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Genetic Predisposition to Male and Female Breast Cancer

■

ACADEMIC DISSERTATION

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GENEETTINEN ALTTIUS MIESTEN JA NAISTEN RINTASYÖVÄSSÄ

Rintasyöpä on yleisin syöpätyyppi naisilla. Noin joka kymmenes nainen sairastuu rintasyöpään elämänsä aikana. Miesten rintasyöpä sen sijaan on harvinainen tauti. Alle 1% miesten syövistä on rintasyöpää, ja alle 1% kaikista rintasyövistä todetaan miehellä. Suurin osa syövistä on sattumalta syntyneitä, mutta arviolta 5-30% johtuu perinnöllisestä alttiudesta. Väitöskirjatyössäni selvitimme perinnöllisten, rintasyövälle altistavien geenien merkitystä miesten ja naisten rintasyövän syntyyn Suomessa.

Aluksi tutkimme suomalaisväestöstä löytyneiden 11 BRCA1- ja 8 BRCA2-mutaation yleisyyttä valikoimattomassa 1035 rintasyöpään sairastuneen naisen joukossa. Tästä otoksesta löysimme 4 BRCA1-geenin (0,4%) ja 15 BRCA2-geenin mutaation kantajaa (1,4%). Mutaation kantajat olivat keskimääräistä nuorempia, heidän sukulaisensa olivat huomattavasti useammin sairastuneet munasarjasyöpään, ja heillä oli useammin ainakin kaksi rintasyöpään sairastunutta sukulaista kuin henkilöillä, joilla ei ollut BRCA1- tai BRCA2-mutaatiota.

Tutkimme seuraavaksi 8 suomalaisessa väestössä todetun BRCA2-mutaation yleisyyttä 154 rintasyöpään sairastuneen miehen otoksessa. Lisäksi tutkimme koko BRCA2-geenin 34 mieheltä. Mutaatio löytyi 7,8% potilaita (12/154). Arvioimme, että kaikkiaan 12-13% suomalaisista miesrintasyöpäpotilaista on BRCA2-mutaation kantajia, kun otetaan huomioon sekä toistuvat että yksittäiset mutaatiot. BRCA2-mutaation kantajat olivat saman ikäisiä kuin ei-kantajat. Lähes puolet (44%) mies-rintasyöpäpotilaista, joiden sukulaisilla esiintyi rinta- tai munasarjasyöpää, olivat BRCA2 mutaation kantajia, kun taas mutaatiot olivat harvinaisia (3,6%) miehillä, joilla sukutaustaa ei ollut. Eri BRCA2-mutaatioiden yleisyys poikkesi suomalaisissa mies- ja naisrintasyöpäväestöissä toisistaan. Tähän saattaa olla syynä jokin BRCA2-mutaation kantajien syöpäriskiin vaikuttava geneettinen tai ympäristötekijä.

Seuraavaksi selvitimme AR-geenimuutosten vaikutusta miesten rintasyöpäriskiin. Tutkimme AR-geenin koodaavan alueen sekä geenissä esiintyvät polymorfiset CAG- ja GGC-toistojaksot 32 rintasyöpään sairastuneelta mieheltä. Lisäksi tutkimme suomalaisilta eturauhassyöpäpotilailta löydetyn Arg726Leu mutaation yleisyyttä 117 rintasyöpään sairastuneen miehen otoksessa. Totesimme, että AR-mutaatiot ovat hyvin harvinaisia rintasyöpään sairastuneilla miehillä ja että toistojaksojen pituudella ei näytä olevan suurta vaikutusta miesten rintasyöpäriskiin.

Äskettäin totesimme, että solusykliä säätelevän kasvurajoitegeenin, CHEK2, mutaatio 1100delC oli huomattavasti yleisempi rintasyöpään sairastuneilla naisilla, joiden suvuissa esiintyi rintasyöpää, kuin kontrolliväestöllä (Vahteristo ym. 2002). Tutkimme neljännessä osatyössä tämän mutaation merkitystä miesten rintasyövän riskitekijänä 114 rintasyöpään sairastuneen miehen kohortissa. Mutaatio esiintyi 1,8% (2/114) miehistä eli yhtä usein kuin terveellä väestöllä (1,4%, 26/1885) (Vahteristo ym. 2002). CHEK2-mutaation kantajat olivat saman ikäisiä kuin koko kohortti keskimäärin, eikä mutaation kantajilla ollut suvuissaan muita syöpiä. Näin ollen

totesimme, että CHEK2 1100delC -mutaatio ei vaikuta miesten rintasyövän riskiin Suomessa.

Yhteenvetona, BRCA2-mutaatiot auttavat selittämään suurimman tunnetun osan miesten rintasyövän perinnöllisestä alttiudesta Suomessa. Toistuvien BRCA2-mutaatioiden yleisyys poikkeaa suomalaisilla mies- ja naisrintasyöpäpotilailla toisistaan. Syynä tähän saattaa olla jokin eri tavoin miesten ja naisten rintasyöpäriskiin vaikuttava geneettinen tai ympäristötekijä. AR-geenimuutokset ja CHEK2 1100delC -mutaatio eivät merkittävästi altista miesten rintasyövälle Suomessa. Osa miesrintasyöpäpotilaista on mahdollisesti BRCA1-mutaatioiden tai vielä tunnistamattomien rintasyövälle altistavien geenien mutaatioiden kantajia.

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

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

I. Syrjäkoski K*, Vahteristo P*, Eerola H, Tamminen A, Kivinummi K, Sarantaus L, Holli K, Blomqvist C, Kallioniemi OP, Kainu T and Nevanlinna H (2000): Population-based study of BRCA1 and BRCA2 mutations in 1035 unselected Finnish breast cancer patients. J Natl Cancer Inst 92:1529-1531. Published earlier in Vahteristo P (2003): Susceptibility genes in hereditary breast cancer. University of Helsinki. * equal contribution

II. Syrjäkoski K, Kuukasjärvi T, Waltering K, Haraldsson K, Auvinen A, Borg Å, Kainu T, Kallioniemi OP and Koivisto PA (2004): BRCA2 mutations in 154 Finnish male breast cancer patients. Neoplasia 6:541-545.

III. Syrjäkoski K, Hyytinen ER, Kuukasjärvi T, Auvinen A, Kallioniemi OP, Kainu T and Koivisto PA (2003): Androgen receptor gene alterations in Finnish male breast cancer. Breast Cancer Res Treat 77:167-170.

IV. Syrjäkoski K, Kuukasjärvi T, Auvinen A and Kallioniemi OP (2004): CHEK2 1100delC is not a risk factor for male breast cancer population. Int J Cancer 108:475-476.

ABBREVIATIONS

A	adenine
AR	androgen receptor
Arg	arginine
ASO	allele-specific oligonucleotide hybridization
ATM	ataxia-telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia-telangiectasia and rad-3 related
BACH1	BRCA1-associated C-terminal helicase
BAP1	BRCA1-associated protein 1
BARD1	BRCA1-associated ring domain gene 1
BLM	Bloom syndrome gene
bp	base pair
BRAF35	BRCA2-associated factor 35
BRC	repeats in BRCA2
BRCA1	breast cancer 1 gene
BRCA2	breast cancer 2 gene
BRCT	BRCA1 carboxy-terminal repeat
BUBR1	human homolog of *S. cerevisiae* budding uninhibited by benzimidazoles1
C	cytocine
c-Abl	abelson murine leukemia viral oncogene homolog 1
Cdc2	cell division cycle 2
Cdc25	cell division cycle 25
Cds1	*S. pombe* cytidine diphosphate -diacylglycerol synthase
CHEK2	checkpoint kinase 2
CpG	cytosine-phosphate-guanine
CtIP	human (adenovirus c-terminal-binding protein) –interacting protein
CYP17	cytochrome P450α17
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
ER	estrogen receptor
FCR	Finnish Cancer Registry
G	guanine
GADD45	growth arrest- and DNA damage-inducible gene
Gln	glutamine
Gly	glycine
GRIP1	glutamate receptor-interacting protein 1
H2A-X	H2A histone family, member X
HFE	hemochromatosis gene
HNPCC	hereditary nonpolyposis colorectal cancer
Leu	leucine
LFS	Li-Fraumeni syndrome
LOH	loss of heterozygosity
Lys	lysine
MBC	male breast cancer
mRNA	messenger RNA
MLH1	human mutator l homolog 1
MRE11	human meiotic recombination homolog 11

MSH2	human mutator s homolog 2
MSH6	human mutator s homolog 6
MYC	avian myelocytomatosis viral oncogene homolog
NBS1	Nijmegen breakage syndrome gene 1
OCCR	ovarian cancer cluster region
PCR	polymerase chain reaction
P/CAF	p300/creb-binding protein -associated factor
Plk1	Polo-like kinase 1
PMS1	postmeiotic segregation increased 1
PMS2	postmeiotic segregation increased 2
PTEN	phosphatase and tensin homolog
PTT	protein truncation test
RAD50	human homolog of *S. cerevisiae* Rad50
RAD51	human homolog of *S. cerevisiae* Rad51
Rad53	*S. cerevisiae* serine/threonine protein kinase Rad53
RB	retinoblastoma
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SQ/TQ	serine glutamine/threonine glutamine
SRC	avian sarcoma (Schmidt-Rupina A-2) viral oncogene
SSCP	single-strand conformation polymorphism
STK11	serine/threonine protein kinase 11
SWI/SNF	human homolog of *S. cerevisiae* mating type switching and sucrose nonfermenting
T	thymine
TP53	tumor protein p53
ZBRK1	zinc finger and BRCA1-interacting protein with a krab domain 1

ABSTRACT

Breast cancer is the most common malignancy among women. It affects one out of every ten women at some point of their lives. Male breast cancer (MBC), on the other hand, is a rare disease that accounts for less than 1% of all cases of cancer in men and less than 1% of all breast cancers. Most breast cancers are sporadic but it has been estimated that 5-30% of breast cancers are due to hereditary predisposition. The aim of this thesis was to estimate the contribution of genetic susceptibility to breast cancer rate on unselected Finnish female and male breast cancer populations.

First, we evaluated the frequency of 11 known Finnish BRCA1 and 8 BRCA2 mutations in 1035 unselected female breast cancer patients. Four BRCA1 (0.4%) and 15 BRCA2 (1.4%) mutation carriers were identified. As compared to non-carriers, mutation carriers tended to be younger in age and more often had a positive family history of ovarian cancer or at least two relatives previously diagnosed with breast cancer.

We then studied the frequency of 8 Finnish BRCA2 mutations in a series of 154 MBC patients. In addition, we screened the entire BRCA2 gene for the presence of novel mutations in a cohort of 34 MBC patients. Mutations were identified in 7.8% (12/154) of the patients. We estimated that the total mutation burden, including both recurrent and sporadic mutations, was approximately 12-13%. Mutation carrier status did not significantly affect the age at which breast cancer diagnoses was made. Patients with a positive family history of breast/ovarian cancer were often BRCA2 mutation carriers (44%), whereas those with no family history showed a lower frequency of involvement (3.6%). The frequency of different BRCA2 mutations varied between male and female breast cancer populations. This suggests that modifying genetic and environmental factors may significantly influence the penetrance of male and female breast cancer in individuals carrying germline BRCA2 mutations.

Next, we evaluated the impact of androgen receptor (AR) gene alterations on the risk of MBC. We screened the coding region of the AR gene for mutations and studied the role of AR CAG and GGC repeat lengths as risk factors for MBC in a cohort of 32 Finnish MBC patients. We also estimated the involvement of the prostate cancer predisposing Arg726Leu germline mutation in a cohort of 117 MBC patients. No germline mutations were found. These data indicate that AR mutations are very rare among MBC patients and that the lengths of the AR repeats do not markedly influence the risk of MBC.

Recently, we observed that the cell-cycle checkpoint kinase (CHEK2) mutation 1100delC was associated with breast cancer patients with a positive family history of breast cancer (Vahteristo et al. 2002). Finally, we evaluated the contribution of this mutation to the MBC risk in a cohort of 114 Finnish MBC patients. The mutation was as common among the patients (1.8%; 2/114) as among the healthy controls (1.4%; 26/1885) (Vahteristo et al. 2002). CHEK2 1100delC did not influence the age at

diagnosis. Mutation carriers did not have a positive family history of cancer. In conclusion, CHEK2 1100delC does not seem to increase the risk of MBC in Finland.

In conclusion, BRCA2 mutations help explain the largest known proportion of hereditary predisposition to MBC in Finland. The frequency of Finnish BRCA2 founder mutations is different among male and female breast cancer patients. There may be modifying genetic and environmental factors that influence the penetrance of male and female breast cancer in individuals carrying germline BRCA2 mutations. AR gene alterations and the CHEK2 1100delC mutation are not significant in the rate of MBC predisposition in Finland. Some of the MBC cases are probably carriers of BRCA1 mutations or mutations of still unidentified novel breast cancer susceptibility genes.

INTRODUCTION

Breast cancer is the most common malignancy among women. It affects one out of every ten women at some point of their lives. Male breast cancer (MBC), on the other hand, is not nearly as common a disease. Therefore, most of the available data on MBC arise from small studies involving a few dozen patients collected from a single hospital.

The majority of breast cancers are sporadic, but 5-30% is estimated to be caused by a genetic predisposition. Few genes, such as BRCA1 and BRCA2, are known to be involved in the predisposition of female and male breast cancer. Mutation frequencies of these genes vary greatly among different populations and among different subgroups of patients, such as young patients, MBC patients and patients with many affected family members. The frequencies of BRCA1 and BRCA2 germline mutations among Finnish breast/ovarian cancer families has been studied but the frequencies among unselected Finnish female and male breast cancer populations have remained unknown.

Gene alterations in AR have also been suggested to predispose to MBC. Only part of the coding region of the AR has been screened for mutations in many studies and the number of subjects has been small. Therefore, some AR gene alterations may have remained undetected. The contribution of the AR gene alterations to MBC incidence in Finland has not been studied.

Recently, a CHEK2 1100delC mutation was associated with increased risk of MBC, but no other studies have been done on CHEK2 and MBC. The frequency of CHEK2 1100delC is elevated in Finnish female breast cancer patients with affected family members compared to population controls. The contribution of the CHEK2 1100delC mutation to MBC predisposition in Finland has not been evaluated.

Although MBC is a rare disease, the number of family members who may carry a hereditary breast cancer predisposing gene alteration merits attention. Furthermore, insights to the genetic predisposition to MBC may also shed light to the causation and predisposition of the much more common female breast cancer.

REVIEW OF THE LITERATURE

1. Male and female breast cancer

1.1 Incidence

Breast cancer is the most common malignancy among women in Finland and other industrialized countries. In the year 2002, 3760 new female breast cancer cases were diagnosed in Finland and this number continues to grow annually (Finnish Cancer Registry 2004).

In contrast, male breast cancer is a rare disease that accounts for less than 1% of all cases of cancer in men and less than 1% of all breast cancers in most countries (Sasco et al. 1993). In the United States, about 1450 new MBC cases will be diagnosed in 2004 and 470 men will die of the disease (Jemal et al. 2004). In Finland, 18 new cases were diagnosed in 2002, and in 2001, 5 men died of the disease (Finnish Cancer Registry 2004).

Age-adjusted MBC incidence is about 1 per 100 000 person-years or less in most countries. Country-specific differences in the incidence of MBC parallel those in women (Sasco et al. 1993, Ewertz et al. 1989). In Finland, the age-adjusted MBC incidence was 0.4 per 100 000 person-years in 2002 (Finnish Cancer Registry 2004). The incidence of MBC has remained stable over the past decades, which is in contrast to the increasing incidence of breast cancer in women (Ewertz et al. 1989, Sasco et al. 1993, Anderson et al. 2004, Finnish Cancer Registry 2004).

