GENETIC EPIDEMIOLOGY OF BREAST CANCER IN CYPRUS:
A CASE -CONTROL STUDY OF DNA REPAIR GENES

A thesis submitted for the degree of
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by

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ABSTRACT

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The occurrence of early-onset breast cancer (EOBC) has been associated with germline mutations in the *BRCA1* and *BRCA2* genes. The first aim of this thesis was to evaluate the frequency and distribution of mutations in these genes, in a group of Cypriot women diagnosed with EOBC. Pathogenic mutations were identified in 6 of the 26 unrelated patients. This study supports a strong correlation between the early onset breast cancer phenotype and the presence of pathogenic *BRCA1/2* mutations. It is of interest that pathogenic mutations were detected in patients without a family history of the disease. Based on these results, we recommend that *BRCA1/2* screening should be offered to patients with a diagnosis of EOBC irrespective of their family history.

The known breast cancer susceptibility genes explain only about 5% of breast cancer cases. Thus, it is likely that other breast cancer susceptibility genes exist. The second aim of the present thesis was to assess whether alterations in DNA repair genes modify breast cancer risk in the Cypriot population. Towards this objective, blood samples were collected and genomic DNA isolated from 1109 Cypriot female breast cancer patients diagnosed between 40-70 years old, and from 1177 age-matched healthy female controls. A total of 79 single nucleotide polymorphisms (SNPs) were genotyped in all samples. Significant associations with breast cancer risk were observed for eight of the SNPs studied. Five SNPs in the *BRCA2*, *MRE11A*, *MUS81*, *PBOV1* and *XRCC1* genes, were associated with an increased risk for breast cancer, while two SNPs in the *NBS1* gene and one SNP in the *MRE11A* gene appeared to be associated with reduced risk for the disease. The data from this study support the hypothesis that genetic variants in DNA repair genes influence breast cancer risk and provides further evidence for the existence of a polygenic model for breast cancer.

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Abbreviations / Glossary

A	adenine
AP site	apurinic and apyrimidinic site
ATF1	activating transcription factor 1
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
BAP1	*BRCA1* associated protein-1
BARD1	*BRCA1* associated RING domain 1
BASC complex	*BRCA1*-associated genome surveillance complex
BER	base excision repair
BIC	breast cancer information core
BLM	Bloom syndrome, RecQ helicase-like
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
BRCC complex	*BRCA1-BRCA2* containing complex
BRCT	*BRCA1* c-terminal
BRIP	*BRCA1* interacting protein C-terminal helicase 1
C	cytosine
C terminal	carboxy terminal
CASP8	caspase 8, apoptosis-related cysteine peptidase
CDH1	cadherin 1, type 1, E-cadherin (epithelial)
CHEK1	*CHK1* checkpoint homolog (S. pombe)
CHEK2	*CHK2* checkpoint homolog (S. pombe)
CI	confidence intervals
CtIP	carboxy-terminal-binding-protein-interacting protein
dATP	deoxyadenosine triphosphate
DBD	DNA-binding domain
DCIS	ductal carcinoma in situ
dCTP	deoxycytosine triphosphate
DDB complex	DNA damage-binding complex
DDB2	damage-specific DNA binding protein 2
dGTP	deoxyguanine triphosphate

DMC1	*DMC1* dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)
DNA	deoxyribonucleic acid
DNA-PKcs	deoxyribonucleic acid dependent protein kinase
dNTPs	deoxyribonucleotide triphosphates
DSB	double strand break
DSS1	deleted in split-hand/split-foot 1
dTTP	deoxythymine triphosphate
EME1	essential meiotic endonuclease 1 homolog 1 (S. pombe)
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2
FANCA	Fanconi anemia, complementation group A
FANCC	Fanconi anemia, complementation group C
FANCD1	Fanconi Anemia complementation group D1
FANCG	Fanconi Anemia complementation group G
FEN1	flap structure-specific endonuclease 1
FGFR2	fibroblast growth factor receptor 2
G	guanine
GADD45	growth arrest and DNA-damage-inducible
GGR	global genomic repair
GWAS	genome-wide association study
HPLC	high performance liquid chromatography
HR	homologous recombination
HRT	hormone replacement therapy
HWE	Hardy-Weinberg equilibrium
IGF-1	insulin like growth factor
IR	ionizing radiation
Kb	kilobase
kDa	kilodalton
KU70	Ku autoantigen, 70kDa
KU80	Ku autoantigen, 80kDa
LD	linkage disequilibrium

LOH	loss of heterozygosity
LSP1	lymphocyte-specific protein 1
MAF	minor allele frequency
MALDI-TOF MS	matrix assisted laser desorption / ionization-time-of-flight mass spectrometry
MAP3K1	mitogen-activated protein kinase 1
MGB	minor groove binder
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
MLPA	multiplex ligation-dependent probe amplification
MRE11A	meiotic recombination 11 homolog A (S. cerevisiae)
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
MSH6	mutS homolog 6 (E. coli)
MRN complex	*MRE11-RAD50-NBS1* complex
mRNA	messenger ribonucleic acid
MUS81	*MUS81* endonuclease homolog (S. cerevisiae)
N terminal	amino terminal
NBS1	Nijmegen breakage syndrome 1 (nibrin)
NER	nucleotide excision repair
NHEJ	non homologous end joining
NCBI	National Center for Biotechnology Information
NIEHS	National Institute of Environmental Health Sciences
NLS	nuclear localization signal
OGG1	8-oxoguanine DNA glycosylase
OR	odds ratio
PALB2	partner and localizer of *BRCA2*
PARP	poly (ADP-ribose) polymerase
PBOV1	prostate and breast cancer overexpressed 1
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PTEN	phosphatase and tensin homolog
RAD23B	*RAD23* homolog B (S. cerevisiae)
RAD50	*RAD50* homolog (S. cerevisiae)
RAD51	*RAD51* homolog (RecA homolog, E. coli) (S. cerevisiae)
RAD51C	*RAD51* homolog C (S. cerevisiae)

RAD51L1	*RAD51*-like 1 (S. cerevisiae)
RAD52	*RAD52* homolog (S. cerevisiae)
RAP80	receptor associated protein 80
RB	retinoblastoma
RFC	replication factor C
RFC1	replication factor C (activator 1) 1, 145kDa
RFLP	restriction fragment length polymorphism
RING	really interesting new gene
RNA	ribonucleic acid
SCD	SQ cluster domain
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
ssDNA	single-stranded DNA
STK11	serine/threonine kinase 11
T	thymine
TCR	transcription coupled repair
TNRC9	trinucleotide repeat containing 9
TP53	tumor protein p53
UV	ultraviolet
XLF	*XRCC4*-like factor
XPB	xeroderma pigmentosum, complementation group B
XPC	xeroderma pigmentosum, complementation group C
XPD	xeroderma pigmentosum, complementation group D
XPG	xeroderma pigmentosum, complementation group G
XRCC	X-Ray cross-complementing
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3

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Summary

The aims of this study/project were two fold: (a) to carry out a mutational analysis of the *BRCA1* and *BRCA2* genes in Cypriot women diagnosed with early onset breast cancer (before the age of 40), who are unselected for a family history of this disease, and (b) to examine the association between common single nucleotide polymorphisms (SNPs) in DNA repair genes and breast cancer risk, in the Cypriot population.

The thesis is divided into three parts, which comprise five chapters in all as outlined below. **Part I** provides a literature review on breast cancer incidence, mortality and epidemiology as well as the high-penetrance breast cancer genes, *BRCA1* and *BRCA2* (**Chapter 1**). This is followed by background information on lower penetrance breast cancer susceptibility alleles, the methods that are used to identify them, as well as a review of DNA repair mechanisms and the role that genes involved in these mechanisms play in breast cancer susceptibility **(Chapter 2)**.

Part II describes the experimental work performed and the two principal studies involved in the project; these comprise Chapters 3 and 4. **Chapter 3** presents the first study, in which the contribution of *BRCA1* and *BRCA2* germline mutations to the incidence of early-onset breast cancer in Cyprus was investigated. For this study, twenty-six consecutive incident female breast cancer cases diagnosed before the age of 40, between the years 2003 and 2004, were recruited. The entire coding regions, including splice sites, of the *BRCA1* and *BRCA2* genes were sequenced using cycle sequencing. Four pathogenic mutations: two in *BRCA1* and two in the *BRCA2* gene, which confer high risks of breast and ovarian cancer, were identified in six of the twenty-six unrelated patients investigated. The *BRCA2* Cypriot founder mutation c.8755delG (8984delG) was detected in three unrelated patients. Of the six *BRCA1/2* mutation carriers, only four had a family history. This was the first study evaluating the frequency and distribution of mutations in these genes in Cypriot women with early onset breast cancer who are unselected for a family history of the disease. The results of this study show that the prevalence of *BRCA1* and *BRCA2* mutations in this group of women is high. Hence, Cypriot women with early-onset breast cancer should be offered *BRCA1/2* genetic testing, irrespective of their family history. Future clinical management of these early onset breast cancer patients will depend

on the result of this genetic testing. Furthermore, genetic counselling can help women at risk to make informed decisions about possible prevention strategies they may wish to pursue.

The primary aim of the second study described in **Chapter 4,** was to investigate the association between SNPs in DNA repair genes and breast cancer risk in the Cypriot population. Towards this effort, blood samples were collected and genomic DNA was isolated from 1109 Cypriot female breast cancer patients diagnosed between 40-70 years old and 1177 age-matched healthy female controls. A total of 79 SNPs were genotyped using three methods: PCR followed by restriction fragment length polymorphism analysis, real-time PCR with Taqman SNP genotyping assays, and SNP genotyping using the Sequenom MassARRAY iPLEX Gold assays and matrix assisted laser desorption / ionization-Time-of-Flight mass spectrometry (MALDI-TOF MS) for detection. Significant associations with breast cancer risk were observed for eight of the SNPs studied. Five SNPs in the *BRCA2*, *MRE11A*, *MUS81*, *PBOV1* and *XRCC1* genes were associated with an increased risk for breast cancer, while two SNPs in the *NBS1* gene and a SNP in the *MRE11A* gene appeared to be associated with reduced risk for the disease. This study provides support for the hypothesis that genetic variants in DNA repair genes influence breast cancer risk and provides further evidence for the current hypothesis that favours a polygenic model of breast cancer.

In **Part III**, the results of the two studies are discussed (**Chapter 5**). In addition, directions for future research are presented, including the elucidation of the functional impact of the breast cancer associated SNPs, the expansion of the SNP study as well as the initiation of a genome-wide association study in the Cypriot population.

Part I

Literature Review

Chapter 1

Introduction to breast cancer and the *BRCA1* and *BRCA2* genes

1.1 Breast Cancer

Breast cancer is a major public health burden throughout the world. It is by far the most common type of cancer in women, comprising 23% of all female cancers. In year 2002, there were an estimated 1.15 million new cases of breast cancer and 411,000 deaths resulting from this disease (Parkin et al., 2005). Despite the common occurrence, the exact aetiology of breast cancer is still unknown. It is believed that breast cancer is a multifactorial disease and it is a result of the interaction of genetic and environmental factors (Ponder, 2001). Over the past decade, significant progress has been made in defining risk factors determining susceptibility of individuals to developing breast cancer as well as the genetic factors that contribute to this risk. Despite this improved knowledge, the unravelling of the complex genetic and environmental influences on this disease is still at an early stage. An even better understanding of the genetic mechanisms underlying the development and progression of breast cancer would be a major advance for improved prevention, detection and treatment strategies.

1.2 Incidence and mortality

According to the latest cancer incidence and mortality estimates of the International Agency for Research on Cancer (IARC), available in the GLOBOCAN series, breast cancer is the second most frequent cancer in the world with 1.15 million cases in 2002, and the most common malignancy in women, accounting for 23% of all new cases (Parkin et al., 2005).

As described by Parkin (2004), incidence rates of breast cancer vary between different parts of the world with a significant difference observed between the low-risk areas, which include the Far East and Africa, and the high-risk areas, mainly North America and Western Europe (Figure 1). The highest age-standardized incidence rates are in North America with 99.4 per 100,000 and the lowest are in Central Africa with 16.5 per 100,000. Together, the USA and Europe roughly account for 16% of the worldwide population and 60% of the worldwide incidence of breast cancer. Incidence rates of breast cancer are increasing in most countries, and the changes are usually greater where rates were previously low. Overall, there is an annual worldwide increase of breast cancer incidence rates of about 0.5%. However, cancer registries in Eastern Asia are recording annual increases in

incidence of up to 3% (China). Assuming a 3% growth in East Asia, there would be around 1.5 million new breast cancer cases in 2010 (Parkin, 2004; Parkin et al., 2005).

Breast cancer death rates have been decreasing steadily because of earlier detection and more effective treatments. Worldwide figures show that the ratio of mortality to incidence is about 0.35 and despite breast cancer being the second most common cancer overall, it ranks fifth as a cause of death (Parkin et al., 2005).

In Cyprus, breast cancer is the most frequent type of cancer in women, with approximately 400 new cases diagnosed every year; about 20–30 of these occur in patients younger than 40 years of age (Bank of Cyprus Oncology Centre Database). Based on the Middle East Cancer Consortium (MECC) Monograph (Freedman et al. 2006), the age standardized incidence rate for the years 1998-2001 was 57.7 per 100,000. Although breast cancer incidence rates are increasing annually, mortality rates are stable, representing an improved survival rate. This improvement can be attributed to effective means of early detection, mainly mammography, as well as to significant improvement in treatment options with the availability of efficient hormonal and chemotherapeutic adjuvant and neoadjuvant therapy along with radiotherapy.

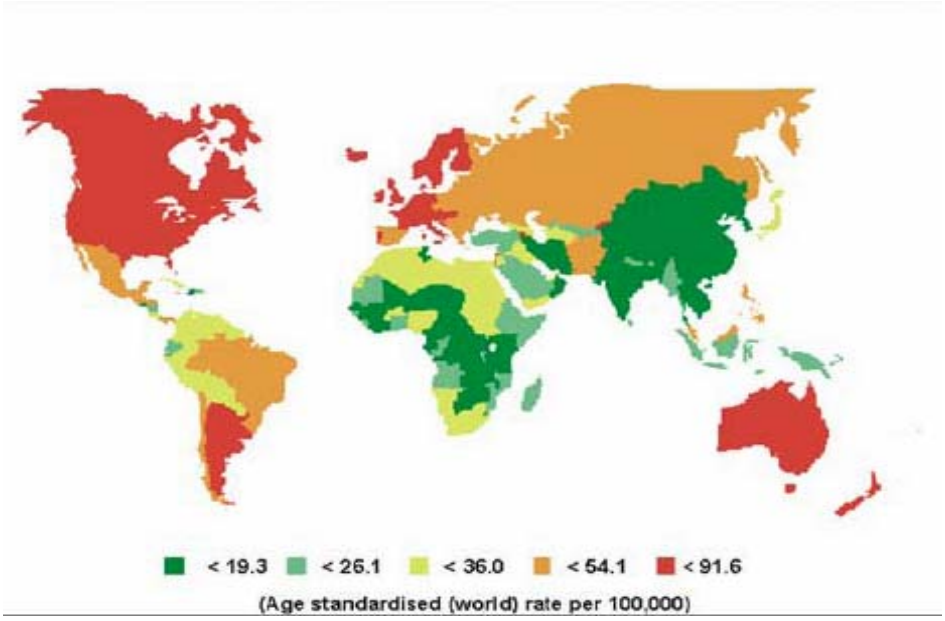


Figure 1: Incidence of female breast cancer worldwide (per 100,000; all ages) (Taken from Parkin, 2004)

1.3 Breast morphology

Breast development begins about 7-8 weeks after conception and at the later stages of gestation the nipples, areola and ducts are formed. Complete morphologic maturation of the breast tissue only occurs following pregnancy.

In brief, as illustrated in Figure 2, the mammary gland consists of around twenty lobes, each of which has a branching duct system ending in terminal ducts. At the beginning of menarche, and with the influence of estrogen and progesterone, lobules are formed which replace pre-existing terminal ducts. Lobules are clusters of epithelial cells which radiate from the nipple and terminate in dozens of tiny bulbs that can produce milk (alveoli). They are bound together by fairly dense connective tissue septa. As a result of the replacement of terminal ducts by lobules, there is an increase in the volume and elasticity of the connective tissue as well as in the vascularity. The contour of the breast is filled out by fat tissue (Boron and Boulpaep, 2003).

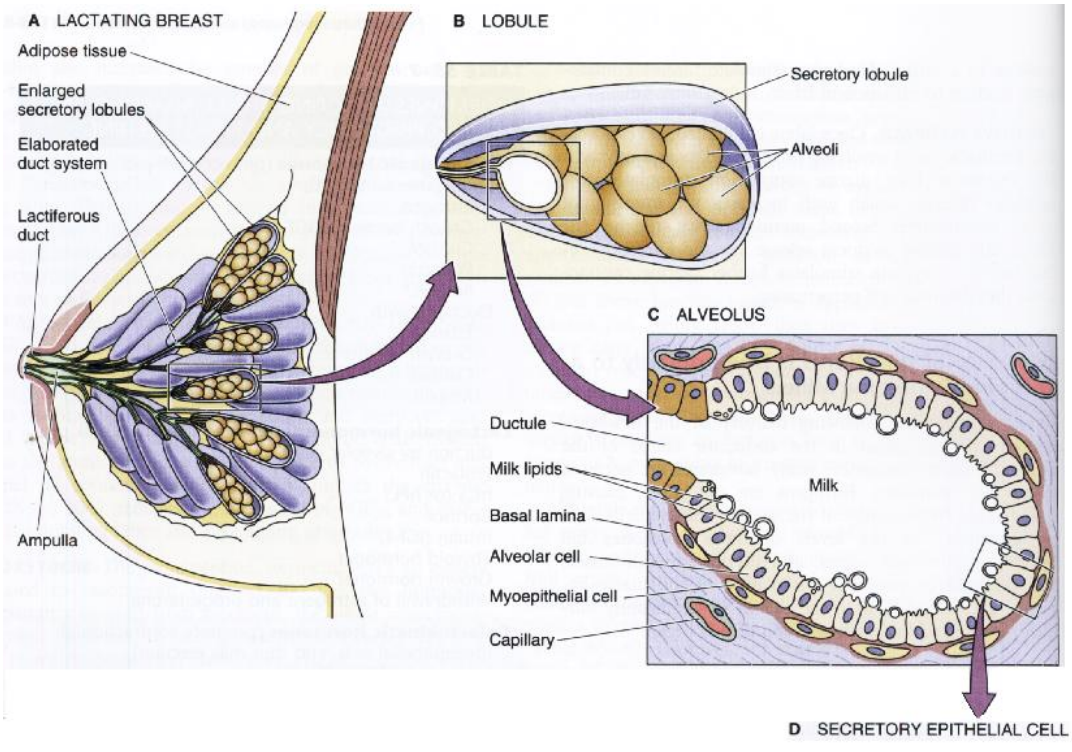


Figure 2: Architecture of the human breast

A. Schematic representation of the branching duct-to-lobule structure of the breast during lactation. B. Each breast lobule contains the alveoli, where the milk-secreting cells are located. C. The epidermal cells lining the alveoli secrete milk. Taken from (Boron and Boulpaep, 2003)

1.4 Breast carcinoma

Breast carcinoma is a neoplastic condition that affects the breast tissue. In the past, it was believed that breast cancer is a result of a well-defined sequence of histological changes in the mammary epithelium. It was suggested that breast carcinogenesis is a multi-step process which starts with hyperplasia, progressing through atypical hyperplasia to in situ carcinoma and finally to invasive malignant carcinoma (Vogelstein and Kinzler 1998). The advancement of molecular biology and genetics has led to an abundance of data in relation to breast carcinogenesis. As a result of the new knowledge generated, it is nowadays believed that breast cancer is a complex, multi-factorial, polygenic and multi-step process (Beckmann et al., 1997; Ponder, 2001; Antoniou and Easton, 2003).

The most common breast cancers have an epithelial origin and are divided into two main categories: in situ carcinomas and invasive carcinomas, which account for 13-30% and 70-85% of all cases respectively (Tavassoli and Devilee, 2003; Kumar et al., 2005). The majority of in situ (non-invasive) breast carcinomas are ductal carcinomas in situ (DCIS). The number of DCIS cases has increased significantly in the past two decades, because of the application of mammographic screening. It is noted that almost half of the mammographically detected cancers are DCIS. Invasive carcinomas are the most common breast carcinomas. The majority of invasive carcinomas (79%) are ductal carcinomas (Tavassoli and Devilee, 2003; Kumar et al., 2005).

1.5 Epidemiology and risk factors

The epidemiology of breast cancer has been more extensively studied, than any other human disease. To date, several risk factors have been identified and table 1 summarizes factors that increase breast cancer risk, as well as protective factors.

Breast cancer incidence correlates with ethnic origin and shows age specific patterns. A substantial variation in breast cancer incidence and mortality is observed between different countries, with the difference between the Far East and Western countries being fivefold. Studies on breast cancer rates of immigrants, moving from low to high-risk countries, have shown that women assume the rate of their host countries within one or two generations, indicating that environmental factors are of greater

importance than genetic factors (McPherson et al., 2000). Breast cancer incidence increases with age and doubles almost every ten years until menopause, when the rate of increase slows dramatically, suggesting an important role of reproductive hormones in breast cancer aetiology (Collaborative Group on Hormonal Factors in Breast Cancer, 1996).

One of the most established risk factors for breast cancer is lifetime exposure to hormones, both endogenous hormones that are related to menstrual cycle, as well as exogenous hormones which are derived from contraceptives, hormone replacement therapy (HRT) and diet (Dumitrescu and Cotarla, 2005). The hormone(s) that are responsible for breast cancer initiation remain unidentified as yet, but it is believed that estrogen plays a major role in modifying breast cancer risk. Two mechanisms have been proposed which correlate estrogens with breast carcinogenesis. The first mechanism suggests that estrogen metabolites, and in particular, the hydroxylated (catechol) estrogens, are genotoxic through induction of oxidative DNA damage, and long periods of exposure increase breast cancer risk (Cavalieri et al., 2000). The second mechanism is the receptor-mediated hormonal activity, which is generally related to stimulation of cellular proliferation, resulting in more opportunities for accumulation of genetic damage leading to carcinogenesis (Russo et al., 2000).

Women with early age at menarche or late menopause have an increased risk of developing breast cancer. A woman who began menstruating when she was younger than 12 years old has a 10-20% increased risk of breast cancer compared to one whose menstruation started when she was older than 14 years of age (Berkey et al., 1999; Kelsey and Horn-Ross, 1993). In addition, women who experience a delayed natural menopause (after the age of 55) are twice as likely to develop breast cancer compared to women who experience menopause before the age of 45 (McPherson et al., 2000). The protective effect of menopause is also seen in women who undergo bilateral prophylactic oophorectomy (McPherson et al., 2000). There is a link between usage of exogenous hormones such as HRT and oral contraceptives and breast cancer. Women who take HRT, mainly estrogen combined with progestin, are more likely to develop breast cancer after four to five years of therapy compared to women who never used HRT (Rossouw et al., 2002). Some studies have shown an increased risk of breast cancer in women taking oral contraceptives. A small increase

in the relative risk of breast cancer has been reported among women taking oral contraceptives and for a period of 10 years after stopping them. However, after this period of 10 years, a woman's risk of developing breast cancer returns to the same level as if she had never used oral contraceptives. Since most women take birth control at a young age, when breast cancer is rare, the increase in risk is thought to have little effect on overall incidence rates (Collaborative Group on Hormonal Factors in Breast Cancer, 1996).

Pregnancy seems to have a dual effect on breast cancer risk. On the one hand, breast cancer risk is temporarily increased for a few years after a woman gives birth and on the other hand, this excess risk is reduced gradually and in the longer term, the effect of a birth is protective against the disease (Beral and Reeves, 1993). Furthermore, an early age of full-term pregnancy is protective for breast cancer, irrespective of the total number of pregnancies. In more detail, the younger a woman is when she has her first child, the lower her risk of developing breast cancer during her lifetime. A woman who gives birth to her first child after the age of 30, has approximately twice the risk of developing breast cancer, compared to a woman who has a child before the age of 20 (McPherson et al., 2000). Research also suggests that protection against breast cancer increases with the number of full term pregnancies; women with five or more children have about half the risk of nulliparous women (Ewertz et al., 1990; Layde et al., 1989). After pregnancy, breastfeeding for a long period of time (a year or longer) further reduces breast cancer risk by a small amount (Collaborative Group on Hormonal Factors in Breast Cancer, 2002).

Breast density has also been related to breast cancer risk. Studies have shown that increased breast cancer density is associated with increased breast cancer risk in both premenopausal and postmenopausal women. Women with more than 75% increased breast density on mammography have an approximately five times increased risk of developing breast carcinoma compared to women with less than 5% increased breast density (Byrne et al., 1995; Boyd et al., 1995). In addition, there is evidence that nulliparity and high breast density act synergistically and breast cancer risk increases to sevenfold, in women who have never had a child and have dense breasts (van Gils et al., 2000). Studies have also indicated that there is a relationship between the presence of benign breast disease and breast cancer risk. Women diagnosed with

atypical hyperplasia, have a fourfold to fivefold increase in breast cancer risk. (McPherson et al., 2000).

Obesity is another known risk factor for breast cancer. Obese women have an increased risk for postmenopausal but not premenopausal breast cancer. The association between obesity and breast cancer risk has been proposed to be largely due to increased estrogenic activity in overweight women. Increased body weight results in elevated circulating estrogens from peripheral aromatization of androgens in adipose tissue. Breasts, which are estrogen-sensitive tissues, are therefore exposed to more estrogen stimulation in obese women, leading to an increased risk for breast cancer. A second possible mechanism is that obesity, which is associated with metabolic syndrome, increases the levels of circulating insulin and insulin like growth factor (*IGF*–1) (Lorincz and Sukumar, 2006). Insulin and *IGF1* have been implicated in breast tumorigenesis because of their ability to stimulate mitogenesis, and their key role in mammary gland cell proliferation and survival (Imagawa et al., 2002; Deming et al., 2007).

It has also been demonstrated that moderate physical activity in adolescence and young adulthood reduces the risk of breast cancer. This may be due to the fact that exercise can delay the age of menarche and modify the bio-available hormone levels (Monninkhof et al., 2007).

Numerous epidemiological studies investigated the relationship between lifestyle factors such as diet, alcohol consumption and smoking and breast cancer risk. Data regarding the role of soy remains conflicting. A recent meta-analysis suggests that soy food intake in the amount consumed in Asian populations may have protective effects against breast cancer (Wu et al., 2008). In addition, high fat diet seems to be weakly associated with breast cancer risk, whereas a diet rich in fruit and vegetables, that are good sources of natural antioxidants, seems to protect women from breast cancer (DeBruin and Josephy, 2002; Gerber et al., 2003).

Contradictory results have been reported regarding cigarette smoking and breast cancer risk. Most studies found no or little association, between smoking and breast cancer risk (Terry and Rohan, 2002). However, more recent studies suggest that

smoking at young ages and particularly during the period between menarche and first childbirth may increase breast cancer risk. This sensitivity of the female breast to tobacco carcinogens decreases after the first childbirth when the breast tissue is terminally differentiated (Ha et al., 2007). The results of most studies indicate that there is a link between regular alcohol consumption and breast cancer risk (Singletary and Gapstur, 2001).

Radiation exposure is another well-established risk factor for breast cancer. The best evidence that associates radiation with increased breast cancer risk comes from survivors of atomic bomb explosions in Japan, who have been shown to have a significant increase in the incidence of breast cancer compared to unexposed persons (Land et al., 2003). Furthermore, a significant increased risk for breast cancer is seen in patients with Hodgkin's disease who have been treated with radiation therapy (Travis et al., 2003).

Family history of breast cancer is one of the most important and well-established risk factors for breast cancer. The first reports documenting family history of breast cancer were written by two French surgeons, Le Dran in 1757 and Broca in 1866 (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Eisinger et al., 1998). Since then, many studies have addressed this issue and concluded that a woman's risk is approximately doubled if she has a first-degree relative with breast cancer. The risk is elevated significantly by increasing number of affected relatives and is greater in women with relatives affected at a young age (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Thompson and Easton, 2004).

Table 1: Summary table of factors that influence breast cancer risk (modified from Dumitrescu and Cotarla 2005)

FACTORS THAT MODIFY BREAST CANCER RISK		MAGNITUDE OF RISK
Factors that increase breast cancer risk		
Well-confirmed factors	Increasing age	++
	Geographical region (USA & Western countries)	++
	Family history of breast cancer	++
	Mutations in *BRCA1* and *BRCA2* genes	++
	Mutations in other high-penetrance genes	++
	Ionizing radiation exposure (in childhood)	++
	History of benign breast disease	++
	Late age of menopause (>54)	++
	Early age of menarche (<12)	++
	Nulliparity and older age at first birth	++
	High mammographic breast density	++
	Hormonal replacement therapy	+
	Oral contraceptives recent use	+
	Obesity in postmenopausal women	+
	Tall stature	+
	Alcohol consumption (~1 drink/day)	+
	High insulin-like growth factor (IGF-I) levels	++
	High prolactin levels	+
Probable factors	High-saturated fat and well-done meat intake	+
	Polymorphisms in low-penetrance genes	+
	High socioeconomic status	+
Factors that decrease breast cancer risk		
Well-confirmed factors	Geographical region (Asia and Africa)	--
	Early age of full-term pregnancy	--
	Higher parity	--
	Breast feeding (longer duration)	--
	Obesity in premenopausal women	-
	Fruit and vegetables consumption	-
	Physical activity	-
	Chemopreventive agents	-
Probable factors	Non-steroidal anti-inflammatory drugs	-
	Polymorphisms in low-penetrance genes	-

++ (moderate to high increase in risk) - odds ratios/relative risk: 1.5 - >3.0 (1.5 to greater than 3 times risk of disease)

+ (low to moderate increase in risk) - odds ratios/relative risk: 1.1 - <1.5 (10% to 1.5 times greater risk of disease)

-- (moderate to high decrease in risk) - odds ratios/relative risk: <0.4 (greater than 60% reduced risk of disease)

- (low to moderate decrease in risk) - odds ratios/relative risk: 0.4 - <0.9 (10% to 60% reduced risk of disease)

1.6 Inherited predisposition to cancer

It is well established that most genetic alterations leading to cancer are somatic. However, a number of cancers (around 5-10%) are due to inherited cancer predisposition syndromes and arise from inherited germline mutations in a cancer-susceptibility gene (Garber and Offit, 2005). Inherited cancer predisposition syndromes are characterized by multiple affected family members usually at an early age of cancer onset, multiple primary cancers and for some rare syndromes, congenital abnormalities (Fearon, 1997). To date, over 200 hereditary cancer susceptibility syndromes have been described, the majority of which are inherited in an autosomal dominant manner (Nagy et al., 2004). The lifetime risk of cancer for individuals carrying a mutation in a cancer predisposition gene is high and ranges between 50% and 80% (Ponder, 2001). The likelihood of developing cancer depends on the actual gene and the mutant allele as well as on other modifying risk factors, both genetic and non-genetic. Furthermore, it also depends on the complex gene-environment interactions which are currently under intense investigation, but at the moment remain poorly understood (Ponder, 2001).

Recently, a number of genome-wide association studies (GWAS) have identified common SNP-based variants conferring low to moderate risk for cancer. These findings have brought us a step closer to a polygenic model for cancer. However, these small effects of multiple genes only explain a small proportion of the observed familial clustering for cancer and an extended analysis with a more complete range of potential susceptibility variants is needed (McCarthy et al., 2008).

1.7 Inherited predisposition to breast carcinoma

Family history of breast cancer constitutes one of the most important risk factors for the disease. The Ancient Romans were the first who observed the presence of familial clustering of breast cancer (Steel et al., 1991) but, as already mentioned, it was not until the late 18th and mid 19th century, that the first formal reports were produced by two French surgeons, Le Dran and Broca, who noted an association between family history of breast cancer and disease (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Eisinger et al., 1998).

In the years that followed, epidemiological studies provided strong evidence that women who have breast cancer patients as first-degree relatives, have an elevated risk for the disease. This observation led to the suggestion, that there are genetic factors which are associated with breast cancer. In addition to epidemiological evidence, the existence of families prone to breast cancer that displayed multiple affected members with an early age of onset, stood as proof of the existence of genetic susceptibility to the disease (Thompson and Easton, 2004).

The first evidence in favour of the existence of an autosomal dominant breast cancer susceptibility gene came in 1984, from a segregation analysis study of families with multiple cases of breast cancer (Williams and Anderson, 1984). Two additional studies which followed a few years later provided further support for this (Claus et al., 1991; Newman et al., 1988).

In 1990, Mary-Claire King and her group studied a large group of multiple-case early-onset families and by linkage analysis assigned, a chromosomal region (17q21) which appeared to be the locale of a gene responsible, for inherited susceptibility to breast cancer in families (Hall et al., 1990). Three years later, the first breast-ovarian cancer-susceptibility gene *BRCA1* (breast cancer 1) was identified by positional cloning (Miki et al., 1994). During the same year, the second breast cancer-susceptibility locus, *BRCA2* (breast cancer 2), was localized to chromosome 13q12–q13 by linkage studies of families with multiple cases of early-onset breast cancer that were not linked to *BRCA1* (Wooster et al., 1994). The *BRCA2* gene was cloned in 1995 (Wooster et al., 1995) and its complete coding sequence and exonic structure were described in 1996 (Tavtigian et al., 1996).

BRCA1 and BRCA2 are the most important breast cancer susceptibility genes in the context of large multiple case families, segregating both early onset breast cancer cases and ovarian cancer cases. It is estimated that mutations in these genes explain approximately 40% of familial breast cancer and account for 5% to 20% of the total breast and ovarian cancers respectively (Chen et al., 2006). Other more conservative estimates indicate that mutations in the high-risk breast cancer genes explain around 20-25% of the overall excess familial risk and less than 5% of total breast cancer incidence (Oldenburg et al., 2007).

There are also a number of other inherited cancer predisposition syndromes which include breast cancer in their clinical presentation. These are Li-Fraumeni syndrome [*TP53* (tumor protein p53) mutations], Cowden disease [*PTEN* (phosphatase and tensin homolog) mutations], Peutz-Jeghers syndrome [*STK11/LKB1* (serine/threonine kinase 11) mutations] and hereditary diffuse gastric cancer syndrome [*CDH1* (cadherin 1) mutations]. Overall, hereditary breast cancer accounts for 5-10% of all cases but less than 25% of hereditary cases can be explained by germline mutations in the currently identified breast cancer susceptibility genes (Bradbury and Olopade, 2007; Stratton and Rahman, 2008).

Recently, genome-wide linkage analyses were carried out using a large number of families with multiple cases of breast cancer which were not carrying mutations in the *BRCA1* or *BRCA2* genes. This approach has not mapped any additional breast cancer susceptibility loci (Smith et al., 2006). However, this does not entirely exclude the possibility of the existence of additional high-penetrance breast cancer susceptibility genes but it strongly suggests that if they exist, they account for only a small proportion of the excess familial risk (Stratton and Rahman, 2008).

1.8 *BRCA1* and *BRCA2* genes

1.8.1 *BRCA1* and *BRCA2* penetrance

Germline mutations in the two breast cancer susceptibility genes confer strong lifetime risks of breast and ovarian cancer. *BRCA1* germline mutations also confer increased risks of pancreatic and perhaps uterine and cervical cancer (Thompson and Easton, 2002b) and *BRCA2* mutations may also predispose to prostate, pancreatic cancer and perhaps gallbladder, bile duct cancer and melanoma (Breast Cancer Linkage Consortium, 1999; van Asperen et al., 2005).

BRCA1 mutations are strongly associated with families with ovarian cancer whereas *BRCA2* mutations are strongly associated with families with male breast cancer cases. The calculated breast cancer risks for individuals' harbouring a *BRCA1*/*BRCA2* mutation depends on the method of ascertainment of the families studied. In high-risk families with multiple cases of breast and/or ovarian cancer, the cumulative risk of breast cancer at age 70 years was 85% for *BRCA1* and 84% for *BRCA2* mutation carriers. Furthermore, *BRCA1* and *BRCA2* mutations were

estimated to cause a cumulative lifetime risk of ovarian cancer at age 70 years of 63% and 27% respectively. (Ford et al., 1998). However, the average estimates of breast cancer risks at age 70 of a more recent meta-analysis of 22 population-based and hospital-based studies between *BRCA1* and *BRCA2* mutation carriers were 65% and 45% respectively. In addition, the average cumulative risks for ovarian cancer by age 70 years were 39% for *BRCA1* mutation carriers and 11% for *BRCA2* mutation carriers (Antoniou et al., 2003). Another observation made in studies focusing on *BRCA1* mutation carriers, was that their relative risks for breast cancer declined significantly with age (Antoniou et al., 2003; Chen et al., 2006). The same trend was observed for *BRCA2* mutation carriers by Chen et al. (2006) but not by Antoniou et al. (2003).

From the above, it is quite evident that there is controversy regarding the penetrance of *BRCA1* and *BRCA2* mutations. Ever since the identification of the two genes more than a decade ago, the estimation of cancer risks in individuals who test positive for a mutation is still an area of intense research. Mutations in *BRCA1* and *BRCA2* genes are rare and therefore penetrance estimates from individual studies often lack precision. It should be noted that penetrance estimates are based on multiple-case families and vary between countries because of the influence of non-genetic factors. Nevertheless, it is widely accepted that the lifetime breast cancer risk for *BRCA1* mutation carriers ranges between 46% and 85% and for *BRCA2* mutation carriers between 43% and 84%, depending on the population studied (Oldenburg et al., 2007).

As interest in genetic testing increases and more women become aware that they are *BRCA1/BRCA2* mutation carriers, the penetrance of these genes will decline because of preventive measures that will be undertaken such as prophylactic mastectomies and oophorectomies (Narod and Foulkes, 2004). Furthermore, it should be noted that risk estimates for *BRCA1* and *BRCA2* mutation carriers do not take into account the modifying effects of other genes on risk. One example is the presence of a SNP in the 5' untranslated region of *RAD51* [*RAD51* homolog (RecA homolog, E. coli) (S. cerevisiae] gene, 135G>C, which was proven that to modify breast cancer risk in *BRCA2* mutation carriers by altering the expression of *RAD51* (Antoniou et al., 2007). Microarray studies in irradiated lymphoblastoid cell lines from breast cancer

patients have identified potential *BRCA1/2* modifier genes which should be further investigated in the future (Walker et al., 2008).

Cancer risks are also modified depending on the position of the mutation in the gene sequence. Carriers of a mutation within the central region of *BRCA1* were found to have a lower breast cancer risk compared to carriers of mutations elsewhere in the gene. Furthermore, for *BRCA2* mutations it was found that women with mutations in the central region of the gene (ovarian cancer cluster region), had a lower breast cancer risk than women with mutations outside this region (Thompson and Easton, 2001; Thompson et al., 2002).

1.8.2 *BRCA1* and *BRCA2* structure and expression

The *BRCA1* and *BRCA2* genes do not share any obvious sequence homology but have common features. Both genes have an extremely large exon 11 which comprises 61% and 48% of the whole coding sequence of *BRCA1* and *BRCA2* respectively. Furthermore, both genes have translational start sites at exon 2 and in humans, the highest levels of expression are observed in testis, thymus and ovaries (Miki et al., 1994; Tavtigian et al., 1996). *BRCA1* and *BRCA2* genes are relatively poorly conserved between species with the exception of a few small domains.

1.8.2.1 *BRCA1* structure

The *BRCA1* gene is located on chromosome 17q21 and spans approximately 100 kb of genomic DNA (Figure 3). It consists of 24 exons of which 22 are encoding a 1863 amino acid protein. *BRCA1* exon 1 is non-coding, and the region originally identified as exon 4 is an Alu repeat not generally included in the transcript (Miki et al., 1994; Smith et al., 1996).

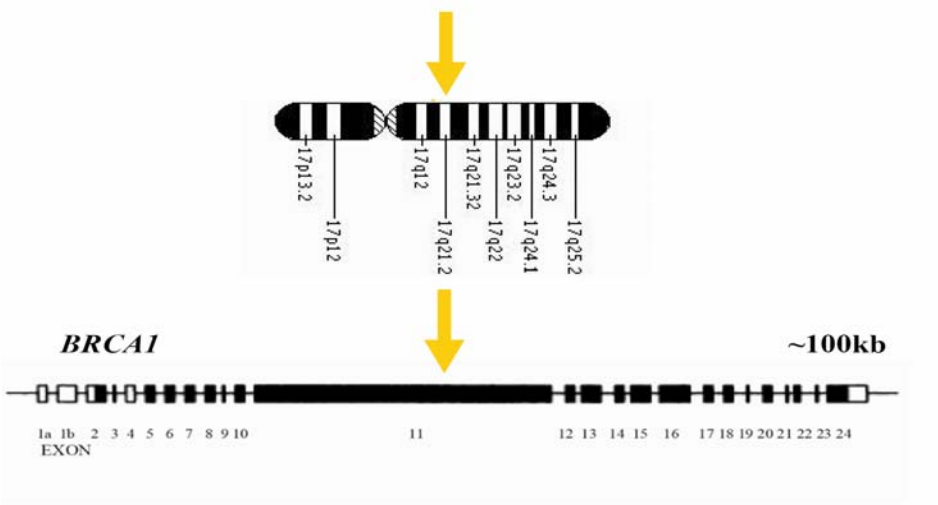


Figure 3: Genomic structure of the *BRCA1* gene which spans around 100 kb of genomic sequence and consists of 24 exons. Modified from Hakansson et al. 1997 and Genetics Home Reference website (http://ghr.nlm.nih.gov)

1.8.2.1.1 *BRCA1* protein

BRCA1 is a 220 kDa protein which shows a predominantly nuclear localization and forms nuclear "dots," or foci, during S phase of the cell cycle and following DNA damage (Chen et al., 1995; Scully et al., 1996; Scully et al., 1997). The *BRCA1* protein contains important functional domains that interact with a range of proteins. In summary, *BRCA1* contains a zinc-binding RING finger motif in its amino terminal region (Miki et al., 1994), 2 nuclear export signals near its N terminus (Rodriguez and Henderson, 2000; Thompson et al., 2005), 2 nuclear localization signals (NLS) (Chen et al., 1996), a DNA binding domain in the central region of the protein (Paull et al., 2001), an SQ-cluster domain (SCD) between amino acids 1280 and 1524 (Cortez et al., 1999) and two carboxy-terminal BRCT domains (Bork et al., 1997; Callebaut and Mornon, 1997; Koonin et al., 1996) (Figure 4).

