HENNA MATTILA

Hereditary Prostate Cancer in Finland

From genetic linkage to susceptibility genes

■

ACADEMIC DISSERTATION
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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following communications, referred in the text by their Roman numerals (I-IV). In addition, some unpublished results are presented.

I. Syrjäkoski K.*, **Fredriksson H.***, Ikonen T., Kuukasjärvi T., Autio V., Matikainen M.P., Tammela T.L.J., Koivisto P.A., Schleutker J., Hemochromatosis gene mutations among Finnish male breast and prostate cancer patients. *Int J Cancer*, 2006; 118:518-20.
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II. Hebring S.J.*, **Fredriksson H.***, White K.A.*, Maier C.*, Ewing C.*, McDonnell S.K., Jacobsen S.J., Cerhan J., Schaid D.J., Ikonen T., Autio V., Tammela T.L.J., Herkommer K., Paiss T., Vogel W., Gielzak M., Sauvageot J., Schleutker J., Cooney K.A., Isaacs W., Thibodeau S.N., Role of the Nijmegen breakage syndrome 1 gene in familial and sporadic prostate cancer. *Cancer Epidemiol Biomarkers Prev*, 2006; 15:935-8.
* equal contribution

III. **Fredriksson H.***, Ikonen T.*, Autio V., Matikainen M.P., Helin H.J., Tammela T.L.J., Koivisto P.A., Schleutker J., Identification of germline *MLH1* alterations in familial prostate cancer. *Eur J Cancer*, 2006; 42:2802-6.
* equal contribution

IV. **Mattila H.**, Ikonen T., Vihinen M., Isotalo J., Oja H., Tammela T., Wahlfors T., Schleutker J., Non-sense mediated mRNA decay and microRNA array analysis of Finnish *HPCX*-linked families. Submitted.

ABBREVIATIONS

ABL	V-abl Abelson murine leukemia viral oncogene homolog
AI	Allelic imbalance
Ala	Alanine
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AR	Androgen receptor
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATM	Ataxia telangiectasia mutated
BCL2	B-cell leukemia/lymphoma 2
B-CLL	B-cell chronic lymphocytic leukemia
BCR	Breakpoint cluster region
BHLHB2	Basic helix-loop-helix domain containing, class B, 2
bp	Base pair
BPH	Benign prostate hyperplasia
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
CABP	Prostate cancer/brain cancer susceptibility locus
CD40LG	CD40 ligand
CDC25A	Cell division cycle 25A
CHEK2	CHK2 checkpoint homolog (S. pombe)
CHL1	Cell adhesion molecule with homology to L1CAM
CLL	Chronic lymphocytic leukemia
CNTN4	Contactin 4
CNTN6	Contactin 6
CSAG2	*Homo sapiens* CSAG family, member 2
CTBP2	C-terminal binding protein 2
Cy-3	Cyanine-3
Cy-5	Cyanine-5
Cys	Cysteine
del	Deletion
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonuclease acid
dNTP	Deoxyribonucleotide
DSB	DNA doublestrand break
EDTA	Ethylenediaminetetraacetic acid

EHBP1	EH domain binding protein 1
ELAC2	elaC homolog 2 (E. coli)
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERG1	ETS-related gene 1
ETS	The E twenty-six
ETV	ETS variant
FDH	Finnish disease heritage
FDR	False discovery rate
FHIT	Fragile histidine triad gene
Glu	Glutamic acid
GWAS	Genome-wide association study
HER2	Human epidermal growth factor receptor 2
HFE	Hemochromatosis
HH	Hereditary hemochromatosis
His	Histidine
HLOD	Heterogeneity logarithm of odds
HPC	Hereditary prostate cancer
HPC1	Hereditary prostate cancer 1
HPC2	Hereditary prostate cancer 2
HPC20	Hereditary prostate cancer 20
HPCX	Hereditary prostate cancer X
HNF1B	HNF1 homeobox B
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Homologous recombination
ICPCG	International consortium for prostate cancer genetics
Ile	Isoleucine
IL5RA	Interleukin 5 receptor, alpha
ins	Insertion
IVS	Intron
KCLOD	Kong and Cox exponential allele sharing model LOD score
KLK2	Kallikrein-related peptidase 2
KLK3	Kallikrein-related peptidase 3
LDOC1	Leucine zipper, down-regulated in cancer 1
Leu	Leucine
LMTK2	Lemur tyrosine kinase 2
LOD	Logarithm of odds
LOH	Loss of heterozygosity
MAGEA1	Melanoma antigen family A, 1
MAGEA11	Melanoma antigen family A, 11
MAGEC1	Melanoma antigen family C, 1
MAGEC3	Melanoma antigen family C, 3
MAGED1	Melanoma antigen family D, 1
Mb	Megabase

MBC	Male breast cancer
MBNL3	Muscleblind-like 3, (*Drosophila*)
Met	Methionine
miRNA	MicroRNA
MLH1	mutL (*E. coli*) homolog L
MMR	Mis-match repair
mRNA	Messenger RNA
MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)
MSH6	MutS homolog 6 (*E. coli*)
MSI	Microsatellite instability
MSMB	Microseminoprotein, beta
MSR1	*Macrophage scavenger receptor 1*
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NBN (NBS1)	Nibrin (Nijmegen breakage syndrome 1)
NBS	Nijmegen breakage syndrome
NCOA3	Nuclear receptor coactivator 3
NHEJ	Non-homologous end-joining
NMD	Nonsense-mediated decay
NMM	No male-to-male
NPL	Nonparametric multipoint linkage
NUDT10	Nudix (nucleoside diphosphate linked moiety X)-type motif 10
NUDT11	Nudix (nucleoside diphosphate linked moiety X)-type motif 11
OGG1	8-oxoguanine DNA glycosylase
OR	Odds ratio
OXTR	Oxytocin receptor
p	Short arm of the chromosome
PALB2	Partner and localizer of BRCA2
PCAP	Predisposing for prostate cancer
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PIN	Prostatic intraepithelial neoplasia
PMS2	PMS2 postmeiotic segregation increased 2 (*S. cerevisiae*)
Pro	Proline
PSA	Prostate specific antigen
PTC	Premature termination codon
q	Long arm of the chromosome
RAP2C	*Homo sapiens* RAP2C, member of RAS
RB1	Retinoblastoma 1
RBMX	RNA binding motif protein, X-linked
RNA	Ribonuclease acid
RNASEL	Ribonuclease L (2´,5´-oligoisoadenylate synthetase-dependent)
Ser	Serine

SLC22A3	Solute carrier family 22 (extraneuronal monoamine transporter), member 3
SNP	Single nucleotide polymorphism
SOX3	SRY (sex determining region Y)-box 3
SPANX	Sperm protein associated with the nucleus, X-linked
SSCP	Single strand conformational polymorphism
SSR4	signal sequence receptor, delta
TAR	Transformation-associated recombination
TLOD	Theta logarithm of odds
Thr	Threonine
TKTL1	Transketolase-like 1
TMPRSS2	Transmembrane protease, serine 2
TP53	Tumor protein p53
tRNA	Transfer RNA
TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1
Tyr	Tyrosine
U66046	hypothetical protein FLJ44451
UTR	Untranslated region
Val	Valine
VBP1	Von Hippel-Lindau binding protein 1
VHL	Von Hippel-Lindau syndrome gene
X	Stop codon
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
ZNF75	Muscleblind-like 3, (*Drosophila*)

ABSTRACT

The burden that prostate cancer causes to the public health care system is remarkable. A total of 4189 new cases of prostate cancer and 793 deaths from the disease were registered in Finland in 2007, making it the most frequent nondermatologic cancer among Finnish males. Prostate cancer is a very heterogeneous disease with multiple factors contributing to the susceptibility of males to this disease. In addition to age and race, positive family history is one of the strongest epidemiological risk factors. However, despite the localization of many susceptibility loci, there has been limited success in identifying high-risk susceptibility genes.

In this thesis study, the aim was to further characterize the role of the *HPCX* locus on chromosome Xq27-q28, which seems to explain a large fraction of the Finnish hereditary prostate cancer families. Nonsense-mediated messenger RNA decay and microRNA expression array analysis of prostate cancer patients and their healthy brothers was performed and the results suggested a role for *MAGEC1* in genetic prostate cancer susceptibility, especially in the *HPCX*-linked form of the disease. A start codon missense variation Met1Thr in the *MAGEC1* gene was significantly associated with prostate cancer risk in both hereditary and unselected prostate cancer. Furthermore, two different statistical analyses of microRNA microarrays declared 27 microRNAs significantly differentially expressed in the lymphoblastoid cells between hereditary prostate cancer patients and their healthy brothers. Hsa-miR-770-5p, hsa-miR-19b-2*, hsa-miR-767-3p, hsa-miR-220a, and hsa-miR-151-3p were the most intriguing for further studies, as some of them were also suggested to have binding sites in *MAGEC1* gene.

Furthermore, the role of *HFE*, *NBN*, and *MLH1* genes in prostate cancer predisposition was evaluated in the present study. None of the examined genetic variations in these genes showed significant associations to prostate cancer risk suggesting that they do not have a causative role in the etiology of prostate cancer. However, further studies in different population cohorts are needed to reliably exclude these genes as prostate cancer susceptibility genes.

In summary, several genes and genetic alterations potentially involved in prostate carcinogenesis were thoroughly analyzed in this thesis study. The results from the *HPCX* study warrant further analyses, especially related to genes in *MAGEC* family and certain microRNAs.

YHTEENVETO

Eturauhassyöpä on tällä hetkellä miesten yleisin syöpä länsimaissa, myös Suomessa. Kuitenkin taudin etiologia tunnetaan varsin huonosti, mikä tekee taudin torjumisen ja hoitamisen haasteelliseksi. Useimmat eturauhassyöpätapauksista ovat sporadisia, mutta on olemassa vahvaa tieteellistä näyttöä siitä, että osa eturauhassyövästä on perinnöllistä. Uskotaan, että kaikista syöpätapauksista noin 5-10 % on varsinaisia perinnöllisiä syöpiä. Rintasyövän, kolorektaalisyövän ja melanooman perinnöllisten muotojen taustalta on löydetty muutamia erityisen suuren riskin aiheuttavia geenejä. Eturauhassyövän kohdalla tilanne ei kuitenkaan näytä yhtä selkeältä. Tauti on ilmeisesti varsin monitekijäinen.

Kytkentäanalyysi on perinteinen lähestymistapa etsiä tautien alttiusgeenejä perheistä ja paikallistaa niitä tietyille kromosomialueille. Tähän mennessä on pystytty paikallistamaan useita kromosomialueita ko. menetelmällä perinnöllisiä eturauhassyöpäperheitä tutkien. Kuitenkaan alttiusgeenejä näiltä alueilta ei ole pystytty löytämään kuin kolme, *ELAC2* kromosomissa 17p11, *MSR1* kromosomissa 8p22-23 ja *RNASEL* kromosomissa 1q24-q25. Näillä geeneillä ei tutkimuksien mukaan ole suurta kausaalista merkitystä suomalaisessa väestössä. Vahvasti eturauhassyövälle altistavien, perhekasaumia aikaansaavien geenimuutosten lisäksi syöpäriskiin vaikuttanevat osaltaan myös hormonimetaboliaan liittyvät ja mahdollisesti ympäristötekijöiden kanssa yhdessä vaikuttavat ns. matalan penetranssin geenien polymorfismit. Tällaisia geenejä ovat esim. androgeenireseptorigeeni (*AR*), 5-alfa-reduktaasigeeni (*SRD5A*) ja vitamiini-D-reseptorigeeni (*VDR*).

Väitöskirjatyössä selvitettiin kolmen kytkentäalueilla sijaitsevan geenin, *HFE* kromosomissa 6p21.3, *NBN* kromosomissa 8q21 ja *MLH1* kromosomissa 3p21.3, osuutta eturauhassyöpäriskiin suomalaisessa väestössä. Kaikkien kolmen geenin suhteen tutkimustulokset olivat hyvin samansuuntaiset. *HFE*-geenin kahden yleisimmän, perinnöllistä hemokromatoosia aiheuttavien geenimuutosten (Cys282Tyr ja His63Asp), osuudet tutkittiin valikoimattomien eturauhassyöpäpotilaiden, miesrintasyöpäpotilaiden ja verrokkien joukoissa. Tulosten perusteella nämä *HFE*-geenin variaatiot eivät ole voimakkaasti eturauhassyövälle altistavia geenimuutoksia.

NBN-geenin mutaation 657del5 on havaittu olevan eturauhassyövälle altistava geenimuutos, erityisesti slaavilaista alkuperää olevassa väestössä. Tässä *NBN*-geenin tutkimustyössä oli mukana tutkimuskeskuksia Suomesta, Yhdysvalloista ja Saksasta, jotka ovat osa kansainvälistä eturauhassyövän tutkimuskonsortiota (International Consortium for Prostate Cancer Genetics, ICPCG). Mutaation

657del5 osuus selvitettiin laajassa aineistossa, joka koostui 1819 eturauhassyöpäperheisiin kuuluvasta potilaasta, 1218 valikoimattomasta eturauhassyöpäpotilasta ja 697 verrokista. Perhenäytteiden joukosta löytyi neljä mutaation kantajaa (0,22 %) ja valikoimattomien eturauhassyöpäpotilaiden joukossa kantajia oli 0,25 %. Verrokkiryhmästä mutaation kantajia ei löytynyt. Lisäksi suomalaisessa väestössä koko geenin proteiinia koodaava alue sekvensoitiin 20 potilaalta ja kahden löydetyn aminohappoa vaihtavan geenimuutoksen, Glu185Gln ja Asp95Asn, osuudet selvitettiin isommassa aineistossa, mutta kumpikaan muutoksista ei liittynyt kohonneeseen eturauhassyöpäriskiin. Näin olleen *NBN* ei näyttäisi olevan merkittävä eturauhassyövän alttiusgeeni suomalaisessa väestössä, eikä muissakaan tutkituissa ei-slaavilaisissa väestöissä.

MLH1 on hyvin tunnettu perinnöllisen ei-polypoottisen paksusuolisyövän (HNPCC) alttiusgeeni ja HNPCC aiheutuu suomalaissuvuissa pääasiassa *MLH1*-geenin mutaatioista. Geeni sijaitsee lähellä suomalaista eturauhassyövän kytkentäaluetta 3p25-p26. Tässä tutkimuksessa Tampereen yliopistollisen sairaalan poistorekisteristä etsittiin kaikki potilaat, joilla oli eturauhassyövän lisäksi jokin muu syöpä. Näitä löytyi 11, joista kahden näytteessä immunohistokemiallinen värjäys oli poikkeuksellinen. Vain toisesta potilaasta (potilas A) oli riittävästi näytettä jatkotutkimukseen. *MLH1*-geenin proteiinia koodaava alue sekvensointiin potilaalta A ja tuloksena löytyi kaksi aminohappoa vaihtavaa pistemutaatiota ja kaksi hiljaista mutaatiota. Lisäksi *MLH1*-geenin proteiinia koodaavat alueet seulottiin sekä SSCP-menetelmällä (single strand conformation polymorphism) että suorasekvensoinnilla suomalaisissa eturauhassyöpäperheissä. Kolmen löytyneen mutaation osuudet määritettiin isommassa näyteaineistossa. Tulosten perusteella mikään mutaatioista ei liity kohonneeseen eturauhassyöpäriskiin, mutta Ile219Val-mutaatiota kantavat valikoimattomat eturauhassyöpäpotilaat olivat tilastollisesti merkitsevästi nuorempia kuin verrokkiryhmän mutaation kantajat.

Lisäksi tässä väitöskirjatyössä tarkoituksena oli tutkia ja tarkemmin karakterisoida genomin laajuisessa kytkentäanalyysissa havaittua *HPCX*-aluetta kromosomissa Xq27-q28, joka näyttäisi olevan voimakkaimmin kytkeytynyt eturauhassyöpään juuri suomalaisessa väestössä. Tutkimuksessa hyödynnettiin kahta uutta mikrosirutekniikkaa, NMD-sirutekniikkaa ja mikroRNA-sirutekniikkaa, *HPCX*-kytkeytyneiden perheiden analyysissa. Tulokset antavat aihetta jatkotutkimuksille koskien *MAGEC1*-geenin aloituskodonimuutosta Met1Thr, joka liittyi tilastollisesti merkitsevästi kohonneeseen eturauhassyöpäriskiin, sekä eturauhassyöpäperheissä että valikoimattomissa eturauhassyöpätapauksissa. Lisäksi mikroRNA-analyysissa nousi esiin 27 mikroRNA:ta, joiden ilmentyminen oli tilastollisesti merkitsevästi erilainen eturauhassyöpäpotilaiden ja heidän terveiden veljiensä välillä.

INTRODUCTION

Prostate cancer is the most common male malignancy in the Western world and, after lung cancer, the second most common cause of cancer related deaths in Finland (Finnish Cancer Registry, 2008). Like most cancers, prostate cancer is a complex disorder in which disease initiation is the result of an interaction between genetic and non-genetic factors. The three most important risk factors for prostate cancer are age, race, and family history. Approximately one-tenth of prostate cancer cases are believed to be caused by high-risk inherited genetic factors. The results from a large Scandinavian twin study suggested that even 40% of the risk of prostate cancer could be explained by heritable factors (Lichtenstein et al., 2000). However, the identification of causative genes for prostate cancer has been challenging in spite of evidence that supports the existence of one or more hereditary prostate cancer genes. Traditionally, genetic linkage analysis has been a fruitful method to associate genes that affect phenotype to their location on chromosomes. These studies have shown linkage of prostate cancer susceptibility genes to multiple loci on different chromosomes, including chromosomes 1, 3, 8, 17, 20, and X. However, differences from the mode of inheritance to the target genes exist. To date, three prostate cancer susceptibility genes have been proposed from the linked regions, ribonuclease L (*RNASEL*) at 1q25 (*HPC1*) (Carpten et al., 2002), macrophage scavenger receptor 1 (*MSR1*) at 8p22 (Xu et al., 2002a), and elaC *E. coli* homolog 2 (*ELAC2*) at 17p11 (*HPC2*) (Tavtigian et al., 2001), but mutations in these genes are rare and explain only a small portion of prostate cancer susceptibility.

Recently, chromosome 8q24 has emerged as a potentially important region in prostate cancer genetics. Many studies involving linkage, admixture mapping, and whole genome associations have identified multiple risk variants in this region associated with susceptibility to prostate cancer (Amundadottir et al., 2006, Freedman et al., 2006, Gudmundsson et al., 2007b, Haiman et al., 2007b, Yeager et al., 2007, Witte, 2007). The 8q24 region is relatively gene-poor and the risk variants are located in nonprotein coding regions. A strong candidate is the proto-oncogene *MYC*, even though the variants found in *MYC* do not seem to be associated with prostate cancer (Gudmundsson et al., 2007b, Yeager et al., 2007). However, the associated variants at 8q24 could hypothetically affect *MYC* expression by altering its regulation and, in turn, affect disease risk. Further work is needed to elucidate the biological mechanisms underlying these associations at 8q24.

In addition to high-risk susceptibility genes, numerous studies on low to moderate penetrance candidate genes together with environmental and dietary factors have been performed. These studies have usually been focused on genes

involved in androgen metabolism, DNA repair, immunity, or drug metabolism. Especially, the role of the androgen receptor (*AR*) has been studied extensively, as the function of *AR* is important in the development and progression of prostate cancer.

At the moment, it is assumed that prostate cancer results from a complex interaction of all the above mentioned risk factors and the puzzle of prostate cancer is much more complicated than initially anticipated. In this present study, the aim was to investigate genetic variation in three candidate genes (*HFE*, *NBN*, and *MLH1*) and further characterize the prostate cancer susceptibility locus, *HPCX*, at Xq27-q28 by different array methods. The overall aim of the study was to provide essential new information on the genetic risk factors leading to prostate cancer, which would help to better understand the molecular basis of the disease.

REVIEW OF THE LITERATURE

1. Cancer genetics

The destiny of every cell is dependent on its genetic material, DNA. Changes in DNA will influence the expression and function of genes, the basic units of heredity. Usually cancer emerges from single somatic cells and their progeny, which acquire genetic and epigenetic changes and therefore have a growth advantage over other cell populations (Stratton et al., 2009). Hanahan and Weinberg (2000) suggested that most cancers need to acquire six functional capabilities during their development: 1) self-sufficiency in growth signals, 2) insensitivity to anti-growth signals, 3) capability to invade and metastasize, 4) limitless replicative potential, 5) sustained angiogenesis, and 6) capability to evade apoptosis. Genetic changes found in cancers typically affect two classes of genes. Cancer-promoting oncogenes are typically activated in cancer cells while tumor suppressor genes are inactivated.

1.1 Tumor suppressor genes and Knudson's two-hit hypothesis

Experiments using cell fusion demonstrated that malignancy can be suppressed when malignant cells are fused with certain non-malignant ones (Harris et al., 1969). This provided important evidence that tumor formation requires recessive loss of function mutations in certain genes. These genes are now called tumor suppressor genes. The first identified tumor suppressor gene was *RB1* (*retinoblastoma 1*). Retinoblastoma is a rapidly developing cancer that develops in the cells of the retina and affects one out of 20 000 children. About 40% of all cases are caused by mutations in *RB1,* located on chromosome 13 (Sabado Alvarez, 2008). Knudson (1971) based his two-hit hypothesis on his observations on 48 cases of retinoblastoma. The model suggests that two hits are needed to inactivate both alleles of a tumor suppressor gene (Figure 1). The first hit can be either sporadic or inherited. If the altered allele is inherited, it is found in all body cells that contain genetic material. When the second allele of the gene pair becomes inactivated in a particular somatic cell, this can lead to loss of control of cell growth and unchecked cell proliferation.

Tumor suppressor genes have many important control functions in normal cellular processes, for example proliferation, DNA repair and cell cycle. One tumor suppressor having a very crucial role in the control of cell cycle is p53 encoded by the *TP53* gene (Matlashewski et al., 1984). If *TP53* is damaged, tumor suppression is severely reduced. People who inherit only one functional copy of *TP53* will most

likely develop tumors in early adulthood, a disease known as Li-Fraumeni syndrome (Chompret, 2002). Over half of human tumors contain a mutation or deletion of *TP53* (Hainaut et al., 1997).

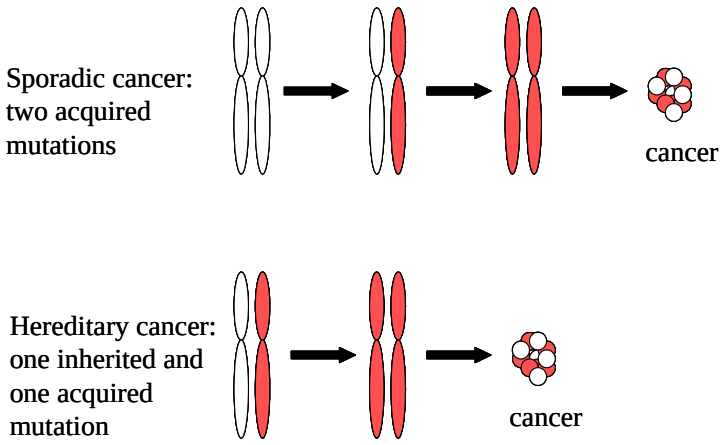

Figure 1. *Knudson's two-hit hypothesis for tumorigenesis. In the hereditary form of the cancer, the affected individual inherits a mutated allele from one parent and a somatic mutation in the target tissue inactivates the normal allele. In sporadic cancers both inactivating mutations have to occur in the same somatic cell. Cancer is therefore more likely to occur in individuals who carry the mutation in their germline. (Knudson, 1971)*

Tumor suppressor genes can further be divided into subclasses based on their function (Kinzler and Vogelstein, 1997). Caretaker genes encode products that stabilize the genome and cancer occurs indirectly because inactivation leads to genetic instabilities. This increases the number of mutations in all genes. In contrast, gatekeeper genes directly regulate tumor growth since mutations altering these genes lead to irregular growth regulation and differentiation. A third subclass of genes in which mutations lead to a significant susceptibility to cancer is composed of the landscaper genes (Kinzler and Vogelstein, 1998). Proteins encoded by landscaper genes contribute to neoplastic growth by controlling the microenvironment in which cells grow.

1.2 Oncogenes

Numerous genes have been identified as proto-oncogenes. Many of these are responsible for providing positive signals that lead to cell division. Defective versions of these genes, known as oncogenes, can cause a cell to divide in an unregulated manner. In contrast to tumor suppressor genes, a key feature of oncogene activity is that a single altered copy leads to unregulated growth (Todd and Wong, 1999), and the proto-oncogene can become an oncogene by a relatively small modification of its original function. There are three basic forms of activation:

1) a mutation within a proto-oncogene can cause a change in protein structure, 2) an increase in protein concentration caused by an increase in gene copy number, or 3) a chromosomal translocation (Croce, 2008). The products of oncogenes are typically transcription factors (*MYC*), chromatin remodelers (*ALL1*), growth factors (*PDGF*), growth factor receptors (*ERBB* gene family), signal transducers (*PI3K*), and apoptosis regulators (*BCL2*). It is possible that targeted inactivation of oncogenes could be a specific and effective treatment for cancer. In fact, there are many anticancer drugs in use, which target oncogenic proteins. The best known anticancer drugs are Trastuzumab (Herceptin), targeted against human epidermal growth factor receptor 2 (HER2) tyrosine kinase receptor in breast cancer (Carter P. et al., 1992), and Imatinib (Gleevec), which is a tyrosine kinase inhibitor targeted against the BCR-ABL fusion protein in chronic myelogenous leukemia (Druker and Lydon, 2000).