Men of all ages can be affected by breast cancer (Sasco et al. 1993). Mean age at diagnosis of MBC (61-68 years) is 5 to 10 years higher than that of female breast cancer (Sasco et al. 1993, Cutuli et al. 1995, Donegan and Redlich 1996, Hill et al. 1999, Anderson et al. 2004). Incidence rates for MBC increase steadily as a function of age, whereas rates for women increase rapidly until the age of 50 years and then continue to rise more slowly (Ewertz et al. 1989, Anderson et al. 2004).

1.2 Histopathology

Breast carcinoma originates from the epithelial cells of the terminal duct lobular unit (reviewed in Sainsbury et al. 2000). *In situ* breast cancer or non-invasive breast cancer remains within the basement membrane. Invasive cancer spreads outside the basement membrane and is divided into two major types, ductal and lobular carcinoma, ductal invasive breast cancer being the most common. Rare invasive breast cancer types include medullary, mucinous, papillary, tubular and cribriform.

Diagnostic evaluation and staging of breast cancer is similar in both sexes. All of the histologic subtypes of breast cancer that have been described in women have also been reported in men (Donegan and Redlich 1996, Giordano et al. 2002). About 90% of all breast tumors in men are invasive carcinomas and only 10% are noninvasive (Stalsberg et al. 1993, Cutuli et al. 1995, 1997). Almost all of the non-invasive cancers are ductal carcinoma *in situ*. Lobular carcinoma *in situ* is extremely rare. Invasive ductal carcinoma accounts for more than 80% of all tumors and papillary carcinoma about 5%. Invasive lobular carcinoma represents only 1-3% of all cases (Goss et al. 1999). The more rare subtypes account for the rest of the cases.

Tumors in males are often in the central subareolar region and involve the nipple (Goss et al. 1999). A slight tendency towards the left breast has been suggested (Sasco et al. 1993, Donegan and Redlich 1996). Bilateral breast cancer is rare among men (Donegan and Redlich 1996, Goss et al. 1999). Male breast cancers tend to be of a higher grade compared to the female breast cancers although contradictory results have been published (Willsher et al. 1997, Muir et al. 2003, Anderson et al. 2004). Male breast carcinomas are more often estrogen (about 80% of cases) and progesterone (70-75%) receptor positive than female carcinomas (Donegan and Redlich 1996, Cutuli et al. 1995, Giordano et al. 2002, Muir et al. 2003, Bärlund et al. 2004). Expression of molecular markers associated with favorable (Bcl-2) or with poor prognosis (ERBB2, p53, cyclin D1) is quite similar between the two sexes in many studies, although some studies have reported differences (Anelli et al. 1995, Weber-Chappuis et al. 1996, Wick et al. 1999, Shpitz et al. 2000, Bärlund et al. 2004, Bloom et al. 2001, Giordano et al. 2002, Wang-Rodrigues et al. 2002, Muir et al. 2003, Rudlowski et al. 2004). In a study by Tirkkonen et al. (1999), accumulation of somatic genetic changes during tumor progression of sporadic and BRCA2-associated male breast tumors was almost identical to those identified in the corresponding sporadic and BRCA2-associated female breast cancers. It has been suggested that MBC resembles more postmenopausal than premenopausal female breast cancer (Anderson et al. 2004).

1.3 Risk factors

Breast cancer is caused by both environmental and genetic factors. Family history of breast and/or ovarian cancer is one of the strongest risk factors for female and male breast cancer. Other risk factors that have been associated with female breast cancer include age, early menarche, nulliparity, late age at first birth, late menopause, obesity, hormone replacement therapy, oral contraceptives, radiation exposure, and being born in developed countries (reviewed in McPherson et al. 2000).

Many of the risk factors for MBC involve increased estrogen to androgen levels, indicating that breast cancer in men, as in women, may be hormonally driven. Risk factors associated with MBC include infertility, liver disease, obesity, orchiectomy, orchitis, testicular injury, and undescended testes (Sasco et al. 1993, D'Avanzo and La Vecchia 1995, Hsing et al. 1998, Sorensen et al. 1998, Ewertz et al. 2001). Men

with the Klinefelter's syndrome, characterized by the 47,XXY karyotype, small testes, azospermia, and gynecomastia, may have up to a 50-fold increased risk of breast cancer (Hasle et al. 1995, Hultborn et al. 1997, Swerdlow et al. 2001). Between 3 to 20% of MBC patients have the Klinefelter's syndrome, compared to only 0.1% in the general population (Hultborn et al. 1997). Benign breast conditions, race and ethnic background, age, life-style variables such as high social class, occupational exposures, radiation and certain drugs e.g. estrogens, digoxin and methyldopa have an impact on breast cancer risk (Sasco et al. 1993, D'Avanzo and La Vecchia 1995, Ganly and Taylor 1995, Cocco et al. 1998, Pukkala and Weiderpass 1999, Ewertz et al. 2001, Anderson et al. 2004). Gynecomastia does not likely represent a significant risk factor (Goss et al. 1999, Yildirim and Berberoglu 1998, Braunstein 1993, Ewertz et al. 2001, Giordano et al. 2002).

Family history of breast/ovarian cancer is a strong risk factor for MBC. Approximately 15-20% of MBC patients have a positive family history compared to 7% of the general male population (Goss et al. 1999, Hill et al. 1999, Giordano et al. 2002). Men with a female relative with breast cancer have an odds ratio of 2.17 for developing breast cancer and those with an affected male relative have an even higher risk (odds ratio 3.98) (Rosenblatt et al. 1991). The risk increases with an increasing number of first-degree relatives affected and with a young age at diagnosis of affected relatives (Rosenblatt et al. 1991). The age at presentation, the duration of symptoms, the stage of the disease at presentation or the overall survival do not seem to be influenced by family history (Goss et al. 1999, Hill et al. 1999).

Second primary malignancies affect 5-15% of men and correspond to neoplastic disease patterns expected in the male population: prostate, gastrointestinal tract, lung and skin (Donegan and Redlich 1996, Auvinen et al. 2002). In a large study based on the Surveillance, Epidemiology, and End Results program, no overall increased risk of subsequent cancer was seen among MBC patients (Auvinen et al. 2002). Although bilateral breast cancer is rare among men, the risk of subsequent contralateral breast cancer was strongly elevated. Men with a primary cancer other than breast cancer did not have an increased risk of subsequent breast cancer (Auvinen et al. 2002). Recently, it has been suggested that there is an association between MBC and prostate cancer but not all studies are in agreement (Grabrick et al. 2003, Leibowitz et al. 2003, Thellenberg et al. 2003).

1.4 Treatment and prognosis

Breast cancer treatment options include surgery, radiation, chemotherapy and hormone treatment. Because of the rarity of MBC, treatment recommendations have been extrapolated from those of women (Giordano et al. 2002, Volm 2003).

The prognosis of breast cancer has greatly improved during the last decades. The five-year relative survival rate for female breast cancer patients, based on the Finnish Cancer registry data, is now approximately 80% (Dickman et al. 1999). Axillary

lymph node status, tumor size, histologic grade and hormone receptor status have been shown to be significant prognostic factors in women as well as in men with breast cancer (Cutuli et al. 1995, Giordano et al. 2002). The 5-year survival rate for MBC patients with a stage I disease is 55-100%, stage II 41-78%, stage III 16-62% and stage IV 0-14%. Survival rates are 57-100% for lymph node negative and 25-65% for lymph node positive cases (Donegan and Redlich 1996, Giordano et al. 2002). Clinical outcome for both sexes is similar when matched with major prognostic factors (Cutuli et al. 1995, Willsher et al. 1997, Goss et al. 1999, Hill et al. 1999, Vetto et al. 1999, Giordano et al. 2002). The overall survival rates for men are lower than for women, but this is probably due to later stage at presentation, more advanced age, and higher rates of death from intercurrent illness (Cutuli et al. 1995, Donegan and Redlich 1996, Goss et al. 1999)

2. Genetics of cancer

Cancer is a genetic disease. It is thought to originate from a single cell that has acquired a series of genetic and epigenetic changes, providing the cell with a growth advantage. Capabilities of cancer cells include self-sufficiency in growth signals, insensitivity to antigrowth signals, escape from apoptosis, unlimited replication potential, angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2000).

Two types of genes are involved in cancer predisposition, oncogenes and tumor suppressor genes. Proto-oncogenes often encode secreted growth factors, cell surface receptors, components of the intracellular signaling, nuclear transcription factors and cell cycle regulators. Proto-oncogenes, when inappropriately activated by a point mutation, translocation or amplification, turn into oncogenes. Activation of one allele is sufficient to give a growth advantage. Over 100 oncogenes have been identified so far but inherited mutations in oncogenes are rare.

Tumor suppressor genes are divided into gatekeepers, caretakers or landscapers (Kinzler and Vogelstein 1997, 1998). Their inactivation leads to cancer. Gatekeepers directly regulate tumor growth by regulating proliferation, promoting differentiation or by accelerating cell death. Caretakers guard genome integrity through DNA repair and replication. Landscapers transform adjacent cells by abnormal intercellular signaling (Kinzler and Vogelstein 1998). According to the Knudson's two-hit hypothesis, both copies of the tumor suppressor gene have to be inactivated for cell transformation (Knudson 1971, Knudson 2001). In hereditary cancer, one defective allele is inherited and the other allele is often lost by a somatic inactivating alteration such as a loss of heterozygosity (LOH) by a large deletion. The second hit can also be an epigenetic silencing of the remaining allele by hypermethylation of a CpG island in the promoter region or by controlling the acetylation status of histones (reviewed in Plass 2002). Sometimes loss of a single tumor suppressor allele, haploinsufficiency, is enough to cause tumor progression (reviewed in Balmain et al. 2003).

3. Hereditary predisposition to breast cancer

Most breast cancers are sporadic and are caused by somatic genetic alterations. It has been estimated that 5-30% of breast cancers are caused by hereditary predisposition (Lynch et al. 1984, Claus et al. 1996, Lichtenstein et al. 2000). Only a minority of women belong to high-risk families with multiple cases of breast cancer, ovarian cancer, bilateral breast cancer, male breast cancer and/or cases diagnosed at an early age. Mutations in high-penetrance susceptibility genes, such as BRCA1 or BRCA2, cause predisposition to cancer in some of these families. In many of the families, clustering of cancers is believed to be caused by defects in one or more low- to moderate-penetrance genes (Antoniou et al. 2002, Narod and Foulkes 2004). Also, clustering of sporadic cases in a family is often taking place due to the high incidence of the disease in the general population.

Increased risk of breast cancer is also associated with a few rare hereditary cancer syndromes, such as ataxia-telangiectasia (MIM 208900; caused by mutations in ATM), Cowden disease (MIM 158350; PTEN), hereditary nonpolyposis colorectal cancer (MIM 114500; MLH1, MSH2), Li-Fraumeni syndrome (MIM 151623; TP53 and CHEK2) and Peutz-Jeghers syndrome (MIM 175200; STK11) (Boardman et al. 1998, Bell et al. 1999, Allinen et al. 2001, Birch et al. 2001, Fackenthal et al. 2001, Olsen et al. 2001, Vahteristo et al. 2001c, Allinen et al. 2002, Chenevix-Trench et al. 2002, Concannon 2002, Borresen-Dale 2003, Eng et al. 2003, Lim et al. 2003).

Only few genes have been suggested to be involved in the etiology of MBC. These include the high-penetrance breast cancer susceptibility genes BRCA1 and BRCA2. A CHEK2 1100delC mutation has been associated with MBC (Meijers-Heijboer et al. 2002). Gene alterations in AR, MLH1, PTEN and CYP17 genes have also been reported among MBC patients (Wooster et al. 1992, Lobaccaro et al. 1993a, b, Risinger et al. 1996, Boyd et al. 1999, Young et al. 1999, Borg et al. 2000, Fackenthal et al. 2001, Gudmundsdottir et al. 2003).

3.1 BRCA1

3.1.1 BRCA1 gene and protein

The first high penetrance breast cancer susceptibility gene Breast cancer 1 gene (BRCA1; MIM 113705) was identified using linkage analysis and positional cloning in early-onset breast cancer families (Hall et al. 1990, Miki et al 1994). BRCA1 is located on chromosome 17q21. These 81 kilobases of genomic DNA have an unusually high density (41.5%) of Alu repetitive DNA (Smith et al. 1996). BRCA1 has two alternative transcription initiation sites in non-translated exons 1a and 1b and several splice variants (Xu et al. 1995, Miki et al. 1994). Exon 4 is not translated (Miki et al. 1994). The very large exon 11 encodes 61% of the 1863 amino acids long protein and the rest of the protein is encoded by 21 exons.

The BRCA1 protein exhibits only weak similarities with other protein sequences. It has an N-terminal RING-finger, which is involved in protein-protein interactions such as between BRCA1-homodimers and BRCA1-BARD1 and BRCA1-BAP1 heterodimers and which exhibits ubiquitin protein ligase activity (Figure 1) (Miki et al 1994, Wu et al. 1996, Brzovic et al. 1998, Jensen et al. 1998, Ruffner et al. 2001). Two nuclear localization signals are located in exon 11 (Chen et al. 1996). BRCA1 also contains a putative transactivation domain (Chapman and Verma 1996). The carboxy-terminal end contains two BRCA1 C-terminal repeats (BRCT domains) that interact with multiple transcription activators and repressors (Koonin et al. 1996). The BRCT motif is often found in proteins involved in DNA repair and metabolism (Callebaut and Mornon 1997). BRCA1 is expressed in numerous tissues, including breast and ovary, with the highest transcript levels in testis and thymus (Miki et al. 1994). Mouse Brca1 is expressed in rapidly proliferating cells and in differentiating tissues, including mammary epithelial cells during puberty and pregnancy (Marquis et al 1995). BRCA1 expression is cell cycle dependent (Gudas et al. 1996, Rajan et al. 1996, Vaughn et al. 1996b).

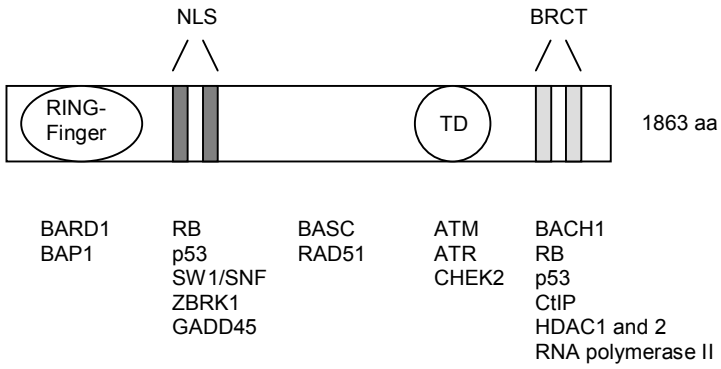

Figure 1. BRCA1 functional domains and interacting proteins. NLS, nuclear localization signals; TD, transactivation domain; BRCT, BRCA1 C-terminal repeats.

3.1.2. Function of BRCA1

The BRCA1 protein has several proposed functions (reviewed in Venkitaraman 2002). BRCA1 works in preserving chromosome structure, based on its role in double-strand DNA break repair by homologous recombination. After DNA damage or a replication block, BRCA1 is phosphorylated at specific sites by ATM, CHEK2 and ATR kinases (Cortez et al. 1999, Lee et al. 2000, Tibbetts et al. 2000). BRCA1 migrates to the site of DNA damage, marked by phosphorylated H2A-X histones. BRCA1 binds DNA directly and interacts with enzymes that alter chromatin and DNA structure, including SWI/SNF chromatin-remodeling complex, regulators of histone acetylation/deacetylation and DNA helicases BLM and BACH1 (Yarden and Brody 1999, Bochar et al. 2000, Wang et al. 2000, Paull et al. 2001, Cantor et al. 2001). BRCA1 is also involved in centrosome duplication and in controlling the cell cycle (Xu at al. 1999, Deng 2002, Yarden et al. 2002).