The N-terminal RING domain is a zinc-binding domain that encompasses the first 109 amino acids of the *BRCA1* protein. The *BRCA1* RING domain exhibits an ubiquitin ligase activity. It interacts with the *BRCA1*-associated RING domain protein (*BARD1*) which is required for ubiquitin-ligase activity (Brzovic et al., 2001). Studies using *BRCA1* and *BARD1* mutant cell lines and knockout mice have shown that these two proteins have a shared functional role in vivo (Deng, 2002; McCarthy et al., 2003; Shen et al., 1998b). Indeed, the *BRCA1* RING domain does

not bind directly to DNA; it forms an interaction surface which facilitates the formation of a heterodimer with the *BARD1* RING domain (Brzovic et al., 2001). This interaction surface is targeted for mutations in the *BRCA1* gene. Mutations in the *BRCA1* RING domain predispose to cancer because they inactivate *BRCA1* ubiquitin ligase activity by reducing or abolishing heterodimerization *in vivo* (Ruffner et al., 2001). Studies have shown that the stability of the *BRCA1* and *BARD1* proteins is depended upon their heterodimerization *in vivo* (Brzovic et al., 2003; Joukov et al., 2001). Overall, the formation of the *BRCA1*/*BARD1* heterodimer, along with the structural integrity of the *BRCA1* RING domain, are of critical importance for the tumour-suppressor function of *BRCA1*. The RING finger of *BRCA1* also interacts with *BAP1* (*BRCA1*-associated protein 1) and has a role in *BRCA1* homodimer formation (Brzovic et al., 1998; Jensen et al., 1998).

BRCA1 contains two nuclear localization signals, a nuclear import and a nuclear export signal. This suggests that *BRCA1* protein has the ability to shuttle between the nucleus and the cytoplasm and may have implications for the regulation and function of this tumour suppressor (Rodriguez and Henderson, 2000).

The DNA binding domain of *BRCA1* comprises amino acids 452-1079 and contributes to the DNA-repair-related functions of the protein. A large number of DNA repair proteins including those making up the *BRCA1*-Associated Genome Surveillance Complex (BASC) bind to this region (Wang et al., 2000). This region also contains interaction sites for *RAD51* (Scully et al., 1997), an important component of DNA repair by homologous recombination.

The 244 amino acids between 1280 and 1524 contain 10 SQ/TQ sites [clusters of serines (S) and threonines (T) followed by glutamine (Q)] and comprise the *BRCA1* SCD domain. SQ or TQ sequences are the preferred sites of *ATM* phosphorylation. In response to DNA damage, *BRCA1* is phosphorylated at multiple serine residues within the SCD domain by *ATM* (Cortez et al., 1999).

The BRCT domain of the *BRCA1* protein consists of two individual repeats, the N-terminal BRCT and the C-terminal BRCT. The two motifs are about 110 residues long, are similar in structure and are packed together in a head-to-tail arrangement

(Williams et al., 2001). *BRCA1* interacts with many proteins through its BRCT domain including *CtIP* (carboxy-terminal-binding-protein-interacting protein), *BRIP* (*BRCA1*-interacting protein), *p300*, *RNA polymerase II*, *TP53* and *RB* (retinoblastoma). It is believed that this part of the protein functions as a multipurpose protein-protein interaction module (Cantor et al., 2001; Deng and Brodie, 2000). The BRCT motifs are also involved in phosphopeptide binding by either stabilizing or forming the phosphopeptide binding site (Clapperton et al., 2004). Like the *BRCA1* RING domain, the BRCT motifs of the protein are of critical importance for its tumour suppression function since truncating and missense mutations which predispose to breast and ovarian cancer were found in this domain. Mutations within the BRCT domain inhibit the interaction with partner DNA damage protein *BRIP* and as a result of this, damaged DNA escapes the checkpoints during the G2/M phase of the cell cycle (Yu et al., 2003).

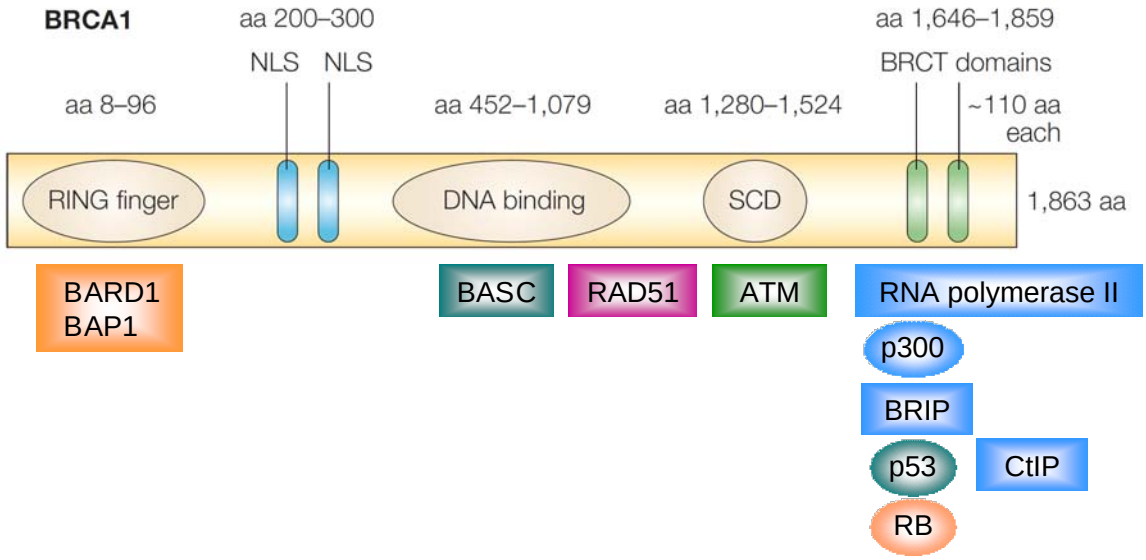


Figure 4: *BRCA1* functional domains and selected binding partners. Modified from Narod and Foulkes 2004

1.8.2.2 *BRCA2* structure

The *BRCA2* gene is located on chromosome 13q12 and spans approximately 70 kb of genomic DNA (Figure 5). It consists of 27 exons of which 26 encode a 3418 amino acid protein (Tavtigian et al., 1996; Wooster et al., 1995).

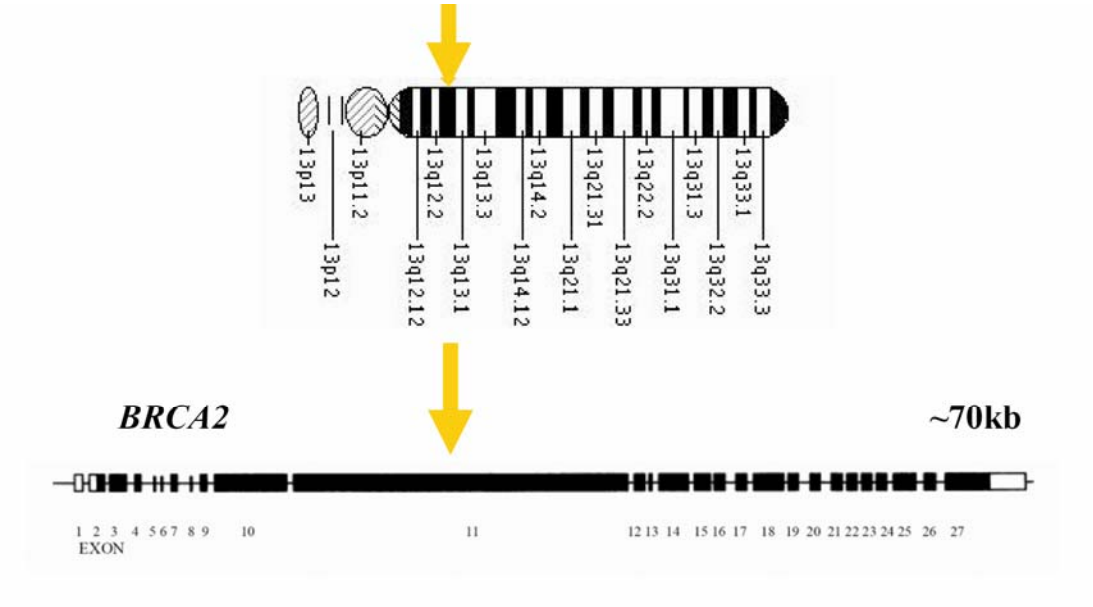


Figure 5: Genomic structure of the *BRCA2* gene which spans around 70 kb of genomic sequence consists of 27 exons. Modified from Hakansson et al. 1997 and Genetics Home Reference website (http://ghr.nlm.nih.gov)

1.8.2.2.1 *BRCA2* protein

BRCA2 protein contains two known functional domains, the BRC-repeats motifs and the DBD binding domain. The middle region of the protein, which is encoded by exon 11, contains eight BRC-repeat motifs that are conserved among mammalian species suggesting an important function. The BRC repeats are essential for *BRCA2* function in DNA repair by mediating direct binding to the DNA recombinase *RAD51*, a protein that is essential for DNA repair and genetic recombination. It is currently believed that in human *BRCA2*, six of the eight motifs can bind directly to *RAD51* (Chen et al., 1998; Wong et al., 1997). Mutations in *BRCA2* BRC repeats are associated with cancer predisposition. Experiments in mice have demonstrated that deletions of all *BRCA2* BRC domains are embryonically lethal whereas deletions of several BRC repeats lead to cancer (Donoho et al., 2003).

The *BRCA2* C-terminal region contains the DBD binding domain, which interacts with *DSS1* (deleted in split-hand/split-foot 1), a highly conserved 70 amino-acid protein. *DSS1* binding protein is essential for *BRCA2* function (Gudmundsdottir et al., 2004; Kojic et al., 2003; Li et al., 2006). Studies in mammalian cells have demonstrated that *DSS1* is required for the formation of DNA damage-induced *RAD51* foci suggesting that it has a role in *BRCA2* and *RAD51* dependent repair by homologous recombination (Gudmundsdottir et al., 2004; Li et al., 2006). Furthermore, studies implicate *DSS1* in maintaining the correct conformation of the *BRCA2* protein. The exact mechanism of how *DSS1* depletion induces degradation of *BRCA2* remains unclear but it was observed that *BRCA2* was largely insoluble in the absence of *DSS1* (Yang et al., 2002). The carboxy-terminal region of *BRCA2* also includes two nuclear localization signals, which enable *BRCA2* to enter the nucleus and also facilitate *RAD51* transport into the nucleus (Davies et al., 2001; Spain et al., 1999) (Figure 6).

A second *RAD51* binding site also exists in the extreme C-terminus of the *BRCA2* protein (Mizuta et al., 1997; Sharan et al., 1997). This region is also phosphorylated at residue 3291 by cyclin-dependent kinases (Esashi et al., 2005). It is still unclear exactly how the two *RAD51*-binding regions of the *BRCA2* work synergistically to control *RAD51* function.

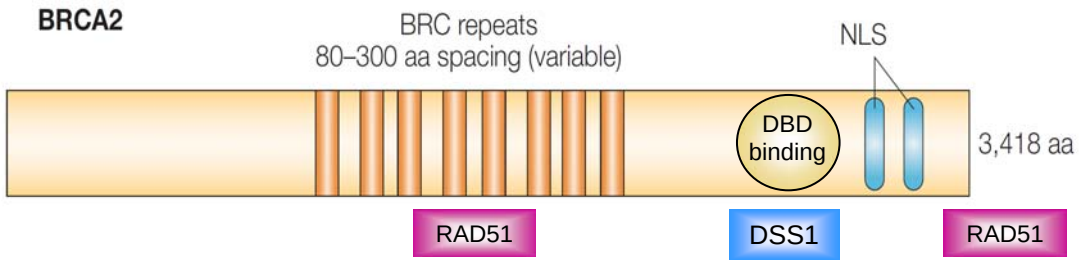


Figure 6: *BRCA2* functional domains and selected binding partners. Modified from Narod and Foulkes 2004

1.8.3 *BRCA1* and *BRCA2* proteins and their proposed functions

The *BRCA1* and *BRCA2* genes were discovered by positional cloning with no prior knowledge of their functional roles. Since their discovery, the elucidation of their biology has been the focus of intensive research. We now know that both genes are key players in DNA-repair pathways and are considered as "caretaker" genes (genes that act as sensors of DNA damage and participate in the repair process) (Kinzler and Vogelstein, 1997). Until recently, it was believed that *BRCA1* and *BRCA2* genes are classical tumour suppressor genes for which the Knudson two hit hypothesis holds true. Individuals who have a genetic predisposition to hereditary breast and ovarian cancer carry a deleterious germline mutation in the *BRCA1* or the *BRCA2* gene, in every one of their cells. According to Alfred Knudson's two hit hypothesis of cancer causation (Knudson, 1971), a second hit in the wild-type *BRCA1* or *BRCA2* allele is required for the development of *BRCA*-associated cancer. A number of studies have documented loss of heterozygosity (LOH) in *BRCA*-associated tumours with retention of the disease predisposing allele (Smith et al., 1992; Neuhausen and Marshall, 1994; Collins et al., 1995; Gudmundsson et al., 1995).

Recently, there has been some controversy about this. A study by King et al. (2007) reported that breast cancer initiation in *BRCA*-mutation carriers might result from *BRCA* haploinsufficiency and not from loss of the wild-type allele. In contrast, data from the same group suggest that loss of the wild-type *BRCA* allele is required for ovarian carcinogenesis. An editorial in "*Annals of Surgical Oncology*" journal outlines a number of reasons, which can explain the variability of these findings compared to previous studies (Meric-Bernstam, 2007). Meric-Bernstam suggests that the variability may be due to differences in methodology or sampling as well as to studying only two genotypes: 185delAG in *BRCA1* and 617delT in *BRCA2*. Furthermore, King et al. have not evaluated changes in methylation, which could cause epigenetic silencing of the wild-type allele. Additional studies using larger numbers of samples with different genotypes are warranted in order confirm or refute the hypothesis that LOH affecting the wild-type *BRCA* allele is not obligatory for breast tumorigenesis in *BRCA1*/*2* mutation carriers.

Despite the progress that has been made in understanding the functions of the *BRCA1* and *BRCA2* genes, a complete picture has not yet been attained and there is still much more to learn. Based on the current information, we know that *BRCA1* has multiple biological functions and is involved in DNA damage repair, transcriptional regulation, cell cycle checkpoint control, protein ubiquitylation and chromatin remodelling (Narod and Foulkes, 2004). On the other hand, *BRCA2* plays an important role in homologous recombination, both in meiosis and repair of double-strand breaks. It also has a role in centrosome regulation and maintenance of chromosomal stability (Narod and Foulkes, 2004).

BRCA1 and *BRCA2* participate in the biological response to DNA damage that includes the activation of cell cycle checkpoints and the recruitment of the DNA damage repair machinery. Both *BRCA1* and *BRCA2* are implicated in DNA repair by homologous recombination, a repair mechanism in which a homologous chromatid serves as a template to guide repair of the damaged strand. *BRCA1* and *BRCA2* proteins have distinct roles in double-strand break repair (Narod and Foulkes, 2004).

1.8.3.1 *BRCA1* protein functions

BRCA1 plays a key role in DNA double strand break repair and in the maintenance of genomic integrity. It has been demonstrated that *BRCA1* facilitates DNA repair through its involvement in homologous recombination (HR), non-homologous end joining (NHEJ) and nucleotide excision repair (NER). An overview of these DNA repair mechanisms will be presented in section 2.2 of this thesis. *BRCA1* protein serves as a scaffold that organizes and coordinates a number of proteins that are involved in maintaining genomic integrity (Deng and Brodie, 2000).

The most deleterious form of DNA damage is double strand breaks. There are two main pathways that are used for repairing these breaks: HR and NHEJ. There is evidence that *BRCA1* is implicated in both these pathways. It is believed that *BRCA1* protein is involved in NHEJ via its interaction with the MRN [*MRE11A* [meiotic recombination 11 homolog A (S. cerevisiae)] - *RAD50* [*RAD50* homolog (S. cerevisiae)]- *NBS1* [Nijmegen breakage syndrome 1 (nibrin)] complex. The MRN complex plays an important role in both HR and NHEJ. The exact mechanism of *BRCA1* interaction with the MRN complex and its involvement in NHEJ is not yet

fully known. There is conflicting evidence on the role of *BRCA1* in NHEJ, which is summarized, in a recent review by Bau et al. (2006). Many studies have provided evidence that *BRCA1* deficient cells have decreased NHEJ fidelity. Moreover, *BRCA1*-deficient mouse embryonic fibroblasts were found to have significantly reduced NHEJ activity. On the other hand, other studies have demonstrated that *BRCA1* can promote only specific subtypes of NHEJ and has no effect on others. This may be a reflection of the different roles of *BRCA1* in sub-pathways of NHEJ (Bau et al., 2006).

BRCA1 is also involved in DNA repair by homologous recombination. The first indication that *BRCA1* participates in DNA repair was the observation that it associates and co localizes with *RAD51* in subnuclear clusters (Scully et al., 1997). *RAD51* is the major component of the HR pathway. The nature of interaction between *BRCA1* and *RAD51* is unknown but it is believed that the association is likely to be indirect and possibly mediated by *BRCA2*. In the event of DNA damage, both *RAD51* and *BRCA1* localize to the region of damage and *BRCA1* also undergoes phosphorylation. The subnuclear localization and the phosphorylation of *BRCA1* protein, suggest that it is involved in DNA-damage dependent replication checkpoint response (Scully et al., 1997b; Thomas et al., 1997). Further evidence that *BRCA1* is involved in DNA repair by homologous recombination comes from the observation that *BRCA1* deficiency results in decreased *RAD51* foci formation in cultured cells after γ-irradiation (Huber et al., 2001). Based on the fact that *BRCA2* also interacts with *RAD51*, it was suggested that a complex consisting of *BRCA1*, *BRCA2* and *RAD51* is formed and functions to repair damaged DNA (Chen et al., 1999). There is an indication that this complex functions during or after DNA replication, since the levels of *BRCA1*, *BRCA2* and *RAD51* expression increase in cells when they enter the S phase of the cell cycle (Venkitaraman, 2002).

BRCA1 has also been linked to a number of other DNA repair processes due to its interaction with other proteins that are involved in response to and in the repair of DNA damage. *BRCA1* together with *BRCA2*, *RAD51*, *BARD1* and other proteins is part of the BRCC (*BRCA1*-*BRCA2*-Containing Complex) that constitutes an E3 ubiquitin ligase that enhances cellular survival following DNA damage (Dong et al., 2003). Furthermore, *BRCA1* forms part of the BASC (*BRCA1*-Associated Genome

Surveillance Complex) super complex. The BASC complex includes the DNA mismatch repair proteins *MLH1* [mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)], *MSH2* [mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)] and *MSH6* [mutS homolog 6 (E. coli)], the MRN complex proteins *MRE11A-RAD50-NBS1*, the Bloom syndrome helicase *BLM*, *ATM* (ataxia telangiectasia mutated) kinase, DNA replication factor C, *RFC* (replication factor C) and *PCNA* (proliferating cell nuclear antigen). It is believed that this complex acts as a sensor for DNA damage and is also directly involved in repairing DNA damage by DNA-replication associated repair (Wang et al., 2000). The involvement of *BRCA1* in repairing double strand breaks is supported by its participation in the BASC complex and its interaction with the MRN complex. The MRN complex plays a critical role in DNA damage sensing, signalling and repair mechanism, as well as in the maintenance of chromosomal integrity of the cell (Assenmacher and Hopfner, 2004).

BRCA1 also functions in signalling the response to DNA damage. Following DNA damage, *ATM* and *ATR* (ataxia telangiectasia and Rad3 related) protein kinases phosphorylate *BRCA1* in response to different stimuli (Cortez et al., 1999; Gatei et al., 2000; Okada and Ouchi, 2003; Tibbetts et al., 2000). Furthermore, in response to γ-irradiation *ATM* phosphorylates and activates *CHEK2* [*CHEK2* checkpoint homolog (S. pombe)], which in turn phosphorylates *BRCA1* (Lee et al., 2000). Hence, *ATM*, *ATR* and *CHEK2* kinases regulate *BRCA1* function and in turn cell cycle regulation and DNA repair by phosphorylation.

Recently there has been progress in elucidating the mechanism by which *BRCA1* recognizes double-strand breaks. *BRCA1*, through its C-terminal domains forms three distinct complexes with *Abraxas*, *BACH1* and *CtIP* proteins. *RAP80* (receptor associated protein 80) recruits *BRCA1-Abraxas* and *BRCA1-CtIP* complexes to damaged DNA. (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007). In addition, the *BRCA1-CtIP* complex interacts with the MRN complex to facilitate double-strand break resection and to activate homologous recombination mediated repair of DNA (Chen et al., 2008; Sartori et al., 2007).

BRCA1 also plays a role in DNA repair by NER and is involved in both transcription coupled repair and global genome repair. In detail, it was reported that *BRCA1*

deficiency leads to blockage of RNA polymerase II transcription machinery at the site of repair of oxidative 8-oxoguanine residues (Le Page et al., 2000). Moreover, *BRCA1* has been reported to be involved in global genomic repair and more specifically in the transcriptional activation of genes that are involved in recognition of damaged DNA. In detail, *BRCA1* induces expression of *XPC* (xeroderma pigmentosum, complementation group C), *DDB2* (damage-specific DNA binding protein 2) and *GADD45* (growth arrest and DNA-damage-inducible) genes in the absence of *TP53* (Hartman and Ford, 2002). Another link between *BRCA1* and the NER pathway comes from its association with *MSH2* and *MSH6* (as part of the BASC complex) which are required for transcription-coupled repair (Wang et al., 2000).

Recently *BRCA1* has been found to have an enzymatic function as a ubiquitin ligase together with its interacting protein *BARD1*, via the formation of a RING/RING heterodimer. Ubiquitylation is the process during which proteins are tagged for degradation by the proteasome. The *BRCA1/BARD1* heterodimer targets proteins involved in cell cycle regulation and DNA repair, for degradation (Irminger-Finger and Jefford, 2006).

Another important function that is associated with the *BRCA1* protein is chromatin remodelling. *BRCA1* is involved in chromatin remodelling around the sites of DNA damage via its direct interaction with a protein complex that contains the chromatin remodelling proteins *SW1*/*SNF*. As a result of this interaction, a number of other proteins that are implicated in response to DNA damage, namely *KU70* (Ku autoantigen, 70kDa) and *GADD45*, are activated (Bochar et al., 2000). Also, *BRCA1* via its C-terminal BRCT repeat directly interacts with *BACH1* (also known as *FANCJ* or *BRIP1*), a DNA helicase that plays a critical role in maintenance of genome stability (Cantor et al., 2001) . Another indication that *BRCA1* is involved in chromatin remodelling comes from its participation in the BASC complex, which is known to be involved in the chromatin remodelling process that facilitates DNA repair (Wang et al., 2000).

1.8.3.2 *BRCA2* protein functions

The exact role of the *BRCA2* protein still remains elusive. It has been demonstrated that *BRCA2* plays an important role in homologous recombination, both in meiosis and in the repair of double-strand breaks. The major role of the *BRCA2* protein is to assist in organizing *RAD51* function and facilitate homologous recombination. *BRCA2* binds *RAD51* recombinase directly and regulates recombination-mediated double strand break repair. *BRCA2* is required for the efficient nuclear localization of *RAD51* and mediates the recruitment of *RAD51* to the sites of double strand breaks. Hence it is essential for the cellular function of *RAD51* (Davies et al., 2001; Sharan et al., 1997; Yuan et al., 1999). *BRCA2*-deficient cell lines are very sensitive to DNA damaging agents and exhibit a genomic instability phenotype that includes accumulation of double-strand breaks and in turn chromosomal breaks (Kraakman-van der Zwet et al., 2002; Moynahan et al., 2001). In addition, *BRCA2* has been identified as the *FANCD1* (Fanconi Anemia complementation group D1) gene. When both *BRCA2* alleles are inactivated, a Fanconi anemia phenotype can occur (Howlett et al., 2002; Offit et al., 2003).

Not long ago, a nuclear partner of *BRCA2*, namely *PALB2* was identified. *PALB2* (partner and localizer of *BRCA2*) provides stability to the *BRCA2* protein to perform its cellular functions namely DNA repair by homologous recombination and checkpoint control. *PALB2* is also required for *BRCA2* intranuclear localization (Xia et al., 2006).

Recently, it has been demonstrated that *BRCA2* plays a critical role in meiotic recombination through its direct interaction with *DMC1* recombinase (Thorslund et al., 2007). Moreover, *BRCA2* controls mitotic checkpoint activity (Tutt et al., 1999; Yu et al., 2000), maintains normal centrosome number and function and has been implicated in regulation of cytokinesis in the final stages of cell division (Daniels et al., 2004). Finally, *BRCA2* has been shown to contribute to activation of transcription (Milner et al., 1997; Shin and Verma, 2003), G2/M checkpoint control (Yuan et al., 1999), suppression of tumour development by inhibition of cancer cell proliferation (Wang et al., 2002) and mammalian gametogenesis (Sharan et al., 2004).

1.8.3.3 *BRCA1* and *BRCA2* protein functions and targeted therapy

Since the identification of *BRCA1* and *BRCA2* genes, extensive research has been carried out to elucidate their functions and identify the pathways in which they are involved. Recently, the knowledge gained on *BRCA1* and *BRCA2* function in DNA repair has been exploited to therapeutic advantage.

As it was discussed extensively in this chapter, heterozygous germline mutations in the *BRCA1* and *BRCA2* genes predispose their carriers to breast and ovarian cancers (Wooster and Weber, 2003). These individuals have a wild type allele, which expresses the *BRCA1*/*2* protein and therefore their cells have relatively normal *BRCA1*/*2* function. However, at some point in their lifetime, somatic loss of heterozygosity of the wild-type *BRCA1*/*2* allele occurs which is believed to foster cancer progression by promoting genomic instability and mutation. As a result of this, the wild type allele is lost in tumours and only the mutant protein is expressed (Gudmundsdottir and Ashworth, 2006).

BRCA1 and *BRCA2* proteins play an important role in repairing DSBs by HR. *BRCA1*/*2* mutation carriers are deficient in the homologous recombination repair pathway and cells are forced to use alternative repair pathways which are error prone and lead to genomic instability and eventually cell death (Moynahan et al., 1999; Moynahan et al., 2001b; Tutt et al., 2001).

A novel therapeutic approach, which is based on the concept of "synthetic lethality" (Hartwell et al., 1997) and is targeted towards women who carry *BRCA1* or *BRCA2* mutations is currently being evaluated. The principle of this therapy is to kill tumour cells, which are *BRCA1*/*2* – deficient, but not harm cells with normal *BRCA1*/*2* function. Scientists hypothesized that loss of an additional DNA repair pathway (BER pathway) in *BRCA1/2* mutant cells would result in synthetic lethality whereas cells with functional *BRCA1*/*2* alleles would be unaffected and able to survive (Bryant et al., 2005; Farmer et al., 2005). The base excision repair (BER) pathway was targeted, by reducing the activity Poly (ADP-ribose) Polymerase (*PARP*) enzyme which is critical for BER (Boulton, 2006). Experiments using small molecule inhibitors of *PARP1* have demonstrated that *BRCA1* or *BRCA2* deficient cells were selectively killed whereas cells with functional *BRCA1* or *BRCA2* had

remained unaffected (Farmer et al., 2005; Tutt et al., 2005).

The efficacy of *PARP* inhibitors led to a phase I clinical trial aiming to assess the use of *PARP* inhibitors in the treatment of cancer in *BRCA1* or *BRCA2* mutation carriers. The preliminary results of phase I trial showed significant efficacy and modest toxicity and phase II trials are currently underway (Ratnam and Low, 2007). A considerable amount of testing is still required before *PARP* inhibitors are used in a clinical setting for the treatment of *BRCA1*- or *BRCA2*-associated cancers.

This approach represents a good example of how basic research into the biology of *BRCA1* and *BRCA2* can lead to novel therapeutic strategies and new tailored therapies. It is anticipated that in the future, the expansion of our knowledge on the functions of *BRCA1* and *BRCA2* will enhance our ability to treat *BRCA1*- and *BRCA2*-associated cancers.

1.8.4 *BRCA1* and *BRCA2* mutation spectrum

Genetic testing to identify the presence of *BRCA1* and *BRCA2* mutations has become an integral part of contemporary clinical practice in many countries. Mutation screening of the two breast cancer predisposition genes is also carried out all over the world for research purposes, and more specifically for estimating the prevalence of these genes in different ethnic groups.

More than 3000 distinct sequence variants have been described in the *BRCA1* and *BRCA2* genes to-date. The Breast Cancer Information Core (BIC) database had recorded (as of December 2008) more than 1600 distinct germline *BRCA1* mutations and more than 1800 *BRCA2* mutations. It is interesting that there are no hot spots in these two genes and mutations are evenly distributed across their entire coding sequences (Figure 7). This makes mutation screening of *BRCA1* and *BRCA2* technically challenging and requires that each gene is screened in its entirety.

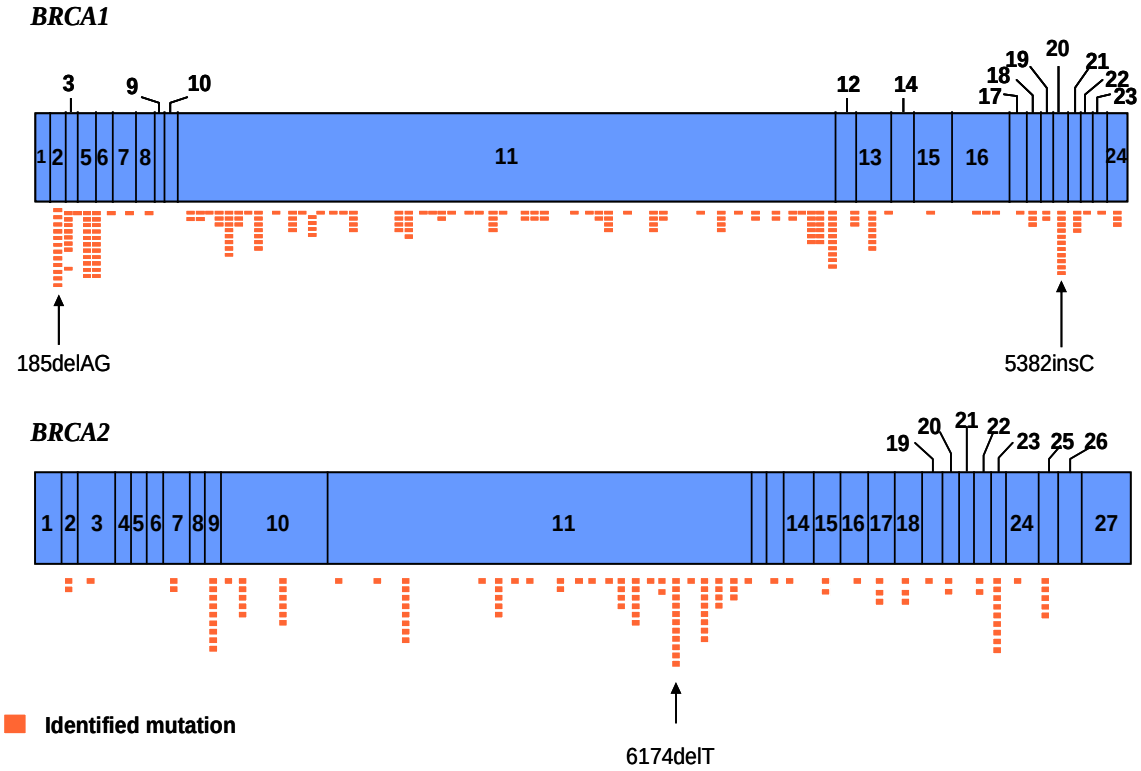


Figure 7: Mutation spectrum of *BRCA1* and *BRCA2* genes spanning across their exons in a condensed map modified from the BIC database (http://research.nhgri.nih.gov/bic/). The three most common mutations in the *BRCA1* (185 delAG, 5382 insC) and *BRCA2* (6174delT) genes are pointed out with arrows.

Initial reports on disease-associated *BRCA1* and *BRCA2* mutations documented genetic alterations that resulted in protein truncations, mainly small deletions and insertions as well as nonsense mutations leading to premature stop codons (Castilla et al., 1994; Friedman et al., 1994; Miki et al., 1994; Simard et al., 1994; Wooster et al., 1995; Couch et al., 1996; Neuhausen et al., 1996; Tavtigian et al., 1996). Deleterious mutations generate truncated non-functional *BRCA1* or *BRCA2* proteins. Nowadays, the most common types of deleterious *BRCA1* and *BRCA2* mutations identified remain small frameshift insertions and deletions as well as nonsense mutations and splice-site mutations.

With the advancement of technology and the development of sensitive quantitative techniques, it is now possible to screen both genes for the presence of genomic rearrangements i.e. large exonic deletions or insertions. Studies on homogeneous ethnic populations have revealed that the frequency of *BRCA1* genomic

rearrangements in high-risk breast cancer families ranges between 1.3% and 4.4%, depending on the ethnicity as well as on the study eligibility criteria. The frequency of *BRCA2* genomic rearrangements is lower than *BRCA1* and ranges between 0% and 2.4%, depending again on the population studied and the study eligibility criteria. Overall, large *BRCA1* rearrangements account for between 8% and 19% of the total number of *BRCA1* mutations whereas large *BRCA2* deletions/duplications contribute between 0% and 11%, of all *BRCA2* mutations (Palma et al., 2008). Based on these data, it can be concluded that genomic rearrangements contribute significantly to the spectrum of identifiable mutations of the *BRCA1* and *BRCA2* genes and screening for genomic rearrangements should become an integral part of the routine mutational analysis of *BRCA1/2* genes (Palma et al., 2008).

In addition to protein truncating mutations, splice site mutations and genomic rearrangements, large numbers of missense mutations have been identified in both *BRCA1* and *BRCA2* (BIC Database). These mutations cause single amino acid changes. In some cases, depending on the location of the mutation and the actual amino acid substitution missense mutations can disrupt protein function, whereas in others they are neutral polymorphic variants. The classification of missense mutations as disease-associated or neutral is difficult and given that the status of the majority of missense mutations identified so far is uncertain, they are reported as variants of uncertain significance or unclassified variants (BIC Database).

Geneticists are currently using a number of approaches to classify variants of uncertain significance in the *BRCA1* and *BRCA2* genes and to assess their contribution to breast cancer risk. These include analysis of segregation of mutations with disease in families (Thompson et al., 2003), evaluation of the frequency of variants in unaffected controls (Deffenbaugh et al., 2002) , analysis of clinical and histopathological data (Gomez-Garcia et al., 2005), loss of heterozygosity analysis (Osorio et al., 2002), bioinformatics analysis to predict effect of the amino acid change on protein structure (Mirkovic et al., 2004) and cross-species sequence variation analysis (Abkevich et al., 2004; Tavtigian et al., 2006). Recently, integrated models which combine several approaches for classification of DNA sequence variants of unknown clinical significance, into deleterious or neutral, have been proposed (Chenevix-Trench et al., 2006; Easton et al., 2007; Goldgar et

al., 2004; Lovelock et al., 2007; Osorio et al., 2007; Phelan et al., 2005; Spurdle et al., 2008). The application of these methods has resulted into the classification of a number of *BRCA1* and *BRCA2* unclassified variants. These methods have the disadvantage that they use high-risk mutations as reference. For this reason, the possibility that some missense mutations classified as being of low clinical significance, are in fact associated with moderate risk of cancer cannot be excluded (Waddell et al., 2008).

The most reliable method for the classification of *BRCA1* and *BRCA2* alterations is via the development of specific functional assays that take advantage of biochemistry and cell biology in order to assess the influence of unclassified variants on protein function. It is expected that these assays will be adapted in the future in conjunction with other sources of information for clinical purposes and more specifically for the development of validated likelihood models which will provide reliable risk assessment to individuals at high risk (Carvalho et al., 2007).

1.8.5 Ethnic differences in *BRCA1* and *BRCA2* mutation spectra

Germline disease-causing *BRCA1* and *BRCA2* mutations have been identified in individuals from all over the world, originating from different races and ethnic groups. Many studies have demonstrated that certain *BRCA1* and *BRCA2* mutations are restricted to certain ethnic populations and geographical areas. This observation is partly the result of the presence of founder mutations within these genes (Neuhausen, 1999; Szabo and King, 1997).

The most common and well-characterized mutations are those detected in individuals of Ashkenazic Jewish descent. It is estimated that around 2.5% of Ashkenazi Jews carry one of the three founder mutations (185delAG and 5382insC in *BRCA1* and 6174delT in *BRCA2*) (Fodor et al., 1998; Roa et al., 1996; Struewing et al., 1997). Another population, whose *BRCA1* and *BRCA2* mutational spectrum is well characterized due to the availability of samples, is the Icelanders. Around 0.4-0.6% of the Icelandic population carry a single *BRCA2* founder mutation (999delTCAAA) (Johannesdottir et al., 1996; Thorlacius et al., 1997).

Several other recurrent mutations have been described in a number of ethnic groups and populations and are summarized in a recent review by Fackenthal and Olopade (2007). Briefly, the small number of detected *BRCA1* and *BRCA2* pathogenic mutations characterizes some populations, whereas others have a broader and more diverse mutational spectrum (Figure 8). It should be noted that some of the recurrent mutations were found in a number of different populations and ethnic groups. The number of distinct recurrent mutations in a population depends on the population history and more specifically on influences of migration, population structure, geographical and cultural isolation (Szabo and King, 1997).

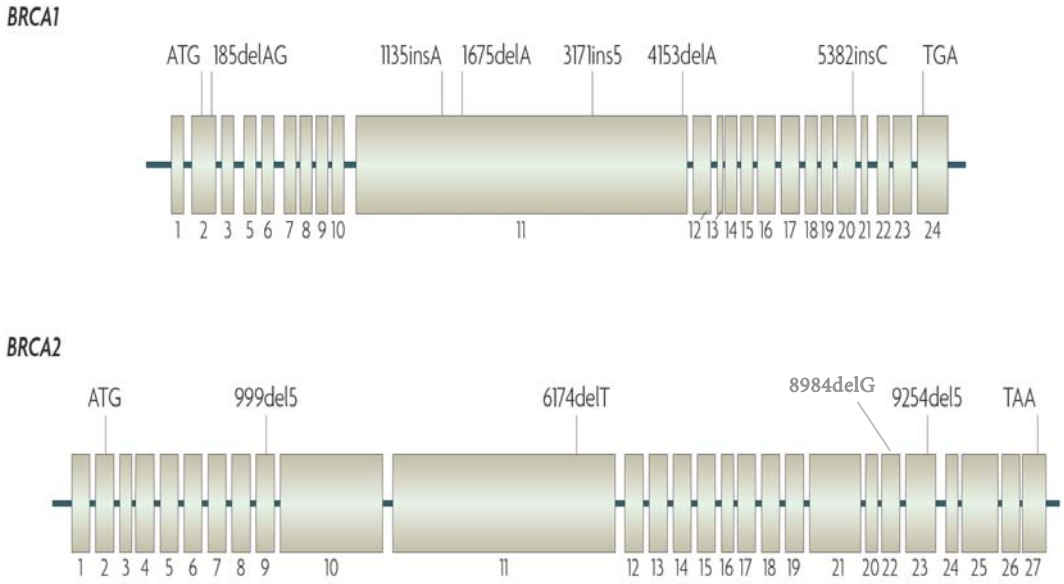


Figure 8: Position of several founder mutations within *BRCA1* and *BRCA2* genes. *BRCA1* 185delAG and 5382insC and *BRCA2* 6174delT are founder mutations for Ashkenazi Jews, *BRCA1* 1135insA and 1675delA are founder mutations for the Norwegian population and *BRCA1* 3171ins5 and 4153delA are founder mutations for the Swedish and Polish population respectively. Furthermore *BRCA2* 9254del5 is a founder mutation of the Spanish population and 8984delG is a founder mutation of the Cypriot population [modified from Fackenthal and Olopade (2007)].

In Cyprus, 10 distinct disease-causing *BRCA1* and *BRCA2* mutations were identified in 150 Cypriot breast/ovarian cancer families subjected to genetic testing (Hadjisavvas et al., 2001; Hadjisavvas et al., 2003; Hadjisavvas et al., 2004; unpublished data). Two out of the ten mutations, 8984delG in *BRCA2* gene and

5429delG in *BRCA1* gene, have been detected in more than one Cypriot family. These two mutations account for the majority of *BRCA1* and *BRCA2* mutation-positive families identified so far in Cyprus. Furthermore, four of the truncating mutations that have been identified are novel and characterise the Cypriot population. It seems that the *BRCA2* gene plays a more important role in familial breast cancer than *BRCA1* in the Cypriot population. It is noted that frameshift mutation 8984 delG in the *BRCA2* gene is the most frequent mutation identified in the Cypriot population and was detected in nine unrelated families. Haplotype analysis revealed that this is a founder mutation for the Cypriot population (Hadjisavvas et al., 2004).

1.8.6 *BRCA1* and *BRCA2* in early onset breast cancer

Early age of diagnosis is a useful marker of genetic susceptibility to breast cancer. Familial breast cancer occurs at a considerably younger age compared to the typical age of onset in the general population (Claus et al., 1991). The occurrence of early-onset breast cancer has been associated with mutations in the *BRCA1* and *BRCA2* genes (Langston et al., 1996; Krainer et al., 1997).

In the general population, women with an early age of breast cancer diagnosis are more likely than others, to have genetic predisposition to the disease. Studies on the contribution of *BRCA1* and *BRCA2* mutations to the incidence of breast cancer have primarily focused on individuals coming from high-risk families and large founder effect populations. In contrast to the plethora of family based studies, few population based studies examining the prevalence of *BRCA1* and *BRCA2* mutations in women who were diagnosed with breast cancer at a young age, have been carried out.

Chronologically, the first population-based studies that were performed had concentrated on selected populations with highly recurrent founder mutations i.e. the Icelanders and the Ashkenazi Jews. Data from these studies revealed that the prevalence rates of *BRCA1* and *BRCA2* mutations amongst early onset breast cancer patients can be as high as 30% for Ashkenazi Jews (Abeliovich et al., 1997) and around 25% for Icelanders (Johannesdottir et al., 1996; Thorlacius et al., 1997). This high percentage is a result of the presence of founder mutations in these two ethnic populations. In detail, 25% of women in Iceland diagnosed with breast cancer below

the age of 40 carried a single *BRCA2* mutation (999delTCAAA) while among unselected Jewish breast cancer cases diagnosed below the age of 40, 30% were found to carry one of the three founder *BRCA1/2* mutations (*BRCA1* 185delAG, 5382insC and *BRCA2* 6174delT).

In addition to Icelanders and Ashkenazi Jews, the contribution of *BRCA1* and *BRCA2* mutations to the population incidence of early-onset breast cancer has been examined in various other geographical regions and ethnic groups. In detail, studies were carried out in North America, United Kingdom, Spain, the Netherlands, Sweden, Germany, Philippines, Korea and Australia (Langston et al., 1996; Krainer et al., 1997; Peto et al., 1999; Southey et al., 1999; Anglian Breast Cancer Study Group, 2000; Loman et al., 2001; Malone et al., 2000; Papelard et al., 2000; Loman et al., 2001; De Leon Matsuda et al., 2002; Antoniou et al., 2003; de Sanjose et al., 2003; Hamann et al., 2003; Choi et al., 2004). In these geographical regions, the prevalence of *BRCA1* and *BRCA2* mutations among early onset breast cancer patients' ranges between 5% and 10%. Mutations in the two cancer predisposition genes make approximately equal contributions to the incidence of early onset breast cancer, with the exception of the Philippines, where *BRCA2* plays a more significant role compared to *BRCA1* (De Leon Matsuda et al., 2002). The differences observed in the mutation frequencies between various populations may be explained by the different study selection criteria as well as by differences in the sensitivity of the genotyping methods. However, it is also possible that these are real differences since the patient groups that were studied, come from different ethnic and racial backgrounds.

