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University of Iceland, Faculty of Medicine**

**Mapping of genomic aberrations in
non-BRCA1/2 familial breast cancer
using array-CGH**

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Meistararitgerð við Læknadeild Háskóla Íslands

**Kortlagning erfðabrenglana með hjálp
örflögutækni í brjóstæxlum sjúklinga
úr ættum með hækkaða tíðni meinsins**

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ÁGRIP

Brjóstakrabbamein er næst algengasta krabbamein í heiminum og greinist ein af hverju tíu konum í hinum vestræna heimi með sjúkdóminn á lífsleiðinni. Auk aldurs er fjölskyldusaga um brjóstakrabbamein einn helsti áhættuþátturinn sem staðfestur hefur verið. Tvö æxlisbæligen, BRCA1 og BRCA2, hafa verið einangruð og hefur verið sýnt fram á að kímlínustökkbreytingar í þeim auki áhættuna á að fá brjóstakrabbamein 10-20-falt. Samhliða þess konar stökkbreytingu sjást í æxlunum ummerki þess að samstæði mótlitningurinn hafi misst erfðaefnisbút sem innihélt heilbrigða eintak gensins, í samræmi við kenningu Knudson um tveggja þrepa æxlismyndun. Margar fjölskyldur eru með ættlægni sem ekki skýrist af BRCA1 eða BRCA2 en fram að þessu hafa engin fleiri gen með ámóta þýðingu fyrir ættlæg brjóstakrabbamein verið einangruð.

Þessi rannsókn nýtir array-CGH (örflögutækni) til að finna magnbrenslanir litningshluta í æxlum og kortleggja hvaða litningssvæðisvæði sýna áþekka brenglun þegar meðlimir hvernar fjölskyldu eru bornir saman. Slík litningssvæði geta borið í sér gen sem ráða framvindu krabbameinsins og jafnvel orsaka ættlægnina en það er þá sannreynt frekar með erfðatengslaprófi. Sýni úr 131 æxli með ættlægni án kímlínubreytinga í BRCA1 eða BRCA2 voru tekin í örflöguskoðun og af þeim voru 61 úr 15 fjölskyldum með þrjú eða fleiri sýni. Fjögur svæði á litningi 16 reyndust hafa algengar magnbrenslanir í fimm fjölskyldum, nánar tiltekið í a.m.k. 19 af 23 æxlum. Tvö svæðanna eru með magnaukningu og eru á stutta armi

litningsins (16p13.3 og 16p11.2), hin tvö sýna magntap og eru á langa arminum (16q21 og 16q23.2). Tengslagreining á völdum erfðamörkum í viðkomandi svæðum á litningi 16 sýndi ekki marktæk tengsl við brjóstakrabbamein í þessum fjölskyldum en í einni þeirra reyndust þær fimm konur sem fengið höfðu brjóstakrabbamein hafa erfð sameiginlega setröð á 16q21. Með stikalausum fjölpunkta tengslaprófi á þeirri fjölskyldu fengust hámarkslogdildi 1,6 til 1,8. Í svæði sameiginlegu setraðarinnar er að finna SNP-erfðapátt, rs3803662, sem nýlega var sýnt fram á að bæri erfðasamsætu með væg áhrif til brjóstakrabbameinsmyndunar. Þessi erfðapáttur var greindur í viðkomandi fjölskyldu og líka hinum fjórum og reyndist ekki skýra þá háu tíðni brjóstakrabbameins sem finnst í fjölskyldunum. Annarsstaðar á litningi 16 (16p12) er að finna genið PALB2 sem sýnt hefur verið fram á að tengist brjóstakrabbameinsáhættu í Finnlandi. Það er staðsett í 10 cM fjarlægð frá svæðinu sem sýndi tíða magnaukningu á 16p11.2. PALB2 sýndi ekki marktæk erfðatengsl í íslensku fjölskyldunum. Rannsóknin í heild bendir til að ættlægnin sem skoðuð var stafi ekki af almennum áhrifum æxlisbæligens né gens sem af einhverjum orsökum kynni að einkennast af því að sjást magnbreytt í æxli.

ABSTRACT

Breast cancer is the second most frequent cancer in the world affecting one out of every ten women in the Western world. Besides age the best established risk factor for breast cancer is family history of the disease. Two main breast cancer susceptibility genes have been identified, BRCA1 and BRCA2, with an increased relative risk of 10- to 20- fold. Both genes are tumour suppressor genes found to be disabled by germline mutation and somatic loss in tumour cells in accordance to Knudson's two-hit model. A proportion of high-incidence breast cancer families is not explained by these two genes but despite extensive research no other breast cancer susceptibility genes of comparable significance have been identified.

In the current study we use array CGH to map DNA copy-number changes in an effort to reveal chromosome regions with aberrations that breast tumours derived from the same family have in common. Such an observation would suggest the location of a gene influencing breast cancer development and possibly the susceptibility gene, subject to further tests by linkage analysis. Of 131 non-BRCA1/2 familial breast tumours included in the array-CGH study, 61 originate from 15 families with three or more studied family members each. Copy-number changes are seen at four chromosome-16 regions in at least 19 of 23 tumours deriving from five families. These four regions are 16p13.3 and 16p11.2 (copy number gain) and 16q21 and 16q23.2 (copy number loss). Linkage analysis with microsatellite markers in these four regions indicated no evidence of breast cancer susceptibility

genes in four of the five families. In one family haplotype sharing was observed at chromosome region 16q21 in all five breast cancer cases. Multipoint analysis under a non-parametric model gave a maximum lod score ranging from 1.6 to 1.8 for this family. SNP rs3803662 is located within the shared haplotype region and recently one of its alleles has been identified as a low-penetrance risk allele of breast cancer. Genotyping the family members of this and the other four families this marker was not seen to explain the high frequency of breast cancer among the families. The PALB2 gene located at 16p12 has been identified as a breast cancer susceptibility gene in Finnish breast cancer cases. It is located approximately 10cM from the region showing frequent copy number gain at 16p11.2. Linkage analysis showed no evidence of PALB2 linkage in the breast cancer families analysed. The results of this study indicate that the analysed high risk breast families are not likely to be explained by a tumour suppressor gene or other possible forms of a common autosomal dominant effect characterized by copy-number changes.

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ABBREVIATIONS

BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
TP53	Tumour protein 53
PTEN	Phosphatase and tensin homolog
CGH	Comparative genomic hybridization
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
SNP	Single-nucleotide polymorphism
dCTP	Deoxycytidine triphosphate
SSC	Saline-sodium citrate
SDS	Sodium dodecyl sulfate
TRIS	tris(hydroxymethyl)aminomethane
MgCl ₂	Magnesium chloride
NaCl	Sodium chloride
ETDA	ethylenediaminetetraacetic acid
dNTP	deoxyribonucleotide
ATM	Ataxia telangiectasia mutated
BRIP1	BRCA1 interacting protein C-terminal helicase 1
CHEK2	CHK2 checkpoint homolog (<i>S. pombe</i>)
CDH8	Cadherin 8, type 2
CDH11	Cadherin 11, type 2
FRA16D	Fragile site 16D
WWOX	WW domain containing oxidoreductase
RET	ret proto-oncogene
MEN2	Multiple endocrine neoplasia type 2

TNRC9	TOX high mobility group box family member 3
FGFR2	Fibroblast growth factor receptor 2
ATR	ataxia telangiectasia and Rad3 related
MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1
MSH6	mutS homolog 6
p21	Cyclin-dependent kinase inhibitor 1A
GADD45	Growth Arrest and DNA Damage
RAD51	RAD51 homolog (RecA homolog, <i>E. coli</i>) (<i>S. cerevisiae</i>)
LKB1/STK11	serine/threonine kinase 11
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
MYC	v-myc myelocytomatosis viral oncogene homolog
CCND1	cyclin D1
MDM2	Mdm2 p53 binding protein homolog

INTRODUCTION

Breast cancer is the second most frequent cancer in the world, after lung cancer, affecting one out of every ten women in the Western world during their lifetime. It is the most frequent cancer among women, accounting for 22% of all cancers whereas less than 1% of breast cancer cases occur in males (Hill *et al.*, 2005; Parkin, 2004). The incidence rates are much higher in the developed countries compared to the developing countries with Europe and North America together accounting for almost 60% of worldwide cases (Parkin, 2004; Parkin *et al.*, 1999). Environmental and cultural factors clearly play a major role in this international variation. Studies have shown that breast cancer incidence increases in immigrants from regions with lower breast cancer incidence who move to regions with higher incidence, and within one or two generations the risk is on level to that of the native population (Kliewer & Smith, 1995; Ziegler *et al.*, 1993). Various factors besides environmental and cultural factors have been linked to increased breast cancer risk e.g. family history of breast cancer, mutations in tumour suppressor genes and socioeconomic status. Factors modulating the exposure to hormones including age at menarche and menopause, early pregnancy and use of oral contraceptives are strongly associated to increased breast cancer risk (reviewed in (Dumitrescu & Cotarla, 2005)).

Familial breast cancer and predisposing genes

Besides age the best established risk factor for breast cancer is family history of the disease. Women with an affected first-degree relative are twice as likely to develop breast cancer and the risk is increased with the number of affected relatives (Collaborative.Group.on.Hormonal.Factors.in.Breast.Cancer, 2001). About 5-10 % of breast cancer cases are due to high penetrance germline mutations in breast cancer susceptibility genes (reviewed in (Balmain *et al.*, 2003; Fackenthal & Olopade, 2007; Oldenburg *et al.*, 2007)). Lifestyle and endogenous factors together with moderate- and common low-penetrance cancer susceptibility genes probably account for the rest of breast cancer cases (reviewed in (Dumitrescu & Cotarla, 2005) (Stratton & Rahman, 2008)).

High-penetrance breast cancer susceptibility genes

To date, two main breast cancer susceptibility genes have been identified, BRCA1 and BRCA2, with an increased relative risk of breast cancer of 10- to 20- fold (Fackenthal & Olopade, 2007; Stratton & Rahman, 2008). The estimated overall familial risk of breast cancer due to BRCA1 and BRCA2 is about 15-20%, and the genes account for nearly half of high-incidence breast cancer families (Anglian.Breast.Cancer.Study.Group, 2000; Peto *et al.*, 1999). Germline mutations in other genes such as TP53, PTEN and LKB1/STK11 also confer a high risk of breast cancer but are very rare and are associated with the multi-cancer syndromes Li-Fraumeni

syndrome, Cowden disease and Peutz-Jegher syndrome (reviewed in (Oldenburg *et al.*, 2007)).

BRCA1 and BRCA2

In 1990 BRCA1 was located on chromosome 17q21 through linkage analysis on early-onset breast cancer families and finally isolated in 1994 (Hall *et al.*, 1990; Miki *et al.*, 1994). In 1994, applying linkage analysis on early-onset breast cancer families, BRCA2 was located on chromosome 13q12 and isolated the following year (Wooster *et al.*, 1995; Wooster *et al.*, 1994). The lifetime risk of developing breast or ovarian cancer, for women carrying germline mutation in BRCA1 is 65-85% and 39-63%, and for BRCA2 45-84% and 11-27% (Antoniou *et al.*, 2003; Ford *et al.*, 1998). BRCA2 is also associated with male breast cancer, estimating carriers to have an 80-fold higher risk of developing breast cancer, accounting for about 10% of all male breast cancers (Thompson & Easton, 2004). There is also evidence of increased risk of other types of cancer among BRCA1 and BRCA2 mutation carriers, including colon, pancreatic and cervical cancers for BRCA1 and prostate and pancreatic cancer for BRCA2 (Breast.Cancer.Linkage.Consortium, 1999; Thompson & Easton, 2002).

Germline mutations in the BRCA1 and BRCA2 genes are being recorded by the Breast Cancer Information Core (BIC) (<http://research.nhgri.nih.gov/bic/>) and according to the database a large number of them have been reported only once. The majority of

the mutations are nonsense or frame-shift mutations causing truncation of the protein products making them non-functional.

Although BRCA1 and BRCA2 germline mutations are rare in the general population certain mutations are observed frequently within specific populations. This being the case, the reason could be a shared common ancestry (Narod & Foulkes, 2004; Thompson & Easton, 2004). In Iceland only one recurrent mutation has been described for each of the BRCA genes. The BRCA1 (G5193A) mutation is relatively uncommon in Iceland (Bergthorsson *et al.*, 1998) whereas a much more common mutation in the BRCA2 gene, 999del5, is estimated to account for about 6-8% of both female breast and ovarian cancers in the population and the overall frequency being around 0.6% (Johannesdottir *et al.*, 1996; Rafnar *et al.*, 2004; Thorlacius *et al.*, 1997). Population based studies on the Ashkenazi Jews have also shown two recurrent germline mutations in the BRCA1 gene (185delAG and 5382insC) and one in the BRCA2 gene (6174delT) accounting for almost all of the BRCA1 and BRCA2 mutations found in the Ashkenazi Jewish population; the overall frequency exceeding 2% in the population (reviewed in (Ferla *et al.*, 2007; Thompson & Easton, 2004)).

BRCA1 and BRCA2 gene and protein structure

The BRCA1 gene spans over 81 kb of DNA and has 22 coding exons and a protein product consisting of 1863 amino acids. It contains several functional domains including: The RING-finger domain, that binds to BARD1 (BRCA1-associated RING domain 1 protein)

enhancing the ubiquitin-ligase function of BRCA1, a DNA-binding domain, which contributes to the DNA-repair related functions of BRCA1 and a BRCT domain which is found in many proteins involved in DNA repair and binds to many proteins, including RNA polymerase II and p53 (reviewed in (Narod & Foulkes, 2004; Venkitaraman, 2008)).

The BRCA2 gene spans over 82 kb of DNA, has 26 coding regions and a protein product consisting of 3418 amino acids. It contains 2 distinct domains, BRC repeats and a binding site at the C-terminal region of BRCA2, that bind with RAD51, a key component in which DNA is repaired by homologous recombination (reviewed in (Narod & Foulkes, 2004; Venkitaraman, 2008)).

BRCA1 and BRCA2 function

BRCA1 and BRCA2 are tumour suppressor genes in accordance to the two-hit model proposed by *Knudson* in 1971 where he proposed that a tumour suppressor gene requires the loss of both of its functional alleles for oncogenesis to occur. In familial cancer, one germline mutation is inherited (first hit) and the second mutation occurs by somatic mutation (second hit). In the nonhereditary form, both mutations occur in somatic cells (Knudson, 1971). Somatic mutations in BRCA1 and BRCA2 are extremely rare and generally not found in sporadic tumours. However, epigenetic changes in BRCA1 in the form of promoter hypermethylation and loss of expression have been reported in sporadic tumours (Birgisdottir *et al.*, 2006; Catteau *et al.*, 1999; Russell *et al.*, 2000; Staff *et al.*, 2003). The significance of

silencing genes by epigenetic changes is similar to carrying a germline mutation since both events lead to loss of the protein.

The main function of BRCA1 and BRCA2 lies in preserving the integrity and stability of the chromosomal structure as has been shown by studies of BRCA2-deficient murine cells accumulating gross chromosomal rearrangements (Yu *et al.*, 2000) and cancer cells from BRCA1 and BRCA2 mutation carriers using CGH (Tirkkonen *et al.*, 1997; Tirkkonen *et al.*, 1999) and later array CGH (Jonsson *et al.*, 2005).

BRCA2 along with its association with RAD51 is central to the repair of DNA by homologous recombination. The BRC motifs of BRCA2 bind directly to RAD51 (Sharan *et al.*, 1997; Wong *et al.*, 1997) and a model proposed by Venkitaraman outlines that BRCA2 holds RAD51 inactive until phosphorylation of the BRCA2-RAD51 complex by DNA damage-signaling kinases such as ATM or ATR triggers the inactive complex to an active state at the site of DNA damage (Venkitaraman, 2002). BRCA1 is reported to interact with RAD51 and BRCA2 (Chen *et al.*, 1998), however, the mechanism by which it interacts with the complex is unclear. The functions of BRCA1 are not as restricted to mainly taking part in homologous recombination at sites of DNA repair as those of BRCA2 are. BRCA1 has various functions in response to DNA breakage or replication arrest. It can exist as a part of the BASC (BRCA1-associated genome-surveillance complex), a complex that includes many proteins involved in DNA repair including ATM, MSH2, MSH6 and the RAD50-MRE11-NBS1 complex, which is suggested to serve as a sensor to DNA damage

(Wang *et al.*, 2000). BRCA1 has been reported to associate with proteins that remodel chromatin, such as SW1 and SNF (Bochar *et al.*, 2000), which is a process necessary for DNA repair, recombination and transcription as well as having a role in the expression of cell cycle related genes, such as p21 and GADD45 (Harkin *et al.*, 1999; Somasundaram *et al.*, 1997). BRCA1 has also been shown to act as a ubiquitin ligase through its interaction with BARD1 (Hashizume *et al.*, 2001).