BRCA1 is part of a BRCA1-associated genome surveillance complex, which contains several proteins (e.g. MSH2, MSH6, MLH1 and ATM) that are able to recognize abnormal DNA structures (Wang et al. 2000). BRCA1 interacts with the double-strand DNA break repair protein complex MRE11/RAD50/NBS1, proteins that also migrate to sites of phosphorylated H2A-X (Zhong et al. 1999, Wang et al. 2000). A small fraction (2-5%) of BRCA1 is complexed with RAD51 recombinase, an essential enzyme for double-strand DNA break repair by homologous recombination (Scully et al. 1997). BRCA1 regulates, together with a transcription factor ZBRK1, the expression of GADD45, a tumor suppressor gene that is also a target of the p53 pathway (Harkin et al. 1999, Li et al. 2000, Zheng et al. 2000).

BRCA1 interacts with the RNA polymerase II holoenzyme through RNA helicase A (Anderson et al. 1998). Other proteins involved in transcription and RNA metabolism with BRCA1 include AR, p53, RB, ER-α, c-MYC and CtIP (Chapman and Verma 1996, Scully et al. 1997, Anderson et al. 1998, Wang et al. 1998, Zhang et al. 1998, Fan et al. 1999, Deng and Brodie 2000, Yeh et al. 2000, Zheng et al. 2001). The BARD1/BRCA1 complex, like other RING proteins, functions as an ubiquitin ligase of unknown specificity (Hashizume et al. 2001, Ruffner et al. 2001). BARD1 is also suggested to inhibit RNA processing following DNA damage, possibly together with BRCA1 and a polyadenylation factor (Kleiman and Manley 1999, 2001, Xu et al. 1999). BRCA1 has also been linked with X-chromosome inactivation in females and with Fanconi's anemia (Buller et al, 1999, Garcia-Higuera et al. 2001, Ganesan et a. 2002). Its role in early embryogenesis is crucial since knockout mice die during gestation (Gowen et al. 1996).

3.1.3 BRCA1 mutations and cancer

Over 2000 distinct mutations, polymorphisms and variants of unknown function have been identified in the BRCA1 gene (Breast Cancer Information Core: http://research.nhgri.nih.gov/bic/), most of the data arising from studies of female breast-ovarian cancer families. Mutations are distributed throughout the coding sequence without any mutational hotspots. The majority of the disease causing mutations is frameshift mutations (small insertions or deletions), causing a premature stop codon. Other disease-associated mutations include nonsense and splice-site mutations. Large genomic rearrangements have also been reported. The relevance of many of the BRCA1 missense variants to the cancer predisposition is unclear.

Although most mutations are unique, recurrent founder mutations have been identified in many populations, e.g. Ashkenazi Jews, Belgians, Dutch, Finns, French Canadians, Germans, Hungarians and Norwegians and among Polish and Scottish/Northern Irish people (Petrij-Bosch et al. 1997, Struewing et al. 1997, Vehmanen et al. 1997a, Huusko et al. 1998, Tonin et al. 1998, Claes et al. 1999, van der Looij et al. 2000, Meindl et al. 2002, Heimdal et al. 2003, Janiszewska et al. 2003, Scottish/Northern Irish BRCAI/BRCA2 Consortium 2003). In Finland, 6 recurrent BRCA1 and 5 BRCA2 founder mutations account for the majority (84%) of

mutation positive female breast cancer families (Vehmanen et al. 1997a, b, Huusko et al. 1998).

Mutations in BRCA1 predispose carriers to breast and ovarian cancers. Tissue-specificity of BRCA1 mutation-associated cancers has been proposed to be due to the estrogen responsiveness of BRCA1 (Hilakivi-Clarke 2000). The risk of breast and ovarian cancer among BRCA1 and BRCA2 mutation carriers vary greatly in different reports. This variation may depend on the population, the difference in mutations and modifying genetic factors. The first studies of large breast cancer families found the cumulative breast cancer risk for BRCA1 mutation carriers to be 75-87% and the ovarian cancer risk 44-63% by the age of 70 (Ford et al. 1994, Easton et al. 1995 Narod et al. 1995b). More recent risk estimates, especially in population-based studies, have shown lower breast and ovarian cancer penetrance estimates (Struewing et al. 1997, Fodor et al. 1998, Hopper et al. 1999, Anglian Breast Cancer Study Group et al. 2000, Satagopan et al. 2001, Antoniou et al. 2003). BRCA1 mutations possibly also predispose carriers to cancers of the fallopian tube, prostate, pancreas, uterine, cervix, colon, stomach, invasive squamous cell cancer of the skin and leukemias/lymphomas (Ford et al. 1994, Struewing et al. 1997, Johansson et al. 1999, Warner et al. 1999, Lal et al. 2000, Risch et al. 2001, Brose et al. 2002, Thompson et al. 2002b, Liede et al. 2004).

Early studies on high-risk families indicated that about 80% of hereditary susceptibility to female breast cancer was due to mutations in BRCA1 or BRCA2 (Easton et al. 1993, Narod et al. 1995a, Ford et al. 1998). Recently much lower frequencies among breast cancer families, around 20-50%, have been reported (Håkansson et al. 1997, Vehmanen et al. 1997a, b, Frank et al. 1998, Tonin et al. 1998, Wagner et al. 1998, Santarosa et al. 1999, Ikeda et al. 2001, Verhoog et al. 2001, De La Hoya et al. 2002, Meindl et al. 2002, Perkowska et al. 2003). About 21% of Finnish high-risk families are mutation positive, carrying either a BRCA1 (10%) or a BRCA2 (11%) mutation (Vehmanen et al. 1997a, b).

Early age of breast cancer diagnosis has been another criterion to select patients for mutation screening. Mutation frequencies in the cohorts of early-onset breast cancer have been 0.8-12% for BRCA1 and 2.1-6.6% for BRCA2 (Langston et al. 1996, Krainer et al. 1997, Malone et al. 1998, Hopper et al. 1999, Peto et al. 1999, Malone et al. 2000, Loman et al. 2001, Tonin et al. 2001, Yassaee et al. 2002, Hamann et al. 2003, Martinez-Ferrandis et al. 2003).

Mutation frequencies among unselected breast cancer patients or in the general population are much less studied due to the challenges in mutation screening of the BRCA1 gene. The largest studies have been done in isolated populations with founder effects such as the Ashkenazi Jews, where 3.7-8.3% of unselected breast cancer patients and 1.1% of the general population carry a BRCA1 founder mutation (Struewing et al. 1997, Fodor et al. 1998, Hartge et al. 1999, Warner et al. 1999). In the studies where the entire coding region of BRCA1 has been analyzed, only a few hundred patients have been included (Garcia-Patino et al. 1998, Newman et al. 1998,

Tang et al. 1999). The mutation frequencies in these studies have ranged from 1.4 to 5.7%.

BRCA1-associated breast cancers are often aggressive, ductal invasive, high-grade carcinomas with prominent lymphocyte infiltration (Breast Cancer Linkage Consortium 1997, Phillips et al. 1999, Chappuis et al. 2000). They are aneuploid, estrogen and progesterone receptor negative and p53 positive. Gene-expression profiles and accumulation of somatic genetic changes during tumor progression of tumors with BRCA1 mutations and sporadic tumors differ from each other (Tirkkonen et al. 1997, Hedenfalk et al. 2001). Often BRCA1-associated cancers are diagnosed at a younger age than sporadic cancers and the risk of bilateral cancer is higher (Easton et al. 1993, Ford et al. 1994, Cornelis et al. 1995, Noguchi et al. 1999, Hamann and Sinn 2000, Bergthorsson et al. 2001, Haffty et al. 2002). An association with medullary or atypical medullary carcinoma has also been seen (Phillips et al. 1999). Prognosis of breast cancer patients with a BRCA1 mutation is the same as in sporadic cases (Chappuis et al. 1999, Hamann and Sinn 2000, Eerola et al. 2001b) but a worse outcome has also been suggested (Foulkes et al. 1997, Foulkes et al. 2000, Stoppa-Lyonnet et al. 2000, Robson et al. 2004).

3.1.4 BRCA1 and male breast cancer

BRCA1 mutations in MBC patients are quite rare (Table 1). After the linkage of BRCA1 to chromosome 17q was identified, it was reported that familial male breast cancer is not linked to BRCA1 (Stratton et al. 1994). Since then, a few cases of MBC patients with BRCA1 mutations have been reported (Table 1). It has been estimated that 19% of large breast carcinoma families with one or more cases of male breast cancer are attributable to BRCA1 (Ford et al. 1998). In a recent study of 76 MBC cases referred for clinical evaluation, as many as 8 (10.5%) carried a BRCA1 mutation, indicating that BRCA1 mutations might be more important to MBC predisposition than previously thought (Frank et al. 2002). The association of BRCA1 with MBC is also supported in studies by Brose et al. (2002) and Thompson et al. (2002a). In a clinic-based study of 483 BRCA1 mutation carriers, a 58-fold risk increase of MBC was observed for BRCA1 carriers (Brose et al. 2002). Seven cases of MBC were observed in 356 BRCA1 families (Thompson et al. 2002a). BRCA1 mutations are more frequent among young MBC patients and among patients with a positive family history (Ottini et al. 2003, Frank et al. 2002).

Table 1. BRCA1 mutations among male breast cancer patients by percentage.

Country, Study cohort	No. of MBC cases	Screening strategy	No. of BRCA1 mutation carriers (%)	Reference
USA, BC/OC families	8	Entire gene	2 (25)	Serova et al. 1997
Different origins, clinical patients	76	Founder/ Entire gene	8 (11)	Frank et al. 2002
Italy, MBC population	25	Entire gene	1 (4)	Ottini et al. 2003
Israel, MBC population	124	Founder	4 (3)	Struewing et al. 1999
Israel, MBC high risk families / unselected MBC	31	Founder	1 (3)	Sverdlov et al. 2000
USA, BRCA1 families	4	Founder/ Entire gene	4 (ND)	Brose et al. 2002
Different origins, BRCA1 families	7	Founder/ Entire gene	3 (ND)	Thompson et al. 2002a
Sweden, HNPCC families	2	Entire gene	1 (ND)	Borg et al. 2002
USA, high risk BC/OC families	1	Entire gene	1 (ND)	Struewing et al. 1995
UK, MBC population	94	Entire gene	0	Basham et al. 2002
USA, MBC population	54	Entire gene	0	Friedman et al. 1997
Hungary, MBC population	18	Entire gene	0	Csokay et al. 1999
Canada, MBC population	14	Entire gene	0	Wolpert et al. 2000

MBC, male breast cancer; BC, breast cancer; OC, ovarian cancer; ND, not determined; HNPCC hereditary nonpolyposis colorectal cancer

3.2 BRCA2

3.2.1 BRCA2 gene and protein

The location of the second high penetrance breast cancer susceptibility gene was uncovered using linkage analysis on 15 high-risk breast cancer families that were unlinked to BRCA1 (Wooster et al. 1994). The Breast cancer 2 gene (BRCA2; MIM 600185) at 13q12 has 27 exons, of which 26 encode a protein of 3418 amino acids (Wooster et al. 1995, Tavtigian et al. 1996). Although BRCA1 and BRCA2 are quite different in sequence, there are many similarities between them. For example, the large exons 10 and 11 encode 59% of the BRCA2 protein.

Like BRCA1, BRCA2 exhibits only little resemblance to other proteins. It has a possible transactivation domain in the amino-terminus (Figure 2) (Milner et al. 1997). Eight BRC-sequences that bind RAD51 are in the central part of the protein (Bork et

al 1996, Wong et al. 1997, Chen et al. 1998). Three oligonucleotide binding folds that bind single-stranded DNA directly and two nuclear localization signals are located in the carboxy-terminus (McAllister et al 1997, Yang et al. 2002). BRCA2 is expressed in several tissues, including breast, ovary, testis, thymus and lung, and its expression is cell cycle dependent (Tavtigian et al. 1996, Rajan et al. 1996, Vaughn et al. 1996a).

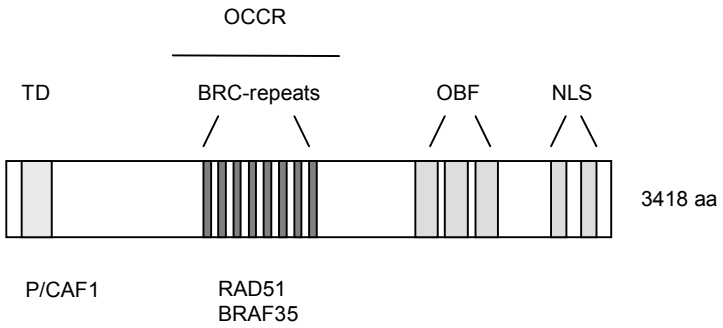

Figure 2. BRCA2 functional domains and interacting proteins. OCCR, ovarian cancer cluster region; TD, transactivation domain; BRC, repeats in BRCA2; OBF, oligonucleotide binding folds; NLS, nuclear localization signals.

3.2.2 Function of BRCA2

BRCA2, just like BRCA1, functions in maintaining genome integrity (reviewed in Venkitaraman 2002). Functioning BRCA2 is crucial for embryogenesis since knockout mice die early in embryogenesis (Sharan et al. 1997). The major role of BRCA2 is to regulate the availability and activity of RAD51 (Sharan et al. 1997, Wong et al. 1997, Davies et al. 2001). RAD51 is essential for double-strand DNA break repair by homologous recombination. It coats single-stranded DNA to form a nucleoprotein filament that invades and pairs with a homologous DNA duplex, initiating strand exchange between the paired DNA molecules (Baumann and West 1998). The BRCA2-RAD51 interaction involves a substantial portion of the proteins.

BRCA2 has been suggested to take part in cell cycle control. BRCA2 possibly participates in G2/M control through a direct interaction with BRCA2-associated factor 35 (BRAF35), a novel protein that preferentially binds *in vitro* to branched DNA structures (Marmorstein et al. 2001). BRAF35 and BRCA2 co-localize to condensing chromosomes. BRCA2 is phosphorylated by a mitotic Polo-like kinase (Plk1) in the G2/M phase of the cell cycle and BRCA2 interacts with and is phosphorylated by a mitotic checkpoint protein hBUBR1 (Futamura et al. 2000, Lin et al. 2003, Lee et al. 2004)

BRCA2 also has a putative role in transcriptional regulation. The product of BRCA2 exon 3 (the putative transactivation domain), when fused to a DNA-binding domain, activates transcription in yeast (Milner et al. 1997). A germline deletion of exon 3 only in a breast-ovarian cancer family implies that a lack of transcriptional activation

function might predispose to tumor development (Nordling et al. 1998). BRCA2 may activate transcription by modulating the acetylation of histones. BRCA2 interacts with the transcriptional co-activator protein P/CAF that has histone acetylation activity (Fuks et al. 1998, Shin and Verma 2003). BRCA2 also synergizes with the nuclear receptor co-activator p160 GRIP1 to enhance transcriptional activation by androgen receptor (Shin and Verma 2003). The transcriptional activation domain of BRCA2 interacts with replication protein A, a protein essential for DNA repair, replication and recombination. Therefore, the transcriptional activation domains within BRCA2 may provide links to replication protein A and DNA repair processes rather than transcription (Wong et al. 2003).