In these population-based studies it was observed that the chances of finding a pathogenic mutation in either *BRCA1* or *BRCA2* genes increased by increasing numbers of first degree affected relatives. Thus the frequency of deleterious mutations in patients with a strong family history was greater compared to patients without a family history. A family history was observed in more than 80% of patients from the Icelandic population and 40% to 90% in other populations.

However, studies from Korea and Australia (Southey et al., 1999; Choi et al., 2004) challenged this paradigm. In these studies it was found that the majority of women

with deleterious *BRCA1/2* mutations did not have a family history of breast or ovarian cancer. Therefore, it is possible in the clinical setting that the proportion of early onset breast cancer patients with family history is smaller compared to patients without family history. Hence, the majority of early onset breast cancer cases that are *BRCA1/2* mutation carriers in these countries are not "hereditary" but "sporadic" since they occur in individuals without a family history of breast cancer. This highlights the importance of a population-based perspective for breast cancer genetics which not only focuses on families with multiple cases of breast cancer but also on women with early onset breast cancer irrespective of their family history.

Chapter 2

Genetic susceptibility to breast cancer and the role of DNA repair genes

2.1 Genetic susceptibility to breast cancer

2.1.1 Moderate risk breast cancer genes

Breast cancer susceptibility genes can be split into 2 main categories: "high-risk" genes and "moderate risk" genes. As discussed extensively in Chapter 1, *BRCA1 and BRCA2* are the main breast cancer susceptibility genes and together with *PTEN, TP53, CDH1 and LKB1/STK11* comprise the high-risk susceptibility group. These highly penetrant genes were first identified in the 1990s by genetic linkage studies (Oldenburg et al., 2007). Nowadays, it is widely accepted that among all populations, around 5% to 10% of all breast cancer cases, arise in individuals who inherit a highly penetrant mutation in a breast cancer susceptibility gene such as the *BRCA1* and *BRCA2* (Thompson and Easton, 2004).

Over the past five years a number of genes conferring a moderate increase in breast cancer risk have been discovered. These genes were identified through candidate gene resequencing of genes whose encoded proteins are involved in biological pathways that include *BRCA1* and *BRCA2*. Rare coding variants in the *ATM*, *CHEK2*, *BRIP1* and *PALB2* genes confer a two to three times higher breast cancer risk to their carriers (Foulkes, 2008). It is estimated that the contribution of this class of genetic variants to familial risk is less than 3%. This estimation is based on their relatively low frequency and the modest increases in risk that they cause (Pharoah et al., 2008).

2.1.1.1 *ATM*

The *ATM* gene encodes a checkpoint kinase which plays a central role in sensing and signalling in the presence of DNA double-strand breaks. Mutations in the *ATM* gene (OMIM number 607585) are known to cause Ataxia telangiectasia, a rare recessive disorder characterized by neurologic deterioration, telangiectasias, immunodeficiency states, and hypersensitivity to ionizing radiation. Initially there was controversy regarding the role of heterozygous *ATM* mutations in breast cancer predisposition. A number of studies provided conflicting results with estimated relative risk ratios ranging from 1.3 to 12.7 (Angele and Hall, 2000; Ahmed and Rahman, 2006; Renwick et al., 2006). *ATM* is currently classified as a moderate risk breast cancer gene based on the results of a large epidemiological study, which has demonstrated that heterozygous *ATM* mutation carriers have an estimated relative

risk for breast cancer of 2.37 (Renwick et al., 2006).

2.1.1.2 *CHEK2*

CHEK2 is a checkpoint kinase which plays an important role in the DNA damage repair response pathway. A number of *CHEK2* mutations were studied in relation to breast cancer risk but no clear relationship was found (Bell et al., 2007). *CHEK2* 1100delC mutation appears to be the only mutation associated with an increase in breast cancer risk. Heterozygous 1100delC mutation carriers have a two fold increased risk for breast cancer (*CHEK2* Breast Cancer Case-Control Consortium, 2004). In addition, a recent meta-analysis has reported a 4.8 relative risk of developing breast cancer for heterozygous *CHEK2* 1100delC mutation carriers, who have a family history of breast cancer (Weischer et al., 2008). The clinical applicability of this finding still remains uncertain because of population-specific differences in mutation frequency. Offit and Garber (2008) suggested that it is not justified to test for the *CHEK2* 1100delC mutation because of the low mutation frequency. In contrast, Weischer et al. (2008) proposed that *CHEK2* 1100delC should be considered together with *BRCA1* and *BRCA2* mutation screening in women with a family history of breast cancer.

2.1.1.3 *BRIP1*

BRIP1, also known as *BACH1* directly interacts with *BRCA1* and has a role in DNA repair and cell cycle checkpoint. Monoallelic *BRIP1* gene mutations are associated with an increased risk of breast cancer whereas biallelic *BRIP1* mutations cause Fanconi Anemia (Levran et al., 2005; Seal et al., 2006). In a recent study, Seal et al. (2006) screened 1212 individuals with breast cancer from *BRCA1*/*BRCA2* mutation free families and 2081 controls and calculated that the relative risk of breast cancer for heterozygous *BRIP1* mutation carriers is 2.0. It is noteworthy that incomplete segregation of *BRIP1* mutations with breast cancer was observed in this study, which is consistent with a low penetrance allele.

2.1.1.3 *PALB2*

PALB2 is a gene that directly interacts with *BRCA2* and plays a role in homologous recombination and DNA double strand break repair. Biallelic *PALB2* mutations cause Fanconi Anemia and predispose to childhood cancer (Reid et al., 2007).

Monoallelic *PALB2* mutations confer a 2.3-fold higher risk of breast cancer. As in the case of *BRIP1*, incomplete segregation of *PALB2* mutations in families with hereditary breast cancer was observed making predictive screening of unaffected women difficult (Rahman et al., 2007).

In general, the moderate-penetrance breast cancer susceptibility genes make a relatively small contribution to the overall familial risk of breast cancer. Current estimates suggest that carriers of moderate-penetrant mutant alleles in one of the four genes have approximately 6-10% risk of developing breast cancer by age 60, compared to around 3% in the general population (Stratton and Rahman, 2008).

2.1.2 Association studies for discovering low penetrance breast cancer genes

As mentioned above, it is estimated that around 5-10% of all breast cancers and less than 25% of the inherited component of breast cancer can be explained by germline mutations in the known breast cancer susceptibility genes (Easton, 1999; Oldenburg et al., 2007). However, despite the large body of evidence which supports the fact that breast cancer has a strong genetic component, most genes underlying the disease remain unknown. Attempts to localize additional highly penetrant breast cancer susceptibility genes have failed and it was concluded that if any additional high penetrance genes exist, they account for only a small proportion of the excess familial risk (Smith et al., 2006). This led to the suggestion that the remaining breast cancer susceptibility is polygenic in nature and that a number of low penetrance alleles, each conferring a small risk, are involved (Antoniou et al., 2002; Pharoah et al., 2002).

Linkage studies have been very successful in mapping disease genes for monogenic disorders but have limited power to detect low to moderate risk alleles that predispose to polygenic diseases such as breast cancer (Pharoah et al., 2004). An alternative method for mapping disease genes are association studies. Association studies are based on the "common disease-common variant" hypothesis which proposed that common variants possibly in association with environmental factors, underlie most common diseases (Chakravarti, 1999; Reich and Lander, 2001). In association studies the frequency of a genetic variant is compared between a group of cases (affected individuals) and a group of controls (unaffected individuals)

(Cardon and Bell, 2001; Risch, 2000). Allelic association is present when the distribution of the genetic marker differs between the two groups i.e. a variant is associated with disease susceptibility if it is over-represented in the cases group and vice-versa; it is protective for the disease if it is under-represented in the cases group. All the currently known low-penetrance breast cancer susceptibility alleles have been discovered through association studies.

At present, mostly due to technical ease, the most commonly studied form of genetic variation are single nucleotide polymorphisms; SNPs. Single nucleotide polymorphisms (from the Greek meaning "having multiple forms") are classified as commonly occurring (>1%) single base pair genetic substitutions in at least one population. It has been estimated that around 90% of the sequence variants in the human genome are SNPs (Collins et al., 1998). Using the classic neutral theory of population genetics is was inferred that there are about 11 million SNPs in the human genome (Kruglyak and Nickerson, 2001).

Over the last 10 years, considerable research effort has been expended into searching for low penetrance breast cancer susceptibility alleles, via the conduction of association studies. The majority of the initial association studies for breast cancer had concentrated on testing functional SNPs in candidate genes, involved in important biological pathways such as DNA repair, carcinogen metabolism, cell-cycle control and hormone synthesis and metabolism. Recently, studies have shifted towards studying minimal sets of tagging SNPs which can capture common genetic variation in the region / gene of interest (Pharoah et al., 2004).

Numerous individual studies evaluating candidate genes have reported associations which failed to replicate across studies. The failure to replicate these associations has led to scepticism and the majority of these results are nowadays considered to be false-positive findings (Hirschhorn et al., 2002; Ioannidis et al., 2001; Ioannidis et al., 2006; Lohmueller et al., 2003; Wacholder et al., 2004). The lack of replication is mainly due to two factors: insufficient power of individual studies to detect small contributions to risk and low prior probability of a disease association for a given variant. All genetic associations demand a high level of proof and thus replication of findings in large independent data sets is of paramount importance and is regarded as

a prerequisite for convincing evidence of association (Chanock et al., 2007). Recently, scientists from all over the world have joined forces by forming multigroup collaborations and combining data from thousands of breast cancer cases and controls from different ethnics groups, in order to overcome limitations of individual studies and have sufficient power to detect small effects on breast cancer risk (Breast Cancer Association Consortium, 2006).

This collaboration proved to be successful and the Breast Cancer Association Consortium has identified, the only well-validated breast cancer susceptibility locus to emerge so far, using the candidate gene approach. In detail, they identified a SNP in the *CASP8* gene D302H (rs1045485) which is associated with a reduction in breast cancer risk ($P_{trend} = 1.1 \times 10^{-7}$) in this large multiethnic cohort (Cox et al., 2007).

Thus far, candidate gene studies on breast cancer have examined only a small fraction of the more than 25000 genes across the human genome. However, the majority of these studies performed only limited evaluations of genetic variation in a proportion of these genes. It is therefore possible that additional breast cancer susceptibility loci can be identified by the candidate gene approach, in large highly powered studies, involving more comprehensive evaluations of genetic variation. Furthermore, it is believed that this approach will assist scientists in identifying rare variants in candidate genes which are associated with disease risk (Garcia-Closas and Chanock, 2008).

Recently, a new approach which involves the study of genetic variation across the entire human genome by rapid genotyping of hundreds of thousands of SNPs, in many samples aiming to identify genetic associations with a particular disease has emerged. Scientists are taking advantage of the new genotyping platforms, which allow simultaneous genotyping of more than 1.8 million markers for genetic variation and enable high-performance, high-powered and low-cost genotyping to carry out genome-wide association studies (GWAS). With GWAS it is possible to scan the human genome for associations without prior knowledge of gene function or position.

Over the past 2 years, four GWAS for breast cancer have been published. These studies have reported a small number of well-validated, statistically unimpeachable, low penetrance breast cancer susceptibility alleles. Easton et al (2007b) identified five SNPs that were associated with breast cancer risk. The largest effect was observed with a SNP in intron 2 of the *FGFR2* gene (rs2981582) with a per allele relative risk of 1.26 as compared with the low-risk allele. In addition, loci within or near the *TNRC9* (recently renamed *TOX3*), *MAP3K1* and *LSP1* genes as well as a locus in 8q24 that does not contain any known protein-coding genes were associated with disease risk at the genome wide level of association. Subsequent GWAS from the Nurses Health study, an Icelandic study and a familial Ashkenazi Jewish cohort study have replicated the associations observed with the *FGFR2* and *TNRC9* loci and have also identified additional novel susceptibility loci at chromosomal locations 2q35, 5p12 and 6q22.33 (Hunter et al., 2007; Stacey et al., 2007; Gold et al., 2008; Stacey et al., 2008).

The population prevalence of the low risk breast cancer alleles is high and ranges from 28% to 87% (Stratton and Rahman, 2008). It is of interest that for some of these newly identified loci the higher risk allele is the more common. Based on their high frequency and despite the low risks that these predisposition alleles confer, it was estimated that they account for a substantial proportion of familial breast cancer (Stratton and Rahman, 2008).

GWAS for breast cancer are still at an early stage. It is likely that many more breast cancer susceptibility loci, which exhibit weaker effects than the ones identified so far, exist (Easton and Eeles, 2008). It is expected that the next generation of GWAS studies, that will be larger in size and will combine analysis across multiple scans, will identify these additional susceptibility loci (Easton and Eeles, 2008). As more risk alleles are identified, our knowledge on the biological pathways involved in carcinogenesis will improve. It is anticipated that by increasing the number of susceptibility loci identified, the ability to predict risk will improve and single common low penetrance genes may become clinically useful (Easton and Eeles, 2008).

2.1.3 Summary of the three classes of known breast cancer susceptibility alleles

In the last twenty years major advances have been made in understanding susceptibility to breast cancer. Current knowledge supports the existence of three classes of breast cancer susceptibility alleles as outlined below. It is now well accepted that *BRCA1* and *BRCA2* genes are possibly the only highly penetrant breast cancer susceptibility genes. Mutations in these two breast cancer susceptibility genes are rare (population carrier frequency ≤0.1%) and confer a 10-20 fold increased risk for breast cancer. The *BRCA1* and *BRCA2* genes account for a substantial proportion of the familial risk for breast cancer. The second class of breast cancer susceptibility genes consists of genes that confer moderate increases in breast cancer risk. These genes are *ATM*, *BRIP1*, *CHEK2* and *PALB2* which are all involved in *BRCA1*- and *BRCA2*-related biological pathways. Heterozygous mutations in these genes are rare (population carrier frequency ≤0.6%) and their carriers have a two to three fold relative risk for breast cancer. The possibility that other moderate-penetrance breast cancer susceptibility alleles exist cannot be excluded since mutations in these genes are quite uncommon. Resequencing studies which will include large families may help towards this direction. The third class of genetic risk alleles for breast cancer has emerged more recently and consists of low penetrance susceptibility alleles, mainly SNPs which confer risks of 1.3-fold or less. These SNPs were identified by GWAS and are very common (population carrier frequency 5-50%). It is very likely that a number of additional low penetrance breast cancer susceptibility alleles exist. It is anticipated that these additional susceptibility alleles will be identified within the next few years by large scale GWAS (Stratton and Rahman, 2008). Figure 9 summarizes the genetic landscape of breast cancer susceptibility.

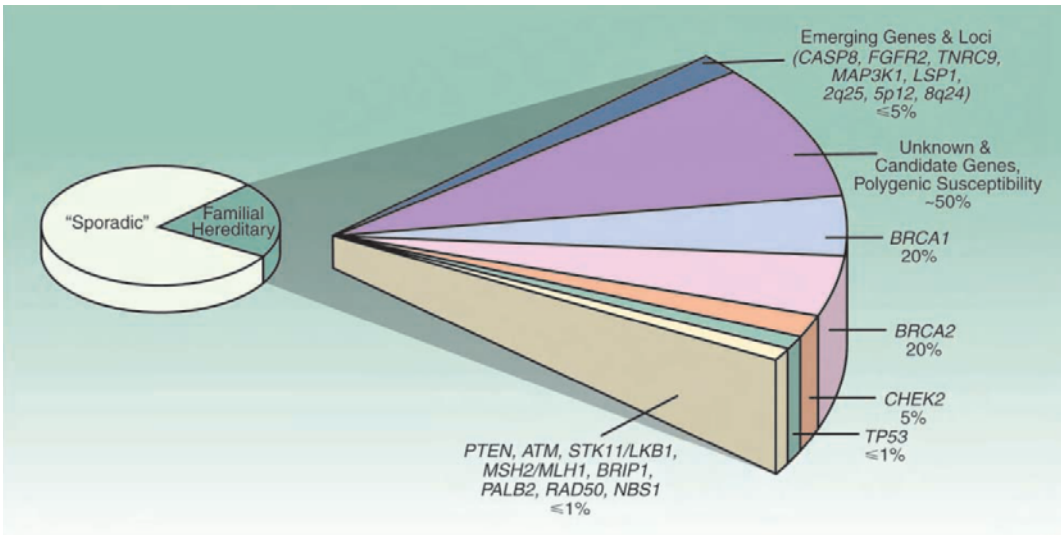


Figure 9: Summary of genes predisposing to breast cancer (taken from Olopade et al., 2008)

2.2 DNA repair

Mammalian cells are under constant mutagenic attacks from both endogenous reactive metabolites as well as a plethora of exogenous agents, including chemical carcinogens, ionizing radiation, ultraviolet rays and chemotherapeutic drugs that impact its integrity. Consequently, multiple mechanisms for repairing DNA damage and maintaining genomic integrity have evolved. DNA repair is essential for survival of mammalian cells. Deficiencies in the cell's DNA repair system can cause hypersensitivity to DNA damaging agents, accumulation of mutations and chromosomal aberrations which in turn lead to cell malfunctioning, cell death and tumorigenesis (van Gent et al., 2001).

Inherited deficiencies in the DNA repair mechanism are responsible for a number of highly penetrant genomic instability syndromes that have cancer as a predominant phenotype. Examples include Bloom and Werner's syndromes, Fanconi Anemia and Xeroderma Pigmentosum. These syndromes are characterized by hypersensitivity to DNA damaging agents leading to mutation accumulation and in turn to tumour formation. (Hamosh et al., 2005). The link between inherited DNA repair deficiencies and susceptibility to cancer highlights the importance of safeguarding genomic integrity via efficient DNA repair processes.

The human genome has about 150 genes that are associated with DNA repair. The DNA repair genes can be split into two main categories: those which are associated with signalling and regulation of DNA repair and those which are associated with distinct repair mechanisms such as base excision repair (BER), nucleotide excision repair (NER) and DNA double-strand break (DSB) repair (Wood et al., 2005).

The evolutionary process has endowed us, with a sophisticated DNA repair system. In mammals there are at least five main repair pathways that protect us against DNA damaging agents - BER, NER, homologous recombination (HR), non homologous end joining (NHEJ) and mismatch repair (MMR) which are illustrated in Figure 10. The pathway choice is dependant on the type of damage (Friedberg et al.,1995; Lindahl and Wood, 1999).

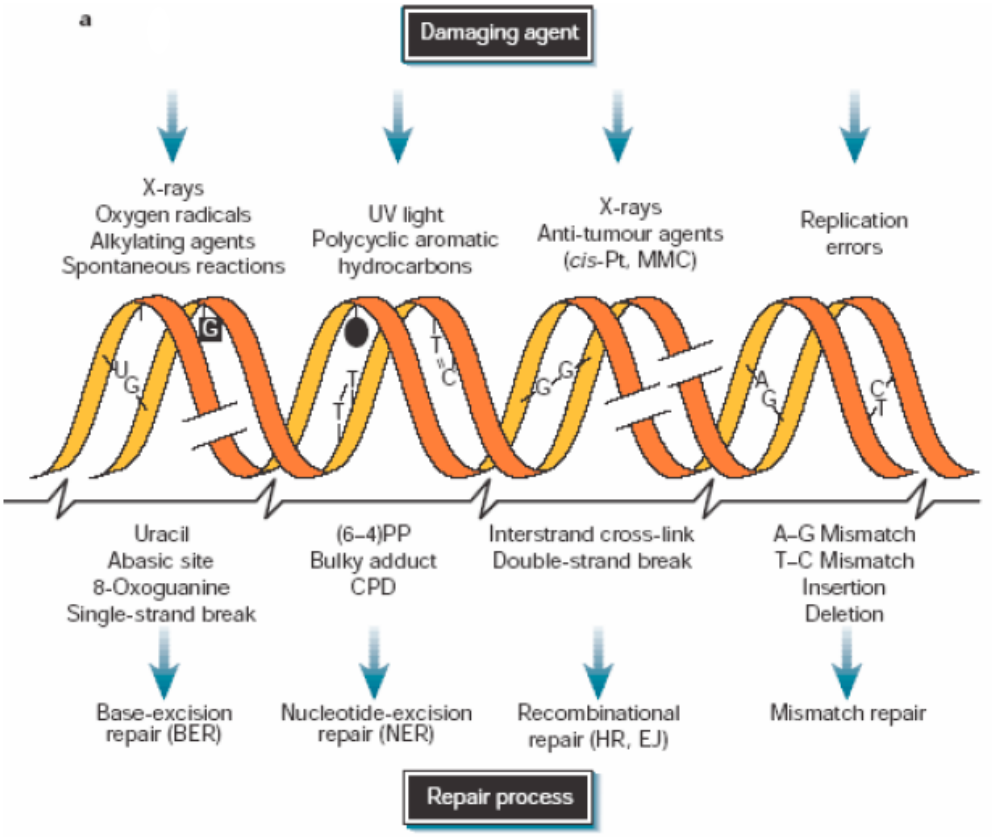


Figure 10: Common DNA damaging agents, examples of DNA lesions induced by agents, and most relevant DNA repair mechanism responsible for removal of lesion. Taken from Hoeijmakers (2001).

2.2.1 Base excision repair

BER is the main mechanism that repairs oxidized DNA bases arising either spontaneously within the cell, due to reactive oxygen radicals, methylation, deamination and hydroxylation or from attack by exogenous agents including ionizing radiation and long-wave UV light. Hence, BER is mostly responsible for recognizing and repairing minor damages to DNA bases and sugars Christmann et al. (2003).

There are at least 12 different DNA damage specific glycosylases as well as about 20 other proteins which participate in the BER process (Christmann et al., 2003) . The first step of BER is carried out by specific DNA glycosylases that recognize the specific base modification and excise the lesion by hydrolysis of the N-glycosylic bond, which connects the base to the sugar-phosphate backbone, forming an AP site (Scharer and Jiricny, 2001). The abasic site is subsequently recognized by *APE1* endonuclease protein, which cleaves the phosphodiester bond 5' to the abasic sugar site, leaving behind a nick with a normal 3' hydroxyl group and an abnormal 5' abasic terminus (Wilson and Barsky, 2001). "Short-patch" BER that is the dominant mode in mammals, proceeds with DNA polymerase β removing the 5'-abasic residue and filling in the single nucleotide gap (Wiebauer and Jiricny, 1990; Dianov et al., 1992; Sobol et al. 1996). Next, the nick is sealed by either the *XRCC1*/*Ligase III* complex or DNA *ligase I* (Kubota et al., 1996). An alternative BER sub pathway referred to as “long-patch” BER exists. “Long-patch” BER repair mode occurs by the excision of at least 2 nucleotides by *FEN1* (flap structure-specific endonuclease 1) protein (Klungland and Lindahl, 1997). DNA synthesis is catalyzed by either a *PCNA*-stimulated, *Pol β*-directed pathway or a *PCNA*-dependent, *Pol δ/ε* -directed pathway (Stucki et al., 1998). Finally, DNA *ligase I* seals the nick to complete the repairing process (Tomkinson et al., 2001). A summary of the BER mechanism is depicted in Figure 11.

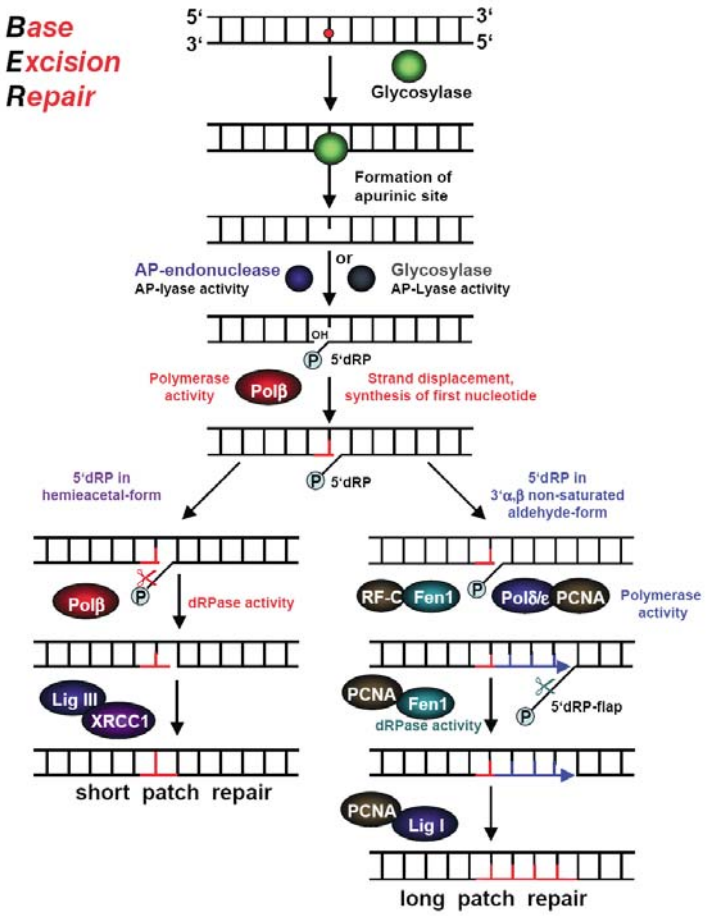


Figure 11: Mechanism of base excision repair. Taken from Christmann et al. (2003)

2.2.2 Nucleotide excision repair

Enzymes involved in NER recognize and repair chemical modifications to DNA bases which cause bulky distortions in the shape of the DNA double helix. Consequently this pathway is critical for protecting against agents that cause structural distortion in DNA including pyrimidine dimers, photodimers resulting from UV irradiation and DNA intra-strand cross-links. There are about 30 proteins which are involved in the NER pathway (Christmann et al. 2003).

NER consists of 2 distinct sub-pathways termed global genomic repair (GGR) and transcription coupled repair (TCR). GGR is responsible for the removal of lesions from the non-transcribed domains of the genome. In GGR, several sensing proteins including the DDB and *XPC-RAD23B* [*RAD23* homolog B (S. cerevisiae)] complexes screen the genome and recognize DNA helix distortions (reviewed in

Balajee and Bohr, 2000; Hanawalt, 2002; Mullenders and Berneburg, 2001). TCR focuses on repairing the damage that blocks elongating RNA-polymerases in the transcribed strand of active genes (Bohr et al., 1985; Mellon et al., 1987). In response to damage, RNA polymerases are displaced by the action of TCR-specific factors *CSA* and *CSB* as well as NER proteins *XPB*, *XPD* and *XPG* in order to make the area accessible for repair (Schaeffer et al., 1993; Le Page et al., 2000). The subsequent steps of GGR and TCR are identical. Upon identification of the DNA damage site more than 25 NER proteins are recruited to the site of the lesion to excise the damaged DNA surrounding the lesion and synthesize and ligate the resulting gap (Hoeijmakers, 2001; Christmann et al. 2003). In detail, *XPD* and *XPB* helicases of the *TFIIH* transcription factor open around 30 bases of DNA in the area surrounding the damage and *RPA* binds to the undamaged strand (Schaeffer et al.1993 ; Schaeffer et al., 1994; Yokoi et al., 2000). Next, *ERCC1*/*XPF* endonuclease cuts the damaged strand 5' of the damaged base while *XPG* endonuclease performs the same action 3' of the damaged base (Habraken et al., 1994 ; O'Donovan et al., 1994 ; Sijbers et al., 1996). The cleaved nucleotides, which contain the damage, are then removed and the regular DNA replication machinery is recruited to fill in the gap (Hoeijmakers, 2001). A summary of the NER mechanisms is shown in Figure 12.

In section 1.8.3.1 of this thesis, the role of *BRCA1* in NER mechanisms was discussed. *BRCA1* is implicated in both transcription-coupled repair and global genomic repair (Le Page et al., 2000; Wang et al. 2000; Hartman and Ford; 2002).

There are at least three syndromes, which are associated with defects in NER pathway: Xeroderma Pigmentosum, Cockayne syndrome and Trichothiodystrophy. All three syndromes are characterized by extreme ultraviolet sensitivity and an increased risk of sun-induced skin cancer (Christmann et al., 2003; Hoeijmakers, 2001; Mohrenweiser et al., 2003).

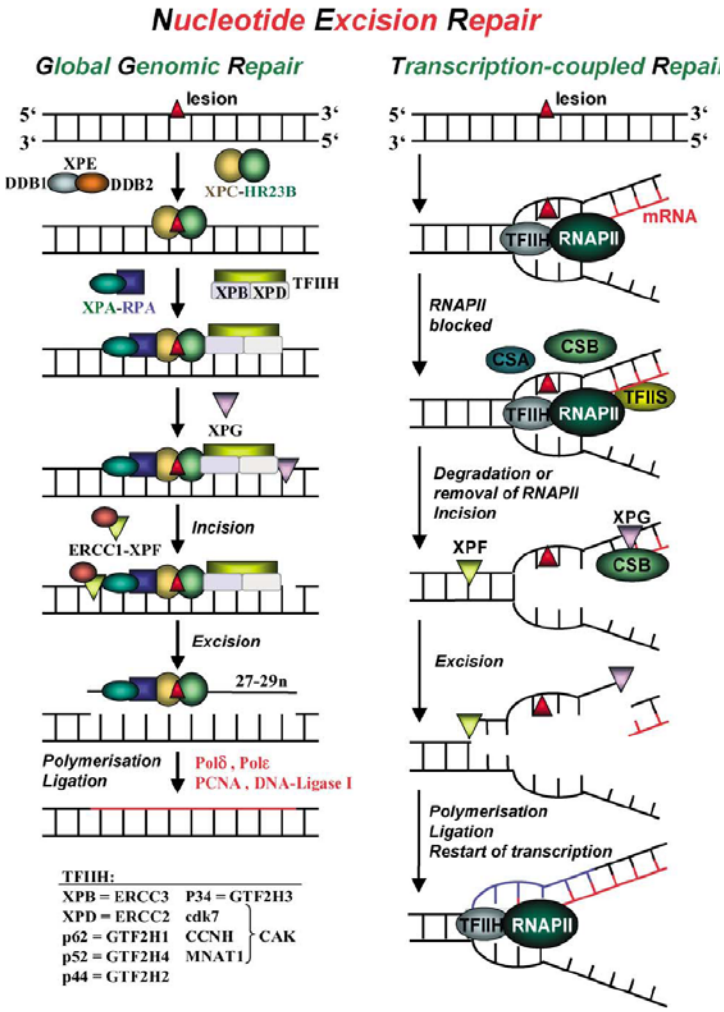


Figure 12: Mechanisms of nucleotide excision repair. Taken from Christmann et al. (2003). *BRCA1* is implicated in both GGR and TCR. *BRCA1* enhances GGR repair by inducing expression of *XPC*, *DDB2* and *GADD45* (not pictured) genes which are involved in recognition of damaged DNA (Hartman and Ford, 2002). *BRCA1* is also implicated in TCR via the activation of the RNA polymerase II transcription machinery. It is essential for the repair of the 8-oxoguanine oxidative damage, located on the transcribed strand in human cells. (Le Page et al., 2000).

2.2.3 Double strand break repair

DNA double-strand breaks are potentially harmful damages that can arise either directly from exposure to DNA-damaging agents such as ionizing radiation, X-rays, free radicals and chemicals or may spontaneously arise due to replication failure (Hoeijmakers, 2001). It is noted that only one non-repaired DSB in an essential gene

is sufficient for inducing cell death via apoptosis. This highlights the importance of the existence of efficient DSB repair mechanisms (Rich et al., 2000).

There are two different pathways involved in DSB repair: HR and NHEJ. In mammals the predominant DSB repair pathway is NHEJ whereas in simple eukaryotes, HR is the main pathway (Cromie et al., 2001; Haber, 2000). Moreover, the occurrence of HR or NHEJ also depends on the cell cycle phase. HR takes place during late S and G2 phases whereas NHEJ takes place mainly in G0/G1 phases (Takata et al., 1998; Johnson and Jasin, 2000).

2.2.3.1 Homologous Recombination

HR uses nucleotide sequence complementarity between the intact and the damaged chromatid, as a basis for properly repairing DSB. HR is an error-free pathway since it entails copying the missing information from an undamaged homologous chromosome (Sonoda et al., 2001).

Briefly, in HR the DSB is first resected in the 5' to 3' direction by the MRN complex (*MRE11-RAD50-NBS1*). The resulting 3' single-stranded tails are bound by a heptameric ring complex formed by the *RAD52* proteins in order to be protected against exonucleolytic digestion (Stasiak et al., 2000). The resulting 3' single-stranded tails then invade the DNA double helix of a homologous, undamaged partner molecule. These strand-exchange events are catalyzed by *RAD51* that has the ability to bind single-stranded DNA and promote ATP-dependent and *RPA*-stimulated interactions, with homologous regions on undamaged DNA molecules. In detail, *RAD51* forms filaments along the unwound DNA strand to facilitate strand invasion. *RAD51* nucleoprotein filament includes five *RAD51*-related proteins: *XRCC2*, *XRCC3*, *RAD51B*, *RAD51C* and *RAD51D*. Following DSB recognition and identification of the homologous undamaged sequence, the broken ends are extended by the action of DNA polymerase, which copies information from the intact double-stranded copy. Finally the ends are ligated by DNA ligase I and the interwound DNA strands, known as Holliday junctions, are resolved by resolvases according to the classical model of Holliday (Haber, 2000; Khanna and Jackson, 2001; van Gent et al., 2001). The mechanism of DNA repair by HR is summarized in Figure 13.

As it was already mentioned in section 1.8.3 of this thesis, the proteins encoded by the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are integral components of the HR pathway due to their interactions with *RAD51* protein. *BRCA2*, via its BRC repeats and a C-terminal domain interacts directly *RAD51* and plays a major role in the control, localization and function of this key recombinase, whereas *BRCA1* has an indirect association with *RAD51* which is possibly mediated by *BRCA2*. Furthermore, HR is mediated by the BASC complex. *BRCA1* is part of this complex (Narod and Foulkes, 2004). Studies have shown that *BRCA2* deficiency leads to impaired DSB repair by HR (Moynahan et al., 2001; Tutt et al., 2001; Xia et al., 2001). In addition, *BRCA1* mutant cells have an impaired ability to repair DNA damage by HR (Deng and Wang, 2003; Moynahan et al., 2001; Snouwaert et al., 1999).

Deficiencies in HR have been strongly linked to carcinogenesis. Malfunctioning copies of helicase genes involved in HR regulation cause a number of cancer-related syndromes including Bloom's, Werner's and Rothmund-Thomson (Kitao et al., 1999).

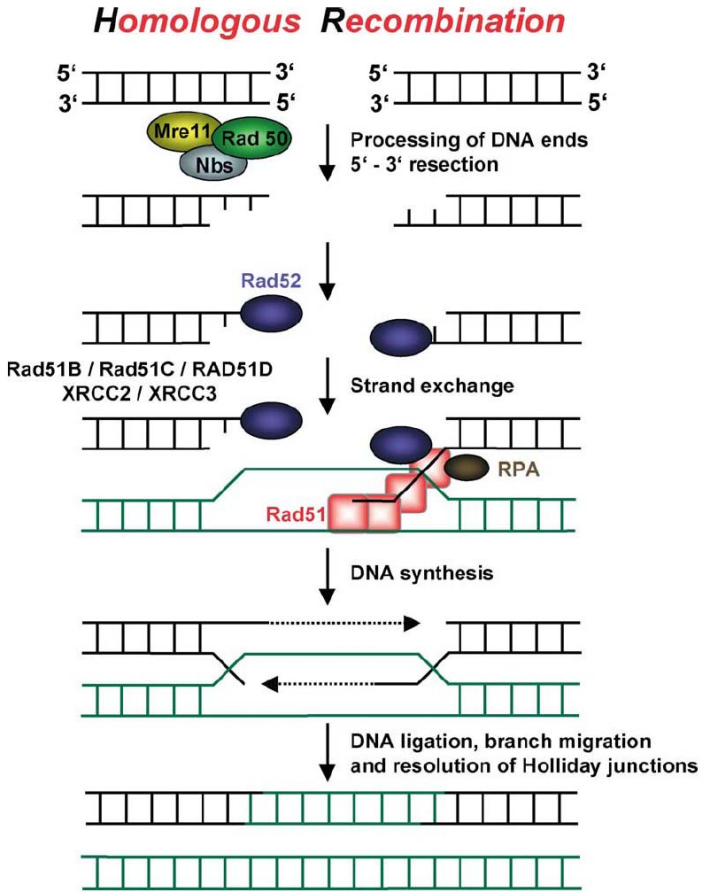


Figure 13: Mechanism of homologous recombination repair. Taken from Christmann et al. (2003). HR is initiated by single-stranded DNA (ssDNA) generation. This process is promoted by a number of proteins including the MRN complex (*MRE11-RAD50-NBS1*). Next, ssDNA invades the undamaged template, in events catalyzed by *RAD51*, *BRCA1* and *BRCA2*. Following the actions of specific polymerases, helicases and other essential components, DNA ligation and substrate resolution occur.

2.2.3.2 Non-homologous End Joining

In humans, the predominant mechanism of DSB is NHEJ. In contrast to HR, the NHEJ system does not require sequence homology between the DNA ends. DSB are repaired by directly ligating the two DSB ends (Critchlow and Jackson, 1998). NHEJ mechanism involves 5 steps: detection of DSB, molecular bridging of broken DNA ends, processing of the DNA ends to make them compatible and ligatable, gap filling and ligation (Figure 14). The processing of broken DNA ends in most cases, results in loss of a few nucleotides at the broken ends, making NHEJ pathway error-prone (Christmann et al. 2003).

There is growing evidence suggesting that there are multiple pathways for NHEJ. The best understood pathway involves *Ku* proteins, DNA-dependent protein kinase, and the *DNA ligase IV-XRCC4* complex (Jazayeri and Jackson, 2002). However, it was demonstrated that cell extracts lacking *DNA-PKcs*, as well as *Ku*-depleted extracts could still perform efficient end joining. This indicates the existence of additional *Ku*-independent pathways of NHEJ (Wang et al., 2003). Moreover, cell extracts from Fanconi Anemia fibroblasts, have a deficiency in NHEJ process that appears to be independent of *DNA-PKcs* and *Ku* (Lundberg et al., 2001).

The first step of *Ku*-dependent NHEJ pathway involves the recognition and binding of the DNA ends by the *Ku70* and *Ku80* heterodimer. This step protects DNA from exonuclease digestion. Next, the *Ku* heterodimer recruits and activates the catalytic subunit of DNA-dependent protein kinase (*DNA-PKcs*) together forming the DNA-PK holoenzyme. *Ku* proteins and *DNA-PKcs* are both capable of attaching to the DNA ends. *Ku* translocates internally upon binding of the *DNA-PKcs* to the DNA end. The DNA ends need to be processed and trimmed for proper annealing. A fraction of DSB ends are processed by *Artemis* nuclease and *DNA-PKcs*. *Artemis* forms a complex with *DNA-PKcs*, which acts as an endonuclease at both 5'and 3' overhangs degrading single-strand overhangs and hairpins. Processing of DSB is also performed by the MRN complex that removes excess DNA at 3' flaps as well as by *FEN1*, which removes 5' flaps. Polymerases *Pol* μ and *Pol* λ possibly fill in all the gaps. Finally, ligation to form a single DNA molecule is performed by *XRCC4* - *DNA ligase IV* complex. This complex is recruited by and interacts with *Ku* and *DNA-PKcs*. It binds to the ends of DNA molecules and links together duplex DNA molecules with complementary but not ligatable ends (Khanna and Jackson, 2001; van Gent et al., 2001; Christmann et al., 2003; Schulte-Uentrop et al., 2008).

As it was already mentioned in section 1.8.3.1 of this thesis, *BRCA1* is involved DNA repair by NHEJ. BRCA1 binds *in vivo* and *in vitro* to the MRN complex which is an important component of the NHEJ pathway (Bau et al. 2006).

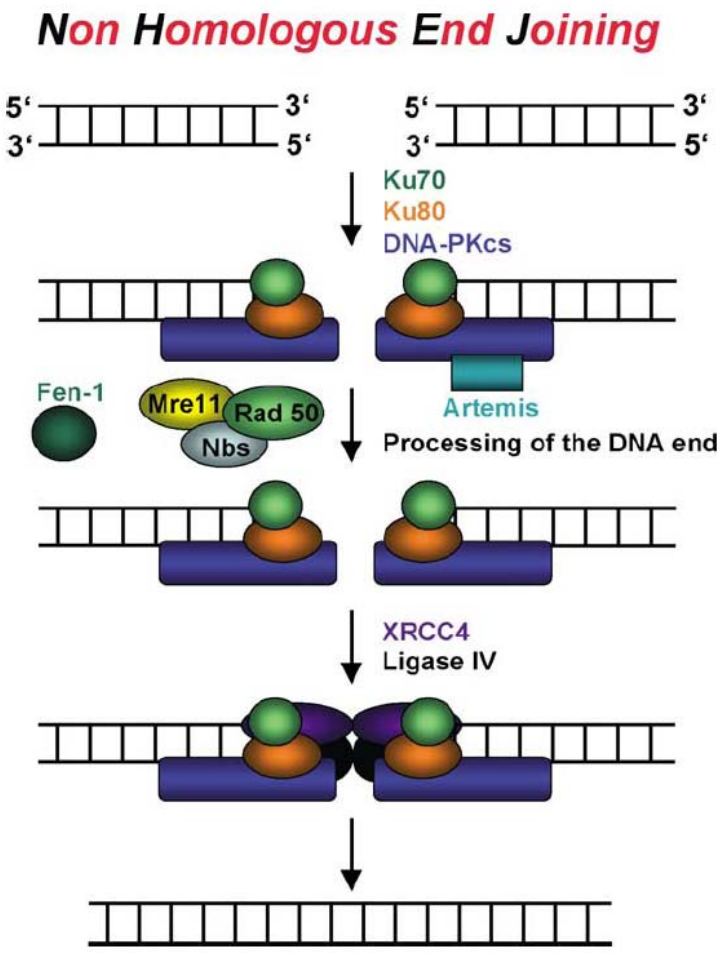


Figure 14: Mechanism of non-homologous end joining. Taken from Christmann et al. (2003). NHEJ pathway is initiated by the binding of *Ku*70/Ku80 heterodimer to the broken DNA ends. The DSB-bound *Ku* then recruits the catalytic subunit *DNA-PKcs*, which forms a complex with *Artemis* nuclease. The DNA ends are rejoined by *XRCC4*/ *DNA ligase IV* complex. Recently, *XLF* (*XRCC4*-like factor; also called Cernunnos), a new factor of the NHEJ pathway has been identified (Ahnesorg et al., 2006; Buck et al., 2006). *XLF* has at least 2 distinct functions NHEJ. The major role of *XLF* protein in NHEJ is the stimulation of the ligase activity of the *XRCC4*/ *DNA ligase IV* complex (Hentges et al., 2006). *XLF* also has a function to the early process of NHEJ. It responds to DSB induction and accumulates at the damaged site within a few seconds (Yano and Chen, 2008).