Chromosomal translocations often activate oncogenes in lymphoid cancers (Nambiar et al., 2008), but that also happens in solid tumors. An example of that mechanism in prostate cancer is the fusion of *TMPRSS2* gene with *ERG1* and *ETV*, which belong to the family of ETS transcription factors (Tomlins et al., 2005). In the original study, 23 out of 29 prostate cancer samples contained rearrangements in *ERG* or *ETV*. *TMPRSS2* has androgen responsive promoter elements, and its fusion with ETS-related genes creates a fusion protein that increases proliferation and inhibits apoptosis of prostate gland cells. This will facilitate their transformation into cancer cells. The confirmation of gene rearrangements in prostate cancer may in the future mean that the fusion status can be used for detection, classification, and treatment of the disease (Morris et al., 2008).

1.3 MicroRNAs as tumor suppressors and oncogenes

MicroRNAs (miRNA) are single-stranded RNA molecules of 19-24 nucleotides in length, which downregulate gene expression during various crucial cell processes (Fabbri et al., 2008). MiRNAs are encoded by genes but they are not translated into proteins (non-coding RNA); instead each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional miRNA (Denli et al., 2004). Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules. As miRNAs play a key role in diverse biological processes, altered miRNA expression is likely to contribute to cancer (Table 1.).

The first evidence of the involvement of miRNAs in cancer was deduced from the findings that miR-15a and miR-16-1 are downregulated or deleted in the majority of patients with chronic lymphocytic leukemia (CLL) (Calin et al., 2002). This result led to the finding that 50% of the known miRNAs are located in or very close to fragile sites, regions of loss of heterozygosity (LOH), regions of

Table 1. *Overexpressed and downregulated miRNAs in cancer. Adapted and modified from Spizzo et al.* (2009).

MiRNAs	Deregulation in cancer
let-7 family	Downregulated in lung, breast, gastric, ovary, prostate and colon cancers, CLL, and leiomyomas. let-7a-3 gene hypomethylated in lung adenocarcinoma; overexpressed in AML.
miR-10b	Downregulated in breast cancer. Overexpressed in metastatic breast cancer.
miR-15a, miR-16-1 cluster	Downregulated in CLL, DLBCL, multiple myeloma, pituitary adenoma, prostate and pancreatic cancer. Germline mutations in B-CLL patients. Upregulated in nasopharyngeal carcinoma.
miR-17, miR-18a, miR19a, miR-20a, miR-19b-1, miR-17-92 cluster	Overexpression in lung and colon cancer, lymphoma, multiple myeloma and medulloblastoma. LOH at miR17-92 locus in melanoma (20%), ovarian (16.5%) and breast (21.9%) cancer.
miR-106b-93-25	Overexpression in gastric, colon, and prostate cancer, neuroblastoma, and multiple myeloma.
miR-21	Overexpression in glioblastoma, breast, lung, prostate, colon, stomach, esophageal, and cervical cancer, uterine leiomyosarcoma, DLBCL, and head and neck cancer.
miR-29 family	Downregulation in CLL, colon, breast, and lung cancer, and cholangiosarcomas. Upregulation in breast cancer.
miR-34 family	Downregulated in pancreatic cancer and Burkitt's lymphoma without MYC translocation. Hypermethylation of miR-34b,c in colon cancer
miR-101	Downregulation in prostate cancer, hepatocellular carcinoma, and bladder cancer.
miR-122a	Downregulation in hepatocellular carcinoma.
miR-124a family	Hypermethylation in colon, breast, gastric, and lung cancer, leukemia and lymphoma.
miR-125a, miR-125b	Downregulation in glioblastoma, breast, prostate, and ovarian cancer. Upregulation in myelodysplastic syndrome, AML, and urothelial carcinoma.
miR-127	Hypermethylation in tumor cell lines.
miR-143, miR-145 cluster	Downregulated in colon adenoma/carcinoma, in breast, lung, and cervical cancer, and in B cell malignancies.
miR-155	Overexpressed in Burkitt's lymphoma, Hodgkin's lymphoma, DLBCL, breast, lung, colon, and pancreatic cancer.
miR-181	Overexpressed in breast, pancreas, and prostate cancer.
miR-221, miR-222	Overexpressed in CLL, thyroid papillary carcinoma, and glioblastoma. Downregulated in AML.
miR-200 family	Downregulated in clear-cell carcinoma and metastatic breast cancer.
miR-372, miR-373 cluster	Overexpression of miR-373 in testicular cancer.

amplifications, and common breakpoints associated with cancer (Calin et al., 2004). For example, miR-142 is located 50 nucleotides from the breakpoint region that involves chromosome 17 and *MYC*. This translocation induces an abnormal *MYC* overexpression associated with lymphomas by juxtaposing the *MYC* gene close to the miR-142 promoter (Tsujimoto et al., 1984, Garzon et al., 2006).

Functional studies have shown that miRNAs act as tumor suppressors and oncogenes (Sassen et al., 2008). He et al. (2005) showed that the miR17-92 cluster was upregulated in 65% of the B-cell lymphoma samples tested. The contribution of this cluster to cancer formation was tested with a well-characterized mouse model of MYC-induced B-cell lymphoma. The miR-17-92 cluster was overexpressed in hematopoietic stem cells from mice having the *MYC* transgene and as a result, tumor development was accelerated. O'Donnell et al. (2005) independently identified the same cluster of miRNAs to be regulated by *MYC*. Chromatin immunoprecipitation experiments showed that MYC binds directly to this locus and *MYC* induces expression of E2F1 growth factor. The mir-17-92 cluster which is also induced by *MYC* does, in contrast, inhibit E2F1 expression. Based on this, a novel regulatory mechanism is suggested by which *MYC* regulates gene expression by activating the transcription of target genes and at the same time, inducing inhibitory miRNAs that block their translation. This proves that the same miRNAs may have oncogenic or tumor suppressor activity. Costinean et al. (2006) were able to show that selective overexpression of miR-155 in B cells of Eμ-miR-155 transgenic mice induces early B cell polyclonal proliferation that results in high-grade lymphoma-pre-B leukemia. This was the first evidence that a miRNA itself can induce cancer. Also miR-21 has been shown to work as an oncogene. This miRNA is upregulated in glioblastoma (Ciafrè et al., 2005), pancreas (Volinia et al., 2005), and breast cancer (Iorio et al., 2005). Chan et al. (2005) demonstrated that knock-down of miR-21 in cultured glioblastoma cells triggers activation of caspases and this in turn leads to increased apoptosis.

The fast development of microarray technology has made it possible to investigate the miRNA expression profiles in patient material on a large scale. Differential miRNA expression profiles have been identified between normal and tumor samples (Calin and Croce, 2006) and these cancer specific miRNA profiles exist in every analyzed cancer type. These include for example breast cancer (Iorio et al., 2005), lung cancer (Takamizawa et al., 2004), and colon carcinoma (Michael et al., 2003). There are also reports from altered miRNA expression in prostate cancer compared to normal prostate tissue. Mattie et al. (2006) investigated the expression patterns of amplified miRNA from a small set of clinical prostate specimens. Unsupervised clustering analysis performed on the different miRNA samples unequivocally differentiated the prostate cancer tissues from the normal samples and non-malignant precursor lesions. Porkka et al. (2007) studied miRNA expression profiles in six prostate cancer cell lines, nine prostate cancer xenograft samples, four benign prostatic hyperplasia (BPH) samples, and nine prostate cancer samples using an oligonucleotide array hybridization method. Differential

expression of 51 miRNAs was detected between benign tumors and carcinoma tumors. Thirty-seven of them were down-regulated and 14 were up-regulated in cancer samples. These represent the miRNAs that presumably have a significant role in prostate cancer development. Similar results were obtained in another study where a set of miRNAs, including miR-125b, miR-145, and let-7c, were downregulated in clinically localized prostate cancer compared to benign tissue (Ozen et al., 2008).

1.4 Nonsense-mediated mRNA decay in cancer

Messenger RNAs are controlled for errors that arise during gene expression by a mechanism called nonsense-mediated mRNA decay (NMD) (Culbertson, 1999). As a result, most mRNAs that cannot be translated along their full length are degraded. This process ensures that truncated proteins are seldom made, and this in turn reduces the accumulation of faulty proteins that might be deleterious.

NMD has a significant role in the etiology of human genetic diseases and inherited cancers. For example, 89% of mutations in the *ATM* gene that cause ataxia-telangiectasia (Gilad et al., 1996) and 77% of mutations in *BRCA1* that are associated with breast cancer lead to premature chain termination (Couch and Weber, 1996). The identification of tumor suppressor genes and mutations in genes in solid tumors by classical cancer genetics methods has proven to be difficult. Manipulation of NMD can be exploited to identify premature termination codons in cancer. This method, proposed by Noensie and Dietz (2001), is used for the discovery of mutations without any prior information on the genes of interest by blocking the NMD pathway in cells with a translational inhibitor, such as emetine. As a result, mutated transcripts containing premature termination codons (PTCs) are stabilized and they accumulate in the cells. This enrichment in mRNA levels can be measured by gene expression microarrays. The manipulation of NMD together with expression array analysis has proven to be a powerful tool for detecting novel gene mutations in prostate cancer (Huusko et al., 2004, Rossi et al., 2005), melanoma (Bloethner et al., 2008), and colon cancer cell lines (Ivanov et al., 2007).

2. Prostate cancer

The prostate is a part of the male reproductive system and its function is to produce and store seminal fluid. In adult men a typical prostate is about three centimeters long and weighs about twenty grams. It is located in the pelvis, under the bladder and in front of the rectum. The normal prostate shows a high degree of cellular organization with three different cell populations: secretory luminal, basal, and neuroendocrine cells. A stem cell model of prostate cancer suggests that prostate cancer derives from transformed stem cells located in the basal cell layer achieving

secretory luminal characteristics under androgenic stimulation (Bonkhoff and Remberger, 1996, Schalken and van Leenders, 2003). Approximately 70% of prostate cancers originate in the peripheral zone of the gland and of the remaining cancers 15% derive from the central zone and 10-15% from the transitional zone. Almost all prostate cancers are adenocarcinomas. The remaining cases consist of squamous cell carcinoma, signet-ring carcinoma, transitional carcinoma, neuroendocrine carcinoma, or sarcoma (Bracarda et al., 2005).

2.1 Incidence and mortality

With an estimated 186 320 new prostate cancer cases in 2008, prostate cancer is the most common malignancy in the USA (excluding basal and squamous cell skin cancers) (American cancer society, 2008). In Finland the corresponding figure for the year 2007 is 4198 new cases and the incidence of prostate cancer was 85.9 per 100 000 men (Finnish Cancer Registry, 2008). The incidence of prostate cancer in Finland has increased since the 1960s and a very rapid increase was seen in 1990s (Finnish Cancer Registry, 2008) (Figure 2). This observed increase in incidence is most likely due to better access to health care and more frequent use of PSA testing (Kvale et al., 2007), but some unknown factors also exists. The use of PSA testing in asymptomatic men is controversial, as prostate cancer is detected in men who would not have been diagnosed during their lifetime in the absence of testing.

Even though the incidence has been rising, the mortality of prostate cancer in Finland has been quite steady. The peak was seen in 1996-2000 and since then, the mortality has slowly declined (Figure 2.). This can also be explained by the use of massive PSA testing because a much higher proportion of early stage cancer cases are being diagnosed than with lower levels of testing and this naturally leads to higher survival rates overall and lower mortality rates relative to incidence.

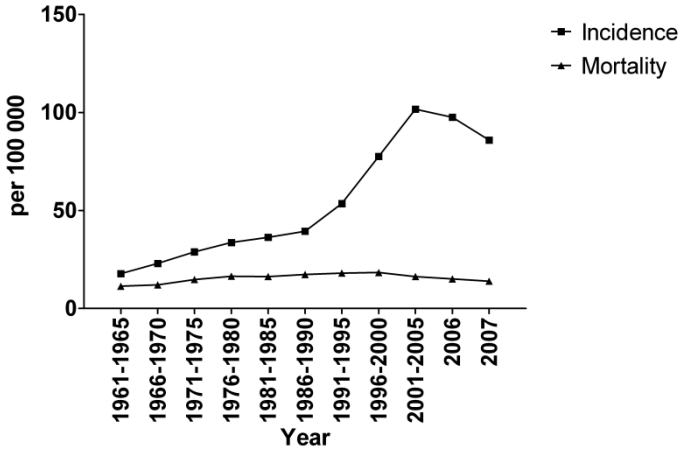

Figure 2. *Incidence and mortality of prostate cancer in Finland 1961-2007. Data modified from Finnish Cancer Registry (2008).*

2.2 Etiology and risk factors

The causes of prostate cancer are essentially unknown. Epidemiological studies have suggested various factors that might have a role in prostate cancer risk, for example history of benign prostate hyperplasia (BPH) (Chokkalingam et al., 2003, Guess, 2001), history of high-grade prostatic intraepithelial neoplasia (PIN) (Bostwick and Qian, 2004), inflammation (Nelson et al., 2004, Klein and Silverman, 2008), androgen hormones (Lucia et al., 2007), sexual activity (Dennis and Dawson, 2002, Dimitropoulou et al., 2009), consumption of vegetables and different vitamins (Chan et al., 2009), consumption of tea and coffee (Lee et al., 2009), obesity (MacInnis and English, 2006, Wallström et al., 2009), and alcohol consumption (Sommer et al., 2004), but the results are inconsistent. The only well-documented risk factors for prostate cancer are age, ethnicity, and family history (Crawford, 2003).

2.2.1 Age and ethnicity

The risk of prostate cancer is largely determined by age. It is believed that all men, given the proper amount of time, will eventually develop prostate cancer, but that many die of other causes, such as other diseases and accidents, before developing the disease. Autopsy studies suggest that most men aged older than 85 years have histological prostate cancer (Sakr et al., 1993). About 85% of the prostate cancer cases occur in men over the age of 65 (Grönberg, 2003). Prostate cancer is very rare in young men and there are no available statistical data for the incidence of the disease in men under 35 (Finnish Cancer Registry, 2008).

Prostate cancer incidence varies considerably between different ethnic groups. In the USA the incidence of prostate cancer is the highest in the world and African-American men have the highest incidence rate as well as the highest mortality rates associated with prostate cancer, followed consecutively by Caucasians, Hispanics, Asians and Pacific Islanders, then American Indians and Alaska Natives (Hsing et al., 2000, Weir et al., 2003). One explanation for the high incidence of prostate cancer among African-Americans is the vitamin D hypothesis (Schwartz, 2005). In black men the densely pigmented skin absorbs UV rays, making it more difficult for those individuals to synthesize vitamin D from UV light (Matsuoka et al., 1991). The incidence of prostate cancer in Asian countries is the lowest in the world. However, the rates have risen rapidly in the past years and nowadays prostate cancer is the most common cancer among males in many Asian countries. It is suggested that the increased incidence is associated with westernization of the lifestyle (Pu et al., 2004). In Africa the incidence rates of prostate cancer are not fully reliable, but studies from Uganda and Nigeria report that prostate cancer is very common and in Nigeria it is the most common cancer in males (Wabinga et al., 2000, Ogunbiyi and Shittu, 1999). The differences seen in prostate cancer incidence among different

ethnic groups are most probably caused by various factors including exposure to external risk factors, differences in cancer registration and health care and genetic susceptibility (Grönberg, 2003). To evaluate the impact of environmental and genetic factors on prostate cancer etiology, Beiki et al. (2009) compared the risk of prostate cancer in Sweden among foreign-born men to that of Swedish-born men. The results showed that overall foreign-born men had a significantly decreased risk of prostate cancer, but the risk was increased among those who stayed 35 years or longer. This confirms the assumption that both environmental and genetic factors are involved in the etiology of prostate cancer (Beiki et al., 2009).

2.2.2 Family history

The clustering of prostate cancer in families was already reported in the 1950s (Morganti et al., 1956) when it was noted that a higher proportion of prostate cancer patients reported a close relative with prostate cancer compared to the controls. A few years later, Woolf et al. (1960) reported that deaths due to prostate cancer were threefold higher among the fathers and brothers of men dying from prostate cancer compared with relatives of men dying from other causes. Steinberg et al. (1990) performed a case-control study to estimate the relative risk of prostate cancer in men with a positive family history of the disease. Results showed that there was a trend of increasing risk with increasing number of affected family members (five-fold increased risk with two first degree relatives and 11-fold with three first degree relatives). In addition, an early diagnosis age increases the risk of prostate cancer in the relatives of the affected (Keetch et al., 1995).

The observed clustering of prostate cancer in families can be a result of genetic origin, exposure to environmental factors, or chance alone as prostate cancer is so common. Familial prostate cancer is usually defined as a family where there are two affected first-degree relatives. This group of prostate cancer patients is thought to account for 10 to 20 percent of all cases (Stanford and Ostrander, 2001). A more strict definition of familial prostate cancer is hereditary prostate cancer, which characterizes families in which a pattern of Mendelian inheritance of susceptibility genes is seen (Carter BS et al., 1992, Carter et al., 1993). The hereditary prostate cancer families are characterized by at least one of the so called Carter criteria (Carter et al., 1993): 1) three or more first-degree relatives with prostate cancer, 2) prostate cancer in three successive generations through maternal or paternal lineage, or 3) two first-degree relatives diagnosed at a young age ($\leq$ 55 years). This form of prostate cancer is estimated to account for 5 to 10 percent of all cases (Stanford and Ostrander, 2001). Interestingly, a large Scandinavian twin study reported that 42% of the risk of prostate cancer could be explained by heritable factors (Lichtenstein et al., 2000). This proportion is the highest ever reported for a common malignancy. Nevertheless, sporadic prostate cancer cases constitute the major part of all cases in

the general population. Prostate cancer is considered to be sporadic if the patient has no close relatives with the disease.

Segregation analysis is a statistical test to determine the pattern of inheritance for a given trait. Several segregation analyses of prostate cancer in different populations have been performed and most of them support the autosomal dominant mode of inheritance (Grönberg et al., 1997a, Schaid et al., 1998, Verhage et al., 2001, Valeri et al., 2003), but also a multifactorial (Gong et al., 2002, Conlon et al., 2003), recessive (Monroe et al., 1995), and X-linked (Monroe et al., 1995, Cui et al., 2001) modes of inheritance have been suggested. In Finland, the segregation of prostate cancer was studied for two cohorts, 557 early-onset and 989 late-onset families (Pakkanen et al., 2007). In the Finnish population the familial aggregation of prostate cancer is best explained by a complex model that includes a major susceptibility locus with recessive inheritance and a significant paternal regressive coefficient. Interestingly, this is the first study where recessive inheritance is estimated to fit in all data sets. Parallel results were seen in a large segregation study from the UK and Australia, where 4390 families were analyzed for genetic models of susceptibility to prostate cancer (MacInnis et al., 2009). The best-fitting model was the mixed recessive model, suggesting that one or more genes having strong recessively inherited risk together with gene variants having small multiplicative effects on cancer risk may account for the genetic susceptibility to prostate cancer.

3. Genetic predisposition to prostate cancer

The hereditary component of prostate cancer risk is obvious, but the identification of highly penetrant prostate cancer genes has still been particularly difficult; several factors contribute to that issue. First, prostate cancer is typically diagnosed at a late age, so collecting DNA samples from living affected men for more than one generation is often a great problem. Second, it is often difficult to distinguish between hereditary and sporadic forms of the disease (phenocopies) which introduce misleading results in the analyses. The third major problem is the apparent genetic heterogeneity of the disease and the minor effect of each of the individual variants contributing to the cancer risk. Therefore, there is a great need for methods of statistical analysis that can take into account multiple predisposing genes with moderate penetrance (Ostrander and Stanford, 2000).

3.1 Linkage studies

Traditionally, the search for a disease gene starts with linkage analysis. In this method, the aim is to find out the rough location of the gene relative to another DNA sequence, which has its position already known (Altshuler et al., 2008). Prostate cancer linkage studies have been used to localize rare and highly penetrant

susceptibility genes. In the beginning the hopes were high that finding the genes predisposing to prostate cancer would be as effortless as finding the genes for colon (Peltomäki, 2001) and breast cancer (Miki et al., 1994, Wooster et al., 1995). Over the years there have been many published reports of possible linkage of prostate cancer susceptibility to different chromosomes, but the signals have not always been reproducible between studies (Table 2.).

Table 2. *Prostate cancer susceptibility loci and candidate genes.*

Locus	Chr location	Candidate gene	References	Study size	Result
HPC1	1q24-q25	*RNASEL*	Smith et al., 1996	66 families	LOD=3.65
			Grönberg et al., 1997c	91 families	LOD=3.67
			McIndoe et al., 1997	49 families	LOD=0.48
			Eeles et al., 1998	136 families	LOD<0
			Goode et al., 2000	149 families	LOD<0
			Hsieh et al., 1997	92 families	NPL Z=1.83
			Neuhausen et al., 1999	41 families	LOD=2.82
			Brown et al., 2004	33 families	NPL Z=1.12
PCAP	1q42.2–q43	None	Berthon et al., 1998	47 families	LOD=2.70
			Cancel-Tassin et al., 2001a	64 families	LOD=2.56
			Whittemore et al., 1999	97 families	LOD<0
			Suarez et al., 2000	49 families	NPL Z=0.5-1.0
			Goddard et al., 2001	254 families	LOD=2.84
			Easton et al., 2003	1293 families	LOD<1.0
			Brown et al., 2004	33 families	NPL Z=1.48
CAPB	1p36	None	Gibbs et al., 1999	12 families	LOD=3.22
			Berry et al., 2000b	13 families	LOD<0
			Badzioch et al., 2000	207 families	LOD<0
			Goode et al., 2001	149 families	LOD=0.21
			Matsui et al., 2004	44 families	LOD=2.24
HPCX	Xq27-q28	None	Xu et al., 1998	360 families	LOD=4.60
			Lange et al., 1999	153 families	NPL Z=1.06
			Schleutker et al., 2000	57 families	LOD=2.05
			Peters et al., 2001	186 families	LOD=0.63
			Bochum et al., 2002	104 families	NPL Z=1.20
			Brown et al., 2004	33 families	NPL Z=1.20
HPC2	17p11	*ELAC2*	Tavtigian et al., 2001	33 families	LOD=4.50
			Lange et al., 2003	175 families	LOD=2.36
HPC20	20q13	None	Berry et al., 2000b	162 families	LOD=2.69
			Bock et al., 2001	172 families	LOD=0.09
			Zheng et al., 2001	159 families	NPL Z=1.02
			Cancel-Tassin et al., 2001b	66 families	LOD<0
			Cunningham et al., 2003	160 families	LOD=4.77
			Brown et al., 2004	33 families	NPL Z=1.17
			Schaid et al.,2005	1234 families	LOD=0.06

8p	8p21–p23	*MSR1*	Xu et al., 2001b	254 families	LOD=1.84
			Wiklund et al., 2003	57 families	LOD=1.08
3p	3p25-p26	None	Schleutker et al., 2003	13 families	LOD=2.57
			Rökman et al., 2005	16 families	LOD=3.39
			Chang et al., 2005a	188 families	LOD=1.75
			Chang et al., 2005b	188 families	LOD=3.08
8q	8q24	None	Amundadottir et al., 2006	323 families	LOD=2.11
			Freedman et al., 2006	1597 cases, 873 controls	LOD=7.1
			Yeager et al., 2007	1172 cases, 1157 controls	OR=1.42, $p=9.75x10^{-5}$
			Haiman et al., 2007b	4266 cases, 3252 controls	$p=7.9x10^{-19}$
			Gudmundsson et al., 2007b	1453 cases, 3064 controls	OR=1.71, $p=1.6x10^{-14}$

3.1.1 HPC1 and RNASEL

The first linkage study of prostate cancer completed by Smith et al. (1996) in 66 high-risk prostate cancer families provided evidence of linkage to the long arm of chromosome 1 (1q24-25), named as *HPC1* (OMIM #601518). Afterwards, several confirmatory and/or supportive studies were performed (Cooney et al., 1997, Grönberg et al., 1997b, Hsieh et al., 1997, Neuhausen et al., 1999, Goode et al., 2000, Xu, 2000, Goddard et al., 2001, Xu et al., 2001a) but also many studies failed to confirm the linkage (Eeles et al., 1998, Berthon et al., 1998, Suarez et al., 2000, Berry et al., 2000b), which was surprising as the initial linkage was very strong (maximum HLOD 5.43) (Smith et al., 1996).