Moderate-penetrance breast cancer susceptibility genes

Although the frequency of mutations in moderate breast cancer susceptibility genes such as ATM, BRIP1, CHEK2 and PALB2 are more common than those in high-risk genes they have a lower penetrance (2-4 fold risk) and total familial relative risk (<1% each) compared to BRCA1 and BRCA2 (Erkko *et al.*, 2007; Meijers-Heijboer *et al.*, 2002; Rahman *et al.*, 2007; Renwick *et al.*, 2006; Seal *et al.*, 2006). Many different mutations have been reported to cause breast cancer susceptibility in these four genes, most resulting in premature protein truncation (reviewed in (Stratton & Rahman, 2008)).

PALB2

Although predisposing PALB2 mutations generally appear to cause moderate risk of breast cancer (Rahman *et al.*, 2007) mutations have also been found in strong hereditary breast cancer families (Foulkes *et al.*, 2007; Tischkowitz *et al.*, 2007). Two founder mutations in PALB2

have been reported. In Finland the founder mutation, 1592delT, has been reported with an associated 4-fold increased risk for female breast cancer (Erkko *et al.*, 2007) which is notably higher than the ~2-fold increased risk of other mutations among moderate breast cancer susceptibility genes. A founder mutation has also been reported among French-Canadian women with early onset breast cancer. It was found in 2 of 356 (0.5%) unselected French-Canadian women diagnosed with breast cancer under the age of 50 compared to none of the 6440 controls tested (Foulkes *et al.*, 2007).

Biallelic mutations in PALB2, as well as BRIP1, also cause rare subtypes of Fanconi anemia (Levitus *et al.*, 2005; Levran *et al.*, 2005; Reid *et al.*, 2007; Xia *et al.*, 2007). The phenotype of PALB2-caused Fanconi anemia is identical to a rare subtype of Fanconi anemia caused by biallelic mutations in BRCA2 hence reflecting the functional relationship between BRCA2 and PALB2, as PALB2 acts as a binding factor that ensures the tumour-suppressor function of BRCA2 (Reid *et al.*, 2007; Xia *et al.*, 2007; Xia *et al.*, 2006).

Common low-penetrance breast cancer susceptibility alleles

In recent years common low-penetrance breast cancer susceptibility alleles have been identified through large case versus control series of genome-wide association studies using SNPs (single nucleotide polymorphisms). So far, several such common low-penetrance alleles have been reported (table 1). The disease-causing variants underlying the associated alleles may not be easily identifiable since the SNPs are often in tight linkage disequilibrium with many nearby variants and

often do not reside near coding or promoter regions of genes as is the case with rs13387042 on 2q35 that lies many kilobases from the nearest gene (Stacey *et al.*, 2007). However, the disease-causing variant may also be the allele itself affecting a gene or a nearby gene as is the case for the SNP rs2981582 in the FGFR2 locus, although it is located in a non-coding region of the gene (Easton *et al.*, 2007). For low-penetrance alleles the associated relative risk of breast cancer is estimated to be up to about 1,25-fold for a heterozygous genotype or about 1,65-fold for a homozygous genotype (reviewed in (Stratton & Rahman, 2008)).

Table 1. List of published genome-wide association studies and their results.

SNP	Region	SNP	CC	TC	TC/CC	OR (95%CI)	OR (95%CI)	P-value	Study
10q26	FGFR2	rs2981582	21.668	20.973	1.23 (1.18-1.28)	1.63 (1.53-1.72)	1.26 (1.23-1.30)	2 x 10 ⁻⁷⁶	Easton et al (2007)
		rs1219648	2.921	3.214	1.20 (1.07-1.42)	1.64 (1.42-1.90)		1.1 x 10 ⁻¹⁰	Hunter et al (2007)
16q12	TNRC9 LOC643714	rs3803662	21.668	20.973	1.23 (1.18-1.29)	1.39 (1.26-1.45)	1.20 (1.16-1.24)	1 x 10 ⁻³⁶	Easton et al (2007)
		4.554	17.577	1.27 (1.19-1.36)	1.64 (1.45-1.85)	1.28 (1.21-1.35)	5.9 x 10 ⁻¹⁹	Stacey et al (2007)	
2q35		rs13387042	4.533	17.513	1.11 (1.03-1.20)	1.44 (1.30-1.58)	1.20 (1.14-1.26)	1.3 x 10 ⁻¹³	Stacey et al (2007)
5p12	MRPS30	rs10941679	5.028	32.090			1.19 (1.13-1.26)	2.9 x 10 ⁻¹¹	Stacey et al (2008)
5q11	MAP3K1 MGC33648 MIER3	rs889312	21.668	20.973	1.13 (1.09-1.18)	1.27 (1.19-1.36)	1.13 (1.10-1.16)	7 x 10 ⁻²⁰	Easton et al (2007)
2q33	CASP8	rs1045485	17.109	16.423	0.89 (0.85-0.94)	0.74 (0.62-0.87)	0.88 (0.84-0.92)	1.1 x 10 ⁻⁷	Cox et al (2007)
8q24		rs13281615	21.668	20.973	1.06 (1.01-1.11)	1.18 (1.1-1.25)	1.08 (1.05-1.11)	5 x 10 ⁻¹²	Easton et al (2007)
11p15	LSP1	rs3817198	21.668	20.973	1.06 (1.02-1.11)	1.17 (1.08-1.25)	1.07 (1.04-1.11)	3 x 10 ⁻⁹	Easton et al (2007)
6q25.1	ESR1	rs2046210	6.472	3.962	1.36 (1.24-1.49)	1.59 (1.40-1.82)		2 x 10 ⁻¹⁵	Zheng et al (2009)
1p11.2		rs11249433	10.263	9.335	1.16 (1.09-1.24)	1.30 (1.19-1.41)		6.7 x 10 ⁻¹⁰	Thomas et al (2009)
14q24.1	RAD51L1	rs999737	10.298	9.395	0.94(0.88-0.99)	0.70 (0.62-0.80)		1.7 x 10 ⁻⁷	Thomas et al (2009)
6q22.33	ECHDC1 RNF146	rs2180341			1.53 (1.32-1.77)	1.51 (1.10-2.08)	1.41 (1.25-1.59)	2.9 x 10 ⁻⁸	Gold et al 2008
		rs6569479			1.50 (1.29-1.74)	1.48 (1.07-2.04)	1.39 (1.23-1.57)	1.2 x 10 ⁻⁷	
		rs7776136	1.442	1.465	1.51 (1.31-1.76)	1.42 (1.05-1.90)	1.39 (1.23-1.57)	6.6 x 10 ⁻⁸	
		rs6569480			1.51 (1.30-1.75)	1.51 (1.10-2.08)	1.40 (1.24-1.58)	6.1 x 10 ⁻⁸	

*Targeted candidate association study

Finding additional high-risk breast cancer susceptibility genes

By employing genetic models, segregation analysis is used to predict the patterns of cancers by comparing them with observed frequencies to select for the best fitting model. These analyses have had great influence in the molecular approaches with which genetic predisposition factors have been investigated. Early segregation analysis of breast cancer families favoured a highly penetrant autosomal dominant genetic model which was confirmed by locating BRCA1 and BRCA2 through linkage analysis (reviewed in (Turnbull & Rahman, 2008)). Subsequent studies have not been able to identify additional dominant high-penetrant major tumour suppressor genes such as BRCA1 and BRCA2 and it is unlikely that such genes will be found (Nathanson & Weber, 2001).

Segregation analyses based on both population-based cases of breast cancer and large familial clusters, without BRCA1 or BRCA2 mutations, favour a polygenic model that suggests that there are large numbers of susceptibility factors, each conferring only small risks of the disease. However, the possibility has not been excluded that some of the risk could be due to common recessive effects or rare autosomal dominant effects (Antoniou *et al.*, 2001; Antoniou *et al.*, 2002; Antoniou *et al.*, 2004; Cui *et al.*, 2001).

The causal factor for the risk most likely also depends on the population investigated. A study based on the results of many genome-wide association studies on many different complex diseases, involving multiple genetic and environmental determinants, suggests

that to ensure the success of such a study it is best to increase the number of individuals studied and/or to study a sample drawn from only one ethnic group (Altmuller *et al.*, 2001).

Linkage studies

Linkage analysis is highly suitable to locate high-penetrance cancer-susceptibility genes in families in which the majority of the affected individuals carry a germline mutation. Linkage analysis tests whether co-segregation of genomic markers is of significant linkage to a causative gene, using DNA samples from multiple members of large families. As previously mentioned genome-wide linkage analyses were applied to map BRCA1 and BRCA2 (Wooster 1994, Hall 1990). Genome-wide and targeted linkage studies using microsatellite markers on non-BRCA1/BRCA2 families have proposed several chromosomal regions as the possible site of a breast cancer susceptibility gene (table 2). Three studies have by linkage analysis suggested chromosome 8p21-p22 as a region harbouring a breast cancer susceptibility gene (Kerangueven *et al.*, 1995; Seitz *et al.*, 1997a; Seitz *et al.*, 1997b) although results from another independent study targeting the region did not support the hypothesis (Rahman *et al.*, 2000). Chromosome 13q21 has also, by linkage analysis, been suggested to harbour a breast cancer susceptibility gene (Kainu *et al.*, 2000) but has not been confirmed by other independent studies targeting the region (Thompson *et al.*, 2002). A genome-wide linkage analysis on 14 high-risk breast cancer families from Finland indicated linkage to chromosome 2q32 (Huusko *et al.*, 2004) and a study

performed by the Breast Cancer Linkage Consortium on 149 multiple case breast cancer families suggested several regions of linkage to breast cancer susceptibility (Smith *et al.*, 2006). Two genome-wide linkage analyses using SNP markers identified several regions of possible susceptibility but as with many other studies failed to detect statistically significant linkage signals (Bergman *et al.*, 2007; Gonzalez-Neira *et al.*, 2007). Significant linkage to 9q21-q22 (Oldenburg *et al.*, 2008; Rosa-Rosa *et al.*, 2009) and to 21q22 (Oldenburg *et al.*, 2008; Rosa-Rosa *et al.*, 2009) have recently been reported but they have not been verified.

Several reasons might explain why previous studies have failed to identify new breast cancer susceptibility genes in high risk families. It could be that the genetic model used for linkage analysis is not correct and a polygenic model would possibly fit better (Oldenburg *et al.*, 2007). Heterogenic study populations might also help to explain the failure of detecting strong linkage signals in some previous genome-wide linkage studies where multiple susceptibility loci each only explain only a small proportion of families (Oldenburg *et al.*, 2008). As breast cancer families characterized by ovarian and male breast cancer cases helped identifying the BRCA1/2 genes so could other common pathological or genetic features such as chromosomal aberrations or gene expression help to identify new subgroups among the non-BRCA1/2 breast cancer families. In an effort to increase the statistical linkage power the use of subsets of families from more homogenous populations would prove more effective as proposed by Altmuller *et al.* (Altmuller *et al.*, 2001).

Table 2. Linkage studies from non-BRCA1/2 multiple-case breast cancer families.

Locus	Model	Nbr of families	Lod	Study
8p12-22	dominant	8	2.51	Kenangueven et al 1995
8p12-22	dominant	4	2.97	Seitz et al 1997
8p12-22	dominant	31	0.03	Rahman et al 2000
13q21	dominant	77	3.46	Kainu et al 2000
13q21	dominant	128	-0.11	Thompson et al 2002
2q32	non parametric	14	3.2	Huusko et al 2004
4q13	dominant	149	1.8	Smith et al 2006
2p25	dominant		2.38	Smith et al 2006 (4 ≤ cases < 50 years)
2p22.3	non parametric	19	2.25	
4p14-q12	non parametric	19	2.22	Gonzales-Neira et al 2007*
	non parametric	19	2.22	Gonzales-Neira et al 2007*
7q21	non parametric	19	2.59	Gonzales-Neira et al 2007*
11q13-q14	non parametric	19	2.51	Gonzales-Neira et al 2007*
14q21	non parametric	19	2.41	Gonzales-Neira et al 2007*
10q23-q25	dominant	14	2.34	Bergman et al 2007*
12q14-q21	dominant	14	2.16	Bergman et al 2007*
19p13-q12	dominant	14	2.1	Bergman et al 2007*
9q21-22	non parametric	85	3.96	Oldenburg et al 2008
3q25	dominant	41	3.01	Rosa-Rosa et al 2009*
6q24	dominant	41	2.26	Rosa-Rosa et al 2009*
21q22	dominant	41	3.55	Rosa-Rosa et al 2009*

*Based on SNPs

Genomic aberrations in breast tumours

Chromosomal aberrations, whether they affect the structure or the number of chromosomes, are generally characteristic of tumour cells. Structural aberrations ranging from single nucleotide substitutions to gross chromosomal re-arrangements, including genomic translocations, amplifications, deletions and inversions, are related to defects in the DNA repair pathway that arise during DNA replication.

However numerical aberrations that lead to altered ploidy occur when the segregation of cells during mitosis goes awry (Venkitaraman, 2008).

Loss of heterozygosity

A common type of somatic aberration found in primary breast tumours is deletion revealed by loss of heterozygosity (LOH) since by affecting one of a pair of chromosomes, an allele appears missing at each heterozygous locus within the aberrated chromosomal region. In line with Knudson's two-hit model, consistent LOH in a genomic region implicates the presence of a tumour suppressor gene, given that the retained chromosome may contain an independent mutation, either germline or somatic. But despite many LOH-studies of sporadic breast cancer through the years, the number and identity of tumour suppressor genes is largely unknown (reviewed in (Oldenburg *et al.*, 2007)). A pooled analysis of 151 published LOH-studies of breast tumours observed a preferential loss in specific regions of chromosomes 7q, 16q, 13q, 17p, 8p, 21q, 3p, 18q, 2q and 19p (in descending order of significance) (Miller *et al.*, 2003). Another LOH study consisting of 100 tumour samples from 42 selected non-BRCA1/BRCA2 breast cancer families found LOH frequencies of 40% or greater at chromosomes 1q41, 4p16, 11q23.3, 16p13, 16q24, 17p12, 21q22, 22q11 and 22q13. Many of these chromosomal regions have also been highlighted in the analyses of sporadic breast tumours (Oldenburg *et al.*, 2006).

Gene amplifications

Gene amplification is an important mechanism for oncogene over-expression in malignant tumours and occurs frequently in breast cancer. Amplification has been observed in various regions across the genome both including regions of known oncogenes and where no oncogenes have been identified. Among common oncogenes in breast cancer are ERBB2, MYC, CCND1 and MDM2. Each of these genes are amplified in 5-20% of all human breast cancers (Al-Kuraya *et al.*, 2004).

Comparative genomic hybridization

Comparative genomic hybridization (CGH) of metaphase chromosomes allows for the screening of the entire genome for DNA copy-number aberrations giving a resolution of about 10-20 Mb (Kallioniemi *et al.*, 1992). More recently, CGH performed on DNA microarrays (array CGH) has been applied to detect copy number changes at much higher resolution. There are different kinds of array-CGH platforms, including bacterial artificial chromosome (BAC) clones, cDNA clones and oligonucleotides (reviewed in (Kallioniemi, 2008)). Many studies, both CGH and array-CGH, on breast cancer have been reported in recent years.

Two separate studies applying conventional metaphase CGH on unselected primary invasive tumours (305 and 55 tumours) observed chromosomal copy number aberrations in more than 90% of the tumours. Common gains were at chromosomes 1q, 5p, 8q, 16p, 17q, 19p, 19q and 20q (18%-67%) and common losses on 8p, 9p, 13q and

16q (18%-38%). Both studies observed the most frequent aberrations to be gains of DNA on 1q (67% and 55%) and 8q (49% and 41%) (Rennstam *et al.*, 2003; Tirkkonen *et al.*, 1998). Another study of sporadic breast cancer applying array CGH of 47 primary tumours, hence giving much higher resolution (~0.9 Mb), also detected high levels of aberrations in the same regions as the two previous studies reported as well as in several new regions, including gains at chromosome 4p and 9q and losses at 4q, 17p, 18p and 21q. The highest aberrations were gains at 1q32.1 (66%) and 8q24.3 (79%) (Naylor *et al.*, 2005).