BRCA1 is implicated in the NHEJ pathway via its interaction with the MRN complex. The exact mechanism of interaction and its involvement in NHEJ is not yet fully know. MRN complex proteins are involved in processing broken DNA ends, making them available for ligation by the *XRCC4*/ *DNA ligase IV* complex.

2.3 Genetic polymorphisms, DNA repair capacity and breast cancer risk

2.3.1 Genetic polymorphisms

As it was mentioned in section 2.2, mammalian cells are equipped with various DNA repair mechanisms, to preserve genomic integrity. However, from an evolutionary perspective, genetic variation is the raw material of evolution. A certain level of continuous mutagenesis may be deemed necessary to allow individuals to cope with environmental or lifestyle shifts and challenges. Sequence changes take place throughout the human genome. The vast majority of these alterations are non-coding changes which have no or little effect on cellular function (Shastry, 2007).

Humans share more than 99.9% of their genome sequence. Single nucleotide polymorphisms explain around 90% of all sequence variants in the human genome (Collins et al., 1998). It is estimated that approximately 11 million SNPs exist in the human genome with a minor allele frequency (MAF) of 1% and approximately 5 million SNPs with a MAF of 10% (Ladiges et al., 2004). However, only a small number (50,000–250,000) of these SNPs are functionally important, resulting in small/moderate alterations on phenotypes that are related to disease risk (Chanock, 2001). The National Center for Biotechnology Information (NCBI) has established the Single Nucleotide Polymorphism Database (dbSNP) in response to a need for a general catalog of genome variation (Sherry et al., 2001). According to data obtained from the dbSNP database on December 2008, there are 19,125,432 registered SNPs in the database which are distributed throughout the 3.2 billion bases of the human genome.

2.3.2 Reduced DNA repair capacity and breast cancer risk

There is growing evidence that human cancer can be initiated by DNA damage caused by endogenous and exogenous mutagens. It was estimated that in normal human cells approximately 1% of single-strand lesions are converted to approximately 50 endogenous double-strand breaks per cell per cell cycle (Vilenchik and Knudson, 2003). These double strand breaks are usually repaired with a high fidelity. Unrepaired DNA double-strand breaks, due to either a decrease in HR accuracy or to shunting repair events in NHEJ pathway that are inherently mutagenic, contribute significantly to the rate of cancer. A series of additional

mutations and epigenetic changes are needed in order to lead to tumour formation. Disruption of the caretaker functions of DNA repair genes leads to the inactivation of gatekeeper genes that regulate growth or promote cell death. These events result in acceleration of the multi-step process of carcinogenesis and give rise to early onset cancer. Consequently, women with a reduced DSB repair capacity are thought, to be at an increased risk of breast cancer (Ralhan et al., 2007).

It is evident that the DNA repair process has a critical role in protecting the genome against mutations that lead to cancer. Research focusing on genetic variation in DNA repair genes is driven by the hypothesis that SNPs in DNA repair genes can reduce their capacity to repair DNA damage and thereby lead to increased cancer susceptibility. This idea has been fuelled by skin, colorectal and breast cancers since a number of cases are attributed to hereditary defects in NER, mismatch repair and the *BRCA1/2* genes, respectively (Foulkes, 2008).

It has been suggested that inter-individual differences in DNA repair capacity modify cancer risk (Berwick and Vineis, 2000). Evidence supporting this comes from epidemiological studies, focusing primarily on lung and skin cancer, which have reported that deficiencies in DNA repair capacity lead to accumulation of DNA damage and accelerate the carcinogenesis process. (Berwick and Vineis, 2000; Michiels et al., 2007). To test the hypothesis that reduced DNA repair capacity underlies breast carcinogenesis, many studies have examined lymphocytes that were treated *in vitro* with DNA-damaging agents. DNA damage was evaluated using a number of phenotypic assays for cancer risk including Comet assay for measuring DNA strand breaks and mutagen sensitivity assay (Berwick and Vineis, 2000; Rajeswari et al., 2000; Ramos et al., 2004; Shi et al., 2004; Smith et al., 2003b). All studies have reported an increased risk of breast cancer in individuals with greater sensitivity to mutagens or poorer DNA repair capacity. Characteristically, a Puerto Rican study by Ramos et al. (2004) reported that the DNA repair capacity of breast cancer patients was severely reduced (36%) compared to healthy controls. The study concluded that a 1% decrease in the DNA repair capacity corresponded to a 22% increase in breast cancer risk. In summary, the findings of these studies support the hypothesis that reduced DNA repair capacity is associated with an increased risk of breast cancer.

Recent studies have adopted a family based design in order to assess the relationship between DNA repair capacity and breast cancer risk. Sister sets discordant for breast cancer were used in order to reduce potential confounding due to population admixture, differences in genetic susceptibility as well as lifestyle factors that cluster within families. Large differences in DNA repair capacity were observed between these sisters' sets, which had other breast cancer risk factors in common. This observation further supports and strengthens the hypothesis that deficient DNA repair capacity is associated with susceptibility to breast cancer (Kennedy et al., 2005; Machella et al., 2008).

It has also been suggested that deficient DNA repair capacity predisposes to familial and sporadic breast cancer. Ionizing radiation that is an established risk factor for breast cancer as well as other suspected risk factors such as chemical carcinogens, alcohol and diet result in reactive oxygen species, bulky DNA adducts and DNA strand breaks (Ron, 1998; Johnson-Thompson and Guthrie, 2000). As a result of this, women may acquire modifications in critical oncogenes and tumour suppressor genes leading to cellular transformation and carcinogenesis (Gonzalez et al., 1999; Katsama et al., 2000). A number of studies have reported that breast cancer patients as well as healthy women with a family history of breast cancer, are hypersensitive to IR and may have deficient DNA repair capacity compared to healthy women without a family history. Hence, a combination of exposure to genotoxic agents with genetic factors may elevate breast cancer risk (Helzlsouer et al., 1996; Parshad et al., 1996; Patel et al., 1997; Scott et al., 1999; Hu et al., 2002).

The importance of DNA repair mechanisms in carcinogenesis is emphasized by familial cancer syndromes, which are linked to aberrant DNA repair activity. The two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* play an important role in the HR pathway for repairing DSB. This further supports the hypothesis that DNA repair mechanisms are of particular etiological importance during breast carcinogenesis. Consequently, genes involved in DNA repair pathways, are possible breast cancer susceptibility candidates. Notably, the 10 established breast cancer susceptibility genes, which are implicated in inherited predisposition to breast cancer, are all involved in pathways critical to genomic integrity (Walsh and King, 2007).

In addition to familial breast cancer, genes involved in the DNA repair pathway also play a role in sporadic breast cancers. A number of studies have shown that there was reduced or absent expression of *BRCA1*, in 30-40% of breast cancers, indicating a more general role of *BRCA1* in breast carcinogenesis (Thompson et al., 1995; Wilson et al., 1999). Additionally, there is evidence that low or absent expression of *ATM*, *CHEK2* and *TP53* occurs in sporadic breast cancers (Angele and Hall, 2000; Sullivan et al., 2002). Loss of heterozygosity (LOH) of *TP53*, *BRCA1*, *BRCA2*, and *ATM* genes has been demonstrated to occur early in sporadic breast tumorigenesis and to contribute to genetic heterogeneity during tumour development (Meng et al., 2004). The study of sporadic breast tumours has also revealed loss of heterozygosity in a number of additional genes that are involved in DNA repair. LOH was found in the *RAD51*, *RAD52* and *RAD54* regions in 32%, 16% and 20% respectively, of the breast specimens investigated, suggesting that LOH in these regions, could be related to breast cancer and poor tumour prognosis (Gonzalez et al., 1999).

Taken together, these data support the hypothesis that common variation in genes involved in DNA repair pathways may influence breast cancer risk. A large number of such common variants have been identified by resequencing DNA repair genes, in samples from unrelated individuals (Shen et al., 1998a; Kuschel et al., 2002; Mohrenweiser et al., 2002). Novel SNPs are being identified continuously by large-scale genotyping efforts such as the National Institute of Environmental Health Sciences (NIEHS) SNPs Program. Candidate genes are continuously being sequenced to identify common sequence variation for functional analysis and population-based studies.

2.3.2.1 DNA repair SNPs and association with breast cancer risk

The reporting of SNPs that are associated with breast cancer risk has increased exponentially in recent years. Consequently, the number of genetic epidemiology studies examining associations between DNA repair SNPs and breast cancer risk has also increased rapidly. In the last seven years, several studies have identified variant alleles, which are associated with breast cancer risk. In this section, some of these studies, which have found associations between SNPs in DNA repair genes and breast cancer risk will be outlined. It is not the intention in this section, to provide a complete review of the related literature as the list of SNPs studied to date in relation

to breast cancer risk is exhaustive, and is increasing rapidly.

One of the first large-scale studies analysing SNPs in DNA repair genes in relation to breast cancer risk, was conducted in the U.K by Kuschel et al. (2002). The study consisted of 2205 breast cancer cases and 1826 healthy controls. Associations were found for two SNPs in the *XRCC3* gene, namely T241M and IVS5 A>G at nucleotide 17893. Homozygous carriers of the 241M allele had an increased risk for breast cancer. In addition, the rare allele of IVS5 A>G was associated with a dominant protective effect. A marginally significant association was also observed for the rare variant in R188H of the *XRCC2* gene. Furthermore, a SNP in the *LIG4* gene (T>C at nucleotide 1977) was associated with a decrease in breast cancer risk. In brief, this study provided evidence that SNPs in *LIG4*, *XRCC2* and *XRCC3* genes alter breast cancer risk and that variation in genes involved in DNA repair pathways, plays a role in breast carcinogenesis.

Recently, a collaborative study has evaluated the association of 19 SNPs in 7 genes involved in DSB DNA repair and breast cancer risk. The basis of this study were 2 population-based case-control studies in the USA (3,368 cases and 2,880 controls) and Poland (1,995 cases and 2,296 controls). Weak associations with breast cancer were found for *XRCC3* T241M and IVS7-14A>G variants (pooled odds ratio (95% CI): 1.18 (1.04-1.34) and 0.85 (0.73-0.98) for homozygous variant vs. wild-type genotypes, respectively), as well as for a rare variant in *ZNF350* S472P [1.24 (1.05-1.48)], with no evidence for study heterogeneity. A meta-analysis of studies in the Caucasian population which provided support that *BRCA2* N372H variant is also weakly associated with breast cancer risk (1.13 (1.10-1.28); total of 13,032 cases and 13,314 controls) was also conducted (Garcia-Closas et al., 2006).

One of the bigger studies on the contribution of genetic variation in DNA repair genes in relation to breast cancer risk, is the one carried out by Haiman et al. (2008). The study examined common genetic variation across 60 genes in a multiethnic cohort consisting of five different racial/ethnic populations. Over 2600 SNPs were genotyped in each population and LD patterns at each candidate locus were identified. A total of 2093 cases and 2303 controls participated in that study. Validations of the top allelic associations were performed in additional 6483 cases

and 7309 controls. A SNP in the *FANCA* gene (rs1061646) was associated with an increased risk for breast cancer in both the initial as well as in the replication studies. This SNP yielded an 8% increase in risk per allele. No other statistically significant associations were observed. The study concluded that common variation in the examined DNA repair genes is not strongly associated with breast cancer risk.

Johnson et al. (2007) have reported that combinations of rare coding variants in the *BRCA1, BRCA2, ATM, TP53* and *CHEK2* genes are significantly associated with breast cancer risk among women with bilateral breast cancer. The study analyzed 1037 potentially functional SNPs in 473 women with bilateral breast cancer and 2463 controls. For 21 SNPs, with a MAF of less than 10%, a significant trend in risk was observed with increasing numbers of variant alleles (P_{trend} = 0.00004, odds ratio for 3 or more SNPs = 2.90, 95% CI 1.69–4.97). The study suggested that the combined effects of functional variants in the *BRCA1, BRCA2, ATM, TP53, CHEK2* genes as well as a few other candidate genes such as *PALB2* and *BRIP1* may account for a substantial proportion of breast cancer.

In January 2009, a field synopsis on low-penetrance variants in DNA repair genes and cancer susceptibility was published (Vineis et al., 2009). The aim of this study was to identify all articles published between 1985 and August 2007 reporting frequencies of DNA repair gene variants in cancer patients and healthy cancer-free controls. A summary of these articles is available on the DNA repair web site of the Institute for Scientific Interchange foundation (http://www.episat.org). The systematic searches identified 82 studies that referred to breast cancer risk and DNA repair gene variants. The meta-analysis of these studies failed to identify many signals with strong credibility. In detail, statistically significant associations were found for 7 SNPs in the *BRCA2*, *ERCC4*, *PARP1*, *TP53* and *XRCC3* genes. However, all SNPs lost their statistical significance after excluding studies in which the requirement for HWE was not met or after excluding the first published studies on the respective association. From this study it was concluded that the genetic effects, if any, of the DNA repair gene SNPs that were part of the analyses in breast cancer, are small in magnitude. Additional variants in DNA repair genes should be studied in order to help us improve our understanding, on the role and effects of DNA repair genes in the aetiology of breast cancer. For this reason Chapter 4 of this thesis involves the study of genetic variation in selected DNA repair genes in

relation to breast cancer risk.

Part II

Experimental Work

Chapter 3

Early onset breast cancer study

3.1 Materials and Methods

3.1.1 Subjects

Twenty-six consecutive incident female breast cancer cases diagnosed before the age of 40, between the years 2003 and 2004, participated in this study. These Cypriot patients were selected on the basis of a diagnosis of early-onset breast cancer, under age 40, irrespective of their family history. All patients had a histological diagnosis of breast cancer and were clinically managed by the Bank of Cyprus Oncology centre, which operates as a referral centre and offers treatment and follow-up for 80-90% of all breast cancer cases diagnosed in Cyprus. Patients received genetic counselling and were informed about the aims of the study. All 26 women agreed to undergo genetic testing, gave an informed consent and provided blood samples. Information on family history of cancer, with emphasis on breast or ovarian cancer incidence, was obtained and pedigrees were constructed. Cancer diagnoses for patients and their affected relatives were verified by reviewing histological reports.

3.1.2 Control Samples

A control group that consisted of 50 DNA samples from 50, age-matched, unrelated healthy Cypriot women, with no family history of breast or ovarian cancer, was recruited. This was used to estimate the frequency of the detected *BRCA1* and *BRCA2* variants in the general population and to assess whether nucleotide variants were likely polymorphisms.

3.1.3 Preparation of total genomic DNA

Genomic DNA was isolated from peripheral-blood lymphocytes using a standard extraction protocol (phenol-chloroform method). Briefly, red blood cells were lysed using RBC lysis buffer (Sambrook et al., 1989). White blood cells were separated from the lysed red blood cells by centrifugation. An extraction buffer containing SDS was used together with proteinase K to lyse the white blood cells by overnight incubation at 37^0C. This was followed by a phenol-chloroform extraction step and the DNA was precipitated using ethanol. DNA samples were resuspended in 400 μl of HPLC grade water.

3.1.4 Polymerase chain reaction (PCR)

Polymerase chain reaction was used to amplify the entire coding sequence and intron- exon junctions of the *BRCA1* and *BRCA2* genes. Briefly, preparation of the PCR included 30 ng of genomic DNA in total volume of 25 μl with 0.25 mM of each appropriate primer, in the presence of Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; at pH 8.7 (Qiagen 10X PCR buffer), 0.25 mM of dATP, dCTP, dGTP, dTTP (Invitrogen) and 1 unit of Taq DNA polymerase (Qiagen). Amplification was carried out on an Applied Biosystems 9700 thermocycler. The thermocycling profile involved an initial denaturing step at 94^0C 5 min followed by 35 cycles of a denaturing step for 20 sec at 94^0C, an annealing step for 20 sec at 55^0C, and an extension step for 20 sec at 72^0C, with a final extension for 7 min at 72^0C. The primers used for amplification were designed in the department of Electron Microscopy / Molecular Pathology of the Cyprus Institute of Neurology and Genetics using Primer 3 software. A total of 31 primer pairs for *BRCA1* and 41 primer pairs for *BRCA2* were used for mutation screening (Hadjisavvas et al., 2004).

Following PCR amplification, the DNA products were electrophoresed on a 2% agarose gel as described by Sambrook et al. (1989). Following gel electrophoresis, DNA was visualized under short wavelength UV light and a digitalized photograph was taken for reference.

Next, the PCR products were purified through a Montage™ PCRm96 plate (Millipore) according to manufacturer's recommendations. The purification protocol included a filtration step followed by resuspension and recovery of the samples in a final volume of 40 μl.

3.1.5 Cycle Sequencing

Three μl of the purified PCR products were used for cycle sequencing. The reactions were carried out according to manufacturer's recommendations using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). PCR products were sequenced using the same primers used for PCR amplification. On completion of the reaction, dye terminators were removed using Montage SEQ96 Sequencing Reaction Cleanup plates (Millipore). Samples were placed on an ABI 310 Genetic Analyzer (Applied Biosystems) and were electrophoresed according to manufacturer's

instructions. When a mutation was identified, a new PCR product using a second DNA sample, obtained from the same patient, was sequenced in order to confirm the result.

3.1.6 Multiplex ligation-dependent probe amplification (MLPA)

In order to detect large genomic rearrangements in the *BRCA1* and *BRCA2* genes, MLPA (MRC Holland) using the P087 and P045 kits was carried out, following the manufacturer's protocols. Fragment analysis was carried out on an ABI 310 Genetic Analyzer using ROX-500 as a size standard. Peak profiles were analyzed using Coffalyser software (MRC Holland).

3.1.7 In silico sequence analysis tools

To evaluate potential alternative splicing effects, three in silico sequence analysis tools, namely NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html), SpliceSiteFinder (http://violin.genet.sickkids.on.ca/ali/splicesitefinder.html) and NetGene (http://www.cbs.dtu.dk/services/NetGene2/) were used. In order to classify missense variants, the Polyphen algorithm (http://coot.embl.de/PolyPhen.) was used.

3.2 Results

3.2.1 *BRCA1* and *BRCA2* mutation analysis

Mutation analysis revealed the presence of six pathogenic mutations (two in *BRCA1* and four in *BRCA2*) in 6 out of the 26 women diagnosed with early-onset breast cancer (Figure 15). In total, mutational analysis of the two genes revealed the presence of 20 variants in the *BRCA1* gene and 26 variants in the *BRCA2* gene. In describing individual variants, mutation nomenclature guidelines of the HGVS (Human Genome Variation Society) (http://www.hgvs.org/rec.html) and the nomenclature used in the BIC database (http://research.nhgri.nih.gov/bic/), which appears in brackets, were used.

The 20 variants identified in the *BRCA1* gene include two truncating mutations, six missense mutations, five polymorphisms and seven intronic variants (Table 2). The two truncating mutations are a nonsense mutation c.1840A>T (1959A>T), at codon 614 in exon 11, a lysine to a STOP (K614X) and a frameshift mutation at position c.5310delG (5429delG) at codon 1770 in exon 21, which introduces a STOP 22

amino acids downstream (p.Phe1772Serfsx21). These mutations were detected in two unrelated patients. The six missense mutations which were detected are c.1067A>G (Q356R), c.1984C>T (H662Y), c.2612C>T (P871L), c.3113A>G (E1038G), c.3348A>G (L1183K) and c.4837A>G (S1613G). Finally, of the 20 variants identified, 7 occur within intronic sites of the *BRCA1* gene, namely in introns 5, 8, 9, 12, 17 and 18. It is noted that missense mutation c.1984C>T (H662Y), polymorphism c.1482A>G (Q494Q) and intronic variants c.126-23C>A (IVS5-23C>A) and c.4185+3A>G (IVS12+3A>G) are novel while the remaining variants have been reported by others in the BIC database.

Table 2: Details of the 20 variants detected in *BRCA1* in the patient and the control group

Exon	Sequence variant	Amino acid variant	Mutation type	Mutation effect	Frequency in the patient group (%)	Frequency in the control group (%)
		Truncating Mutations				
11	c.1840A>T (1959A>T)	p.Lys614X	N	N	2	0
21	c.5310delG (5429delG)	p.Phe1772Serfsx21	F	F	2	0
		Missense Mutations				
11	c.1067A>G (1186A>G)	p.Glu356Arg (Q356R)	M	P	15	5
11	**c.1984C>T (2103C>T)**	**p.His662Tyr (H662Y)**	**M**	**UV**	**2**	**0**
11	c.2612C>T (2731C>T)	p.Pro871Leu (P871L)	M	P	29	50
11	c.3113A>G (3232A>G)	p.Glu1038Gly (E1038G)	M	P	37	41
11	c.3348A>G (3467A>G)	p.Leu1183Lys (L1183K)	M	P	31	42
16	c.4837A>G (4956A>G)	p.Ser1613Gly (S1613G)	M	P	37	41
		Polymorphisms				
9	c.591C>T (710C>T)	p.Cys197Cys (C197C)	P	P	2	0
11	**c.1482A>G (1601A>G)**	**p.Gln494Gln (Q494Q)**	**P**	**P**	**2**	**0**
11	c.2082C>T (2201C>T)	p.Ser694Ser (S694S)	P	P	37	39
11	c.2311 T>C (2430T>C)	p.Leu771Leu (L771L)	P	P	37	34
13	c.4308T>C (4427T>C)	p.Ser1436Ser (S1436S)	P	P	37	47
		Intronic Variants				
5	**c.126-23C>A (IVS5-23C>A)**		**UV**	**UV**	**2**	**0**
8	c.302-34T>C (IVS7-34T>C)		UV	P	60	100
9	c.442-57delT (IVS8-57delT)		UV	P	31	29
12	**c.4185+3A>G (IVS12+3A>G)**		**UV**	**UV**	**2**	**0**
17	c.4676-68A>G (IVS16-68A>G)		UV	P	31	31
17	c.4676-94A>G (IVS16-94A>G)		UV	P	31	31
18	c.5152+66G>A (IVS18+66G>A)		UV	P	37	29

F = Frameshift; N = nonsense; UV = unclassified variant;

M = missense; P = polymorphism; Boldface = novel variants

Mutation nomenclature is according to GenBank accession number U14680 (*BRCA1*) with numbering starting at the A of the first ATG. The nomenclature as used in the BIC database is shown in parentheses.

The 26 *BRCA2* variants identified in the Cypriot early onset breast cancer cases include two truncating mutations, seven missense mutations, six polymorphisms and 11 intronic variants (Table 3). The first truncating mutation which was detected is a novel frameshift, c.3531-3534 delCAGC (p.Asp1177Glufsx19) in exon 11, which introduces a STOP codon (1196X). This mutation was detected in one patient. The second frameshift mutation detected is c.8755delG (8984delG) in exon 22 which introduces a STOP codon 7 amino acids downstream (p.Gly2919Valfsx8). This mutation was detected in three unrelated patients. The seven missense mutations are c.865A>C (N289H), c.1114C>A (H372N), c.1889C>T (T630I), c.2971A>G (N991D), c.4258 G>T (D1420Y), c.5744 C>T (T1915M) and c.7544C>T (T2515I). Finally of the 26 variants identified, 11 occur within intronic sites of the BRCA2 gene, namely in introns 2, 4, 8, 10, 11, 14, 17, 25 and 27. It is noted that polymorphism c.7140T>C (H2380H) and intronic variant c.681+43A>G (IVS8+43A>G) are novel while the remaining variants have been reported by others in the BIC database.

The MLPA analysis of *BRCA1* and *BRCA2* genes was negative for all 26 DNA samples evaluated in this study indicating that large deletions or duplications were not present in the study cohort.

Three different theoretical splicing and skipping prediction methods were applied in an attempt to predict aberrant splicing based on the DNA sequence of the unclassified variant *BRCA1* c.4185+3A>G (IVS12+3A>G). All three software applications used predicted that this intron variant might result in deleterious alterations at the mRNA level. Furthermore, the Polyphen algorithm predicted that the missense mutation *BRCA1* c.1984C>T (H662Y) is “probably damaging”.

Table 3: Details of the 26 variants detected in *BRCA2* in the patient and the control group

Exon	Sequence variant	Amino acid variant	Mutation type	Mutation effect	Frequency in the patient group (%)	Frequency in the control group (%)
		Truncating Mutations				
11	**c.3531_3534del4 (3758del4)**	**p.Asp1177Glufsx19**	**F**	**F**	**2**	**0**
22	c.8755delG (8984delG)	p.Gly2919Valfsx8	F	F	6	0
		Missense Mutations				
10	c.865A>C (1093A>C)	p.Asn289His (N289H)	M	P	12	11
10	c.1114A>C (1342A>C)	p.His372Asn (H372N)	M	P	21	22
10	c.1889C>T (2117C>T)	p.Thr630Ile (T630I)	M	UV	2	0
11	c.2971 A>G (3199A>G)	p.Asn991Asp (N991D)	M	P	12	7
11	c.4258G >T (4486G>T)	p.Asp1420Thr (D1420Y)	M	P	2	2
11	c.5744 C>T (5972C>T)	p.Thr1915Met (T1915M)	M	P	2	0
15	c.7544C>T (7772C>T)	p.Thr2515Ile (T2515I)	M	P	2	0
		Polymorphisms				
10	c.1365A >G (1593A>G)	p. Ser455Ser (S455S)	P	P	12	10
11	c.2229T.C (2457T>C)	p.His743His (H743H)	P	P	8	10
11	c.3396A>G (3624A>G)	p.Lys1132Lys (L1132L)	P	P	27	26
11	c.3807jC>T (4035C>T)	p.Val1269Val (V1269V)	P	P	54	57
14	**c.7130T>C (7358T>C)**	**p.His2380His** (H2380H)	**P**	**P**	**2**	**1**
14	c.7242A>G (7470A>G)	p.Ser2414Ser (S2414S)	P	P	23	20
		Intronic Variants				
2	c.1-203G>A (203G>A)		P	P	23	25
4	c.425+36A>G (IVS4+36A>G)		UV	UV	2	0
8	**c.681+43A>G (IVS8+43A>G)**		**UV**	**UV**	**2**	**0**
8	c.681+56C>T (IVS8+56C>T)		UV	P	12	17
10	c.1909+12delT (IVS10+12delT)		UV	P	100	100
11	c.1910-51G>T (IVS11-51G>T)		UV	P	2	11
11	c.6841+80del4 (IVS11+80del4)		UV	P	29	51
14	c.7435+53C>T (IVS14+53C>T)		UV	P	2	11
17	c.7618-14T>C (IVS16-14T>C)		UV	P	46	51
25	c.9118-16T>C (IVS24-16T>C)		UV	UV	2	1
27	c.10333A>G (IVS27+76A>G)		UV	UV	2	1

F = Frameshift; N = nonsense; UV = unclassified variant;

M = missense; P = polymorphism; Boldface = novel variants

Mutation nomenclature is according to GenBank accession number U43746 (*BRCA2*) with numbering starting at the A of the first ATG. The nomenclature as used in the BIC database is shown in parentheses.

Out of the 26 patients recruited, 15 women reported a family history of at least one breast or ovarian cancer, whereas 11 women had a negative history. Furthermore, of the six *BRCA1/2* mutation carriers detected, two had a negative family history (two of 11, approximately 20%). A summary of the family history of breast and ovarian cancer for the six carriers of the pathogenic mutations, as well for the one case carrying the variant c.4185+3A>G (IVS12+3A>G) is presented in Table 4. The family history of women who were not found to carry a pathogenic *BRCA1/2* mutation ranged from one first degree relative with breast cancer, to a maximum of four breast cancer cases in the family.

Table 4: Details of the *BRCA1* and *BRCA2* pathogenic mutation carriers

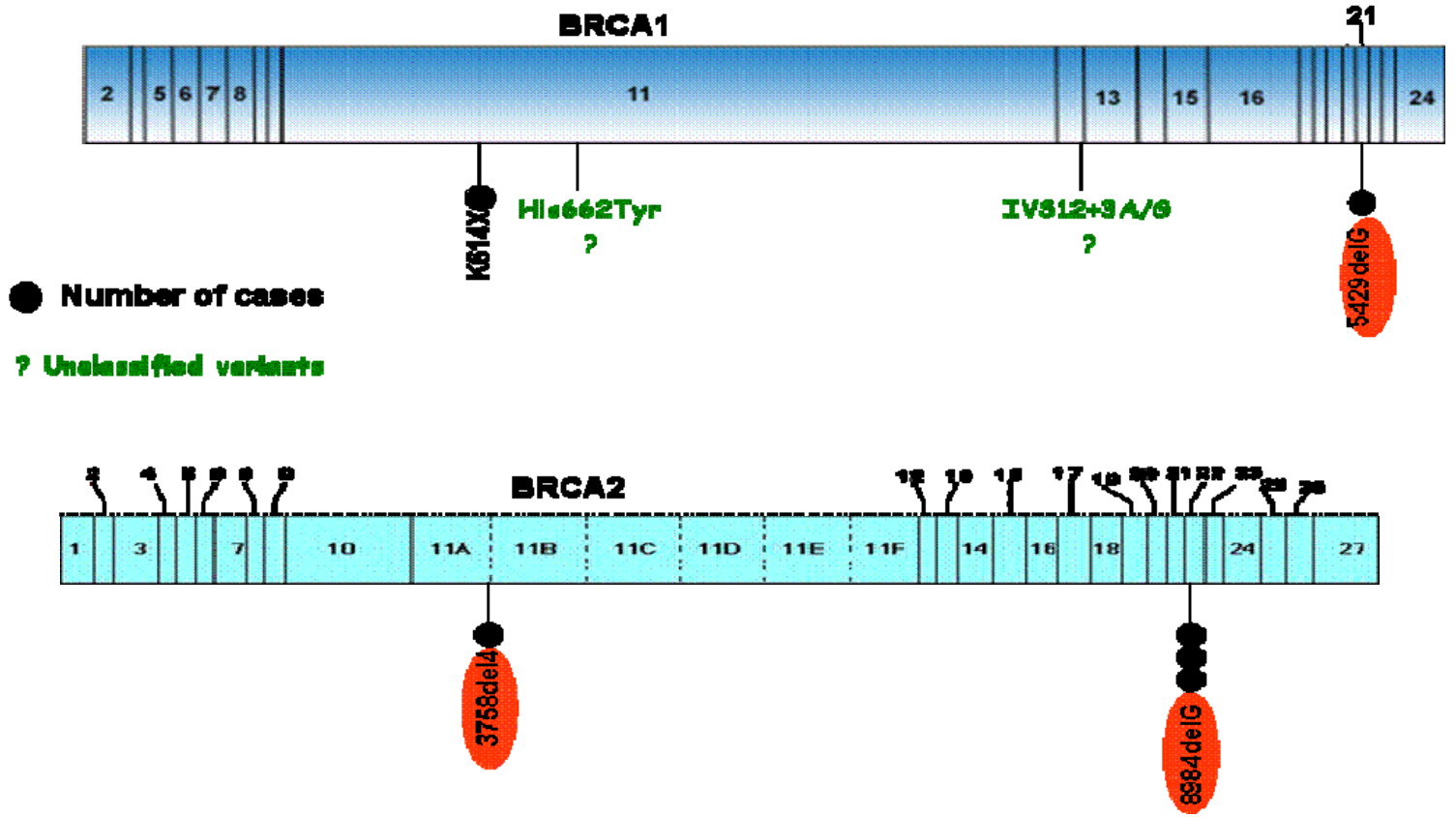
Gene	Exon	Mutation	Amino acid variant	Age at Diagnosis	BC/OV Family History
BRCA1	11	c.1840A>T (1959A>T)	p.Lys614X	40	1 MBC
BRCA1	21	c.5310delG (5429delG)	p.Phe1772Serfsx21	33	-
BRCA1	12	c.4185+3A>G (IVS12+3A>G)		27	-
BRCA2	11	c.3531-3534delCAGC (3758del4)	p.Asp1177Glufsx19	32	3 BC, 1 OC
BRCA2	22	c.8755delG (8984delG)	p.Gly2919Valfsx8	33	-
BRCA2	22	c.8755delG (8984delG)	p.Gly2919Valfsx8	34	3 BC
BRCA2	22	c.8755delG (8984delG)	p.Gly2919Valfsx8	30	9 BC

BC=breast cancer; M=male; OC=ovarian cancer

3.2.2 Results of *BRCA1* and *BRCA2* gene analysis in the control group

In order to evaluate the significance of *BRCA1* and *BRCA2* sequence variants detected in the patient group, general population frequency analysis was conducted using a group of 50 unrelated age-matched healthy women (section 3.1.2). Details of the results from analyzing the 20 *BRCA1* and the 26 *BRCA2* variants in the healthy group and frequencies of these variants in the patient group are displayed in Tables 2 and 3.

Figure 15: Summary of the pathogenic mutations as well as the unclassified variants detected in the Cypriot early onset breast cancer group. Frameshift mutations are represented by red circles, nonsense mutations appear in black and unclassified variants appear in green. Black circles represent the number of early onset breast cancer patients carrying each mutation.



3.3 Discussion

In Cyprus, molecular studies of germline *BRCA1* and *BRCA2* mutations to date, had focused on women from high-risk families with multiple cases of breast or ovarian cancer. Results of the molecular analysis of these high-risk Greek Cypriot families revealed a different spectrum of *BRCA1/2* mutations compared to other Mediterranean countries. In addition, a founder Cypriot mutation in the *BRCA2* gene has been identified (Hadjisavvas et al., 2004).

Family studies have proved invaluable for understanding the significance and contribution of *BRCA1* and *BRCA2* genes in breast cancer among Greek Cypriot women. However, observations in such families regarding the nature and penetrance of *BRCA1* and *BRCA2* mutations may not reflect the full spectrum of alterations present in the general population. It is currently accepted that a variable proportion of early-onset breast cancer is associated with mutations in highly penetrant dominant genes such as *BRCA1* and *BRCA2* and that the proportion may be higher in populations harbouring founder mutations (Robson et al., 1998).

In Cyprus, the prevalence of *BRCA1* and *BRCA2* mutations in patients with breast cancer who were unselected for a family history of this disease, has not been determined. The aim of this study was to evaluate the frequency and distribution of mutations in the *BRCA1* and *BRCA2* genes, in a cohort of Greek Cypriot women with early-onset breast cancer.

Breast cancer is the most common cancer among women in Cyprus, with about 400 new cases diagnosed annually. About 20–30 of these newly diagnosed cases occur in patients younger than 40 years of age. As described in section 3.1.1, the cases investigated in this study were consecutive cases diagnosed with early-onset breast cancer, by age 40, between the years 2003 and 2004. This number represents the annual expected incidence of this category of cases, as indicated by the accumulated data from the Cyprus Cancer Registry.

A total of 26 DNA samples from women who were given a diagnosis of early onset breast cancer, before the age of 40, and who were not selected on the basis of family history, were screened for *BRCA1/2* mutations. In the 26 breast cancer cases

investigated, two pathogenic mutations in *BRCA1* affecting two patients and two pathogenic mutations in *BRCA2* affecting four patients were detected. All characterized disease-associated mutations were truncating mutations causing premature stop codons and were detected in six unrelated patients.

Of the 26 recruited patients, 15 reported a positive family history of at least one incidence of breast or ovarian cancer. In this group, four patients were found to carry pathogenic *BRCA1/2* mutations. The remaining 11 patients had no relevant family history, but it is of interest that two of these were found to carry pathogenic *BRCA1/2* mutations. In addition, in this group of 11 patients, a possible splice-site variant c.4185+3A>G (IVS12+3A>G) was detected in one patient raising the number of pathogenic mutation carriers to a possible three (3 of 11, approximately 30%).

A total of six missense mutations in the *BRCA1* gene and seven missense mutations in the *BRCA2* gene have been detected in the study population. It is noted that five out of the six missense mutations identified in the *BRCA1* gene are polymorphisms, since they were also present in more than 5% of the control group. Similarly, three out of the seven missense mutations identified in the *BRCA2* are polymorphisms. In addition, missense mutations c.4258G>T (D1420Y), c.5744C>T (T1915M) and c.7544C>T (T2515I) have been previously reported as polymorphisms (Deffenbaugh et al., 2002; Sigurdson et al., 2004; Wu et al., 2005).

The unclassified variant c.4185+3A>G (IVS12+3A>G) in the *BRCA1* gene is of particular interest. This specific variant involves nucleotide +3 in the consensus sequence of the donor splice site of exon 12. It is well characterized that mutations in the regions required for correct RNA splicing can result in exon skipping or in activation of cryptic splice-sites, which in turn lead to deletion of exon sequences or retention of intronic sequences (Shapiro and Senapathy, 1987). Hence, intronic alterations that are located within or near intron-exon boundaries of the *BRCA1* and *BRCA2* may have a potential impact on mRNA splicing fidelity. A number of splice-site prediction programs, that use highly conserved sequences as a basis of predicting the effects of mutations on RNA splicing have been developed.

Three different theoretical splicing and skipping prediction software applications were applied in an attempt to predict the effect of the *BRCA1* c.4185+3A>G variant on RNA splicing. All three software applications, predicted that this intronic variant might result in deleterious alterations at the mRNA level. Unfortunately, an RNA sample from this patient, which would allow the assessment of the transcript that is produced by this variant allele, was not available.

The novel missense mutation c.1984C>T (H662Y) in the *BRCA1* gene may also be pathogenic. Amino acid 662 is located in the DNA-binding region of the *BRCA1* gene, and contributes to the DNA repair- related functions of *BRCA1* (Narod and Foulkes, 2004). Mutation c.1984C>T (H662Y) alters the amino acid histidine from a basic polar, positively charged molecule, to the aromatic, non-polar amino acid tyrosine. It is well known that the stoichiometry and the charges of the amino acids play a role in the conformation and the function of proteins. The Polyphen algorithm predicted that this variant is "probably damaging". Polyphen is an algorithm that classifies the functional effect of each missense variant into three categories (probably damaging, possibly damaging, and benign). This classification is based on comparing homologous sequences for conservation and examination of the structural and physicochemical aspects of the substitution. Polyphen has been reported to have the smallest false positive rate among the various online algorithms (Ng and Henikoff, 2006). In silico analysis of missense mutations provides a useful first line analysis method for newly observed substitutions. Based on Polyphen algorithm, missense mutation c.1984C>T (H662Y) in the *BRCA1* gene may lead to the synthesis of a dysfunctional *BRCA1* protein which in turn predisposes to the breast cancer phenotype. Although bioinformatics methods can predict the impact of missense mutations, the predictions cannot be applicable in the clinical setting unless they are complemented by functional studies. Hence, in the case of H662Y missense mutation in the *BRCA1* gene, it would be interesting to carry out functional studies in order to determine the pathogenicity of this variant. This is part of future work, and it is our intention to study the effect of this variant further by expressing it *in vivo* and investigating its influence on protein function. The experimental plan involves subcloning the *BRCA1* H662Y unclassified variant in a mammalian expression vector followed by transiently transfecting the expression construct in mammalian cell lines and studying both the functional activity of the protein as well

as its biological effects on the cell. The *BRCA1* C61G variant that is known to be pathogenic (Brzovic et al., 1998) will be used as a positive control in our experimental plan.

Little is known about the contribution of large *BRCA1* and *BRCA2* rearrangements to early onset breast cancer. So far, very few studies have examined this. In a recent study from Germany, large rearrangements were detected in 3 out of the 103 (2.9%) early-onset breast cancer patients screened. All three gross alterations were detected in the *BRCA1* gene (Engert et al., 2008). In an Indonesian series of early-onset breast cancer cases, *BRCA1* large rearrangements accounted for 1% (1/102) of these cases (Purnomosari et al., 2007). Furthermore, when screening 59 Singapore-Asian early onset breast and ovarian cancer patients, 2 genomic rearrangements were detected in 2 unrelated breast cancer patients, 1 in each gene (Lim et al., 2007). Based on the published results of these studies, it can be concluded that large *BRCA1/2* rearrangements account for only a small fraction of early onset breast cancer.

It is interesting that no large genomic rearrangements were detected in the Cypriot early-onset breast cancer cohort. This could be attributed to the small number of samples screened but the most likely explanation is ethnic background differences. The percentage of large genomic rearrangements in the *BRCA1* and *BRCA2* genes has been reported to vary significantly between different populations (Palma et al., 2008). At this point it should be noted that studies performed in high-risk Cypriot families, have also not detected any large *BRCA1* or *BRCA2* rearrangements (unpublished data). However, due to the small number of individuals included in this investigation, no conclusive recommendations for large-rearrangements testing in Cypriot early onset breast cancer cases can be drawn.

The histopathology of *BRCA1*-mutation carriers' breast tumours is distinct from sporadic cases. Most *BRCA1*-associated tumours are infiltrating ductal carcinomas (Narod and Foulkes, 2004). Medullary breast carcinomas are more common among *BRCA1*-mutation carriers compared to controls. In addition, carriers of *BRCA1* mutations show low rates of carcinoma in situ (Breast Cancer Linkage Consortium, 1997). Over-representation of the "triple-negative" phenotype [(negative for

estrogen-receptor, progesterone-receptor and epidermal growth factor receptor 2 (HER2)] is another feature of *BRCA1*-associated tumours (Lakhani et al., 2002). In contrast, the histopathology of *BRCA2* mutation carriers is more heterogeneous and similar to non-carriers (Breast Cancer Linkage Consortium, 1997). In the present study, infiltrating ductal carcinoma was the predominant tumour type in both *BRCA1*/*2* mutation carriers and non-carriers. It is noted that two of the infiltrating ductal carcinomas were comedo type and were diagnosed in women with truncating *BRCA1*/*2* mutations.

Previous population-specific studies revealed that the contribution of *BRCA1* and *BRCA2* mutations to the incidence of early-onset breast cancer ranges between 5% and 10% (Langston et al., 1996; Peto et al., 1999; Malone et al., 2000; Anglian Breast Cancer Study Group, 2000; Hamann et al., 2003). In contrast, the findings of the present study show that 23% (6 of 26) of Cypriot early-onset breast cancer cases are associated with a germline mutation in either the *BRCA1* or the *BRCA2* genes. This figure of 23% is higher than most studies but compares favourably with data from two other ethnic populations, for which a higher proportion of *BRCA*-associated early-onset breast cancers has been reported. Data show that the prevalence rates can be as high as 30% for Ashkenazi Jews (Abeliovich et al., 1997) and around 25% for Icelanders (Johannesdottir et al., 1996; Thorlacius et al., 1997). This high percentage is a result of the presence of founder mutations in these ethnic populations. More specifically, three ancestral mutations [*BRCA1* c.68_69delAG (185delAG), c.5266insC (5382insC) and *BRCA2* c.5946delT (6174delT)] appear in about 2% of Ashkenazi Jews (Struewing et al., 1997), while about 0.5% of Icelanders carry the c.771delTCAAA (999del5) mutation in the *BRCA2* gene (Johannesdottir et al., 1996; Thorlacius et al., 1997). The high prevalence rates of *BRCA1* and *BRCA2* mutations in the Cypriot cohort of patients may also be explained by the presence of the Cypriot founder mutation c.8755delG (8984delG) in the *BRCA2* gene (Hadjisavvas et al., 2004). Although the effect of this founder mutation is not as striking as the case of the Icelanders or Ashkenazi Jews, the results show that it makes a substantial contribution to the incidence of early-onset breast cancer in the Cypriot population. In addition, it is likely that the contribution of these genes to early-onset breast cancer might be even higher given that certain unclassified variants might also be causative.