The *RNASEL* gene, which maps to *HPC1*, encodes the 2´-5´-oligoadenylate-dependant RNase L. It functions as a mediator of the interferon induced RNA degradation pathway, involved in defense against viral infections (Zhou et al., 1997). It is supposed to be a tumor suppressor gene (Lengyel, 1993). In 2002 it was reported that two germline mutations, Glu262X and Met1Ile, in the *RNASEL* gene segregate in HPC families that show linkage to *HPC1*, and *RNASEL* could possibly be the candidate gene for *HPC1* (Carpten et al., 2002). In a follow-up study from Finland, the *RNASEL* gene was screened in 66 patients with HPC and the variant Glu256X was associated with prostate cancer risk, especially in families with four or more affected (OR=5.85, 95% CI=1.20-28.87) (Rökman et al., 2002). Similar results were obtained in a study from USA where 95 affected men in 75 prostate cancer families were tested and Glu256X was found in one family with two of three affected brothers being heterozygous carriers (Chen H et al., 2003). In a study among Ashkenazi Jews a novel frameshift mutation (471delAAAG) was detected, which leads to premature truncation of the protein (Rennert et al., 2002). The mutation was estimated to be as frequent as 4% in that population and the frequency was higher in patients with prostate cancer than controls (OR=3.0, 95% CI=0.6-

15.3). The carriers were also diagnosed at an earlier age (P<0.001). A subsequent study among the Ashkenazi Jew population in Montreal confirmed the presence of this founder mutation, but association with prostate cancer risk was not detected (Kotar et al., 2003). In Sweden, the effect of *RNASEL* on prostate cancer risk was analyzed in hereditary, familial, and sporadic prostate cancer (Wiklund et al., 2004). The prevalence of Glu256X carriers was almost identical among cancer patients and controls and evidence for segregation was not observed in any HPC family. These authors found a marginally significant inverse association between mutation Asp541Glu and prostate cancer risk (OR=0.77, 95% CI=0.59-1.00), which had previously been associated with an increased risk of prostate cancer in Japan (Nakazato et al., 2003).

In summary, many studies provide data to support that *RNASEL* plays a role in HPC, but opposing results have also been presented. It seems that genetic variants of this gene may account for only a part of prostate cancer, and the effects are seen especially in families with many affected family members.

3.1.2 PCAP and CABP

There are two other susceptibility loci in chromosome 1. *PCAP*, located 60 centimorgans downstream from *HPC1*, was reported in a linkage scan of 47 French and German families (Berthon et al., 1998). *CABP* locus (Cancer of the Prostate and Brain) was identified in a study in which 12 families with a history of both prostate and primary brain cancer were screened for linkage (Gibbs et al., 1999). Both of these linkages have been difficult to replicate. Xu et al. (2001a) performed a multipoint linkage analysis spanning chromosome 1 in 159 HPC families. The strongest linkage was seen at 1q24-q25, but also elsewhere on chromosome 1 some evidence of linkage was observed. This strengthens the impression that there are multiple loci on chromosome 1 for prostate cancer.

3.1.3 HPCX

Originally, linkage to chromosome Xq27-q28 was observed in a combined study of 360 prostate cancer families collected at four research centers in USA, Finland and Sweden, named as *HPCX* (Xu et al., 1998). The maximum two-point lod score of 4.60 was seen in the combined dataset at marker *DXS1113*, but also significant evidence for locus heterogeneity was observed. Many studies attempting to confirm these findings have been published. Lange et al. (1999) performed a linkage study with 153 families and the maximum two-point HLOD of 0.15 was seen at marker *DXS1108* in families without male-to-male transmission, although it was not statistically significant. In order to estimate the role of *HPCX* in German prostate cancer families, 104 families were genotyped at six markers spanning the original

linkage region (Bochum et al., 2002). A maximum NPL Z score was seen at marker *DXS984* (1.20) and significant evidence was obtained in the group of families with early-onset disease (diagnosis age ≤ 65 years). The first significant confirmation of the original finding came from Utah prostate cancer families, where linkage to *HPCX* was seen in a dataset containing families having no more than five generations (multipoint TLOD of 2.74; P=0.0002) (Farnham et al., 2005). Chang et al. (2005a) identified a subset of 244 men with aggressive prostate cancer (Gleason score ≥7, tumor stage T2c or higher, primary PSA ≥20 ng/ml) in 188 HPC families and performed a genome-wide scan. The strongest evidence for linkage was observed at *DXS8043* (HLOD 2.54, P=0.0006), 2 Mb centromeric to the *HPCX* locus originally reported by Xu et al. (1998). In contrast to these positive findings, negative studies showing no evidence of linkage have also been performed (Peters et al., 2001, Brown et al., 2004).

In Finland, the original finding was confirmed in a set of 57 HPC families with at least two living prostate cancer patients (Schleutker et al., 2000). Analysis was carried out with 22 markers for the *HPCX* region and the maximum two-point LOD score was 2.05 at *DXS1205*. Subgroup analyses revealed that no male-to-male (NMM) transmission and late age at diagnosis (>65 years) accounted for the most of the cases (maximum two-point LOD score 3.12). Subsequently, the region around the best linkage marker was found to be in strong linkage equilibrium in the Finnish HPC families (Baffoe-Bonnie et al., 2005). Equal results were obtained in a study by Yaspan et al. (2008), where an association study was conducted to identify risk variants within the *HPCX* locus. The haplotype extending from rs5907859 to rs1493189 was concordant with the region within the Finnish population and was associated with prostate cancer (OR=3.41, 95% CI, 1.04–11.17, P = 0.034).

Despite the significant linkage information, the susceptibility gene for *HPCX* has not been identified. Although the androgen receptor gene (*AR*) is located on the X chromosome and would be a likely candidate gene, it is located more than 50 cM from the region of linkage. One obvious reason for unsuccessful candidate gene identification is the fact that the chromosomal region of *HPCX* has an extremely complex genomic structure (Stephan et al., 2002). The region contains duplicated segments and an inversion of substantial size, which makes the traditional methods of positional cloning unusable. Of interest in the *HPCX* region is the presence of multiple gene family clusters. The family of human melanoma-associated antigens (*MAGE*) encodes tumor-specific antigens recognized by autologous cytotoxic T lymphocytes (Chomez et al., 2001). The first member of the human *MAGE* family (*MAGE-A1*) was identified as a gene encoding a tumor-specific-antigen (van der Bruggen et al., 1991). It was later found to belong to a cluster of 12 *MAGE-A* genes located in the Xq28 region (De Plaen et al., 1994, Rogner et al., 1995). A sequencing project at the Xp21 region led to the discovery of a second cluster named *MAGE-B* and a *MAGE-C* cluster was localized to Xq26-q27 (Muscatelli et al., 1995). The genes belonging to *MAGE-A*, *-B*, and *-C* subclusters are expressed in malignant tumors and testis but not in other normal tissues. Therefore, they are also

named as cancer/testis (CT) antigen and tumor specific antigen (Xiao and Chen, 2004). In contrast, other *MAGE* subfamilies are expressed in various normal adult tissues. Although the subgroups are expressed in different tissues, they share the MAGE homology domain (MHD), suggesting functional conservation. It is speculated that this family of proteins functions during embryonic development and then the genes are inactivated, probably by methylation (Xiao and Chen, 2004). During tumor development, the genes are re-activated, expressed, and they may become antigenic targets that are recognized by the immune system (Zhao et al., 2002). In other words, *MAGE* genes take part in the immune process by targeting early tumor cells for immune destruction and their defective function might lead to tumor progression.

The narrowed *HPCX* region spans ~ 750 kb and contains the cluster of *SPANX* (Sperm Protein Associated with the Nucleus on the X chromosome) genes (*SPANX-A1*, *-A2*, *-B*, *-C*, and *-D*) (Figure 3). They encode proteins that are expressed in the normal testis, non-gametogenic tissues, and certain tumors (Westbrook et al., 2000, Westbrook et al., 2004). SPANX-A/D genes are transcribed postmeiotically and they are thought to have a role in reproduction (Westbrook et al., 2004). Evolutionary analysis revealed that the *SPANX- A/D* genes are rapidly amplifying and they are the most rapidly evolving gene family in the hominoid lineage and the existence of the *SPANX-N* cluster at Xq27 was revealed based on analysis of *SPANX-A/D* homologs in nonhuman primates (Kouprina et al., 2004a). N-cluster includes *SPANX-N1*, *-N2*, *-N3*, and *-N4* at Xq27 and *-N5* is located at chromosome Xp11. The proteins encoded by these genes are small proteins highly expressed in spermatozoa. Kouprina et al. (2005) set out to study the entire cluster of *SPANX* genes in families with prostate cancer. Due to the fact that these genes are very similar and reside within chromosomal duplications, a routine PCR method could not be used. Instead, a transformation-associated recombination (TAR) cloning technique was applied, which allows direct isolation of genes up to 250 kb (Kouprina et al., 2004b). A comprehensive analysis of *SPANX-C*, *SPANX-B*, and *SPANX-D* loci was performed and the results showed an extensively complex and dynamic organization of the *SPANX* genes but no variation could be associated with prostate cancer risk. In a further study, variations in *SPANX-A1*, *SPANX-A2*, *SPANX-C*, and *SPANX-N1/N4* genes were analyzed, but none of the sequence variations in the coding regions were associated with susceptibility to prostate cancer (Kouprina et al., 2007). Therefore, it can be concluded that genetic variation in the *SPANX* genes does not explain HPCX. However, it might be reasonable to assume that *SPANX* genes have a modifying role in the predisposition to prostate cancer, probably through some complex recombinational interaction (Kouprina et al., 2007).

The region around *HPCX* also contains the leucine zipper, down-regulated in cancer 1 gene (*LDOC1*). It was isolated as a gene encoding a leucine-zipper protein whose expression was down-regulated in pancreatic and gastric cancer cell lines (Nagasaki et al., 1999). It also induces apoptosis (Inoue et al., 2005) and possibly

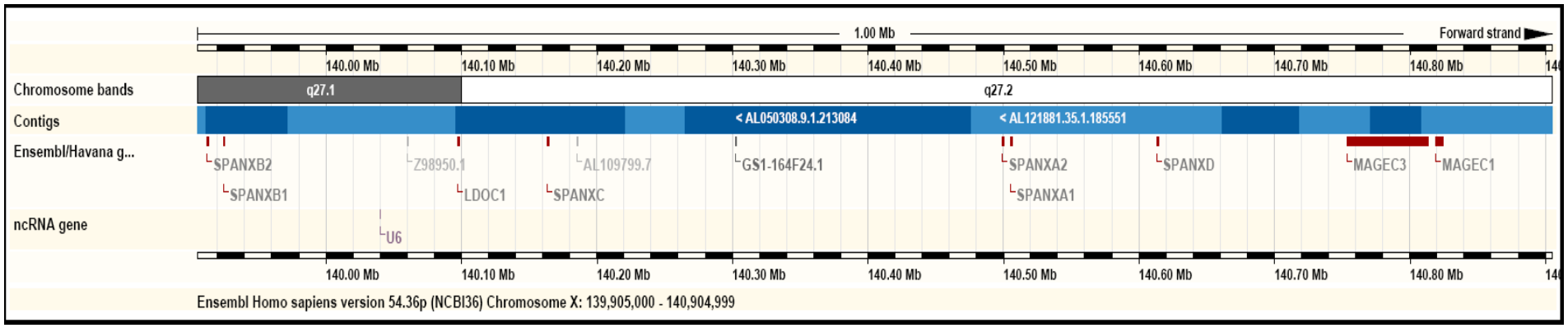

Figure 3. *Mapping of the SPANX gene family to the human X chromosome between 139.9M and 140.1M (Ensembl Homo sapiens version 54.36p at http://www.ensembl.org)*

inhibits the degradation of p53 protein (Mizutani et al., 2005). Recently, it was also found to be down-regulated also in esophageal cancer (Ogawa et al., 2008). Kouprina et al. (2005) sequenced the promoter and coding sequences of *LDOC1* and no nucleotide changes were observed in 17 prostate cancer patients and 22 unaffected controls, except for one polymorphism in the promoter region 720 bp upstream of the start codon. Presumably, *LDOC1* is not a predisposing factor to prostate cancer, even though it may have an important role in the development and progression of some cancers.

3.1.4 HPC20

Linkage to chromosome 20q13 emerged from an analysis performed with 162 North American families with ≥3 affected family members (Berry et al., 2000a). The highest two-point LOD score was 2.69 at marker *D20S196* and the maximum multipoint NPL score was 3.02 (P=0.002). For further analysis, the families were stratified according to male-to-male transmission, average age at diagnosis, and number of affected individuals. Strongest linkage was seen with the families having <5 affected family members. This finding was partially replicated when linkage was seen in a subset of 16 black families among 172 unrelated prostate cancer families (LOD=0.86, P=0.023) (Bock et al., 2001). Afterwards, two independent studies confirmed the linkage at chromosome 20 (Zheng et al., 2001, Cunningham et al., 2003), but also negative findings have been published (Cancel-Tassin et al., 2001b). Interestingly, the International Consortium for Prostate Cancer Genetics (ICPCG) was not able to confirm the linkage to *HPC20* in an analysis of 1234 families with multiple cases of prostate cancer (Schaid, Chang and International Consortium For Prostate Cancer Genetics, 2005), which represents the most profound attempt to replicate the original finding.

3.1.5 HPC2 and ELAC2

Originally, the linkage scan with eight high-risk prostate cancer families from Utah with 300 polymorphic markers provided suggestive evidence for linkage at chromosome 17p11 near marker *D17S520* (Tavtigian et al., 2001). When the number of families was increased to 33, the analysis yielded a maximum two-point LOD score of 4.5 at marker *D17S1289* and maximum three-point LOD score of 4.3 at the markers *D17S1289* and *D17S921*. This interval was selected as a target for a positional cloning project. An additional 94 families were included in to the analysis and surprisingly, the linkage disappeared. However, a common haplotype was detected and the region could be narrowed down to a 1.5 Mb fragment. The *ELAC2* gene was identified from that region and it was the first candidate gene for prostate cancer based on linkage results (Tavtigian et al., 2001). *ELAC2* (elaC homolog 2 *E.*

coli) encodes a protein of 826 amino acids, which functions as a tRNA 3´ processing endoribonuclease (3´ tRNase), an enzyme responsible for the removal of a 3´trailer from precursor tRNA (Takaku et al., 2003). It has also been shown to interact with γ-tubulin complexes (Korver et al., 2003), which might suggest a role in cell division.

In the original study by Tavtigian et al. (2001), sequencing analysis of *ELAC2* revealed two mutations (1641insG and Arg781His) and two common missense variants (Ser217Leu and Ala541Thr). Ser217Leu and Ala541Thr were found to be associated with prostate cancer risk and furthermore, 1641insG segregated with the disease status in a Utah prostate cancer family. The results were confirmed in an independent study, where 359 prostate cancer patients and 266 male controls were genotyped for Ser217Leu and Ala541Thr variants (Rebbeck et al., 2000). In this study, the highest risk for prostate cancer was observed among men who carried the Leu217/Thr541 variants (OR=2.37, 95% CI=1.06-5.29). In addition, the *ELAC2* variations were estimated to cause 5% of prostate cancer in the general population. In contrast, a meta-analysis of six studies did not find any association for Thr541 and Leu217 alleles with prostate cancer risk (Camp and Tavtigian, 2002). In the Finnish population the *ELAC2* gene was sequenced in the probands of the 66 HPC families (Rökman et al., 2001a). Seventeen variants were found, including the previously characterized missense mutations Ser217Leu and Ala541Thr and a previously unreported missense mutation, Glu622Val. Only Glu622Val was significantly associated with prostate cancer in the Finnish population (OR=2.94, 95% CI=1.05-8.23).

In another study from Finland, the exons 7 and 17 of *ELAC2* were screened among 26 primary untreated and 13 locally recurrent hormone-refractory prostate carcinomas, three cell lines (LNCaP, DU-145 and PC-3), and ten human prostate cancer xenografts (Nupponen et al., 2004). The results do not support the hypothesis that *ELAC2* would be a commonly mutated gene in sporadic prostate cancer. A study by Minagawa et al. (2005) diminished the role of *ELAC2* predisposition to cancer by reporting that the 3´ processing activity of ELAC2 protein is not affected by the missense variations and there is no causality between the enzymatic properties of the protein and prostate cancer risk.

3.1.6 8p22-p23 and MSR1

Loss of heterozygosity in the short arm of chromosome 8 has frequently been observed in prostate cancer (Visakorpi et al., 1995, Cunningham et al., 1996, Rökman et al., 2001b, Saramäki et al., 2006). Xu et al. (2001b) performed a linkage study in 159 HPC families with 24 markers on chromosome 8p. In this set of families, evidence for linkage was found at 8p22-p23 with a HLOD of 1.84 (P=0.004). The finding was replicated in two independent studies (Wiklund et al., 2003, Maier et al., 2005) and consequently, seven mutations, co-segregating with

the disease status, in the macrophage scavenger receptor 1 gene (*MSR1*) at 8p22 were observed in HPC families (Xu et al., 2002a). *MSR1* gene encodes the class A macrophage scavenger receptors. These receptors are macrophage-specific trimeric integral membrane glycoproteins and have been implicated in many macrophage-associated physiological and pathological processes, which include atherosclerosis, Alzheimer's disease, and host defense (Peiser and Gordon, 2001). In a study from Finland, the youngest affected patient from 120 prostate cancer families was screened for *MSR1* sequence variations (Seppälä et al., 2003a). Five variants were identified, but the carrier frequencies did not differ significantly between patients and controls. The only significant finding was that the mean age at diagnosis of the Arg293X mutation carriers was lower compared to non-carriers (55.4 *vs.* 65.4, P=0.04). Sun et al. (2006) meta-analyzed eight published studies in order to evaluate the effect of three rare mutations and five common variants of *MSR1* on prostate cancer risk. Several variants were associated with sporadic disease, but the association was not seen when the results of the original study were excluded. However, the frequency of Asp175Tyr mutation was higher among African-American prostate cancer patients. The authors suggested that the genetic variation of *MSR1* may produce a moderate risk of prostate cancer, especially in African-American men (Sun et al., 2006). More recently, two studies from Poland and India reported negative results showing no evidence for the *MSR1* contribution to prostate cancer risk (Rennert et al., 2008, Cybulski et al., 2007).

3.1.7 3p25-p26 and MLH1

Three linkage analyses, including the one carried out on Finnish prostate cancer families, have shown positive linkage on chromosome 3p (Schleutker et al., 2003, Chang et al., 2005a, Chang et al., 2005b). In the Finnish study, 87 individuals from 13 prostate cancer families were genotyped for 413 different microsatellite markers with an average spacing of 10 cM (Schleutker et al., 2003). The highest two-point LOD scores were observed at 3p25-p26 (2.57) and at 11q14 (2.97). The region at 11q14 also reached a suggestive level of significance in a pooled ICPCG study of aggressive prostate cancer (LOD=2.4) (Schaid et al., 2006). Afterwards, these two regions were fine-mapped at high resolution in 16 best Finnish families, including new multiplex families, which were not included in the original scan (Rökman et al., 2005). Fine-mapping validated 3p26 as a susceptibility locus for prostate cancer in Finland (maximum multipoint HLOD = 3.39) but in contrast, the results decreased evidence in the 11q14 region. Ten genes from 3p26, including *CHL1*, *CNTN6*, *CNTN4*, *IL5RA*, *TRNT1*, *BHLHB2*, *OXTR*, *VHL*, *CDC25A*, and *FHIT*, were screened for mutations, but nothing significant emerged from these studies (Rökman et al., 2005 and unpublished data).

As MutL protein homolog 1 gene (*MLH1*) is a known tumor suppressor gene located at 3p22, it therefore represents a possible candidate gene for the detected

locus. Originally, *MLH1* was associated with the Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC), which is the most common dominantly inherited colorectal cancer syndrome characterized by the development of colorectal, endometrial and various other cancers at an early age (Lynch and de la Chapelle, 2003). It is caused by a mutation in one of the DNA-mismatch repair genes (MMR) (*MSH2*, *MLH1*, *MSH6*, and *PMS2*) (Peltomäki and Vasen, 2004). A defect in these genes results in multiple mistakes in microsatellite sequences throughout the genome. This is called microsatellite instability (MSI) and is the hallmark of the Lynch syndrome. In prostate cancer, varying degrees of MSI (20% - 65%) and loss of the MMR proteins and down-regulation of MMR enzyme activity have also been detected (Gao et al., 1994, Egawa et al., 1995, Watanabe et al., 1995, Yeh et al., 2001, Chen Y et al., 2003), but also opposite results have been published demonstrating strong MLH1 nuclear immunopositivity (Chuang et al., 2008). Nevertheless, the accumulated data suggest that *MLH1* may have a role in prostate tumorigenesis. Supporting that hypothesis, the variant Ile219Val in *MLH1* showed an association with prostate cancer in a candidate single nucleotide polymorphism (SNP) analysis (Burmester et al., 2004).

3.1.8 Linkage scans incorporating prostate cancer aggressiveness

It is a well known fact that the aggressiveness of prostate cancer varies greatly. Some tumors stay latent for long periods of time and are present only as small foci detected at autopsy in men that die from other causes (Sakr et al., 1994). At one extreme is prostate cancer that has metastasized and is really a life-threatening disease. What is not so clear is whether these cancers differing in severity also have different etiologies. If this is true, mixing the two types (or more) in genetic linkage studies may produce false results and also reduce the power to detect major gene effects.

For the first time, Witte et al. (2000) performed a genome-wide scan with 513 brothers with prostate cancer using the Gleason score as a measure of aggressiveness. The results suggested candidate regions on chromosomes 5q31-q33, 7q32, and 19q12. Neville et al. (2002) further examined the region on chromosome 7q32-q33 and were able to confirm the linkage and narrow down the region to 1.1 Mb. They also reported a high frequency of allelic imbalance (AI) in a set of 48 primary prostate tumors, which was very interesting as AI of 7q has previously been associated with poor outcome in prostate cancer patients (Takahashi S. et al., 1995). Further confirmation came from a German study where 100 prostate cancer families were genotyped using eight markers on chromosome 7q and the evidence of linkage emerged from families with aggressive and late onset disease (Paiss et al., 2003).

In 2005, Chang et al. (2005a) reported a re-evaluation study of 623 men in 188 prostate cancer families. Men were stratified according to the clinical/pathologic criteria of the disease and 244 men were classified as having aggressive prostate

cancer. A repeat genome-wide scan revealed strong evidence of linkage in Xq27-q28, 3p26, 22q13 and 9p21, regions that were not seen as potent in the original study with all 623 men. Stanford et al. (2006) performed a genome-wide scan in 123 families taking into account the disease characteristics. By this means, two regions on chromosome 22q were highlighted (22q11.1, dominant HLOD=2.75; 22q12.3-q13.1, recessive model HLOD=1.90). The region in 22q11.1 has not been previously identified, but a signal in region 22q12.3-q13.1 was noted by Lange et al. (2003) in a subset of African-American prostate cancer families and in families with four or more affected members. In addition, ICPCG reported a significant linkage at 22q12 (dominant model LOD=3.57) in 269 families with five or more affected members (Xu et al., 2005). A subset of families from Utah, some of which were also included in the ICPCG analysis, produced borderline evidence for linkage on chromosome 22q (dominant model HLOD=2.42) (Camp et al., 2005).

In a study from the US, chromosome region 15q12 was noted to be significantly linked to prostate cancer risk when only men with aggressive disease were coded as affected (LOD=3.49, P=0.005) (Lange et al., 2006). This linkage increased when only Caucasian-American families were included into the analyses (n=65, LOD=4.05). This region overlaps with region 15q11, which was identified in the ICPCG linkage study on 1233 prostate cancer families (Xu et al., 2005). Interestingly, in a recent analysis of newly collected Finnish families (≥3 affected cases per family) the same area is seen (George et al., unpublished data).

3.2 Genome-wide association studies

Besides linkage studies, another important approach for finding prostate cancer genes has been association studies in cohorts of men with and without prostate cancer, and without information regarding family history of the disease. In these studies, allele or genotype frequencies of tagging SNPs are compared between cases and controls in order to find a variation that is associated with the disease. While linkage analysis is best suited for finding rare variants with high penetrance, genome wide association (GWA) studies will provide stronger power to detect small and modest effects on cancer risk. This seems very suitable as it is believed that most of the genetic basis for prostate cancer arises from multiple low-risk gene variants (Easton et al., 2003). In the past years, GWA studies have been successful in identifying common sequence variants associated with a modest increase in prostate cancer risk (Table 3.) (Duggan et al., 2007, Gudmundsson et al., 2007a, Gudmundsson et al., 2007b, Haiman et al., 2007b, Yeager et al., 2007, Witte, 2007, Gudmundsson et al., 2008, Eeles et al., 2008, Thomas et al., 2008). If present in combinations, these SNPs may result in a higher risk among the proportion of men who carry many variants. However, it seems that these recently established prostate cancer susceptibility variants are not associated with disease outcome (Fitzgerald et al., 2009, Wiklund et al., 2009).

Table 3. *Summary of loci that are found by recent GWAS and that are modestly associated with prostate cancer risk. Adapted and modified from Witte* (2009).