CGH on hereditary breast cancer tumours

A high-frequency of copy number alterations has been described for BRCA1 and BRCA2 associated breast cancer, possibly reflecting the importance of BRCA1 and BRCA2 in DNA repair (Jonsson *et al.*, 2005; Tirkkonen *et al.*, 1997; Tirkkonen *et al.*, 1999; van Beers *et al.*, 2005; Wessels *et al.*, 2002). A metaphase CGH study showed frequent losses (>50%) at chromosomes 4p, 4q, 5q and 13q in BRCA1 associated tumours and the losses of 4q (81%) and 5q (86%) seemed especially characteristic for the BRCA1 tumours compared to a sporadic group of breast cancer tumours. Frequent gains were reported on chromosomes 1q, 6p, 8q, 10p, 16p and 17q. For BRCA2-associated tumours, frequent losses were reported on 3p, 6q, 8p, 11q and 13q and frequent gains on 1q, 8q, 17q and 20q. The region showing the highest frequency of loss was on 13q (73%) and it included the site of the BRCA2 gene. Knowledge of this high frequency of somatic loss

might have helped, at an earlier stage, to direct linkage studies of non-BRCA1 families to this region. This suggests that CGH studies on non-BRCA1/BRCA2 breast cancer susceptibility families could be advantageous in pinpointing candidate regions for the search of genetic predisposition loci (Tirkkonen *et al.*, 1997). The CGH technique has also been applied to try to classify breast cancer into subgroups. A study by Wessels *et al.* classified BRCA1-associated breast cancer from sporadic with an accuracy of 84%. Somatic aberrations on 3p (loss), 3q (gain) and 5q (loss) were used to distinguish between the groups (Wessels *et al.*, 2002). However, a study aiming to classify BRCA2-associated breast cancer from sporadic breast cancer using the same classification method as Wessels *et al.* could not reliably distinguish between the groups (van Beers *et al.*, 2005). Another study based on array CGH identified 169 significant BAC clones which enabled discrimination between BRCA1, BRCA2 and sporadic breast tumours using hierarchical clustering. All BRCA1 tumours tightly clustered separately from sporadic tumours. The BRCA2 tumours however showed a somewhat higher similarity with the sporadic tumours but still displayed a distinct genomic profile of their own (33% of BRCA2 tumours clustered within the sporadic or BRCA1 group) (Jonsson *et al.*, 2005). Two studies on non-BRCA1/BRCA2 hereditary breast cancer applying metaphase CGH and one array-CGH study revealed frequent gains on chromosomes 1q, 8q, 16p, 17q, 19p, 19q and 20q and frequent losses on 6q, 8p, 9p, 11q and 13q (Gronwald *et al.*, 2005; Kainu *et al.*, 2000; Melchor *et al.*, 2007). Kainu *et al.* reported a

minimal region of common copy number loss at 13q21-22 and subsequently performed linkage analysis on that region obtaining a two-point lod score of 2.76, suggesting 13q21 to be a putative novel breast cancer susceptibility locus. Although these results have not been verified they could be due to population heterogeneity since most of the families showing linkage are of Finnish origin. Thus, applying CGH on non-BRCA1/BRCA2 hereditary breast cancer tumours may pinpoint novel susceptibility loci by subsequently determining common chromosomal regions lost in numerous tumours from the same pedigree, followed by genotyping and linkage analysis (Kainu *et al.*, 2000).

Further means to subgroup families to aid linkage analyses

Results from expression profiling have indicated that it is possible to classify breast cancer families into distinct subgroups based on whether they carry a germline mutation in either BRCA1 or BRCA2 or neither gene (Hedenfalk *et al.*, 2003; Hedenfalk *et al.*, 2002). Profiling tumours by gene expression shows that tumours from individuals of the same family tend to group together, indicating that such grouping is based on hereditary factors and not by chance (Hedenfalk *et al.*, 2002). Hence both CGH and gene expression profiling offer the possibility to find distinct subgroups of families that share the same genetic etiology, due to a shared predisposition to breast cancer.

Information provided by sub-grouping of breast tumours could be used to enhance the power of linkage analysis by reducing the

heterogeneity of the families involved. If they differ considerably in their underlying genetic explanation, with only few families matching each explanation, the study will suffer from lack of power to detect linkage. In order to avoid this, this study takes advantage not only of the supposedly relatively low genetic heterogeneity of the Icelandic population but also of first applying array CGH on non-BRCA1/BRCA2 tumours before selecting families. Selection of families and of chromosomal loci for genotyping and linkage analysis will be preferentially based on regions that overlap with respect to DNA copy number aberration in the tumours of the family members. In Iceland there are possibilities that such common regions of aberration in a subset of the families would tie with a recurrent mutation in a common susceptibility gene analogous to what was seen in BRCA1 and BRCA2. A single germline mutation in either BRCA1 or BRCA2 has been identified among the Icelandic population. The 999del5 mutation in the BRCA2 gene is found in all known BRCA2-families in Iceland despite distant relations (Arason *et al.*, 1998; Johannesdottir *et al.*, 1996; Thorlacius *et al.*, 1996). This founder mutation most likely originated in a common ancestor as haplotype analysis of neighbouring alleles revealed the region with identical allele sizes between BRCA2 families (Gudmundsson *et al.*, 1996).

AIMS OF THE STUDY

The aim of this study was to map common genomic aberrations in breast tumours derived from selected high-risk non-BRCA1/BRCA2 breast cancer families (BRCAX) with the further aim of locating a breast cancer susceptibility gene. In order to do this, array CGH was applied to 131 tumour samples from members of families whose selection was based on frequency of breast cancer within the family and availability of samples. The BRCAX array-CGH results were analysed based on family origin. BRCAX subgroup analysis was performed based on age and number of family members diagnosed with breast cancer. The data from the BRCAX tumours were also analysed in comparison to array-CGH data from groups of sporadic, BRCA1 and BRCA2 tumours. Since the results from the BRCAX array-CGH revealed common aberrations in 4 regions of chromosome 16 in tumours from members of five high-risk breast cancer families, it followed that a total of 26 microsatellite markers in the aberrated regions of chromosome 16 were genotyped and the results applied to linkage analysis to verify if there was evidence of linkage to breast cancer.

As chromosome 16 was highlighted by the array-CGH results, previously reported indications of breast cancer linkage to chromosome 16 were also considered. The SNP rs3803662 on 16q12 that has been associated with increased risk of breast cancer was genotyped to see if it played a possible role in these five high-risk breast cancer families. The PALB2 gene on 16p12.1 has also been

associated with increased risk of breast cancer. Linkage analysis of the five high-risk families from the array-CGH study and four additional high-risk non-BRCA1/2 breast cancer families was undertaken, as well as screening of a Finnish 1592delT founder mutation in the family members and a large group of unselected breast cancer patients.

MATERIALS AND METHODS

Array-CGH analysis

Sample selection

The samples for the array-CGH part of the study consisted of 113 fresh-frozen tumour samples and 18 paraffin-embedded tumour tissue samples from members of high-risk non-BRCA1/BRCA2 breast cancer families (BRCAX families) (table 3). The tumours in the BRCAX families were from families with either: at least 3 breast cancer cases (proband plus two first, second or third degree relatives) or 2 if either was diagnosed by age 40 or both by age 50. All tumour samples come from the Department of Pathology at Landspítali University Hospital from patients diagnosed with breast cancer in the years 1952-2003. The research is a part of a larger research project at the Department of Pathology LSH and was approved by the Icelandic Data Protection Authority and the National Bioethics Committee.

Table 3. The number of BRCAX families in the array-CGH study and the number of tumours per family.

Tumours per family	Nbr. of families	Total nbr. of tumours
1	48	48
2	11	22
3	7	21
4	3	12
5	3	15
6	1	6
7	1	7
Total	74	131

DNA extraction

Fresh-frozen tumour tissue was treated with proteinase K (20 mg/ml) overnight at 55°C. Using the Wizard[®] Genomic DNA purification Kit (Promega), RNA was degraded by addition of 3 µl of RNase solution followed by 15-30 min incubation at 37°C. Then 200 µl of a protein precipitation solution were added and the samples were vortexed to facilitate the precipitation of the proteins and centrifuged for 15 minutes at 15000 g. The supernatant containing the DNA was added to 600 µl of isopropanol to precipitate the DNA which after centrifugation was resuspended in 200 µl of TE buffer (10 mM TRIS pH 8 and 1 mM EDTA pH 8) followed by a phenol-chloroform purification. The DNA was then precipitated by mixing with 2 volumes of absolute EtOH (ethanol) and centrifuged for 30 min. The liquid was discarded and 70% EtOH added to wash the pellet and centrifuged for 30 min. The EtOH was removed and the DNA pellet resuspended in 20-100 µl TE buffer.

DNA extraction from the paraffin-embedded tissue was preceded by xylene treatment to remove paraffin, and then carried out using Proteinase K digestion and phenol-chloroform purification. Finally, the DNA was precipitated by EtOH, centrifuged for 45 min and the liquid discarded leaving a DNA pellet which was suspended in TE buffer.

DNA labelling and hybridization

Tumour DNA and normal male reference DNA (Promega), 2 µg of each, were labelled with cyanine 3 (Cy3) (sample) and cyanine 5

(Cy5) (reference) fluorescent dyes using the Array CGH labelling kit (Invitrogen Life Technologies). Sample DNA and reference DNA, 21 μl of each at concentration 95 ng/ μl , were put in separate tubes along with 20 μl of 2.5X concentrated random primers, denatured at 95°C for 5 min and annealed on ice for 5 min after which 5 μl of 10X concentrated dCTP, 2 μl of Cy3-dCTP or Cy5-dCTP and 1 μl of Exo-Klenow fragment were added. The DNA was amplified in a polymerization reaction at 37°C for 2 h. Next, the differentially labelled sample and reference DNA were mixed together and 100 μg of human COT-1 DNA added, to reduce unspecific binding and finally the sample was dried down.

Since DNA isolated from paraffin-embedded tissue is generally of lesser quality than from fresh-frozen tissue the labelling of DNA from paraffin embedded samples was performed using “dye-swap”, i.e. the tumour and reference DNA were labelled as usual for one array and then the dyes were switched between the tumour and reference DNA for a second array. Thus each spot is represented by two replicates to reduce variability in the dye labelling and fluorescence emission (noise) (Yang *et al.*, 2002).

The DNA was resuspended in 50 μl of formamide-based hybridization buffer, then denatured at 70°C for 15 minutes followed by reannealing for 30 minutes at 37°C and applied to microarray slides and incubated under coverslips for 72 h at 37°C (Snijders *et al.*, 2001). Finally the arrays were washed in four steps in solutions containing: 1) 2X SSC and 0.1% SDS, 2) 2X SSC and 50% formamide, 3) 2X SSC and 0.1% SDS and finally 4) 0.2X SSC. Next, they were dried by centrifugation

and scanned using an Agilent microarray scanner (Agilent Technologies) to detect fluorescent signals. Before the hybridization the microarrays were UV-crosslinked at 500 mJ/cm² and pre-washed using the Universal Microarray Hybridization Kit (Corning) according to the manufacturer's instructions. The microarrays used are high-resolution tiling arrays (CHORI BACPAC Resources, (<http://bacpac.chori.org/genomicRearrays.php>) containing 32,433 clones with an average resolution of 80 kb over the genome (Ishkanian *et al.*, 2004). They were produced at the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University, Sweden (<http://swegene.onk.lu.se>) as previously described by Jönsson *et al.* (Jonsson *et al.*, 2007).

Data analysis

The microarray scanner detects two high resolution images, one of each dye. Using GenePixTM Pro 5.0.1.24 (Molecular Devices), a microarray image analysis software, the two images are merged into one showing both Cy3 and Cy5 fluorescent intensities. The colour of each spot corresponds to the difference between the sample and reference DNA where a red spot might indicate proportionally more sampleDNA, yellow spot an equal amount of sample and reference DNA and a green spot less of sample DNA. Each spot is automatically assigned the corresponding clone identity by the software. Spots of poor quality are flagged manually since some events such as dust or accidental merging of spots may affect the quality of the spots leading to false signals. The relative ratios of the spots are calculated and a

quantified data matrix produced. Figure 1 displays a simplified schematic view of the array-CGH work process.

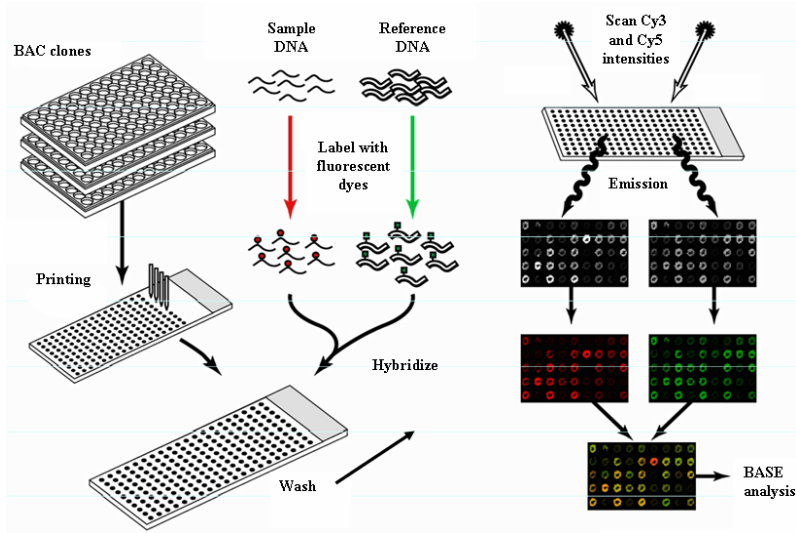


Figure 1. A schematic overview of the array-CGH work process. BAC clones are printed to microarray slides. Sample DNA and reference DNA are labelled with fluorescent dyes, hybridized and finally washed to remove unspecific binding. A microarray scanner detects two high-resolution images, one of each dye for each spot. The two images are merged and edited and a quantified data matrix produced. Further data analysis is then performed in BASE (<http://base2.thep.lu.se/onk/>)

Further data analysis of the quantified data matrix from GenePix was done in the web-based BioArray Software Environment (BASE; <http://base2.thep.lu.se/onk/>) (Saal *et al.*, 2002). Background correction of the Cy3 and Cy5 intensities for each spot was done using the median foreground (overall intensity of the spot) minus the median local background (ambient signal) intensities from the quantified data matrix and \log_2 ratios were calculated from the corrected intensity values. The data was filtered for spots that had been flagged because of bad quality and for spots with a signal-to-noise ratio <5 . After

applying the filters, average intensity ratios were calculated for spots present in dye-swap hybridizations (paraffin samples) and spots whose ratio was greater than 2 standard deviations from the mean were eliminated. The data was normalized using intensity-based Lowess (Locally weighted least squares regression) which uses a locally fitted regression curve to center the data with a smoothing factor of 0.33 and excludes the X and Y chromosome clones (Chen *et al.*, 2003). A moving average smoothing algorithm with a 250 kbp sliding window was applied to reduce experimental noise over the chromosome profile and an R (<http://www.r-project.org>) implementation in BASE, the CGH-plotter software, was used to determine breakpoints (gains/losses) on each chromosome (Autio *et al.*, 2003). In the CGH-plotter, a moving median sliding window of three clones and a noise constant of 15 were applied. The limit for gain or loss was set to \log_2 ratio of ± 0.2 . The value for each clone is given as loss, normal or gain, providing a ternary scale of the data. Using the values given by the CGH-plotter software, the total number of altered clones in each tumour was extracted from the data and a two-sided Mann-Whitney test was used to calculate significant differences in the number of altered clones between groups. The ternary scale of the data for each clone was used to detect significant differences in genomic profiles between defined subgroups of BRCAX using a multi-Fisher's exact test adjusted for multiple-testing using the step-down permutation procedure maxT, providing strong control of the family-wise Type I error rate (FWER). Clones with P-value of less than 0.01 were considered significant (Natrajan *et al.*, 2009).

Genotyping and linkage analysis

Sample selection

For linkage analysis on chromosome 16, DNA samples from 66 individuals in 5 high-risk non-BRCA1/BRCA2 breast cancer families were subjected to genotyping (table 4). To evaluate population allele frequencies in the linkage analysis, 38 control samples were also genotyped. The sample set for the PALB2 region genotyping consisted of 111 individuals from nine high-risk non-BRCA1/BRCA2 breast cancer families (see table 1 in (Gunnarsson *et al.*, 2008), plus the 38 controls to evaluate allele frequencies, and 638 unselected breast cancer cases diagnosed in the period 1987-2003 who were screened for the Finnish PALB2 founder mutation, 1592delT to detect the frequency of the mutation in the Icelandic population.

The research is a part of a larger research project at the Department of Pathology LSH and was approved by the Icelandic Data Protection Authority and the National Bioethics Committee.

Table 4. Summary of the main clinical characteristics of the 5 high-risk breast cancer families included in the linkage analysis on chromosome 16.

Family	Number of affected individuals	Number of typed individuals (affected)	Mean age at first diagnosis years (range)
70386	5	11 (5)	49.4 (44-64)
70236†	10*	14 (10)	57.4 (34-88)
70234†	9*	17 (8)	54.2 (38-75)
70080	5	11 (5)	49.6 (30-66)
70070	9	13 (9)	51.8 (30-77)
Total	38	66 (37)	52.7 (30-88)

†Co-occurrence of ovarian cancer in the family (one family member diagnosed). *One additional family member diagnosed with breast cancer not included because of bilinear family history. In family 70234 the bilinear case carries a BRCA2 999del5 mutation inherited from the other parental side.