In the group of patients studied, the highest proportion of mutations was reported in the *BRCA2* gene, 15% (4 of 26 positive) vs. 7.7% (2 of 26 positive), in the *BRCA1* gene. Evidently, *BRCA2* accounts for a considerably larger proportion of early-onset breast cancer in Cyprus than does *BRCA1*. This is in agreement with previous data on familial breast/ovarian cancer studies in Cypriot families (Hadjisavvas et al., 2001; Hadjisavvas et al., 2004). It appears that in most other populations studied, mutations in the two genes make approximately equal contributions to early-onset breast cancer (Langston et al., 1996; Peto et al., 1999; Anglian Breast Cancer Study Group, 2000; Hamann et al., 2003) with the exception of Iceland where *BRCA2* accounts for most of the early-onset breast cancer cases, and the Ashkenazi Jews where *BRCA1* accounts for the majority of cases.

In the majority of western world countries, single cases of breast cancer are not generally accepted for genetic testing for hereditary breast cancer genes. Given the current constraints on health care resources, *BRCA1* and *BRCA2* screening in these countries is limited to women with a strong family history of breast cancer. International guidelines have as a prerequisite for breast cancer genetic screening before the age of 50, the presence of at least one first-degree relative diagnosed with breast cancer under the age of 40 (Blamey, 1998; Eccles et al., 2000) or 50 (Hoskins et al., 1995). Hereditary breast ovarian cancer syndrome is characterized by an autosomal dominant pattern of inheritance and as a result of this, half of *BRCA* gene mutation carriers are expected to be men. Sometimes, it is very difficult to recognize this inheritance pattern, due to the very low penetrance (<6%) of *BRCA*-associated breast cancer in men, especially in families with limited numbers of women old enough to develop breast and ovarian cancer. This is also the case for small families, families which lack paternal aunts or families with premature mortality (Weitzel et al., 2007).

Overall, the current study data indicate that *BRCA* screening policies based on family history would miss a substantial proportion of early onset mutation carriers in Cyprus. *BRCA1/2* pathogenic mutations were detected in 4 of 15 patients with a family history, as well as in 2 of 11 patients without a family history. These findings support a strong correlation between the early onset breast cancer phenotype and

BRCA1/2 gene analysis, since the prevalence of *BRCA1/2* mutations in young Cypriot patients is relatively high.

Although no definitive recommendations can be made from this study regarding genetic testing of Cypriot women with early onset breast cancer, it is clear that the prevalence of *BRCA1/2* mutations in this population of young women is high. The analysis of early-onset breast cancer in the Cypriot population suggests that young age of diagnosis alone, could be a sufficient predictor of finding a pathogenic *BRCA1/2* mutation. Based on these results, it is recommended that *BRCA1/2* screening should be offered to Cypriot patients, with a diagnosis of early-onset breast cancer irrespective of their family history.

3.4 Publications resulting from work described in this Chapter

- Loizidou, M., Marcou, Y., Anastasiadou, V., Newbold, R., Hadjisavvas, A., and Kyriacou, K. (2007). Contribution of *BRCA1* and *BRCA2* germline mutations to the incidence of early-onset breast cancer in Cyprus. Clin Genet *71*, 165-170.

Chapter 4

Case-control study of DNA repair genes

4.1 Materials and Methods

4.1.1 Study population

To investigate the associations between genetic factors and breast cancer risk in the Cypriot population, we conducted a population-based case-control study, with the acronym *MASTOS* (Greek word for breast). The study population for this *PhD* project were women participating in the *MASTOS* study. Blood samples were collected between 2004-2006 from 1109 female breast cancer patients diagnosed between 40-70 years old and 1177 aged-matched healthy controls. Cases participating in the study were women who were previously diagnosed with breast cancer between January 1999 and December 2006. The majority of cases were ascertained from the Bank of Cyprus Oncology Centre, which operates as a referral centre and offers treatment and follow-up for 80-90% of all breast cancer cases diagnosed in Cyprus. The rest of the patients, were recruited at the Oncology Departments of the Nicosia, Limassol, Larnaca and Paphos district hospitals. The control group consisted of healthy women who were participating in the National program for breast cancer screening with the use of mammography. Volunteers were enrolled in the study during the same calendar period as the cases, from the 4-district mammography screening centres that operate in Cyprus. Eligible controls were women with no previous history of breast cancer who had a negative mammography result. All study participants, both cases and controls, were of Greek Cypriot Caucasian origin, thus reducing any potential bias due to population stratification. In addition, the study population was representative of the whole island population and thus consisted of women who resided in all five districts of the country, minimizing potential selection bias. The participation rate of cases and controls was very high covering around 98% of eligible cases and controls. In addition to blood samples, a risk factor questionnaire, which included extensive demographic, epidemiological and pathological data, was obtained from each participant through a standardized interview. Breast cancer cases were verified by reviewing histological reports. The study was reviewed and approved by the National Bioethics Committee of Cyprus. All participants provided written informed consent.

4.1.2 Gene and SNP selection

The study concentrated on genes that are important components of the DNA repair pathway. One of the most extensively studied group of genes in relation to breast cancer risk, are the *XRCC* (X-Ray Cross-Complementing) genes. These genes were initially discovered through their role in DNA damage response caused by ionizing radiation. *XRCC* genes are important components of various DNA repair pathways contributing to DNA-damage processing and genetic stability (Thacker and Zdzienicka, 2004). In summary, *XRCC1* gene is an important component of the BER pathway acting as a scaffold for other BER enzymes (Caldecott, 2003). *XRCC2* and *XRCC3* genes are necessary for HR repair and are required for *RAD51* focus formation (Bishop et al., 1998; O'Regan et al., 2001).

This section of the thesis had two primary aims as explained below. The first aim of this study, was to test the hypothesis that common variants in the *XRCC* genes modify susceptibility to breast cancer. The investigation focused on evaluating five potentially functional SNPs in the *XRCC1*, *XRCC2* and *XRCC3* genes. The five SNPs that were studied are: *XRCC1* R194W (rs1799782), *XRCC1* R280H (rs25489), *XRCC1* R399Q (rs25487), *XRCC2* R188H (rs3218536) and *XRCC3* T241M (rs861539).

The second and primarily aim of this study was to investigate the association between SNPs in genes that interact with *BRCA1* and *BRCA2* and breast cancer risk in the Cypriot population. Genetic variation in 72 SNPs in 35 genes (see Table 5 for a list of DNA repair genes in which SNP(s) were studied), which interact with *BRCA1* and/or *BRCA2* genes and their association with breast cancer, was assessed in a case-control study of Cypriot women. Furthermore, the role of 2 additional SNPs in the *PBOV1* (*UROC28*) and *DBC2* genes which are both upregulated in breast cancer was assessed (An et al., 2000; Hamaguchi et al., 2002).

Table 5:: Details of the genes studied and their DNA repair activity (Wood et al., 2005)

Gene	Activity
Base Excision Repair (BER)	
XRCC1	Ligase accessory factor
OGG1	8-oxoG opposite C
Mismatch excision repair (MMR)	
MLH1	MutL homologs, forming heterodimer
MSH2	Mismatch and loop recognition
MSH6	Mismatch and loop recognition
Nucleotide excision repair (NER)	
DDB2	Complex defective in XP group E
ERCC2	5' to 3' DNA helicase
XPC	Binds damaged DNA as complex
Homologous recombination (HR)	
BRCA2	Cooperation with *RAD51*, essential function
MRE11A	3' exonuclease
DMC1	Rad51 homolog, meiosis
EME1	Structure-specific DNA nuclease
MUS81	Structure-specific DNA nuclease
NBS1	Mutated in Nijmegen breakage syndrome
RAD50	ATPase in complex with *MRE11A*, *NBS1*
RAD51	Homologous pairing
XRCC2	DNA break and crosslink repair
XRCC3	DNA break and crosslink repair
DNA polymerases (catalytic subunits)	
PCNA	Sliding clamp for pol δ and pol ε
Genes defective in diseases associated with sensitivity to DNA damaging agents	
ATM	ataxia telangiectasia
BLM	Bloom syndrome helicase
BRIP1	*BRCA1*-associated DNA helicase
FANCA	Involved in tolerance or repair of DNA crosslinks
FANCC	Involved in tolerance or repair of DNA crosslinks
FANCD2	Involved in tolerance or repair of DNA crosslinks
FANCE	Involved in tolerance or repair of DNA crosslinks
PALB2	Involved in tolerance or repair of DNA crosslinks
Other conserved DNA damage response genes	
ATR	*ATM*- and *PI-3K*-like essential kinase
CHEK1	Effector kinase
CHEK2	Effector kinase
TP53	Regulation of the cell cycle

The genetic variants were selected based on three main criteria: (1): all SNPs chosen belong to genes which interact with either *BRCA1* and/or *BRCA2*; (2) the SNPs chosen are either functional SNPs (based on potential protein changes, evolutionary conservation and location in putative functional regions (Nakken et al., 2007; Rudd et al., 2005; Savas et al., 2004) or (3) SNPs which were reported by other groups to modify cancer risk (Barroso et al., 2006; Beiner et al., 2006; Gaudet et al., 2008; Johnson et al., 2007; Justenhoven et al., 2004; Kilpivaara et al., 2004; Landi et al., 2006; Lu et al., 2007; Poplawski et al., 2005; Rahman et al., 2006; Sigurdson et al., 2004; Webb et al., 2005). For *MRE11A* and *RAD50,* the tagging SNPs in (Allen-Brady and Camp, 2005) were genotyped and for *NBS1*, the tagging SNPs in Lu et al. (2006) were genotyped. SNPs in the *PBOV1* and *DBC2* genes were selected based on their minor allele frequency (MAF) >0.05.

In total, a panel of 79 SNPs, which are summarized in Table 6, were genotyped and analyzed in the Cypriot case-control population.

4.1.3 Preparation of total genomic DNA

Blood samples were collected from all 2286 study participants in 10 ml EDTA tubes. Genomic DNA was isolated from peripheral-blood lymphocytes using the phenol chloroform method (as described in section 3.1.3).

4.1.4 SNP genotyping

SNPs were genotyped using one of the following three methods:

1) PCR followed by restriction fragment length polymorphism (RFLP) analysis, with genotypes being determined by agarose gel electrophoresis

2) real-time PCR with Taqman SNP genotyping assays and allelic discrimination using an ABI PRISM 7900HT instrument (Applied Biosystems) and

3) SNP genotyping using the Sequenom MassARRAY iPLEX Gold assays and matrix assisted laser desorption / ionization-Time-of-Flight mass spectrometry (MALDI-TOF MS) for detection (Sequenom).

At the start of this study, the Department of Electron Microscopy/ Molecular Pathology of the Cyprus Institute of Neurology and Genetics was not equipped with a high-throughput genotyping technology. Consequently, the only SNP genotyping

method which could be used, based on available equipment, was PCR-RFLP analysis. PCR-RFLP is a poor choice for high throughput analysis since it is a relatively slow and expensive genotyping method. Not a long time after the commencement of this study, the Department purchased a real time PCR instrument. Taqman SNP genotyping is a PCR-based method which is relatively easy to implement and provides high-confidence results. It does not require post-PCR processing and can be used for high throughput genotyping (Syvanen, 2001). Hence, based on the clear advantages of Taqman SNP genotyping over PCR-RFLP genotyping, in terms of higher-throughput and lower cost, it was decided to genotype the rest of the SNPs under investigation, using this technology. SNP genotyping technology is progressing rapidly and novel, faster and cheaper methods are emerging. In order to keep up with the latest developments, the Cyprus Institute of Neurology and Genetics purchased a Sequenom MassArray MALDI-TOF MS system. This methodology is one of the cheapest and most error free technologies for high throughput SNP typing. It uses samples arrayed in 384 well plates and allows multiplexing with simultaneous genotyping of up to 40 SNPs (Sequenom). The accuracy of this method, combined with the high data acquisition speed and the capability of multiplexing led to the abandonment of Taqman technology and the application of Sequenom MassARRAY iPLEX Gold assays for genotyping the rest of the SNPs under investigation.

Table 6: Details of the single nucleotide polymorphisms investigated

Gene	rs number	Base change	Amino acid change	Genotyping method	Gene	rs number	Base change	Amino acid change	Genotyping method
ATF1	rs2230674	G>C	P191A	iPLEX	***FANCE***	rs9462088	G>A	A502T	iPLEX
ATM	rs1800057	C>G	P1054R	iPLEX	***MLH1***	rs1799977	A>G	I219V	TAQMAN
	rs2234997	T>A	D126E	iPLEX		rs1800149	C>G	L729V	iPLEX
	rs2235000	G>A	G514D	iPLEX		rs2020872	A>G	I32V	iPLEX
	rs3218688	C>T	L942F	iPLEX		rs2308317	G>A	V213M	iPLEX
	rs3218695	C>A	D814E	iPLEX		rs1800734	G>A	-	iPLEX
	rs3218708	C>T	T935M	iPLEX	***MRE11A***	rs1009456	G>T	-	iPLEX
	rs4987945	C>G	L546V	iPLEX		rs10831234	C>T	-	iPLEX
ATR	rs2227928	T>C	T211M	iPLEX		rs556477	G>A	-	iPLEX
	rs2229032	A>G	R2425Q	iPLEX		rs601341	G>A	-	TAQMAN
BARD1	rs2070094	G>A	V507M	iPLEX	***MSH2***	rs2059520	A>G	-	TAQMAN
	rs2229571	G>C	R378S	iPLEX		rs2303428	T>C	-	iPLEX
	rs3738888	C>T	R658C	iPLEX	***MSH6***	rs1042821	C>T	G39E	iPLEX
	rs1048108	C>T	P24S	TAQMAN		rs1800935	T>C	D180D	iPLEX
BLM	rs11852361	C>T	P868L	iPLEX	***MUS81***	rs545500	C>G	P180R	iPLEX
	rs7167216	G>A	I1321V	iPLEX	***NBS1***	rs1805787	C>G	-	iPLEX
BRCA2	rs1799944	A>G	N991D	TAQMAN		rs1805794	C>G	E185Q	iPLEX
BRIP1	rs4986764	T>C	S919P	iPLEX		rs6413508	C>T	P672L	iPLEX
CHEK1	rs506504	T>C	I471V	iPLEX		rs769416	G>T	Q216K	iPLEX
CHEK2	rs17879961	T>C	I157T	iPLEX		rs769420	G>A	P266L	iPLEX
DBC2	rs2241261	C>T	-	iPLEX		rs12677527	C>T	-	iPLEX
DDB2	rs830083	G>C	-	iPLEX		rs13312840	T>C	-	iPLEX
DMC1	rs2227914	A>G	M200V	iPLEX	***OGG1***	rs1052134	C>G	S326C	TAQMAN
EME1	rs12450550	T>C	I350T	iPLEX	***PALB2***	rs45494092	T>C	L337S	iPLEX
ERCC2	rs13181	A>C	K751Q	TAQMAN		rs45532440	G>C	E672Q	iPLEX
FANCA	rs1800282	T>A	V6D	iPLEX		rs45478192	T>G	L939W	iPLEX
	rs2239359	C>T	G501S	TAQMAN		rs45551636	G>A	G998E	iPLEX
	rs7190823	T>C	T266A	iPLEX	***PBOV1***	rs6927706	A>G	I73Y	iPLEX
	rs9282681	A>G	T1328A	iPLEX	***PCNA***	rs3626	G>C	-	iPLEX
FANCC	rs1800364	A>T	D195V	iPLEX	***RAD50***	rs2299015	A>C	-	iPLEX
FANCD2	rs2272125	A>C	L1366L	iPLEX		rs2522406	G>A	-	iPLEX

Gene	rs number	Base change	Amino acid change	Genotyping method	Gene	rs number	Base change	Amino acid change	Genotyping method
RAD50	rs2706377	A>G	-	iPLEX	***TP53***	rs1042522	G>C	P72R	TAQMAN
	rs3187395	G>A	E925K	iPLEX	***XPC***	rs2228000	C>T	A499V	iPLEX
RAD51	rs1801320	G>C	-	TAQMAN		rs2227999	G>A	R492H	iPLEX
	rs1801321	G>T	-	TAQMAN	***XRCC1***	rs1799782	C>T	R194W	RFLP
RAD51C	rs28363317	A>G	T287A	iPLEX		rs25487	G>A	R399Q	RFLP
RAD51L1	rs28908468	C>G	P365R	iPLEX		rs25489	G>A	R280H	RFLP
RAD52	rs7487683	C>T	G180R	TAQMAN	***XRCC2***	rs3218536	G>A	R188H	RFLP
RFC1	rs2066791	A>G	I598V	iPLEX	***XRCC3***	rs861539	C>T	T241M	RFLP

4.1.5 Polymerase chain reaction (PCR)

Briefly for screening of the 5 *XRCC* gene SNPs, preparation of the PCR included 30 ng of genomic DNA in total volume of 25 µl with 0.25 mM of each appropriate primer, in the presence of Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; at pH 8.7 (Qiagen 10X PCR buffer), 0.25 mM of dATP, dCTP, dGTP, dTTP (Invitrogen) and 1 unit of Taq DNA polymerase (Qiagen). Amplification was carried out on an Applied Biosystems 9700 thermocycler. Details of the primers as well as the PCR conditions for each SNP can be found in table 7. All PCR reactions were carried out in 96-well plates. Control samples were included in each plate, representing the 3 possible genotypes (wild type, heterozygous, homozygous mutant). Each plate also contained a negative (no template) control.

4.1.6 Restriction enzyme DNA digestion

On completion of the amplifications, enzyme digestions were carried out to determine genotypes of the 5 *XRCC* gene SNPs investigated. Restriction enzymes (New England Biolabs) were diluted with their appropriate dilutent buffers (as recommended by the manufacturer), down to 1 unit/µl. One unit of the appropriate enzyme was added directly to the plates with the PCR products. Reactions were incubated for at least 2 hours at the optimal temperature for each specific enzyme activity to ensure complete digestion. Details of the restriction enzymes used as well as the digestion conditions can be found in Table 8. Table 9 provides details of the sizes of the expected digest products for each SNP analyzed.

4.1.7 Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook et al. (1989), in a 1x TBE buffer. 2% gels were prepared with the addition of 0.5 mg ethidium bromide. 5 µl of loading dye (Sambrook et al, 1989) were added to the reactions which were subsequently loaded onto agarose gels. Gels electrophoresis was carried out at 1-10 volts/cm of gel for 30 min. Following gel electrophoresis, DNA was visualized under short wavelength UV light and a digitalized photograph was taken for reference. The different genotypes were recorded by observing RFLP patterns and comparing each sample with the 3 controls. Overall success rate for the genotyping assays was 99%. For quality control, random samples were genotyped in

duplicate and had identical genotyping assignments. Direct sequencing was also used to confirm these calls.

Table 7: *XRCC* SNPs screened, primer sequences used, annealing temperatures and references where they were obtained

Gene / SNP	Nucleotide change	Primer F (5'-3')	Primer R (5'-3')	PCR fragment size	Annealing Temperature (^{o}C)	Primer Reference
XRCC1 rs1799782	C/T	GCCCCGTCCCAGGTA	AGCCCCAAGACCCTTTCACT	491	58	Hu et al., 2001
XRCC1 rs25489	G/A	GTCTGAGGGAGGAGGGTCTG	CAGAGGAGCTGGGGAAGATC	240	65	Loizidou et al., 2008 *
XRCC1 rs25487	A/G	TCTCCCTTGGTCTCCAACCT	AGTAGTCTGCTGGCTCTGG	403	60	Hu et al., 2001
XRCC2 rs3218536	G/A	CACCCATCTCTCTGCCTTT	CCTCTCGACGACTGTGTGAT	237	55	Loizidou et al., 2008 *
XRCC3 rs861539	C/T	GGTCGAGTGACAGTCCAAAC	TGCAACGGCTGAGGGTCTT	456	55	Hu et al., 2001

* Primers were designed in the department of EM / Molecular Pathology of the Cyprus Institute of Neurology and Genetics

Table 8: Details of the restriction enzymes used, their restriction sites as well as the digestion conditions

Gene / SNP	Enzyme	Restriction site
XRCC1 rs1799782	*Pvu II*	5'…CAG▼CTG…3' 3'…CTC▲GAC…5'
XRCC1 rs25489	*RsaI*	5'…GT▼AC…3' 3'...CA▲TG…5'
XRCC1 rs25487	*Msp I*	5'…C▼CGG…3' 3'…GGC▲C…5'
XRCC2 rs3218536	*SexAI*	5'…A▼CCWGGT…3' 3'…TGGWCC▲A…5'
XRCC3 rs861539	*Nla III*	5'…CATG▼…3' 3'…▲GTAC…5'

Table 9: Expected sizes of PCR digest products

Gene / SNP	Restriction Fragments		
	Wild type (NN)	**Heterozygous (NM)**	**Homozygous (MM)**
XRCC1 rs1799782	491	491,197,294	197,294
XRCC1 rs25489	123,117	240,123,117	240
XRCC1 rs25487	403	403,270,133	270,133
XRCC2 rs3218536	237	237,146,91	146,91
XRCC3 rs861539	456	456,246,210	246,210

4.1.8 Taqman SNP genotyping assays

The 12 SNPs: *BARD1* rs1048108 (Pro24Ser), *BRCA2* rs1799944 (Asn991Asp), *ERCC2* rs13181 (Lys751Gln), *FANCA* rs2239359 (Gly501Ser), *MLH1* rs1799977 (Ile219Val), *MRE11A* rs601341, *MSH2* rs2059520, *OGG1* rs1052134 (Ser326Cys), *RAD51* rs1801320 and rs1801321 (135G>C-5' UTR and 172 G>T-5'UTR), *RAD52* rs11226 (2259 C>T-3'UTR) and *TP53* rs1042522 (Pro72Arg) were genotyped in all subjects participating in the study using Taqman SNP genotyping assays from Applied Biosystems. All SNPs studied had a minor allele frequency of over 0.05. For genotyping SNP rs1801320 the primers and probes described previously by Kuschel et al. (2002) were used.

The TaqMan SNP Genotyping Assays are single tube PCR assays that were developed by Applied Biosystems. The assays include two PCR primers which are designed against a conserved region of the genome flanking the SNP of interest and two TaqMan probes designed across the locus of interest, one for each allele (refer to Figure 16). Each probe is labelled with a different fluorescent reporter dye at the 5' end (usually VIC dye is linked to the 5' end of allele X probe and FAM dye to the 5' end of allele Y probe). Each probe also has a non-fluorescent quencher and a minor groove binder (MGB) at its 3' end. MGB offers stability to the double-stranded probe template structure and increases the probe Tm without increasing its length. Hence, it allows the design of shorter probes resulting in greater differences in melting temperature values between matched and mismatched probes. This produces greater accuracy in allelic discrimination. The quencher and the fluorophore dye are in close proximity to each other, and this provides efficient quenching of an intact probe (De la Vega et al., 2005). Taqman SNP analysis utilizes the 5' exonuclease activity of DNA Taq polymerase and the quenching effects of specific fluorescent dyes to determine the relative frequency of each allele within an individual genome. During thermocycling, each Taqman MGB probe anneals to the locus of interest in an allele specific manner. As Taq DNA polymerase extends the primers, it also degrades the annealed probe, releasing the fluorescent dye that is not longer quenched and thus become detectable. The use of two probes, one specific to each allele of the SNP, which are labelled with two different fluorophores, allows detection of both alleles in a single tube. Following PCR, the fluorescent signal is read for each reporter dye, in each well of the reaction plate. Genotype calls for each

sample are made by plotting the normalized intensity of the reporter dyes in each well on a cluster plot (Figure 17). The analysis software uses a clustering algorithm and each sample (well) is allocated to one of three possible genotype clusters (De la Vega et al., 2005).

Each assay was carried out using 10 ng genomic DNA in a 5μL reaction using Taqman Universal PCR Master Mix (Applied Biosystems), forward and reverse primers, and FAM and VIC-labelled probes purchased from Applied Biosystems (ABI Pre-Designed assays). All assays were carried out in 384-well plates. The fluorescence profile was determined on an ABI PRISM 7900HT instrument and the results analyzed with Sequence Detection Software (Applied Biosystems). All the appropriate quality control measures were taken in order to ensure reliable genotyping and to avoid false positive or false negative results due to genotyping errors. In detail, duplicates of 48 DNA samples and water controls were genotyped and had identical genotyping assignments. In addition, the placement order of DNA samples on the 384-well plates was randomized to ensure the same study conditions for samples from cases and controls. Genotype call rates ranged from 99% to 100% and duplicate concordance rates were higher than 99%.

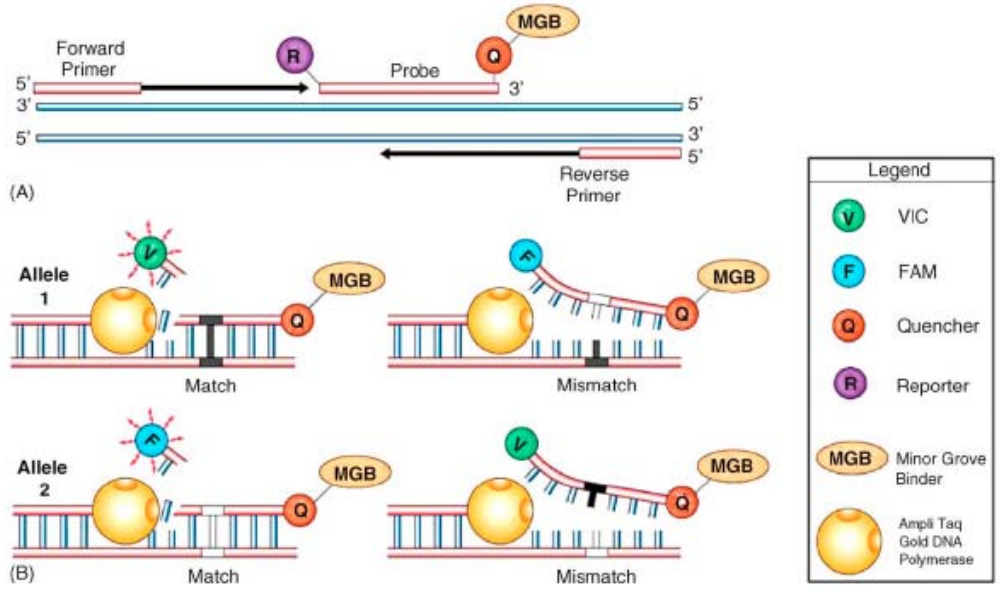


Figure 16: (A) Probe binding and primer extension in a Taqman SNP Genotyping Assay. (B) Allelic discrimination is achieved by the selective annealing of matching probe and template sequences, which generates an allele specific fluorescent signal. Figure taken from De la Vega et al. (2005).

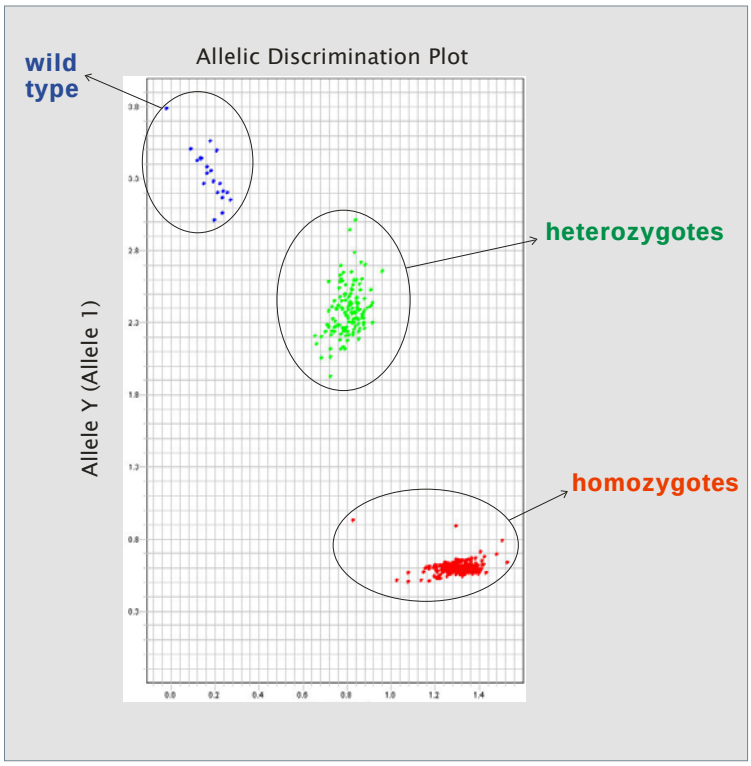


Figure 17: Representative example of a Taqman allelic discrimination plot showing 3 clusters of samples (wild type, heterozygous, homozygous mutant)

4.1.9 MassARRAY iPLEX Gold assay

The remaining 62 SNPs were genotyped using MassARRAY iPLEX Gold technology (Sequenom). This technology combines the benefits of allele-specific single-base primer extension biochemistry and the sensitivity and accuracy of MALDI-TOF MS detection (Figure 18).

Briefly, this technology involves amplifying a region containing the SNP of interest by PCR, followed by extension of an oligonucleotide probe over the SNP site in this PCR product, with a mixture of deoxynucleotides and dideoxynucleotides, to generate allele-specific DNA products. Extended PCR products are subsequently separated and analyzed using chip-based MALDI-TOF MS. The time-of-flight is proportional to mass, permitting precise determination of the size of products generated, which can be converted into genotype information. Because the mass resolution of this method is very high, one can routinely perform multiplexed assays to permit analysis of up to 40 SNPs in one PCR reaction/tube. The entire process permits complete automation including assay development, PCR setup, post-PCR treatment, nanoliter transfer of products onto silicon chips, serial reading of chip positions in the mass spectrometer, and final analytical interpretation. This approach is very reliable since allele calling, depends on mass, and spurious associations due to genotyping errors are far less likely, compared to other commonly used genotyping methods (van den Boom and Ehrich, 2007).

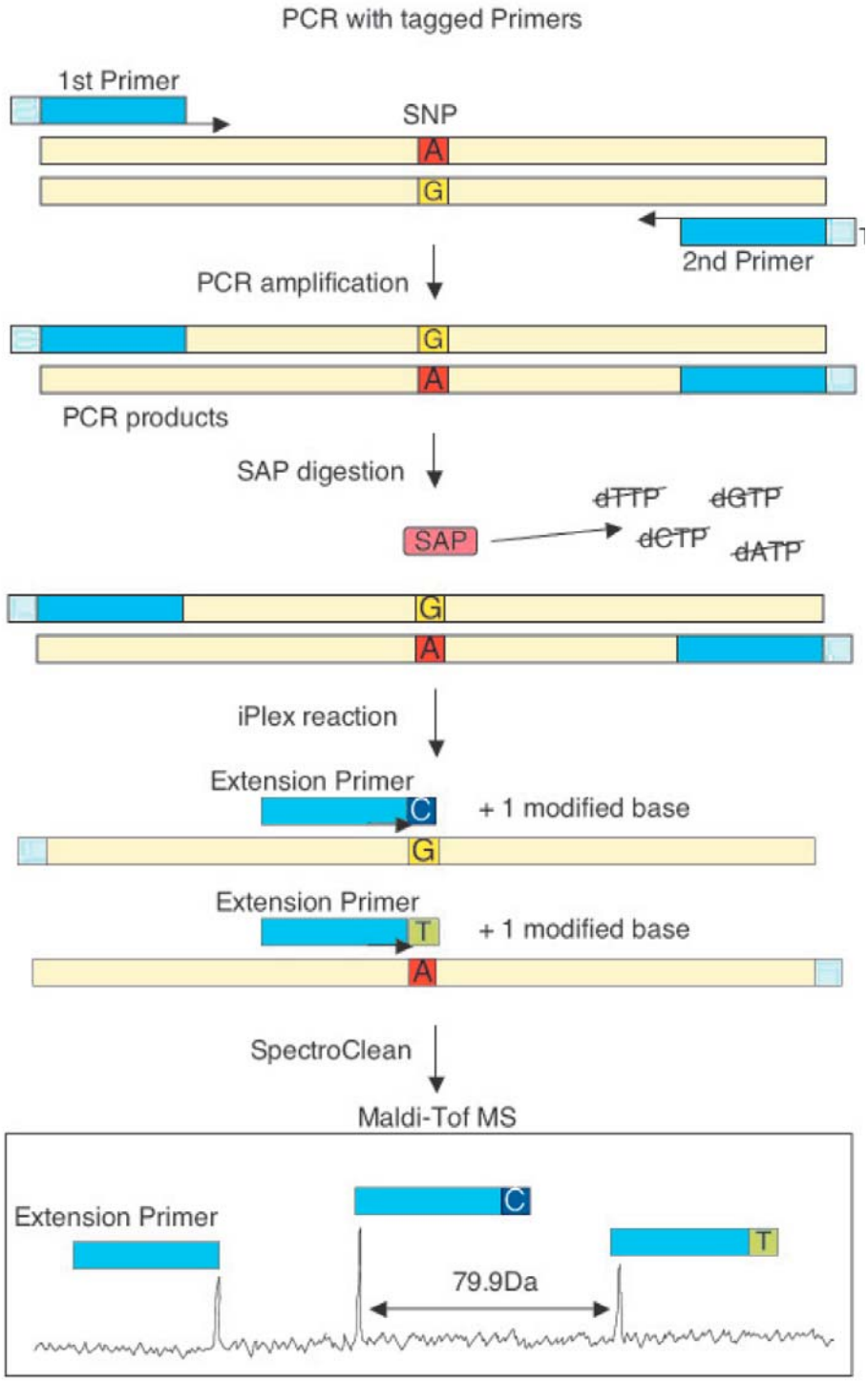


Figure 18: Schematic representation of amplification, SAP digestion, primer extension and analyte detection using iPLEX chemistry and MALDI-TOF MS (taken from Medigenomix website: http://www.medigenomix.de/en/)

Sixty-two SNPs in *ATF1*, *ATM*, *ATR*, *BARD1*, *BLM*, *BRIP1*, *CHEK1*, *CHEK2*, *DDB2*, *DMC1*, *EME1*, *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *MLH1*, *MRE11A*, *MSH2*, *MSH6*, *MUS81*, *NBS1*, *PALB2*, *PCNA*, *RFC1*, *RAD50*, *RAD51C*, *RAD51L1*, *RAD52* and *XPC* genes were genotyped using this method. Assay design was based on published sequences retrieved from the National Center of Biotechnology Information (NCBI) databases. A 34-plex and a 28-plex multiplex assay (table 10) were designed using the Sequenom MassARRAY Assay Design software (version 3.0). SNPs were genotyped using Sequenom iPLEX chemistry on a MALDI-TOF Compact Mass Spectrometer (Sequenom).

Briefly, PCR reactions were carried out in a final volume of 5 µl in standard 384-well plates. PCR was performed with 5 ng of genomic DNA, 1 unit of HotStarTaq DNA polymerase (Qiagen), 500 µmol of each dNTP and 100 nmol of each PCR primer. PCR thermal cycling was carried out in an ABI-9700 instrument (Applied Biosystems) for 15 min at 94°C, followed by 44 cycles of 20 s at 94°C, 30 s at 56°C and 60 s at 72°C. Next, PCR products were treated with 0.5 units of shrimp alkaline phosphatase for 40 minutes at 37^{o}C to dephosphorylate unincorporated dNTPs, followed by enzyme inactivation for 5 min at 85^{o}C. After adjusting the concentrations of the extension primers to equilibrate signal-to-noise ratios, the post-PCR primer extension reaction of the iPLEX gold assay was performed in a final 10 µl volume extension reaction containing 0.2 µl of termination mix, 0.0041 µl of iPLEX enzyme (Sequenom) and 700–1400 nM of extension primers. A two-step 200 short cycles program was used for the iPLEX reaction: initial denaturation was for 30 s at 94°C followed by five cycles of 5 s at 52°C and 5 s at 80°C. An additional 40 annealing and extension cycles were then looped back to 5 s at 94°C, 5 s at 52°C and 5 s at 80°C. Final extension was carried out at 72°C for 3 min. The iPLEX reaction products were desalted by diluting samples with 16 µl of water and adding 6 mg of clean resin. Following a quick centrifugation (3200 g for 5 minutes), reaction products were spotted on a 384-format SpectroChip (Sequenom). SpectroCHIPs were processed in a MassARRAY Compact Analyzer (Bruker Daltonics) by MassARRAY Workstation (version 3.3) software (Sequenom). Acquisition data were analysed using MassARRAY TYPER 3.4 software (Sequenom). Genotypes were differentiated on the basis on the mass of each allele (Figures 19 and 20).