Loci	SNP	Odds ratio	p value	Size of study (cases/ controls)	Nearby gene	Reference
2p15	rs721048	1.15	$7.7x10^{-9}$	12500/29034	*EHBP1*	Gudmundsson et al., 2008
3p12	rs2660753	1.30	$2.7x10^{-8}$	1854/1894	Intergenic	Eeles et al., 2008
6q25	rs9364554	1.21	$5.5x10^{-10}$	1854/1894	*SLC22A3*	Eeles et al., 2008
7q21	rs6465657	1.19	$1.1x10^{-9}$	1854/1894	*LMTK2*	Eeles et al., 2008
8q24 I	rs16901979	1.52	$1.1x10^{-12}$	1453/3064 4266/3252 1172/1157 1854/1894	Intergenic	Gudmunsson et al., 2007b Haiman et al., 2007b Yeager et al., 2007 Eeles et al., 2008
8q24 II	rs6983267	1.25	$9.4x10^{-13}$	4266/3252 1172/1157 1854/1894	Intergenic	Haiman et al., 2007b Yeager et al., 2007 Eeles et al., 2008
8q24 III	rs1447295	1.42	$6.4x10^{-18}$	1453/3064 4266/3252 1172/1157 1854/1894	Intergenic	Gudmunsson et al., 2007b Haiman et al., 2007b Yeager et al., 2007 Eeles et al., 2008
10q11	rs10993994	1.38	$8.7x10^{-29}$	3941/3964 1854/1894	*MSMB*	Thomas et al., 2008 Eeles et al., 2008
10q26	rs4962416	1.18	$2.7x10^{-8}$	3941/3964	*CTBP2*	Thomas et al., 2008
11q13	rs7931342	1.21	$1.7x10^{-12}$	3941/3964 1854/1894	Intergenic	Thomas et al., 2008 Eeles et al., 2008
17q12	rs4430796	1.22	$1.4x10^{-11}$	3493/14348 3941/3964 1854/1894	*HNF1B*	Gudmunsson et al., 2007a Thomas et al., 2008 Eeles et al., 2008
17q24	rs1859962	1.20	$2.5x10^{-10}$	3493/14348 1854/1894	Intergenic	Gudmundsson et al., 2007a Eeles et al., 2008
19q13	rs2735839	1.37	$1.5x10^{-18}$	1172/1157 1854/1894	*KLK2*, *KLK3*	Yeager et al., 2007 Eeles et al., 2008
Xp11	rs5945619	1.29	$1.5x10^{-9}$	12500/29034 1854/1894	*NUDT10*, *NUDT11*	Gudmundsson et al., 2008 Eeles et al., 2008

3.2.1 8q21-24 and NBN (NBS1)

One of the most interesting findings in recent GWAS is that at least three distinct loci on chromosome 8q24 within 1 Mb have SNPs that associate with prostate cancer risk (Gudmundsson et al., 2007b, Haiman et al., 2007b, Severi et al., 2007, Yeager et al., 2007). A meta-analysis performed by Cheng et al. (2008) tested 10 SNPs on 8q24 and the results suggested that these variations would increase the risk of prostate cancer up to 50%, especially the risk of advanced disease. In addition, SNPs in that region have been associated with colorectal, breast, ovarian, and bladder cancers (Haiman et al., 2007a, Zanke et al., 2007, Ghoussaini et al., 2008, Kiemeney et al., 2008). There are no known genes in the 8q24 region. Interestingly, the *MYC* oncogene is located approximately 200 kb downstream, but recent studies

have shown that 8q24 SNPs do not affect *MYC* expression (Gudmundsson et al., 2007b, Kiemeney et al., 2008).

In addition to recent GWAS, positive signals for region 8q21 have been detected in several linkage studies. The first report was published in 2000 by Gibbs et al. (2000). In that study, 94 families were included and analyzed as a single set and then stratified by mean age at diagnosis. When the screening was performed under a recessive model, a positive linkage signal was observed at marker *D8S2324* with a LOD score of 2.17. The same region was detected in a linkage study of aggressive prostate cancer families from Utah (Christensen et al., 2007). In the analysis, 8q at marker *D8S1132* showed nominal linkage evidence with HLOD score of 1.67 in a subset of early onset patients. Very recently, Stanford et al. (2009) completed a genome-wide single nucleotide polymorphism (SNP) linkage scan in 2072 individuals from 307 HPC families with 5867 SNPs. The analysis included clinical features of prostate cancer to produce a more refined disease phenotype. Suggestive evidence for linkage was found at 8q22 with a KCLOD of 1.88 between SNPs rs1449233 and rs1483457 (Stanford et al., 2009).

DNA damage may increase cancer risk, and DNA double-strand breaks (DSBs) are the most serious risk to genomic integrity. If DSBs are unrepaired, it might lead to genomic instability and cancer (Kuschel et al., 2002). The *NBN* gene (also known as Nijmegen breakage syndrome 1, *NBS1*), located on chromosome 8q21, is a key DNA repair protein in the homologous recombination repair pathway (Carney et al., 1998). Individuals having biallelic mutations in *NBN* gene suffer from Nijmegen breakage syndrome (NBS), which is a rare autosomal recessive condition of chromosomal instability that is clinically characterized by microcephaly, immunodeficiency, radiation sensitivity, and a strong predisposition to malignancies (Demuth and Digweed, 2007). In fact, *NBN* has been suggested to be a susceptibility gene for many cancers (Plisiecka-Halasa et al., 2002, Debniak et al., 2003, Gorski et al., 2003, Resnick et al., 2003, Soucek et al., 2003, Steffen et al., 2004, Buslov et al., 2005) and nine mutations localized in the coding sequence of the *NBN* gene have been identified in cancer patients. Cybulski et al. (2004a) reported a study from Poland stating that *NBN* plays a role in the etiology of prostate cancer. The frequency of the 657del5 founder mutation was compared between 56 HPC cases, 305 sporadic cases, and 1500 controls and the frequencies were 9%, 2.2%, and 0.6%, respectively. This founder mutation was significantly associated with prostate cancer risk among familial cases (OR=16, P<0.0001) and sporadic cases (OR=3.9, P=0.01). In addition, LOH of wildtype *NBN* was detected in seven of eight 657del5 carriers but only in one of nine non-carriers.

3.3 Role of other cancer predisposing genes in prostate cancer

A third approach to identifying prostate cancer susceptibility genes is to investigate the role of genes in which a predisposing effect in other cancers or cancer

syndromes is well-established. Most of the studied genes have previously been associated with breast cancer, since a link between prostate and breast cancer etiology has been suspected for many years (Thiessen, 1974, McCahy et al., 1996, Ekman et al., 1997). Many of the genes are involved in DNA repair indicating that defects in the ability to repair DNA damage and maintain genomic integrity predispose to many types of malignant transformation.

3.3.1 BRCA1 and BRCA2

Multiple studies have demonstrated a clear association between *BRCA1* and *BRCA2* and increased prostate cancer risk in mutation carriers (Ford et al., 1994, Easton et al., 1997, Sigurdsson et al., 1997, Struewing et al., 1997, Friedenson, 2005). Probably the most striking evidence comes from a study where 263 men with prostate cancer diagnosed before the age of 55 were analyzed for *BRCA1* and *BRCA2* mutations (Edwards et al., 2003). Truncating mutations in *BRCA2* were found in 2.3% of the patients and the relative risk for prostate cancer development was 23-fold. In Finland, epidemiological analysis of breast cancer families identified an excess risk of prostate cancer in *BRCA2* mutation-positive families (Eerola et al., 2001). To further assess the contribution of *BRCA2* germline mutations to prostate cancer susceptibility in Finland, Ikonen et al. (2003) screened seven Finnish *BRCA2* founder mutations from 444 prostate cancer patients and from 104 patients with HPC. Also, the role of *BRCA1* in causation of prostate cancer was studied by screening five unique and six founder *BRCA1* mutations from 46 Finnish patients with HPC. Surprisingly, no mutations were found, which implicates a limited role for *BRCA1* and *BRCA2* mutations in predisposition to prostate cancer in Finland.

3.3.2 CHEK2

CHK2 checkpoint homolog (S. pombe) (*CHEK2*), located on 22q12.1 functions as an upstream regulator of *p53* and in the ATM-dependent DNA signaling pathways (Zhou and Bartek, 2004). Initially, it was identified in Li-Fraumeni syndrome, in which identified mutations, especially c.1100delC, were shown to result in truncated proteins (Bell et al., 1999). Li-Fraumeni syndrome is a rare disorder that increases the risk of developing multiple types of cancer, particularly in children and young adults (Varley, 2003). The cancers that are most often associated with Li-Fraumeni syndrome include breast cancer, osteosarcoma, and soft tissue sarcomas. Other cancers commonly seen in this syndrome include brain tumors, leukemias, and adrenocortical carcinoma. The first study providing evidence of *CHEK2* mutations in prostate cancer was performed by Dong et al. (2003). In that study, a total of 28 (4.8%) germline *CHEK2* mutations were found among 578 patients. In addition,

screening for *CHEK2* mutations in 149 families with familial prostate cancer revealed 11 mutations in nine families. Eighteen of the found mutations were unique and 16 of them were identified in both sporadic and familial cases, but were not detected among 423 unaffected men. Previously, the 1100delC mutation has been associated with an elevated risk of breast cancer, particularly in families with two affected relatives (Vahteristo et al., 2002). Seppälä et al. (2003b) reported that the frequency of 1100delC, was significantly higher among 120 patients with HPC (OR=8.24, 95% CI=1.49–45.54, P=0.02) compared to population controls. In addition, the Ile157Thr variant had a significantly higher frequency among HPC patients (OR=2.12, 95% CI=1.06–4.27, P=0.04) than the frequency seen in the population controls. The results suggested that *CHEK2* variants may be considered as low-penetrance prostate cancer predisposition alleles in the Finnish population. Parallel results were obtained in a study from Poland where three *CHEK2* (IVS2+1G, 1100delC, and Ile157Thr) variants associated with prostate cancer risk (Cybulski et al., 2004b).

3.3.3 PALB2

The Partner and localizer of BRCA2 (*PALB2*) gene encodes a recently identified protein that interacts with BRCA2. It colocalizes with BRCA2 and the interaction is essential for the double-stranded break repair functions of BRCA2 (Xia et al., 2006). In addition, multiple *BRCA2* missense mutations without biological consequence identified in breast cancer patients seem to disrupt the *PALB2* binding site and disable the BRCA2 double-stranded break repair function. Indeed, it has been shown that *PALB2* is a breast cancer susceptibility gene (Rahman et al., 2007, Tischkowitz et al., 2007). Erkko et al. (2007) screened the entire *PALB2* gene in 113 *BRCA1*/*BRCA2* mutation-negative breast cancer families from Northern Finland. Six exonic variants were found from which four were also detected in the control group. One variation, 1592delT, was found to be significantly associated with the disease (OR=11.3, 95% CI=1.8-57.8, P=0.005). This variation resulted in a truncated protein product and had a significantly lower BRCA2 binding affinity. Subsequently, 164 HPC cases and 475 unselected prostate cancer cases were screened for this particular mutation and it was found in one HPC family. Segregation analysis was performed on that mutation positive family and the results indicated high penetrance of the mutation in the two generations that were studied. To further assess the role of *PALB2* variants in Finnish prostate cancer families, Pakkanen et al. (unpublished data) screened 178 HPC cases and 285 unselected prostate cancer cases with complete clinical data for variants in the coding region and splice sites of *PALB2*. A total of six variants were identified in *PALB2*, but no novel variants among Finnish prostate cancer cases were found. None of the detected *PALB2* variants was associated with prostate cancer at a population level. However, it cannot be excluded that some of these variants contribute to cancer

susceptibility at an individual level. The role of *PALB2* variants was also evaluated in a study from Canada (Tischkowitz et al., 2008). *PALB2* was sequenced in probands from 95 prostate cancer families, 77 of which had two or more cases of early onset prostate cancer and the remaining 18 had one case of early onset prostate cancer and five or more total cases of prostate cancer. Two previously unreported variants, Lys18Arg and Val925Leu, were identified, but are unlikely to be pathogenic. No truncating mutations were identified. These results showed that damaging *PALB2* mutations are unlikely to contribute to hereditary prostate cancer risk.

3.3.4 HFE

Hereditary hemochromatosis type 1 (HH) is a hereditary disease characterized by excessive absorption of dietary iron resulting in an increase in body iron stores, which is harmful due absence of a means to excrete excess iron in humans. This surplus iron accumulates in tissues and organs disrupting their normal function. The most vulnerable organs include the liver, adrenal glands, the heart and the pancreas with patients suffering from cirrhosis, adrenal insufficiency, heart failure or diabetes (Fix and Kowdley, 2008). The hereditary form of the disease is most common in Caucasians, in particular those of Irish descent (Byrnes et al., 2001). The *HFE* gene is located in chromosome 6p21.3, and the two main missense alterations in the gene in HH are His63Asp and Cys282Tyr (Feder et al., 1996).

Patients with HH are at high risk for developing hepatocellular carcinoma (Cauza et al., 2003), and carriers of *HFE* variants have been reported to bear an increased risk for cancer, including prostate cancer (Geier et al., 2002), leukemia (Dorak et al., 2005), malignant glioma (Martinez di Montemuros et al., 2001), and colorectal and gastric cancer (Nelson et al., 1995, Geier et al., 2002, Dorak et al., 2005). An increased frequency of breast cancer has been detected in carriers of the Cys282Tyr variant (Kallianpur et al., 2004) and the same is true for His63Asp carriers (Barton et al., 2004). Two independent linkage studies on prostate cancer have suggested the chromosomal region of *HFE* to harbor a prostate cancer susceptibility gene. In an ICPCG study, from 1233 prostate cancer families with genome wide linkage data available, those that had at least three members with clinically aggressive prostate cancer were selected, resulting in 166 pedigrees. A linkage signal reaching a suggestive level of significance was found on chromosome 6p22.3 (LOD = 3.0) (Stanford et al., 2006). In genome-wide linkage analysis for aggressive prostate cancer in Utah high-risk families the late-onset subset showed suggestive linkage on chromosome 6p (HLOD=2.37) (Christensen et al., 2007).

Male breast cancer (MBC) is a very rare disease affecting only a few men every year (Finnish Cancer Registry, 2008). MBC is similar to breast cancer in females in its etiology, family history, prognosis, and treatment. In approximately 30% of MBC cases, the family history is positive for the disease. Germ-line mutations in

BRCA2 gene are known to predispose to MBC (Fentiman et al., 2006). Male carriers of *BRCA2* mutations have an 80–100 times higher risk for the development of breast cancer than the general male population thus making *BRCA2* the strongest presently known MBC linked gene (Thompson, Easton and Breast Cancer Linkage Consortium, 2001). However, only a proportion of MBC cases (4–40% depending on the population) can be explained by mutations in *BRCA2* (Venkitaraman, 2002). Therefore, it is most likely that also other susceptibility genes for MBC are still to be found. Interestingly, a possible link between hemochromatosis gene *HFE* and MBC has been suggested (Thomas, 1999).

AIMS OF THE STUDY

The general aim of this study was to increase the knowledge of genetic predisposition to prostate cancer by studying susceptibility loci previously associated with prostate cancer risk and variation in genes located on those chromosomal regions. The specific aims were:

1. to study the role of genetic variation in *HFE* gene in prostate and male breast cancer in Finland (I).

2. to assess the contribution of *NBS1* variants in prostate cancer predisposition (II).

3. to identify and investigate the *MLH1* alterations in prostate cancer in Finland (III).

4. to further characterize the *HPCX* locus by searching for truncating mutations in lymphoblastoid cell lines with the NMD array method and by studying miRNA expression variation with miRNA microarrays in Finnish *HPCX*-linked prostate cancer families (IV).

MATERIALS AND METHODS

1. Study subjects

1.1 Families with prostate cancer (II-IV)

Finnish prostate cancer families have been collected by the study group Genetic Predisposition to cancer in the Laboratory of Cancer Genetics at the University of Tampere and Tampere University Hospital. Identification of the families was accomplished through nation-wide registry based searches, referrals from urologists in Finland and advertisements in newspapers, television and radio (Schleutker et al., 2000). Diagnoses and the family histories were obtained by questionnaire and they were confirmed from the Finnish Cancer Registry, individual patient records, and parish records. Families used in different studies are presented in Table 4.

1.2 Unselected prostate cancer patients (I-IV)

Since the year 1996, samples and written informed consents have been collected from consecutive prostate cancer patients diagnosed in the Tampere University Hospital, which is a regional referral center in the Pirkanmaa area for all patients with prostate cancer. This results in unselected, population-based collection of the patients. Clinical data were collected from hospital records. The number of patients used in Studies I-IV is presented in Table 4.

1.3 Patients with male breast cancer (I)

All 237 male breast cancer (MBC) patients diagnosed in the Finland between 1967 and 1996 were identified from the Finnish Cancer Registry. A total of 116 MBC cases (49%) were available for the study. Seventy-nine of those (33%) were alive and were approached through the attending physicians. Blood samples were collected and a written informed consent was obtained from 37 patients. Paraffin-embedded tissue samples were available from 79 patients. The clinical information on the MBC patients included histological subtype of the breast cancer, age at diagnosis, and the attending hospital. All patients had been screened for previously identified Finnish breast cancer 2 gene (*BRCA2*) mutations (Syrjäkoski et al., 2004).

Table 4. *Summary of samples used in studies I-IV.*

Sample group	Study I	Study II	Study III	Study IV
Affected familial cases	-	164/121/20[a]	121/18[d]	6/14/163[e]
Unaffected familial cases	-	-	-	6/14[f]
Unselected prostate cancer patients	843	380/613/200[b]	200	757
BPH patients	-	-	202	375
MBC patients	116	-	-	-
Prostate cancer and colon cancer patients	-	-	11	-
PSA controls	-	-	-	746
Population controls, male	480	440/200[c]	200	757
Population controls, female	-	-	-	764

[a]657del5 genotyping/D95N,E185Q genotyping/entire gene sequencing
[b]657del5 genotyping/D95N genotyping/E185Q genotyping
[c]D95N genotyping/E185Q genotyping
[d]SSCP screening/entire gene sequencing
[e]NMD array/miRNA array/genotyping
[f] NMD array/miRNA array

1.4 Patients with prostate and colon cancer (III)

A total of 355 prostate cancer patients were found from the discharge registry of the Tampere University Hospital having an additional solid primary tumor (excluding skin malignancies except melanoma) from 1st January 1970 until 31st December 1999. Fifteen of them had both prostate cancer and colon cancer, and paraffin embedded samples were available for analyses from 11 of them.

1.5 Patients with benign prostate hyperplasia (III-IV)

Patients diagnosed with benign prostate hyperplasia (BPH) were also collected from the Tampere University Hospital. 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 prostate specific antigen (PSA) was elevated the patients underwent biopsies to exclude prostate cancer. The indication for biopsy was total PSA ≥4 ng/ml or total PSA of 3.0-3.9 ng/ml with the proportion of free PSA <16%. The number of BPH patients used in Studies I-IV is presented in Table 4.

1.6 PSA controls (IV)

In Study IV, a set of men who had a PSA level less than 1.0 ng/ml (named as PSA controls), were used as a control group. These PSA controls were obtained from the Finnish population-based prostate cancer screening trial (third round), which aims to

evaluate the effect of screening with PSA testing on death rates from prostate cancer (Määttänen et al., 1999, Mäkinen et al., 2004, Schröder et al., 2009).

1.7 Population controls (I-IV)

The population controls were male or female blood donors obtained from the Finnish Red Cross in the cities of Tampere, Turku and Kuopio. The blood donors in Finland are 18 to 65 year-old healthy and voluntary individuals. The number of controls used in different studies is presented in Table 4.

1.8 Other populations (II)

In study II, in addition to Finnish sample population, four separate study populations were used from Mayo Clinic (USA), University of Michigan (USA), Universitätsklinikum Ulm (Germany) and Johns Hopkins University (USA). These centers, including University of Tampere, are participating in the International Consortium for Prostate Cancer Genetics (ICPCG). Description and amounts of the samples are presented in Table 5.

Table 5. *Other study populations used in study II*.

	Affected familial	Unaffected familial	Sporadic	Controls
Johns Hopkins	194	-	-	-
Michigan	734	182	8	-
Ulm	299	111	338	208
Mayo Clinic	428	-	492	489

1.9 Ethical aspects (I-IV)

Permission to collect families throughout Finland and use the data from Finnish Cancer Registry was granted by the Ministry of Social Affairs and Health on June 20th, 1995 (license 59/08/95). Permission to collect and use blood samples and clinical data from prostate cancer patients treated in the Pirkanmaa Hospital District was granted by the Institutional Review Board of Tampere University Hospital (licenses 95062 and 99228) on March 8th, 1995 (latest extension December 30th, 2003). Permission to collect and use tumor samples for medical research was granted by the National Authority for Medicolegal Affairs on February 1st, 2006 (license 5569/32/300/05). Permission to collect and use blood samples and clinical data from prostate cancer patients treated in Hatanpää City Hospital was granted on July 1st, 1996 by the Institutional Review Board of the City of Tampere (license

8595/403/2005). All individuals participating in this study provided a written informed consent for use of their samples and medical records.

2. Methods

2.1 DNA extraction (I-IV)

Genomic DNA was extracted from blood lymphocytes using a commercially available kit (Puregene, Gentra systems, Inc., Minneapolis, MN & Wizard®, Promega Corporation, Madison, WI). DNA yields were quantified using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

2.2 RNA and miRNA extraction (IV)

Total RNA and miRNAs were extracted from patients' lymphoblastoid cell lines using the TRIzol® reagent (Invitrogen, Carlsbad, CA). RNA yields were quantified by agarose gel electrophoresis and by using a ND-1000 spectrophotometer (Nanodrop Technologies).

2.3 SSCP analysis (III)

Single-strand conformational polymorphism (SSCP) analysis (Orita et al., 1989) was used for screening the entire coding sequence of the *MLH1* gene using primers designed to include all intron-exon boundaries (Table 6.). 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. 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 0.5x mutation-detection-enhancement gel (FMC BioProducts, Rockland, ME) with 1% glycerol in 0.5x Tris-borate EDTA. After electrophoresis, gels were dried and exposed to Kodak BioMax MR films for 6 h. Samples that created aberrantly moving bands as well as two to three normally moving bands per run were analyzed by sequencing, using the original PCR primers.

2.4 Minisequencing (I-III)

Minisequencing was used as a genotyping method to determine the frequencies of *HFE* His63Asp and Cys282Tyr, *NBS1* Asp95Asn, and *MLH1* Ile219Val and Val647Met variants. DNA amplification was performed with 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. Minisequencing was performed as described by Syvänen (1998). Primer sequences are presented in Table 7.

2.5 Sequencing (II-IV)

Direct sequencing was used for mutation screening and as a genotyping method to determine the frequencies of *NBS1* Glu185Gln and 657del5, *MLH1* Pro434Leu, and *MAGEC1* Met1Thr variants in studies II-IV. 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 according to the instructions of the manufacturer using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with the ABI 3100 and 3130xl sequencers (Applied Biosystems, Foster City, CA). Sequence analysis was performed with different versions of Sequencher software (Gene Codes Corporation, Ann Arbor, MI). Primer sequences used in sequencing are presented in Tables 6 and 7.

2.6 Immunohistochemistry (III)

Immunohistochemistry for MLH1, MSH2 and MSH6 was performed on paraffin sections in a LabVision Autostainer instrument (Labvision Corporation, Fremont, CA). The sections were subjected to four cycles (7 min + 3 x 5 min) of heating in a microwave oven at 850 W in Tris-EDTA buffer, pH 9.0 for epitope retrieval. The primary antibodies used were: clone G168-15 (BD Biosciences Pharmingen, San Diego, CA) at 1:25 for MLH1, clone FE11 (Oncogene Sciences, Uniondale, NY) at 1:150 for MSH2, and clone 44 (BD Transduction Laboratories, Lexington, KY) at 1:200 for MSH6. Visualisation of the primary antibody was done with the two-step Envision™ polymer kit (DakoCytomation Denmark A/S, Glostrup, Denmark) using diaminobenzidine as chromogen. The recommendations of the International Collaborative Group of Hereditary NonPolyposis Colorectal Cancer (Müller et al., 2001) (HNPCC) were followed in the microscopic evaluation.

