate cancer were more than five times more likely to carry the *CYP19A1* T201M T allele than population-based controls (OR, 5.42; 95% CI, 2.33-12.6; P < 0.0001; Table 3). This association was still significant after the conservative Bonferroni correction (eight independent genes overall; n = 8). We used the same categories in the further analysis of *CYP17A1* −34T>C. No association was seen to clinically less significant prostate cancer (OR, 1.09; 95% CI, 0.75-1.59; P = 0.65). However, this alteration increased the risk for moderate cancer

Table 2. Variants found by SSCP analysis

Gene	Amino acid change	Nucleotide*	Exon/intron
SRD5A2	A49T	145G>A	Exon 1
	V89L	265G>C	Exon 1
		281+15T>C	Intron 1
HSD17B3		154+(30_31)insT	Intron 1
		201+38T>C	Intron 2
		278−67G>A	Intron 3
		672+33A>G	Intron 9
	Premature STOP codon	729_735delGATAACC	Exon 10
	G289S	865G>A	Exon 11
HSD3B1	R71I	212G>T	Exon 2
	L338L	1012C>T	Exon 3
	N367T	1100A>C	Exon 3
HSD3B2	N207N	621C>T	Exon 3
CYP11A		269+49C>A	Intron 1
	F313F	939C>T	Exon 5
	P389P	1167C>T	Exon 7
CYP17A1		−34T>C	5′-UTR
	H46H	138C>T	Exon 1
	S65S	195G>T	Exon 1
		1139+19T>G	Intron 6
CYP19A1	V80V	240A>G	Exon 2
		451+31T>C	Intron 3
		451+(35_36)insTTT	Intron 3
	T201M	602C>T	Exon 4
		743+36A>T	Intron 5
	R264C	790C>T	Exon 6
		858+26C>T	Intron 6
		1512+19C>T	3′-UTR
KLK3		−158G>A	5′-UTR
		−252A>G	5′-UTR
		−205_206insA	5′-UTR
		−285G>A	5′-UTR
	G16G	48T>C	Exon 2
	A18A	54A>G	Exon 2
	Q39Q	117G>A	Exon 2
	S79S	237C>T	Exon 3
	D102N	304G>A	Exon 3
	L132I	394C>A	Exon 3
	I179T	536T>C	Exon 4
		786+15C>T	3′-UTR
AKR1C3	Q5H	15C>G	Exon 1
	P30P	90G>A	Exon 2
	K104K	312A>G	Exon 3
		370−14T>C	Intron 3
	P180S	540C>T	Exon 5
		681−20C>G	Intron 6
		929+40A>G	Intron 8
		930−4T>G	Intron 8
		972+8G>A	3′-UTR
HSD17B2	A111T	331G>A	Exon 2

*Nucleotide numbering begins at the A in the start codon except for 5′-UTR variants of the *KLK3* gene in which numbering starts at the beginning of the first exon.

Table 3. Association of the *CYP19A1* T201M variant with prostate cancer

Dependent variable	*n* (CT and TT)	OR (95% CI)	*P*
T stage			
Controls (*n* = 923)	13	1.00	
T_1-T_2 (*n* = 542)	20	2.68 (1.32-5.43)	0.006
T_3-T_4 (*n* = 305)	4	0.93 (0.30-2.87)	0.90
Prostate-specific antigen at diagnosis			
Controls (*n* = 923)	13	1.00	
<20 ng/mL (*n* = 625)	19	2.19 (1.07-4.47)	0.031
≥20 ng/mL (*n* = 217)	5	1.65 (0.58-4.68)	0.88
WHO grade			
Controls (*n* = 923)	13	1.00	
I (*n* = 165)	10	4.51 (1.94-10.5)	<0.0001
II (*n* = 526)	11	1.49 (0.66-3.36)	0.33
III (*n* = 132)	2	1.08 (0.24-4.82)	0.92
Gleason score			
Controls (*n* = 923)	13	1.00	
2-7 (*n* = 349)	12	2.49 (1.12-5.51)	0.024
7-10 (*n* = 432)	9	1.49 (0.63-3.51)	0.36
Combined WHO and T			
Controls (*n* = 923)	13	1.00	
WHO I and T_1-T_2 (*n* = 139)	10	5.42 (2.33-12.6)	<0.0001
WHO II-III and T_1-T_2 (*n* = 403)	10	1.78 (0.77-4.10)	0.17
WHO II-III and T_3-T_4 (*n* = 305)	4	0.93 (0.30-2.87)	0.90

NOTE: The unselected prostate cancer cases were classified according to T stage, prostate-specific antigen value at diagnosis, WHO grade, and Gleason score.

(OR, 1.42; 95% CI, 1.09-1.83; P = 0.007). The association was marginally significant after the conservative Bonferroni correction (n = 8).

We further tested a hypothesis of a joint effect of *CYP19A1* T201M with five common polymorphism (*LHB* I15T, *CYP17A1* −34T>C, *KLK3* −252A>G, *AKR1C3* Q5H, and *SRD5A2* V89L) in prostate cancer risk (Supplementary Table S2). Increased risk was noted in individuals carrying both *CYP19A1* T201M and *KLK3* −252A>G (OR, 2.87; 95% CI, 1.10-7.49; P = 0.03).