DNA extraction

DNA was extracted from blood samples, paraffin-embedded tissue and fresh-frozen tumour tissue. Blood samples were lysed and DNA extracted from nuclei according to *Miller et al* (Miller *et al.*, 1988). In short, 18 ml of ice-cold lysis buffer (0.32 M sucrose, 10 mM TRIS pH 7.4, 5 mM MgCl₂ and 1% Triton X-100) were added to 9 ml of whole blood followed by 30 min centrifugation at 1400 g. After discarding the supernatant, the precipitate was washed with 20 ml of lysis buffer by vortexing and centrifugation repeated. The supernatant was discarded again and the precipitate resuspended in 3 ml of nuclei lysis buffer (10 mM TRIS pH 8.2, 400 mM NaCl and 2 mM EDTA pH 8). The cell lysates were digested overnight at 37°C with 200 µl of 10% SDS and 50 µl of ProteinaseK (20mg/ml). After digestion, 1.5 ml of saturated (~6M) NaCl was added and the tube vortexed for 15 s and centrifuged for 15 min. The precipitated protein was left at the bottom

of the tube and the supernatant containing the DNA transferred to a new tube. The DNA was precipitated by adding 2 volumes of absolute EtOH and then resuspended in TE buffer. DNA from paraffin-embedded tissue and from fresh-frozen tumour tissue was extracted as previously described in relation to the array-CGH analysis.

PCR reactions

A total of 26 microsatellite markers and 1 TaqMan[®] SNP genotyping assay (Applied Biosystems) were selected from 4 distinct regions on chromosome 16 (table 5) and genotyped.

For genotyping the region surrounding the PALB2 gene, 4 microsatellite markers were used (table 6). To screen for the 1592delT mutation 1 forward primer and 2 reverse primers were used, one designed for shorter PCR products and preferentially used in case of paraffin DNA (table 7).

Table 5. List of markers at chromosome 16 used in linkage analysis of five high-risk breast cancer families selected on the basis of array-CGH results.

Cytogenic Location	Marker	Distance between markers (cM)
16p13.3	D16S521	
16p13.3	D16S291	3.22
16p13.3	D16S475	3.31
16p12.1	D16S3068*	
16p12.1	D16S3100*	3.73
16p11.2	D16S298	2.74
16p11.2	D16S685	2.79
16q12.1	D16S3136*	
16q12.2	D16S415*	5.51
16q12.2	D16S3034*	1.43
16q12.2	D16S771*	1.64
16q13	D16S3057*	6.44
16q21	D16S3089	2.87
16q21	D16S514	1.15
16q21	D16S265	2.4
16q21	D16S503*	~0.1
16q21	D16S3043	1.2
16q23.1	D16S515*	
16q23.1	D16S3101	2.68
16q23.1	D16S3049*	3.25
16q23.1	D16S516	3.36
16q23.2	D16S3040	4.06
16q23.2	D16S3098	3.87
16q23.2	D16S505*	0.64
16q23.3	D16S3091*	2.16
16q24.1	D16S520*	14.7

*Markers added to the genotyping at a second stage, their selection based on the initial results.

Table 6. List of markers in the PALB2 region used in linkage analysis of 9 high-risk breast cancer families. PALB2 is located on 16p12.1 between D16S412 and D16S420.

Cytogenic Location	Marker	Distance between markers (cM)
16p12.3	D16S3036	
16p12.1	D16S412	3,77
16p12.1	D16S420	1,64
16p12.1	D16S3068	4,08

Table 7. Primers used for PALB2 1592delT mutation screening.

Primer	Forward	Reverse
PALB2_ex4e	5'HEX - ACTGAAGATAATGACTTGTC TAGGAA	GGCAAATAGTAATTGTTAACTTTCA TC TGGACCTGTTAACAATCGACA*

*Alternative for PALB2_ex4eR, providing shorter PCR products and used for analysing DNA samples extracted from tissue preserved in paraffin.

Microsatellite markers: The primers were purchased from MWG Biotech and Applied Biosystems. Each forward primer from MWG Biotech was labelled with either FAM or HEX fluorophore reporter dyes at its 5' end. The forward primers from Applied Biosystems were 5' labelled with FAM, VIC or NED fluorophore reporter dyes.

The reactions were carried out in a total of 10 µl volumes, in 96 well plates, using 25 ng of DNA, 10mM of each dNTP, 0.3 U of *Taq* DNA polymerase (Fermentas), 10X *Taq* reaction buffer, 25 mM MgCl₂ and dH₂O. Each reaction contained either 20 pmol of each primer (MWG) or 5 pmol (Applied Biosystems primers). The PCR conditions for all primer pairs, except for 1592delT primers, were: 3 min incubation at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 45 s and a final extension at 72°C for 10 min. PCR conditions for the 1592delT primers were the same except that the annealing temperature was 64°C instead of 55°C.

TaqMan[®] SNP genotyping assays: The assays consist of two sequence-specific primers and two allele-specific TaqMan[®] MGB (Minor Groove Binder) probes to detect the polymorphism of interest. The MGB probes are labelled at the 5' end with either VIC or FAM reporter dyes (Afonina *et al.*, 1997; Kutayavin *et al.*, 1997). The PCR

reactions were carried out in 10 µl volumes, in 48 well plates sealed with an optical adhesive film, using 20 ng of DNA, 2X concentration of TaqMan[®] Genotyping MasterMix, 20X concentration of TaqMan[®] SNP Genotyping Assay and dH₂O. The PCR conditions were: 30 s Pre-PCR read at 60°C, 10 min holding stage at 95°C, 40 cycles at 92°C for 15 s and 60°C for 1 min 30 s followed by a 30 s post-PCR read at 60°C.

Genotyping

Microsatellite markers: The microsatellite PCR products were pooled, if possible, by taking 2 µl of each product and mixed together in a microcentrifuge tube and the total volume brought up to 40 µl with dH₂O. Next, 0.5 µl of the total volume were added to a 9 µl mix of Hi-Di[™] formamide (Applied Biosystems) and either GeneScan[™] – 600 LIZ or GeneScan[™] – 500 ROX size standards (Applied Biosystems). The samples were heated at 95°C for 5 minutes and placed immediately on ice for at least 5 min before being loaded into the automated ABI PRISM 3130xl Genetic Analyzer where different fluorescent signals were detected and alleles were distinctively called by size. The alleles were automatically called using the GeneMapper software v4.0 and checked manually.

TaqMan[®] SNP genotyping assays: The PCR reactions and the genotyping were performed on a StepOne[™] Real-Time PCR System (Applied Biosystems). The alleles were automatically called at the end of the PCR process using the StepOne[™] software v2.0.

Linkage analysis

The Genetic Analysis System software (GAS) and MERLIN (Abecasis *et al.*, 2002) were used to check the genotyping data for Mendelian inconsistencies within the families. Genotypes which were inconsistent with Mendelian inheritance were reviewed. Any ambiguous genotypes were dropped. Allele frequencies for the microsatellite markers were calculated using founder individuals from the families as well as the control individuals. Evidence for linkage was evaluated using parametric and non-parametric linkage analysis methods. Two-point lod (logarithm of odds) scores for 13 markers were calculated using the FASTLINK program. Multi-point lod scores for 26 markers using MERLIN were calculated using parametric (dominant and recessive models) and non-parametric (linear- and exponential models (Kong & Cox, 1997)) methods. The dominant model assumed a rare (population frequency = 0.0033) dominantly inherited disease allele. Age-dependant liability classes were defined using the modified CASH model (Claus *et al.*, 1991; Easton *et al.*, 1993). The recessive model assumed the same liability classes but the susceptibility allele population frequency was 0.08 (Smith *et al.*, 2006).

LOH analysis

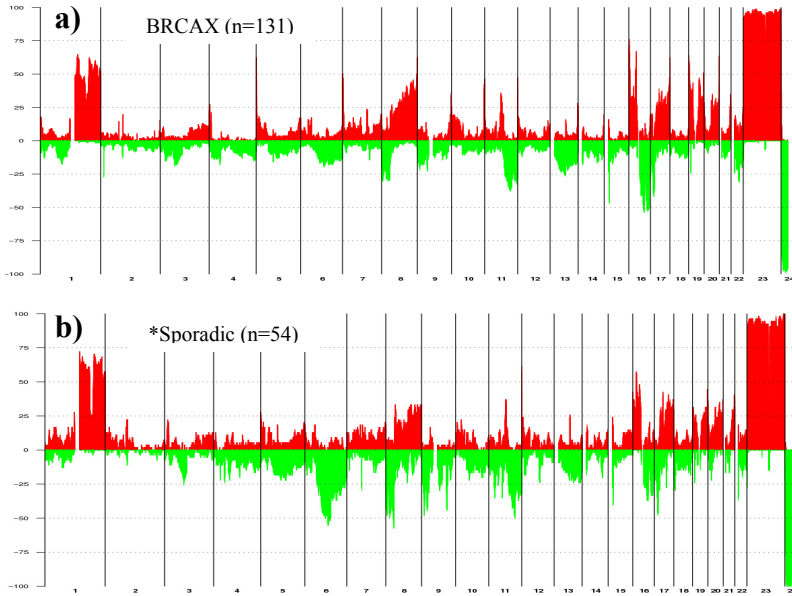
LOH (loss of heterozygosity) analysis was performed for family 70386 using markers D3136, D16S771, D16S514 and D16S3043. Normal DNA from blood samples and tumour DNA from fresh-frozen tumour tissue and paraffin-embedded tissue was previously

extracted as described earlier and PCR reactions carried out as previously described using 5' fluorescence labelled primers. The PCR products were run and analyzed in the automated ABI PRISM 3130xl Genetic Analyzer where different fluorescent signals were detected and called using the GeneMapper software v4.0. Allelic imbalance/LOH was evaluated from the ratio of allele peak heights in normal vs. tumour DNA. LOH was considered to exist if allelic imbalance was <0.67 or >1.50 (an imbalance of 33% or more).

RESULTS

Array-CGH study

In order to map genomic aberrations in breast tumours derived from selected non-BRCA1/BRCA2 breast cancer families (BRCAX families) with the aim of locating a chromosomal region commonly aberrated within families and thus possibly harbouring a breast cancer susceptibility gene, array CGH was performed on breast tumour samples from 131 patients in 74 BRCAX-families with multiple cases of breast cancer. Figure 2a shows the frequency of aberrations, gain or loss of DNA over the entire genome for the 131 tumour samples. Regions displaying the highest frequency of gains include chromosome regions on 1q, 8q, 11q13.1-q13.4, 16p, 17q, 19p, 19q and 20q. Regions showing the highest frequency of loss of DNA include 8p, 11q, 13q, 16q, 17p12 and 22q12.3. Chromosome 16 displays both the regions of highest gain and loss of DNA. Gains of DNA are seen on 16p13.3 in about 75% of tumours and on 16p11.2 in >65% of tumours. Loss of DNA can be seen in >50% of tumours on 16q21 and 16q23.1-23.3.



*The data is from the array-CGH database at BASE

Figure 2a-b. Whole genome aberration profiles for the BRCAX and sporadic groups. Red shows copy number gains (%) and green copy number loss (%). From left, chromosome 1 to Y (24).

Two previous CGH studies on hereditary tumours have reported that recurrent losses of DNA in a common chromosomal region can indicate a possible location of a susceptibility locus (Hemminki *et al.*, 1997; Kainu *et al.*, 2000). So far only the study by Hemminki *et al.* on the Peutz-Jeghers syndrome has been confirmed by other studies and a possible tumour suppressor gene identified (Amos *et al.*, 1997; Hemminki *et al.*, 1998).

Gain-of-function mutations in an oncogene, some of which might theoretically compromise the integrity of DNA by e.g. reducing the cell's capabilities of DNA repair in some way, could also be a factor in hereditary cancers. Studies have shown that hereditary cancers can be caused by autosomal dominant gain-of-function mutations in proto-

oncogenes as is evident in the MEN-2 tumour syndrome which is caused by germline activating mutations of the RET proto-oncogene (reviewed in (Kouvaraki *et al.*, 2005; Raue & Frank-Raue, 2007).

When looking at the frequency and location of aberrations, within and among the high-risk breast cancer families, two regions on chromosome arm 16q show a high frequency of loss in families 70234, 70386, 70070, 70080 and 70236 (fig. 3). One is located at 16q21 and has the smallest region of overlap about 4 Mb in size, showing loss of DNA in 20 of 23 tumours in the five families. For the other region, at 16q23.1-q23.2, the smallest region of overlap is about 3 Mb in size and shows loss of DNA in 19 of the 23 tumours. Families 70386 and 70080 show loss of DNA at both regions in all the tumours. In family 70234, 6 of 7 tumours show loss in both regions of interest, the exception possibly explained by higher proportion of normal cells in the seventh sample (CI001) as it had indications of loss in both regions, almost crossing the threshold limit ($\log -0.2$) set for loss in the CGH-plotter software (fig. 4).

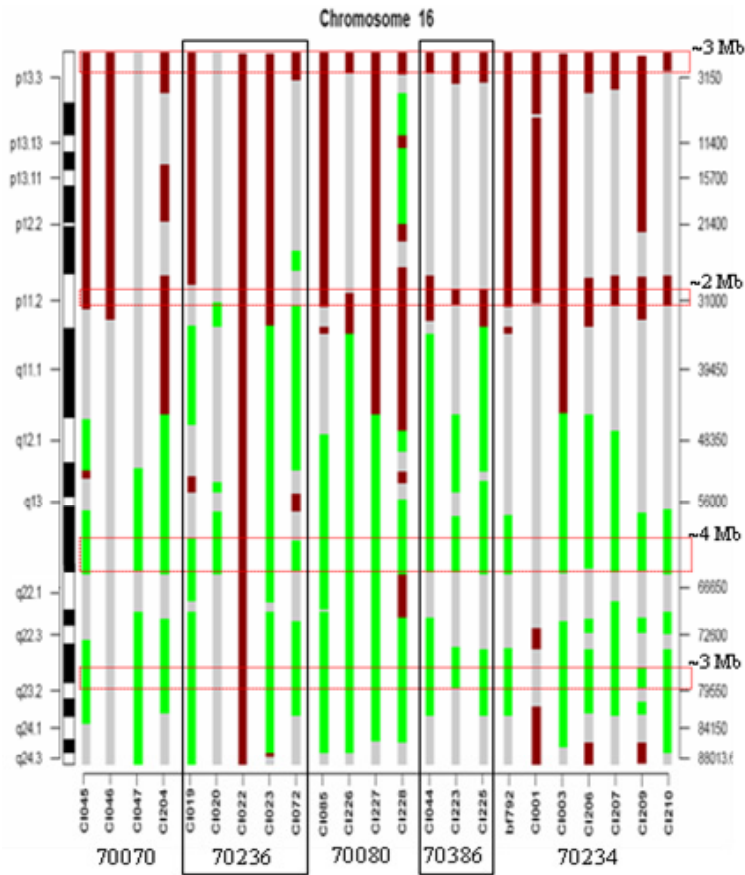


Figure 3. Location of aberrations on chromosome 16 for families 70070, 70236, 70080, 70386 and 70234. Copy-number loss is indicated by green colour and gain by red. The red dotted boxes indicate the smallest region of overlap for the 4 regions showing high frequency of aberrations. The y-axis shows the genomic location in kilobases and the location of cytobands. The x-axis shows the tumour samples and which families they originated from.

For families 70234, 70386, 70070, 70080 and 70236 gain of DNA on 16p13.3 is seen in 21 and on 16p11.2 in 19 of 23 samples. The smallest region of overlap on 16p13.3 is about 3 Mb in size and about 2 Mb on 16p11.2. Families 70080, 70386 and 70234 show gain of DNA in both regions in all the tumours (fig. 3).