3.2.3 BRCA2 mutations and cancer

Almost 1900 mutations, polymorphisms and variants of unknown function are distributed throughout the coding sequence in BRCA2 (Breast Cancer Information Core: http://research.nhgri.nih.gov/bic/). Little over half of the reported mutations are missense mutations with unclear relevance to the cancer predisposition. Disease-associated mutations include frameshift, nonsense and splice-site mutations.

Most of the reported mutations are unique. BRCA2 founder mutations for female breast cancer have been found in several populations, such as in Finland, Germany, Holland, Hungary, Iceland, Sardinia, Scotland/Northern Ireland, Slovenia, Spain, Sweden, and among Ashkenazi Jews and French Canadians (Thorlacius et al. 1996, Håkansson et al. 1997, Vehmanen 1997a, b, Huusko 1998, Tonin et al. 1998, Pisano et al. 2000, van der Looij et al. 2000, Verhoog et al. 2001, Krajc et al. 2002, Meindl et al. 2002, Campos et al. 2003, Scottish/Northern Irish BRCAI/BRCA2 Consortium 2003).

Just like in BRCA1, mutations in BRCA2 predispose carriers to breast and ovarian cancers. Risk of breast cancer, 26-84% by the age of 70 years, is as high as among BRCA1 carriers (Ford et al. 1998, Thorlacius et al. 1998, Breast Cancer Linkage Consortium 1999, Warner et al. 1999, Anglian Breast Cancer Study Group et al. 2000, Satagopan et al. 2001, Antoniou et al. 2002, Antoniou et al. 2003). Risk of ovarian cancer is lower, 10-31%, among BRCA2 carriers and is influenced by the position of the mutation (Gayther et al. 1997, Ford et al. 1998, Breast Cancer Linkage Consortium 1999, Anglian Breast Cancer Study Group et al. 2000, Antoniou et al. 2000, Satagopan et al. 2002, Antoniou et al. 2003). Ovarian cancer risk may be higher if the mutation is in the central part of BRCA2, in the so-called ovarian cancer cluster region (OCCR) (Gayther et al. 1997, Thompson et al. 2001). Other cancers that have been associated with BRCA2 mutations include melanoma and cancers of the cervix, colorectum, gallbladder and bile ducts, liver, pancreas, prostate and stomach (Sigurdsson et al. 1997, Stuewing et al. 1997, Breast Cancer Linkage Consortium 1999, Johannsson et al. 1999, Eerola et al. 2001a, Risch et al. 2001, Scott et al. 2002, Tulinius et al. 2002, Edwards et al. 2003, Hahn et al. 2003, Jakubowska et al. 2003, Liede et al. 2004).

The large size of the gene and the lack of mutational hotspots make large-scale BRCA2 mutation screening a challenge. Many studies have concentrated on high-risk families or young patients. BRCA2 mutation frequencies of 3-76% have been reported among breast cancer families with the highest frequency found in Iceland (Håkansson et al. 1997, Vehmanen et al. 1997a, b, Frank et al. 1998, Tonin et al. 1998, Santarosa et al. 1999, Wagner et al. 1999b, Ikeda et al. 2001, Verhoog et al. 2001, De La Hoya et al. 2002, Meindl et al. 2002, Perkowska et al. 2003). About 2.1% to 6.6% of early-onset breast cancer patients have been found to harbor a BRCA2 mutation (Krainer et al. 1997, Hopper et al. 1999, Peto et al. 1999, Loman et al. 2001, Tonin et al. 2001, Hamann et al. 2003, Martinez-Ferrandis et al. 2003). Some studies have only analyzed previously identified founder mutations in cohorts of unselected breast cancer patients (Thorlacius et al. 1997, Thorlacius et al. 1998, Warner et al. 1999, van der Looij et al. 2000, Chappuis et al. 2001). BRCA2 founder mutation frequencies have been 0.2-10.4% in these studies. In Iceland, 0.6% of the general population carries a single BRCA2 999del5 founder mutation that is found in 7.7-10.4% of the unselected female breast cancer cases and in 76% of breast cancer families (Thorlacius et al. 1996, 1997, 1998).

Clinicopathological features of BRCA2-associated breast tumors do not differ significantly from sporadic tumors, although increased frequency of lobular subtype and frequent expression of estrogen receptor has been seen (Marcus et al. 1996, Armes et al. 1998, Noguchi et al. 1999, Chappuis et al. 2000, Vahteristo et al. 2001b). Prognosis of BRCA2 mutation carriers does not differ significantly from sporadic cases (Verhoog et al. 2000, Eerola et al. 2001b). Just like carriers of a BRCA1 mutation, BRCA2 carriers have an increased risk of bilateral cancer and an earlier age of onset than sporadic cases (Noguchi et al. 1999, Bergthorsson et al. 2001, Haffty et al. 2002). A later age at breast cancer diagnosis of BRCA2 carriers compared to BRCA1 carriers has been reported (Ford et al. 1998, Scottish/Northern Irish BRCAI/BRCA2 Consortium 2003). In a study by Hedenfalk et al. (2001) gene-expression profiles of tumors with BRCA2 mutations, BRCA1 mutations, and sporadic tumors differed significantly from each other. Accumulation of somatic genetic changes during tumor progression has also been suggested to follow a unique pathway in individuals carrying a BRCA2 mutation (Tirkkonen et al. 1997).

3.2.4 BRCA2 and male breast cancer

Mutations in BRCA2 represent the strongest known risk factor for MBC. The lifetime risk of male breast cancer in BRCA2 mutation carriers (6.9%) is approximately 80 to 100 times higher than in the general population (Thompson et al. 2001). Frequency of BRCA2 mutations in different studies of MBC patients vary from 4% to 40% (Table 2). The small size of study cohorts, different patient ascertainment criteria, as well as different mutation detection methodologies may explain some of this variation.

Table 2. BRCA2 mutations among male breast cancer patients by percentage.

Country, Study cohort	No. of MBC cases	Screening strategy	No. of BRCA2 mutation* carriers (%)	Reference
Iceland, MBC population	30	Founder	12 (40)	Thorlacius et al. 1996
UK, Families with MBC	33	Entire gene	12 (36)	Evans et al. 2001
Hungary, MBC population	18	Entire gene	6 (33)	Csokay et al. 1999
Belgium, BC/OC families	16	Entire gene	5 (31)	Claes et al. 2004
France, BC families	12	Entire gene	3 (25)	Pages et al. 2001
Sweden, MBC population	34	Entire gene	7 (21)	Haraldsson et al. 1998
Different origins, clinical patients	76	Founder/ Entire gene	14 (18)	Frank et al. 2002
Spain, Families with MBC	17	Entire gene	3 (18)	Diez et al., 2000
USA, MBC population	50	Entire gene	7 (14)	Couch et al. 1996
Canada, MBC population	14	Entire gene	2 (14)	Wolpert et al. 2000
Israel, MBC population	124	Founder	15 (12)	Struewing et al. 1999
Italy, MBC population	25	Entire gene	3 (12)	Ottini et al. 2003
Poland, MBC population	37	Entire gene	4 (11)	Kwiatkowska et al. 2001
UK, MBC population	28	Entire gene	2 (7)	Mavraki et al. 1997
UK, MBC population	94	Entire gene	5 (5)	Basham et al. 2002
USA, MBC population	54	Entire gene	2 (4)	Friedman et al. 1997

MBC, male breast cancer; BC, breast cancer; OC, ovarian cancer
* missense mutations with unknown significance excluded

BRCA2 mutations are more prevalent in MBC patients with a positive family history as compared to those with no family history (Couch et al. 1996, Diez et al. 2000, Wolpert et al. 2000, Evans et al. 2001, Frank et al. 2002). Large breast cancer families with at least one MBC case have a 60-76% chance of carrying BRCA2 mutations (Ford et al. 1998, Osorio et al. 2000). A few studies have reported a high frequency of mutations also among patients with a negative family history (Haraldsson et al. 1998, Csokay et al. 1999). In many studies, age at MBC diagnosis and clinicopathological characteristics of the tumors do not differ significantly between BRCA2 mutation carriers and non-carriers (Friedman et al. 1997, Haraldsson et al. 1998, Csokay et al. 1999, Struewing et al. 1999, Wolpert et al. 2000, Basham et al. 2002, Frank et al. 2002, Kwiatkowska et al. 2003).

3.3 AR

3.3.1 AR gene, protein and diseases

The Androgen receptor gene (AR; also known as dihydrotestosterone receptor; MIM 313700) is located on the chromosome region Xq11-12 and spans about 90 kb of DNA (Lubahn et al. 1988, Brown et al. 1989). Its eight exons encode a protein of 919 amino acids (Lubahn et al. 1989). The AR gene has highly polymorphic polyglutamine (CAG) and polyglycine (GGC) repeats in the coding area of the first exon.

The AR, like other members of the steroid hormone receptor family, has four functional domains (Figure 3). The amino-terminal transactivation domain is encoded by the large first exon (58% of the gene). Two zinc fingers of the DNA-binding domain are encoded by exons 2 and 3. The carboxy-terminus contains the nuclear targeting signal and the ligand-binding domain (Lubahn et al. 1988, Chang et al. 1988, Lubahn et al. 1989, Tilley et al. 1989). Mutations in the highly conserved DNA- and ligand-binding domains can cause complete or partial androgen insensitivity syndrome. This syndrome is characterized by complete or partial inhibition of normal development of both internal and external male sex organs in 46,XY individuals (Quigley et al. 1995, Gottlieb et al. 1998).

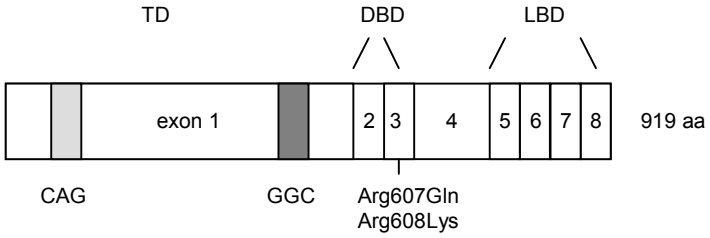

Figure 3. AR functional domains, exons, CAG and GGC repeats, and mutations found in male breast cancer patients. TD, transactivation domain; DBD, DNA-binding domain; LBD, ligand-binding domain.

AR exists in two isoforms and is expressed in many tissues (Faber et al. 1991, Wilson and McPhaul 1994). *In vitro*, the length of the polymorphic CAG repeat in exon one correlates inversely with the transcriptional activity of the AR even within the normal range (6-34 repeats) (Chamberlain et al. 1994, Kazemi-Esfarjani et al. 1995). Longer CAG repeat length has also been reported to reduce AR mRNA and protein expression (Choong et al.1996). Men with exceptionally long CAG repeats suffer from varying degrees of androgen insensitivity. Spinal and bulbar muscular atrophy (Kennedy's disease; MIM 313200), a neurodegenerative disease, is also caused by CAG repeat expansion (La Spada et al. 1991). GGC repeat together with the preceding $(GGT)_3GGC(GGT)_2$ sequence codes for about 10 to 30 glycine repeats

(Lubhan et al. 1988, Tilley et al. 1989). Even though deletion of the polyglycine tract reduces transcriptional activity of the AR by about 30% in transient transfection assays, its function has remained unclear (Gao et al. 1996).

3.3.2 Function of AR

AR mediates biological effects of androgens to different downstream genes. After binding a ligand, AR is dissociated from heat shock chaperone proteins, phosphorylated, dimerized and translocated into the nucleus. Ligand-bound AR has inhibitory effects on cell death processes also in the cytoplasm (Kousteni et al. 2001). In the nucleus, AR binds to androgen responsive elements at the promoter regions of target genes, including many genes that regulate cell growth, male sexual differentiation and sexual maturation (Heinlein and Chang 2002). Transcription regulation requires several other proteins such as coactivators and corepressors (Heinlein and Chang 2002).

3.3.3 AR germline alterations and cancer

Germline alterations in the AR gene that affect its activity have been suggested to affect prostate cancer risk. Short CAG repeat lengths have been associated with increased disease risk, more aggressive cancers, an earlier age of onset and likelihood of recurrence, although these results are debated (Irvine et al. 1995, Giovannucci et al. 1997, Bratt et al. 1999, Edvards et al. 1999). It has also been proposed that the size of the GGC repeat might influence prostate cancer risk (Irvine et al. 1995, Giovannucci et al. 1997, Stanford et al. 1997, Chang et al. 2002). Germline point mutations in the AR predisposing to prostate cancer are rare. An Arg726Leu (CGC to CTC) substitution was found to increase the risk of prostate cancer in the Finnish population (Mononen et al. 2000). This mutation was found in nearly 2% of prostate cancer patients, almost six times more often than in the general population (0.33%).

AR germline alterations have also been suggested to influence breast cancer risk among women and men. Long CAG repeats have been proposed to increase breast cancer risk in the general population and among women with a family history of breast cancer, probably by decreasing the capacity of the receptor to activate transcription (Giguere et al. 2001, Haiman et al. 2002). In the study by Rebbeck et al. (1999) women with BRCA1 mutations were at a significantly increased risk of breast cancer if they carried at least one AR allele with 28 or more CAG repeats. An association of shorter CAG repeat lengths with more aggressive forms of breast cancer has also been proposed (Yu et al. 2000). Influence of CAG repeat lengths on female breast cancer risk has remained controversial (Spurdle et al. 1999, Given et al. 2000, Kadouri et al. 2001, Menin et al. 2001).

Two studies have been published on CAG polymorphisms and MBC risk. Haraldsson et al. (1998) found no significant difference in the number of CAG repeats in 29 unselected Swedish MBC cases when compared to 30 healthy male blood donors, but

30 or more repeats were observed only among the MBC patients. In another study, no difference was seen between the median CAG repeat length of 59 consecutive MBC patients and 79 controls from the UK (Young et al. 2000). However, the control group had 28 or less CAG repeats, whereas two MBC patients had alleles with 29 and 30 repeats.

AR germline point mutations are rare among breast cancer patients. Two such mutations have been found in MBC patients. An Arg607Gln substitution was found in two brothers with infiltrating ductal breast cancer and with partial androgen insensitivity syndrome (Wooster et al. 1992). An Arg608Lys substitution was also found in a MBC patient with partial androgen insensitivity syndrome (Lobaccaro et al. 1993a, b). Both of these mutations are located in the DNA-binding domain. *In vitro* studies revealed normal androgen-binding affinities and weaker DNA-binding, which resulted in reduced transactivation efficiency (Poujol et al. 1997).

3.4 CHEK2

3.4.1 CHEK2 gene and protein

Checkpoint kinase 2 (CHEK2; also known as CHK2, CDS1; MIM 604373) is a human homolog of the *Saccharomyces cerevisiae* Rad53 and *Schizosaccharomyces pombe* Cds1 protein kinases. Matsuoka et al. (1998), Blasina et al. (1999), Chaturvedi et al. (1999) and Brown et al. (1999) independently identified CHEK2. CHEK2 spans 50 kilobases of genomic DNA on chromosome region 22q12.1 and contains 14 exons. Exons 11-14 have six homologous fragments that are located on chromosomes 7, 10, 15, 16, 22 and X (Sodha et al. 2000).