Table 10: PCR and extension primers for the 34- and the 28-plex assays designed for SNP genotyping

SNP	Forward Primer (5'-3')	Reverse Primer (5'-3')	Extension Primer (5'-3')	Assay
rs2308317	ACGTTGGATGTCGACATACCGACTAACAGC	ACGTTGGATGGTTAGGACACTACCCAATGC	CCCAATGCCTCAACC	34 plex
rs1800935	ACGTTGGATGACAAACTGCCAATTCAAGCC	ACGTTGGATGATACTGAGAGCAATGCAACG	AATGCAACGTGCAGA	34 plex
rs7487683	ACGTTGGATGTCCTGTACTCTCTCTTCTCC	ACGTTGGATGTGTCTGTGCACTCGCAGTAG	GAGTGGGAAGGCCTC	34 plex
rs28908468	ACGTTGGATGTGGGTATGGTGGCACATCTG	ACGTTGGATGACTGAACTGGGCTCCAGAAA	GCTCCAGAAATCCTCC	34 plex
rs2227999	ACGTTGGATGTCCAAGAGTGCCTCCAGGAC	ACGTTGGATGTGAAGAGCTTGAGGATGCCG	GCAAGCTTGGGTCCTTA	34 plex
rs2303428	ACGTTGGATGTATTAGTAGCAGAAAGAAG	ACGTTGGATGGTTGATTTACCTCCCATATTG	gCATATTGGGGCCTACA	34 plex
rs45551636	ACGTTGGATGTGGGACCCTTTCTGATCAAC	ACGTTGGATGAAAATCAATCAATGCTTTTC	CAATGCTTTTCTTACCCT	34 plex
rs1042821	ACGTTGGATGAGCCTCGCTCCAGGCCGCAT	ACGTTGGATGTCACGCGAAGGCGGCCGT	acGCCGCCGCTGCCCCCG	34 plex
rs506504	ACGTTGGATGCCTGAAGATTAAAGGGAAGC	ACGTTGGATGATGGTCCGATCATGTGGCAG	ATGTGGCAGGAAGCCAAA	34 plex
rs2070094	ACGTTGGATGCTCACCACTTCACGATGCAG	ACGTTGGATGCATTTCTGGAGGCTCCATAG	AACAGCTTGACTATATCCA	34 plex
rs2020872	ACGTTGGATGACTCCCTCCGTACCAGTTCT	ACGTTGGATGAAGTTATCCAGCGGCCAGC	tcatCGGCCAGCTAATGCT	34 plex
rs1805787	ACGTTGGATGTACTCTGTGCATGTATGTTG	ACGTTGGATGTTCAGCCAGATGGCAGACTC	tGCAGACTCGAGAATAGAA	34 plex
rs7167216	ACGTTGGATGAAGAAGTGCCGCTGAGGAG	ACGTTGGATGTTTACTTGCAAAGTAGTGGG	GCAAAGTAGTGGGAAGATA	34 plex
rs1800282	ACGTTGGATGCCTGGGTCCTGGCCCGAG	ACGTTGGATGTGTAGGCGCCAAGGCCATGT	ggggATGTCCGACTCGTGGG	34 plex
rs7190823	ACGTTGGATGTTACCAAGATGGTAACTCAC	ACGTTGGATGTCAGCATTCTCTGCAGTACA	ctctaTGCAGTACATCAACCG	34 plex
rs12450550	ACGTTGGATGAAGGGAAGGAAACGCTTCAG	ACGTTGGATGACAATCACCAGTGACAGAGC	ggaaCTGCTGTCTTTGCTGTG	34 plex
rs6413508	ACGTTGGATGTTAGTTGACCATAATCATC	ACGTTGGATGGACTGAATTTAGATCACTGG	cAAAACTCTACTTCCAGAAATC	34 plex
rs2272125	ACGTTGGATGAGAGTGAGCATAGCTTTGAC	ACGTTGGATGACTCACCCAACATGTGCCTC	ggagCAAAAAGACCCTGGAACT	34 plex
rs3218688	ACGTTGGATGCTTCATTTAACGTAACTCACC	ACGTTGGATGGATTCTAGCACGCTAGAACC	tCACGCTAGAACCTACCAAATCC	34 plex
rs2229571	ACGTTGGATGCGTAAAGTTGGTGGTACATC	ACGTTGGATGTGGTGTACCTGGTGAAAGAC	gCATCGGACATGTTACTGTTTTT	34 plex
rs45494092	ACGTTGGATGTCTCTGTTTGATTTTGTTC	ACGTTGGATGGTTCTCTAAATGAACTCACC	tcccTGAACTCACCTACAATAACT	34 plex
rs556477	ACGTTGGATGGTCATTGAATAGTTCACGCC	ACGTTGGATGGATGAACTGACATAATGTGTG	ggggTGTGTGAGAAAGTCACTGA	34 plex
rs3218695	ACGTTGGATGTAGCAAAACAGGAAGCATAC	ACGTTGGATGGCGATTGTTAACATCAAAGC	tTTAACATCAAAGCTAATGAATGA	34 plex
rs28363317	ACGTTGGATGCCACCTAATGCAGGAACAAG	ACGTTGGATGTTGTAGGTAATTTTAACCAA	gAGGTAATTTTAACCAATCAGATG	34 plex
rs2522406	ACGTTGGATGATGACCCAAAGTAGCATAAG	ACGTTGGATGGGGTACTTCATGCTAAGACC	cctcCTTCATGCTAAGACCAGAGAC	34 plex
rs1009456	ACGTTGGATGAATCTGGAGCTCCAGATGTG	ACGTTGGATGTGAAAATAGCCAGGTTTGGG	gggcAGAGTGGCTGCCACCGCGAGA	34 plex
rs1800057	ACGTTGGATGTTACAGGAAAGTCTTTTCCC	ACGTTGGATGCCACAGTTCTTTTCCCGTAG	cacatTTCTTTTCCCGTAGGCTGATC	34 plex
rs3626	ACGTTGGATGGTTTTCTGTAAATAACCTAT	ACGTTGGATGGACCAGATCTGACTTTGGAC	TTGGACTTTATTCTTTAAACAAATTG	34 plex
rs4987945	ACGTTGGATGCCTGCAGTATGCTGTTTGAC	ACGTTGGATGGCTCTATTCCCATTTTTACCG	ggggCCTGGAACTATACTGGTGGTCA	34 plex
rs9462088	ACGTTGGATGACCACCTCCATGGCCTATG	ACGTTGGATGCCAAGGCCCTATCAATACTC	ggAAGGCCCTATCAATACTCACGTTAG	34 plex
rs1800734	ACGTTGGATGATCAATAGCTGCCGCTGAAG	ACGTTGGATGAGTGCCTCGTGCTCACGTTC	acatTGCCTCGTGCTCACGTTCTTCCTT	34 plex
rs11852361	ACGTTGGATGCTAGGCAATCAAATGCCACC	ACGTTGGATGTAGCATGAGCTTTAACAGAC	cGACATAATCTGAAATACTATGTATTAC	34 plex
rs13312840	ACGTTGGATGCCATGATCTGATAAAGGAC	ACGTTGGATGGATGTCATGGGGAAATGTTC	aaacgTTACCTTAAGTATAACGTAAACT	34 plex
rs2227928	ACGTTGGATGGCAGTTGATGAGTATGCAAA	ACGTTGGATGCACAATTGCAATAATACGAG	gaaAATTGCAATAATACGAGTAAGAACC	34 plex
rs1805794	ACGTTGGATGGGACGTCCAATTGTAAAGCC	ACGTTGGATGTCAATTTGTGGAGGCTGCTT	GCTGCTTCTTGGACT	28 plex
rs2228000	ACGTTGGATGAGCCATCGTAAGGACCCAAG	ACGTTGGATGTCGCTGCACATTTTCTTGCC	GAGCTTGAGGATGCC	28 plex

SNP	Forward Primer (5'-3')	Reverse Primer (5'-3')	Extension Primer (5'-3')	Assay
rs1800364	ACGTTGGATGACAGATGAGGAGAGCCTCCA	ACGTTGGATGCGTCCCTGTCACGAGTTTGT	ACGAGTTTGTGTCCCA	28 plex
rs545500	ACGTTGGATGTTCTGATCCACAGGTAGCCC	ACGTTGGATGGAGGACCAGGTTCCTGTGAA	aAGGGCTGGCCAGGGT	28 plex
rs1800149	ACGTTGGATGAGGTTAGCAAGCTGCAGGAT	ACGTTGGATGGTCTATAAAGCCTTGCGCTC	CCTTGCGCTCACACATT	28 plex
rs830083	ACGTTGGATGCAACGTGACAAAACCCCATC	ACGTTGGATGTCAGCCTCCCAAGTGCGTG	CATGCCCAGCTAATTTTT	28 plex
rs2299015	ACGTTGGATGAGAGGAATCCACAAATAGGG	ACGTTGGATGCTGAATCCCCACACTATTCC	gcttGCGTGGACACTCTGC	28 plex
rs2230674	ACGTTGGATGTTGTCTGAGAGGTGAGAGTC	ACGTTGGATGGATCCGAACTACACCTTCAG	ccCCTTCAGCTACTTCTCTG	28 plex
rs769420	ACGTTGGATGGCTAGGTTGATAACAGAAGAG	ACGTTGGATGTTCCTGTATCAACAACACAC	tataAACAACACACGTTCCC	28 plex
rs6927706	ACGTTGGATGGTCAAAAGGCAACAGAGTTC	ACGTTGGATGTGTGTCTGCAAGGGTGTGAG	ggtCATGGTGTGACTGTTCT	28 plex
rs3738888	ACGTTGGATGGTGAACAGGAAGAAAAGTATG	ACGTTGGATGACCAGCTGTTCTCTGTTGAG	ctccGTTGAGCCTGCTTCTGC	28 plex
rs2234997	ACGTTGGATGGCTAAAATGTCAAGAACTC	ACGTTGGATGATCAGCTCCGTAAATAGCAC	ATTAGATGAATCTTTCACTGT	28 plex
rs3218708	ACGTTGGATGTTAGGGCAGCTGATATTCGG	ACGTTGGATGCAGGTGGAGGGATTTGGTAG	atGGATTTGGTAGGTTCTAGC	28 plex
rs45478192	ACGTTGGATGGCCTGATGTGTATAATCTCG	ACGTTGGATGCCTTGGGAATTACATACCTG	ccaTCTGATTTCCAAATTTCCC	28 plex
rs2066791	ACGTTGGATGGTAGGAGTTTGTTGGCACAG	ACGTTGGATGAATATAAGCCAACCTCGCTC	ctaatCCAACCTCGCTCAAGACC	28 plex
rs2229032	ACGTTGGATGAGGATGCCTGGGCAGGAGA	ACGTTGGATGGCCAGTGTATGCTACCAAAG	ccCTGAAAAACTCAAAGTATTCC	28 plex
rs17879961	ACGTTGGATGCAAAGGTTCCATTGCCACTG	ACGTTGGATGAGGAAGTGGGTCCTAAAAAC	AGTGGGTCCTAAAAACTCTTACA	28 plex
rs10831234	ACGTTGGATGAACCTCTGCCACAAAGCATC	ACGTTGGATGCGTGACAACAGGATCTTGAG	aGCTTTAGTTTATGTTGTGATCT	28 plex
rs2241261	ACGTTGGATGTAACCTCTGGTCATTAGGTC	ACGTTGGATGTCCAGGAAAACGCTGGTTAG	gaAGCTAACCTGAAACAAAGGTAG	28 plex
rs2227914	ACGTTGGATGTGCCAGCTTCTTCATGGAAC	ACGTTGGATGACTGCATTTCCCATCAGGTG	cccTTTCCCATCAGGTGAACATCAG	28 plex
rs769416	ACGTTGGATGGATGAACCATCTATTGGAAG	ACGTTGGATGGTTTTCCCTTTGAAGATTTG	aCTTTGAAGATTTGTTTTCTTTCCT	28 plex
rs45532440	ACGTTGGATGACTGAGTCCTAAACGCATGG	ACGTTGGATGGGATGTGATTTTCCTGGTAG	gcgGGTAGAACAATAAGGTCCTCTT	28 plex
rs2235000	ACGTTGGATGTGGCTTACTTGGAGCCATAA	ACGTTGGATGAGGCTGACCCAGTAAATAAC	ccctTCTGTCAACCTCAACTAAACTA	28 plex
rs2706377	ACGTTGGATGTTTATCCTATTAAACTTTGC	ACGTTGGATGGCTCTTATCATTTATTTTTT	TTTAAATCTAAACTTATAAACCATTTC	28 plex
rs3187395	ACGTTGGATGCCCTTTGGAAACAACATTGG	ACGTTGGATGCCTGTGCTATTTTGTTGCTTG	gTTTTTTGTTGATTAATTCTTCTTTTT	28 plex
rs9282681	ACGTTGGATGGTAAAAAGCGAAAGGCAGCA	ACGTTGGATGTGTTGCAGACCTCAGGCTGG	ggggTCCGTGTGGCCCCGGATCAGCAC	28 plex
rs12677527	ACGTTGGATGGCTAATATTGTGCTTAGGAG	ACGTTGGATGAAAAAGGCTGGTTTACCAA	gggagAAAGGCTGGTTTACCAATCTCTT	28 plex
rs4986764	ACGTTGGATGAGTCTACACTTGAAGTGACC	ACGTTGGATGTCTGGTGATAGATGACTTGC	ggaagTGCTGCTTCCAGTAAATAAGGTG	28 plex

For quality control, 48 random samples were genotyped in duplicate. Furthermore, ten samples were sequenced to confirm genotype calls from the MALDI-TOF platform. The genotype concordance rate between platforms was 99%. The order of the DNA samples on 384-well plates was randomized in order to ensure the same study conditions for samples from cases and controls. Genotyping call rates ranged from 95% to 99% and duplicate concordance rates were higher than 99%. The SNP that had 20% missing data was excluded from further analysis.

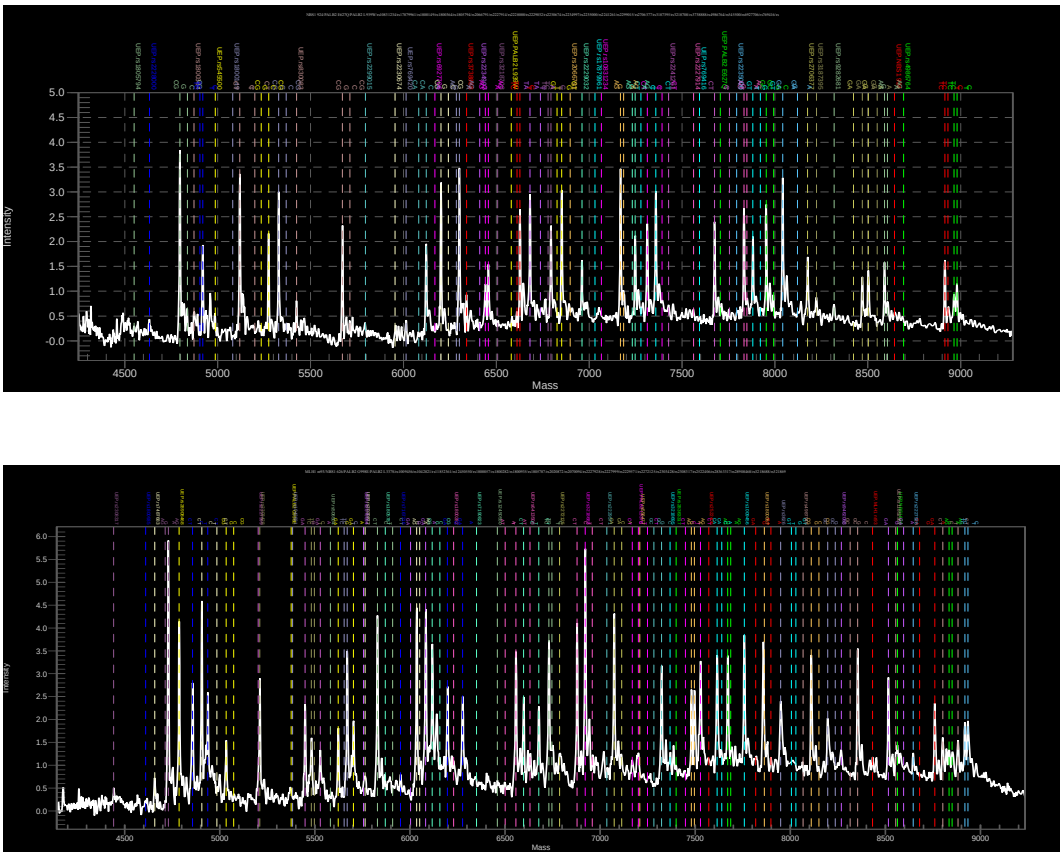


Figure 19: Examples of a 28-plex (top) and a 34-plex (bottom) reaction . The positions of all probes and possible analyte peaks are labelled. Mass is in daltons (Da).

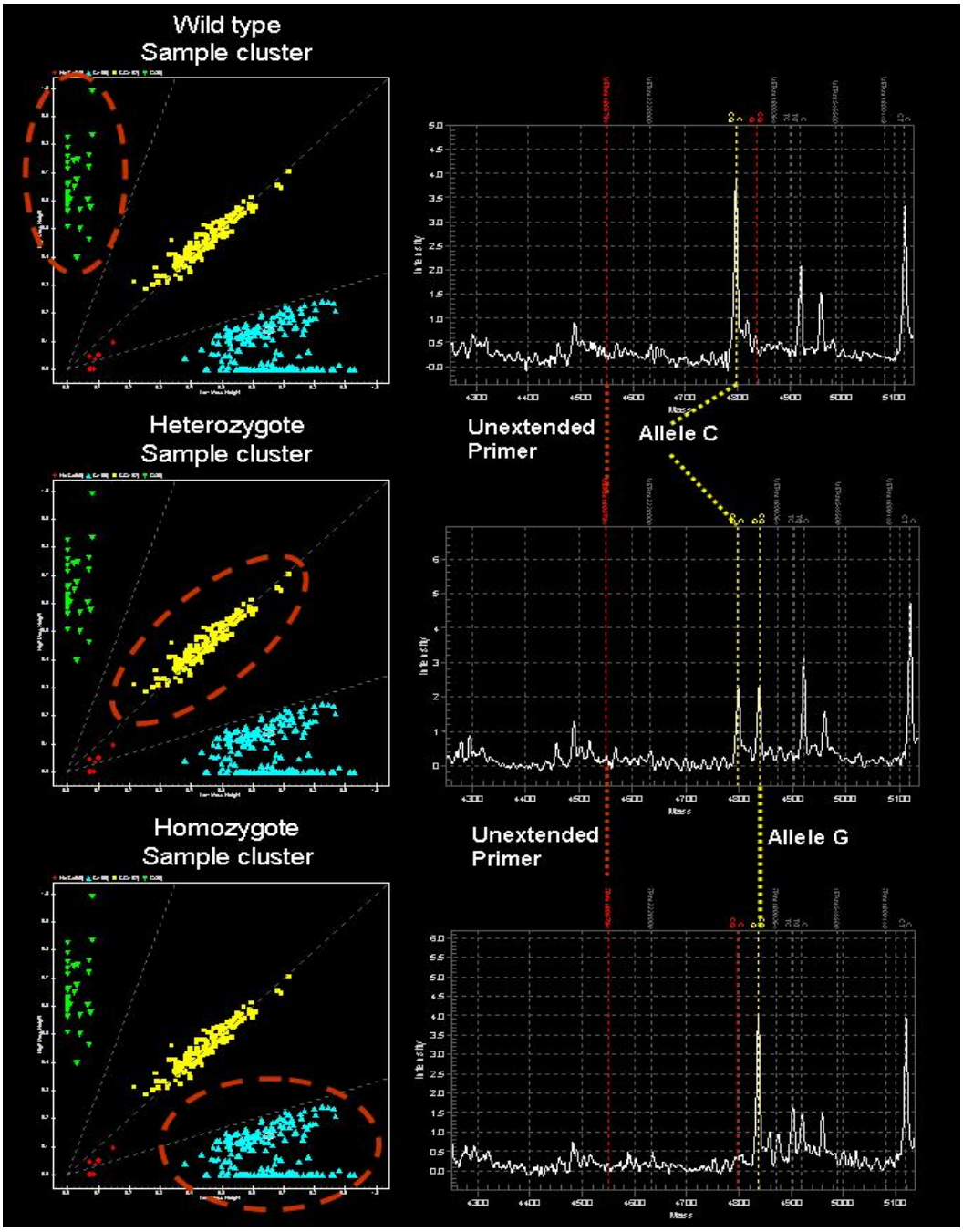


Figure 20: Typical raw data output from the multiplex MassARRAY spectrometry iPLEX assay (rs1805794). Graphs on the left show clusters of all samples on the chip, with non-template controls at the bottom left (in red). The sample spectrum is shown on the right, with the unextended primer marked on the left with the dotted red line, and the three potential products marked right with dotted yellow lines. A homozygote wild type is shown in (a), while the heterozygote rs1805794 is shown in (b) and the homozygote mutant in (c).

4.1.10 Statistical Analysis

Statistical analysis was performed using SNPStats, a web-based application designed for analysis of association studies. SNPStats has a user-friendly interface and utilizes PHP (Personal Home Page) server programming language for building input forms, uploading data, calling the statistical analysis procedures and processing the output. Statistical analyses are performed using the *R genetics* package (R Development Core Team, 2005, http://www.R-project.org) as well as the *haplo.stats* package (http://mayoresearch. mayo.edu/mayo/research/biostat/schaid.cfm) (Sole et al., 2006).

The validity of genetic association studies depends considerably on the use of appropriate controls. In theory, a control sample from a homogeneous randomly mating population should follow Hardy–Weinberg equilibrium (HWE). HWE for a locus depends on a number of assumptions about the tested population. Conformity with HWE suggests that these assumptions are met including, for example, absence of recent mutations, no selective survivorship among genotypes, conformance with Mendelian segregation and random mating. Testing for HWE is used primarily as a data quality check. Departures from HWE, may point to genotyping error or other biases. Loci which deviate from HWE among controls are usually discarded (Khoury et al., 1993). All SNPs that were genotyped in this study, were assessed for conformance with HWE in the control samples, by applying an exact test.

The effect of each SNP on breast cancer risk, was assessed by applying a number of statistical tests. The primary tests of association were the univariate analyses between each SNP and breast cancer. Genotype frequencies in cases and controls were compared using the X^2 test. The association between breast cancer and each SNP was examined using logistic regression with the SNP genotype tested, under models of complete dominance and recessive inheritance, as well as under the log-additive model. Associations between the different genotypes and the risk of breast cancer were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from logistic regression analyses with and without adjustment for age (under or over 55 years), menopause status (pre- or post-menopausal), family history of breast cancer (first degree relative with breast cancer) and use of hormone replacement therapy. The significance level was set at $P<0.05$.

Associations between breast cancer and common haplotypes of the *ATM*, *MRE11A, NBS1* and *XRCC1* genes were also investigated using SNPStats, which allows the estimation of maximum likelihood estimates of haplotype frequencies using the Expectation-Maximization (EM) algorithm. Logistic regression was performed to test the association between haplotypes and breast cancer risk. Haplotypes with a frequency of less than 1% were not considered further for analysis since they are likely to be a result of rare recombination events.

4.2 Results

4.2.1 Association analysis of SNPs and breast cancer

The characteristics of the cases and controls enrolled in this study are summarized in Table 11. The mean ± SD age of cases and controls were 51.6±9.2 and 56.4±9.2 years respectively with a median age of 56 years for both groups. There were no statistically significant differences, between cases and controls in terms of level of education and marital status. It is noted that the numbers of cases and controls were also representative of the geographical distribution of the Cypriot population.

As shown in Table 11, there was a substantial, statistically significant difference in the frequency of cases and controls reporting a first-degree relative with breast cancer. Women who had a first degree relative with breast cancer were at a higher risk for breast cancer (OR=1.64, 95% CI 1.26 to 2.12), compared to women without a family history of the disease.

Breast cancer risk was significantly greater in obese women (BMI >30) (OR 1.29, 95% CI 1.03 to 1.60) when compared with women with a BMI<25 respectively.

A statistically significant trend with age at menarche and breast cancer risk was observed. Women who started menstruating earlier than the age of 11 had ~50% increased risk for breast cancer, compared to women who started menstruating after the age of 15 years (OR=1.56, 95% CI 1.14 to2.13). Furthermore women with an age of menarche between 12-14 years were also at an increased risk for breast cancer compared to women who started menstruating after the age of 15 years (OR 1.27, 95% [CI] 1.01-1.6).

Some associations of reproductive factors with breast cancer were observed in the study sample. In detail, cases were more likely than controls to have never been pregnant. This difference is statistically significant (OR 0.69, 95% [CI] 0.50-0.96). Furthermore, women with three or more children and women who had breastfed for more than 1 year had significantly reduced ORs compared with women who had never had a child and never breastfed respectively (Table 11). There were no notable case-control differences in the age at first pregnancy.

Equal percentages of cases (25.32%) and controls (25.13%) had ever used birth control pills. However, significantly more controls than cases had ever taken HRT and were at a reduced risk for breast cancer (OR =0.45, 95% CI= 0.37-0.55).

Table 11: Demographic and risk factor characteristics in the Cypriot population

Variables	Number		OR (95% CI)	*P*
	Cases	Controls		
Age (years)				
Mean ± SD	51.6±9.2	56.4±9.2		
Median	56	56		
Education				
Primary School	419	463	1.00 (ref.)	
High School	412	434	1.05 (0.87-1.3)	0.62
College or other post HS	131	147	0.98 (0.75-1.29)	0.91
Bachelor's	135	122	1.22 (0.93-1.62)	0.16
Marital status				
Married	924	1012	1.00 (ref.)	
Widowed / divorced	130	117	1.22 (0.93-1.59)	0.15
Never married	57	42	1.49 (0.99-2.24)	0.06
Family history of breast cancer				
No	952	1068	1.00 (ref.)	
Yes	156	107	1.64 (1.26-2.12)	0.0001
Age at menarche (years)				
≤11	157	134	1.56 (1.14-2.13)	0.005
12-14	798	838	1.27 (1.01-1.6)	0.043
15 or more	154	205	1.00 (ref.)	
Pregnancy				
No	88	66	1.00 (ref.)	
Yes	1023	1110	0.69 (0.50-0.96)	0.028
Number of children				
None	103	88	1.00 (ref.)	
One	90	82	0.94 (0.62-1.42)	0.76
Two	480	492	0.83 (0.61-1.14)	0.25
Three or more	436	515	0.72 (0.53-0.99)	0.04
Age at 1st pregnancy				
Before 30	877	991	1.00 (ref.)	
Over 30	119	103	1.30 (0.99-1.73)	0.06
Breast feeding				
Never	393	346	1.00 (ref.)	
6 months or less	331	381	0.76 (0.62-0.94)	0.01
7-12 months	166	181	0.81 (0.63-1.04)	0.10
More than 1 year	222	267	0.73 (0.58-0.92)	0.0075
Oral contraceptive use				
No	826	876	1.00 (ref.)	
Yes	280	294	1.01 (0.87-1.16)	0.92
HRT use				
No	926	815	1.00 (ref.)	
Yes	174	341	0.45 (0.37-0.55)	<0.00001

A total of 79 SNPs were studied in relation to breast cancer risk in the Cypriot population. Of these 79 SNPs, 78 were genotyped successfully whereas one had more than 20% missing data and was excluded from further analysis.

Table 12 summarizes the distribution of genotypes in cases and controls, as well as the allele frequencies, for the 79 SNPs, of which the 78 were successfully genotyped. Seven SNPs (rs1800149, rs1801320, rs2706377, rs1800282, rs7487683, rs3626, rs28908468) deviated significantly from HWE in controls ($p<0.01$) and were excluded from further analysis. SNPs which deviated slightly from HWE, ($0.01<$HWE $p<0.05$) were tested for association with breast cancer risk despite not conforming to the HWE condition. The deviations observed are likely to be chance results rather than genotyping errors, since the homozygotes for these SNPs are very rare in the Cypriot population. It is notable that the frequencies of these SNPs are similar to those reported by the HapMap Project. These small deviations may also be due to hidden population structures that specifically affect the genes of interest. Of the remaining 71 SNPs, 8 were monomorphic in both groups.

Significant differences in genotype frequencies between breast cancer patients and controls were observed in 8 of the 71 SNPs analyzed. The associations of SNPs and breast cancer risk in Cypriot women are shown in Table 13. Eight of the 71 SNPs were associated at a p value of less than 0.05. Three SNPs were protective for breast cancer while the remaining five, were associated with an increased risk for the disease.

The *NBS1* gene SNPs rs13312840 and rs769416 and the *MRE11A* SNP rs556477 were associated with a reduced risk for breast cancer. In detail, the variant allele of *NBS1* rs13312840 (924 T>C) was associated with a reduced risk of disease (OR TT *vs.* TC/CC = 0.58; 95% CI, 0.37 to 0.92; p = 0.019). Carriers of the *NBS1* rs769416 rare allele also had a reduced risk of breast cancer (OR GG *vs.* GT/TT = 0.23, 95% CI 0.06–0.85, P = 0.017). Furthermore, the variant allele of *MRE11A* rs556477 was associated with a reduced risk of developing the disease (OR AA *vs.* AG/GG = 0.76; 95% CI, 0.64 to 0.91; p = 0.0022).

The variant alleles of *MUS81* rs545500, *PBOV1* rs6927706, *XRCC1* rs25489, *BRCA2* rs1799944 and *MRE11A* rs601381 were associated with an increased risk of breast cancer. In detail, the variant allele of *MUS81* rs545500 was associated with an increased risk of developing breast cancer (OR GG *vs.* GC/CC= 1.21, 95% CI, 1.02 to 1.45; p = 0.031). In addition, the rare allele of *PBOV1* rs6927706 was also associated with an increased risk of developing breast cancer (OR AA *vs.* AG/GG = 1.53, 95% CI, 1.07 to 2.18; p = 0.019). The rare allele of *XRCC1* rs25489 was associated with an increased risk for breast cancer. Homozygous carriers of *XRCC1* A allele were found to have an increased risk of breast cancer (OR AA *vs.* GG/GA = 4.41, 95% CI 0.93–20.99, P = 0.035). Significant associations with breast cancer risk were also found for the variant alleles of *BRCA2* rs1799944 and *MRE11A* rs601341. Carriers of *BRCA2* rs1799944 G allele were found to have an increased risk of breast cancer (OR AA *vs.* AG/GG = 1.58, 95% CI 1.19–2.09, P = 0.0013) with Ptrend = 0.0012. Homozygous carriers of the *MRE11A* rs601341 A allele had an increased risk of breast cancer (OR AA/GA *vs.* GG = 1.39, 95% CI 1.09–1.77, P = 0.0081) with Ptrend = 0.027.

No significant associations with breast cancer were observed for the other 55 SNPs studied.

Table 12: Genotype frequencies in cases and controls for the 79 SNPs studied

Gene		Controls				Cases				MAF		HWE
	rs number	AA	Aa	aa	Total	AA	Aa	aa	Total	Controls	Cases	Controls
ATF1	rs2230674	1071	86	2	1159	1021	81	1	1103	0.04	0.04	0.69
ATM	rs1800057	1087	85	2	1174	1015	85	0	1100	0.04	0.04	0.68
	rs2234997	1153	4	1	1158	1093	10	0	1103	0	0	0.01
	rs2235000	1160	1	0	1161	1102	2	0	1104	0	0	1
	rs3218688	979	2	0	981	926	0	0	926	0	0	1
	rs3218695	1177	0	0	1177	1109	0	0	1109	0	0	Monomorphic
	rs3218708	1177	0	0	1177	1109	0	0	1109	0	0	Monomorphic
	rs4987945	1169	1	0	1170	1100	1	0	1101	0	0	1
ATR	rs2227928	401	520	229	1150	344	517	218	1079	0.43	0.44	0.011
	rs2229032	899	242	17	1158	833	253	16	1102	0.12	0.13	0.89
BARD1	rs1048108	514	520	138	1172	515	445	138	1098	0.33	0.34	0.7
	rs2070094	466	551	156	1173	461	485	159	1105	0.37	0.36	0.75
	rs2229571	341	580	249	1170	316	540	241	1097	0.46	0.47	0.95
	rs3738888	1150	7	0	1157	1099	4	0	1103	0	0	1
BLM	rs11852361	1123	46	2	1171	1046	52	2	1100	0.02	0.03	0.094
	rs7167216	1127	44	2	1173	1055	47	2	1104	0.02	0.02	0.081
BRCA2	rs1799944	1058	108	4	1170	945	133	8	1086	0.05	0.07	0.52
BRIP1	rs4986764	475	534	161	1170	465	502	141	1108	0.37	0.35	0.57
CHEK1	rs506504	1064	105	5	1174	986	116	2	1104	0.05	0.05	0.19
CHEK2	rs17879961	1158	0	0	1158	1101	1	0	1102	0	0	1
DBC2	rs2241261	342	560	252	1154	288	546	264	1098	0.46	0.49	0.26
DDB2	rs830083	871	271	15	1157	803	277	21	1101	0.13	0.14	0.3
DMC1	rs2227914	1146	3	0	1149	1099	1	1	1101	0	0	1
EME1	rs12450550	918	221	24	1163	838	239	23	1100	0.12	0.13	0.021
ERCC2	rs13181	383	585	208	1176	331	603	171	1105	0.43	0.43	0.59
FANCA	rs1800282	978	149	19	1146	883	189	16	1088	0.08	0.1	<0.0001
	rs2239359	433	543	186	1162	387	524	190	1101	0.39	0.41	0.46
	rs7190823	573	476	113	1162	541	462	98	1101	0.3	0.3	0.33
	rs9282681	1096	51	0	1147	1065	42	2	1109	0.02	0.02	1
FANCC	rs1800364	1159	1	0	1160	1104	1	0	1105	0	0	1
FANCD2	rs2272125	787	346	38	1171	710	346	43	1099	0.18	0.2	1

Gene		Controls				Cases				MAF		HWE
	rs number	AA	Aa	aa	Total	AA	Aa	aa	Total	Controls	Cases	Controls
FANCE	rs9462088	1051	115	4	1170	990	106	4	1100	0.05	0.05	0.56
MLH1	rs1800149	1156	0	1	1157	1101	0	2	1103	0	0	0.00043
	rs2020872	1177	0	0	1177	1109	0	0	1109	0	0	Monomorphic
	rs2308317	1177	0	0	1177	1109	0	0	1109	0	0	Monomorphic
	rs1800734	494	496	137	1127	446	505	136	1087	0.34	0.36	0.47
	rs1799977	568	497	110	1175	543	449	98	1090	0.31	0.3	0.95
MRE11A	rs1009456	1040	105	0	1145	992	89	5	1086	0.05	0.05	0.17
	rs10831234	949	190	12	1151	899	193	6	1098	0.09	0.09	0.48
	rs556477	444	550	167	1161	494	473	130	1097	0.38	0.33	0.9
	rs601341	452	566	156	1174	385	530	190	1105	0.37	0.41	0.32
MSH2	rs205920	562	489	119	1170	512	471	108	1091	0.31	0.31	0.41
	rs2303428	929	217	18	1164	870	211	16	1097	0.11	0.11	0.22
MSH6	rs1042821	653	451	64	1168	646	388	70	1104	0.25	0.24	0.24
	rs1800935	655	428	90	1173	608	413	83	1104	0.26	0.26	0.094
MUS81	rs545500	673	430	55	1158	589	435	77	1101	0.23	0.27	0.22
NBS1	rs1805787	548	483	104	1135	549	447	103	1099	0.3	0.3	0.94
	rs1805794	543	502	109	1154	511	497	96	1104	0.31	0.31	0.68
	rs6413508	1167	6	1	1174	1097	4	0	1101	0	0	0.012
	rs769416	1141	10	0	1151	1098	3	0	1101	0	0	1
	rs769420	1177	0	0	1177	1109	0	0	1109	0	0	Monomorphic
	rs12677527	546	505	115	1166	512	497	96	1105	0.32	0.31	0.95
	rs13312840	1096	55	0	1151	1063	32	1	1096	0.02	0.02	1
OGG1	rs1052134	647	455	72	1174	615	422	71	1108	0.26	0.25	0.54
PALB2	L337S	1170	1	0	1171	1097	4	0	1101	0	0	1
	E672Q	1035	120	4	1159	972	126	4	1102	0.06	0.06	0.77
	L939W	1177	0	0	1177	1109	0	0	1109	0	0	Monomorphic
	G998E	1076	92	2	1170	1010	92	2	1104	0.04	0.04	1
PBOV1	rs6927706	1083	63	1	1147	1017	83	2	1102	0.03	0.04	0.61
PCNA	rs3626	827	242	36	1105	838	196	34	1068	0.14	0.12	0.0012
RAD50	rs2299015	742	370	45	1157	743	323	37	1103	0.2	0.18	1
	rs2522406	1064	41	1	1106	1044	31	0	1075	0.02	0.01	0.34
	rs2706377	1052	66	21	1139	1046	37	14	1097	0.05	0.03	<0.0001
	rs3187395	1177	0	0	1177	1109	0	0	1109	0	0	Monomorphic

Gene		Controls				Cases				MAF		HWE
	rs number	AA	Aa	aa	Total	AA	Aa	aa	Total	Controls	Cases	Controls
RAD51	rs1801320	952	216	0	1168	915	193	0	1108	0.09	0.09	<0.0001
	rs1801321	400	530	236	1166	340	522	236	1098	0.43	0.45	0.014
RAD51C	rs28363317	1164	6	0	1170	1095	6	0	1101	0	0	1
RAD51L1	rs28908468	572	201	0	773	979	75	0	1054	0.13	0.04	<0.0001
RAD52	rs11226	568	494	108	1170	561	448	92	1101	0.3	0.29	1
	rs7487683	1135	35	3	1173	1072	33	0	1105	0.02	0.01	0.0043
RFC1	rs2066791	1177	0	0	1177	1109	0	0	1109	0	0	Monomorphic
TP53	rs1042522	638	438	97	1173	555	463	85	1103	0.27	0.29	0.088
XRCC1	rs1799782	973	182	9	1164	914	175	8	1097	0.09	0.09	0.88
	rs25487	520	516	140	1176	506	479	122	1107	0.34	0.33	0.49
	rs25489	959	207	2	1168	923	177	9	1109	0.09	0.09	0.01
XRCC2	rs3218536	999	177	1	1177	972	135	1	1108	0.08	0.06	0.02
XRCC3	rs861539	351	600	226	1177	312	560	220	1092	0.45	0.46	0.32
XPC	rs2228000	673	402	64	1139	653	379	65	1097	0.23	0.23	0.68
	rs2227999	1040	128	4	1172	963	138	4	1105	0.06	0.07	1

AA, common homozygote; Aa, heterozygote; aa, rare homozygote.

Table 13: Genotypic specific risk (OR and 95% CI)

Gene	Rs number	Dominant OR (95%CI)[a] ; p value[a]	Recessive OR (95%CI)[a] ; p value[a]	Log-additive OR (95%CI)[a] ; p value[a]
ATF1	rs2230674	1.02 (0.74-1.42); 0.89	0.60 (0.05-7.34); 0.68	1.01 (0.73-1.40); 0.94
ATM	rs1800057	1.10 (0.79-1.52); 0.57	-	1.07 (0.78-1.47); 0.69
	rs2234997	2.31 (0.75-7.12); 0.13	-	1.83 (0.68-4.91); 0.23
	rs4987945	1.27 (0.07-21.89); 0.87	-	-
ATR	rs2227928	1.14 (0.95-1.38); 0.15	1.04 (0.84-1.30); 0.71	1.07 (0.95-1.21); 0.25
	rs2229032	1.09 (0.89-1.34); 0.42	1.01 (0.48-2.15); 0.97	1.07 (0.89-1.30); 0.45
BARD1	rs1048108	0.92 (0.78-1.10); 0.37	1.07 (0.82-1.40); 0.6	0.97 (0.86-1.11); 0.68
	rs2070094	0.98 (0.82-1.17); 0.8	1.07 (0.83-1.37); 0.6	1.01 (0.89-1.14); 0.93
	rs2229571	0.99 (0.82-1.20); 0.93	0.96 (0.78-1.18);0.69	0.98 (0.87-1.11); 0.77
	rs3738888	0.46 (0.13-1.66); 0.23	-	-
BLM	rs11852361	1.28 (0.85-1.95); 0.24	0.95 (0.13-7.12); 0.96	1.25 (0.84-1.85); 0.27
	rs7167216	1.26 (0.82-1.94); 0.3	0.95 (0.13-7.14); 0.96	1.22 (0.81-1.84); 0.34
BRCA2	rs1799944	**1.57 (1.19-2.07); 0.015**	1.95 (0.56-6.76); 0.28	**1.53 (1.17-1.99); 0.014**
BRIP1	rs4986764	0.94 (0.79-1.12);0.49	0.96 (0.75-1.25);0.78	0.96 (0.85-1.09); 0.53
CHEK1	rs506504	1.19 (0.89-1.59); 0.24	0.48 (0.09-2.59); 0.37	1.15 (0.87-1.51); 0.34
DBC2	rs2241261	1.18 (0.97-1.43); 0.095	1.16 (0.94-1.43); 0.17	1.12 (0.99-1.27); 0.061
DDB2	rs830083	1.14(0.93-1.39);0.2	1.51 (0.74-3.07);0.25	1.14 (0.95-1.37); 0.15
DMC1	rs2227914	0.63 (0.09-4.39);0.64	-	1.10 (0.25-4.73); 0.9
EME1	rs12450550	1.15 (0.94-1.42); 0.18	0.94 (0.52-1.72); 0.85	1.11 (0.92-1.33); 0.27
ERCC2	rs13181	1.08 (0.89-1.30); 0.43	0.80 (0.63-1.00); 0.054	0.97 (0.85-1.10); 0.61
FANCA	rs7190823	1.02 (0.86-1.22); 0.8	0.91 (0.68-1.23); 0.56	1.00 (0.87-1.14); 0.95
	rs9282681	0.84 (0.54-1.30);0.44	-	0.89 (0.58-1.35); 0.57
	rs2239359	1.13 (0.94-1.35); 0.2	1.05 (0.83-1.33); 0.67	1.07 (0.95-1.22); 0.26
FANCC	rs1800364	1.25 (0.07-21.55); 0.88	-	-
FANCD2	rs2272125	1.12 (0.93-1.34); 0.24	1.08 (0.68-1.72); 0.74	1.09 (0.93-1.28); 0.27
FANCE	rs9462088	0.95 (0.71-1.26); 0.71	0.73 (0.15-3.53); 0.7	0.94 (0.71-1.24); 0.67
MLH1	rs1799977	0.97 (0.81-1.15); 0.73	1.02 (0.76-1.38); 0.88	0.99 (0.86-1.13); 0.81

Gene	Rs number	Dominant OR (95%CI)[a] ; p value[a]	Recessive OR (95%CI)[a] ; p value[a]	Log-additive OR (95%CI)[a] ; p value[a]
	rs1800734	1.09 (0.91-1.31); 0.33	1.01 (0.77-1.31); 0.96	1.05 (0.92-1.20); 0.46
MRE11A	rs1009456	0.93 (0.68-1.26);0.63	-	0.98 (0.73-1.32); 0.9
	rs10831234	1.04 (0.83-1.31);0.72	0.69 (0.25-1.93);0.47	1.02 (0.82-1.26); 0.85
	rs556477	**0.76 (0.64-0.91); 0.0022**	0.81 (0.62-1.05); 0.11	**0.82 (0.72-0.93); 0.0027**
	rs601341	1.12 (0.93-1.34); 0.23	**1.38 (1.08-1.76); 0.0089**	**1.15 (1.02-1.31); 0.027**
MSH2	rs2059520	0.98 (0.82-1.17); 0.83	0.93 (0.70-1.25); 0.64	0.98 (0.85-1.11); 0.71
	rs2303428	1.02 (0.82-1.26); 0.89	0.93 (0.45-1.89); 0.83	1.01 (0.83-1.22); 0.94
MSH6	rs1042821	0.85 (0.71-1.01); 0.066	1.23 (0.84-1.79); 0.29	0.92 (0.80-1.07); 0.27
	rs1800935	1.09 (0.91-1.30); 0.34	1.07 (0.77-1.48); 0.69	1.07 (0.93-1.22); 0.36
MUS81	rs545500	**1.21 (1.02-1.45); 0.031**	1.43(0.98-2.08);0.06	**1.21(1.04-1.39); 0.012**
NBS1	rs1805787	0.92 (0.77-1.10); 0.36	0.97 (0.72-1.31); 0.84	0.95 (0.83-1.08); 0.43
	rs1805794	1.08 (0.91-1.28); 0.4	0.93 (0.69-1.26);0.65	1.03 (0.90-1.18); 0.65
	rs6413508	0.46 (0.13-1.66); 0.23	-	0.46 (0.14-1.50); 0.18
	rs769416	**0.23 (0.06-0.85); 0.017**	-	-
	rs12677527	1.04 (0.88-1.24); 0.64	0.88 (0.65-1.19); 0.4	1.00 (0.87-1.14); 0.98
	rs13312840	**0.58 (0.37-0.92); 0.019**	-	**0.61 (0.39-0.95); 0.028**
OGG1	rs1052134	0.98 (0.82-1.17); 0.82	1.15 (0.80-1.64); 0.45	1.01 (0.88-1.16); 0.91
PALB2	L337S	3.75 (0.40-35.04); 0.2	-	-
	E672Q	1.06 (0.81-1.40); 0.66	0.73 (0.18-3.04);0.67	1.05 (0.80-1.36); 0.74
	G998E	1.04 (0.76-1.43); 0.8	0.67 (0.09-4.89); 0.69	1.03 (0.76-1.4); 0.85
PBOV1	rs6927706	**1.53 (1.07-2.18); 0.019**	1.63(0.12-21.60);0.71	**1.51(1.06-2.13); 0.02**
RAD50	rs2299015	0.89 (0.74-1.07); 0.21	0.90 (0.56-1.43); 0.65	0.91 (0.77-1.06); 0.22
	rs2522406	0.80 (0.49-1.31); 0.37	-	0.78 (0.48-1.26); 0.31
	rs3187395	0.32 (0.06-1.85); 0.19	-	-
RAD51	rs1801320	0.91 (0.73-1.14); 0.41	-	-
	rs1801321	1.12 (0.93-1.35); 0.22	1.04 (0.84-1.29); 0.69	1.07 (0.94-1.20); 0.31
RAD51C	rs28363317	0.93 (0.28-3.10); 0.91	-	-
RAD52	rs11226	0.88 (0.74-1.05); 0.16	0.91 (0.67-1.24); 0.54	0.91 (0.80-1.04); 0.18
TP53	rs1042522	1.10 (0.93-1.31); 0.27	0.85 (0.62-1.16); 0.3	1.03 (0.90-1.18); 0.67

Gene	Rs number	Dominant OR (95%CI)[a] ; p value[a]	Recessive OR (95%CI)[a] ; p value[a]	Log-additive OR (95%CI)[a] ; p value[a]
XRCC1	rs1799782	1.05 (0.83-1.33); 0.66	1.03 (0.38-2.78); 0.95	1.05 (0.84-1.31); 0.67
	rs25487	0.97 (0.82-1.16); 0.77	0.91 (0.69-1.19); 0.49	0.96 (0.85-1.21); 0.58
	rs25489	0.97 (0.77-1.22); 0.78	**4.41 (0.93-20.99); 0.035**	1.01 (0.81-1.26); 0.92
XRCC2	rs3218536	0.79 (0.61-1.02); 0.069	0.44 (0.02-8.00); 0.58	0.79 (0.61-1.01); 0.064
XRCC3	rs861539	1.05 (0.87-1.35); 0.59	1.08 (0.87-1.35); 0.48	1.05 (0.93-1.19); 0.45
XPC	rs2228000	1.01 (0.84-1.21); 0.91	0.96 (0.66-1.40); 0.85	1.00 (0.87-1.16); 0.99
	rs2227999	1.12 (0.86-1.46); 0.42	1.10 (0.26-4.64); 0.42	1.11 (0.86-1.43); 0.43

Data in bold highlight the statically significant results; *BRCA2* rs1799944 dominant model (AA vs AG/GG), *MRE11A* rs556477 dominant model (AA vs AG/GG), rs601341 recessive model (GG/GA vs AA), *MUS81* rs545500 dominant model (GG vs CG/CC), *NBS1* rs769416 dominant model (GG vs GT), *NBS1* rs13312840 dominant model (TT vs TC/CC), *PBOV1* rs6927706 dominant model (AA vs AG/GG) and *XRCC1* rs25489 recessive model (GG/GA vs AA)

[a] Adjusted for age, menopause status, family history of breast cancer and use of hormone replacement therapy (HRT)

4.2.2 Haplotype analyses

Data analysis suggests that the *NBS1* haplotype GGCGCAC (rs769416, rs769420, rs13312840, rs1805794, rs6413508, rs12677527, rs1805787), which contains the *NBS1* rs13312840 C allele, is associated with a reduced breast cancer risk compared with the most frequent haplotype GGTCCGC (OR=0.62; 95% CI= 0.39 to 0.97). This haplotype was found more frequently among controls (2.25%) than cases (1.47%) in Cypriot women (p=0.037). A reduced risk for breast cancer was also associated with a rare haplotype in the *NBS1* gene (OR=0.42; 95% CI= 0.26 to 0.66; $p=2 \times 10^{-4}$). In addition, the *MRE11A* haplotype AGCG (rs556477, rs601341, rs10831234, rs1009456) is associated with a significantly increased risk for breast cancer (OR=1.32; 95% CI= 1.13 to 1.54; p=0.0004). This haplotype was found more frequently among cases (27.52%) than controls (23.84%) in Cypriot women (p=0.037). None of the common *ATM* and *XRCC1* haplotypes were associated with breast cancer (Table 14).