Table 6. *Primer sequences used in SSCP analyses and direct sequencing.*

Gene	Exon	Forward (5´>3´)	Reverse (5´>3´)
NBN[a]	1	GTCAGCAGCCCCGGTTAC	CGCCCATGCTAACTTCCT
	2	TGAGGCTTTACTGAAAACACAA	ACTGGTACCACTGCCACAAT
	3	AATTGTTGTCTGCCGTGTTG	GGCCCACTCAAACTCTCATC
	4	TTGCCATCTCTGCAACTCTG	CTTCTCGGTGGAAGGACAAC
	5	GCAGTGACCAAAGACCGACT	CAACAAAGAAATTTGGGGAAC
	6	CGCGATTAGATGCTTTTTGTC	AGAAACCCCGTAATCACAGC
	7	CCGCAGAGACCTGTTGAACT	TTTACATTGTTAGGTGAAAAGC
	8	GCCCCAGCGAGTAAGCTATT	TGGTGAATATGGTCACCCCTA
	9	TTGGAGAAAACCATGTGCAG	AGGAGCTGGGACAGAGATCA
	10	CGATCTTTGTTTCTCTATTAAAGTTGC	CTGCAGCAGCAGAAGCATAC
	11a	TGTGAACTAAATCGGAGGGAGTG	ATCCATCCTTGGCCTTTTTC
	11b	GAAATGGATGATGTGGCCAT	TTACCATTTACCTTATCAACC
	12	TGGATTTAGATCGCTTCCAA	ATGAGATGACAGTCCCCGTA
	13	AGATTCCCAAATGACAAGTGA	ATCTTTGTTTAGCATCACTGG
	14	AGAAGGGCAAAAACAGATGG	ATTAATGCTCTGTAACTCAGGA
	15	GATGTGGTGACCTCCAGGAT	ACCGTCTTTTTGCCTGAATG
	16	TGTCATTCCCATCCTATTTGC	CAATGGTGGAAGGGTGACTT
MLH1[a]	1	AATCAATAGCTGCCGCTGAA	GGGGAGAGCGGTAAAGAAAC
	2	AGAAATGATGGTTGCTCTGC	TGCAAAAGCCTAGTTTCCAG
	3	CTGGGTGACAGAGCAAGACT	TTTGCTCAGATTTGCATACATT
	4	TGGAAGCAGCAGTTCAGATA	GTTGAGACAGGATTACTCTG
	5	TCTCTTTTCCCCTTGGGATT	GCTCTAACCCATGCCTTCTG
	6	GCCCCAGTCAGTGCTTAGAA	TTGGTGGCTAAACCTTGACC
	7/8	AAAAGGGGGCTCTGACATCT	TCCAAAATAATGTGATGGAATGA
	9	TGGGTGAACAGACAAATGGA	AACCAAACTTTGCCATGAGG
	10	CTTTCTTCCTGGGGATGTGA	GCCAGTGGTGTATGGGATTC
	11	TCGATCCTGAGGTTTTGACC	GCAAAAATCTGGGCTCTCAC
	12	ACAGACTTTGCTACCAGGAC	GCCAAAGTTAGAAGGCAGTTTT
	13	AGTTGCTTGCTCCTCCAAAA	TGCCCAGCAAAACTGTAGTG
	14	TGTGTTCGTTTTCACCAGGA	ATGGGAAATGGTCGAACTTG
	15	CACAGCCAGGCAGAACTATT	CCGGCCGAGTATCAGTAGAT
	16a	CCTGCCATTCTGATAGTGGA	AACAGAAGTATAAGAATGGCT
	16b	GGATGCTCCGTTAAAGCTTG	CTCCCAAAGTGCTGGGATTA
	17	ATTTTTGGGGCTCTCCATCT	TTCCAGATCAAAGGGTGGTC
	18	CCTGCTCTCATCCCCACTAA	GATGGGCAAGTTTCATCTCC
	19	GCACATCCCATCAGCCAGGA	TAAGTCTTAAGTGCTACCAACAC
MLH1[b]	1	ACAGCTGAAGGAAGAACGTG	AGTCGTAGCCCTTAAGTGAG
	2	CATTAGAGTAGTTGCAGACTG	AAGGTCCTGACTCTTCCATG
	3	AGTAACATGATTATTTACTCATC	AATGACAGACAATGTCATCAC
	4	CAGCAGTTCAGATAACCTTTC	GATTACTCTGAGACCTAGGC

	5	ATTAGTATCTATCTCTCTACTG	AAAGCTTCAACAATTTACTCTC
	6	TCACTATCTTAAGACCTCGC	TTGATGACAAATCTCAGAGAC
	7	GGCTCTGACATCTAGTGTG	CCTTATCTCCACCAGCAAAC
	8	TTCAGTCTCAGCCATGAGAC	GTGATGGAATGATAAACCAAG
	9	TTTTGTAATGTTTGAGTTTTGAG	GTGGATTTCCCATGTGGTTC
	10	GACAGTTTTGAACTGGTTGC	ACATCTGTTCCTTGTGAGTC
	11	CACATACACCATATGTGGGC	AAAATCTGGGCTCTCACGTC
	12a	TCTTATTCTGAGTCTCTCCAC	ATCTGTCTTATCCTCTGTGAC
	12b	ATGCATTTCTGCAGCCTCTG	AATAAAGGAGGTAGGCTGTAC
	13	TTGCTCCACCAAAATGCAACC	CAGTTGAGGCCCTATGCATC
	14	GTGCTTTGGTCAATGAAGTGG	CCATTGTTGTAGTAGCTCTGC
	15	TATCTCAAGCATGAATTCAGC	ATCAGTTGAAATTTCAGAAGTG
	16	GGATGCTCCGTTAAAGCTTG	AACAGAAGTATAAGAATGGCTG
	17	GGAAAGCACTGGAGAAATGG	TCCAGCACACATGCATGTAC
	18	TGTGATCTCCGTTTAGAATGAG	TGTATGAGGTCCTGTCCTAG
	19	GTATGTTGGGATGCAAACAG	ACTTTGTATCGGAATACAGAG
MAGEC1[a]	1	ACCCACTGTCATTCCTGGTG	GCAGCAGGTAAACGTGTGAAC
	2	TTGGTAGATGCAGAGGATCC	CTGTGTTCTCTCCAGCCTCA
MAGEA1[a]	5´UTR	GGTTCCCGCCAGGAAACATC	TTGATGCCTGGCAGAGCCTG
	5´UTR/1a	GGCCCGTGGATTCCTCTTCC	TGTTGGGCCTCAAGGGCTTC
	1b	GGCCTGTGGGTCTTCATTGC	CATAGCGTGCGGGATCACTG
	1c	CCTTTCCCACTACCATCAAC	CATAGCGTGCGGGATCACTG
	1d	TTGACGTGAAGGAAGCAGACCC	TGAGAACACTGACCGCTCTCTTCAG
	1e	TCCCGCACGCTATGAGTTCC	CCACTGCTGTTATTATCCCAATTCACA
	1f	TGAAGAGAGCGGTCAGTGTTCTCA	GCCACCTCGATTACGTGACTGC
MAGEA11[a]	5´UTR	ACACGGGCAGAATCGGGTTC	CTCAGCCTGATAATTTGG
	5´UTR/1	GGGCTTGGTATCATGAGAAAGACCT	TCTCTCCTGCTGAACTGTGGATGA
	2	TGGGAAACCTTCAGGGAGATGA	TGCTGACCTAAGTGCAGCCCTC
	3	TCACCCTTAATCTACAAATGGCCC	GCATCCATGGCAGTGGGAGA
	4a	CCCAGAGGATCACTGGAGGAGA	TGCGGAGCAATAAATGAACCAA
	4b	TCTCCCACTGCCATGGATGC	GGCACCTGCCGGTACACCA
	4c	AGCATGCCCAAGTCTGGCCT	TCTCCAAGTCACCCGATGGAA
	4d	AGGGCCACACCCAGCAGTTT	CCAGGACATCTCAGAGAATCTGCAA
MAGEC3[a]	1	TCTTTGCCTTCTGGGCTATCAGTG	CATGAACATCAAGGCTGGGCA
	2	TTTGAAAGCCCTTTCTGTATTCTGGA	CACAGGGAGGGCAGGGCTAA
	3	CAGAGTCCTGCCTTGGGCCT	TCGTCCATATTTCCCTGCCCA
	4	CCAGAATCCTCCTGAGAGTCCTCC	TGACAAGCTCCCTGGGCTCC
	5	CTGGAAGGAGTAAAACCTTG	ACCTTTTCCTACCCACCTCC
	6/7	GGGTAGGGAGGTGGGCACAA	GCAGGGTGGGAATCTCTGGG
	8	GAGGACGGAGAGGTCCCACG	GGATGTGAGAAAGCACCTCGGG
	9	CTGCCATTCCTGGTGCCTCA	AGAGGTGGCATCCCAGCAGC
	10	CCCGAGTGTGACAGAGGACTTGG	TCAGTCAGGGCAATGCCAAA
	11	GGCATGCCTTGCCAGAAAGTG	GCTGGCCTCTGAATGGGCTC

	12	CCCGAGGAGGTCATCTGGGA	AGCAATTGGAAGGAGAAGCATTTG
MAGED1[a]	5´UTR	CAGGACGAAGCTTTTGTTGG	CAGAATCCAGGGTGAAGGGA
	1	CCGTTCCTCCTCACATTCAC	CCCTTCGATAAACGATGGGAA
	2	AGCCATCAGTCCCTGCTCA	CCAGGCGGGTACTAAACATG
	3a	TGCCACCCGGGCTCCCTATT	CTGCCTGGGAAAAATCATAGGC
	3b	AATGCCAAGGATGTGCCCAA	CTATAGCCTAGCTGAATACAG
	4a	TGGGTCAGGCTGTCACTACA	AGATCACTGGGTTCTGCCAG
	4b	ACGTCAGAGCCCTCCAGC	TGAATTACAGATGAGAGGATG
	5	GGAAAGAGTAAGAGCTGTAAC	GTCATACAAGCAGAAGAATGC
	6	AGGACCTTGACTGTGCTACC	ATGACCTGCTATGTGACCCT
	7	AGTTTAGGGCAAGTCTCAGG	ATGACCTGCTATGTGACCCT
	8/9	TAGATGGGCAAGCTGGTTGG	CCAAACCAAACCCTCCAGAC
	10	CATCTCTGACCTCTGTTCTG	GGATTACAGGCATGAGCCAT
	11	ATGGTGTTCAGGAATGAGCC	ACATGTTAAGTTTACCTAACAGC
	12	ACATTGTCCTTATCCATTCATC	CACATAAATACAACCCCATGG
	3´UTR	GCATGAGCTAGAAGTATTAGG	TACCCTTCAAACTACAGCTG
RBMX[a]	1	TGTCCAATAAACTTGAGGAAC	GCTCAGAACTTCTTAGAGGA
	2	TCCTCTAAGAAGTTCTGAGC	TGTCCTAGACCAGACTTGGA
	3	GAGCAACACTTACCCATAAC	GGTAGATGAACAAGTGTTGTC
	4	AAGCCACTGCACTCCAGCCT	CAAAGTGCTAGGATTACGGG
	5/6	TCATAGCCTGAATGTAAGTTC	GAAACCTTTAAGTCCCAGAG
	7	GTCATTCCAGTTCACGTGAT	GCAAGCACCACACAGCCTAA
	8	CCCAGTTGAGAAAGTAAACT	AGTCCTTGGGTAGTTATGAT
CSAG2[a]	1	CTAGATGTTGCTGTGAAGGTA	TCATAGAGAAATGGTGCAGG
	2	CCTGCACCATTTCTCTATGA	TGATTGAGTTCCTCGTTCGG
RAP2C[a]	1	ACTCTCCTCCCCAGTTCGCA	GTAACCTCAAAAATGCGTCATG
	2	GTATACTCACTGAGGTATGAC	CAGTGCCATATGTATTAACACT
SOX3[a]	1a	ATCACGGGTCCTCCGGGTTG	CTGCGTTCGCACTACTCTTGC
	1b	CGGCAATGTACAGCCTTCTG	TTTCTTGAGCAGCGTCTTGG
	1c	AGAAGCGACCATTCATCGAC	CGACGTTCATGTAGCTCTGA
	1d	CCGATGCACCGCTACGACAT	GGTGGCAGGTACATGCTGAT
	1e	CCATCGCATCGCACTCTCAG	GCAACAGTCCCAGGCAAGCA
MBNL3[a]	1	TAATCCTTTAATAGTCCACAG	TACTCATTTCAAAGCACATCA
	2	AGTTTTGGAGCTTAGATTGTC	AGTGCACAGCTCTGTAGTGT
	3	CAATAGTAAATTTTGATGTGGC	GGGTTCTCTTATTTAAAACAAC
	4	CAGTCAGCTTCCAGTCATCA	CCTTGACTGTTCATATTAAGG
	5	CATACCCTTACGGAGGATTC	GTCGACAGTCCTCCTATACA
	6	TGCATGTGCTGAGTAATTGAT	ATACTCAGAAAATTTCCAGCC
	7	CCTACCACACCTCATTCCAT	GAAACACATAAGAATCTCACCA
	8a	GCACTGTTAACAGACAGAAC	GCTTAATACATTGACTGCAAC
	8b	GGAACCATGCTTATAGACTAA	ACATCACAAGCAATGTCTATG
ZNF75[a]	1	TTGGTACCAACCTGATGCAC	CTCCTCCATTCTGATATATCC
	2	CTCTAATGTCATGCATTCCC	ATCATAGCTGCTAGCATGGC

	3	TTTACTGAAGGCATCCTGATG	TGCTGGACACTATGAGTCCT
	4	ATCTTCAGCACCTGGCCTAC	TGGAACAGTGGGACTCTCTC
	5a	TCCAGACTGGACCAGAAATC	CCAGTGTGAATTCTGTGGTG
	5b	CCAAAACTTATGGATCTTCATG	ACATGTACCCTTCCCAAATG
U66046[a]	1a	GAAAGATAAGGGCTGTGTTC	TTTACGTGCGATGCAATAGG
	1b	AGCAGCCACAGGAACCTTGA	TGGTGCCTGACTTGAGAATA
	1c	GGTGGCATTCTCATCCATGA	CCTGGTCTCAGAGGCTGAAC
	1d	GGGAGCTCTGAAGGGTTAGG	TTGCTCCTGTATAAATGGGAAG
	1e	TAACCATTACCCACATTCCT	ACTGGAGGGAAATATACCAA
SSR4[a]	1	GCTGCCAGAGACGTCACAAT	TGGGTCAGAGGCTCGCAAGA
	2	CTGATCCGTGCTATGAGGCA	CTCCCTCTATCGACACAGGT
	3	ACCTGCAGGCCGTGTGAGCA	TGCCGACACTGCAGCACTCC
	4	GGAGTGCTGCAGTGTCGGCA	TGCCAGCACCTCCCAACCCA
	5	CCGGTGTTCCTACCTGTCTTTC	GACATGCTGGCCAATCACGG
	6	AAGGTGACCAGGGCTGGCTG	TCTGCTGGCCAGGCAGACAAG
VBP1[a]	1	CCAATGAATGTGCATGGAGATG	TCAGCTCCTTCCAACAGTCG
	2	ATCTGAGTGGCATGAATTCT	GACTGAACACTTGACATCTG
	3	GCAAAGTTAGTAAGACCGTGT	AGAAGGCAGACTGGATTCCAC
	4	GTTCAACTGTGCAATCTCTC	ACATATCTCGAGCAATACTAC
	5	AAGGCAGCAAGCTGGATTTG	GGTTTGCTGCACCTATCAAC
	6	TTGTGTGAGTGGAGTGAATAC	GTCAACCTGTAAGGATAAAGG
LDOC1[a]	1a	ACCGTCCGTCCGAATGGCCT	GCATGTAAGACGCCGTCTGC
	1b	CAGGTACGTCCGCCGAGCTG	GCAGTGGCTCCTGGCGGGGT
TKTL1[a]	1	AGGGAGCTGCACCGACATCA	TTCACCACACGGCCTCATGG
	2	CCGCTTCTATGAGGAGACCATG	TGTTCCCACACGGTGGCTGT
	3	AGGAGCAGCCTGCACTCAGT	TCACGGAGATAGGTGGCTGC
	4	TTGGTTGGCCCAGATGATCC	GGAGCAGGCTGGGTTACAAG
	5	CTATCAGAGGCGCTGCGAAG	AATGAGTCATCTGCCTAGGCC
	6	GGTGACCTCATAGGCACTCAC	GGTTCGTCCATGGAGACCAG
	7	GATAGATGATAATTTGTCATTCTAC	GACTCCACCTGGCTACTTGC
	8	AGTTCAGCAGGTGCAGAATG	CACATGCTAATCTTTCTCTGTG
	9	TCAGAGCTAGAAGTGGGTGG	GTTCAGTATCCTGTACTGTTGG
	10	GTCCACTGGTTGTAGATGCT	CTGAAGATAGCTGGTGATAGA
	11	ATAGCGGCATAGCAAAGTGC	AGAGACTCAGAATAACATGCAG
	12	GAGCATTGAAGTTCAAGCTG	AAAGCCATGCTGGGTGAGAG
	13	CCTGGGAAGCTGCAGATTCA	AGAAACAAAATGTGACAGTAGAG
CD40LG[a]	1	GAAGCACATTTTCCAGGAAG	GGTTTCTACCATCATCCATC
	2	TGATGCCGTGGAAATGAATG	TGTCAGTTTCCCGATCTAGC
	3	GACAGGATCTGAGTCTATATGA	GATGCAACAACACTGGGTTG
	4	CAGTTGTAGAACTGGACCAG	AGGGAATAGGAGAAGTGTAG
	5a	CATGGCTCTGTCTGACTCTG	TGCAGCTCTGAGTAAGATTC
	5b	GTCAAGCTCCATTTATAGCC	CTACATGCCTTGGAGTGTAT

[a]Primers for sequencing, [b]Primers for SSCP

Table 7. *Primer sequences used in genotyping by minisequencing or direct sequencing in Studies I-IV.*

Gene	Variant	Forward (5´>3´)	Reverse (5´>3´)	Detection (5´>3´)
HFE	Cys282Tyr	Biotin-TACTACCCCCAGAACATCAC	GGCTCTCATCAGTCACATAC	GGCCTGGGTGCTCCACCTGG
	His63Asp	Biotin-AGGTTCACACTCTCTGCACT	CTGGCTTGAAATTCTACTGG	CTCCACACGGCGACTCTCAT
NBN	Asp95Asn	TCCCTGTATTGACATTAAAAGA	Biotin-GCTGAAACAAAG CTGTCCA	CCCGAACTTTGAAGTCGGGG
	Glu185Gln	GCAGTGACCAAAGACCGACT	CAACAAAGAAATTTGGGGAAC	-
	657del5	CGCGATTAGATGCTT TTTGTC	AGAAACCCCGTAATCACAGC	-
MLH1	Ile219Val	Biotin-TCAGCAAGGAGAGACAGTAG	GTGATGGAATGATAAACCAAG	ACTAACAGCATTTCCAAAGA
	Pro434Leu	ACAGACTTTGCTACCAGGAC	GCCAAAGTTAGAAGGCAGTTTT	-
	Val647Met	GGAAAGCACTGGAGAAATGG	Biotin-TCCAGCACACATGCATGTAC	CCCTTCTGATTGACAACTAT
MAGEC1	Met1Thr	ACCCACTGTCATTCCTGGTG	GCAGCAGGTAAACGTGTGAAC	-

2.7 Microarrays (IV)

2.7.1 NMD oligonucleotide array protocol

Nonsense-mediated decay (NMD) microarray technology was used in Study IV to distinguish post-transcriptional shifts in mRNA stability and identify nonsense mutations. The cell lines were derived by Epstein-Barr virus transformation of peripheral mononuclear leucocytes from patients and their healthy brothers (controls). Lymphoblastoid cell lines were grown in RPMI-1640 medium (Lonza Group Ltd., Basel, Switzerland) supplemented with 10% fetal bovine serum (Lonza) and antibiotics. The emetine treatment protocol was used as described previously (Ionov et al., 2004). Briefly, cells were incubated for 10 h in culture medium containing 100 μg/ml of emetine (Sigma-Aldrich, Saint Louis, MO) and then for 4 h with actinomycin D (Sigma-Aldrich) at a concentration of 5 μg/ml. Patient and control cells were treated in a similar manner; half of the cells were treated with emetine and actinomycin D and the rest were controls treated only with actinomycin D. Total RNA was extracted from treated and untreated cells with Trizol according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). Ten micrograms of total RNA was used to generate fluorescent Cy-3 labeled cRNA (untreated cells) and Cy-5 labeled cRNA (treated cells) using Agilent Fluorescent Direct Label Kit (Agilent Technologies, Palo Alto, CA). Equal amounts of both Cy-3 and Cy-5 labeled cRNA were hybridized to the Agilent 44K Whole Human Genome oligo microarrays (Agilent Technologies) according to the manufacturer's protocol.

2.7.2 MicroRNA array protocol

Agilent Human miRNA V2 Oligo Microarray Kit (Agilent Technologies) was used for analyzing the miRNA expression levels in patient lymphoblastic cell lines cultured as described in section 2.7.1 (without drug treatments). Total RNA labeled with Agilent miRNA labeling kit (Agilent Technologies) was hybridized to Agilent miRNA arrays with eight identical arrays per slide.

2.7.3 Array data analysis

Microarray slides were scanned (Agilent Microarray Scanner) after hybridization and data were extracted using Feature Extraction software, versions A.7.5.1 and 9.5.1 (Agilent Technologies). For the oligonucleotide array the raw microarray expression values of the rMeanSignal and gMeanSignal variables were first background adjusted and the natural logarithm of a ratio of the variables rMeanSignal and gMeanSignal was calculated. The quantile normalization method was used to normalize the log-ratio values between arrays. Then, a linear mixed

model was used to identify the set of differentially expressed genes. The normalized expression values of each gene at Xq27-28 were separately modeled by the linear mixed model that included the treatment effect (i.e. person being affected versus healthy) as a fixed effect, and family effect as a random effect. In the model analysis, genes were considered to be differentially expressed if the calculated estimate for the parameter associated with the fixed treatment effect was greater than zero, and if, at the same time, the p-value in the t-test for the null hypothesis concerning the fixed effect parameter being zero was smaller than the cut-off value 0.025.

The miRNA array analysis was performed by two different methods. The first analysis was performed by following the same principles as in oligonucleotide arrays, with the exception that the cut-off for p-value was 0.05. In addition, only those miRNAs that had the mean expression value above the median expression level of all miRNAs were selected into the final set of differentially expressed miRNAs. In the second analysis, the variable gMeanSignal was used. First, the data were log2 transformed and quantile normalized between all the arrays without any background subtraction. Then, an extension of Friedman test statistics was used for each miRNA separately. The statistics is based on the regression rank scores (Gutenbrunner et al., 1993, Schindler, 2008) and compares two treatments (healthy *vs*. cancer) taking into account the family effects. There are 16 observations for every individual and the test statistic is designed to test the null hypothesis that in each family the average expression of healthy brothers is equal to the average expression of cancer patients in the family. This statistics also takes into account a different number of cancer and healthy brothers in the family. The hypothesis was tested against both one-sided alternatives and to obtain a good approximation of the exact p-values, the brothers were randomly permutated inside of every family. P-values were then calculated based on 6000 such permutations and the the false discovery rate (FDR) was set to a level of 25% to avoid false positive results. MiRNAs with p-values less than 0.005 were declared as having higher expression in cancer patients compared to healthy controls only when testing against the alternative that the average expression is higher in cancer patients.

2.8 Bioinformatics tools (IV)

For finding genomic targets for miRNAs in Study IV, the miRanda algorithm was used (Enright et al., 2003). This algorithm finds target genes for every miRNA based on three properties: sequence complementarity, free energies of RNA-RNA duplexes, and conservation of target sites in related genomes. The effect of Met1Thr variant for the MAGEC1 protein functionality was tested with PolyPhen (http://coot.embl.de/PolyPhen/).

2.9 Statistical analyses (I-IV)

Association of the different variants with prostate cancer (Studies I-IV) and male breast cancer (Study I) was tested by logistic regression analysis using different versions of the SPSS statistical software package (SPSS, Chicago, IL). Association with clinical and pathological features of prostate cancer (Studies I,III, and IV) was tested among unselected prostate cancer cases by Student's t-test, Mann-Whitney test, Kruskal-Wallis test, Pearson chi-square test, and Fisher's exact test included in the different versions of the SPSS.

RESULTS

1. Common *HFE* variants in prostate and male breast cancer (I)

In order to investigate the role of *HFE* variants Cys282Tyr and His63Asp in prostate and male breast cancer predisposition, the frequencies of these variants were determined among 843 unselected prostate cancer patients, 116 male breast cancer patients, and 480 male population controls. First, the carrier frequencies of Cys282Tyr and His63Asp were compared between male prostate cancer patients, and population controls. No significant differences were detected (His63Asp, P=0.37; Cys282Tyr, P=0.13). The odds ratios were calculated to estimate the prostate cancer risk, and a borderline result for a lower prostate cancer risk among heterozygous Cys282Tyr carriers was seen (Table 8.). The associations between the variations and clinical, pathological, and demographic features of the disease were also analyzed but no associations were observed.

In similar manner, the carrier frequencies of Cys282Tyr and His63Asp were compared between male breast cancer patients and controls. Again, no significant differences in genotype frequencies between different sample groups were observed. In addition, there were no significantly altered risks for male breast cancer among these two variants (Table 8.) However, male breast cancer patients who were homozygous for Cys282Tyr and His63Asp variants were younger (49 and 54 years) at the time of diagnosis compared to the average age of diagnosis of the rest of the patients (64.9 years), but no statistical analysis was performed due to the small number of mutation carriers. His63Asp heterozygotes were older than wild type cancer patients (67.2 *vs.* 64.3 years, P=0.20) but the difference was not statistically significant.

In a subanalysis, the frequencies of the Cys282Tyr and His63Asp variants were compared between *BRCA2* mutation carriers and non-carriers. Patients who had the most common BRCA mutation (9346(-2)A>G) were more often heterozygous for *HFE* His63Asp or Cys282Tyr than male breast cancer patients without *BRCA2* mutations (50% *vs.* 26%), but the difference was not statistically significant (P=0.21).

Table 8. *Association of the HFE Cys282Tyr (G845A) and His63Asp (C187G) variants with unselected prostate cancer or MBC.*

Sample and variation	Carrier freq.	OR	95% CI	P
Controls				
His63Asp heterozygotes (CG)	88/480 (18.3%)	1.00		
His63Asp homozygotes (GG)	7/480 (1.5%)	1.00		
Cys282Tyr heterozygotes (GA)	45/480 (9.4%)	1.00		
Cys282Tyr homozygotes (AA)	3/480 (0.6%)	1.00		
PC patients				
His63Asp heterozygotes (CG)	177/843 (21.0%)	1.19	0.90-1.59	0.23
His63Asp homozygotes (GG)	17/843 (2.0%)	1.44	0.59-3.50	0.42
Cys282Tyr heterozygotes (GA)	55/843 (6.5%)	0.68	0.45-1.02	0.06
Cys282Tyr homozygotes (AA)	9/843 (1.1%)	1.66	0.45-6.16	0.45
MBC patients				
His63Asp heterozygotes (CG)	26/116 (22.4%)	1.29	0.79-2.11	0.32
His63Asp homozygotes (GG)	1/116 (0.9%)	0.59	0.07-4.82	0.62
Cys282Tyr heterozygotes (GA)	5/116 (4.3%)	0.44	0.17-1.12	0.09
Cys282Tyr homozygotes (AA)	1/116 (0.9%)	1.38	0.14-13.41	0.78
MBC patients with *BRCA2* 9346(-2)A>G				
His63Asp heterozygotes (CG)	2/8 (25.0%)	1.49	0.29-7.48	0.63
His63Asp homozygotes (GG)	0/8 (0%)			
Cys282Tyr heterozygotes (GA)	2/8 (25%)	3.22	0.63-16.44	0.16
Cys282Tyr homozygotes (AA)	0/8 (0%)			

2. *NBN* as a candidate gene for familial and sporadic prostate cancer (II)

To explore the relevance of Nibrin gene (*NBN*, also known as Nijmegen breakage syndrome 1, *NBS1*), which is associated with the repair of DNA double strand breaks, in prostate cancer initiation a number of analyses were performed in five centers participating in the International Consortium for Prostate Cancer Genetics (ICPCG). First, the frequency of the *NBN* 657del5 mutation in familial (n=1819) and sporadic (n=1218) prostate cancer cases was compared to that found in controls (n=697). Four mutation carriers were identified among familial cases in two different families and the frequency for the probands was 0.22%. Among sporadic cases, the carrier frequency was 0.25%. The mutation was not detected among unaffected family members or in controls. To find out possible previously unidentified mutations, the entire coding region of the *NBN* gene was sequenced with primers designed to include all of the intron-exon boundaries in 20 of the youngest affected cases from the subset of Finnish prostate cancer families. A total of 13 changes were found. Two of the changes were missense variants, three were silent changes, and 8 of the variants took place in introns or 3´-untranslated regions (UTRs). For the entire Finnish subset of patients and controls, the frequencies of the two found missense variants, Asp95Asn and Glu185Gln, were determined and for the Ulm subset of samples, the frequency of a rare alteration (Arg215Trp, 643 C>T)

was determined but no association was seen with the variants and prostate cancer risk between cases and controls (Table 9.).