CHEK2 is 543 amino acids long and contains several evolutionarily conserved elements (Figure 4). The aminoterminal SQ/TQ motif contains a series of serine or threonine residues followed by glutamine (Matsuoka et al. 1998). This domain has a potential regulatory function. After DNA damage or replication blockage, this site is phosphorylated by ATM/ATR kinases (Matsuoka et al. 2000, Liu et al. 2000). The fork head-associated domain is involved in protein-protein interactions by binding phosphothreonine residues (Matsuoka et al. 1998, Blasina et al. 1999, Chaturvedi et al. 1999, Brown et al. 1999, Durocher et al. 2000). The kinase domain at the carboxy-terminal of CHEK2 contains the activation loop, a conserved structural motif in kinase domains (Matsuoka et al. 1998, Blasina et al. 1999, Chaturvedi et al. 1999, Brown et al. 1999). In addition, mammalian CHEK2 contains a c-Abl SRC homology-3 (SH3) domain -consensus binding site that is not conserved in lower eukaryotes (Brown et al. 1999). CHEK2 is widely expressed with stronger expression in human testis, spleen, colon, and peripheral blood leukocytes (Matsuoka et al. 1998, Brown et al. 1999).

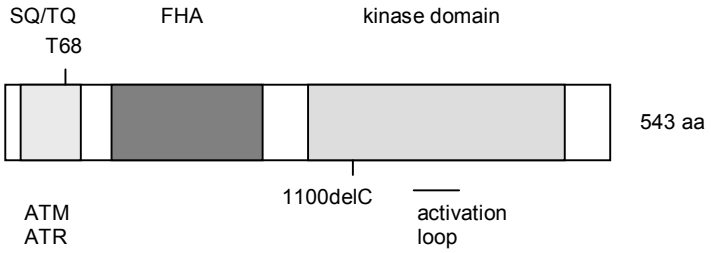

Figure 4. CHEK2 functional domains and interacting proteins. SQ/TQ, serine-glutamine/threonine-glutamine pairs; FHA, forkhead-associated domain.

3.4.2 Function of CHEK2

Protein kinases covalently modify proteins by transferring phosphate groups from ATP to the substrate. This phosphorylation can rapidly alter activity, protein-protein interactions or subcellular localization of the substrate. Maintenance of chromosomal DNA integrity is constantly monitored by cell-cycle checkpoints. CHEK2 can respond to DNA damage throughout the cell cycle (Matsuoka et al. 1998). It acts downstream of ATM/ATR in mediating the DNA damage checkpoint (Matsuoka et al. 1998, Blasina et al. 1999, Brown et al. 1999, Chaturvedi et al. 1999, Bartek et al. 2001). CHEK2 is phosphorylated at Thr68 by ATM *in vivo* and *in vitro* (Matsuoka et al. 1998, Ahn et al. 2000, Matsuoka et al. 2000, Melchionna et al. 2000). Activation of CHEK2 leads to the inhibitory phosphorylation of the Cdc25 phosphatase, excluding it form the nucleus and preventing it from activating cyclin-dependent kinase Cdc2, hence causing G2 arrest (Matsuoka et al. 1998, Blasina et al. 1999, Brown et al. 1999, Chaturvedi et al. 1999, Falck et al. 2001b). CHEK2 stabilizes p53 and upregulates its function by phosphorylation upon DNA damage leading to cell cycle arrest in G1 (Chehab et al. 2000, Hirao et al. 2000, Shieh et al. 2000, Falck et al. 2001a). CHEK2 also regulates the function of BRCA1 (Lee et al. 2000, Zhang et al. 2004).

3.4.3 CHEK2 mutations and cancer

Li-Fraumeni syndrome (LFS; MIM 151623) is a rare, autosomal, dominant disease characterized by the occurrence of bone and soft-tissue sarcomas, breast cancer, brain tumors, leukemia and adrenocortical tumors in multiple relatives at an early age (Li and Fraumeni 1969, Li et al. 1988). Mutations in the highly penetrant TP53 are known to cause LFS (Malkin et al. 1990, Srivastava et al. 1990).

Recently, CHEK2 alterations (1100delC, I157T and R145W) were found in a subset of LFS families that lack TP53 mutations (Bell et al. 1999, Lee et al. 2001). Functional analyses have revealed that 1100delC and R145W mutations affect the function of CHEK2 (Lee et al. 2001, Falck et al. 2001b, Wu et al. 2001). The significance of the I157T variant to the function of CHEK2 and to the cancer predisposition is somewhat unclear (Lee et al. 2001, Falck et al. 2001b, Wu et al.

2001, Kilpivaara et al. 2004b). Two 1100delC mutations (4.5%) were found among 44 Finnish families that fulfilled the criterion of LFS, Li-Fraumeni like syndrome, or that were phenotypically suggestive for LFS (Vahteristo et al. 2001c). I157T was detected in 8.9% (7/79) of Finnish breast cancer families (Allinen et al. 2001). Twenty-one of the families also fulfilled the criteria for Li-Fraumeni like or LFS. This mutation was present in 6.5% (13/200) of cancer-free blood donors but only in 3.9% (10/259) of unselected breast cancer cases. The differences between the groups were statistically insignificant indicating that I157T is rather a polymorphism than a deleterious mutation.

The significance of 1100delC mutation in predisposition to breast cancer has been widely evaluated (Meijers-Heijboer et al. 2002, Sodha et al. 2002, Vahteristo et al. 2002, Meijers-Heijboer et al. 2003, Offit et al. 2003, Oldenburg et al. 2003, Broeks et al. 2004, Dufault et al. 2004, Osorio et al. 2004, CHEK2 Breast Cancer Case-Control Consortium 2004). Recently CHEK2 1100delC mutation was found in 5.1% of individuals with breast cancer from non-BRCA1/BRCA2 breast cancer families, including 13.5% of individuals from families with MBC (Meijers-Heijboer et al. 2002). CHEK2 1100delC was shown to confer an approximately 2-fold increase of breast cancer risk in women and a 10-fold increase of risk in men lacking BRCA1 and BRCA2 mutations and it was estimated to account for 1% of breast cancers in women and as much as 9% of breast cancers in men (Meijers-Heijboer et al. 2002). An approximately 2-fold increased risk of breast cancer among CHEK2 1100delC carriers was seen in a large case-control study of 10 860 breast cancer patients and 9065 controls (CHEK2 Breast Cancer Case-Control Consortium 2004). Oldenburg et al. (2003) reported that the 1100delC variant acts as a breast cancer risk modifier in non-BRCA1/BRCA2 multiple-case families.

Frequency of 1100delC was studied in an unselected Finnish population-based breast cancer cohort and in an independent cohort of familial breast cancer patients (Vahteristo et al. 2002). Frequency of the mutation was higher in breast cancer patients with a positive family history of breast cancer (3.1%) as compared to population controls (1.4%). Patients with bilateral breast cancer were six times more likely to be 1100delC carriers than patients with unilateral cancer (Vahteristo et al. 2002). Broeks et al. (2004) also detected a significant excess risk for CHEK2 1100delC mutation carriers to develop a contralateral breast tumor. A trend for a larger tumor size and a higher grade was seen among 1100del carriers than among non-carriers (Kilpivaara et al. 2004a). The CHEK2 1100delC mutation was shown to identify families with a hereditary breast and colorectal cancer phenotype (Meijers-Heijboer et al. 2003). Simultaneously with our study (IV), two reports on CHEK2 1100delC and MBC were published. No mutations were found among 188 unselected cases of non-BRCA1/BRCA2 MBC patients from UK and the USA nor among 54 MBC cases from Israel (Neuhausen et al. 2004, Ohayon et al. 2004)

In some populations, CHEK2 1100delC is quite rare and the clinical applicability of germline testing for the mutation is limited (Offit et al. 2003, Dufault et al. 2004, Osorio et al. 2004). There are reports that common CHEK2 polymorphisms and

variants other than 1100delC do not make a major contribution to breast cancer susceptibility, but more studies are needed (Kuschel et al. 2003, Schutte et al. 2003, Dufault et al. 2004). Sodha et al. (2002) suggested that tumorigenesis in association with CHEK2 mutations does not involve loss of the wild type allele. Somatic CHEK2 mutations are rare in breast cancer (Ingvarsson et al. 2002, Sullivan et al. 2002).

Cancers, other than breast, associated with CHEK2 mutations include sarcoma, brain, prostate, colorectal, stomach, ovarian and lung cancer, leukemia and non-Hodgkin's lymphoma (Bell et al. 1999, Hofmann et al. 2001, Tavor et al. 2001, Aktas et al. 2002, Ingvarsson et al. 2002, Hangaishi et al. 2002, Miller et al. 2002, Tort et al. 2002, Dong et al. 2003, Meijers-Heijboer et al. 2003, Seppälä et al. 2003). Ovarian cancer was not associated with CHEK2 1100delC in the Finnish population (Vahteristo et al. 2002).

3.5 Other MBC susceptibility genes

Hereditary nonpolyposis colorectal cancer (HNPCC; MIM 114500) syndrome is an autosomal dominantly inherited disease characterized by predisposition to colorectal cancer and other tumor types such as endometrial, stomach, biliary tract, urinary tract, ovarian and small bowel carcinoma (Lynch et al. 1993). Germline mutations in MLH1 (3p21.3), MSH2 (2p22-p21), PMS1 (2q31-q33), PMS2 (7p22), and MSH6 (2p16) are known to cause HNPCC (Fishel et al. 1993, Leach et al. 1993, Bronner et al. 1994, Papadopoulos et al. 1994, Nicolaides et al. 1994, Miyaki et al. 1997). Although most studies find no association between HNPCC and breast cancer, a few reports on MBC cases with MLH1 mutations in typical HNPCC families exist (Risinger et al. 1996, Boyd et al. 1999, Borg et al. 2000).

Cowden disease (MIM 158350) is an autosomal dominant cancer predisposing syndrome caused by mutations in the PTEN tumor suppressor gene located on 10q23 (Li et al. 1997, Liaw et al. 1997, Steck et al. 1997). The disease is characterized by hamartomas, benign, hyperplastic, disorganized growths. Cowden disease increases risk of thyroid cancer and female breast cancer (Liaw et al. 1997, Eng 2003). Women in Cowden's disease families have a 25-50% lifetime risk of developing breast cancer (Eng 2003). PTEN mutations have only a minor role, if any, in breast cancer predisposition outside the context of Cowden disease (FitzGerald et al. 1998, Carroll et al. 1999, Chen et al. 1999, Lauge et al. 1999, Figer et al. 2002). Two male Cowden disease patients with breast cancer have been reported (Fackenthal et al. 2001).

CYP17 gene (MIM 202110) on chromosome 10q24.3 codes for a cytochrome P450c17α enzyme, which is involved in the synthesis of androgens and estrogens (Picado-Leonard and Miller 1987, Brentano et al. 1990). A polymorphic T to C substitution has been described in the 5′-untranslated region of the gene, 34 bp upstream from the translation initiation site (Carey et al. 1994). The substitution creates an additional promoter motif (CCACC), which has been suggested to lead to increased transcriptional activity and enhanced steroid hormone production. Elevated

levels of sex hormones in serum of women with the C allele have been observed but the association between the CYP17 polymorphism and breast cancer risk in females is controversial (Dunning et al. 1998, Feigelson et al. 1997, Feigelson et al. 1998, Haiman et al. 1999). The C polymorphism has been associated with an increased risk of male breast cancer and the frequency of CC genotype has been reported to be higher among MBC BRCA2 999del5 mutation carriers than non-carriers (Young et al. 1999, Gudmundsdottir et al. 2003).

AIMS OF THE STUDY

The aim of this thesis was to investigate the molecular basis of hereditary predisposition to breast cancer in the male and female populations.

The specific aims were:

1. To determine the prevalence of BRCA1 and BRCA2 mutations in unselected Finnish female breast cancer patients (I);
2. To study the prevalence of BRCA2 mutations in Finnish male breast cancer patients (II);
3. To investigate the effects of AR gene alterations to the risk of male breast cancer (III); and
4. To study the prevalence of the CHEK2 1100delC mutation in male breast cancer patients in Finland (IV).

MATERIALS AND METHODS

1. Ethical issues

The Ministry of Social Affairs and Health, the Ethical Committee of Tampere University Hospital, and the Ethical Committees of the Departments of Oncology and Obstetrics and Gynecology, Helsinki University Central Hospital granted their permissions to conduct these studies. Patients were approached by attending physicians. Relatives were contacted only if the proband and the relative in question gave their permissions. All study participants signed a written informed consent before donating a blood sample. The study did not alter treatment protocols or patient management. No data from the research work were provided to the patients or family members, but genetic counseling was offered at the Department of Clinical Genetics, Tampere University Hospital if a participant contacted the research group.

2. Collection of study cohorts

2.1 Finnish female breast cancer population

For study I, 1035 consecutive, newly diagnosed female breast cancer cases were collected. These patients were treated at the Departments of Oncology at the Tampere University Hospital (n=408) between January 1997 and May 1999 and at the Helsinki University Central Hospital (n=627) between April 1997 and March 1998. These cohorts account for 82% of all breast cancer cases at these clinics during the study period. The two university hospitals attend about 30% of all Finnish breast cancer patients. Because of recent migration patterns within Finland, the ancestral origins of these patients were of all parts of Finland.

Written informed consent and a blood sample were obtained from the patients. All study participants filled out a short questionnaire on breast, ovarian and other cancers in their families. When the participant reported one or more cancers among her relatives, she was asked to complete a more detailed questionnaire with respect to her family members. However, only patients reporting breast and/or ovarian cancers in first- or second-degree relatives were considered to have a family history of the disease.

2.2 Finnish male breast cancer population

The Finnish Cancer Registry (FCR) was utilized to identify all MBC patients diagnosed in Finland between 1967 and 1996 (Figure 5). The FCR covers 99% of solid tumors diagnosed in Finland (Teppo 1994). The 237 MBC cases with microscopically confirmed diagnosis of carcinoma were eligible for the study

(Figure 5). Information obtained on the MBC patients included date of birth, age at diagnosis and the attending hospital. The average age at diagnosis of the patients was 65.4 years (range 30-94 years). Additional information on vital status and possible date of death was obtained by linking patients' personal identification codes to the centralized, nationwide, and computer-based Finnish Population Register Centre. First- and second-degree relatives were identified from the Population Register Centre and from church records. Incident cancer cases among these relatives were identified from the FCR.

Seventy-nine living patients (33%; 79/237) were contacted through the attending hospitals. Thirty-seven patients who were willing to participate provided a written informed consent, a blood sample, and a detailed questionnaire on their family members, as well as the presence of breast, ovarian and other cancers in their families. Additionally, formalin-fixed, paraffin-embedded tissue samples were obtained from the archives of the attending hospitals for 160 patients. An experienced pathologist (T. Kuukasjärvi, MD, PhD) confirmed the breast cancer diagnosis in 147 of these cases.

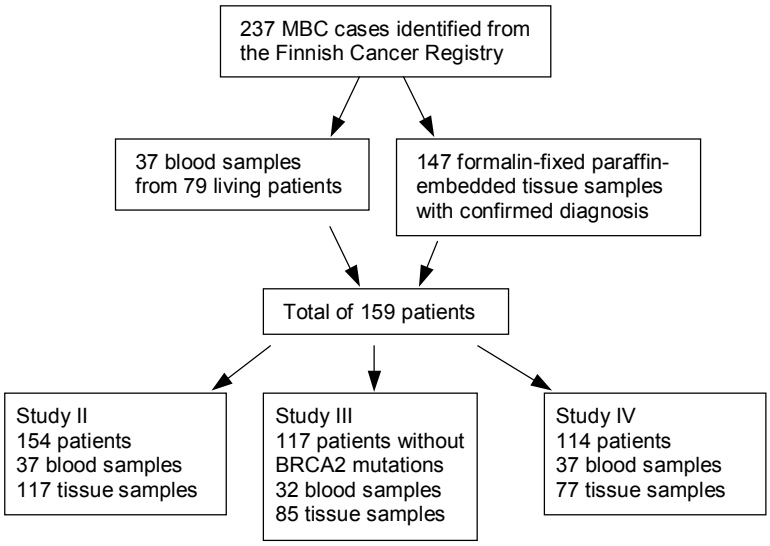

Figure 5. Collection of the MBC cohort and the number of patients used in studies II-IV.