The effect of the T201M mutation on the structure and function of aromatase was investigated with numerous bioinformatic methods and tools. Based on sequence database searches, position 201 is not highly conserved in aromatases and in P450 family. The residue most common in this position in the aromatase family is arginine. T201 is predicted to be close to the COOH-terminal end of a long α-helix by several methods including PHD, PROF, and Jpred. The mutation was predicted not to increase disorder or aggregation tendency of the protein. Predictions of surface accessibility of T201 with programs PHD, PROF, and SADM are somewhat contradictory. It is possible that the residue is on the buried surface of the helix. Program SIFT (sorting intolerant from tolerant) uses multiple sequence information to predict whether an amino acid substitution affects protein function. According to SIFT prediction, T201M mutation is not tolerated. SIFT predictions have been shown to be very accurate in detecting deleterious mutations (24).

Discussion

Cancer growth depends on the ratio of cells proliferating to those dying. In prostate gland, androgens are the main regulator of this ratio by both stimulating proliferation and inhibiting apoptosis. Here, we carried out a comprehensive evaluation of the role of selected androgen pathway candidate genes in prostate cancer risk and in the clinical characteristics of the disease in large study cohorts involving almost 2,000 samples from patients and controls. First, prostate cancer patients were screened for genetic variation to identify those alterations that may be enriched in the prostate cancer population. Then 13 missense variants, one deletion resulting in a truncated protein and two 5′-untranslated region (UTR) variants, were selected for further analyses. In addition, the *AR* R726L and *LHB* I15T variants were included in the study based on information on disease association from the previous literature (14, 15).

Our results indicate that most of these alterations are not significantly associated with prostate cancer. We found no disease association for the *LHB* I15T, *HSD3B1* N367T, *SRD5A2* V89L, and *HSD17B3* G289S variants which have been previously reported to increase prostate cancer risk (10, 15, 25, 26). In addition, we confirmed our earlier report in an extended cohort that the *SRD5A2* variant A49T does not increase prostate cancer risk (27). We have previously reported an increased risk among the *AR* R726L carriers (14). In the previous study, the allele frequency of the R726L mutation was 0.3% (3 of 900) in controls and 1.9% (8 of 418) among the unselected cases. In the present study, the allele frequencies were 0.9% (8 of 923) and 1.3% (11 of 847), respectively, and no statistically significant association was seen.

In a large meta-analysis of 10 studies by Ntais et al. (28) including samples from subjects of European, Asian, and African descent, no overall association between *CYP17A1* −34T>C and prostate cancer risk was observed. However, in a subgroup of African descent, the C allele increased the prostate cancer risk, unlike in subjects of European and Asian descent. In this study, we saw no overall association between *CYP17A1* −34T>C and prostate cancer risk in our Finnish sample set. On the other hand, after classifying the patients according to clinical data, we observed that carriers of the C allele have increased risk for moderate cancer. In contrast to Modugno et al. (8) and Suzuki et al. (29), we saw no association between the *CYP19A1* R264C alteration and prostate cancer risk. However, the T allele of another alteration, T201M, in the same *CYP19A1* gene showed a mild, statistically significant increase for prostate cancer among the unselected prostate cancer cases (OR, 2.04; P = 0.04). Interestingly, stratified analysis revealed a strong association with clinically less significant cancer (OR, 5.42; P < 0.0001). The observation that *CYP19A1* T201M variation did not associate with familial prostate cancer may be due to the fact that in prostate cancer families, the possible effect may be masked by some other stronger genetic component.

The results from previous studies of prostate cancer predisposition have often been conflicting. The discrepancy between the studies may be due to sampling bias (e.g., small number of cases, often from highly selected hospital-based series), the choice of controls (benign prostate hyperplasia or non-prostate cancer controls, blood donors, etc.), or population stratification. However, the Finnish population is genetically very homogeneous (30) and, therefore, ideal for unbiased allele association studies. Our study was based on a large sample of unselected prostate cancer cases from the whole Pirkanmaa Hospital District, which represents

unbiased sampling of the entire population. The same is true for the control samples, which were collected from central, west-southern, and eastern areas in Finland. Obviously, a small fraction of population controls will get prostate cancer later in life. According to the statistics by the Finnish Cancer Registry, the cumulative crude probability of a prostate cancer diagnosis up to 84 years of age is 8.5% based on current incidence rates.

Despite the small percentage of patients with *CYP19A1* T201M mutation, we were able to carry a preliminary gene-gene interaction study of this alteration due to the large overall study cohorts. A significant association to prostate cancer was seen when the patient carried both *CYP19A1* T201M and *KLK3* −252A>G variants.

Due to the extensive use of prostate-specific antigen screening, the most typical prostate cancers today are small organ–confined cancers. Thus far, no diagnostic biomarkers indicating disease aggressiveness and progression have been discovered. The *CYP19A1* gene codes for a cytochrome *P*450 enzyme complex, called aromatase, engaged in the biosynthesis of estrogens from androgens in the testis. The T201M mutation of this protein could, through altered enzyme function, affect testosterone levels in the body. Based on the bioinformatic analyses, we can assume that T201 is in structurally important α-helix, most likely at least partly buried to the core of the protein. Substitution by methionine affects the packing of the helix and, consequently, the local and global fold of the enzyme. The effect may also rise from losing stabilizing polar interaction(s) formed by the hydroxyl group of threonine. Apparently, the change is not completely altering the function of the protein, which could lead to modified activity and phenotype. A higher activity of the variant enzyme could result in lower levels of androgens in carriers as androgens are more efficiently converted into estrogens.

Our findings suggest that the rare *CYP19A1* T201M variant may be associated with less aggressive prostate cancer and may serve as a marker for individuals with clinically less significant disease. The biochemical significance of the T201M alteration in aromatase function warrants further studies. In addition, a multigenic model of prostate cancer susceptibility is supported.

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