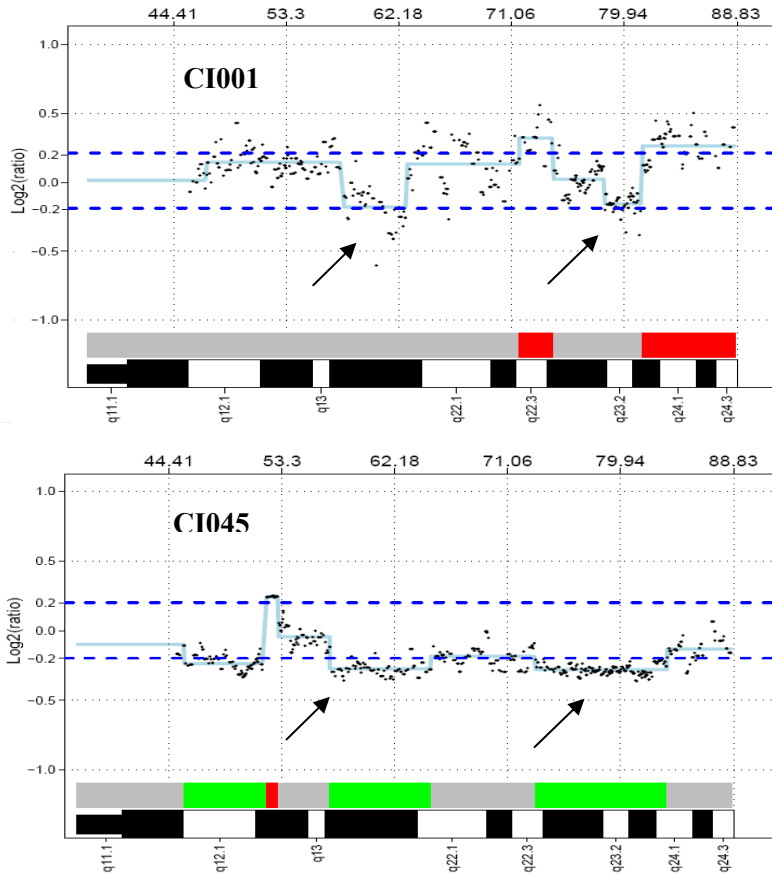


Figure 4. Comparison of chromosome arm 16q between samples CI001 from family 70234 and sample CI045 from family 70070. Arrows in sample CI001 point at regions of possible loss of DNA compared to regions of loss in sample CI045. The x-axis gives the chromosomal location in megabases and shows cytobands. The blue dotted line shows the threshold of $\log \pm 0.2$.

Genotyping and linkage analysis – Chromosome 16

Regions of common loss or gain of DNA in breast tumours belonging to members of the same families could indicate a possible location of a susceptibility locus. In light of this and based on the results from the array-CGH analysis of families 70234, 70386, 70070, 70080 and 70236 on chromosome 16, microsatellite markers along two regions of

common genomic loss, 16q21 and 16q23.1-q23.2, for the five families were genotyped to test whether this loss of DNA could possibly be due to the location of a tumour suppressor gene. Also markers along regions 16p13.3 and 16p11.2, which both show high frequencies of gain, were genotyped to test if this might reflect a location of a proto-oncogene. Originally, 13 markers were selected for genotyping in the four regions of interest on chromosome 16 (table 5). Figures 5 and 6 show the pedigree drawings of the five families.

For all five families combined, two-point lod scores indicated no evidence of linkage for any of the markers (table 9). None of the markers showed combined lod scores higher than 0.4, the highest being 0.395 ($\theta = 0.2$) for marker D16S3101; however the next flanking marker, D16S516, is negative (-0.174). The highest lod score for a single family (70070) at D16S3101 is 0.770 ($\theta = 0.00001$) but again the lod score of the next flanking marker, D16S516, is negative (-0.776). Interestingly, in family 70386, breast-cancer affected cases shared the same haplotype at 3 adjacent markers (D16S3089, D16S514 and D16S265) in the 16q21 region (fig. 5). The maximum lod scores for these markers were 0.341, 0.438 and 0.434 ($\theta = 0.00001$), respectively. In light of this we decided to add more markers in this region and also add markers in the surrounding regions of 16p11.2 and 16q23.1-23.2 where in some cases haplotypes were indeterminable and requiring additional information. Haplotype analysis using both the original 13 markers and an additional 13 markers further extended the shared haplotype seen in family 70386 spanning a region of about 10.5 Mb in 16q12-q21, from marker

D16S3034 to D16S503 (fig. 5). The results from the linkage analysis can be seen in figure 7. Multi-point lod scores, under the dominant model, for family 70386 ranged from 0.689 to 0.825 for markers D16S3057 to D16S503 (reaching maximum at D16S3089). A non-parametric model for the same markers in 70386 gave lod scores ranging from 1.61 to 1.76 (D16S3089 again displaying the maximum score).

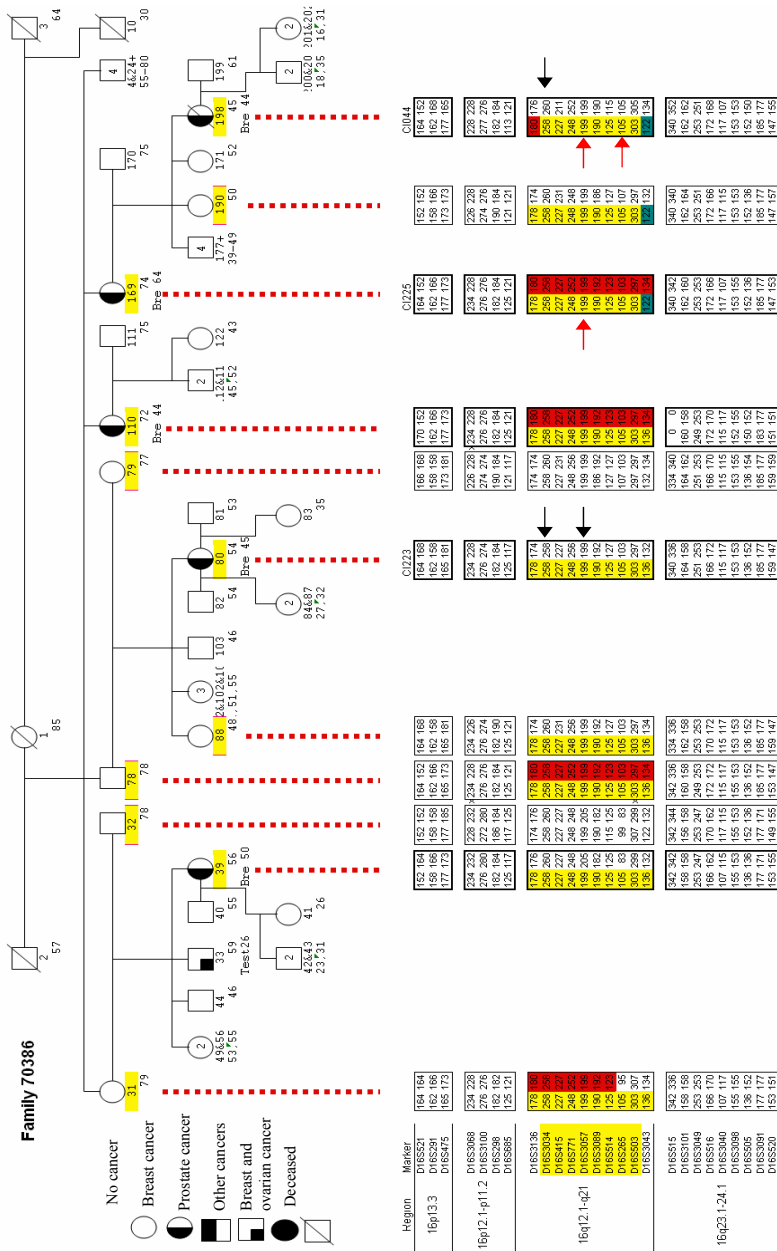


Figure 5. Pedigree structure of family 70386 and genotyping results. Black arrow indicates loss of a supposed wild-type allele, red arrow loss of a supposed mutant allele in LOH study of family 70386. Explataion of symbols also applies to figure 6.

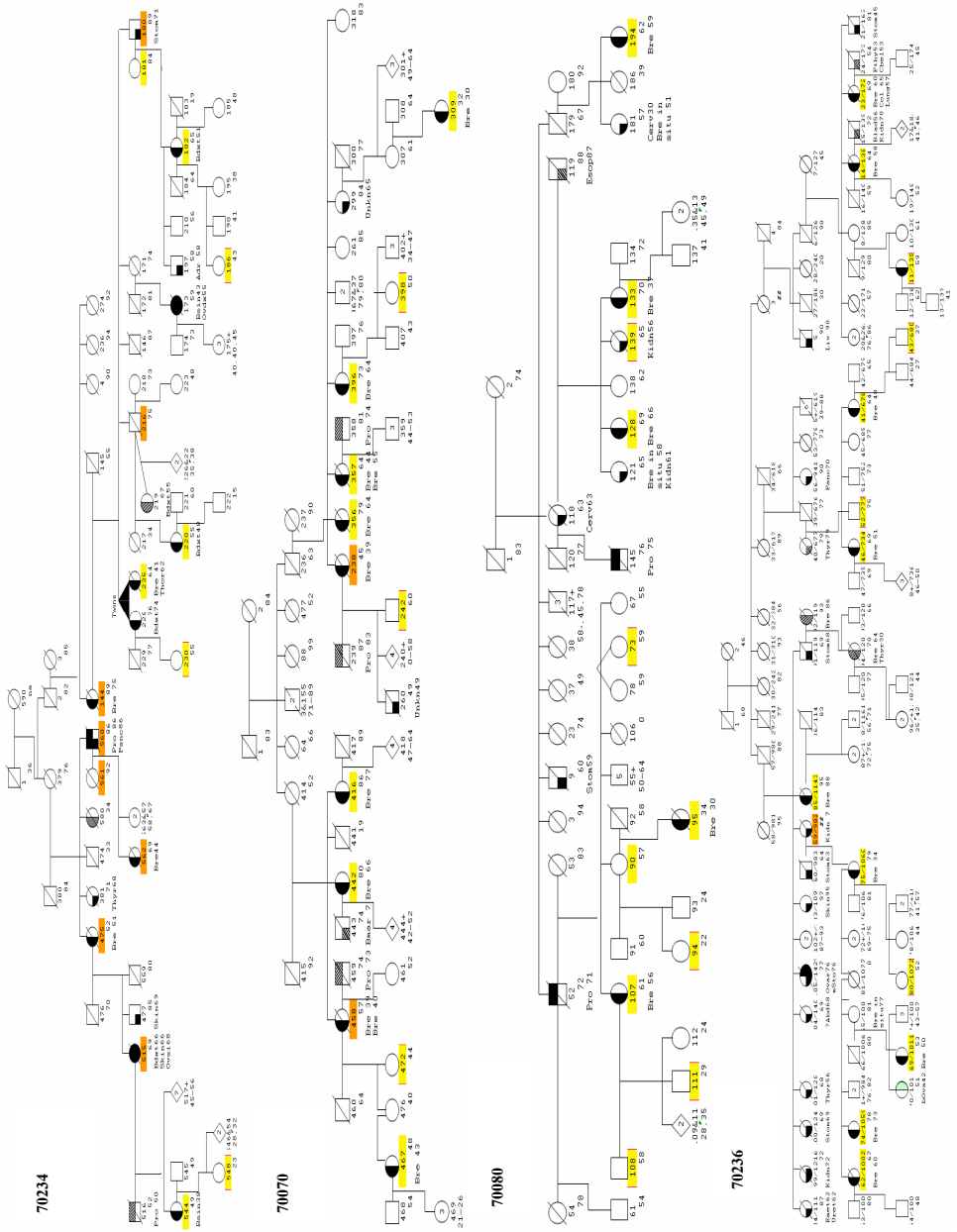


Figure 6. Pedigree structure of families 70234, 70070, 70080 and 70236. Explanation of symbols can be seen in figure 5.

Table 8. Two-point lod scores for microsatellite markers on chromosome 16. Recombination fractions (θ) 0.00001 and 0.2 (gender averaged) are displayed.

16p13.3	Marker	D16S521		D16S291		D16S475			
	θ	0.00001	0.2	0.00001	0.2	0.00001	0.2		
	70386	-0.29	0.00	0.00	0.07	-0.21	-0.05		
	70236	-0.10	-0.05	0.02	0.01	-0.03	-0.02		
	70234	-0.08	0.19	-0.66	-0.02	-0.83	0.01		
	70080	-0.23	-0.08	-0.26	-0.09	-0.27	-0.01		
	70070	-0.86	-0.02	-0.08	0.06	-1.02	-0.21		
SUM	-1.40	0.04	-0.98	0.03	-2.36	-0.28			
16p11.2	Marker	D16S298		D16S685					
	θ	0.00001	0.2	0.00001	0.2				
	70386	0.26	0.10	-0.22	-0.01				
	70236	-0.19	-0.08	-0.05	-0.03				
	70234	-1.24	-0.07	0.53	0.26				
	70080	-0.36	-0.07	-0.37	-0.12				
	70070	-0.84	0.02	-0.85	-0.21				
SUM	-2.37	-0.10	-0.96	-0.10					
16q21	Marker	D16S3089		D16S514		D16S265		D16S3043	
	θ	0.00001	0.2	0.00001	0.2	0.00001	0.2	0.00001	0.2
	70386	0.34	0.23	0.44	0.17	0.43	0.18	-0.38	-0.08
	70236	-0.10	-0.04	-0.19	-0.08	-0.15	-0.05	-0.10	-0.04
	70234	-1.05	0.02	-0.56	-0.02	-0.76	-0.11	0.38	0.29
	70080	-0.31	-0.01	-0.37	-0.10	-0.29	-0.04	-0.25	0.06
	70070	0.07	0.14	-0.83	-0.11	-1.11	-0.17	-0.11	0.09
SUM	-1.04	0.24	-1.52	-0.15	-1.88	-0.18	-0.46	0.32	
16q23.1-2	Marker	D16S3101		D16S516		D16S3040		D16S3098	
	θ	0.00001	0.2	0.00001	0.2	0.00001	0.2	0.00001	0.2
	70386	-0.49	-0.07	-0.44	-0.16	-0.42	-0.11	-0.42	-0.12
	70236	0.03	0.01	-0.18	-0.05	-0.18	-0.05	0.16	0.07
	70234	-0.88	0.18	-0.90	-0.05	-0.61	0.01	-0.43	-0.05
	70080	-0.18	-0.04	0.01	0.07	-0.21	-0.08	-0.31	-0.04
	70070	0.77	0.31	-0.78	0.01	-1.18	-0.17	-1.52	-0.27
SUM	-0.75	0.39	-2.29	-0.17	-2.60	-0.39	-2.52	-0.40	

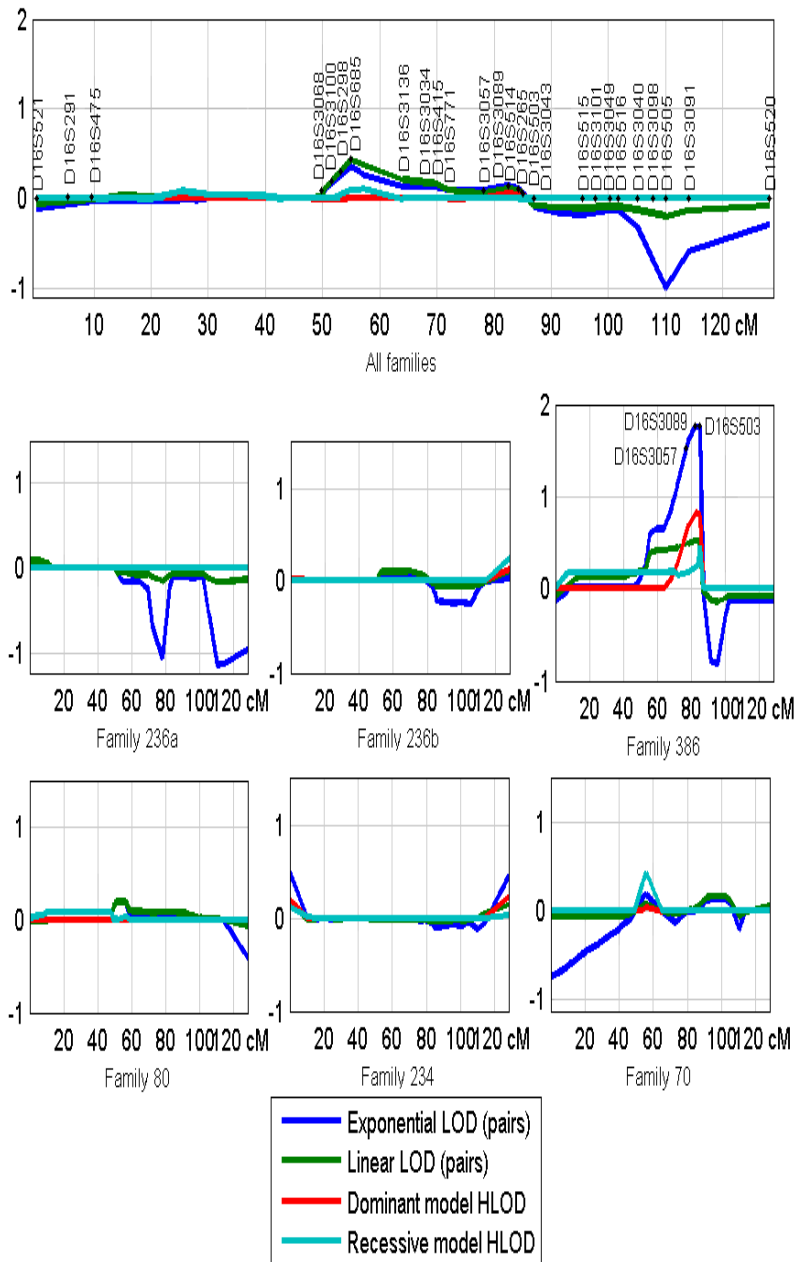


Figure 7. Multipoint lod scores for markers from selected regions on chromosome 16 in five breast cancer families. The families display very high frequency of copy number changes at these chromosome regions in tumours from the family members.