Table 9. *Association of the NBN 657del5, Asp95Asn, Glu185Gln, and Arg215Trp variants with unselected or familial prostate cancer.*

Variant and sample	Carrier freq.	OR	95% CI	P
657del5[a,*]				
Controls	0/697 (0%)			
Unselected or sporadic prostate cancer	3/1218 (0.25%)			
All affected familial	4/1819 (0.21%)			
Probands	2/909 (0.22%			
Unaffected familial	0/293 (0%)			
Asp95Asn[b,*]				
Controls	0/440 (0%)			
Unselected prostate cancer	1/613 (0.16%)			
Affected familial	1/121 (0.83%)			
Glu185Gln[b]				
Controls heterozygous	79/200 (39.5%)	1.00		
Controls homozygous	32/200 (16.0%)	1.00		
Unselected prostate cancer heterozygous	94/200 (47.0%)	1.59	0.86-2.91	0.14
Unselected prostate cancer homozygous	24/200 (12.0%)	1.23	0.67-2.26	0.51
Affected familial heterozygous	63/121 (52.1%)	1.42	0.73-2.76	0.30
Affected familial homozygous	18/121 (14.9%)	0.80	0.40-1.59	0.52
Arg215Trp[c]				
Controls	3/208 (1.44%)	1.00		
Sporadic prostate cancer	6/338 (1.78%)	1.24	0.31-4.99	0.77
Affected familial	2/139 (1.44%)	1.0	0.17-6.05	1.0
Unaffected familial	1/111 (0.90%)			

[a]All samples
[b]Tampere samples
[c]Ulm samples
[*]Testing for association was not possible because the alteration was not detected in the control population.

3. *MLH1* alterations and risk for prostate cancer (III)

To assess the role of *MLH1*, a known predisposing gene for Lynch syndrome, in prostate cancer causation in Finland, 11 patients with tumor samples available were identified through the discharge registry of Tampere University Hospital having both prostate cancer and colon cancer. Immunohistochemistry of MLH1, MSH2, and MSH6 proteins showed abnormal staining in two of the patients (Table 10.). The coding region of *MLH1* was sequenced from Patient A, and two silent and two missense variations were found (Table 10.).

Table 10. *Patients with abnormal staining in immunohistochemistry analysis of MLH1, MSH2, and MSH6.*

Patient	Cancers	Abnormal staining	Variations
A	Locally advanced prostate cancer Dukes B mucinous carcinoma in descending colon	*MLH1*	Ile 219Val Pro434 Leu Lys471 Phe626 1558+58,G>A
B	Prostate cancer Mucinous carcinoma of the cecum	*MSH2*	-[*]

[*]No DNA available for sequencing.

In addition, the *MLH1* gene was screened by SSCP among 121 probands from HPC families and re-sequenced among 18 affected persons from 6 families with the best multipoint HLOD scores (>0.5) per family at chromosome 3p. Twelve variants were identified including Ile219Val variation already found from the Patient A. Three of the variants were exonic, three were located in the UTR regions and six of them were intronic. Frequencies of the three found missense mutations in the *MLH1* gene (Ile219Val, Pro434Leu, and Val647Met) were determined among prostate cancer and HPC patients and in two different control groups. For Ile219Val, association with prostate cancer risk was analyzed, but no statistically significant differences in the carrier frequencies were observed and therefore, no association was seen between the variant Ile219Val and prostate cancer or HPC (Table 11.). The only interesting finding was that the mean age of diagnosis of the Ile219Val carriers among unselected prostate cancer patients was statistically higher when compared with non-carriers (68.1 *vs*. 65.9; P=0.03). Other statistically significant associations of the Ile219Val variant with demographic, clinical, or pathological features of the disease did not emerge.

Table 11. *Association of the MLH1 gene variants with unselected prostate cancer and HPC.*

Sample and variant	Carrier freq.	OR	95% CI	P
Ile219Val				
Population controls	110/200 (55.0%)	1.00		
Unselected prostate cancer patients	108/200 (54.0%)	0.96[*]	0.65-1.42[*]	0.84[C]
		1.00[**]	0.68-1.48[**]	0.99[**]
HPC patients	66/121 (54.5%)	0.98[*]	0.62-1.55[*]	0.94[*]
		1.02[**]	0.65-1.61[**]	0.92[**]
BPH patients	109/202 (54.0%)			
Pro434Leu[†]				
Val647Met[†]				

[*]Population controls as a control group.
[**]BPH patients as a control group.
[†]Testing for association was not possible because the alteration was not detected in the control group.

4. *HPCX*, a susceptibility locus for prostate cancer (IV)

4.1 NMD oligoarray analysis and genotyping

To investigate the role of *HPCX* in Finnish cancer patients and controls in more detail, novel array technologies were exploited to overcome the problems associated with traditional methods for finding candidate genes in the *HPCX* region. As a first step, a NMD microarray analysis with Agilent 44K Whole Human Genome Oligonucleotide microarrays was performed in the families (five families, six prostate cancer patients and six healthy brothers) showing the strongest linkage to *HPCX* in order to identify genes containing nonsense mutations. From the microarray expression analysis performed with RNA isolated from patient lymphoblastoid cell lines, 17 genes were selected for resequencing based on three distinct criteria (Table 12.). Nonsense variations were not detected, but altogether 34 changes were identified, including 8 missense variants, 6 silent changes and 20 variants taking place in intronic regions or 5´UTR and 3´UTR.

Table 12. *Genes selected for resequencing based on NMD oligoarray analysis.*

Gene ID	Gene name	Cytogenetic band	Selection criteria
RBMX	RNA binding motif protein, X-linked	Xq26.3	p<0.05
CSAG2	*Homo sapiens* CSAG family, member 2	Xq28	p<0.05
RAP2C	*Homo sapiens* RAP2C, member of RAS	Xq25	p<0.05, fold change >1.5
SOX3	SRY (sex determining region Y)-box 3	Xq27.1	p<0.05
MBNL3	Muscleblind-like 3, (*Drosophila*)	Xq26.2	p<0.05
ZNF75	Zinc finger protein 75	Xq26.3	p<0.05
MAGEC1	Melanoma antigen family C, 1	Xq26	p<0.05
MAGEA1	Melanoma antigen family A, 1	Xq28	p<0.05, fold change >1.5
MAGEA11	Melanoma antigen family A, 11	Xq28	location
MAGEC3	Melanoma antigen family C, 3	Xq27.2	location
MAGED1	Melanoma antigen family D, 1	Xp11.23	p<0.05
U66046	hypothetical protein FLJ44451	Xq28	p<0.05
SSR4	signal sequence receptor, delta	Xq28	p<0.05
VBP1	von Hippel-Lindau binding protein 1	Xq28	p<0.05
LDOC1	leucine zipper, down-regulated in cancer, 1	Xq27	p<0.05
TKTL1	transketolase-like 1	Xq28	p<0.05
CD40LG	CD40 ligand	Xq26	p<0.05

One of the most interesting variations for follow-up, Met1Thr (2T>C) in *MAGEC1*, was selected for large-scale genotyping in the Finnish population (757 patients with unselected prostate cancer, 163 patients with HPC, 375 patients with BPH, 746 PSA controls, 757 male population controls, and 764 female population controls). The

frequency of the variation among different sample groups was 1.72%, 2.45%, 0.53%, 1.21%, 0.92%, and 0.65%, respectively. A statistically significant difference was observed in the carrier frequencies of the Met1Thr variant between the sample groups, and subsequently, an association was seen between the variant and unselected prostate cancer and HPC. The association was strongest when male and female blood donors and BPH patients were used as a control group (OR=2.35, 95% CI=1.10-5.02 for unselected prostate cancer; OR=3.38, 95 % CI=1.10-10.40 for HPC). The association between the frequency of the variant and the disease phenotype (tumor WHO grade, Gleason score, T-stage, age at diagnosis, and primary PSA) was also analyzed among the unselected prostate cancer cases. No significant associations were found from these studies. The segregation of the Met1Thr variant with disease status was analyzed in three prostate cancer families carrying the variation, but cosegregation was incomplete.

4.2 miRNA array analysis

Since no causative mutations emerged from the resequencing project and no such mutations have been previously found in other studies from the *HPCX* region, it is possible that the defect occurs at the regulatory level. Therefore, miRNA expression levels in lymphoblastoid cell lines were detected using an Agilent Human miRNA V2 Oligo Microarray Kit (Agilent Technologies). At this step, the number of *HPCX*-linked families was increased by 6 (altogether 14 prostate cancer patients and 14 healthy brothers). The array data were analyzed by two different methods. In the first analysis, significantly altered expression between the affected brother and the healthy brother from *HPCX* families was observed in 15 miRNAs (hsa-miR-138, hsa-miR-146a*, hsa-miR-29c, hsa-miR-29c*, hsa-miR-346, hsa-miR-34c-3p, hsa-miR-421, hsa-miR-487b, hsa-miR-519e*, hsa-miR-623, hsa-miR-631, hsa-miR-650, hsa-miR-769-3p, hsa-miR-769-5p, and hsa-miR-770-5p). To analyze this further, the patients and controls were divided into three different subgroups based on the disease status (healthy individuals, patients with non-aggressive prostate cancer, and patients with aggressive prostate cancer having primary PSA $\geq$ 20 ng/ml or Gleason score $\geq$7). Hsa-miR-770-5p showed a significant difference in expression between the sample groups and had the highest expression in the group of healthy controls and the lowest expression in the group of aggressive cancer cases (healthy versus non-aggressive cancer, p=0.05; healthy versus aggressive cancer, p=0.01). The miRanda algorithm was utilized to find out whether the variant sites found in 17 genes from *HPCX* region cause a miRNA target site to appear or disappear. Interestingly, hsa-miR-770-5p was also on the top 5% of the list produced by the miRanda algorithm together with 11 of those 15 miRNAs, which had significantly altered expression between patients and healthy individuals. In addition, 9 out of those 12 miRNAs were predicted to have a target site in the *MAGEC1* gene, including hsa-miR-770-5p (Table 13a.). In the second analysis, significantly altered

expression between the affected brother and the healthy brother was observed in 12 miRNAs (hsa-miR-296-5p, hsa-miR-767-3p, hsa-miR-220a, hsa-miR-133a, hsa-miR-548b-5p, hsa-miR-595, hsa-miR-519c-3p, hsa-miR-129*, hsa-miR-223, hsa-miR-19b-2*, hsa-miR-151-3p, and hsa-miR-133b). Interestingly, three miRNAs are located in the Xq25-28 region (hsa-miR-19b-2*, hsa-miR767-3p, and hsa-miR-220a) and one miRNA is located in the 8q24 region (hsa-miR-151-3p). Ten out of twelve miRNAs were on the top 5% on the list produced by the miRanda algorithm and five out of those ten miRNAs were predicted to have a target site in the *MAGEC1* gene in the site of the variant (Table 13b).

Table 13. *Differentially expressed miRNAs between prostate cancer patients and their healthy brothers and possible miRNA target genes and sites.*

a) First analysis

miRNA	**p-value**	**Chr location**	**Target gene and variant site**
hsa-miR-770-5p	0.002	14q32.2	*MAGEC1*, Glu1058 *MAGEC3*, c.-189C>T *ZNF75*, Thr478
hsa-miR-421	0.011	Xq13.2	*MAGEC1*, c.-2008T>C *MAGEC1*, c.5-44T>C
hsa-miR-769-3p	0.016	19q13.32	*MAGEC1*, Glu1058 *MAGEC3*, Leu294Val
hsa-miR-29c*	0.025	11q32.2	-
hsa-miR-346	0.026	10q23.2	*MAGEA11*, Leu359
hsa-miR-769-5p	0.027	19q13.32	*MAGEC1*, His467Gln
hsa-miR-138	0.034	16q13	*MAGEC1*, Cys25Tyr
hsa-miR-631	0.034	15q24.2	*MAGEA1*, c.-2924G>T *MAGEA1*, c.-264G>A *MAGEC3*, c.259-66G>A
hsa-miR-146a*	0.036	5q33.3	-
hsa-miR-487b	0.038	14q32.31	*MAGEC1*, c.*53C>T *MAGEC3*, c.259-66G>A
hsa-miR-29c	0.039	1q32.2	*MAGEC1*, Leu443Val *MAGEC1*, His709Tyr
hsa-miR-650	0.039	22q11.22	*MAGEC1*, Glu1058
hsa-miR-519e*	0.040	19q13.41	-
hsa-miR-623	0.044	13q32.3	*LDOC1*, c.-62C->G *MAGEC1*, c.*53C>T
hsa-miR-34c-3p	0.048	11q23.1	*MAGEC3, c.-189C>T*

b) Second analysis

miRNA	p-value	Chr location	Target gene and variant site
hsa-miR-133b	0.0043	6p12.2	*MAGEC1*, c.-2008T>C *MAGEC3*, Leu320 *SUHW3*, p.Gly617
hsa-miR-548b-5p	0.0023	6q22.31	*MAGEC1*, c.*53C>T
hsa-miR-595	0.0027	7q36.3	*CD40LG*, Leu50Ser
hsa-miR-151-3p	0.0038	8q24.3	*CD40LG*, Leu50Ser *MAGEC1*, c.5-44T>C *MAGEC1*, c.*53C>T *MAGEC3*, Leu294Val
hsa-miR133a	0.0017	18q11.2	*MAGEC3*, Leu320
hsa-miR-519c-3p	0.0027	19q13.41	*MAGEC1*, Cys25Thr *MAGEC1*, Leu443Val
hsa-miR-296-5p	0.0002	20q13.32	*MAGEA1*, c.-264G>A
hsa-miR-223	0.0033	Xq12	*CD40LG*, Leu50Ser *LDOC1*, c.-62C>G *MAGEC3*, Leu294Val
hsa-miR-220a	0.0015	Xq25	*MAGEC1*, c.*53C>T *MAGEC1*, Met1Thr
hsa-miR-767-3p	0.0002	Xq28	*MAGEC3*, Leu320
hsa-miR-129*	0.0033	7q32.1	-
hsa-miR-19b-2	0.0033	Xq26.2	-

DISCUSSION

1. *HFE* gene variants in prostate and male breast cancer risk in Finland

High serum iron concentrations and transferrin saturation are common events seen among *HFE* heterozygotes (Bulaj et al., 1996) and that often leads to free radical formation and DNA damage. Patients with hereditary hemochromatosis are at high risk for hepatocellular carcinoma, and an increased risk for other cancers has also been detected (Dorak, Burnett and Worwood, 2005). Mutated BRCA2 protein has decreased or no capacity for DNA repair (Boulton, 2006). As the presence of modifier factors for breast cancer penetrance has been suggested (Thorlacius et al., 1996) and BRCA2 9346(-2)A>G mutation carriers were two times more often heterozygous for His63Asp or Cys282Tyr variants than the rest of the MBC cases, a possible modifier role for *HFE* in male breast cancer penetrance can be postulated based on our results. However, these results must be interpreted cautiously, as the number of samples in this study was quite low and no statistical significance was reached. In addition, the findings should be verified in cell line studies. Cardoso et al. (2006) also proposed a modifier role for the *HFE* gene in viral-related neoplasia such as cervical carcinoma by a dual role on iron metabolism and immunology, but the results of this study show an opposite effect. These authors examined the frequency of Cys282Tyr and His63Asp in 346 individuals including 201 women with cervical cancer and 146 controls and found that the His63Asp carriers had a significantly lowered risk for developing cervical cancer compared to non-carriers (OR=0.56, 95% CI=0.35-0.92, P=0.01). This issue is made even more confusing by a study from Turkey that reports that His63Asp mutation frequencies were increased in a group of breast cancer patients compared to healthy control individuals (22.2% *vs*. 14%, P=0.02) (Gunel-Ozcan et al., 2006). Ideally, genotyping of *HFE* variants should be performed in large cohorts in order to obtain more reliable results and to rule out the chance factor.

In contrast to male breast cancer risk, our results do not support a major role for the *HFE* mutations in the causation of prostate cancer in Finland at the population level. The frequencies of the His63Asp and Cys282Tyr mutations did not differ between prostate cancer patients and controls and no association was seen with the cancer risk and the variants. This is opposite to the results of Geier et al. (2002) who reported an increased risk of prostate cancer among HH patients. However, the number of prostate cancer patients in that study was very low (n=3), so any conclusions are dangerous to draw. Larger cohorts of subjects with HH overload disorders are needed to confirm these findings. Our study is to our knowledge the

first where associations of *HFE* variants are analyzed in large sample sets but analyses in different populations are still warranted.

In the present study the samples were not screened for new *HFE* mutations so we can not totally rule out the possibility that *HFE* variants are associated with prostate or male breast cancer risk. There are also other iron metabolism gene polymorphisms that could influence the risk of cancer, for example transferrin receptor mutations, but their role was not assessed in this study.

2. Contribution of variants in genes involved with DNA repair to prostate cancer risk (II, III)

Defects in DNA damage signaling and repair pathways are fundamental to the etiology of most human cancers. DNA mismatch repair (MMR) is a system for repairing incorrect insertions, deletions and mis-incorporations of bases that can arise during DNA replication and recombination. Loss of MMR has been reported in a wide variety of human malignancies and is associated with instability of microsatellite repeat sequences throughout the genome (Heinen et al., 2002). The MMR system consists of various types of proteins such as the MutL homologues (MLH) and the MutS homologues (Hsieh and Yamane, 2008).

DNA double strand breaks (DSBs) represent the most serious DNA damage, which can result in genomic instability, including chromosome rearrangements or gene mutations, and can finally lead to malignancy. There are two complementary mechanisms for DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Haber, 2000, Karran, 2000).

Currently, there are over 100 known DNA repair genes, and most of them are known to carry genetic variation in humans (Wood et al., 2001). However, only a few published studies on DNA repair genotypes and prostate cancer exist. Xu et al. (2002b) found that two variants in the DNA repair gene *OGG1* were associated with both sporadic and familial prostate cancer. The *XRCC1* Arg399 genotype has been shown to be associated with elevated prostate cancer risk in those individuals with low vitamin E or lycopene intake (Van Gils et al., 2002). In the present study, the role of two genes, *MLH1* and *NBN*, located at known prostate cancer susceptibility loci and involved in DNA repair mechanisms, was evaluated in prostate cancer predisposition in the Finnish population.

2.1 NBN is not a major susceptibility gene for prostate cancer in Finland

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive condition of chromosomal instability. It is caused by mutations in the *NBN* gene located at chromosome 8q21. The *NBN* gene product, nibrin, has been found to interact with at least two other proteins, hMre11 and Rad50 and is involved in end-processing of both physiological and mutagenic DNA DSBs (Kobayashi et al., 2004). The first

evidence of a possible correlation between *NBN* mutation carriers and cancer risk came from family studies indicating that relatives of NBS patients with the 657del5 founder mutation had a high risk of developing cancer (Seemanova, 1990). Since then, several studies have examined the frequency of the *NBN* mutations in cancer patients and nine mutations localized in the coding sequence of the *NBN* gene have been identified in cancer patients (http://nijmegenbreakagesyndrome.net). A Polish study by Cybulski et al. (2004a) postulated that the *NBN* gene predisposes to prostate cancer, as the 657del5 founder mutation was significantly associated with the prostate cancer risk among familial cases (OR=16, P<0.0001) and sporadic cases (OR=3.9, P=0.01) in Poland. In our study, an extensive amount of prostate cancer patients, both familial and sporadic cases participating in the ICPCG collaboration, were genotyped for the 657del5 mutation. The data indicate no major role for the *NBN* gene in prostate cancer predisposition as the 657del5 mutation was very rare. This is probably due to the fact that 657del5 is a Slavic founder mutation, and the frequency of this mutation is very low in other populations. Much larger sample cohorts would be needed to achieve statistical power for such analysis. Other studies, not mainly focused on prostate cancer, have also failed to confirm the original finding (Stanulla et al., 2000, Rischewski et al., 2000, Stumm et al., 2001, Gorski et al., 2005, Suspitsin et al., 2009).

In our study, the *NBN* gene was also screened for novel mutations among twenty patients from Finnish prostate cancer families. Two missense variants, Asp95Asn and Glu185Gln, were identified. In further genotyping, Asp95Asn turned out to be a very rare mutation and neither did the Glu185Gln show any association with increased prostate cancer risk. In other reported studies, the role of Glu185Gln in cancer risk is mixed. It has been associated with lung cancer risk (Medina et al., 2003), but not with breast cancer risk (Kuschel et al., 2002, Zhang et al., 2005) or bladder cancer risk (Sanyal et al., 2004). On the other hand, a recent meta-analysis study by Lu et al. (2009) combining 16 case-control studies (9 734 cancer patients and 10 325 controls) suggested that the Glu185Gln variant is mildly associated with an increased risk of cancer (OR=1.06, 95% CI=1.00-1.12) and the association is most pronounced in Caucasians.

The frequency of the variation Arg215Trp was examined within the Ulm subset of patients, but no significant associations were identified in our study. This variation was previously thought to be merely a neutral polymorphism of *NBN*, but its pathogenicity emerged with the identification of compound heterozygous 657del5/ Arg215Trp NBS patients. Arg215Trp was first described in acute lymphoblastic leukemia (ALL) and has also been detected among several cancer patients. A study conducted in Poland reported that carriers of the Arg215Trp variant have an increased risk of developing colorectal cancer (OR=5.25, P=0.047) (Steffen et al., 2004).

In our study, several intronic variants and one 3´UTR variation were also detected. However, it is quite difficult to predict their significance in cancer risk and therefore these variants were excluded from further analysis, even though there are

many reports stating that intronic variants have functional effects (Enattah et al., 2002, Zhang et al., 2004, Pomares et al., 2009). To conclude the results of the present study, *NBN* does not seem to be a major prostate cancer susceptibility gene in a non-Slavic population. However, the intronic and 3´UTR variants should also be studied in detail to be able to totally exclude the cancer promoting effects of *NBN*.

2.2 MLH1 has no significant role in the causation of prostate cancer in Finland

Several hundred mutations and polymorphisms have been identified for MMR genes (Peltomäki and Vasen, 2004), but interestingly, most of these mutations are observed in the *MLH1* gene making it a high risk susceptibility gene in the process of carcinogenesis. The *MLH1* gene is located on chromosome 3p22, near the suggested Finnish prostate cancer susceptibility locus on 3p25-p26 (Schleutker et al., 2003). As prostate cancer and colorectal cancer have frequently been observed in the same patient, 11 prostate-colon cancer patients were screened by immunohistochemistry for MLH1, MSH2, and MSH6 protein expression defects. SSCP analysis of 121 probands and resequencing of 18 probands from HPC families enabled the search for the *MLH1* variations. No truncating mutations were found, so direct causative role for *MLH1* in HPC could not be established. Several other variations were found, including three missense mutations, Ile219Val, Pro434Leu, and Val647Met. Their frequencies were determined among HPC patients, patients with unselected prostate cancer, patients with BPH, and population controls.

The Ile219Val variation was initially identified in colorectal cancer patients (Liu et al., 1995), but it was not likely to a play causative role in cancer development, as it was found in at least one patient with a different definitive mutation or was found in controls. Later, in a candidate SNP analysis by Burmester et al. (2004) the variant Ile219Val of *MLH1* was significantly associated with prostate cancer risk, even in the age-matched subset. In addition, Ile219Val has been associated with increased risk of refractory ulcerative colitis (Bagnoli et al., 2004). In our study, the frequency of Ile219Val was high among every sample group, but no association with unselected prostate cancer or HPC was seen. Furthermore, the variation did not segregate with the disease status in the HPC families. Recently, Tanaka et al. (2009) analyzed the genetic distribution of *MLH1* polymorphisms Asp132His, Ile219Val, Val384Asp, and Ala723Asp in BPH and sporadic prostate cancer patients, and compared the frequencies to healthy controls from a Japanese population. No differences in frequencies were observed for Ile219Val between controls and BPH patients or cancer cases, which supports our findings. Interestingly, however, a decreased risk for prostate cancer was observed for the heterozygous genotype (T/A) and variant allele (A) on codon 384, a variation that was not detected among the Finnish population. A significant finding in our study was the fact that Ile219Val

carriers among unselected prostate cancer patients were diagnosed at an older age compared to non-carriers, which might suggest that the variation somehow affects the disease onset. Obviously, this needs further investigation, solely because Ile219Val does not seem to have an effect on MMR function (Trojan et al., 2002). The Pro434Leu variation, which was identified from the prostate-colon cancer patient, was not present in other sample groups. Likewise, Val647Met proved to be rare, as it was found only in one HPC family. Altogether, our results do not support a major role for *MLH1* in the causation of prostate cancer and it is not to be considered as a candidate prostate cancer susceptibility gene at the 3p25-p26 region.