Table 14: Estimated haplotype frequencies in cases and controls and haplotypic specific risks

Gene	Haplotype	Control freq	Case freq	OR [95%CI]	χ^2 p-value	Global test p-value
ATM[a]	CCCCCTGC	0.92	0.9225	reference		0.53
	GCCCCTGC	0.0367	0.0349	0.96 (0.69-1.32)	0.8	
	CCCCGTGC	0.0376	0.0329	0.89 (0.34-1.23)	0.48	
	Rare	0.0057	0.0097	1.6 (0.72-3.57)	0.25	
MRE11A[b]	GACG	0.3702	0.326	reference		0.021
	AGCG	**0.2384**	**0.2752**	**1.32 (1.13-1.54)**	**4×10^{-4}**	
	AACG	**0.2478**	**0.255**	**1.17 (1.00-1.36)**	**0.044**	
	AGTG	0.0864	0.0891	1.16 (0.93-1.45)	0.19	
	AGCT	0.0444	0.0438	1.12 (0.84-1.51)	0.44	
	Rare	0.0128	0.0109	0.98 (0.52-1.85)	0.95	
NBS1[c]	GGTCCGC	0.3705	0.3886	reference		0.00019
	GGTCCGG	0.2955	0.2925	0.94 (0.82-1.09)	0.44	
	GGTGCAC	0.2786	0.2905	0.99 (0.86-1.15)	0.92	
	GGCGCAC	**0.0225**	**0.0147**	**0.62 (0.39-0.97)**	**0.037**	
	Rare	**0.0329**	**0.0137**	**0.42 (0.26-0.66)**	**2×10^{-4}**	
XRCC1[d]	CGA	0.5021	0.493	reference		0.83
	CGG	0.3249	0.3308	0.95 (0.83-1.09)	0.48	
	CAA	0.0818	0.0839	0.97 (0.76-1.23)	0.78	
	TGA	0.0804	0.0846	0.98 (0.77-1.24)	0.84	
	Rare	0.0108	0.0077	1.57 (0.66-3.73)	0.31	

Data in bold highlight the statically significant results

[a] *ATM* haplotypes are arranged in the order of rs1800057, rs3218688, rs3218695, rs4987945, rs2230674, rs2234997, rs2235000, rs3218708

[b] *MRE11A* haplotypes are arranged in the order of rs556477, rs601341, rs10831234, rs1009456

[c] *NBS1* haplotypes are arranged in the order of rs769416, rs769420, rs13312840, rs1805794, rs6413508, rs12677527, rs1805787

[d] *XRCC1* haplotypes are arranged in the order of rs1799782, rs25489, rs2548

4.3 Discussion

Breast cancer is a complex disease, with multiple genetic and environmental factors involved in its aetiology. The polygenic model of breast cancer suggests that a proportion of the disease can be explained by multiple loci across the genome which contribute to susceptibility. This model has been proposed as the most likely explanation for the bulk of the genetic component of breast cancer (Antoniou et al., 2002; Pharoah et al., 2002).

DNA repair is essential for maintaining genomic integrity. Deficiencies in the DNA repair pathway lead to genetic instability, which in turn may lead to cancer development. Genetic polymorphisms in DNA repair genes may contribute to differential DNA repair capability between individuals (Mohrenweiser and Jones, 1998). The extent to which genetic variation in the DNA repair genes contributes to breast cancer risk remains unclear. Given the complexity of breast cancer aetiology and the involvement of different DNA repair pathways in breast cancer, this study was designed to investigate the association between common SNPs in DNA repair genes and breast cancer risk.

4.3.1 General considerations regarding the study design and the population studied

The case-control study is the most commonly used approach to study associations between genetic polymorphisms and cancer. This is mainly due to the simplicity of the required sampling and the ease of analysis and interpretation of results. The aim of a case-control association study is to identify genetic components that may contribute to a medical condition, by comparing the distribution of polymorphic markers in candidate genes in groups of unrelated affected and unaffected individuals (Malats and Calafell, 2003).

Besides the advantages of case-control studies, this approach also has some weaknesses. A case-control study that has low participation rates or substantially different ethnicity distribution between cases and controls is subject to selection bias (Rothman et al., 2001; Wacholder et al., 2000). Population stratification is not considered to have caused any bias in the present study, as Cyprus, being an island, presents an example of a genetic isolate. Consequently the Cypriot population is

very homogeneous and this makes Cyprus an ideal location for genetic association studies. As this was a nationwide, population-based study, the number of controls participating in the study was representative of the distribution of the population in the five districts, Nicosia, Limassol, Larnaka, Paphos and Famagusta, as per the official 2004 population estimates. As the results show, in terms of the basic demographic characteristics of the cases and controls recruited into the study, there were no statistically significant differences in the level of education and marital status between the two groups.

The study was designed to be representative of the Cypriot population and towards this effort 1109 Cypriot women diagnosed with breast cancer between the years 1999 and 2006, as well as 1177 age matched healthy controls were recruited. The incidence of breast cancer in Cypriot women is about 400 cases per year and therefore the number of 1109 recruited into this study represents about 40% of the cases diagnosed between the years 1999-2006. Mortality data are not currently available for the Cypriot population. This is mainly due to the fact that until recently, physicians were not obliged to inform the National Cancer Registry on the disease progression and survival of the cases registered into the database. Assuming that the estimated age-adjusted survival from breast cancer for Western Europe, which sums up to 74% of the diagnosed patients (Parkin et al., 2005), also applies for Cyprus, it is estimated that of the approximately 2800 newly diagnosed breast cancer cases between the years 1999 and 2006, around 70 died, before or during the period that the cases were ascertained for the study. This is a relatively small number and the estimation that the present study represents 40% of the cases diagnosed with breast cancer in Cyprus between 1999 and 2006 is greatly not affected.

The participation rate of cases and controls was very high covering around 98% of eligible cases and controls. It has been observed that healthy volunteers who want to participate in an epidemiological study tend to have a higher educational level compared to the remaining population (dos Santos Silva 1999). The present study does not confirm this observation, since the level of education was not significantly different between cases and controls.

The control population consisted of women who were participating in the National

breast cancer-screening program with the use of mammography. All Cypriot women over the age of 50 receive an invitation from the Department of Medical and Public Health Services to attend for a mammogram at their local breast-screening unit, free of charge. Based on the fact that healthy controls were ascertained at the five district breast screening units, the majority of controls participating in the study were postmenopausal women, over the age of 50, who had accepted the invitation for a free mammogram. A smaller proportion of women, under the age of 50, also visit the mammography screening units and hence the study includes a number of premenopausal healthy controls aged below 50 years old. The criteria for ascertaining breast cancer cases were a histologically confirmed diagnosis of breast cancer between the years 1999 and 2006 and an age of diagnosis greater than 40 years. Inevitably, the cases participating in the study were younger than the controls.

Breast cancer is largely viewed as a disease which is predominantly influenced by lifestyle risk factors since only approximately 15% of all breast cancer cases can be attributed to familial and genetic influences (Martin and Weber, 2000). The majority of known risk factors for breast cancer are linked to the hazardous effects of hormonal exposures (ESHRE Capri Workshop Group, 2004). This is also confirmed in the present study. Early age at menarche, small number of children, nulliparity and little or no breastfeeding are all associated with an increased risk of developing breast cancer in the Cypriot population.

Epidemiological studies have demonstrated that use of hormone replacement therapy is associated with an increased risk of breast cancer (Beral, 2003). However, in contrast to current knowledge, a tendency of decreased risk of breast cancer was found among women who received HRT in this study population. This might at least partly be due to the participation of older women in the control population, since HRT is more common among older women.

Another possible source of bias in case-control studies is recall bias. In the current study, breast cancer cases were more likely and more motivated to recall and report exposure to risk factors than the disease-free controls. In addition, the fact that patients are usually more familiar with the study hypothesis than healthy controls could also lead to recall bias. Case-control studies are also vulnerable to information

biases. It is possible that study subjects are misclassified with respect to disease or exposure status. In the current study, cases were verified by reviewing histological reports and controls were women with a negative mammography result hence the possibility of the study being affected by information bias is limited. Furthermore, since the same 5 well-trained scientists conducted the interviews for all the cases and controls, the possibility of information bias is considered to minimal.

However, since genotypes are not modifiable and cannot be affected by recall, information or selection biases, these potential causes of bias are not anticipated to have had any significant effect in the outcomes of the present study.

4.3.2 Genetic variation in the DNA repair genes *XRCC1, XRCC2* and *XRCC3* and risk of breast cancer in the Cypriot population

The *XRCC* genes constitute one of the most well studied groups of genes, in relation to breast cancer risk. These genes were initially discovered through their role in DNA damage response caused by ionizing radiation. *XRCC* genes are important components of various DNA repair pathways contributing to DNA-damage processing and genetic stability (Thacker and Zdzienicka, 2004).

The *XRCC1* gene is an important component of the BER pathway acting as a scaffold for other BER enzymes (Caldecott, 2003). Although many SNPs in the *XRCC1* gene have been documented, three common (variant allele frequency > 0.05) non-synonymous coding SNPs, which are located at codons 194, 280 and 399, have been studied extensively in relation to breast cancer risk (Hu et al., 2005; Saadat and Ansari-Lari, 2008). These SNPs have been shown to alter DNA repair capacity in phenotypic studies and have hence received considerable attention. In detail, rs1799782 (R194W) polymorphism has been associated with an increased BER capacity (Wang et al., 2003) whereas rs25489 (R280H) and rs25487 (R399Q) polymorphisms have been associated with reduced DNA repair capacity (Lunn et al., 1999; Duell et al., 2000; Hu et al., 2001; Matullo et al., 2001; Lei et al., 2002; Takanami et al., 2005; Pachkowski et al., 2006).

The *XRCC2* and *XRCC3* genes are necessary for HR repair and are required for *RAD51* focus formation (Bishop et al., 1998; O'Regan et al., 2001). Rs3218536, a

non-synonymous coding variant in the *XRCC2* gene which changes amino acid 188 from an arginine to a histidine, has been studied extensively in relation to cancer risk but its functional role still remains to be elucidated (Auranen et al., 2005; Danoy et al., 2007; Garcia-Closas et al., 2006; Popanda et al., 2006). Rs861539, a common *XRCC3* variant, comprising a threonine to methionine substitution at amino acid position 241, has been associated with less efficient DNA repair (Matullo et al., 2001) and an increase in the number of centrosomes and binucleated cells (Lindh et al., 2006). This variant has been proposed as a low-penetrance cancer allele for breast and lung cancer (Kuschel et al., 2002 ; Jacobsen et al., 2004) as well as for acute myeloid leukaemia (Seedhouse et al., 2004). However, it has also been demonstrated that the *XRCC3* codon 241 wild type and variant alleles are functionally equivalent in the DSB repair pathway (Araujo et al., 2002) and a number of reports did not find a link between this variant and cancer (Thacker, 2005).

Genetic association studies on breast cancer risk have extensively studied the effects of the above-mentioned, potentially functional, SNPs in the X-Ray cross-complementing genes *XRCC1*, *XRCC2* and *XRCC3* in relation to breast cancer risk. However, the results of these studies have been contradictory and inconsistent (Garcia-Closas et al., 2006; Hu et al., 2005; Saadat and Ansari-Lari, 2008), necessitating the organization of additional case-control studies, for evaluating these variants. In this context, the aim of the present study was to test the association of these common *XRCC* variants and breast cancer in the Cypriot population.

Comparison of the *XRCC1* R280H genotypes between breast cancer cases and healthy controls, revealed that the *XRCC1* 280H allele is associated with an increased risk of breast cancer. In detail, women homozygous for the histidine allele had a 4- to 5-fold increased risk for breast cancer compared to those homozygous for the arginine allele. The association between *XRCC1* R280H SNP and breast cancer is biologically plausible. This SNP is located between the N-terminal and the BRCT I domains of *XRCC1*, in the linker region that separates DNA polymerase b interacting domain, from *PARP* (poly-ADP-ribose polymerase) interacting domain (Kubota et al., 1996; Takanami et al., 2005). There is evidence that this nonconservative amino acid alteration may influence DNA repair capability by

altering the protein-protein interactions between *XRCC1* and other BER proteins. Two recently published functional studies, which investigated the role of the *XRCC1* R280H variant in altering DNA repair capacity, have shown that relative to the wild-type protein, the 280H variant decreases the DNA repair capacity (base excision repair / single strand break repair capacity) of mammalian cells exposed to chemical stresses (Pachkowski et al., 2006; Takanami et al., 2005). In addition, a meta-analysis by Hu et al. (2005) concluded that the 280H variant is associated with an increased cancer risk. These studies support the finding reported here, i.e., the statistically significant association of the 280H allele with breast cancer.

The study by Pachkpwski et al. (2005) has also reported a positive association between the *XRCC1* R280H genotype, breast cancer and smoking. Cells with a reduced DNA repair capacity may not be able to handle DNA damage caused by continuous exposure to genotoxic insults such as smoking. In the current study, three out of the nine women homozygous for the 280His allele were smokers who started smoking at a young age. It is possible that the rest of the women who are homozygous for the 280His allele, were exposed for prolonged durations to passive smoking and that the combination of this exposure with the *XRCC1* 280His genotype, increased their breast cancer risk. Unfortunately information on passive smoking exposure was not collected and this hypothesis cannot be confirmed.

In the current study, there was also some evidence of a dominant protective effect for the *XRCC2* 188His allele carriers. However, the effect of this SNP after adjusting for covariates such as age, menopause status, family history of breast cancer and use of HRT, did not remain statistically significant. There are contradictory reports regarding the role of this rare *XRCC2* variant in breast cancer, with some studies showing an association with increased risk of breast cancer whereas others did not. A recent meta-analysis conducted by the Breast Cancer Association Consortium, concluded that this variant is not associated with breast cancer (Breast Cancer Association Consortium, 2006). Furthermore, a second meta-analysis by Garcia-Closas et al. (2006) in Caucasian populations reached the same conclusion.

This study did not show a statistically significant association between the *XRCC1* R194W, R399Q and *XRCC3* T241M SNPs and breast cancer risk in the Cypriot

population. These results are comparable with those reported from the Breast Cancer Association Consortium (2006) where variants *XRCC1* R399Q and *XRCC3* T241M did not modify breast cancer risk. In addition, a recent meta-analysis supported lack of association between *XRCC1* R194W SNP and breast cancer risk in Caucasian populations (Zhang et al., 2006).

In conclusion the results of this study, suggest that *XRCC1* R280H SNP is associated with breast cancer risk in Cypriot women. Taking into account the important role of *XRCC1* in BER, the statistically significant difference in breast cancer risk in Cypriot women associated with this variant may indicate a true association.

4.3.2 Genetic variation in genes interacting with *BRCA1/2* risk of breast cancer in the Cypriot population Cyprus

Ten different genes, which are involved in pathways critical for the maintenance of genomic integrity, have been implicated in inherited predisposition to breast cancer, including *BRCA1*, *BRCA2*, *p53*, *PTEN*, *CHEK2*, *ATM*, *NBS1*, *RAD50*, *BRIP1* and *PALB2*. The association of germline mutations in DNA repair genes with an increased susceptibility to breast cancer, highlights the importance of these pathways in the development of breast cancer (Walsh and King, 2007).

The DNA repair pathway is clearly involved in familial breast cancer. Thus, it was hypothesized that common SNPs of genes involved in the DNA repair pathway, may influence breast cancer risk. In this context numerous studies have investigated the role of SNPs in DNA repair genes in relation to breast cancer and have reported associations with breast cancer risk (Goode et al., 2002; Kuschel et al., 2002; Garcia-Closas et al., 2006; Haiman et al., 2008).

Analysis of members of the DNA repair pathway appears to be a good rationale for identifying novel susceptibility loci. In particular, genes which have a direct interaction with the *BRCA1* and *BRCA2* genes are very good candidates. Recently, two additional susceptibility genes, namely *BRIP1*/*FANCJ* and *PALB2*/*FANCN*, which interact with *BRCA1* and *BRCA2* genes respectively, have been identified (Seal et al., 2006; Rahman et al., 2007).

The primary aim of this study was to investigate the association between SNPs in genes that interact with *BRCA1* and *BRCA2* and breast cancer risk in the Cypriot population. Genetic variation in 72 SNPs in 35 genes, which interact with *BRCA1* and/or *BRCA2* genes and their association with breast cancer, was investigated in a case-control study of Cypriot women. Furthermore, the role of 2 additional SNPs in the *PBOV1* (*UROC28*) and *DBC2* genes which are both upregulated in breast cancer was also assessed (An et al., 2000; Hamaguchi et al., 2002).

Significant associations with breast cancer risk were observed for eight of the 72 SNPs investigated. Five of these eight SNPs were associated with an increased breast cancer risk, while the remaining three were associated with a reduced risk for breast cancer.

In the current study, there was evidence for an increased breast cancer risk for women carrying the *BRCA2* rs1799944 SNP. The additive model showed a significant trend (P = 0.014) towards increased risk of breast cancer with the number of aspartic acid allele copies among Cypriot women. The N991D variant is located in the conserved BRC repeat region of the *BRCA2* gene, in exon 11 (Rapakko et al., 2006) and many individuals with a family history of breast cancer are carriers of this variant. It has been classified as a variant of no clinical significance in the BIC database. On the other hand, in silico prediction methods suggest that this is a non tolerated amino acid substitution within the limits of confidence in the alignments (Fackenthal et al., 2005). Therefore, until functional data become available, the pathogenicity of this variant cannot be excluded, and it may be a variant that increases risk moderately, but is indeed, not highly penetrant. In this study there was no association between the presence of the *BRCA2* 991Asp allele and family history of breast cancer.

A moderately strong association of this *BRCA2* polymorphism with malignant melanoma has been reported. The presence of this common *BRCA2* variant was associated with malignant melanoma risk (P = 0.002 after Bonferroni correction), in over 9% of the cases studied. The authors suggested that this variant is not a neutral missense mutation and that follow-up studies should be undertaken in melanoma and breast cancer populations to precisely define its pathogenicity (Debniak et al., 2008).

The role of this SNP in breast cancer risk has been investigated in the Multiethnic Cohort study and no association was found (Freedman et al., 2004).

Previous studies that were performed in the Cypriot population revealed a different spectrum of mutations in the *BRCA1* and *BRCA2* genes compared to other populations (Hadjisavvas et al., 2004). In addition, the study of early onset breast cancer cases described in Chapter 3 of this thesis further confirms this finding. The over-representation of the *BRCA2* N991D polymorphism in the breast cancer group supports the notion that this variant is associated with an increased breast cancer risk among Cypriot women and it is possible that this association is characteristic only for the Cypriot population.

The study data also support the notion that *MUS81* rs545500 C allele carriers are at an increased risk for breast cancer. Rs545500 is a non-synonymous SNP located in the coding region of *MUS81*, a structure-specific DNA nuclease which plays an important role in DNA repair by homologous recombination (Osman and Whitby, 2007). This polymorphism results in an amino acid change from a positively charged hydrophilic arginine to an uncharged hydrophobic proline residue, which may have an effect on the 3D structure or a protein-protein binding interface of the *MUS81* protein (Nakken et al., 2007). The role of the *MUS81* gene in breast cancer has not been investigated. However, it was demonstrated that *MUS81* homozygote and heterozygote knockout mice, have a predisposition to develop cancer. Proper bialellic expression of *MUS81* is critical for the maintenance of genomic integrity and tumour suppression (McPherson et al., 2004). Therefore, the rs545500 SNP could predispose individuals to breast cancer, but functional studies need to be performed in order to identify the actual role of this variant in carcinogenesis.

The results of the statistical analysis also suggest that the *PBOV1* rs6927706 polymorphism may be a risk factor for breast cancer. Rs56927706 is a non-synonymous SNP located in the coding region of *PBOV1*, a gene which is up-regulated in prostate, breast, and bladder cancers (An et al., 2000). This polymorphism results in an amino acid change at codon 73 from a hydrophobic isoleucine to a hydrophilic threonine residue. Bioinformatics analysis indicates that this SNP could be involved in splicing regulation (Lee and Shatkay, 2008). However

further work is warranted since the exact roles of the *PBOV1* protein as well as its functional domains are not well known at present.

Analysis of genotyping results revealed that Cypriot women who carry the *NBS1* rs13312840 C and rs769416 T alleles have a reduced risk of breast cancer. The *NBS1* protein is involved in NHEJ pathway that repairs DSBs. The first step of this pathway consists of the recognition of DSBs by the MRN complex whose core contains the *MRE11*, *RAD50* and *NBS1* proteins. *NBS1* is the key regulator of this protein complex (Jazayeri and Jackson, 2002; Kobayashi et al., 2004).

The *NBS1* rs13312840 T>C SNP is located in the 5' UTR (-1120) that is the transcription factor *GATA-1* binding site. The activation domains of *GATA-1* are capable of activating transcription in mammalian cells through *GATA* motifs (Merika and Orkin, 1993). The results of the Cypriot study are in contrast to those of a recent study by Lu et al. (2006), who found an increased risk for breast cancer in non-Hispanic Caucasian women aged 55 years or younger, who were carriers of the rs13312840 C allele. Conflicting evidence for association may be due to population-specific and/or age-specific differences. The protective effect of the *NBS1* rs13312440 SNP observed in this study could be attributed to the SNP itself or to linkage disequilibrium with another variant.

To the best of the author's knowledge this is the first study investigating the role of *NBS1* rs769416 SNP and breast cancer risk. The rs769416 SNP causes an amino acid change (Gly to Lys) at codon 216 of the *NBS1* gene. This SNP is not located within one of the three functional regions of the *NBS1* protein but it may have an alternative splicing regulatory effect, based on the Functional Single Nucleotide Polymorphism (F-SNP - http://compbio.cs.queensu.ca/F-SNP/) database (Lee and Shatkay, 2008). The association of rs769416 SNP and breast cancer needs to be interpreted with caution, since this is a rare SNP in the Cypriot population and the most likely explanation for this association is chance.

Several studies have investigated *NBS1* E185Q SNP (rs1805794) in relation to breast cancer risk (Forsti et al., 2004; Hsu et al., 2007; Kuschel et al., 2002; Lu et al., 2006; Millikan et al., 2005; Zhang et al., 2005). The allele-dose association of this SNP

with breast cancer reported by Lu et al. (2006) was not replicated in this study. A recent large US study of 894 African–American breast cancer cases and 788 controls and 1417 whites breast cancer cases and 1234 controls, reported that rs1805794 is not associated with breast cancer risk (Millikan et al., 2005). In addition, a large-scale case-control study in Europe reported a similar result (Kuschel et al., 2002). Based on the results of these two large scale studies, which are also confirmed by the Cypriot study, it can be concluded that rs1805794 SNP is not associated with breast cancer risk.

Haplotype analysis with the combination of the seven *NBS1* SNPs showed that the frequency of the GGCGCAC haplotype (rs769416, rs769420, rs13312840, rs1805794, rs6413508, rs12677527, rs1805787) was lower in cases than in controls (0.0147 versus 0.0225; p=0.035) suggesting a protective effect. There was also evidence for a protective effect of the rare pooled *NBS1* haplotypes. This protective effect is driven by the difference in frequencies of the pooled rare haplotypes which conferred a low risk (OR=0.42) and had a combined frequency of 3.29% in controls and 1.37% in cases. It is possible that this haplotype is a marker for a single, rare, protective mutation in the Cypriot population. There may be value in sequencing this region, in order to help identify the protective variant(s). Both these findings need to be replicated in independent studies in order to confirm or refute this effect.

The data of the Cypriot breast cancer case-control study suggest that the *MRE11A* rs556477 G allele may be associated with a reduced breast cancer risk. Furthermore, there is evidence for an increased breast cancer risk for women homozygous for the MRE11A rs601341 A allele. The *MRE11A* gene forms a complex with *RAD50* and *NBS1* genes which is involved in the cellular response to DNA double strand breaks. Defects in the members of this tri-complex are linked to increased chromosomal instability which leads to cancer (van den Bosch et al., 2003).

The rs556477 common variant is located in intron 15 of the *MRE11A* gene. The rs556477 MAF is 40% in Caucasians as reported in NCBI's dbSNP database; the same as that observed in our population. The functionality of this SNP is not clear. TFSEARCH webtool (http://www.cbrc.jp/research/db/TFSEARCH.html) was used to search for potential transcription factors binding sites at this position. The

rs556477 SNP is located in a region that is a potential transcription factor binding site of activator protein 1 (*AP-1*), which plays a critical role in signal transduction pathways in many cells. A recent study has shown that inhibition of *AP-1* transcription factors suppresses breast cancer growth. Inhibitors which are capable of blocking *AP-1* activation may be promising agents for the treatment and prevention of breast cancer (Liu et al., 2002). The reduced risk of breast cancer for carriers of rs556477 SNP found in this study is in contrast with the above finding since it is expected that the creation of an *AP-1* binding site will result in an increased breast cancer risk. However it must be taken into account that the prediction that rs556477 A to G substitution results in a gain of an *AP-1* binding site is based on *in silico* analysis and this remains to be proven by *in vitro* data. Furthermore, the *MRE11A* rs556477 polymorphism may not be causal, but could be in linkage disequilibrium with a true protective variant.

The rs601341 A to G substitution results in potential binding of ubiquitous transcription factor Ying Yang 1 (*YY1*) that has a fundamental role in normal biologic processes such as differentiation, replication and cell proliferation. *YY1* overexpression and/or activation results in uncontrolled cellular proliferation, resistance to apoptotic stimuli and tumorigenesis (Gordon et al., 2006). To our knowledge, the role of rs601341 in breast cancer has not been investigated but a protective effect of this SNP against follicular lymphoma has been reported (Rollinson et al., 2006). The increased breast cancer risk associated with homozygous *MRE11A* rs601341 G allele carriers observed in this study could be attributed to the SNP itself or to linkage disequilibrium with another variant in the region. Functional studies will need to be performed in the future to identify the actual causal variant.

The present study presents evidence for an increased breast cancer risk for women carrying the *MRE11A* AGCG (rs556477, rs601341, rs10831234, rs1009456) haplotype. The set of SNPs in the *MRE11A* gene that were genotyped in the present study, were characterized by Allen-Brady and Camp (2005) as the most informative group of SNPs (tagging SNPs) in this gene, which can capture >90% of the intragenetic variation. It has been suggested that haplotype based designs are more powerful compared to single-allele approach studies for association studies (Johnson

et al., 2001). Hence a haplotype based approach where the SNPs being analyzed represent the entire genetic variation of the gene of interest should provide more accurate and definitive findings. It is noteworthy that the effect observed for the *MRE11A* AGCG haplotype in the Cypriot study was stronger than for any individual SNP. This observation suggests that the association is likely to be due to another functional variant or variants, in linkage disequilibrium with the SNPs tested. There may be value in sequencing this region, in order to help identify the causative variant(s). This finding needs to be replicated in independent studies in order to confirm or refute this effect.

There was no evidence of association with breast cancer risk for the rest of the SNPs assessed in the present study. The group of genes evaluated in this study has been analyzed extensively in relation to breast cancer risk and studies have produced contradictory results. However, none of the SNPs for which no association was observed in the Cypriot population, have been marked as major targets which modify breast cancer risk. Furthermore a recent meta-analysis by Vineis et al. (2009), which summarizes the data from genetic association studies for genes involved in DNA repair, has failed to identify associations between a number of SNPs included in the present study (rs4987945, rs2227928, rs2229032, rs4986764, rs13181, rs1052134, rs1801320, rs1801321, rs1042522) and breast cancer risk. The lack of evidence of association indicates that these SNPs are not susceptibility variants for breast cancer. However, there is always the possibility that one or more SNPs could be associated with the disease, but this study does not have sufficient statistical power to detect the association.

It should be noted that the scope of this study was not to comprehensively examine all common genetic variation across these genes for association with breast cancer risk. This study was restricted to a number of SNPs, which are either functional or have been reported by other groups, to modify cancer risk and are located within genes which interact with either *BRCA1* and/or *BRCA2*. The possibility that other variants in these genes are associated with breast cancer risk cannot be excluded. Future large-scale comprehensive candidate gene studies are warranted, to systematically tag all known common variants in these genes and to test the tagging SNPs associations with breast cancer susceptibility.

This study was well powered to detect alleles with modest to high effects at stringent levels of significance. For example, the study has over 80% power to detect a variant with a MAF of 5% conferring a risk of 1.6 under a dominant model. The study had very limited power to detect recessive alleles. The SNP selection for this study was based solely on functionality and their position in genes interacting with BRCA1/2 rather than allele frequency. As a result of this, a number of monomorphic/low-polymorphic SNPs were included in the study. It is noted that this is the first time that these SNPs were studied in the Cypriot population, and their allele frequencies were a priori unknown. Rare SNPs can also contribute to disease risk (Gorlov et al., 2008). However, our study did not have sufficient power to detect such associations, and the possibility that some of the low-polymorphic SNPs studied contribute to breast cancer risk cannot be ruled out.

Since the design of this study knowledge about the *BRCA1* and *BRCA2* genes and their interactions has increased and a similar study starting now would have undoubtedly included more candidate genes and SNPs. In addition, with the completion of Phase III of the HapMap project there is now an unprecedented reference panel for tagging SNP selection which could be studied in addition to the functional SNPs evaluated in the present study. Despite living in the era of genomewide association studies, it is plausible to assume that an expansion of the current candidate gene approach, to more genes and SNPs interacting with *BRCA1* and *BRCA2*, would reveal additional important information regarding breast cancer susceptibility in the Cypriot population. Well-designed candidate genes methods can still achieve higher capture of target genetic variation in candidates genes compared to commercially available SNP arrays. However, this gap is quickly narrowing since it is expected that the new generation of SNP arrays will include a greater number of tagging SNPs, which will provide better coverage as well as significantly increased overall genetic power.

One of the limitations of this study is that the analysis did not consider the possibility of gene-gene interactions. The available data on breast cancer suggest that many cases are likely to be due to co-existence of multiple low penetrance breast cancer susceptibility alleles (Pharoah et al., 2002; Antoniou and Easton, 2006). However, very little is known on how these multiple susceptibility alleles interact

with each other. Since the proteins encoded by the genes selected as candidate susceptibility loci in this study lie in the same pathway, it is possible that the risks observed are the result of interactions which lead to partial abrogation of the DNA repair function. This study has not attempted to assess such effects since the estimate of an interaction effect will be unreliable because of small numbers. For this type of analysis a very large sample size is essential. A review of the literature indicates that this type of analysis is still at its infancy and it is expected that data regarding the interactions of common breast cancer susceptibility alleles, as well as on the interactions among these risk alleles and non-genetic factors will emerge in the near future (Stratton and Rahman, 2008).

It is also possible that while some SNPs displayed marginal or no association with breast cancer risk when studied on an individual basis, they can show evidence of an association with breast cancer risk when combined with other SNPs in other genes. There are examples which point towards this direction. Three studies have observed significant trends in breast cancer risk with increasing numbers of risk genotypes and have suggested that combined SNPs in multiple DNA repair pathways may contribute to breast cancer risk (Smith et al., 2003a; Fu et al., 2003; Smith et al., 2008). Recently, novel high-throughput methods have been established which can determine synthetic gene interactions in multicellular organisms by genome-wide RNA interference (van Haaften et al., 2004). This method was successful in identifying previously unassociated genes with a double-strand-break response that may play a role in human carcinogenesis. The results of such studies can provide useful information on interactions of known DNA repair and related genes, which can be investigated further in genetic association studies.

Gene-environment interactions were also not interrogated in this study. Many DNA repair genes are environmentally-responsive genes and it would be interesting to study the biological implications of genetic variation, in response to an environmental stimulus. However, there are analytic challenges of testing for multiple genetic and environmental risk factors using traditional analytic tools (Thornton-Wells et al., 2004). In genetic studies of complex traits, gene-environment interactions are common but the likelihood of false positive results occurring by chance because of the large number of comparisons being carried out, is a possible

problem which could lead to false positive associations occurring by chance (Bird et al., 2001).

In conclusion, this study provides support for the hypothesis that genetic variants in DNA repair genes influence breast cancer risk and provides further evidence for the polygenic model of breast cancer. However, large-scale genetic epidemiologic studies are warranted to further examine and corroborate the associations observed, between polymorphisms and breast cancer in multi-ethnic groups. In addition, elucidation of the functional impact of the breast cancer associated SNPs is needed, in order to provide further insights into their mechanistic effects on risk.

4.4 Publications resulting from work described in this Chapter

- Loizidou, M. A., Michael, T., Neuhausen, S. L., Newbold, R. F., Marcou, Y., Kakouri, E., Daniel, M., Papadopoulos, P., Malas, S., Kyriacou, K., and Hadjisavvas, A. (2008). Genetic polymorphisms in the DNA repair genes XRCC1, XRCC2 and XRCC3 and risk of breast cancer in Cyprus. Breast Cancer Res Treat *112*, 575-579.

- Loizidou, M. A., Michael, T., Neuhausen, S. L., Newbold, R. F., Marcou, Y., Kakouri, E., Daniel, M., Papadopoulos, P., Malas, S., Hadjisavvas, A., and Kyriacou, K. (2009). DNA-repair genetic polymorphisms and risk of breast cancer in Cyprus. Breast Cancer Res Treat *115*, 623-627.

- Loizidou, M. A., Cariolou, M.A., Neuhausen, S. L., Newbold, R. F., Bashiardes, E., Marcou, Y., Michael, T., Daniel, M., Kakouri, E., Papadopoulos, P., Malas, S., Hadjisavvas, A., and Kyriacou, K. (2009). Genetic variation in genes interacting with *BRCA1*/*2* and risk of breast cancer in the Cypriot population. Breast Cancer Res Treat Epub Aug 28 2009.

Part III

General Discussion, Conclusions and Future work

Chapter 5

General Discussion, Conclusions and Future work

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Breast cancer is a major public health problem throughout the world. It is by far the most commonly occurring cancer among women, accounting for 23% of all female cancers. Despite its common occurrence, and numerous epidemiological and research studies, the exact aetiology of breast cancer is still unknown. It is believed that breast cancer is a multifactorial disease which is a result of interactions between genetic and environmental factors (Ponder, 2001). Over the past decade, significant progress has been made, in defining risk factors that help us to identify individuals who are highly susceptible to developing breast cancer, as well as understanding some of the genetic factors that contribute to this risk. However, the major part of the breast cancer burden is still unexplained, so a lot needs to be done.

The genetic basis of inherited predisposition to breast cancer has been investigated thoroughly during the past two decades and significant discoveries were made. As a result of several years of research, it is now widely accepted that three major groups of breast cancer susceptibility alleles exist, each conferring different levels of risk in the population. These three groups are: the rare high penetrance alleles, the rare moderate penetrance alleles and the common low penetrance alleles (Oldenburg et al., 2007; Pharoah et al., 2008; Stratton and Rahman, 2008).

The two most important components of the high risk breast cancer susceptibility group are *BRCA1* and *BRCA2*. The occurrence of early-onset breast cancer has been associated with mutations in these two genes (Langston et al., 1996; Krainer et al., 1997). Several studies have shown that the genetics of *BRCA1* and *BRCA2* are population specific. A wide spectrum of recurrent and founder mutations have been found in different parts of the world, especially in ethnically defined or isolated populations (Neuhausen, 1999). In this context and based on the fact that Cyprus is an island, in which there has been little population movement, a founder mutation in exon 22 of the *BRCA2* has been identified (Hadjisavvas et al., 2004).

Family studies have proved invaluable for understanding the significance and contribution of *BRCA1* and *BRCA2* genes in breast cancer among Greek Cypriot women. However, observations in such families regarding the nature and penetrance of *BRCA1* and *BRCA2* mutations may not reflect the full spectrum of alterations

present in the general population. It is currently accepted that a variable proportion of early-onset breast cancer is associated with mutations in the *BRCA1* and *BRCA2* genes and that the proportion may be higher in populations harbouring founder mutations (Robson et al., 1998). In Cyprus, the prevalence of *BRCA1* and *BRCA2* mutations in breast cancer patients who were unselected for a family history of the disease was unknown. One of the aims, of this study was to evaluate the frequency and distribution of mutations in the *BRCA1* and *BRCA2* genes, in a group of Greek Cypriot women with early-onset breast cancer.

The findings of the present study support a strong correlation between the early onset breast cancer phenotype and the presence of pathogenic *BRCA1*/*2* mutations. The prevalence of these mutations in the young Cypriot patients (23%), is higher than most countries and compares favourably with data from the Icelandic and the Ashkenazic Jews populations, which display strong founder effects (Johannesdottir et al., 1996; Abeliovich et al., 1997; Thorlacius et al., 1997). It is of interest that pathogenic mutations were detected in patients without a family history of the disease. Based on these results, we recommend that *BRCA1*/*2* screening, should be offered to patients with a diagnosis of early-onset breast cancer irrespective of their family history.

The Department of EM/ Molecular Pathology of the Cyprus Institute of Neurology and Genetics has offered routine genetic testing for the *BRCA1* and *BRCA2* genes in Cyprus since January 2005. Based on the results of this study, the Department offers genetic testing of the *BRCA1* and *BRCA2* genes to all women with an a diagnosis of early onset age of breast cancer (less than 40 years) even in the absence of a family history of breast / ovarian cancer. The experience so far, confirms the results of this study. Genetic testing data from this group of women support the position that early-onset of breast cancer is a sufficient criterion that can be used to identify candidates for *BRCA1* and *BRCA2* mutation screening.

In view of the interesting results obtained from the early onset breast cancer study, my intention is to continue working with this group of breast cancer patients, especially those who were tested negative for *BRCA1*/*2* mutations. Diagnosis of early onset of breast cancer is a strong indicator that the disease has an inherited

genetic component and studying this group of patients may help identify new genes that may be associated with the disease. It would also be worthwhile to participate in a large collaborative genome-wide association study of early-onset female breast cancer, to identify new genes responsible for young cases that are negative for *BRCA1* and *BRCA2* gene mutations.

Mutations in the *BRCA1* and *BRCA2* genes predispose individuals to familial early onset breast cancer and together with other known genes account for no more than 25% of the familial risk of breast cancer (Easton, 1999; Antoniou and Easton, 2006). In the years which followed the identification of the *BRCA1* and *BRCA2* genes, extensive initiatives were undertaken, in order to localize additional highly penetrant breast cancer susceptibility genes, but without any success (Smith et al., 2006). The failure to localize new high penetrance breast cancer susceptibility alleles, has led to the proposal that the remaining breast cancer susceptibility results from the combined effects of many loci each of which confers a small increase in risk (Antoniou et al., 2002).

The most powerful approach to identify these low risk breast cancer variants is through association studies. Association studies are based on the "common disease-common variant" hypothesis (Chakravarti, 1999). These studies compare the frequency of genetic variants in diseased individuals (breast cancer cases) and individuals without the disease (controls) (Risch, 2000; Cardon and Bell, 2001). If the variant under study is over- or under-represented in the cases group, this provides evidence that the locus under study, or a neighbouring locus, is related to disease susceptibility.

In the present thesis, this classical association approach, was followed to assess whether alterations in DNA repair genes modify breast cancer risk in the Cypriot population. The DNA repair pathway is clearly involved in familial breast cancer and thus analysis of members of this pathway and in particular genes which have a direct interaction with *BRCA1* and *BRCA2*, appeared to be a good rationale for identifying novel susceptibility loci.

A number of SNPs were found to be associated with an increased risk for breast cancer in the Cypriot population, whereas others were protective for this disease. The results of this study provide support for the hypothesis that genetic variants in DNA repair genes, influence breast cancer risk and provide further evidence for the existence of common low penetrance variants. However, large-scale genetic epidemiologic studies are warranted to further examine and corroborate the associations observed, between these SNPs and breast cancer in multi-ethnic groups. The scope of this study was to examine a pre-defined set of SNPs in genes that participate in DNA repair and also closely associate with the *BRCA1* and *BRCA2* genes. As both *BRCA1* and *BRCA2* are involved in a multitude of cellular functions, there is still a need to perform a more comprehensive investigation of all common genetic variation across genes which interact with *BRCA1* and/or *BRCA2*. The possibility that other variants in these genes are associated with breast cancer risk cannot be excluded. In the future, it would be worthwhile conducting a comprehensive candidate gene study focusing in this group of genes in order to get a more complete picture of their contribution to breast cancer susceptibility.

The task(s) of validating the biological and functional significance, of the SNPs which were associated with breast cancer risk in the Cypriot population, is a challenging and long-term endeavour. Functional genomics approaches, such as the development of mouse models which harbour these SNPs and subjected to extensive phenotypic analysis, could assist us in understanding the function of these SNPs. Another approach to exploiting the functional significance of these promising SNPs is by using high-throughput tests such as yeast or cell culture systems to look at different aspects of variant function(s), so as to gain further insights into their role(s) and mechanistic effects on disease risk.

The limited success of candidate gene approaches in identifying the genetic background of breast cancer and the recent promising results of GWAS has led many scientists to apply this approach in order to explore further and gain a better insight into breast cancer susceptibility. Currently, GWAS are considered as the most powerful tool to identify common low penetrance disease alleles (Easton and Eeles, 2008). The majority of GWAS on breast cancer conducted to-date, had a strong bias towards North European origin (McCarthy et al., 2008). An extended

analysis with samples from genetically isolated populations with different mutational backgrounds would be advantageous for genome-wide association mapping (Service et al., 2006). In this context, I had the opportunity as part of a fellowship, to receive training in genotyping DNA samples, using SNP arrays and with the aim of conducting a GWAS. A total of 25 samples from Cypriot women were genotyped using the Affymetrix 5.0 chip. My intention is to continue working in this field and provided that the necessary funds are secured by the Department, to conduct a genome-wide association study of breast cancer in the Cypriot population. This GWA approach could possibly identify additional susceptibility loci, as our population is a genetic isolate, and could harbour unique variants not yet disclosed in studies in other populations.

All scientists with an interest in understanding the polygenic basis of breast cancer recognize the necessity of establishing multicenter collaborations, which will enable recruitment of large sample sizes, which will provide more reliable assessments of the risks associated with genetic variation across the genome. Thus the study population of this thesis will be used, for confirmation of associations observed worldwide at genome-wide levels of significance, as well as for the identification of novel susceptibility loci, taking advantage of the sample homogeneity as well as the genetic isolation of the island.