LOH analysis for family 70386

Copy number loss shown by array CGH does not necessarily mean that the wild-type copy of the gene in question is lost. To test the possibility of a tumour suppressor gene being located within the shared haplotype region at 16q12-q21 in family 70386, markers D16S3136, D16S771, D16S514 and D16S3043 were used to study loss of heterogeneity (LOH). LOH analysis was performed comparing genotypes of DNA isolated from blood and tumour samples from the same individual. DNA samples were available from the same 3 members of family 70386 that had tumours included in the array-CGH study. All tested genotypes were heterozygous and thus informative. The results of the study did not support the location of a tumour suppressor susceptibility gene in accordance to Knudson's two-hit theory. Marker D16S771 showed loss of the supposed wild-type allele in 2 of the 3 cases and marker D16S514 in one case. However marker D16S514 also showed loss of the supposed mutant allele in two cases. Marker D16S3136 showed no signs of LOH, which is contrary to the copy number loss seen by CGH. Marker D16S3136 showed no evidence of LOH. Comparing to the array-CGH results, one would have expected to observe some LOH extending to the D16S3136 marker. The reason for not seeing this could lie in technical limitations of the method of LOH-scoring or hidden breakpoints in the array-CGH plot, like e.g. when different subpopulations of cells in the tumour contain different but overlapping copy number changes. Whatever the reason is, marker D16S3136 is not within the haplotype

consistently shared by affected women in the family and therefore of less importance in this respect. The same applies to marker D16S3043 which was also typed for LOH but locates distal to the common haplotype. This marker showed LOH in one genotype, retaining the supposed wild-type allele and therefore not in accordance to Knudson's two-hit model (fig. 5).

Genotyping of SNP rs3803662

A recently published study by Stacey *et al.* (Stacey *et al.*, 2007) associated one of the alleles of SNP rs3803662 on 16q12 with increased breast cancer risk. This SNP is located centromeric but very close to D16S3034 which is within the 16q haplotype segregating with breast cancer in family 70386. We genotyped all available DNA from all five families included in the linkage analysis of chromosome 16. A total number of 59 individuals were typed, of whom 36 had been diagnosed with breast cancer. Stacey *et al.* reported that individuals of European descent that are homozygous for allele T(A) of rs3803662 have a 1.64-fold greater risk of developing breast cancer, with the increased risk being confined to estrogen receptor (ER) positive tumours (Stacey *et al.*, 2007). In the 5 families genotyped for rs3803662, 6 of 59 individuals are homozygous for T (A) allele. Of these 6 homozygotes 5 had been diagnosed with breast cancer which in a sample of unrelated individuals would not be significantly higher than expected ($p=0.39$) since approximately 2/3 of the tested samples were from affected cases (36 of 59). The unaffected homozygote was a sister of an affected one but otherwise the homozygotes were fairly

distributed among the families (one in each of the families 70080, 70236 and 70386 and the remaining one in 70070, being a 4th degree relative of the sisters). The affected homozygotes were diagnosed at an age ranging from 30-64 years and the unaffected one was 44 years old. Four of these tumours were ER positive but the fifth is of unknown ER status.

Genotyping and linkage analysis - PALB2

The PALB2 gene located at 16p12 has been identified as a breast cancer susceptibility gene in Finnish breast cancer cases. It is located approximately 10cM telomeric to the region at 16p11.2 showing frequent copy number gain. We decided to see if it might play a role in breast cancer susceptibility in the Icelandic population.

Table 9. Two-point lod scores for microsatellite markers surrounding the PALB2 gene. Recombination fractions (θ) 0.00001, 0.01 and 0.05 (gender averaged) are shown. The PALB2 gene is located between D16S412 and D16S420, the distance being 0.45Mb and 0.58Mb respectively.

Marker	D16S3036			D16S412		
θ	0.00001	0.01	0.05	0.00001	0.01	0.05
Family						
1	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001
2	-0.287	-0.256	-0.161	0.259	0.251	0.220
3	-0.148	-0.144	-0.128	-0.042	-0.041	-0.035
4	-0.464	-0.377	-0.129	-1.200	-1.084	-0.742
5	-1.094	-0.765	-0.325	-0.491	-0.475	-0.411
6	-0.032	-0.029	-0.020	-0.003	-0.002	-0.002
7	-0.038	-0.036	-0.028	-0.025	-0.023	-0.017
8	-0.247	-0.222	-0.131	0.099	0.096	0.081
9	0.852	0.822	0.703	-1.283	-1.063	-0.632
SUM	-1.460	-1.008	-0.221	-2.686	-2.344	-1.540

Marker	D16S420			D16S3068		
θ	0.00001	0.01	0.05	0.00001	0.01	0.05
Family						
1	-0.002	-0.002	-0.002	-0.001	-0.001	-0.001
2	-0.290	-0.260	-0.167	-0.252	-0.223	-0.138
3	0.111	-0.107	-0.090	-0.212	-0.204	-0.174
4	-1.008	-0.911	-0.615	0.405	0.406	0.395
5	-1.835	-1.537	-1.020	-1.072	-0.927	-0.571
6	-0.024	-0.023	-0.018	0.184	0.173	0.132
7	-0.035	-0.032	-0.024	0.068	0.064	0.049
8	-0.242	-0.228	-0.170	0.179	0.171	0.141
9	-0.634	-0.434	-0.113	-0.136	-0.085	0.029
SUM	-4.182	-3.533	-2.217	-0.837	-0.627	-0.138

Four microsatellite markers in the PALB2 gene region were genotyped but indicated no evidence of linkage in any of the five high-risk breast cancer families with the high incidence of copy number changes at 16p11.2, nor in four additional high-risk non-

BRCA1/2 breast cancer families (table 9). The two markers flanking the *PALB2* gene, D16S420 (~0.58 Mb centromeric to *PALB2*) and D16S412 (~0.45 Mb telomeric to *PALB2*), showed highly negative lod scores of total -4.18 and -2.69 ($\theta = 0.00001$). Furthermore, we screened for the Finnish *PALB2* founder mutation 1592delT. It was not detected in any of the 111 family members of the nine high-risk breast cancer families nor in 638 unselected breast cancer cases. This part of the study has been published in a peer-reviewed journal (Gunnarsson *et al.*, 2008).

BRCAX and BRCA1/2

As previously mentioned this MSc. project is a part of a larger project, including a Nordic collaboration establishing a databank with results from array studies of tumour samples from Nordic breast cancer patients. This databank already includes array-CGH data from several hundreds of breast tumours including BRCA1 and BRCA2 mutated tumours and sporadic tumours. By using array-CGH data from this databank, BRCAX was compared to other tumour groups with respect to the total number of aberrated clones. Bearing in mind that BRCA1- and BRCA2-mutated tumours have a high frequency of aberrations, in line with the role of BRCA1/2 in DNA repair mechanism, a high number of aberrated clones in BRCAX tumours or subgroup of BRCAX tumours might suggest a similar role.

Figure 2a-b shows the aberration profile of the BRCAX and sporadic groups. The copy number frequency of aberrated clones in the BRCAX group was compared to analogous profiles (obtained by the

same methods) of sporadic breast tumours (n=54), BRCA1 germline-mutated breast tumours (n=22) and BRCA2 germline-mutated breast tumours (n=51) (table 10) applying a two-sided Mann-Whitney test. The BRCAX group and the sporadic group do not show a significant difference ($p=0.33$) in the number of aberrated clones, but the difference between BRCAX and the BRCA1/2 groups is significant ($p<0.0001$).

Table 10. Numbers of aberrated clones in different groups of breast tumours. Comparison of total number of aberrated clones between groups was done using a two-sided Mann-Whitney test ($p<0.05$).

	Group			
	BRCAX	Sporadic	BRCA1	BRCA2
Min nr. of aberrated clones	96	151	2.544	859
Max nr. of aberrated clones	19.449	13.013	21.331	20.784
Mean	5.014	5.379	9.136	9.602
Median	3.844	4.093	7.483	9.814
SD	4.043	3.619	5.231	4.027

Comparison of CGH results from different BRCAX subgroups

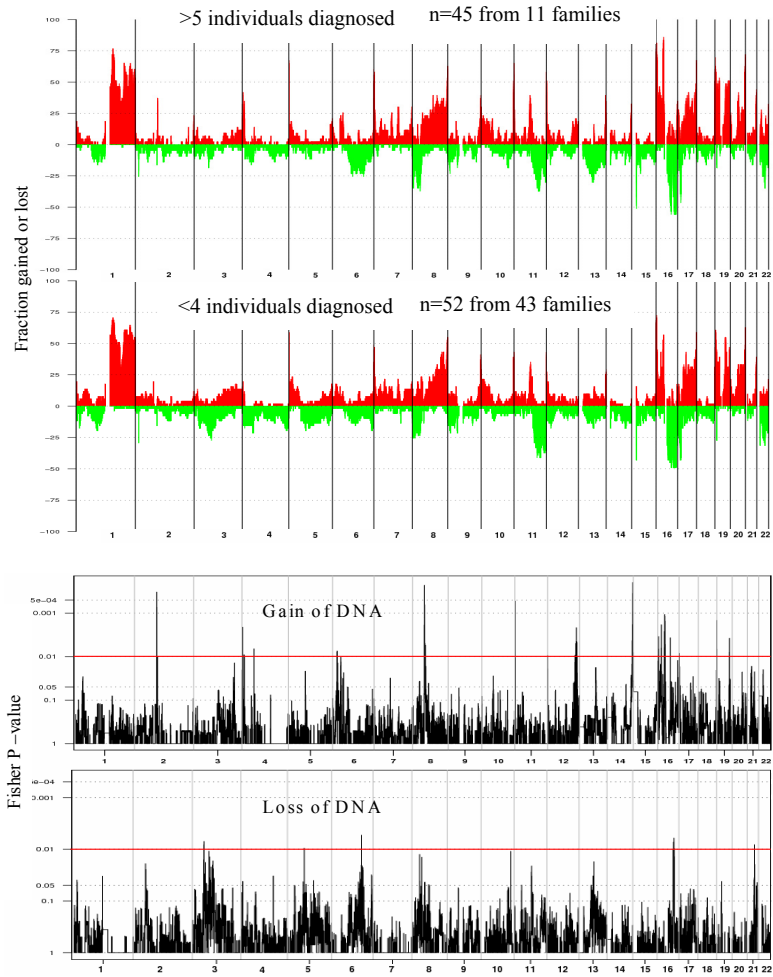
Low age at diagnosis and a high number of affected cases is a common feature within families with a highly penetrant dominantly inherited germline mutation in genes such as BRCA1 and BRCA2. Looking at subgroups within the BRCAX group, based on age at diagnosis and number of diagnosed individuals within families might thus reveal discriminating chromosomal regions, possibly harbouring mutations reminiscent of those of BRCA1/2. More specifically, regions with prevalent aberrations in a subgroup of low age at

diagnosis and/or higher incidences in relatives, as compared to higher age and/or lower incidences, would be of interest in search of high-risk mutations. Therefore a comparison of the whole genomic profile of subgroups within the BRCAX group was done, applying a multi-Fisher's exact test, for groups distinguished by: 1) number of individuals diagnosed with breast cancer in the family (<4 vs. >5 diagnosed), 2) age at diagnosis (≤ 45 vs. ≥ 60 years) and 3) age at diagnosis combined with the number of affected family members (≤ 50 years and ≥ 4 diagnosed vs. >50 years and <4 diagnosed) (fig. 8a-c). The reason for not using the same parameters in the third definition as in the first two is that it would lead to an insufficient sample size in subgroups and reduce the power of the statistical analysis. By the first definition (<4 vs. >5 diagnosed), there are a number of regions of significant difference. Regions showing significantly higher frequencies of gain for the subgroup with >5 individuals diagnosed can be found on 2p11, 4p16, 8q11.21, 12q24, 14p32, 16p13-p11 and 19q13 and higher frequency of loss on 6q22. Regions of higher frequency of loss for the subgroup with <4 individuals diagnosed are on 3p21, 5q13, 16q22 and 21q21 (fig. 8a). For the second definition (≤ 45 vs. ≥ 60 years), regions on 3q25-q26, 9p22-p21, 12p13-p12 and 19q12-q13 were associated with higher frequency of gain in the lower-age subgroup and regions on 2q35, 4p16-p14, 4q33, 4q35, 8p21-p11, 16p13, 17q22-q23 and 17q24 with more frequent losses. No regions were reported to have a significantly higher frequency of aberrations in the subgroup with individuals diagnosed ≥ 60 years compared to those diagnosed ≤ 45 years (fig. 8b). The third definition

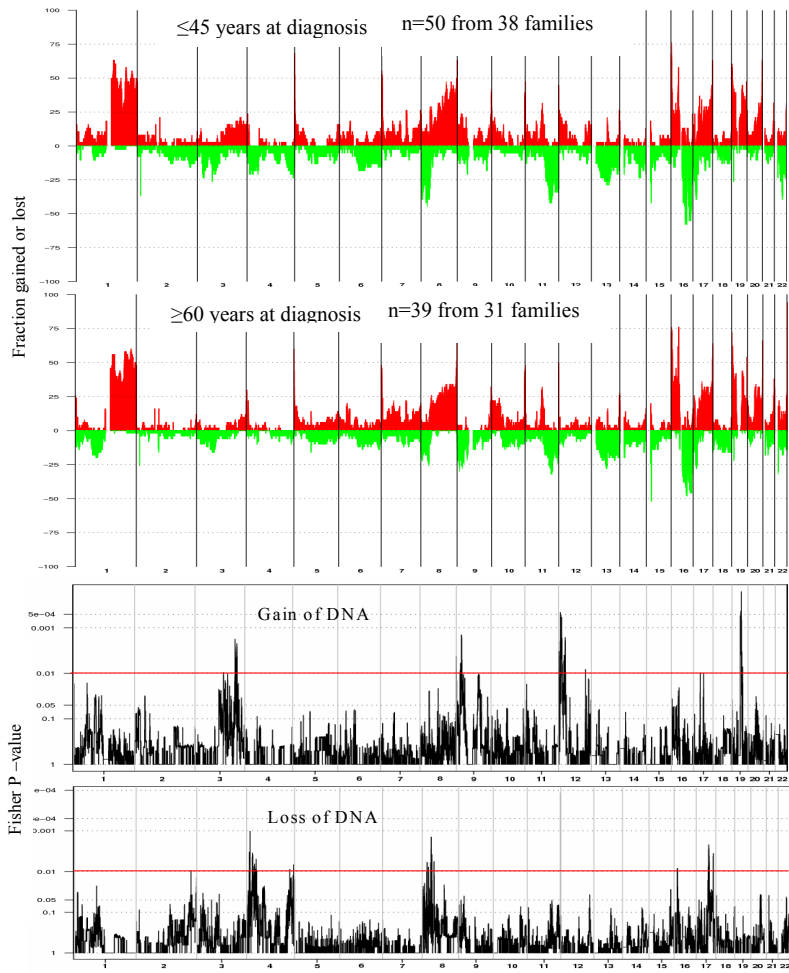
(≤ 50 years and ≥ 4 diagnosed vs. > 50 and < 4 diagnosed) shows the subgroup with younger and more numerous cases to have a significantly higher frequency of gain on 8q11-q23, 12q24 and 19q12 and of losses on 8p23 and 8p22-p11. No other regions were reported to have a significantly higher frequency of aberrations for either subgroup (fig. 8c).

To see whether any of the defined subgroups showed a higher total frequency of aberrations across the genome more akin to BRCA1 and BRCA2 germline mutated tumours, a two-sided Mann-Whitney test was applied. Comparison of the total number of aberrated clones between each defined set of subgroups did not show any significant differences (table 11).