Sufficient amounts of DNA from either blood (n=37) or paraffin-embedded tissues (n=119) of 156 patients were available for the analysis of previously found Finnish BRCA2 mutations (II). After mutation screening, two patients with paraffin-embedded tissue samples were excluded due to conflicting mutation results from different tissue samples. In addition, the entire BRCA2 coding sequence was screened in the blood samples (n=34) that were not found to harbor the founder mutations.

MBC patients without BRCA2 mutations were included in study III. For the study, 117 DNA samples from either blood (n=32) or paraffin-embedded tissues (n=85)

were available. In addition to breast cancer, two patients were diagnosed with prostate cancer. The AR gene was screened for mutations and the lengths of the polymorphic polyglutamine and polyglycine repeats were analyzed in the blood samples. Paraffin-embedded samples were used to study the AR Arg726Leu (CGC to CTC) mutation.

For study IV, all available (n=114) MBC DNA samples were used regardless of the BRCA2 mutation status. Previously, the cohort was screened for the Finnish BRCA2 mutations and included 10 patients with BRCA2 mutations: 9346(-2)A>G (n=6) and one of each 999del5, 4075delGT, 5808del5 and 7708C>T.

3. Mutation analyses

3.1 DNA extraction

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

3.2 Screening for known mutations

3.2.1 Previously identified mutations

At the time of the studies I and II, 11 BRCA1 and 8 BRCA2 mutations were identified from breast and ovarian cancer families in Finland (Vehmanen et al. 1997a, b, Huusko et al. 1998, Sarantaus et al. 2000, Vahteristo et al. 2001a, Sarantaus et al. 2001a, b). Two novel BRCA2 mutations, 4075delGT and 5808del5, were found by screening the entire BRCA2 gene by PTT and DHPLC of 34 MBC cases (II). The new mutations were subsequently screened in all 154 MBC samples (II). These 21 mutations are listed in Table 3.

Recently, an AR Arg729Leu (CGC to CTC) germline mutation was detected in 2% of Finnish prostate cancer patients, almost six times more often than in the general population (0.3%) (Mononen et al. 2000). The possible involvement of this mutation in MBC disposition was investigated in the study III.

A protein truncating mutation 1100delC in the CHEK2 gene has been associated with Li-Fraumeni syndrome, breast cancer families suggestive of LFS, and an increased risk of breast cancer among unselected female and male breast cancer patients (Bell et al. 1999, Vahteristo et al. 2001c, Meijers-Heijboer et al. 2002, Vahteristo et al. 2002). In the study IV, Finnish MBC population was screened for this mutation.

Table 3. BRCA1 (I) and BRCA2 (I, II) mutations screened.

Gene and mutation	Mutation type	Recurrence
BRCA1		
Ex 11; 1924delA	Frameshift	Single patient
Ex 11; 2804delAA	Frameshift	Founder
Ex 11; 3604delA	Frameshift	Founder
Ex 11; 3745delT	Frameshift	Founder
Ex 11; 3904C>A	Nonsense	Single patient
Ex 11; 4154delA	Frameshift	Single patient
Int 11; 4216nt-2A>G	Splice site	Founder
Ex 13; 4446C>T	Nonsense	Founder
Ex 17; 5145del11	Frameshift	Single patient
Ex 20; 5370C>T	Nonsense	Founder
Ex 20; 5382insC	Frameshift	Founder
BRCA2		
Ex 9; 999del5	Frameshift	Founder
Ex 11; 4075delGT*	Frameshift	Single male patient
Ex 11; 4081insA	Frameshift	Founder
Ex 11; 5797G>T	Nonsense	Founder
Ex 11; 5808del5*	Frameshift	Single male patient
Ex 11; 6495delGC,A>C	Frameshift	Single patient
Ex 11; 6503delTT	Frameshift	Founder
Ex 15; 7708C>T	Nonsense	Founder
Ex 18; 8555T>G	Nonsense	Founder
Int 23; 9346nt-2A>G	Splice site	Founder

* only study II

3.2.2 Allele-specific oligonucleotide (ASO) hybridization

The ASO hybridization method is based on the fact that under the appropriate hybridization and washing conditions, a short oligonucleotide probe will hybridize to its PCR-amplified DNA target only when it is a perfect match (Saiki et al. 1986). A single base pair mismatch is often sufficient to prevent stable annealing. In the study I, ASO was used in Tampere to detect 11 known BRCA1 and 8 BRCA2 mutations (Table 3) using conditions described by Friedman et al. (1995). Briefly, for each mutation, two 18 bp long oligonucleotide probes, one for the wild-type allele and one for the mutated allele, were designed so that the mutation site was located in the middle of the probe. Genomic DNA was PCR-amplified using Dynazyme (Finnzymes) or AmpliTaqGold (Applied Biosystems). PCR-products were alkaline denatured, transferred to two Gene Screen Plus nylon filters (DuPont NEN) using a 48-well dot-blot vacuum apparatus and fixed using ultraviolet light. Forty nanograms of oligonucleotide probes were [α-^{32}P]dCTP end-labeled using Terminal deoxynucleotide transferase (Amersham Life Science) and cleaned with NucTrap Probe Purification Columns (Stratagene) according to manufacturers' instructions. Filters were prehybridized for an hour at 54°C before the probes (150 000 cmp/ml) were added. After 3-hour incubation, the filters were washed and exposed to BiomaxMR X-ray films (Kodak) for 1-3 hours.

3.2.3 Restriction fragment length polymorphism (RFLP) analysis

In the RFLP analysis, a PCR-amplified DNA fragment is cleaved with a restriction endonuclease specific for the wild type or the mutated allele. RFLP was used in Helsinki to detect 11 known BRCA1 and 8 BRCA2 mutations in the study I (Tables 3 and 4). After the PCR-amplification and the restriction enzyme digestion (New England Biolabs, Promega, MBI Fermentas), samples were analyzed on 3% ethidium bromide stained agarose gels. The RFLP analyses were designed such that incomplete digestion would lead to a false-positive result, hence minimizing the possibility of a false-negative result. Mutation positive results were also confirmed by sequencing.

Table 4. Restriction endonucleases and digestion conditions used in mutation detection (I).

Gene and Mutation	Enzyme	Digestion conditions
BRCA1		
1924delA, 3745delT, 3904C>A	*Tsp509*I[a]	65°C, overnight
3604delA, 4154delA	*Mbo*II[a]	37°C, 1 h
4216(-2)A>G	*Mse*I[a]	37°C, overnight
5382insC	*Bst*OI[b]	60°C, over night
BRCA2		
5797G>T	*Nla*III[a]	37°C, overnight
6503delTT	*Mbo*II[a]	37°C, 1 h
8555T>G	MseI[a]	37°C, overnight
9346nt-2A>G	*Bfm*I[c]	37°C, overnight

[a] New England Biolabs; [b] Promega; [c] MBI Fermentas

3.2.4 Solid-phase minisequencing

The principle of the solid-phase minisequencing method is to identify a polymorphic nucleotide at a predetermined site in a DNA template by specific extension of a detection primer by a single nucleotide using a DNA polymerase (Syvänen et al. 1990). Solid-phase minisequencing was utilized in the studies II-IV using conditions described by Syvänen et al. (1998). The mutations studied were 21 BRCA2 mutations listed in Table 3, AR Arg729Leu (CGC to CTC) and CHEK2 1100delC. A DNA fragment spanning the polymorphic nucleotide position was amplified with one unbiotinylated and one biotinylated PCR primer using AmpliTaqGold (Applied Biosystems). The amplified biotinylated fragment was captured on a streptavidin-coated microtiter plate manufactured from scintillating plastic (Wallac, Perkin Elmer) and rendered single-stranded using alkaline denaturation. An automatic microtiter plate washer Delfia Platewash (Wallac, Perkin Elmer) was used for all the washes. In a detection-step, a minisequencing primer designed to anneal immediately adjacent to the polymorphism was extended with a single [^{3}H]dNTP complementary to the nucleotide at the polymorphic site using Dynazyme (Finnzymes) at 50°C for 20 minutes. After the washing steps the amount of incorporated [^{3}H]dNTP was measured

directly from the microtiter plate using 1450 MicroBeta PLUS Liquid scintillation counter (Wallac, Perkin Elmer).

3.3 Screening for novel mutations

3.3.1 Protein truncation test (PTT)

PTT is an efficient method to detect mutations, causing an early translation-termination, from large reverse transcribed mRNA or genomic DNA sequences (Roest et al. 1993). PTT was used to screen large exons 10 and 11 of BRCA2 for mutations in a cohort of 34 MBC blood samples (II). Exon 10 was PCR-amplified in 1 and exon 11 in 4 partly overlapping fragments using modified primers that contained a T7 RNA polymerase promoter sequence and a eukaryotic translation initiation sequence (Håkansson et al. 1997). *In vitro* coupled transcription and translation of the PCR products was done using the TNT® T7 Coupled Reticulocyte Lysate system with [^{35}S]methionine (Promega) according to the manufacturer's instructions. The protein products were run on 5% stacking / 15% separating SDS polyacrylamide gels and visualized by exposing the dried gels to BiomaxMR X-ray films (Kodak) for 1-18 hours.

3.3.2 Denaturing high-performance liquid chromatography (DHPLC)

The DHPLC is a sensitive and cost-effective method for automated detection of single base substitutions as well as small insertions and deletions in BRCA1 and BRCA2 genes (Wagner et al 1999a). In the DHPLC analysis, unpurified PCR products are subjected to a denaturing/reannealing step to ensure adequate formation of heteroduplexes. Under partially denaturing conditions, heteroduplices are retained less than their corresponding homoduplices on a unique DNA separation matrix (Oefner and Underhill 1995, Wagner et al 1999a). The DHPLC was used as described by Wagner et al. (1999a) to screen 34 MBC blood samples for novel BRCA2 mutations in exons 2 to 9 and 12 to 27, including also the first 300 bp of exons 10 and 11 (II). PCR-amplified samples were denatured at 95°C for 3 minutes and were allowed to gradually reanneal from 95°C to 65°C for 30 minutes. DHPLC was carried out on automated HPLC instrumentation equipped with a DNASep column (Transgenomic Inc) packed with 2-µm nonporous alkylated poly(styrenedivinylbenzene) particles. The mobile phase was 0.1 M triethylammonium acetate buffer, pH 7.0, 0.1 mM tetrasodium ethylenediamine-tetraacetic acid. Samples were eluted with a linear acetonitrile gradient at the temperatures between 54°C and 60°C. Homo- and heteroduplices were detected by measuring the absorbance of the eluate at 254 nm.

3.3.3 Single-strand conformation polymorphism (SSCP) assay

SSCP was used to screen AR mutations in the study III. SSCP is based on the electrophoretic mobility of single-stranded nucleic acids (Orita et al. 1989). Mobility depends on the size and the sequence of the fragment analyzed and a mutation usually causes a mobility shift in electrophoresis. Conformational change of the single-stranded mutant DNA is believed to be the reason for the mobility shift. SSCP analysis of the coding sequence of AR, excluding fragments containing CAG and GGC repeats (approximately nucleotides 1-385 and 1405-1576, coordinates from Lubahn et al. 1989), was conducted as previously described (Haapala et al. 2001). PCR-amplification of the samples was done using AmpliTaqGold DNA polymerase (Applied Biosystems) in the presence of 0.4µCi [α^{33}P]-dCTP. PCR fragments were heat denatured and run in 0.5x and 0.8x MDE gels (FMC Bioproducts) containing 10% glycerol for 15 to 24 hours at 800V. Dried gels were exposed to BiomaxMR X-ray films (Kodak) for a day.

3.4 DNA sequencing

Direct sequencing was used for mutation screening of the AR fragments containing CAG and GGC repeats (III). These fragments were not screened with SSCP. Nucleotides approximately 250-310 and 1440-1500 (coordinates from Lubahn et al. 1989) were not analyzed. Direct sequencing was also used to confirm mutations detected by ASO, RFLP, minisequencing, PTT, DHPLC and SSCP methods (I-IV). Genomic DNA was reamplified and sequenced using Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI Prism 310 Genetic Analyser according to manufacturer's instructions.

4. Analyses of androgen receptor gene CAG and GGC repeat lengths

The CAG and GGC repeat lengths were determined by denaturing polyacrylamide gel electrophoresis (III). The CAG and GGC repeats were amplified with FastStart Taq DNA polymerase kit (Roche) in the presence of [α^{33}P]-dCTP (Mitchell et al. 2000). Labeled PCR products were heat-denatured in a formamide dye and run in 6% denaturing polyacrylamide gels at 70 W. Samples with known repeat lengths were used as controls and samples with unknown repeat lengths were compared to those.

5. Statistical analyses

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) 8.0 for Windows (Chicago, IL; Study I) or GraphPad InStat version 2.04a (GraphPad Software, San Diego, CA; Studies II-IV). Continuous parametric variables were shown as mean and standard deviation, and comparisons were made with Student's t-test. Confidence intervals for proportions are based on exact confidence

intervals based on the binominal distribution. Associations between categorical variables were analyzed by Fisher's exact test. All the analyses were two-tailed.

RESULTS

1. Histological and clinical features of the Finnish female breast cancer population (I)

A cohort of 1035 consecutive, newly diagnosed breast cancer patients was collected at the Departments of Oncology at the Helsinki University Central Hospital and Tampere University Hospital. The average age at breast cancer diagnosis was 58.9 years in Tampere and 56.2 years in Helsinki, which is comparable to the average age of diagnosis of breast cancer in Finland.

The histological type was available in 998 tumors (96.3%) of the 1035 patients, and the World Health Organization grade was available in 706 (95.3%) of 741 ductal carcinomas (Table 5) (Vahteristo et al. 2001b).

Table 5. Histologic features of the breast cancers (I, Vahteristo et al. 2001b).

Histology	All patients (%)	BRCA1 carriers (%)	BRCA2 carriers (%)
Ductal	741 (74)	4 (100)	12 (80)
Intraductal	55 (6)	0	0
Lobular	148 (15)	0	3 (20)
Medullary	10 (1)	0	0
Other	44 (4)	0	0
Total	998 (100)	4 (100)	15 (100)
Ductal carcinomas, grade known			
Grade 1	189 (27)	1 (25)	0
Grade 2	313 (44)	0	8 (73)
Grade 3	204 (29)	3 (75)	3 (27)
Total	706 (100)	4 (100)	11 (100)

2. BRCA1 and BRCA2 mutations in the Finnish female breast cancer population (I)

All 11 BRCA1 and 8 BRCA2 mutations found previously in Finland were screened using ASO or RFLP (Table 3). The overall frequency of the BRCA1 and BRCA2 mutations in this cohort of unselected female breast cancer patients was 1.8% (19/1035). Four patients (0.4%) carried a BRCA1 mutation and 15 (1.4%) BRCA2 mutation carriers were found (Table 6). Nine of the 11 previous founder mutations were detected, and they accounted for 17 of the 19 mutations of the present study. In addition, one mutation (5797G>T) seen previously only once was found here in two patients (Vahteristo P, Eerola H, Tamminen A, Blomqvist C, Nevanlinna H, unpublished observation).

Table 6. Clinical characteristics and family history of breast and ovarian cancer of the mutation carriers (I).