3. *MAGEC1* variant Met1Thr at *HPCX* significantly associates with prostate cancer risk (IV)

Linkage and epidemiological data support the existence of genetic variants on chromosome X predisposing to prostate cancer. Prostate cancer susceptibility loci on both arms of the X chromosome have been identified, including *HPCX* at Xq27-q28 (Xu et al., 1998, Lange et al., 1999, Schleutker et al., 2000, Cunningham et al., 2003, Brown et al., 2004, Gillanders et al., 2004, Chang et al., 2005a, Farnham et al., 2005, Gudmundsson et al., 2008). Initially, the linkage for *HPCX* was seen in Finnish, Swedish, and US prostate cancer families and further haplotype analysis among Finnish prostate cancer families refined the locus to a candidate interval (Baffoe-Bonnie et al., 2005). However, no causative mutations or variants have been reported in this locus, mostly due to the extremely complex structure of the genomic region (Stephan et al., 2002). In the present study, a novel method of NMD microarray strategy was used to identify transcripts containing nonsense mutations in patients' lymphoblastoid cell lines. Inactivation of autosomal tumor suppressor genes is a two-step process involving the mutation of the target gene and the loss of the wild type allele and in lymphoblastoid cell lines, the normal wild type allele can mask the effect of a germline allele. However, because men have only one X chromosome, tumor suppressor genes may be identified by using patient lymphoblastoid cell lines.

In the array analysis, 17 genes showed significantly altered mRNA expression between patients and their healthy brothers. However, we were not able to identify any truncating nonsense mutations, even though several other variations were detected. Therefore, the false-positive rate in this study was evidently high. One variation from *MAGEC1* gene was selected for follow-up (Met1Thr) and an association was seen between the Met1Thr variant and unselected prostate cancer and HPC. Despite the association with prostate cancer, the segregation of the Met1Thr variant in studied families was incomplete, but that most probably reflects the high phenocopy rate of the disease. *MAGEC1* is a member of the melanoma antigen gene (*MAGE*) family (Lucas et al., 1998). These cancer testis (CT) antigens are expressed in a variety of malignant tumors, but not in normal adult tissues

except for testicular germ cells (Takahashi K. et al., 1995, Van den Eynde and van der Bruggen, 1997). Since their expression is confined to neoplastic cells, they may represent ideal targets for antigen-based vaccination and antigen-directed immunotherapy against malignant tumors (Scanlan et al., 2002, Suri, 2006). To our knowledge this a first study suggesting an association between a *MAGEC1* gene variant and cancer risk. It would be crucial to find out the biological effect of the start codon variation Met1Thr, but in our case it was unfortunately impossible due to the absence of suitable tumor material from prostate cancer patients carrying the variation. It is possible to analyze the possible effect of this variation on protein functionality by different bioinformatics tools, but their results should be taken cautiously, as these programs most often do not take into account the location of the variation in the polypeptide chain. MAGE proteins are very similar with respect to their functions, therefore other members of the gene family may very well compensate for the functions of *MAGEC1* if the start codon mutation abolishes the translation of the gene.

Although Met1Thr variant was associated with the increased risk of both HPC and unselected prostate cancer, we cannot say for sure that *MAGEC1* is the actual candidate gene. It might be that, *MAGEC1* is merely in linkage disequilibrium with the actual causative gene and variant.

In the last few years, miRNAs have been shown to play a key role in the regulation of gene expression, and there is evidence that miRNAs are involved in central biological processes. MiRNAs have come into the focus of molecular research of many diseases, in particular cancer (Fabbri et al., 2008). In the present study, miRNA expression levels from the lymphoblastoid cell lines of patients and their healthy brothers were measured using the Agilent platform. This was done because the defect causing *HPCX*-linked prostate cancer may also be at a regulatory level. By using two different analysis methods, two sets (first 15 miRNAs and second 12 miRNAs) of significantly differentially expressed miRNAs were identified between cancer patients and healthy controls. One of the most interesting miRNAs was hsa-miR-770-5p, the expression of which was highest in healthy controls and lowest in aggressive cancer in the first analysis. In addition, hsa-miR-770-5p placed at the top of the list of miRanda target analysis, where the aim was to identify miRNA targets that either appear or disappear due to the variant site. Interestingly, *MAGEC1* seems to be one of its targets. MicroRNAs recognize their targets based on sequence complementarity and the mature miRNA is partially complementary to one or more messenger RNAs (Brennecke et al., 2005). In humans, the complementary sites are usually within the 3′-untranslated region of the target messenger RNA. All miRNA target finder algorithms return lists of candidate target genes, but the validity of that output in a biological setting needs to be considered. The prediction of miRNA targets by computational approaches is based mainly on miRNAs complementarity to their target mRNAs, and several computer software programs are used to predict miRNA targets (Lindow and Gorodkin, 2007). Among them, TargetScanS, PicTar, and miRanda are the most common

programs. In our study, miRanda was the only algorithm used to identify genomic targets for miRNAs, and the target list might have been different if we had chosen to use a different algorithm instead. In fact, it has been shown that different algorithms produce very different predictions, and the degree of overlap between the lists of predicted targets is often poor (Sethupathy et al., 2006).

In the second analysis, 12 miRNAs were significantly differentially expressed between patients and healthy brothers. Three of the miRNAs, hsa-miR-19b-2*, hsa-miR-767-3p, and hsa-miR-220a, were located in the *HPCX* region. Previously, hsa-miR-767-3p has been shown to be a partner regulatory miRNA of estrogen receptor coregulator *NCOA3* gene (McCafferty et al., 2009) and hsa-miR-19b-2* belongs to a miRNA cluster, miR-106-363, which acts as an oncogene in T-leukemia (Landais et al., 2007). MiRNAs from this cluster have also been reported to be overexpressed in colon and prostate cancer (Volinia et al., 2006) and in leukemia cell lines (Yu et al., 2006). Hsa-miR-151-3p in chromosome 8q24.3 is also an interesting target for further studies based on its location and recent reports. Baffa et al. (2009) were able to show that hsa-miR-151-3p had a 1.4 fold increased expression in metastatic tumors compared to normal tissue. On the contrary, Agirre et al. (2008) reported that hsa-miR-151 is down-regulated in chronic myeloid leukemia (CML).

In this study, two different methods were used for analyzing the miRNA array data. In the first analysis, poor quality data were first filtered out. In addition, the background was subtracted in the first analysis but not in the second analysis. In the first analysis, a linear mixed model was used that models family and individual effects as random. This will work if the data are normally distributed and therefore, in the second analysis, a non-parametric method was used with no assumptions about the underlying distribution and the family and individual effects were modeled as fixed. In the first analysis the null hypothesis was tested only against a two-sided alternative and 0.05 was used as a cut-off point for p-values with no multiple testing procedures. Instead, the second analysis tested the significance of the "treatment" effect against both one-sided alternatives and permutation p-values were calculated, which is more exact. Interestingly, these two methods resulted in totally different lists of significantly differentially expressed miRNAs and they did not overlap at all, but the miRanda algorithm frequently reported *MAGEC1* and *MAGEC3* genes to be target sites for positive miRNAs in both lists. However, the statistically significant differences between sets of data may not necessarily have practical significance, and we therefore cannot draw any conclusions on the superiority of different methods of analysis before we are able to show that the found miRNAs truly have a functional role in prostate carcinogenesis.

Emerging evidence shows that differential miRNA expression is involved in the pathogenesis of prostate cancer. To date, there are several published studies reporting this phenomenon (Mattie et al., 2006, Porkka et al., 2007, Ozen et al., 2008, Devere White et al., 2009). Very recently, Spahn et al. (2009) analyzed the global expression of miRNAs in BPH tissue and high risk primary prostate carcinoma by micro-array analysis and found that the expression of hsa-miR-221

was markedly downregulated in metastasized prostate cancer. In addition, they reported that the expression of miR-221 was associated with prostate cancer progression and clinical recurrence. An interesting study by Epis et al. (2009) reported that hsa-miR-331-3p directly regulates *ERBB-2* mRNA and protein expression in multiple prostate cancer cell lines via two specific *ERBB-2* 3'-UTR target sites. Hsa-miR-331-3p expression was downregulated in *ERBB-2* overexpressing prostate cancer tissue relative to normal adjacent prostate tissue. Furthermore, miR-331-3p blocked the androgen receptor (AR) signaling pathway by reducing transcriptional activity and expression of prostate-specific antigen (PSA), which is an AR target gene.

In contrast to the previously published reports, in our study, lymphoblastoid cell lines established from patient samples were used as a starting material for miRNA isolation. The advantage of using lymphoblastoid cell lines is that they are an unlimited resource of DNA and RNA, and the obtained results are truly patient specific. However, the culturing of the cells is slow and laborious. To our knowledge, the present study is the first ever to study miRNA expression in cancer from lymphoblastoid cells. There is one reported study assessing the relevance of using lymphoblastoid cell lines to study the role of miRNAs in the etiology of autism (Talebizadeh, Butler and Theodoro, 2008). As other studies have used cancer cell lines and tumor tissues, it is not even feasible to compare the expression results between different studies. One thing to bear in mind while analyzing the miRNA expression data from lymphoblastoid cell lines is that the Epstein-Barr virus transformation itself can have drastic effects on miRNA expression profiles. It has been shown that the expression of hsa-miR-155 in Epstein-Barr virus transformed lymphoblastoid cell lines is substantially increased (Jiang et al., 2006). Since one miRNA may regulate many target mRNAs, overexpression of hsa-miR-155 in lymphoblastoid cell lines is likely to result in the downregulation of numerous genes. Obviously, that could have had effects on the NMD array expression analysis.

To summarize, the present study suggests a role for *MAGEC1* in prostate cancer susceptibility, especially in the *HPCX*-linked form of the disease, as a start codon missense variation in the *MAGEC1* gene showed an association between the variant and prostate cancer. Hsa-miR-770-5p also showed significantly altered expression between patients and controls and was predicted to have *MAGEC1* as one of its targets. In addition, three differentially expressed miRNAs, hsa-miR-19b-2*, hsa-miR-767-3p, and hsa-miR-220a, are located near the *HPCX* locus and therefore, represent interesting targets for future investigation. Functional studies are needed to analyze the biological role of these miRNAs and *MAGEC1* and possibly *MAGEC3* genes in prostate carcinoma in detail.

4. Methodological aspects

In Study III, SSCP was used for screening *MLH1* variations among 121 probands from HPC families. This method has been widely used in mutation detection, because it is very simple. However, since conformational states are subject to many experimental conditions and sequence differences that cause minimal changes in strand conformation may not be detected, SSCP is now being replaced by sequencing techniques on account of efficiency and accuracy. Sequencing was used for screening novel variations in Studies II, III, and IV. In addition, sequencing was used for genotyping in Studies II and III. In the future, however, high resolution melt (HRM) analysis will be a powerful technique for the detection of mutations, polymorphisms and epigenetic differences in double stranded DNA samples. This technique has many advantages over other genotyping technologies, namely it is cost effective, fast, and simple. In Study I, solid-phase minisequencing was used to determine sample genotypes (Syvänen, 1998). It is a good method of choice when the analyzed variation is extremely rare, because there is a possibility to pool samples. However, nowadays TaqMan 5´ nuclease assays are an especially attractive choice for large scale genotyping of specific variants.

In the NMD array analysis used in Study IV, the false positive rate was high since no truncating mutations were found, even though 17 genes were screened by sequencing. We used the original method described by Noensie and Dietz (2001) where emetine treatment is followed by actinomycin D. However, it has been observed that in every case the treatment with actinomycin D after emetine incubation does not have a significant effect on treated cells, suggesting that this drug combination is not the best possible method for this type of studies (Wolf et al., 2005). A more recent improvement to the NMD protocol includes a combination of emetine and caffeine treatment (Ivanov et al., 2007). This enables a more efficient identification of false positives produced by cell stress. Although the NMD inhibition method has been successful in identifying mutated genes in many cancers (Huusko et al., 2004, Rossi et al., 2005, Ivanov et al., 2007, Bloethner et al., 2008) contradictory results have also been published. Buffart et al. (2009) specifically inhibited the NMD pathway in two gastric cancer cell lines, GP202 and IPA220 with siRNA directed against *UPF1*. Mutation analysis of 11 candidate genes was performed by sequencing and even though the *UPF1* expression was reduced by over 70% and 80% in the GP202 and IPA220 gastric cancer cell lines, respectively; no nonsense mutations were detected in any of the 11 genes tested. Their result states that the method may actually lead to a high number of false positives.

In Study IV, two different array platforms were used to characterize the *HPCX* locus. Normalization is a standard preprocessing procedure in microarray data analysis to minimize the systematic technological variations and produce more reliable results. Several normalization approaches have been introduced and are widely applied. In our study, the quantile normalization method was utilized as it has been proposed to be preferable to the other methods (Bolstad et al., 2003). DNA and miRNA microarray technologies provide a powerful tool for characterizing

expressions on a genome scale in basic biological research, because these techniques enable the analysis of multiple genetic factors simultaneously. Sincethe development of many diseases, including cancer, and their potential treatment outcomes are determined by the function of multiple genes, DNA microarray will be an important molecular diagnostic technology in the future. In the diagnostics of breast cancer, an array-based diagnostic tool is already in use. A 70-gene tumor expression profile was established as a powerful predictor of disease outcome in breast cancer patients (van ‘t Veer et al., 2002). In order to facilitate its use in a diagnostic setting, the profile was translated into a customized microarray (MammaPrint) containing a set of 1900 probes suitable for high throughput processing, and an extremely high correlation of prognosis prediction between the original data and that generated using the custom array was seen ($p < 0.0001$) (Glas et al., 2006). Hopefully in the future, prostate cancer patients will also have an opportunity to be assessed by similar array-based diagnostic tests.

5. Sample selection and genetic aspects

There is a great deal of evidence suggesting that genetics plays a critical role in prostate cancer predisposition. However, the search for prostate cancer susceptibility genes by linkage studies has not been easy and it has been challenging to replicate the findings. It has become obvious that prostate cancer is genetically a very complex disease and many different factors contribute to the difficulty of identifying high risk genes (Easton et al., 2003, Schaid, 2004). One particularly difficult problem is the locus heterogeneity, meaning that the trait is caused by mutations in genes at different chromosomal loci. That creates significant challenges to discovering the genetic basis of complex genetic diseases. Although the causes of heterogeneity may vary, locus heterogeneity can be particularly damaging. If linkage heterogeneity is ignored in the data analysis, the power to detect linkage is drastically reduced (Schaid et al., 2001).

The nature of prostate cancer itself hampers the genetic studies. Prostate cancer is typically diagnosed at a late age, therefore making it difficult to obtain DNA and tissue samples from affected men for more than one generation, which is a serious problem in linkage analyses. In addition, the lack of clear distinguishing features between hereditary and sporadic prostate cancer is a significant problem. No clinical or pathological characteristics have been found to differ between hereditary and non-hereditary forms of prostate cancer (Carter et al., 1993, Bratt, 2002). The main difference is an earlier age of diagnosis for hereditary prostate cancer, 6–7 years (Bratt, 2002). Hence, based solely on clinical information, it is difficult to discriminate between phenocopies and actual genetic cases within pedigrees. Furthermore, the high prevalence of the disease complicates the identification of genetic risk factors, as many familial clusters of prostate cancer might be caused by chance alone. One possible solution is having large enough study populations so that

well-defined subsets of patients can be produced. In that sense, international collaborative efforts play a crucial role.

The presence of systematic differences in allele frequencies between subpopulations in a population due to different ancestry is called population stratification. This effect can be a real problem for association studies, such as case-control studies, where the association found could be due to the structure of the population or the structure of the population may mask the true associations (Dadd et al., 2009, Freedman et al., 2004). In Studies I, III, and IV, samples of Finnish origin were used and in Study II other populations were also selected as sample material. The Finnish population is considered as a homogeneous isolate, well suited for gene mapping studies because of its reduced diversity and homogeneity. However, recent studies have shown substantial differences between the eastern and western parts of the country, especially in the male-mediated Y chromosome (Palo et al., 2007). Recently, Palo et al. (2009) reassessed the existing data, and the results obtained suggested substantial Scandinavian gene flow into south-western, but not into the eastern, Finland. This might mean that many illnesses belonging to the Finnish disease heritage (FDH) stem from long-term drift, rather than from relatively recent founder effects.

6. Future prospects

The goal in prostate cancer genetics is to find genetic changes that are associated with prostate cancer risk. If we are able to locate the alterations in certain genes that increase the chances of getting prostate cancer, it may be possible in the future to use this knowledge for the screening of family members and for the development of new prostate cancer treatments. From the patients' point of view, developing genetic biomarkers of aggressive cancer would greatly improve the diagnostics and treatment of prostate cancer. Ultimately, the goal would be to be able to develop methods to inhibit the genes that contribute to prostate cancer initiation and development, and thereby stop pre-cancerous cells from forming. In turn, it is important to avoid over treatment and to identify those patients with low-grade prostate cancer who most probably will not benefit from treatment.

Through all these years, it has become evident that prostate cancer susceptibility is a result of complex interaction between several genes and environmental factors. Hopes for identifying single, high-risk prostate cancer susceptibility genes are fading and the significance of single candidate gene analyses are likely to diminish in prostate cancer genetics studies. At present, genome-wide association studies are showing great promise in identifying common, low-penetrance susceptibility alleles for many complex diseases, including prostate cancer. Despite the fact that the statistical evidence for these associations is significant, the biological relevance of the variants and the ways by which they lead to increased risk of prostate cancer still remain unknown and require further genetic and functional characterization. In

addition, these alleles are associated with modest or low risk, and even more risk variants are likely to be identified in the future. This knowledge cannot be used in clinical terms before the influence of these variant alleles on prostate cancer risk is evaluated thoroughly. Linkage analysis is still a valid method for identifying prostate cancer risk loci and increasing our understanding of hereditary prostate cancer, but in the future the stratification of cancer cases by clinical characteristics for linkage studies will have a greater impact.

The fast development of cost-efficient, large-scale genotyping techniques is also setting huge demands on data analysis, computer programs and people who use them. In most cases, the know-how of using analysis softwares is beyond the knowledge of the basic biologist. Therefore, in order to produce good quality data, resources of experts from many different fields and from different research institutes, both national and international, are greatly needed.

Regardless of the direction in which the studies will take us, the future of prostate cancer is in the genes and in the ways that we can manage to control the biological events that they influence.

CONCLUSIONS

The present study was conducted to provide new information on the genetic risk factors leading to prostate cancer by investigating the role of three linkage-based candidate genes, *HFE*, *NBN*, and *MLH1*, and characterizing the prostate cancer susceptibility locus, *HPCX*, in more detail.

The major findings of this study were:

1. Genetic variants, Cys282Tyr and His63Asp, in the *HFE* gene did not show statistically significant association with prostate cancer risk among Finnish unselected prostate cancer patients although a trend towards lower prostate cancer risk among heterozygous Cys282Tyr carriers was seen.

2. *NBN* does not seem to be a prostate cancer susceptibility gene in a non-Slavic population as the 657del5 mutation was present only in less than 1% of the individuals tested.

3. *MLH1* gene variants do not have a causative role in prostate cancer predisposition among Finnish HPC families and unselected prostate cancer families at the population level. However, in rare individual cases, *MLH1* variations may have a predisposing effect.

4. A role for *MAGEC1* in genetic prostate cancer susceptibility, especially in the *HPCX*-linked form of the disease, is suggested. The start codon missense variation, Met1Thr, in the *MAGEC1* gene showed an association between the variant and both HPC and unselected prostate cancer. The roles of hsa-miR-770-5p, hsa-miR-19b-2*, hsa-miR-767-3p, hsa-miR-220a, and hsa-miR-151-3p in prostate cancer predisposition need further study, especially since *MAGEC1* and another gene from the same gene family, *MAGEC3*, were predicted to be common target genes for three of them.

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SHORT REPORT

Hemochromatosis gene mutations among Finnish male breast and prostate cancer patients

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Hereditary hemochromatosis (HH), the most common genetic disease in northern Europeans, is an autosomal recessive disorder of iron metabolism. The association between hepatocellular carcinoma and *HFE* homozygosity is well documented, but recently *HFE* hetero- and homozygosity has also been linked to nonhepatocellular malignancies, including female breast cancer. We hypothesized that C282Y and H63D mutations in the *HFE* gene could contribute to male breast cancer (MBC) and prostate cancer (PC) susceptibility at the population level in Finland. We screened the 2 major *HFE* mutations, H63D and C282Y, from 116 MBC cases diagnosed in Finland between 1967 and 1996, 843 consecutive unselected PC cases diagnosed at the Pirkanmaa Hospital District between 1999 and 2001 and 480 anonymous blood donor controls by minisequencing. Our results indicate that the frequencies of the *HFE* mutations do not significantly differ between MBC and PC patients and the population-based controls. No significantly altered risks for MBC or PC among carriers of the 2 variants were observed. However, *HFE* mutations were seen twice as often among carriers of a common *BRCA2* mutation 9346(−2)A→G compared with the rest of the MBC cases, indicating that *HFE* may be an MBC risk modifier gene among *BRCA2* mutation carriers. In conclusion, our results indicate a minor role for the *HFE* mutations C282Y and H63D in the causation of MBC and PC, but carriers of both *BRCA2* 9346(−2)A→G and an *HFE* mutation may be at an increased risk.
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Key words: hereditary hemochromatosis; C282Y; H63D; male breast cancer; prostate cancer

Prostate cancer (PC) is the most frequently diagnosed malignancy in men in Finland. Male breast cancer (MBC), on the other hand, is a rare disease that accounts for less than 1% of cancers in men (cancer statistics accessed from Finnish Cancer Registry, www.cancerregistry.fi, last updated 21 August 2004). The etiologies of these 2 cancers are not well known, but it has been suggested that they result from an interaction between genetic factors, environmental influences, as well as endogenous hormones and trace elements. Iron is a prooxidizing trace element, which in high levels can lead to free radical formation and DNA damage. Also, iron is important for the proliferation of neoplastic cells. Hereditary hemochromatosis (HH), the most common genetic disease in Caucasians, is an autosomal recessive disorder of iron metabolism that increases iron absorption and results in excessive iron accumulation. The *HFE* gene mutated in HH is located on 6p21.3 and the 2 main missense alterations in *HFE* gene in HH are His63Asp (H63D) in exon 2 and Cys282Tyr (C282Y) in exon 4.[1] The HFE protein, consisting of 343 amino acids, is a transmembrane protein expressed in intestinal and liver cells; it works in conjunction with another small protein called β-2-microglobulin to regulate iron uptake.[1] The HFE protein product binds to the transferrin receptor and reduces its affinity for iron-loaded transferrin by 5- to 10-fold.[2]

It has been shown that *HFE* homozygotes have up to a 19-fold increased risk to develop hepatocellular carcinoma,[3] but also a risk to develop some other malignancies[4] such as breast cancer. An increased frequency of the C282Y allele (1 or 2) in women with breast cancer has been observed and altered iron metabolism among C282Y carriers was suggested to promote the development of breast cancer and possibly more aggressive forms of the disease.[5] An increased risk of female breast cancer has also been reported among carriers of the H63D allele.[6] A possible link between hemochromatosis and MBC has been suggested.[7] Recently, a variation in *BRCA2* mutation spectrum was seen between Finnish male and female breast cancer patients, suggesting that modifying genetic and/or environmental factors may significantly influence the penetrance of breast cancer in individuals carrying germline *BRCA2* mutations.[8] A variation in male and female breast cancer penetrance among *BRCA2* mutation carriers has also been suggested in Iceland.[9]

HFE heterozygosity has also been linked to an increased risk of malignant glioma[10] and colorectal and gastric cancer.[11] The latter observation is of interest, as we have previously detected an association between prostate and gastric cancers in Finland.[12] Based on epidemiologic data and information from a recent genomewide linkage analysis, where a possible PC susceptibility area on chr 6p was detected,[13] we hypothesized that C282Y and H63D mutations in *HFE* gene could also contribute to PC susceptibility. Here, we wanted to evaluate the contribution of *HFE* mutations as a risk factor for both MBC and PC in Finland.

Material and methods

Patients and controls

We identified all 237 MBC patients diagnosed in Finland between 1967 and 1996 from the Finnish Cancer Registry. First- and second-degree relatives, when possible, were identified from the population and parish registries. Incident cancer cases among these relatives were identified through record linkage with the cancer registry. A total of 116 MBC cases (49% of the eligible patients) were available for the study. Seventy-nine patients (33%) were alive and were approached through the attending physicians. We obtained a written informed consent to participate in the study and a blood sample from 37 patients. A questionnaire on malig-

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nancies in the family was also received. Paraffin-embedded tissue samples were available from 79 patients, focusing on those patients who had died of the disease. We had limited access to patients' medical records. The information obtained on the MBC patients included histologic subtype of the breast cancer, date of birth, age at diagnosis and the attending hospital. All patients had been screened for previously identified Finnish *BRCA2* mutations.[8] The cohort included 12 *BRCA2* mutation carriers, 8 9346(−2)A→G carriers and 1 each of 999del5, 4075delGT, 5808del5 and 7708C→T carriers. The mean age at diagnosis for the MBC patients was 64.7 years (range, 30–94) and 10.3% (12/116) of the patients had a positive family history of breast cancer.