8a)



8b)



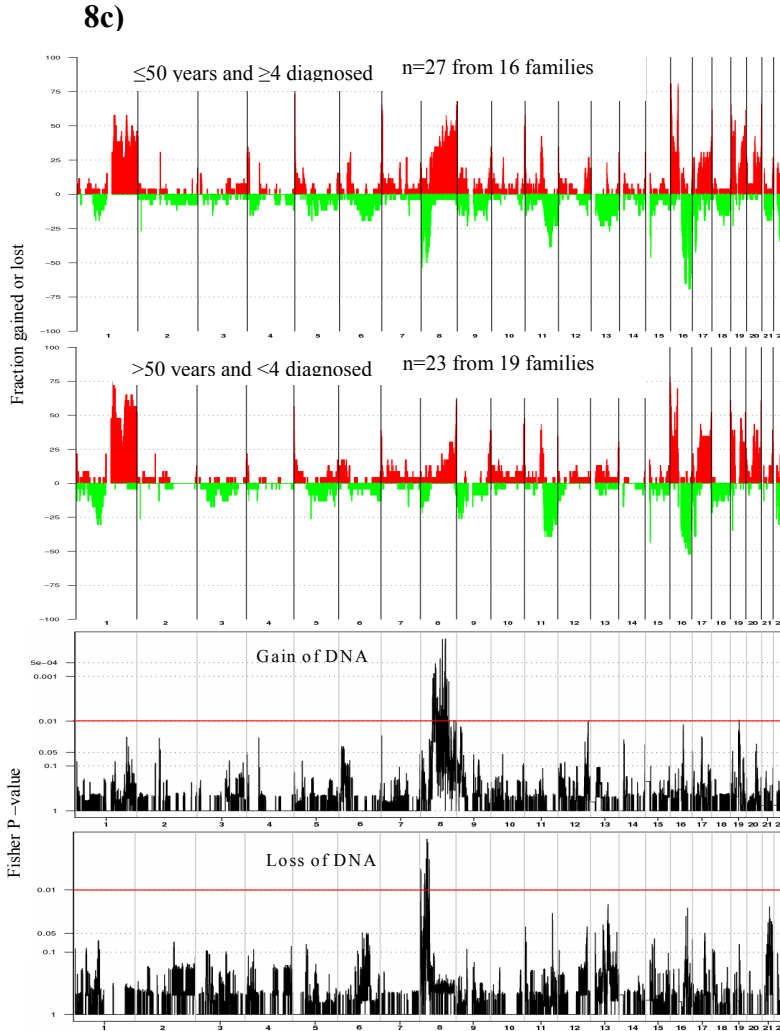


Figure 8a-c. Chromosomal aberrations in different BRCA1/2 subgroups. Grouping based on a) number of individuals diagnosed with breast cancer in the family (<4 diagnosed vs. >4 diagnosed), b) age at diagnosis (≤45 vs. ≥60 years) and c) age at diagnosis combined with the number of affected family members (≤50 years and ≥4 diagnosed vs. >50 years and <4 diagnosed). The proportion of tumours in which each clone is gained (red bars) or lost (green bars) is plotted on y-axis for each BAC clone according to genomic location on x-axis. The two bottom panels illustrate the results of multi-Fisher's exact test performed with the segmented values for each BAC clone to discriminate between regions of significance between the two BRCA1/2 subgroups. The panels show the unadjusted P-value of each clone in regards of gains of DNA and losses of DNA according to the genomic location on the chromosome (x-axis). Clones with a P-value of less than 0.01 are considered significant.

Table 11. Numbers of aberrated clones in different subgroups of BRCAx tumours. Comparison of total number of aberrated clones between subgroups was done using a two-sided Mann-Whitney test ($p < 0.05$).

	Group					
	>5 diagn.	<4 diagn.	≤45 years	≥60 years	≤50 years and ≥4 diagn.	>50 years and <4 diagn.
Min nr. of aberrated clones	513	180	96	305	96	764
Max nr. of aberrated clones	13.869	19.449	19.449	14.103	11.710	14.103
Mean	4.352	5.199	5.089	4.539	4.269	3.824
Median	3.973	3.839	3.996	3.037	3.835	3.693
SD	3.071	5.199	4.568	3.598	3.461	2.806
P-value	0.7225		0.7907		0.9123	

Looking at the number of aberrated clones in individual tumours from the five families with high gains and losses on chromosome 16 and families with 4 or more tumour samples undergoing array CGH (10 families) no single family shows signs of an underlying BRCA1- or BRCA2-like role in DNA-repair, which would be indicated by prevalence of samples with high frequency of aberrations. In 7 of the 10 families a single tumour is seen in each to have similar frequency of aberrated clones as BRCA1/2 mutated tumours, indicating a sporadic effect (table 12).

Table 12. Number of aberrated clones in tumours from families in the BRCAx group having 4 or more tumour samples included in the array-CGH study, and from family 70386.

Family 70070				Family 70440			
Assay	Clones			Assay	Clones		
CI045	1.022	N	4	CI014	11.720	N	4
CI046	2.054	SD	1.993	CI064	96	SD	5.492
CI047	5.419	Mean	2.497	CI199	314	Mean	3.629
CI204	1.494	Median	1.774	CI229	2.385	Median	1.350
Family 70080				Family 70259			
Assay	Clones			Assay	Clones		
CI085	11.974	SD	4.081	bf623	1.611	SD	1.369
CI226	6.654	Mean	6.534	CI057	2.471	Mean	3.170
CI227	5.315	Median	5.985	CI076	4.027	Median	3.249
CI228	2.191			CI077	4.572		
Family 70234				Family si11			
Assay	Clones			Assay	Clones		
bf792	6.544			CI071	4.637		
CI001	5.425	N	7	CI073	3.829	N	6
CI003	10.043	SD	3.251	CI074	513	SD	1.542
CI206	1.472	Mean	4.392	CI212	2.758	Mean	2.681
CI207	725	Median	4.070	CI213	1.302	Median	2.902
CI209	2.466			CI215	3.046		
CI210	4.070						
Family 70236				Family 70138			
Assay	Clones			Assay	Clones		
CI019	2.685	N	5	CI033	4.595	N	5
CI020	6.249	SD	2.803	CI065	5.785	SD	3.790
CI022	10.505	Mean	6.160	CI066	11.710	Mean	5.521
CI023	5.717	Median	5.717	CI221	3.973	Median	4.595
CI072	5.646			CI222	1.542		
Family 70386				Family 70124			
Assay	Clones			Assay	Clones		
CI044	9.603	N	3	CI011	1.239	N	5
CI223	1.205	SD	4.808	CI012	13.869	SD	5.475
CI225	1.349	Mean	4.052	CI013	8.014	Mean	5.401
		Median	1.349	CI218	2.144	Median	2.144
				CI219	1.738		

DISCUSSION

In the current study we report, by use of array CGH, common aberrations in 131 BRCA1 breast tumours. We located four regions of common genomic aberrations on chromosome 16 in five high-risk BRCA1 breast cancer families. Linkage analysis in these five families at the four regions of interest on chromosome 16 indicated no evidence of linkage. However, in one of the families the breast cancer cases shared the same haplotype at one of these loci, 16q21. Additional markers were added to the region and linkage analysis gave higher lod scores in this family, but not to a level of significance. LOH analysis in the tumours from the members of this family did not support the location of a tumour suppressor gene according to Knudson's two-hit theory. The PALB2 gene at 16p12.1 and the SNP rs3803662 at 16q12.1 have previously been implicated in breast cancer susceptibility. Genotyping analysis of rs3803662 in DNA samples from these five families showed higher (but not significantly different) incidence of homozygosity of the risk allele among the breast cancer cases compared to unaffected family members. However, the risk allele of rs3803662 alone cannot explain the high frequency of breast cancer among these families. Linkage analysis on nine high-risk breast cancer families, including the five aforementioned families indicated no evidence of linkage to the PALB2 region. Subgroup analysis of the BRCA1 group, by dividing tumours into groups based on age at diagnosis, number of affected

relatives or both factors, revealed several regions discriminating the subgroups including chromosome 8.

Common regions of aberration in BRCA_X tumours

Five families shared common regions of aberration on chromosome 16 making them candidates for genotyping and linkage analysis. Using CGH to pinpoint a region of a putative tumour suppressor gene by detecting somatic losses of DNA has previously been shown in a study of the Peutz-Jeghers cancer syndrome (Hemminki *et al.*, 1997). A Nordic collaboration study also applied CGH on 61 breast tumours from 37 breast cancer families and detected a high frequency of genomic losses on 13q21-q31. One family with five analysed breast cancer cases showed a minimal region of loss at 13q21-q22. Genotyping revealed a shared haplotype of this region and a targeted linkage analysis for a large set of breast cancer families showed a lod score of 2.76 for one marker at 13q21 (Kainu *et al.*, 2000). Since other studies have not verified the 13q21 findings it is likely that if the putative tumour suppressor locus at 13q21 contains a breast cancer suppressor gene it only accounts for a small proportion of BRCA_X families of Finnish origin (Thompson *et al.*, 2002). It is conceivable that CGH could have aided the isolation of the BRCA2 gene as a CGH study by Tirkkonen *et al.* on BRCA1 and BRCA2 breast cancer tumours did display a loss of DNA of the 13q region, where the BRCA2 gene is located (13q13.1), as the most common aberration in BRCA2 tumours (Tirkkonen *et al.*, 1997).

By looking for common genomic losses among BRCA families with available array-CGH results from at least 3 tumour samples, candidate regions were located at 16q21 and 16q23.1-q23.2, based on loss of DNA in at least 19 of 23 tumours from 5 families. Both regions also display the highest frequency of loss in the BRCA tumour group as a whole, but the decision to select these regions for linkage analyses rested on the fact that these 5 families displayed such a high frequency of common loss without any other region in this study displaying nearly as high frequencies of common DNA losses in more than a single family. Loss of heterozygosity and genomic aberrations of chromosome arm 16q are frequent events in breast cancer suggesting the presence of tumour suppressor genes (reviewed in (Rakha *et al.*, 2006)). An array-CGH study of sporadic tumours reported 34% of the tumours showing loss of DNA at 16q21 (Naylor *et al.*, 2005) and according to the UCSC Genome Browser (genome assembly March 2006) (<http://genome.ucsc.edu>) (Kent *et al.*, 2002) the region harbours only 2 genes, CDH8 and CDH11. Both genes were reported as potential candidate tumour suppressor genes in an array-CGH study on chromosome arm 16q in invasive ductal and lobular breast carcinoma but no pathogenic mutations were discovered (Roylance *et al.*, 2006). Region 16q23.1-q23.2 is within a region frequently reported to display LOH in breast cancer tumours (reviewed in (Rakha *et al.*, 2006)). A common fragile site, FRA16D, is located in 16q23.2 (Mangelsdorf *et al.*, 2000) as well as the suggestive tumour suppressor gene WWOX which is about 1.1 Mb in size and spans the FRA16D site (Bednarek *et al.*, 2000) (reviewed in (Aqeilan & Croce, 2007)).

The WWOX gene has frequently been reported as inactivated in various human cancers suggesting it to be a tumour suppressor. However, it has been suggested that the loss of WWOX expression in cancer is mainly due to its location on the FRA16D site and might be a secondary event and not associated with tumourigenesis (reviewed in (Aqeilan & Croce, 2007)).

Although regions displaying gain of DNA do not suggest a location of a tumour suppressor gene according to *Knudson* (Knudson, 1971), autosomal dominant gain-of-function germline mutations have been shown to cause hereditary cancers. Such is the case of the RET proto-oncogene which was shown to cause hereditary medullary thyroid carcinoma, MEN2 (Donis-Keller *et al.*, 1993; Mulligan *et al.*, 1993) (reviewed in (Kouvaraki *et al.*, 2005; Raue & Frank-Raue, 2007)). This demonstrates that not all hereditary cancer syndromes follow *Knudson's* two-hit-model (Knudson, 1971) and that other mechanisms regarding the cause of inherited cancer are well plausible. One hypothesis could be that an inherited genetic factor which increases susceptibility towards breast cancer development could be further boosted by somatic mutations in the form of gains/amplifications of the mutant allele itself. Such might be the case if the product forms a dimer and only the dimer of the normal allele is active. If the total amount of the product is regulated, amplification of the mutant allele would amount to loss of this normal dimer. Another possibility could be that a promoter region of an oncogene harboured a germline mutation causing increased expression of the oncogene and the risk of cancer would then further increase following a somatic gain or

amplification of that gene. In light of such possibilities the regions of most frequent gain, on 16p13.3 and 16p11.2, in the BRCAX tumour group and the 5 families who also showed common loss of 16q, were considered regions possibly harbouring susceptibility genes. Region 16p13.3 has previously been reported with frequent gains of DNA in both sporadic and hereditary breast cancer tumours (Kainu *et al.*, 2000; Melchor *et al.*, 2007; Naylor *et al.*, 2005) while 16p11.2 has been reported frequently gained in invasive lobular carcinomas (Stange *et al.*, 2006).

Genotyping and linkage analysis on chromosome 16

No linkage has previously been suggested to the regions proposed here on chromosome 16. Based on the results from the array CGH on 5 BRCAX families, genotyping and linkage analysis targeting the candidate sites for the families was undertaken suggesting a rare autosomal dominant effect. Two-point lod scores for the original 13 markers indicated no evidence of linkage. Family 70386 shared a haplotype for 3 markers at 16q21. Additional markers were added to the region as well as for other regions of indeterminable haplotype. Although the addition of the new markers gave higher lod scores for 16q21 in family 70386 the results nevertheless fail to provide significant evidence of linkage. It should be kept in mind that the total number of five breast cancer cases in this family is too low to allow for expecting significant evidence of linkage in this family alone. A non-parametric model gave lod scores of around 1.7, therefore not ruling out the possibility of a susceptibility gene in the region. Several

linkage studies on non-BRCA1/2 breast cancer families (table 2) have been published with lod peaks suggestive of various breast cancer susceptibility loci, but most often without reaching the level of significance.

The lack of evidence of chromosome 16 linkage in the majority of the families analysed could be indicative of other factors in the genome that are the cause of these aberrations making the chromosome 16 events secondary, presenting the possibility of a common susceptibility gene that is located elsewhere in the genome and not within these regions of common aberrations at chromosome 16. If so, the method of array CGH used here is not seen to provide any clue to the chromosomal location of this gene. The alternative possibility exists that a number of genes remain to be identified, each explaining as few as one family.

One thing that must be taken into account when viewing the aberration profile derived from the array-CGH data is normal genome copy number variation that can be falsely interpreted as genuine somatic aberrations. Since this study does not use matching normal and tumour DNA samples, parts or all of the detected aberration in some regions may result from normal variation in the genome and not be due to true somatic aberrations. The Database of Genomic Variants (<http://projects.tcag.ca/variation>) (Iafrate *et al.*, 2004) provides a comprehensive summary of structural variation in the human genome and according to Gorringe *et al.* it is imperative to consult such a database when not using matching of normal and reference DNA (Gorringe & Campbell, 2008). All the four candidate regions on

chromosome 16 have been shown to include normal copy number variants within their size range. According to the Database of Genomic Variants the two regions of gain on 16p overlap with many such variants including a few large variants, >300 kilobases in size. The database also shows the two 16q regions of loss to overlap with a few variants although none are so large (<300 kilobases in size). Thus we can not exclude that some of the supposed aberrations seen in the regions, especially those on 16p, could be due to normal variation rather than somatic aberrations.

Loss of the wild-type allele for markers D16S771 and D16S514, both located within the region of the shared haplotype in family 70386 is observed as well as loss of the supposed mutant allele. Given that only three individuals are studied, these data are not conclusive. However, the frequencies of LOH for the supposed wild-type on 16q12-q21 in family 70386 are not comparable to what is seen for BRCA1/2 germline mutation carriers at the BRCA1/2 loci. LOH is the most common mechanism of inactivation of the wild-type allele for BRCA1/2 germline mutation carriers, occurring in around 90% of informative tumours (Barkardottir *et al.*, 1995; Cornelis *et al.*, 1995; Gudmundsson *et al.*, 1995; Ingvarsson *et al.*, 1998; Osorio *et al.*, 2002).

SNP genotyping

Stacey *et al.* reported that individuals of European descent who are homozygous for allele T(A) at the SNP rs3803662, located on 16q12 near the gene TNRC9, have a 1.64-fold greater risk of developing

breast cancer and that the risk is confined to estrogen receptor-positive tumours (Stacey *et al.*, 2007). SNPs in genes like RAD51, FGFR2, ATM and MDM2 have been implicated as possible modifiers of BRCA1 and BRCA2 associated breast cancer risk (Antoniou *et al.*, 2007; Antoniou *et al.*, 2008; Kadouri *et al.*, 2004; Li *et al.*, 2000; Sliwinski *et al.*, 2005; Yarden *et al.*, 2008). Antoniou *et al.* also provided evidence that rs3803662 is associated with breast cancer risk in both BRCA1 and BRCA2 mutation carriers (Antoniou *et al.*, 2008). Given the location of rs3803662 within the segregating haplotype and the results of previous studies it was conceivable that such a SNP might affect the role of a candidate gene in the 16q12-21 region, in the development of breast cancer. Our results show 6/59 individuals in the 5 families to be homozygous of T (A) allele and 5 of these have been diagnosed with breast cancer, 4 being ER positive and 1 of unknown status. This is in harmony with the published reports and may reflect that the T(A) allele modifies the penetrance of the gene/genes responsible for the familial clustering of breast cancer in the five families. However, the low numbers of individuals here tested would hardly be expected to provide statistically significant results given the low risk increase (1.64-fold) reported for T (A) homozygotes. On the other hand, it can also be said that it is clearly not to be seen that rs3803662 might be the main locus responsible for the high incidence of breast cancer in the five families.