Patient No.	Gene and mutation	Age at diagnosis (years)	Age at diagnosis (years) of 1st and 2nd degree relatives with		Brca <40 years (71/1035)*	>2 brca cases, no ovca cases (82/1053)*
			Brca	Ovca (40/1035)*		
	BRCA1					
K506	3604delA	29	34	50	X	
K382	4216(-2)A>G	39			X	
S4	4446C>T	48	30, 40	45, 45, 68		
P321	5370C>T	71	46, 55, 55	50		
	BRCA2					
K3	999del5	47	27, 33, 59, nk	65		
K82	999del5	57	52, 59			X
P566	5797G>T	30	58		X	
P067	5797G>T	50	nk			
K179	6503delTT	56	71, nk			X
464	7708C>T	36	34, 42, 45, 61 , 95	47	X	
P013	7708C>T	37	34/40§, 34, 38, 54	46	X	
P179	7708C>T	32	49	nk	X	
P625	7708C>T	34	55		X	
P563	7708C>T	61	38, 43			X
P360	8555T>G	59	50	60#		
K79	9346(-2)A>G	50	33, 65			X
K106	9346(-2)A>G	56				
P342	9346(-2)A>G	71				
K323	9346(-2)A>G	73				
				8/19 (42%)	7/19 (37%)	4/19 (21%)

Brca, breast cancer; ovca, ovarian cancer; nk, not known
* 181 patients of the entire cohort of 1035 fulfilled any of the three criteria; § bilateral breast cancer;
# index case

Family history of ovarian cancer was the strongest predictor of a mutation. Only 3.9% of all the patients (40/1035) reported a family history of ovarian cancer (Table 7), but interestingly, as many as 8 of the 19 (42%) mutation carriers were found in this small group. One fifth of the index cases in the 40 ovarian cancer families harbored a mutation. These 8 mutations included 3 of the 4 BRCA1 mutations and 5 of the 15 BRCA2 mutations found in the present study. Over half (56%; 5/9) of the patients belonging to families where the breast-ovarian cancer rates were high (3 or more affected relatives) carried a mutation.

Early age at breast cancer diagnosis was another strong predictor for mutations. Mutation carriers were younger at diagnosis 49.7 years (47.0 years for BRCA1 and 50.5 years for BRCA2) than non-carriers 57.5 years (P=0.026). Seventy-one of the 1035 (6.9%) patients were diagnosed with breast cancer under 40 years of age. These young patients had 8 times higher mutation frequency as compared with those 40 years or older (7/71; 9.9% versus 12/964; 1.2%; $p<0.005$).

Site-specific breast cancer was not a good predictor of a mutation. Only 2.2% (7/318) of the patients with at least 1 relative with breast cancer carried a mutation compared to 1.8% of the entire cohort (19/1035). However, 2 or more relatives with breast cancer and no relatives with ovarian cancer were reported by 82 patients (7.9%), and 4.9% of these (4/82) carried a mutation. No mutations were found among the 15 families with 4 or more cases of breast cancer only. The prevalence of mutations was low (0.6%; 4/677) among the family history negative cases.

Table 7. Number of BRCA1 and BRCA2 mutations among patients with different family history of breast and/or ovarian cancers (I).

Family history (No. of affected relatives)	No. of cases	BRCA1 mutations	BRCA2 mutations	BRCA1 and BRCA2 mutations (%)
Negative	677	1	3	4 (0.6)
Positive	358	3	12	15 (4.2)
1	256	0	4	4 (1.6)
Breast cancer	236	0	3	3 (1.3)
Ovarian cancer	20	0	1	1 (5.0)
2	78	1	5	6 (7.7)
Breast cancer only	67	0	4	4 (6.0)
Ovarian cancer included	11	1	1	2 (18.2)
≥ 3	24	2	3	5 (20.8)
Breast cancer only	15	0	0	0 (0)
Ovarian cancer included	9	2	3	5 (55.6)
Total	1035	4	15	19 (1.8)

3. Finnish males with breast cancer and characteristics of their cancers

The Finnish Cancer Registry was used to identify all MBC patients diagnosed in Finland between 1967 and 1996 (Figure 5). Those 237 MBC cases with microscopically confirmed diagnosis of carcinoma were eligible for our studies. Average age at the breast cancer diagnosis among these 237 patients was 65.4 years (range 30-94). The birthplaces of the MBC patients used in our studies were distributed throughout the country.

Histological type was available for all 147 MBC tissue samples with confirmed diagnosis; 128 were invasive ductal carcinomas (87.1%), 14 invasive lobular carcinomas (9.5%), 4 ductal carcinomas *in situ* (2.7%), and 1 Paget disease (0.8%). Thirty-one per cent of the invasive ductal carcinomas were of World Health Organization grade 3, 56% of grade 2, and 13% of grade 1. Patients' medical records and follow-up data were not available for the studies.

4. BRCA2 mutations in male breast cancer (II)

DNA samples extracted from either blood (n=37) or paraffin-embedded tissues (n=119) of 156 Finnish MBC cases were available for the study II. All 8 BRCA2 mutations found previously in Finland were screened using minisequencing (Table 3). Two patients with paraffin-embedded tissue samples were excluded after mutation screening because of conflicting mutation results from different tissue blocks.

Twelve BRCA2 mutation carriers were found among the 154 (7.8%) patients (Table 8). Three of the 8 different BRCA2 mutations studied were found in 10 patients. In addition, two novel mutations, 4075delGT and 5808del5, were found by screening the entire coding region of BRCA2 in a cohort of 34 patients with blood samples. These 34 patients did not carry any of the previously found BRCA2 mutations. The

two novel mutations were screened in all 154 samples, but no additional mutation carriers were found. Twelve different silent polymorphisms were also detected (203G>A, 1093A>C, 7470A>G, 7663(+53)C>T, 8034(-14)C>T, 8983(-66)T>C, 9485(-141)T>C, 9485(-84)G>A, 9485(-49)T>C, 9485(-16)T>C, 10204A>T and 10462A>G). Founder mutation carriers originated from the same regions in Finland as the female breast cancer patients with the mutations in question (Sarantaus et al. 2000).

Table 8. Clinical characteristics of the MBC cases with BRCA2 mutations (II).

Patient	Mutation	Age at diagnosis (years)	Histological Type (Grade)	Family history of cancer among 1st and 2nd degree relatives Type of relative (age at diagnosis)	
				Breast cancer	Other cancers
Y170	999del5	46	IDC (3)	S (25), S (45), S (54), S (65, 67) *	M stomach (76), F prostate (63), B lung (64), PA uterine (61), PA fallopian tube (79)
Y047	4075delGT	58	ILC	B (78)	S ovarian (67), S cervical (71)
Y099	5808del5	66	IDC (3)	MA (76), PA (83)	MA skin (97), GD leukemia (5), B skin (74), BD chorion (30)
Y021	7708C>T	71	IDC (3)	M (80), S (50)	F skin (NA), S liver (78), SD brain (8)
Y063-17	9346(-2) A>G	67	IDC (2)	SS (71), SD (36), SD (49), SD (50) a, SD (54) b	SD ovarian (58), D uterine (55), So prostate (60), B colon (87), S laryngeal (NA), SD cervical (39), SD cervical (47) a, SD lymphoma (69) b
Y076	9346(-2) A>G	64	IDC (3)		
Y084	9346(-2) A>G	49	IDC (2)	S (73)	F prostate (69), F skin (47), B prostate (58)
Y089	9346(-2) A>G	66	IDC (2)		
Y095	9346(-2) A>G	69	IDC (3)	D (44) c, S (80)	D chorion (33) c, B prostate (69)
Y103	9346(-2) A>G	91	IDC (1)		
Y115	9346(-2) A>G	57	ILC		
Y157	9346(-2) A>G	75	IDC (2)		B stomach (84), So prostate (56)

IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma
M, mother; F, father; S, sister; B, brother; D, daughter; So, son; GD, granddaughter; PA, paternal aunt; PU, paternal uncle; MA, maternal aunt; SD, sister's daughter; SS, sister's son; BD, brother's daughter; NA, not available
* bilateral breast cancer
a, b, c same individuals

Average age at the breast cancer diagnosis was 65.7 years (SD 12.3; range 30-94). Mutation carriers were slightly younger (64.9 years; SD 11.9; range 46-91) than non-carriers (mean 65.7 years; SD 12.4; range 30-94) at the time of diagnosis, but the difference did not reach statistical significance (p=0.83). None of the 4 patients under 40 years and only 2 of the 17 patients under 50 years of age at the time of diagnosis carried a mutation.

Seven of the 16 MBC patients (44%) with at least 1 first- or second-degree relative with female breast cancer had a BRCA2 mutation, whereas only 5 of the 138 (3.6%) patients without family history of breast cancer were mutation carriers. This difference was statistically highly significant ($p<0.0001$). Two of the 3 MBC cases with a first- or a second-degree relative with ovarian cancer carried a BRCA2

mutation, 4075delGT and 9346(-2)A>G. Two mutation positive MBC patients were found to have relatives with MBC. Additionally, two initially unrelated MBC patients without BRCA2 founder mutations were found to be related to each other (second-degree relatives) in a more detailed pedigree analysis. Unfortunately, only tissue samples were available from these patients, and we were not able to screen the entire BRCA2 gene for mutations.

5. AR is not altered in Finnish male breast cancer patients (III)

The entire coding region of the AR gene, with the exception of CAG and GGC repeats (approximately nucleotides 250-310 and 1440-1500, coordinates from Lubahn et al. 1989), was screened for mutations with SSCP or direct sequencing in a cohort of 32 Finnish MBC cases of whom blood samples were available. No mutations were found, but 5 patients (16%) carried previously reported silent polymorphisms (Batch et al. 1992, Hiort et al. 1994, Nordenskjöld et al. 1999). A silent Leu838Leu (CTC to CTT) polymorphism was seen in 1 patient and 4 patients carried a Glu211Glu (GAG to GAA).

A germline mutation Arg726Leu (CGC to CTC) has been detected in 2% of Finnish prostate cancer patients, almost 6 times more often than in the general population (Mononen et al. 2000). This mutation was screened in 117 MBC patients (32 blood samples and 85 paraffin-embedded tissue samples) by solid-phase minisequencing, but none carried the allele.

Lengths of CAG and GGC repeats were also investigated among 32 MBC cases with blood samples. The mean number of CAG and GGC repeats was 21.8 (SD 2.7; median 22; range 14-28) and 16.1 (SD 2.9; median 17; range 4-19), respectively (Figure 6). Similar distributions have been seen among healthy control populations in Finland (Lund et al. 2000, Mononen et al. 2002).

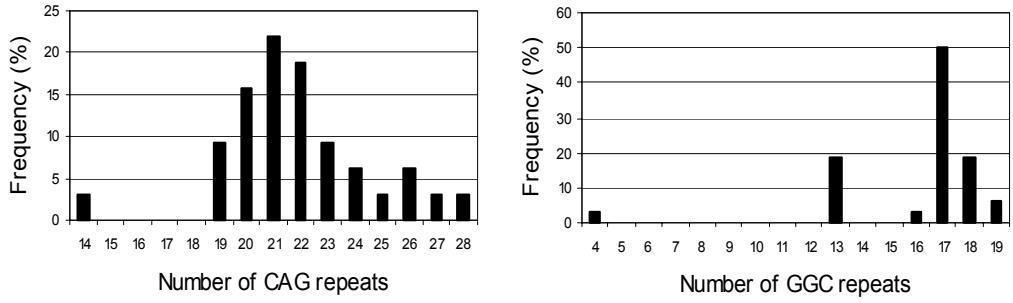

Figure 6. Frequency of androgen receptor CAG and GGC repeats among 32 Finnish male breast cancer patients (III).

6. CHEK2 1100delC mutation is not a great risk factor for male breast cancer in Finland (IV)

CHEK2 1100delC mutation was screened using minisequencing in a cohort of 114 MBC patients, including six BRCA2 9346(-2)A>G and one of each 999del5, 4075delGT, 5808del5 and 7708C>T mutation positive cases. Two CHEK2 1100delC mutation carriers were found. The mutation frequency (1.8%) was similar to that seen in Finnish population controls (26/1885; 1.4%) (Vahteristo et al. 2002) and corresponds to a prevalence ratio of 1.27 (exact 95% confidence interval 0.04-7.92).

Twelve MBC patients had a positive family history of female or male breast cancer or ovarian cancer. The 1100delC mutation carriers did not have a positive family history of cancer. The mutation carriers were slightly older (66 and 67 years) at the time of diagnosis than the cohort in average (65.1 years; standard deviation 12.9; range 30-94). One of the patients with the CHEK2 1100delC mutation carried also the BRCA2 9346(-2)A>G mutation.

DISCUSSION

1. BRCA1, BRCA2 and female breast cancer (I)

The frequency of the 11 previously identified Finnish BRCA1 and 8 BRCA2 mutations was 1.8% among the 1035 unselected Finnish female breast cancer patients. This is much lower than among unselected Icelandic and Ashkenazi breast cancer patients (7-12%) in whom a strong founder effect can also been seen (Johannesdottir et al. 1996, Thorlacius et al. 1997, 1998, Fodor et al. 1998, Warner et al. 1999). This emphasizes the fact that the frequency of BRCA1 and BRCA2 mutations varies in different populations (Szabo and King 1997). A higher prevalence (3.1-6.7%) of BRCA1 and/or BRCA2 mutations among unselected breast cancer patients has also been seen in China, Hungary, Spain, Pakistan and among French Canadians (Garcia-Patino et al. 1998, Tang et al. 1999, van der Looij et al. 2000, Chappuis et al. 2001, Liede et al. 2002). Lower frequencies (0.8-1.6%) have also been reported among unselected breast cancer patients. In these studies, only BRCA1 has been included, and in only one of the four studies the entire gene was analyzed (Emi et al. 1998, Newman et al. 1998, Anton-Culver et al. 2000, Papelard et al. 2000). A large retrospective study of BRCA1 and BRCA2 mutations in early-onset breast cancer cases estimated a similar frequency for both BRCA1 and BRCA2 mutations (2.8%) as obtained in study I (Peto et al. 1999).

The mutation frequency reported in study I is obviously an underestimate, since only the previously identified mutations were screened. However, we believe that the result represents well the BRCA1 and BRCA2 mutation burden since the founder mutations were reported to account for 84% of the BRCA1 and BRCA2 mutations in Finnish families when the entire coding regions were screened (Vehmanen et al. 1997a, b, Huusko et al. 1998). Additionally, linkage analysis suggested possible linkage to either BRCA1 or BRCA2 in only four of 24 additional families with high breast-ovarian cancer rates (T. Kainu, personal communication). Large BRCA1 gene rearrangements found in e.g. the Netherlands and Northern Italy have not been found in Finland (Petrij-Bosch et al. 1997, Lahti-Domenici et al. 2001, Sarantaus et al. 2001b, Montagna et al. 2003). Furthermore, in a study of unselected Finnish ovarian cancer patients 12/13 (92%) and in another study of breast-ovarian cancer families 24/29 (83%) of the mutations were recurrent BRCA1 or BRCA2 mutations (Sarantaus et al. 2001b, Vahteristo et al. 2001a). The screening of the known mutations is therefore an efficient way to estimate the impact of BRCA1 and BRCA2 mutations in Finland. At the end of the year 2003, 20 BRCA1 and 11 BRCA2 mutations were identified in Finland. The new mutations in BRCA1 are 782delAA, 1047C>T, 1201del11, 1731C>T, 1806C>T, 2592insA, 3264delT, 4599G>T and 5622C>T and in BRCA2 1822G>T (H. Nevanlinna, personal communication).