We also obtained samples and written informed consents from 843 (84.5%) of the 998 patients diagnosed with PC in the Pirkanmaa Hospital District during 1999–2001. Tampere University Hospital serves as the local referral area for PC treatment and our sample set resulted in an unselected population-based cohort of PC patients. The mean age at diagnosis for the patients with PC was 68.9 years (range, 45–93). A positive family history of PC was reported by 13.9% (117/843) and of BRCA by 7.8% (66/843) of the patients.

The controls consisted of 480 DNA samples from anonymous, voluntary and healthy male blood donors obtained from the Blood Center of the Finnish Red Cross in Tampere. The research protocols were approved by the ethics committee of the Tampere University Hospital and the Ministry of Social Affairs and Health in Finland.

DNA extraction

DNA was extracted from blood leucocytes using the Puregene kit (Gentra Systems, Minneapolis, MN) and from formalin-fixed, paraffin-embedded tissue samples using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' instructions.

Minisequencing

The frequencies of the *HFE* mutations H63D and C282Y were determined by minisequencing.[14] All primers are available on request. PCR was performed with 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 (PE Biosystems, Foster City, CA) in a final volume of 50 μl at 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 1 min, with a 5-min extension at 72°C after the last cycle.

Statistical analyses

Association of the *HFE* genotypes with MBC and PC was tested by logistic regression analysis using the SPSS statistical software package 11.0 (SPSS, Chicago, IL). Association with demographic, clinical and pathologic features of the disease was tested by Student's *t*-test, Mann-Whitney test, Kruskal-Wallis test, Pearson chi-square test and Fisher's exact test included in the SPSS 11.0.

Results

Carrier frequencies of the *HFE* H63D and C282Y variants among MBC and PC patients and controls are seen in Table I. The carrier frequencies of the H63D and C282Y variants were compared between MBC patients and controls. The frequencies were in Hardy-Weinberg equilibrium among controls and there was no statistically significant difference in the carrier frequencies between MBC patients and controls (H63D: Pearson chi-square test, $p = 0.40$; C282Y: Pearson chi-square test, $p = 0.10$). For comparison, the reported frequencies for C282Y and H63D in normal Finnish population are 10.2% and 20.3%, respectively.[15] This shows that the *HFE* variants are not enriched among blood donors. The ORs for H63D and C282Y mutations were also calculated to estimate the MBC risk (Table I). There were no significantly altered risks for MBC among these 2 variants. Furthermore, there was no association between *HFE* mutations and positive family history of breast cancer since only 6.5% (2/31) of the *HFE* heterozygotes and neither of the 2 homozygotes had relatives with breast cancer compared with 12% (10/83) of the rest of the MBC cases.

TABLE I – *HFE* GENE VARIANTS AMONG MALE BREAST AND PROSTATE CANCER PATIENTS AND CONTROLS

Sample and mutation	Number of carriers/total (%)	OR	95% CI	*p*
H63D				
Controls				
(C/G)	88/480 (18.3)	1.00		
(G/G)	7/480 (1.5)	1.00		
All MBC patients				
(C/G)	26/116 (22.4)	1.29	0.79–2.11	0.32
(G/G)	1/116 (0.9)	0.59	0.07–4.82	0.62
MBC patients with *BRCA2* 9346(−2)A→G				
(C/G)	2/8 (25.0)	1.49	0.29–7.48	0.63
(G/G)	0/8			
PC patients				
(C/G)	177/843 (21.0)	1.19	0.90–1.59	0.23
(G/G)	17/843 (2.0)	1.44	0.59–3.50	0.42
C282Y				
Controls				
(G/A)	45/480 (9.4)	1.00		
(A/A)	3/480 (0.6)	1.00		
All MBC patients				
(G/A)	5/116 (4.3)	0.44	0.17–1.12	0.09
(A/A)	1/116 (0.9)	1.38	0.14–13.41	0.78
MBC patients with *BRCA2* 9346(−2)A→G				
(G/A)	2/8 (25.0)	3.22	0.63–16.44	0.16
(A/A)	0/8			
PC patients				
(G/A)	55/843 (6.5)	0.68	0.45–1.02	0.06
(A/A)	9/843 (1.1)	1.66	0.45–6.16	0.45

MBC patients homozygous for H63D and C282Y were younger (49 and 54 years) at the time of the diagnosis than the rest of the MBC patients in average (64.9 years). In *HFE* C282Y heterozygotes, a slight age difference at diagnosis was seen compared to MBC patients with 2 wild-type alleles (63.2 *vs*. 64.3 years), but the numbers were too small to perform any statistical analysis. H63D heterozygotes were older (67.2 *vs*. 64.3 years; $p = 0.20$) than MBC patients with 2 wild-type alleles.

The carrier frequencies of the 2 *HFE* variants were also compared between *BRCA2* mutation carriers and noncarriers. Patients with the most common *BRCA2* mutation 9346(−2)A→G were 2 times more often heterozygous for *HFE* H63D or C282Y than MBC cases without *BRCA2* mutations (4/8, 50% *vs*. 27/104, 26%; $p = 0.21$).

The carrier frequencies of the H63D and C282Y variants were also compared between PC patients and controls. There was no statistically significant difference in the carrier frequencies between the 2 groups (H63D: Pearson chi-square test, $p = 0.37$; C282Y: Pearson chi-square test, $p = 0.13$). The ORs for H63D and C282Y mutations were also calculated in order to estimate the PC risk (Table I). No significantly elevated or lowered risks for PC were found among these 2 variants. However, a borderline positive result for a lower risk of cancer among C282Y heterozygotes was detected (OR = 0.68; $p = 0.06$). The associations between the mutations and demographic, clinical, or pathologic characteristics of the cases or reported positive family history were calculated (data not shown) and no statistically significant associations were observed.

Discussion

HH and risk of different malignant neoplasia have recently been associated in various studies, including female breast cancer.[5] To assess the contribution of the *HFE* gene mutations to causation of male breast and prostate cancers in Finland, the 2 main variants, H63D and C282Y, were screened from 116 MBC

patients, 843 unselected patients with PC and 480 controls. To our knowledge, this is the first study where the associations of these *HFE* variants to MBC and PC in large sample sets were analyzed. Recently, a variation in *BRCA2* mutation spectrum between Finnish male and female breast cancer patients was seen.[8] The most common *BRCA2* mutation among MBC patients was 9346(−2)A→G, with a frequency of 67% (8/12 *BRCA2* mutation-positive cases). Although this was the second most common mutation among females (27%), the difference in frequencies was significant ($p = 0.03$). Thorlacius *et al*.[9] have also suggested a variation in male and female breast cancer penetrance among *BRCA2* mutation carriers in Iceland. Therefore, the existence of modifier factors has been anticipated but so far none has been identified. In this study, patients with *BRCA2* 9346(−2)A→G mutation were 2 times more often heterozygous for one of the H63D or C282Y variants than the rest of the MBC cases or controls, suggesting a possible modifier role for *HFE* in breast cancer penetrance among male *BRCA2* mutation carriers. However, as *HFE* mutations do not show a gender predilection, the concurrence with *BRCA2* 9346(−2)A→G mutation in MBC does not fully explain the increased penetrance of this *BRCA2* mutation in males compared to females.

Elevated serum iron concentrations and transferrin saturation values are seen among *HFE* heterozygotes compared with normal subjects,[16] leading to free radical formation and DNA damage. Mutated *BRCA2* in turn has no, or at least decreased, capacity for DNA repair. In combination, *HFE* and *BRCA2* aberrations may lead to increased mutation frequency, loss of DNA repair and ultimately malignant formation. However, further studies in cell lines are needed to prove this hypothesis. In addition, it would be interesting to compare the penetrance of the *BRCA2* 9346(−2)A→G mutation in carriers with and without HFE mutations by family screening. Unfortunately, most of the MBC patients are deceased and no samples were available from the family members.

In our study, the samples were not screened for novel mutations, so we cannot rule out that other *HFE* gene mutations are associated with MBC or PC. In addition, it is possible that the interaction of these genetic variants with other iron metabolism gene polymorphisms could influence the cancer risk.

Our results do not support a major role for the *HFE* mutations C282Y and H63D at the population level in the causation of MBC or PC in Finland. The frequencies of the H63D and C282Y mutations do not significantly differ between the cancer patients and the controls and no significantly altered risks for MBC or PC among these 2 variants were found. However, MBC patients with both a *BRCA2* 9346(−2)A→G and an *HFE* mutation are suggested to be at increased risk of breast cancer.

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Identification of germline *MLH1* alterations in familial prostate cancer

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ABSTRACT

Several linkage and loss of heterozygosity (LOH) analyses suggest that the region 3p21-p26, which is a chromosomal location of *MLH1*, could harbour a susceptibility gene for prostate cancer (PRCA). Furthermore, in a recent candidate single nucleotide polymorphism (SNP) analysis the I219V variation of the *MLH1* gene was associated with PRCA. Microsatellite instability (MSI) and germ-line *MLH1* mutations were originally demonstrated in hereditary non-polyposis colorectal cancer (HNPCC) but MSI and loss of *MLH1* function have also been detected in PRCA. To assess the contribution of *MLH1* germline mutations to the development of PRCA in Finland different approaches were used. First, the samples from 11 PRCA-colon cancer patients were screened for MLH1, MSH2 and MSH6 protein expression by immunohistochemistry (IHC). IHC revealed one patient with a putative *MLH1* aberration and sequencing of this sample revealed five sequence variants including two missense variants P434L and I219V. Second, the samples from Finnish hereditary prostate cancer (HPC) families were used for the screening of *MLH1* mutations which produced twelve *MLH1* sequence variants including two missense mutations, I219V, as in the PRCA-colon cancer patient, and V647M. P434L and V647 were both novel, rare variants. Carrier frequencies of the I219V mutation were compared between hereditary prostate cancer (HPC) patients, unselected PRCA cases, patients with benign prostate hyperplasia and controls, but no differences between the sample groups were found. P434L was not present in this study population and V647M was a very rare variant found only in one HPC family. According to the present results, *MLH1* does not have a major role in PRCA causation in Finland.

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1. Introduction

Numerous factors such as race, diet, environmental factors and family history have been considered to be risk factors for prostate cancer (PRCA).[1] Lichtenstein and colleagues[2] claim that approximately 42% of the risk of PRCA could be due to hereditary genetic factors. Despite the tremendous effort that has been put into hereditary prostate cancer (HPC) linkage studies to identify risk genes, only three susceptibility genes have been identified from the chromosomal regions mapped through these analyses: *ELAC2* at 17p11, *RNASEL* at 1q24-q25, and *MSR1* at 8p22-p23.[3–5] Although multiple chromosomal regions have been reported, only few regions have been confirmed in other independent studies. Very recently, three linkage analyses, including the one carried out on Finnish prostate cancer families, have shown positive linkage near the *MLH1* locus on 3p.[6–8] As *MLH1* is a known tumour suppressor gene, it therefore represents an immediate candidate gene for the detected locus. Initially, *MLH1* was associated with hereditary nonpolyposis colorectal cancer (HNPCC), where microsatellite instability (MSI) was observed with defects in the mismatch repair pathway (MMR). Inactivation of the MMR pathway in HNPCC patients is caused by germline mutation in one of the MMR genes, most often in *MLH1*[9] indicating its major role in guarding the genome integrity. Varying degrees of MSI (20–65%) and loss of the MMR proteins and down-regulation of MMR enzyme activityhave also been detected in PRCA,[10–14] suggesting a possible role for *MLH1* in prostatic carcinogenesis. *MLH1* maps to 3p22, a region also showing loss of heterozygosity (LOH) in human PRCA cell line [15] and clinical samples.[16] Furthermore, in a recent candidate single nucleotide polymorphism (SNP) analysis the variant I219V in *MLH1* showed an association with prostate cancer supporting the role for *MLH1* in prostate cancer predisposition.[17] Here, we report a study to evaluate the role of *MLH1* in PRCA predisposition in Finland.

2. Materials and methods

2.1. Patients with PRCA and colon cancer

A search of the discharge registry of the Tampere University Hospital revealed 355 PRCA patients with additional solid primary tumour (excluding skin malignancies except melanoma) from 1 January 1970 until 31 December 1999. Fifteen of the patients had both PRCA and colon cancer. Paraffin embedded prostate and/or colon cancer samples were available for analyses from 11 of them.

2.2. HPC families, patients with unselected PRCA and controls

Identification and collection of the Finnish HPC families have been described elsewhere.[6] For single-strand conformation polymorphism (SSCP) and minisequencing analyses samples from the youngest affected patient available in each of 121 families with HPC were used for the analysis. The families had either two or more affected members who were first- or second-degree relatives. The mean age at diagnosis for the index patients was 64.8 years (range 44–86 years), and the mean number of affected family members was 2.8 (range 2–7). For *MLH1* direct sequencing, genomic DNA samples from altogether 18 affected persons were selected, representing the six families with the best multipoint heterogeneity LOD scores (HLOD) (> 0.5) per family at 3p.[18]

MLH1 mutation frequencies were analysed among 200 unselected, consecutive patients with PRCA, 200 healthy male blood donors, and 202 patients diagnosed with benign prostate hyperplasia (BPH). The mean age at diagnosis for the unselected PRCA patients was 67.1 years (range 47–88). The DNA samples from unselected consecutive PRCA patients were collected from patients diagnosed with PRCA in 1999 in the Tampere University Hospital. Tampere University Hospital is a regional referral centre in the area for all patients with PRCA, which results in an unselected, population-based collection of patients. The population controls consisted of DNA samples from anonymous, voluntary and healthy male blood donors obtained from the Blood Center of the Finnish Red Cross in Tampere. The blood donor must be 18 to 65 years of age, which means that on average 8.5% of them will have a PRCA diagnosis later in life given the current incidence rates according to the Finnish Cancer Registry. The age-matched control group consisted of patients diagnosed with BPH in the Tampere University Hospital. The mean age for the BPH patients was 72.5 (range 48–93), which correlates with the mean age of diagnosis for the PRCA patients (67.1 years; range 47–88). 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 or digital rectal examination showed any abnormality indicative of PRCA the patients underwent biopsies to exclude diagnoses of PRCA, high-grade prostate intraepithelial neoplasia (PIN), atypical small acinar cell proliferation (ASAP), or suspicions of malignancy. Written informed consent was obtained from all living patients and also, for families with HPC, from the unaffected members.

2.3. Immunohistochemistry

MLH1-, MSH2-, and MSH6-immunohistochemistry was performed on paraffin sections in a LabVision Autostainer instrument (LabVision Corporation, Fremont, CA, USA). For epitope retrieval, the sections were subjected to four cycles (7 min + 3 × 5 min) of heating in a microwave oven at 850 W in Tris-EDTA buffer, pH 9.0. The following primary antibodies were used: for MLH1 clone G168-15 (BD Biosciences Pharmingen) at 1:25; for MSH-2 clone FE11 (Oncogene Sciences) at 1:150; for MSH-6 clone 44 (Transduction Laboratories) at 1:200. Visualisation of the primary antibody was done with the two-step Envision™ polymer kit (DakoCytomation Denmark A/S, Glostrup, Denmark) using diaminobenzidine as chromogen. Microscopic evaluation followed the recommendation of the International Collaborative Group of HNPCC.[19]

2.4. SSCP, resequencing and minisequencing

Mutation screening of the coding region of *MLH1* on 121 HPC cases was performed using SSCP analysis as described

previously.[20] For sequencing analysis PCR products were purified in 96-format Acro Prep Filter Plates (Pall Life Sciences, Ann Arbor, MI, USA) using the Perfect Vac Manifold vacuum machine (Eppendorf AG, Hamburg, Germany). Sequencing was performed according to the instructions of the manufacturer using BigDye Terminator v.3.1 Cycle Sequencing Kit and automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed with Sequencher 4.2.2 software (Gene Codes Corporation, Ann Arbor, MI, USA). The frequencies of the three *MLH1* variants were determined in the sample sets of patients and controls described above by minisequencing[21] or by direct sequencing. Primer sequences are available upon request from the authors.

2.5. *Statistical analyses*

Association of the I219V variant with unselected PRCA and HPC was tested by logistic regression analysis using SPSS statistical software package (SPSS 12.0). Association with clinical and pathological features of the disease (age at onset, PSA value at diagnosis, T-stage, WHO grade and Gleason score) was tested among unselected PRCA cases by the Pearson χ^2-test, Kruskal–Wallis test, Fisher's exact test, and *t*-test included in the SPSS statistical software package (SPSS 12.0).

3. Results

Immunohistochemistry (IHC) analysis of MLH1, MSH2 and MSH6 proteins among 11 PRCA-colon cancer patients revealed abnormal staining patterns in two patients; patient A with locally advanced PRCA and Dukes B mucinous carcinoma in descending colon pointing to MLH1 alteration and patient B with PRCA and mucinous carcinoma of the cecum revealed MSH2 abnormality. Enough tissue material for DNA extraction was available from patient A only. Sequencing the *MLH1* coding region from this patient's sample revealed two missense mutations: P434L in exon 12 and I219V in exon 8. Two silent mutations and one intronic variant were also found. SSCP analysis of the probands from 121 HPC families and direct sequencing of the affected cases from the six chromosome 3p-linked families revealed a total of 12 *MLH1* sequence variants (Table 1). None of the variants were truncating mutations. Three of the changes were located in exonic regions, I219V and V647M were missense variants and L653 was a silent change. Three of the variants were located in the 5'UTR or 3'UTR regions, and six of the changes took place in introns. The exonic changes I219V and L653 and intronic variants 453 + 79 A > G, 1558 + 14 G > A, and 1668-19 A > G have been previously reported.[22–25]

The frequencies of the three missense mutations of the *MLH1* gene were determined by minisequencing or direct sequencing among patients with unselected PRCA or HPC and in the two control groups. The P434L missense mutation found in the PRCA-colon cancer patient was not detected in any of those sample groups. The novel missense mutation V647M in exon 17 was found in only one HPC proband. In this family there was no sample from the second affected family member (deceased). V647M was neither detected among patients with unselected PRCA nor among population controls (Table 2). The carrier frequencies for I219V were 54.5%, 54.0%, 55.0%, and 54.0% in the probands with HPC, unselected PRCA cases, population controls and BPH cases respectively. The frequency of I219V was found to be in Hardy–Weinberg equilibrium. No statistically significant differences were observed in the carrier frequencies of I219V between the sample groups (Pearson χ^2 test, $P = 0.996$) and subsequently no association was seen between the variant I219V and HPC (odds ratio (OR), 0.98; 95% confidence interval (CI), 0.62–1.55) or unselected PRCA (OR, 0.96; 95% CI, 0.65–1.42) (Table 2). No association was detected even when the age-matched group of BPH cases was used as a control group (HPC; OR, 1.02; 95% CI, 0.65–1.61 and unselected PRCA; OR, 1.00; 95% CI, 0.68–1.48) (Table 2). The mean age at diagnosis of the I219V variant carriers among patients with unselected PRCA was statistically higher compared with non-carriers (68.1 *versus* 65.9 years; *t*-test, $P = 0.03$). No other statistically significant associations of the I219V variant with clinical or pathological features of the disease were observed (PSA value at diagnosis, T-stage, WHO grade and Gleason score, data not shown).

Table 1 – Summary of MLH1 variations found in Finnish colon-PRCA patients or HPC families

Mutation[a]	Amino acid change	Exon/Intron
−28 A > G[b]		5'UTR
−7 C > T[b]		5'UTR
453 + 79 A > G[c]		Intron 5
454 − 51 T > C[b]		Intron 5
655 A > G[b,c,d]	I219V	Exon 8
885 − 24 T > A[b]		Intron 10
1304 C > T[d]	P434L	Exon 12
1413 G > A[d]	K471	Exon 13
1558 + 14 G > A[b,c]		Intron 13
1558 + 58 G > A[d]		Intron 13
1668 − 19 A > G[b,c]		Intron 14
1878 C > T[d]	F626	Exon 16
1939 G > A[b]	V647M	Exon 17
1959 G > T[c]	L653	Exon 17
1990 − 121 C > T[c]		Intron 17
2271*35_37delCTT[b]		3'UTR

a Numbering is according to the cDNA (NM_000249) starting at the A in the start codon.
b A sequence variant was found by SSCP analysis of 121 HPC families.
c A sequence variant was found by direct sequencing of six 3p-positive HPC families.
d A sequence variant was found by sequencing colon-PRCA patient.

4. Discussion

Chen and colleagues[26] and Yeh and colleagues[14] have reported decreased expression of *MLH1* in PRCA cell lines and primary tumours. Subsequently, it was also shown that loss of the MMR function may result in MSI in secondary genes, like *BAX*, containing microsatellites in their coding regions[13] indicating that the MMR pathway may play an important role in the development of PRCA. Another reason for our interest in *MLH1* is based on the results of our recent genome-wide linkage study, which suggested a Finnish PRCA susceptibility locus

Table 2 – Association of the I219V MLH1 gene mutation with unselected PRCA or HPC

Sample	Carrier frequency (%)	OR (95 % CI)	P
Population controls	110/200 (55.0 %)	1.00	
Patients with unselected PRCA	108/200 (54.0 %)	0.96 (0.65 – 1.42)[a]	0.84[a]
		1.00 (0.68 – 1.48)[b]	0.99[b]
Patients with HPC	66/121 (54.5 %)	0.98 (0.62 – 1.55)[a]	0.94[a]
		1.02 (0.65 – 1.61)[b]	0.92[b]
Patients with BPH	109/202 (54.0 %)		

a Population controls (blood donors) used as a control group.
b BPH cases used as a control group.

to reside on 3p25-p26,[27] near the *MLH1* locus on 3p22. Recently this region has also been suggested to be a susceptibility locus for general cancer susceptibility genes among prostate cancer families[7] and for clinically significant prostate cancer.[8] Furthermore, in candidate SNP analysis from genomic regions that show linkage to prostate cancer susceptibility the I219V variation (rs1799977) of the *MLH1* gene was associated with PRCA.[17] These multiple pieces of evidence support the 3p-area harbouring a tumour suppressor gene.

Although PRCA is not considered to be a common feature of HNPCC, a combination of colorectal and prostate carcinomas has frequently been observed in the same patient[28–30] which could be partly explained by genetic influence. We screened 11 PRCA-colon cancer patients for MLH1, MSH2 and MSH6 protein expression using immunohistochemistry. SSCP analysis of the youngest affected patient available in each of the 121 families with HPC enabled us to search for the mutations from all the Finnish HPC families. Being aware of the decreased sensitivity of the SSCP method to reveal sequence variations we used direct sequencing to study all the affected cases available from six HPC families having positive linkage in 3p25-p26. These three approaches failed to detect any truncating mutations that would indicate a direct causative role for *MLH1* in HPC. Yet, three missense variants were detected. The novel variation P434L found in a PRCA-colon cancer patient was not detected in PRCA patients or controls. The novel missense mutation V647M in exon 17 proved to be extremely rare, as it was found only in one HPC family. The variant I219V behaved more like a polymorphism. Neither of these changes affected the charge status of the amino acid because all of them have nonpolar side chains. In addition, no co-segregation of the variants with the disease was seen in the HPC-families. Although the missense variant I219V found was frequently observed in PRCA patients, we failed to see any association between the variant I219V and HPC or unselected PRCA. In contrast to our results, Burmester and colleagues [17] reported a significant difference in allele frequency between the prostate cancer cases and controls for the I219V variant. The difference remained significant in an age-matched subsample. However, we could not detect any difference in carrier frequency even between the age-matched control group of BPH cases and patients with HPC or unselected PRCA. Interestingly, in the US study the major allele frequency was 0.728 in controls and 0.629 in PRCA cases, while in our study the major allele frequency was 0.661, 0.678, 0.67 and 0.678 in HPC cases, unselected PRCA cases, population controls and BPH cases respectively. Finland has a known genetically homogeneous founder population where ethnic differences in samples are minimised and therefore the allele frequencies should not be distorted due to admixture problems. Our result stands in line with the study by Liu and colleagues [22] that initially identified the changed I219V among colorectal cancer patients and reported it as a polymorphism having no causative role in the disease. On the other hand, Bagnoli and colleagues [31] recently reported an association between the variant I219V and refractory ulcerative colitis. Interestingly, among our unselected PRCA cases the carriers of the variant had a statistically significant tendency ($p = 0.03$) to be diagnosed at older age compared to non-carriers, suggesting that the polymorphism may influence the disease onset. This, however, would warrant further studies on other sample sets. Finally, even though the functional studies of *MLH1* variants by Trojan and colleagues [32] did not reveal any effect for I219V on MMR function, the possibility for that and subsequent PRCA involvement cannot be totally excluded. No such data are available for the rare novel mutations V647M and P434L found in this study.

In summary, no truncating or otherwise clearly deleterious mutations were observed in *MLH1* mutation analysis nor could we associate the I219V, P434L or V647M variants with PRCA in the Finnish population. Therefore, our results do not indicate a significant role for the *MLH1* gene in the causation of PRCA and therefore *MLH1* can most likely be excluded as a candidate gene for the 3p-linkage area, at least in the Finnish population.

Conflict of interest statement

None declared.

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