PALB2 analysis

Several PALB2 mutations associated to breast cancer have been identified and one of them, 1592delT in Finland, has been found to be recurrent (Erkko *et al.*, 2007). PALB2 is located on 16p12.1 and given the possibility of the same ancient origin of the 1592delT mutation similar to what has been hypothesised for the BRCA2 999del5 mutation in the Finnish and the Icelandic population (Barkardottir *et al.*, 2001), linkage analysis targeting the gene was performed as well as screening for the Finnish PALB2 founder mutation in Icelandic breast cancer cases. Our results suggest that PALB2 is not a significant contributor to breast cancer in high-risk breast cancer families in Iceland. Furthermore, the results show that the 1592delT mutation appears not to be associated with breast cancer in the Icelandic population, and if occurring it would be very rare. However the results can not exclude the possibility that other PALB2 mutations causing low or moderate breast cancer risk exist in the Icelandic population. To determine that, it would be necessary to perform a PALB2 mutation screening or SNP analysis in a large cohort of breast cancer cases.

Characterization of BRCAX with respect to limitations of linkage power

The frequency of aberrations and the regions affected in the BRCAX tumours in this study are in concordance to the regions reported by other studies of BRCAX tumours (Gronwald *et al.*, 2005; Kainu *et al.*, 2000; Melchor *et al.*, 2007). BRCAX and sporadic tumours have on average a little over 5000 clones aberrated compared to over 9000

clones in the BRCA1 and BRCA2 tumours (table 10). Before either BRCA1 or BRCA2 genes were isolated the association between ovarian cancer and, in the case of BRCA2, male breast cancer, were recognized and this made the groups more easily defined, hence making it easier to target certain families with high prior probability of linkage in search of the target gene. To date no such phenotype has been associated with a subgroup of BRCA1 families. The possibility of multiple additional genes, each responsible for a small number of families, therefore limits the power of linkage analysis (Nathanson & Weber, 2001). Also the possibility of phenocopies (equivalent to sporadic cases) among relatives of mutation carriers in non-BRCA1/2 breast cancer families causes further problems to the power of detecting a common region of susceptibility. Such cases could well be seen in 7 of the 10 families in table 12 since one tumour in each family differs from the others by displaying a high number of aberrations close to the average of BRCA1 and BRCA2 tumours. It is well plausible that some of these highly aberrated cases are of sporadic nature and caused by events such as promoter hypermethylation of BRCA1 (Birgisdottir *et al.*, 2006; Catteau *et al.*, 1999; Russell *et al.*, 2000; Staff *et al.*, 2003). Such epigenetic changes are analogous to carrying a germline mutation and in case of a gene like BRCA1 would most likely cause high frequencies of aberrations in the genome.

It has been proposed that a way to ensure the success in the search of a susceptibility gene in a complex disease like breast cancer, would be to study a sample set from a homogenic ethnic group (Altmuller *et al.*,

2001) as is the case in this study. Even though this group of BRCAX tumours derives from a relatively genetically homogenic population it is evident that locating a causal gene will be difficult unless common traits can be found for subgroups of families or tumours.

BRCAX subgroup profiling

Detecting regions significantly discriminating between BRCAX subgroups did not reveal a subset of high-risk families with a common region of aberrations comparable to what is seen on chromosome 16 for the five families. No significant difference is seen in the comparison of total number of lost clones whether discriminating by age or number of diagnosis or including both factors. A comparison based on age and number of individuals diagnosed per family separately, shows 19q13 as a region commonly discriminating between groups whereas individuals ≤ 45 years diagnosed and those with >5 family members diagnosed show significantly higher gains in the region. Looking at both factors together, those diagnosed at an earlier age and with higher number of affected relatives show significantly higher frequencies of gain on chromosome regions 8q11-q23, 12q24 and 19q12 and a significantly higher frequency of loss on 8p23-p11. Loss of DNA of chromosome arm 8p and gains of 8q are common events in breast cancer (Gronwald *et al.*, 2005; Rennstam *et al.*, 2003; Tirkkonen *et al.*, 1998) and poor patient outcome has been associated with these aberrations (Weber-Mangal *et al.*, 2003). Linkage studies have previously associated chromosome region 8p12-21 to be a region harbouring a breast cancer susceptibility gene in

non-BRCA1/BRCA2 breast cancer families (Kerangueven *et al.*, 1995; Seitz *et al.*, 1997a; Seitz *et al.*, 1997b) while studies on sporadic and BRCA2 tumours have suggested that tumour suppressor genes on 8p are more likely associated with cancer progression rather than initiation (Sigbjornsdottir *et al.*, 2000; Weber-Mangal *et al.*, 2003). A CGH study comparing genomic aberrations between BRCA1 and sporadic tumours suggested that in BRCA1 tumours the loss of 8p is more likely to be associated with tumour initiation rather than progression as well as 8q gains being early events in BRCA1 tumour development (Gronwald *et al.*, 2005). No subset of BRCA1 families, with 3 or more samples in our array-CGH study, had a common region of high frequency of loss of DNA on 8p. Linkage analysis results from a genome-wide linkage study including some of the families displaying 8p loss showed no evidence of linkage (unpublished data). It is possible that this difference seen on chromosome 8 is related to progression, rather than being an early event, and that losses on 8p and gains on 8q might facilitate earlier onset and increased penetrance.

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Brief report

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Evidence against PALB2 involvement in Icelandic breast cancer susceptibility

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Abstract

Several mutations in the *PALB2* gene (partner and localizer of *BRCA2*) have been associated with an increased risk of breast cancer, including a founder mutation, 1592delT, reported in Finnish breast cancer families. Although most often the risk is moderate, it doesn't exclude families with high-risk mutations to exist and such observations have been reported. To see if high-risk *PALB2*-mutations may be present in the geographically confined population of Iceland, linkage analysis was done on 111 individuals, thereof 61 breast cancer cases, from 9 high-risk non-*BRCA1/BRCA2* breast cancer families, targeting the *PALB2* region. Also, screening for the 1592delT founder mutation in the 9 high-risk families and in 638 unselected breast cancer cases was performed. The results indicate no linkage in any of the high-risk families and screening for the 1592delT mutation was negative in all samples. *PALB2* appears not to be a significant factor in high-risk breast cancer families in Iceland and the 1592delT mutation is not seen to be associated with breast cancer in Iceland.

Background

Breast cancer is among the most frequent human cancers and the most common carcinoma in women in the Western world, where one out of every ten women is affected. A dominant pattern of inheritance is evident in approximately 5–10% of all breast cancers. To date, two main breast cancer susceptibility genes have been identified; *BRCA1* and *BRCA2* accounting for nearly half of high-incidence breast cancer families and an increased relative risk of breast cancer by 10- to 20- fold [1,2]. Other known breast cancer susceptibility genes such as *CHEK2* and *ATM*

have a more moderate penetrance with an increased lifetime risk of about 2- to 3- fold [2].

The *PALB2* gene is a *BRCA2* binding factor that ensures *BRCA2* function as a tumor suppressor and has been shown to cause Fanconi anemia subtype FA-N when biallelic germ-line mutations occur in the gene [3-5]. Recent studies have reported several mutations in *PALB2* to be associated with an increased risk of breast cancer [6-9]. One is the founder mutation 1592delT which has been found to be present at a significantly elevated frequency in

breast cancer families in Finland, resulting in a 4-fold increased risk to mutation carriers [6]. Although predisposing *PALB2* mutations generally appear to cause moderate risk of breast cancer [8], mutations have also been found in strong hereditary breast cancer families [7,9] and might thus be worthwhile searching for by linkage analysis in e.g. geographically confined populations.

Only two *BRCA1* and *BRCA2* mutations have been found in the Icelandic population, *BRCA2* 999del5 and *BRCA1* G5193A, both being founder mutations explaining a large proportion of familial breast cancer in Iceland [10]. The *BRCA2* 999del5 mutation is much more frequent, accounting for around 40% of the hereditary cases and found in about 8% of unselected breast cancer cases and 0,4% of population based control [11]. A *BRCA2* 999del5 mutation is also the most frequently occurring *BRCA1/2* mutation in Finland [12], and haplotype analysis of Finnish and Icelandic *BRCA2* 999del5 families did not exclude the possibility of a common ancient origin of the mutation [13]. The *BRCA1* G5193A mutation however is very rare, found in less than 2,5% of hereditary breast cancer families and in 0,2% of unselected breast cancer cases [14]. The extreme frequency figures of these two founder mutations reflect low genetic diversity in the Icelandic population. The Icelandic population is a very young one, the country being settled by a few thousand founders about 1100 years ago. Low genetic diversity is probably explained by the relatively homogeneous group of settlers, and genetic drift resulting from repeated population bottlenecks due to diseases and famines [15,16].

The aim of this study is to find out if the *PALB2* gene is likely to play a significant role in breast cancer susceptibility in Iceland, by linkage analysis of high-risk non-*BRCA1/2* breast cancer families with markers closely surrounding the *PALB2* gene to test the possibility of a highly penetrant mutation, and by screening for the Finnish 1592delT founder mutation in the family members and a large group of unselected breast cancer patients.

Results and Discussion

No evidence of linkage in the *PALB2* region was found in any of the high risk breast cancer families [see Additional file 1]. The two markers flanking the *PALB2* gene, D16S420 (~0.58 Mb centromeric to *PALB2*) and D16S412 (~0.45 Mb telomeric to *PALB2*), showed highly negative Lod scores of total -4.18 ($\theta = 0,00001$) and -2.69 ($\theta = 0,00001$).

Several *PALB2* mutations have been identified and one of them has been found as a recurrent mutation, 1592delT in Finland [6]. In light of the possible common ancient origin of the *BRCA2* 999del5 mutation in the Finnish and the Icelandic population we decided to screen for the

Finnish *PALB2* founder mutation, 1592delT, in Icelandic breast cancer cases. The mutation was not detected in 638 unselected breast cancer cases nor in any of the 111 family members of the nine high risk breast cancer families.

The results of this study suggest that *PALB2* is not a significant contributor to breast cancer in high-risk breast cancer families in Iceland. Furthermore, the results show that the 1592delT mutation appears not to be associated with breast cancer in the Icelandic population, and if occurring it would be very rare. However the results can not exclude the possibility that other *PALB2* mutations causing low or moderate breast cancer risk exist in the Icelandic population. To determine that, it would be necessary to perform a *PALB2* mutation screening or SNP analysis in a large cohort of breast cancer cases.

Methods

Study population

The sample set consisted of: 111 individuals from nine high-risk non-*BRCA1/BRCA2* breast cancer families (Table 1), 38 controls to evaluate allele frequencies in the linkage analysis, and 638 unselected breast cancer cases diagnosed in the period 1987–2003. All patients contributing to the research have given their informed consent. The research has been approved by the Icelandic Data Protection Authority and the National Bioethics Committee.

Laboratory Analysis

Blood samples were lyzed and DNA extracted from nuclei according to Miller et al [17]. DNA from paraffin-embedded tissue was extracted as described by Jönsson et al [18] and from fresh-frozen tissue using the Wizard Genomic DNA Purification Kit (Promega).

Table 1: Summary of the main clinical characteristics of the 9 high risk breast cancer families

Family	Number of affected individuals	Number of typed individuals (affected)	Mean age at first diagnosis years (range)
1	5	6(4)	61.8(44-76)
2	5	11(5)	49.4 (44–64)
3*	10	14 (10)	57.4 (50–88)
4*	9	17 (8)	54.2 (38–75)
5*	11	20 (9)	49.7 (29–70)
6	6	11 (5)	57.5 (35–79)
7	8	8 (6)	61.1 (42–79)
8	5	11 (5)	49.6 (30–66)
9	9	13 (9)	51.8 (30–77)
Total	68	111 (61)	54.6 (29–88)

*Co-occurrence of ovarian cancer in the family (one family member diagnosed).

To evaluate linkage in the families the following microsatellite markers were used: D16S3036, D16S412, D16S420 and D16S3068 (primer sequences are available in the UCSC Genome Browser, genome assembly March 2006) [19]. Primers to screen for the 1592delT mutation were as in Erkkö et al [6]. All the primers were purchased from MWG Biotech and each forward primer was 5' labeled with either FAM or HEX fluorophore reporter. The PCR conditions for all primer pairs were: 3 minutes incubation at 94°C, followed by 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 45 seconds and a final extension at 72°C for 10 minutes, except for the 1592delT primers for which the annealing temperature was 64°C instead of 55°C. The PCR products were analysed using an automated ABI PRISM 3130 × 1 Genetic Analyzer, alleles were called automatically using the GeneMapper software v4.0 and checked manually.

Statistical analysis

The Genetic Analysis System software (GAS) was used to check the genotyping data for Mendelian inconsistencies within the families. Genotypes which were inconsistent with Mendelian inheritance were reviewed. Any ambiguous genotypes were dropped. Allele frequencies for the microsatellite markers were calculated using founder individuals from the families as well as the control individuals using the Gconvert program [20]. Evidence for linkage was evaluated using parametric linkage analysis methods. Two-point LOD scores were calculated using the FASTLINK program, assuming a rare (frequency = 0.0033) dominantly inherited disease allele. Age-dependant liability classes were defined using the modified CASH model [21,22].

Abbreviations

PALB2: Partner and localizer of *BRCA*; *BRCA1*: Breast cancer susceptibility gene 1; *BRCA2*: Breast cancer susceptibility gene 2; *CHEK2*: *CHK2* checkpoint homolog (S. pombe); *ATM*: Ataxia telangiectasia mutated.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HG conducted the genotyping, wrote the manuscript and participated in the statistical analysis and design of the study. AA did the pedigree analysis, participated in the design of the study and drafting of the manuscript. EMG performed the statistical analysis, participated in the pedigree analysis and in the design of the study. BAA provided biological samples and pathological information. GJ participated in handling of the DNA samples. OTH participated in the recruiting of the cases. RBB initiated the study and coordinated it, and participated in the design and

drafting of the manuscript. All authors read and approved the manuscript.

Additional material

Additional file 1

Two-point Lod scores for microsatellite markers surrounding the *PALB2* gene* at recombination fractions (θ) 0.00001, 0.01 and 0.05 (gender averaged).

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Additional file 1

Additional file 1: Two-point Lod scores for microsatellite markers surrounding the PALB2 gene* at recombination fractions (θ) 0.00001, 0.01 and 0.05 (gender averaged)

Marker	D16S3036			D16S412			D16S420			D16S3068		
	0.00001	0.01	0.05	0.00001	0.01	0.05	0.00001	0.01	0.05	0.00001	0.01	0.05
Family												
1	-0.001	-0.001	-0.001	-0.001	-0.001	-0.001	-0.002	-0.002	-0.002	-0.001	-0.001	-0.001
2	-0.287	-0.256	-0.161	0.259	0.251	0.220	-0.290	-0.260	-0.167	-0.252	-0.223	-0.138
3	-0.148	-0.144	-0.128	-0.042	-0.041	-0.035	0.111	-0.107	-0.090	-0.212	-0.204	-0.174
4	-0.464	-0.377	-0.129	-1.200	-1.084	-0.742	-1.008	-0.911	-0.615	0.405	0.406	0.395
5	-1.094	-0.765	-0.325	-0.491	-0.475	-0.411	-1.835	-1.537	-1.020	-1.072	-0.927	-0.571
6	-0.032	-0.029	-0.020	-0.003	-0.002	-0.002	-0.024	-0.023	-0.018	0.184	0.173	0.132
7	-0.038	-0.036	-0.028	-0.025	-0.023	-0.017	-0.035	-0.032	-0.024	0.068	0.064	0.049
8	-0.247	-0.222	-0.131	0.099	0.096	0.081	-0.242	-0.228	-0.170	0.179	0.171	0.141
9	0.852	0.822	0.703	-1.283	-1.063	-0.632	-0.634	-0.434	-0.113	-0.136	-0.085	0.029
SUM	-1.460	-1.008	-0.221	-2.686	-2.344	-1.540	-4.182	-3.533	-2.217	-0.837	-0.627	-0.138

*The PALB2 gene is located between D15S412 and D16S420, the distance being 0.45Mb and 0.58Mb respectively. The distance from the PALB2 gene to the D16S3036 and D16S3068 markers is 4.1 Mb and 1.9 Mb respectively.