Screening of all breast cancer patients for BRCA1 and BRCA2 mutations remains clinically and ethically unjustified and impossible to perform in practice. Identifying phenotypic clues that would predict the likelihood of finding mutations is therefore vital. In the Study I, family history of ovarian cancer strongly suggested the presence of both BRCA1 and BRCA2 mutations. Fewer than 4% of patients reported a family history of ovarian cancer, but almost one half of all mutations (42%) were found in this group. Early age at breast cancer diagnosis was another strong predictor of mutations. Patients diagnosed under 40 years of age had an eight times higher mutation frequency when compared to those of 40 years or older (9.9% versus 1.2%; P>0.0005). Family history of site-specific breast cancer was not a strong indicator of mutations. Patients with 2 or more relatives with breast cancer had a 4.9% prevalence of mutations and 4/19 (21%) mutation carriers were found in this group. These three criteria distinguished 15 mutation carriers in a subset of 181 patients (8.3%).

2. Male and female breast cancer and BRCA2 (I, II)

Histological type was available for most of the breast cancers studied (I, II). As reported by others, invasive ductal carcinoma was more common among males than females (87% versus 74%) (Cutuli et al. 1995, Giordano et al. 2002). There was a slight trend towards higher World Health Organization grades of male compared with female ductal carcinomas: grade 3 (31% versus 29%), grade 2 (56% versus 44%) and grade 1 (13% versus 27%). A similar trend has been seen in some but not in all studies (Willsher et al. 1997, Muir et al. 2003, Anderson et al. 2004).

The presence of 8 Finnish BRCA2 mutations was studied in 154 Finnish MBC patients (I). In addition, the entire coding sequence of the BRCA2 gene was screened in 34 MBC patients for whom blood samples were available. The overall mutation frequency was 7.8% (12/154), which is somewhat lower than BRCA2 mutation frequencies among MBC patients in most previously published studies (Table 2). However, by accounting for possible sporadic mutations present in the same frequency as among the 34 patients screened (2/34; 5.9%), we can estimate an overall mutation frequency of about 12-13%. This is well in line with previous studies, where the entire coding sequence of all patients was screened for mutations.

Unselected MBC cases were 5.4 times more often carriers of a BRCA2 mutation than unselected female patients (I, II). Very similar figures were seen among unselected Icelandic (4.7-5.2) and Ashkenazi Jewish (4.1-4.9) male and female breast cancer populations where a strong founder effect can also be seen (Johannesdottir et al. 1996, Thorlacius et al. 1996, 1997, Fodor et al. 1998, Struewing et al. 1999, Warner et al. 1999). This suggests that genetic etiology of MBC may be more homogenous than that of female breast cancer.

The spectrum of BRCA2 mutations varied between Finnish male and female breast cancer populations (Figure 7)(I, II). BRCA2 9346(-2)A>G was the most common mutation among the MBC cases with a frequency of 67% (8/12 BRCA2 mutation

carriers). This was the second most common mutation among female breast cancer patients with a much lower frequency (4/15; 27%). The difference in 9346(-2)A>G mutation frequencies was significant between males and females (P<0.0001; odds ratio 14; 95% confidence interval 4-48). Interestingly, only one MBC patient carried the 999del5 mutation, which is the most common BRCA1/2 founder mutation in Finland (Barkardottir et al. 2001). As many as 40% of Icelandic MBC patients carry this mutation (Thorlacius et al. 1996). The low number of 999del5 mutations cannot be explained by simple selection bias, since dozens of samples were available from the two Finnish founder areas of this mutation (Barkardottir et al. 2001). Our findings and those by Thorlacius et al. (1996) therefore point to additional genetic modifier loci, or possibly environmental factors that might differentially influence male and female breast cancer penetrance among carriers of different BRCA2 mutations.

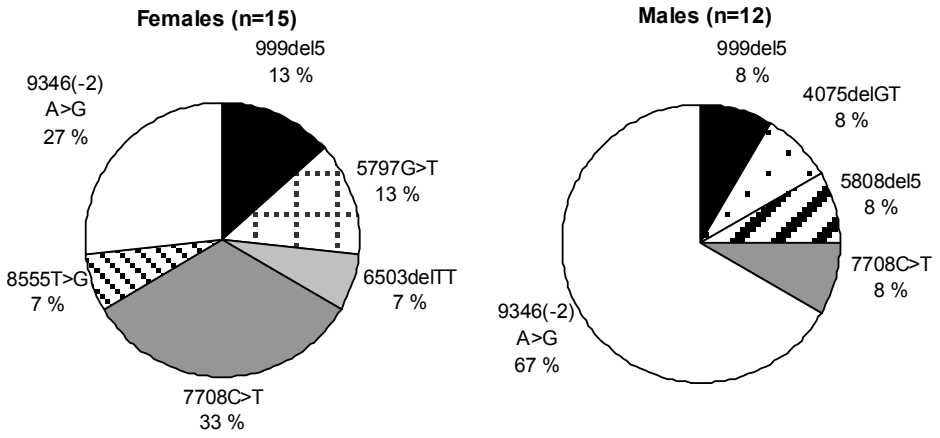

Figure 7. BRCA2 mutation spectrum among mutation positive Finnish female and male breast cancer populations (I, II).

Men were on average 8 years older at the time of breast cancer diagnosis than women (65.7 versus 57.3 years) (I, II). Female BRCA2 mutation carriers were diagnosed earlier than non-carriers (50.5 versus 57.5 years). Such a clear trend was not seen among men with and without a BRCA2 mutation (64.9 versus 65.7 years). None of the 4 male patients under 40 years carried a BRCA2 mutation compared with 5 of the 71 young female patients. Others have also reported that men are 5-10 years older than women at the time of breast cancer diagnosis and that the age at MBC diagnosis is not affected by the BRCA2 mutation status (Sasco et al. 1993, Cutuli et al. 1995, Donegan and Redlich 1996, Csokay et al. 1999, Hill et al. 1999, Struewing et al. 1999, Frank et al. 2002, Anderson et al. 2004).

Family history of ovarian cancer was the strongest predictor of a BRCA2 mutation among MBC cases, just as it was among female breast cancer patients (I, II). Two of the 3 MBC patients with a relative with ovarian cancer carried a BRCA2 mutation. Breast cancer among female relatives was a stronger predictor of a BRCA2 mutation among males than among females. As many as 44% of the MBC patients with at least

one first- or second-degree relative with female breast cancer carried a BRCA2 mutation as compared with 3.3% of females (I, II).

3. AR and male breast cancer (III)

The entire AR coding region, including polyglutamine (CAG) and polyglycine (GGC) tracts, of 32 Finnish MBC patients was screened for alterations. In addition, an AR Arg726Leu (CGC to CTC) germline mutation was studied in 117 MBC samples (85 paraffin-embedded and 32 blood samples). We did not find any mutations. Our results are consistent with the studies by Haraldsson et al. (1998) and Kwiatkowska et al. (2001): AR mutations are rare and are only found in MBC patients with partial androgen insensitivity syndrome (Wooster et al. 1992, Lobaccaro et al. 1993a, b, Haraldsson et al. 1998, Kwiatkowska et al. 2001). Existence of any complete or partial androgen insensitivity syndrome patients in our MBC study cohort is highly unlikely since most men in our cohort had children, a contradiction to androgen insensitivity syndrome. Both of the AR mutations found so far change adjacent amino acids in the DNA-binding domain in exon 3 (Wooster et al. 1992, Lobaccaro et al. 1993a, b). The mutated receptor has reduced transactivation efficiency (Poujol et al. 1997). There might be some unknown mechanism why these two mutations, but not the others causing androgen insensitivity syndrome, lead to MBC. Five patients carried previously reported silent polymorphisms, but these two alterations are unlikely to increase MBC risk (Nordenskjöld et al. 1999, Batch et al. 1992, Hiort et al. 1994). The frequency of Glu221Glu (GAG to GAA) polymorphism is as high as 8-14% in the general population (Batch et al. 1992, Hiort et al. 1994).

The CAG and GGC repeat allele distributions (CAG: mean 21.8; median 22; range 14-28 and GGC mean 16.1; median 17; range 4-19) found in our study were similar to that of healthy control populations in Finland (Lund et al. 2000, Mononen et al. 2002). An association between CAG repeat lengths and risk of female breast cancer has remained controversial (Rebbeck et al. 1999, Spurdle et al. 1999, Given et al. 2000, Yu et al. 2000, Giguere et al. 2001, Kadouri et al. 2001, Menin et al. 2001, Haiman et al. 2002). Rebbeck et al. (1999) suggested that women with BRCA1 mutations are at a significantly increased risk of breast cancer if they carry at least one allele with 28 or more CAG repeats. It has been proposed that long CAG repeats increase the risk of breast cancer in the general population and among women with a family history of breast cancer (Giguere et al. 2001, Haiman et al. 2002). An association of shorter CAG repeats with more aggressive forms of breast cancer has also been suggested (Yu et al. 2000). The two studies on polyglutamine tracts among MBC cases found similar results as we did (Haraldsson et al. 1998, Young et al. 2000). There was no significant difference in the number of CAG repeats in MBC patients when compared to controls in these studies. However, long repeats (over 28 or over 30) were seen only among the MBC cases. Our study did not reveal that long CAG repeats were common among MBC cases. To estimate more comprehensively the significance of AR mutations and polyglutamine and polyglycine tracts to the risk of male and female breast cancer, larger cohorts should be studied.

4. CHEK2 and male breast cancer (IV)

Two of the 114 MBC patients carried the CHEK2 1100delC mutation. One of the carriers also had a BRCA2 9346(-2)A>G mutation. The average age at breast cancer diagnosis was 65.1 years. The two 1100delC carriers were diagnosed with breast cancer at the ages of 66 and 67 years. There is only a weak evidence for a decline in the relative risk of female breast cancer with age among CHEK2 1100del carriers and our study on MBC is in agreement with that (Vahteristo et al. 2002, CHEK2 Breast Cancer Case-control Consortium 2004). CHEK2 1100delC mutation does not greatly influence the onset of breast cancer.

The mutation frequency of 1.8% found in our study is similar to that of Finnish population controls (26/1885; 1.4%) (Vahteristo et al. 2002) and corresponds to a prevalence of 1.27 (95% confidence interval 0.04-7.92). Recently, it was reported that as many as 13.5% of individuals from families with MBC were carriers of 1100delC compared with 1.1% of controls (Meijers-Heijboer et al. 2002). It was estimated that CHEK2 1100delC variant results in a 2-fold increase of breast cancer risk in women and a 10-fold increase of risk in men (Meijers-Heijboer et al. 2002). The mutation was also estimated to account for 1% of breast cancers in women and 9% of breast cancers in men at the population level (Meijers-Heijboer et al. 2002). MBC population-based studies from Israel, the UK and the USA support our results that CHEK2 1100delC is unlikely to account for a significant fraction of MBC cases (Neuhausen et al. 2004, Ohayon et al. 2004). None of the 125 MBC patients from the USA, 79 patients from the UK and 54 patients from Israel carried the mutation (Offit et al. 2003, Neuhausen et al. 2004, Ohayon et al. 2004).

There might be several reasons for the difference in mutation frequencies among MBC patients reported by Meijers-Heijboer et al. (2002) and by the others (Neuhausen et al. 2004, Ohayon et al. 2004, IV). CHEK2 1100delC mutations are more common among female breast cancer patients with at least one first-degree relative with breast cancer and the prevalence increases with the number of affected relatives (Meijers-Heijboer et al. 2002, Vahteristo et al. 2002, CHEK2 Breast Cancer Case-Control Consortium 2004). Meijers-Heijboer et al. (2002) analyzed breast cancer patients from families without BRCA1 or BRCA2 mutations who had at least one case of female and one case of male breast cancer in first- or second-degree relatives. In the other three studies, patients were unselected for the family history of cancer and included many men without positive family history. The two 1100delC carriers in our study did not have a family history of cancer. Additional genetic or environmental factors could increase the risk of MBC among CHEK2 1100del carriers in the cohort of Meijers-Heijboer et al. (2002). The difference in frequencies could also be entirely due to chance. The most probable explanation is that CHEK2 1100delC does not significantly increase the risk of MBC at the population level.

5. Future aspects

Two major breast cancer susceptibility genes, BRCA1 and BRCA2, have been identified so far. BRCA2 mutations explain about 8% of Finnish MBC cases. The role of BRCA1 to the predisposition of Finnish MBC has not been comprehensively studied and requires further investigation.

A substantial percentage of breast cancer families do not carry mutations in BRCA1 or BRCA2, indicating that additional breast cancer susceptibility genes are likely to exist (Antoniou et al. 2002, reviewed in Wooster and Weber 2003, and in Narod and Foulkes 2004). Several candidate regions for a third high-penetrance breast cancer gene (BRCA3) have been proposed, including 2q, 8p12-p22 and 13q21, but these results have not been replicated in independent studies (Kerangueven et al. 1995, Kainu et al. 2000, Rahman et al. 2000, Thompson et al. 2002c, Huusko et al. 2004). Despite rapid advances in new high-throughput techniques, the search for BRCA3 has been difficult. One of the reasons is that no distinctive phenotype for a third class of inherited breast cancer has emerged. It has been suggested that several common, low penetrance genes with multiplicative effects on risk may account for the residual non-BRCA1/2 familial aggregation of breast cancer (Antoniou et al. 2002). Isolated, disease-associated mutations have been found in possible, low-penetrance candidate genes such as BACH1, BARD1 and HFE (Thai et al. 1998, Ghimenti et al. 2002, Kallianpur et al. 2004, Cantor et al. 2004). As new breast cancer predisposing and risk modifier genes are identified their role to MBC susceptibility needs to be studied.

MBC is a rare disease and therefore most of the available data on MBC arise from small studies involving a few dozen patients often collected from a small geographical area. Larger, possible international MBC cohorts should be collected. It is possible that subgroups of families with a specific cancer profiles could be identified from such large cohorts. These families could be used to identify new genes involved in predisposition to male and possible female breast cancer.

SUMMARY AND CONCLUSIONS

The purpose of this thesis was to explore the hereditary predisposition of Finnish male and female populations to breast cancer.

1. Germline BRCA1 and BRCA2 mutations were present in 1.8% of unselected female breast cancer patients. Mutations were more often found among the patients with a family history of ovarian cancer or of early onset breast cancer. Family history of site-specific breast cancer was not a strong predictor of a mutation.

2. BRCA2 mutations were present among 12 (7.8%) of the 154 MBC patients. Patients with a positive family history of breast and/or ovarian cancer were often BRCA2 mutation carriers (44%), whereas those with no family history showed a low frequency of involvement (3.6%).

3. Cancers of men were more often invasive ductal carcinomas than were female carcinomas (87% versus 74%). In average, MBC was diagnosed 8 years later than breast carcinomas in females (65.7 versus 57.3 years). BRCA2 mutation status did not influence the age of MBC diagnosis as it did in the females.

4. The spectrum of BRCA2 mutations varied between Finnish male and female breast cancer populations. Additional genetic modifier loci or environmental factors possibly influence differentially male and female breast cancer penetrance among carriers of different BRCA2 mutations.

5. AR mutations are very rare among MBC patients and might be limited to patients with partial androgen insensitivity syndrome. No difference was seen in the number of highly polymorphic polyglutamine (CAG) and polyglycine (GGC) tracts of AR when compared to population controls.

6. CHEK2 1100delC mutation accounts for only a small fraction (1.8%) of MBC cases in Finland. The two mutation carriers did not have a family history of cancer and the age of breast cancer onset was not influenced by the mutation.

7. Some of the MBC cases might be carriers of BRCA1 mutations or mutations of still unidentified novel breast cancer susceptibility genes. These and possible breast cancer risk modifier genes should be analyzed in further studies.

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