In the last five years major advances in understanding breast cancer susceptibility have been made. Despite this remarkable progress, the majority of inherited breast cancer risk remains unexplained. Pharoah et al. (2002) predicted that, based on the polygenic model of breast cancer, half of breast cancers occur in the 12% of women who are at the highest genetic risk. This highlights the importance of identifying additional susceptibility alleles. Thus more intense research in the field of genetic epidemiology of breast cancer in diverse ethnic groups is still very much needed, in order to shed light on the complex genetic mechanisms, which underlie this common malignancy. To facilitate these endeavours, the study of genetic variation in relation to breast cancer risk in the Cypriot population should continue and be strengthened and expanded. It is expected that in the future, the combination of the effects of multiple low risk breast cancer loci will be useful for risk prediction and subsequently for targeted screening and preventive interventions (Easton and Eeles,

2008). A major challenge for molecular genetics and genetic epidemiology is to continue to identify new breast cancer risk alleles.

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Annex

LIST OF PUBLICATIONS AND PRESENTATIONS

A. Manuscripts appearing in peer review scientific journals

1. **MA Loizidou**, MA Cariolou, SL Neuhausen, RF Newbold, Y. Marcou, T. Michael, E. Kakouri, M. Daniel, P. Papadopoulos, S. Malas, A. Hadjisavvas, K. Kyriacou. Genetic variation in genes interacting with BRCA1/2 and risk of breast cancer in the Cypriot population. **Breast Cancer Res Treat.** 2009 Aug 28. [Epub ahead of print]

2. **MA Loizidou**, T. Michael, SL Neuhausen, RF Newbold, Y. Marcou, E. Kakouri, M. Daniel, P. Papadopoulos, S. Malas, A. Hadjisavvas, K. Kyriacou. DNA-repair genetic polymorphisms and risk of breast cancer in Cyprus. **Breast Cancer Res Treat.** 115(3): 623-7, 2009. Epub 2008 Jun 16.

3. **MA Loizidou**, T. Michael, SL Neuhausen, RF Newbold, Y. Marcou, E. Kakouri, M. Daniel, P. Papadopoulos, S. Malas, K. Kyriacou, A. Hadjisavvas. Genetic polymorphisms in the DNA repair genes *XRCC1*, *XRCC2* and *XRCC3* and risk of breast cancer in Cyprus. **Breast Cancer Res Treat.** 112(3): 575-9, 2008. Epub 2008 Jan 10.

4. **M. Loizidou**, Y. Marcou, V. Anastasiadou, R. Newbold, A. Hadjisavvas, K. Kyriacou. Contribution of BRCA1 and BRCA2 germline mutations to the incidence of early-onset breast cancer in Cyprus. **Clin Genet**, 71(2): 165-170, 2007.

B. Abstracts presented in conferences

1. **M. Loizidou**, M. Cariolou, E. Bashiardes, A. Hadjisavvas, K. Kyriacou, "MALDI-TOF assisted SNP genotyping for finding associations with breast cancer risk in the Cypriot population", **10th International Symposium on Mutation in the Genome**, Paphos, Cyprus, 28 May -1 June 2009 (oral presentation)

2. **M. Loizidou**, T. Michael, Y. Marcou, M. Daniel, E. Kakouri, P. Papadopoulos, S. Malas, K. Kyriacou, A. Hadjisavvas, "DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women", **European Human Genetics Conference 2008**, Barcelona, Spain, 31 May - 2 June 2008 (poster presentation)

3. **M. Loizidou**, Y. Marcou, T. Michael, M. Daniel, P. Papadopoulos, S. Malas, K. Kyriacou, A. Hadjisavvas. "DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women", **American Association for Cancer Research International Conference "Advances in Cancer Research: From the Laboratory to the Clinic"**, Dead Sea, Jordan, 16-19 March 2008 (poster presentation)

4. **M. Loizidou**, T. Michael, SL Neuhausen, RF Newbold, Y. Marcou, E. Kakouri, M. Daniel, P. Papadopoulos, S. Malas, K. Kyriacou, A. Hadjisavvas, "Genetic polymorphisms in the DNA-repair genes and risk of breast cancer in Cyprus". **9th Marianna Lordos Symposium**, Larnaca, Cyprus, 29 February- 2 March 2008 (oral and poster presentation)

5. A. Hadjisavvas, **M. Loizidou**, T. Michael, Y. Marcou, E. Kakouri, M. Daniel, P. Papadopoulos, S. Malas, K. Kyriacou, "Genetic epidemiology of breast cancer in Cyprus; the MASTOS study", **9th Marianna Lordos Symposium**, Larnaca, Cyprus, 29 February- 2 March 2008 (oral presentation)

6. A. Hadjisavvas, **M. Loizidou**, S. Malas, Y. Marcou, K. Kyriacou, "DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women",

American Society of Human Genetics Annual Meeting, San Diego, California, USA, 23-27 October 2007 (poster presentation)

7. A. Hadjisavvas, **M. Loizidou**, T. Michael, Y. Marcou, E. Kakouri, S. Malas, K. Kyriacou, "Searching for molecular targets for breast cancer in genes involved in the DNA-repair pathway among Cypriot women", **Molecular targets for cancer prevention diagnosis and treatment**, Limassol, Cyprus, 7-10 October 2007 (poster presentation)

8. A. Hadjisavvas, **M. Loizidou**, Y. Marcou, Th. Michael, R. Papachristoforou, M. Daniel, P. Papadopoulos, E. Kakouri, S. Malas, K. Kyriacou, "Genetic epidemiology of breast cancer in Cyprus",**3rd Interdisciplinary Cancer Conference**, Athens, Greece, 26-29 April 2007 (poster presentation)

9. A. Hadjisavvas, **M. Loizidou**, M. Daniel, E. Kakouri, S. Malas, Y. Markou, K. Kyriacou "A Preliminary Study on X-ray Repair Cross Complementing (XRCC) Gene Polymorphisms as Possible Biomarkers of Breast Cancer Susceptibility among Cypriot Women", **American Society of Human Genetics Annual Meeting**, New Orleans, Louisiana, USA, 9-13 October 2006 (poster presentation)

10. **M. Loizidou**, Y. Markou, D. Papamichael , M. Televantos, G. Kalakoutis, K. Kyriacou, A. Hadjisavvas "Contribution of BRCA1 and BRCA2 mutations to the incidence of breast and ovarian cancer in young Cypriot women", **EMBO Molecular Medicine Conference: Mammary Gland Development and Breast Cancer Progression**, Dublin, Ireland, 6-8 June 2006 (poster presentation)

11. **M. Loizidou**, Y. Marcou, D. Papamichael, M. Televantos, G. Kalakoutis, K. Kyriacou, A. Hadjisavvas. "Contribution of BRCA1 and BRCA2 germline mutations to the incidence of breast and ovarian cancer in young Cypriot women", **8th Marianna Lordos Cancer Seminar and EU COST Action B20**, Larnaka, Cyprus, 10-12 February 2006 (oral presentation)

C. Invited presentations

1. Association studies for discovering new breast cancer genes / the MASTOS study. **University of Nicosia**, **Department of Life and Health Sciences**, Nicosia, Cyprus, 12 December 2008.

2. DNA repair genetic polymorphisms and the risk of breast cancer in Cyprus. **16th Postgraduate Congress in Clinical Oncology**, Crete, Greece, 12-15 November 2008.

3. Association studies for discovering new breast cancer genes: do they exist? **International forum for the study of Familial and Early Breast Cancer**, Nicosia, Cyprus, 18-21 October 2007.

D. Awards

1. **Fellowship from European Society of Human Genetics (ESHG)**
   For attending the European Human Genetics Conference 2009, Vienna, Austria, 23-26 May 2009

2. **AACR - AstraZeneca International Scholar-in-Training Award**
   For attending the AACR (American Association for Cancer Research) International Conference "Advances in Cancer Research: From the Laboratory to the Clinic", Dead Sea, Jordan, 16-19 March 2008

3. **Best poster award**
   For poster titled "Genetic polymorphisms in the DNA-repair genes and risk of breast cancer in Cyprus" presented at the 9th Marianna Lordos Symposium, organized by the Marianna Lordos Cancer Memorial Fund and the International Collaborative Group (ICG) – Familial Breast Ovarian Cancer, Larnaca, Cyprus, 29 February- 2 March 2008

4. **Fellowship from Fulbright / Amideast**
   Genetic Epidemiology of Breast Cancer at the Department of Epidemiology, School of Medicine, University of California Irvine, Irvine, California, USA, Lab of Prof. Susan Neuhausen, July - August 2007

5. **Fellowship from NIHES (Netherlands Institute for Health Sciences)**
   Erasmus Summer Programme 2006, Training in Genetic Epidemiology, Rotterdam, The Netherlands, 7-25 August 2006

6. **Travel award by Marie Curie Fellowship Association-Irish branch**
   For poster titled: "Contribution of BRCA1 and BRCA2 Germ-Line Mutations to the Incidence of Breast and Ovarian Cancer in Young Cypriot Women" presented at EMBO Molecular Medicine Conference: Mammary Gland Development and Breast Cancer Progression, Dublin, Ireland, June 2006

A1. **MA Loizidou**, MA Cariolou, SL Neuhausen, RF Newbold, E Bashiardes, Y Marcou, T Michael, M Daniel, E Kakouri, P Papadopoulos, S Malas, A Hadjisavvas, K Kyriacou. Genetic variation in genes interacting with BRCA1/2 and risk of breast cancer in the Cypriot population. **Breast Cancer Res Treat.** 2009 Aug 28. [Epub ahead of print]

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EPIDEMIOLOGY

Genetic variation in genes interacting with *BRCA1/2* and risk of breast cancer in the Cypriot population

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Abstract Inability to correctly repair DNA damage is known to play a role in the development of breast cancer. Single nucleotide polymorphisms (SNPs) of DNA repair genes have been identified, which modify the DNA repair capacity, which in turn may affect the risk of developing breast cancer. To assess whether alterations in DNA repair genes contribute to breast cancer, we genotyped 62 SNPs in 29 genes in 1,109 Cypriot women with breast cancer and 1,177 age-matched healthy controls. Five SNPs were associated with breast cancer. SNPs rs13312840 and rs769416 in the *NBS1* gene were associated with a decrease in breast cancer risk (OR TT vs. TC/CC = 0.58; 95% CI, 0.37–0.92; $P = 0.019$ and OR GG vs. GT/TT = 0.23, 95% CI 0.06–0.85, $P = 0.017$, respectively). The variant allele of *MRE11A* rs556477 was also associated with a reduced risk of developing the disease (OR AA vs. AG/GG = 0.76; 95% CI, 0.64–0.91; $P = 0.0022$). *MUS81* rs545500 and *PBOV1* rs6927706 SNPs were associated with an increased risk of developing breast cancer (OR GG vs. GC/CC = 1.21, 95% CI, 1.02–1.45; $P = 0.031$; OR AA vs. AG/GG = 1.53, 95% CI, 1.07–2.18; $P = 0.019$, respectively). Finally, haplotype-based tests identified significant associations between specific haplotypes in *MRE11A* and *NBS1* genes and breast cancer risk. Further large-scale studies are needed to confirm these results.

Keywords Breast cancer · Case–control study · Cyprus · BRCA1/2 interacting genes · Genetic epidemiology · SNP

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Introduction

Breast cancer is the most common malignancy affecting women worldwide, and it is the leading cancer in females in Cyprus, with approximately 400 new cases diagnosed annually [1]. In vitro studies have shown variability in inter-individual DNA repair capacity and have demonstrated that reduced ability to repair DNA is associated with an increased risk for breast cancer [2–4]. It has also been suggested that deficient DNA repair capacity predisposes to both familial and sporadic forms of breast cancer [5–7].

Ten different genes that are involved in pathways critical to genomic integrity have been implicated in inherited predisposition to breast cancer, including *BRCA1*, *BRCA2*,

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p53, *PTEN*, *CHEK2*, *ATM*, *NBS1*, *RAD50*, *BRIP1* and *PALB2*. The association of germline mutations in DNA repair genes with an increased susceptibility to breast cancer highlights the importance of these pathways in the development of breast cancer [8].

The DNA repair pathway is clearly involved in familial breast cancer. Thus, it was hypothesized that common single nucleotide polymorphisms (SNPs) of genes involved in the DNA repair pathway may influence breast cancer risk. Many studies have investigated the role of SNPs in DNA repair genes in relation to breast cancer and have reported associations with breast cancer risk [9–12].

Analysis of members of the DNA repair pathway appears to be a good rationale for identifying novel susceptibility loci. In particular, genes which have a direct interaction with the *BRCA1* and *BRCA2* genes are very good candidates. Recently, two more susceptibility genes, namely *BRIP1*/*FANCJ* and *PALB2*/*FANCN*, which interact with *BRCA1* and *BRCA2* genes, respectively, have been identified [13, 14].

BRCA1 and *BRCA2* participate in the biological response to DNA damage that includes the activation of cell cycle checkpoints and the recruitment of the DNA damage repair machinery. Both *BRCA1* and *BRCA2* are implicated in DNA repair by homologous recombination, and their proteins have distinct roles in double-strand break repair [15].

Despite the progress that has been made in improving our understanding of the functions of the *BRCA1* protein, a complete picture has not yet been attained. It has been hypothesized that *BRCA1* acts as a coordinator of the various functions of DNA damage, recognition, response and repair, and double-strand break repair. *BRCA1* interacts with many DNA repair proteins and protein complexes including the *RAD50-MRE11A-NBS1* (MRN) complex. The proteins associated with *BRCA1* are involved in response to and in the repair of DNA damage in several ways by acting as DNA damage sensors, signal transducers and repair effectors. Hence, these proteins are instrumental in the repair of DNA breakages and in the maintenance of genomic integrity [16–18]. The exact role(s) of the *BRCA2* protein also still remain(s) elusive. It has been demonstrated that *BRCA2* plays an important role in homologous recombination, both in meiosis and in the repair of double-strand breaks. Fewer proteins are known to interact with *BRCA2* compared to *BRCA1* [19]. These include *RAD51*, which mediates DNA repair via homologous recombination (HR) [15], and *PALB2*, which is required for *BRCA2* nuclear localization and stability as well as for some of its functions in HR and double-strand break repair [20]. Overall, *BRCA1* and *BRCA2* act in response to DNA damage and participate in multi-protein complexes that are involved in tumor suppression processes [17].

In this study, we hypothesized that germline variations in genes encoding proteins that interact with *BRCA1/2*, are potential candidates for modifying breast cancer risk in the Cypriot population. Consequently, disturbances in the interactions with *BRCA1* and *BRCA2* may prevent their tumor suppression function(s) and consequently modify inter-individual DNA repair capacity. As part of an ongoing study we assessed genetic variation in 60 SNPs in 29 genes, which interact with *BRCA1* or *BRCA2* genes and their association with breast cancer in a case–control study of Cypriot women. Furthermore, we investigated the role of two additional SNPs in the *PBOV1* (*UROC28*) and *DBC2* genes that are both upregulated in breast cancer [21, 22].

Materials and methods

Study population

To investigate the associations between genetic factors and breast cancer risk in the Cypriot population, we conducted a population-based case–control study, with the acronym MASTOS (Greek word for breast). The population of this study are women participating in the MASTOS study. Blood samples were collected between 2004 and 2006 from 1,109 female breast cancer patients diagnosed between 40 and 70 years old and 1,177 age-matched healthy controls. Participants were women who were previously diagnosed with breast cancer between January 1999 and December 2006. The majority of patients were ascertained from the Bank of Cyprus Oncology Centre which operates as a referral centre and offers treatment and follow-up for 80–90% of all breast cancer cases diagnosed in Cyprus. The rest of the patients were recruited at the Oncology Departments of the Nicosia, Limassol, Larnaca and Paphos district hospitals. The control group consisted of healthy women who were participating in the National program for breast cancer screening with the use of mammography. Volunteers were enrolled in the study during the same calendar period as the cases, from the four district mammography screening centers that operate in Cyprus. Eligible controls were women with no previous history of breast cancer and who had a negative mammography result. All study participants, both patients and controls, were of Greek Cypriot Caucasian origin, thus reducing any potential bias due to population stratification. In addition, the study population was representative of the whole island population and thus consisted of women, who resided in all five districts of the country, minimizing potential selection bias. The participation rate of cases and controls was very high covering around 98% of eligible cases and controls. In addition to blood samples, a risk factor questionnaire, which included extensive demographic, epidemiologic and pathologic data, was obtained from each participant through a standardized interview.

Breast cancer cases were verified by reviewing histological reports. The study was reviewed and approved by the National Bioethics Committee of Cyprus. All participants provided written informed consent.

Gene and SNP selection

Sixty-two SNPs in the *ATF1*, *ATM*, *ATR*, *BARD1*, *BLM*, *BRIP1*, *CHEK1*, *CHEK2*, *DDB2*, *DMC1*, *EME1*, *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *MLH1*, *MRE11A*, *MSH2*, *MSH6*, *MUS81*, *NBS1*, *PALB2*, *PCNA*, *RFC1*, *RAD50*, *RAD51C*, *RAD51L1*, *RAD52* and *XPC* genes were genotyped. The genetic variants were selected based on three main criteria: (1) all SNPs chosen belong to genes that interact with either *BRCA1* or *BRCA2*; (2) the SNPs chosen are either functional SNPs (based on potential protein changes, evolutionary conservation and location in putative functional regions [23–25] or (3) SNPs which were reported by other groups to modify cancer risk [14, 26–32]. For *MRE11A* and *RAD50*, we genotyped the tagging SNPs in Allen-Brady et al. [33], and for *NBS1*, we genotyped the tagging SNPs in Lu et al. [32]. SNPs in the *PBOV1* and *DBC2* genes were selected based on their minor allele frequency (MAF) >0.05.

Genotyping

DNA was isolated from blood samples using standard procedures (phenol–chloroform method). SNPs were genotyped by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of allele-specific primer extension products (Mass Array, Sequenom Inc., San Diego, CA, USA). Assay design was based on published sequences retrieved from the National Center of Biotechnology Information (NCBI) databases. A 34-plex and a 28-plex multiplex assay were designed using the Sequenom MassARRAY Assay Design software (version 3.0). SNPs were genotyped using Sequenom iPLEX chemistry on a MALDI-TOF Compact Mass Spectrometer (Sequenom Inc., San Diego, CA, USA).

Briefly, PCR reactions were carried out in a final volume of 5 µl in standard 384-well plates. PCR was performed with 5 ng of genomic DNA, 1 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 500 µmol of each dNTP and 100 nmol of each PCR primer. PCR thermal cycling was carried out in an ABI-9700 instrument (Applied Biosystems, Foster City, CA, USA) for 15 min at 94°C, followed by 44 cycles of 20 s at 94°C, 30 s at 56°C and 60 s at 72°C. Next, PCR products were treated with 0.5 U of shrimp alkaline phosphatase for 40 min at 37°C to dephosphorylate unincorporated dNTPs, followed by enzyme inactivation for 5 min at 85°C. After adjusting the concentrations of the extension primers to equilibrate signal-to-noise ratios, the post-PCR primer extension reaction of the iPLEX gold assay was performed in a final 10 µl volume extension reaction containing 0.2 ul of termination mix, 0.0041 µl of iPLEX enzyme (Sequenom Inc., San Diego, CA, USA) and 700–1,400 nM of extension primers. A two-step 200 short cycles program was used for the iPLEX reaction: initial denaturation was for 30 s at 94°C followed by five cycles of 5 s at 52°C and 5 s at 80°C. An additional 40 annealing and extension cycles were then looped back to 5 s at 94°C, 5 s at 52°C and 5 s at 80°C. Final extension was carried out at 72°C for 3 min. The iPLEX reaction products were desalted by diluting samples with 16 µl of water and adding 6 mg of clean resin. Following a quick centrifugation (3,200 g for 5 min), reaction products were spotted on a 384-format SpectroChip (Sequenom Inc., San Diego, CA, USA). SpectroCHIPs were processed in a MassARRAY Compact Analyzer (Bruker Daltonics, Bremen, Germany) by MassARRAY Workstation (version 3.3) software (Sequenom Inc., San Diego, CA, USA). Acquisition data were analyzed using MassARRAY TYPER 3.4 software (Sequenom Inc., San Diego, CA, USA).

For quality control, 48 random samples were genotyped in duplicate. Furthermore, ten samples were sequenced to confirm genotype calls from the MALDI-TOF platform. The genotype concordance rate between platforms was 99%. The order of the DNA samples on 384-well plates was randomized in order to ensure the same study conditions for samples from cases and controls. Genotyping call rates ranged from 95 to 99%, and duplicate concordance rates were higher than 99%. The SNP that had 20% missing data was excluded from further analysis.

Data analysis

Hardy Weinberg equilibrium (HWE) was assessed in the control samples by applying an exact test. The primary tests of association were the univariate analyses between each SNP and breast cancer. Genotype frequencies in cases and controls were compared using the χ^2 test. The association between breast cancer and each SNP was examined using logistic regression with the SNP genotype tested under models of complete dominance and recessive inheritance as well as under the log-additive model after adjusting for breast cancer risk factors including age (under or over 55 years), menopause status (pre- or post-menopausal), family history of breast cancer (first degree relative with breast cancer) and use of hormone replacement therapy. Statistical analysis was carried out using SNPStats, a web-based application designed for analysis of association studies [34].

Associations between breast cancer and common haplotypes of the *ATM*, *MRE11A* and *NBS1* genes were also

investigated using SNPStats, which allows the estimation of maximum likelihood estimates of haplotype frequencies using the Expectation-Maximization (EM) algorithm. Logistic regression was performed to test the association between haplotypes and breast cancer risk. For assessing the contribution of the *MRE11A* haplotypes in breast cancer risk, a haplotype tagging SNP genotyped previously was also included in haplotype reconstruction [35]. Haplotypes with a frequency of less than 1% were not considered further for analysis since they are likely to be a result of rare recombination events.

Results

Table 1 shows the genotype frequency in cases and controls for the 62 SNPs, of which the 61 were successfully genotyped. Six SNPs (rs1800149, rs2706377, rs1800282, rs7487683, rs3626, rs28908468) deviated from HWE in controls ($P < 0.01$) and were excluded from further analysis. Of the remaining 55 SNPs, 8 were monomorphic in both groups. Significant differences in genotype frequencies between breast cancer patients and controls were observed in 5 of the 55 SNPs analyzed.

The associations of SNPs and breast cancer risk in Cypriot women are shown in Table 2. Five of the 55 SNPs were associated at a *P* value of less than 0.05. Three SNPs were associated with a reduced risk for breast cancer while the three remaining were associated with an increased breast cancer risk. In detail, the variant allele of *NBS1* rs13312840 (924 T>C) was associated with a reduced risk of disease (OR TT vs. TC/CC = 0.58; 95% CI, 0.37 to 0.92; $P = 0.019$). Carriers of the *NBS1* rs769416 rare allele also had a reduced risk of breast cancer (OR GG vs. GT/TT = 0.23, 95% CI 0.06–0.85, $P = 0.017$). Furthermore, the variant allele of *MRE11A* rs556477 was associated with a reduced risk of developing the disease (OR AA vs. AG/GG = 0.76; 95% CI, 0.64–0.91; $P = 0.0022$). The variant allele of *MUS81* rs545500 was associated with an increased risk of developing breast cancer (OR GG vs. GC/CC = 1.21, 95% CI, 1.02–1.45; $P = 0.031$). In addition, the rare allele of *PBOV1* rs6927706 was also associated with an increased risk of developing breast cancer (OR AA vs. AG/GG = 1.53, 95% CI, 1.07–2.18; $P = 0.019$).

The *NBS1* haplotype GGCGCAC (rs769416, rs769420, rs13312840, rs1805794, rs6413508, rs12677527, rs1805787), which contains the *NBS1* rs13312840 C allele, to be associated with a reduced breast cancer risk compared with the most frequent haplotype GGTCCGC (OR = 0.62; 95% CI = 0.39–0.97; $P = 0.037$). We also found a reduced risk for breast cancer for a rare haplotype in *NBS1* (OR = 0.42; 95% CI = 0.26–0.66; $P = 2 \times 10^{-4}$). In addition, the *MRE11A* haplotype AGCG (rs556477, rs601341, rs1083 1234, rs1009456) is associated with a significantly increased risk for breast cancer (OR = 1.32; 95% CI = 1.13–1.54; $P = 0.0004$). None of the common *ATM* haplotypes were associated with breast cancer (Table 3).

Discussion

Breast cancer is a complex polygenic disease. Published data suggest that a proportion of breast cancer can be explained by common low-penetrance alleles that increase susceptibility [36]. High-penetrance mutations in genes that are involved in DNA repair pathways such as *BRCA1* and *BRCA2* predispose to familial breast cancer [37, 38]. Previously our group characterized novel mutations in these genes in Cypriot families [39, 40]. The importance of common inherited variants in DNA repair genes in relation to breast cancer risk is still being elucidated, but is currently receiving increased attention. Our group as part of an ongoing investigation has studied genetic variation in DNA repair genes in relation to breast cancer risk in the Cypriot population and has reported a number of SNPs that modify breast cancer risk [35, 41]. A number of large studies which focused on the contribution of common SNPs in DNA repair genes in breast cancer, using tagging SNP approaches have also been completed [9, 42, 43]. In this case–control study, we evaluated both functional as well as tagging SNPs in DNA repair genes in relation to breast cancer risk in Cypriot women.

We found that Cypriot women who carry *NBS1* rs13312840 C and rs769416 T alleles have a reduced risk of breast cancer. The *NBS1* protein is involved in non-homologous end-joining (NHEJ) pathway that repairs DNA double-strand breaks (DSBs). The first step of this pathway consists of the recognition of DSBs by the MRN complex whose core contains the *MRE11*, *RAD50* and *NBS1* proteins. *NBS1* is the key regulator of this protein complex [44, 45]. The *NBS1* rs13312840 T>C SNP is located on the 5′ UTR (-1120) of the gene that is the transcription factor *GATA-1* binding site. The activation domains of *GATA-1* are capable of activating transcription in mammalian cells through *GATA* motifs [46]. Our results are in contrast to those of a recent study by Lu et al. who found an increased risk for breast cancer in non-Hispanic Caucasian women aged 55 or younger who were carriers of the C allele [32]. Conflicting evidence for association may be due to population-specific and/or age-specific differences. The protective effect of the *NBS1* rs13312440 SNP observed in our study could be attributed to the SNP itself or to linkage disequilibrium with another variant.

To the best of our knowledge, this is the first study investigating the role of *NBS1* rs769416 SNP and breast cancer risk. The rs769416 SNP causes an amino acid

Table 1 Genotype frequencies in cases and controls for the 62 SNPs studied

Gene	rs Number	Controls				Cases				MAF		HWE
		AA	Aa	aa	Total	AA	Aa	aa	Total	Controls	Cases	Controls
ATF1	rs2230674	1,071	86	2	1,159	1,021	81	1	1,103	0.04	0.04	0.69
ATM	rs1800057	1,087	85	2	1,174	1,015	85	0	1,100	0.04	0.04	0.68
	rs2234997	1,153	4	1	1,158	1,093	10	0	1,103	0	0	0.01
	rs2235000	1,160	1	0	1,161	1,102	2	0	1,104	0	0	1
	rs3218688	979	2	0	981	926	0	0	926	0	0	1
	rs3218695	1,177	0	0	1,177	1,109	0	0	1,109	0	0	Monomorphic
	rs3218708	1,177	0	0	1,177	1,109	0	0	1,109	0	0	Monomorphic
	rs4987945	1,169	1	0	1,170	1,100	1	0	1,101	0	0	1
ATR	rs2227928	401	520	229	1,150	344	517	218	1,079	0.43	0.44	0.011
	rs2229032	899	242	17	1,158	833	253	16	1,102	0.12	0.13	0.89
BARD1	rs2070094	466	551	156	1,173	461	485	159	1,105	0.37	0.36	0.75
	rs2229571	341	580	249	1,170	316	540	241	1,097	0.46	0.47	0.95
	rs3738888	1,150	7	0	1,157	1,099	4	0	1,103	0	0	1
BLM	rs11852361	1,123	46	2	1,171	1,046	52	2	1,100	0.02	0.03	0.094
	rs7167216	1,127	44	2	1,173	1,055	47	2	1,104	0.02	0.02	0.081
BRIP1	rs4986764	475	534	161	1,170	465	502	141	1,108	0.37	0.35	0.57
CHEK1	rs506504	1,064	105	5	1,174	986	116	2	1,104	0.05	0.05	0.19
CHEK2	rs17879961	1,158	0	0	1,158	1,101	1	0	1,102	0	0	1
DBC2	rs2241261	342	560	252	1,154	288	546	264	1,098	0.46	0.49	0.26
DDB2	rs830083	871	271	15	1,157	803	277	21	1,101	0.13	0.14	0.3
DMC1	rs2227914	1,146	3	0	1,149	1,099	1	1	1,101	0	0	1
EME1	rs12450550	918	221	24	1,163	838	239	23	1,100	0.12	0.13	0.021
FANCA	rs1800282	978	149	19	1,146	883	189	16	1,088	0.08	0.1	<0.0001
	rs7190823	573	476	113	1,162	541	462	98	1,101	0.3	0.3	0.33
	rs9282681	1,096	51	0	1,147	1,065	42	2	1,109	0.02	0.02	1
FANCC	rs1800364	1,159	1	0	1,160	1,104	1	0	1,105	0	0	1
FANCD2	rs2272125	787	346	38	1,171	710	346	43	1,099	0.18	0.2	1
FANCE	rs9462088	1,051	115	4	1,170	990	106	4	1,100	0.05	0.05	0.56
MLH1	rs1800149	1,156	0	1	1,157	1,101	0	2	1,103	0	0	0.00043
	rs2020872	1,177	0	0	1,177	1,109	0	0	1,109	0	0	Monomorphic
	rs2308317	1,177	0	0	1,177	1,109	0	0	1,109	0	0	Monomorphic
	rs1800734	494	496	137	1,127	446	505	136	1,087	0.34	0.36	0.47
MRE11A	rs1009456	1,040	105	0	1,145	992	89	5	1,086	0.05	0.05	0.17
	rs10831234	949	190	12	1151	899	193	6	1,098	0.09	0.09	0.48
	rs556477	444	550	167	1,161	494	473	130	1,097	0.38	0.33	0.9
MSH2	rs2303428	929	217	18	1,164	870	211	16	1,097	0.11	0.11	0.22
MSH6	rs1042821	653	451	64	1,168	646	388	70	1,104	0.25	0.24	0.24
	rs1800935	655	428	90	1,173	608	413	83	1,104	0.26	0.26	0.094
MUS81	rs545500	673	430	55	1,158	589	435	77	1,101	0.23	0.27	0.22
NBS1	rs1805787	548	483	104	1,135	549	447	103	1,099	0.3	0.3	0.94
	rs1805794	543	502	109	1,154	511	497	96	1,104	0.31	0.31	0.68
	rs6413508	1,167	6	1	1,174	1,097	4	0	1,101	0	0	0.012
	rs769416	1,141	10	0	1,151	1,098	3	0	1,101	0	0	1
	rs769420	1,177	0	0	1,177	1,109	0	0	1,109	0	0	Monomorphic
	rs12677527	546	505	115	1,166	512	497	96	1,105	0.32	0.31	0.95
	rs13312840	1,122	55	0	1,177	1,075	32	1	1,108	0.02	0.02	1

Table 1 continued

Gene	rs Number	Controls				Cases				MAF		HWE
		AA	Aa	aa	Total	AA	Aa	aa	Total	Controls	Cases	Controls
PALB2	rs45494092	1,170	1	0	1,171	1,097	4	0	1,101	0	0	1
	rs45532440	1,035	120	4	1,159	972	126	4	1,102	0.06	0.06	0.77
	rs45478192	1,177	0	0	1,177	1,109	0	0	1,109	0	0	Monomorphic
	rs45551636	1,076	92	2	1,170	1,010	92	2	1,104	0.04	0.04	1
PBOV1	rs6927706	1,083	63	1	1,147	1,017	83	2	1,102	0.03	0.04	0.61
PCNA	rs3626	827	242	36	1,105	838	196	34	1,068	0.14	0.12	0.0012
RAD50	rs2299015	742	370	45	1,157	743	323	37	1,103	0.2	0.18	1
	rs2522406	1,064	41	1	1,106	1,044	31	0	1,075	0.02	0.01	0.34
	rs2706377	1,052	66	21	1,139	1,046	37	14	1,097	0.05	0.03	<0.0001
	rs3187395	1,177	0	0	1,177	1,109	0	0	1,109	0	0	Monomorphic
RAD51C	rs28363317	1,164	6	0	1,170	1,095	6	0	1,101	0	0	1
RAD51L1	rs28908468	572	201	0	773	979	75	0	1,054	0.13	0.04	<0.0001
RAD52	rs7487683	1,135	35	3	1,173	1,072	33	0	1,105	0.02	0.01	0.0043
RFC1	rs2066791	1,177	0	0	1,177	1,109	0	0	1,109	0	0	Monomorphic
XPC	rs2228000	673	402	64	1,139	653	379	65	1,097	0.23	0.23	0.68
	rs2227999	1,040	128	4	1,172	963	138	4	1,105	0.06	0.07	1

change (Gly to Lys) at codon 216 of the *NBS1* gene. This SNP is not located within one of the three functional regions of the *NBS1* protein, but it may have an alternative splicing regulatory effect, based on the Functional Single Nucleotide Polymorphism (F-SNP) database [47]. Our result on the association of rs769416 SNP and breast cancer needs to be interpreted with caution, since this is a rare SNP in our population and the most likely explanation for this association is chance.

Haplotype analysis with the combination of the seven *NBS1* SNPs showed that the frequency of the GGCGCAC haplotype (rs769416, rs769420, rs13312840, rs1805794, rs6413508, rs12677527, rs1805787) was lower in patients than in controls (0.0147 vs. 0.0225; $P = 0.035$), suggesting a protective effect. There was also evidence for a protective effect of the rare pooled *NBS1* haplotypes. This protective effect is driven by the difference in frequencies of the pooled rare haplotypes that conferred a low risk (OR = 0.42) and had a combined frequency of 3.29% in controls and 1.37% in patients. It is possible that these pooled haplotypes are a marker for a single, rare, protective mutation in the Cypriot population. There may be value in sequencing this region in order to help identify the protective variant(s). Both these findings need to be replicated in independent studies in order to confirm or refute this effect.

Our data support the notion that *MUS81* rs545500 C allele carriers are at an increased risk for breast cancer. Rs545500 is a non-synonymous SNP located in the coding region of *MUS81*, a structure-specific DNA nuclease that plays an important role in DNA repair by homologous recombination [48]. This polymorphism results in an amino acid change from a positively charged hydrophilic arginine to an uncharged hydrophobic proline residue, which may have an effect on the 3D structure or a protein–protein binding interface of the *MUS81* protein [25]. The role of the *MUS81* gene in breast cancer has not been investigated. However, it was demonstrated that *MUS81* homozygote and heterozygote knockout mice have a predisposition to develop cancer. Proper bialellic expression of *MUS81* is critical for the maintenance of genomic integrity and tumor suppression [49]. Therefore, the rs545500 SNP could predispose individuals to breast cancer, but functional studies need to be performed in order to identify the actual role of this variant in carcinogenesis.

Our findings also suggest that the *PBOV1* rs6927706 polymorphism may be a risk factor for breast cancer. Rs6927706 is a non-synonymous SNP located in the coding region of *PBOV1*, a gene which is upregulated in prostate, breast and bladder cancers [21]. The polymorphism results in an amino acid change at codon 73 from a hydrophobic isoleucine to a hydrophilic threonine residue. Bioinformatics analysis indicates that this SNP could be involved in splicing regulation [47]. However, further work is warranted since the exact roles of the *PBOV1* protein as well as its functional domains are not well known at present.

Our current data suggest that the *MRE11A* rs556477 G allele may be associated with a reduced breast cancer risk. The *MRE11A* gene forms a complex with *RAD50* and *NBS1* genes which is involved in the cellular response to DNA double-strand breaks. Defects in the members of this

Table 2 Genotypic specific risk (OR and 95% CI)

Gene	rs number	Dominant OR (95% CI); *P* value[a]	Recessive OR (95% CI); *P* value[a]	Log-additive OR (95% CI); *P* value[a]
ATF1	rs2230674	1.02 (0.74–1.42); 0.89	0.60 (0.05–7.34); 0.68	1.01 (0.73–1.40); 0.94
ATM	rs1800057	1.10 (0.79–1.52); 0.57	–	1.07 (0.78–1.47); 0.69
	rs2234997	2.31 (0.75–7.12); 0.13	–	1.83 (0.68–4.91); 0.23
	rs4987945	1.27 (0.07–21.89); 0.87	–	–
ATR	rs2227928	1.14 (0.95–1.38); 0.15	1.04 (0.84–1.30); 0.71	1.07 (0.95–1.21); 0.25
	rs2229032	1.09 (0.89–1.34); 0.42	1.01 (0.48–2.15); 0.97	1.07 (0.89–1.30); 0.45
BARD1	rs2070094	0.98 (0.82–1.17); 0.8	1.07 (0.83–1.37); 0.6	1.01 (0.89–1.14); 0.93
	rs2229571	0.99 (0.82–1.20); 0.93	0.96 (0.78–1.18); 0.69	0.98 (0.87–1.11); 0.77
	rs3738888	0.46 (0.13–1.66); 0.23	–	–
BLM	rs11852361	1.28 (0.85–1.95); 0.24	0.95 (0.13–7.12); 0.96	1.25 (0.84–1.85); 0.27
	rs7167216	1.26 (0.82–1.94); 0.3	0.95 (0.13–7.14); 0.96	1.22 (0.81–1.84); 0.34
BRIP1	rs4986764	0.94 (0.79–1.12); 0.49	0.96(0.75–1.25); 0.78	0.96(0.85–1.09); 0.53
CHEK1	rs506504	1.19 (0.89–1.59); 0.24	0.48 (0.09–2.59); 0.37	1.15 (0.87–1.51); 0.34
DBC2	rs2241261	1.18 (0.97–1.43); 0.095	1.16 (0.94–1.43); 0.17	1.12 (0.99–1.27); 0.061
DDB2	rs830083	1.14(0.93–1.39); 0.2	1.51(0.74–3.07); 0.25	1.14(0.95–1.37); 0.15
DMC1	rs2227914	0.63 (0.09–4.39); 0.64	–	1.10 (0.25–4.73); 0.9
EME1	rs12450550	1.15 (0.94–1.42); 0.18	0.94 (0.52–1.72); 0.85	1.11 (0.92–1.33); 0.27
FANCA	rs7190823	1.02 (0.86–1.22); 0.8	0.91 (0.68–1.23); 0.56	1.00 (0.87–1.14); 0.95
	rs9282681	0.84 (0.54–1.30); 0.44	–	0.89 (0.58–1.35); 0.57
FANCC	rs1800364	1.25 (0.07–21.55); 0.88	–	–
FANCD2	rs2272125	1.12 (0.93–1.34); 0.24	1.08(0.68–1.72); 0.74	1.09 (0.93–1.28); 0.27
FANCE	rs9462088	0.95 (0.71–1.26); 0.71	0.73 (0.15–3.53); 0.7	0.94 (0.71–1.24); 0.67
MLH1	rs1800734	1.09 (0.91–1.31); 0.33	1.01 (0.77–1.31); 0.96	1.05 (0.92–1.20); 0.46
MRE11A	rs1009456	0.93 (0.68–1.26); 0.63	–	0.98 (0.73–1.32); 0.9
	rs10831234	1.04 (0.83–1.31); 0.72	0.69 (0.25–1.93); 0.47	1.02 (0.82–1.26); 0.85
	rs556477	**0.76 (0.64–0.91); 0.0022**	0.81 (0.62–1.05); 0.11	**0.82 (0.72–0.93); 0.0027**
MSH2	rs2303428	1.02 (0.82–1.26); 0.89	0.93 (0.45–1.89); 0.83	1.01 (0.83–1.22); 0.94
MSH6	rs1042821	0.85 (0.71–1.01); 0.066	1.23 (0.84–1.79); 0.29	0.92 (0.80–1.07); 0.27
	rs1800935	1.09 (0.91–1.30); 0.34	1.07 (0.77–1.48); 0.69	1.07 (0.93–1.22); 0.36
MUS81	rs545500	**1.21 (1.02–1.45); 0.031**	1.43(0.98–2.08); 0.06	**1.21(1.04–1.39); 0.012**
NBS1	rs1805787	0.92 (0.77–1.10); 0.36	0.97 (0.72–1.31); 0.84	0.95 (0.83–1.08); 0.43
	rs1805794	1.08 (0.91–1.28); 0.4	0.93 (0.69–1.26); 0.65	1.03 (0.90–1.18); 0.65
	rs6413508	0.46 (0.13–1.66); 0.23	–	0.46 (0.14–1.50); 0.18
	rs769416	**0.23 (0.06–0.85); 0.017**	–	–
	rs12677527	1.04 (0.88–1.24); 0.64	0.88 (0.65–1.19); 0.4	1.00 (0.87–1.14); 0.98
	rs13312840	**0.58 (0.37–0.92); 0.019**	–	**0.61 (0.39–0.95); 0.028**
PALB2	rs45494092	3.75 (0.40–35.04); 0.2	–	–
	rs45532440	1.06 (0.81–1.40); 0.66	0.73 (0.18–3.04); 0.67	1.05 (0.80–1.36); 0.74
	rs45551636	1.04 (0.76–1.43); 0.8	0.67 (0.09–4.89); 0.69	1.03 (0.76–1.4); 0.85
PBOV1	rs6927706	**1.53 (1.07–2.18); 0.019**	1.63(0.12–21.60); 0.71	**1.51(1.06–2.13); 0.02**
RAD50	rs2299015	0.89 (0.74–1.07); 0.21	0.90 (0.56–1.43); 0.65	0.91 (0.77–1.06); 0.22
	rs2522406	0.80 (0.49–1.31); 0.37	–	0.78 (0.48–1.26); 0.31
	rs3187395	0.32 (0.06–1.85); 0.19	–	–
RAD51C	rs28363317	0.93 (0.28–3.10); 0.91	–	–
XPC	rs2228000	1.01 (0.84–1.21); 0.91	0.96 (0.66–1.40); 0.85	1.00 (0.87–1.16); 0.99
	rs2227999	1.12 (0.86–1.46); 0.42	1.10 (0.26–4.64); 0.42	1.11 (0.86–1.43); 0.43

Data in bold highlight the statically significant results

[a] Adjusted for age, menopause status, family history of breast cancer and use of hormone replacement therapy (HRT)

Table 3 Estimated haplotype frequencies in cases and controls and haplotypic specific risks

Gene	Haplotype	Control freq	Case freq	OR [95% CI]	χ^2 *P* value	Global test *P* value
ATM[a]	CCCCCTGC	0.92	0.9225	1.00		0.53
	GCCCCTGC	0.0367	0.0349	0.96 (0.69–1.32)	0.8	
	CCCCGTGC	0.0376	0.0329	0.89 (0.34–1.23)	0.48	
	Rare	0.0057	0.0097	1.6 (0.72–3.57)	0.25	
MRE11A[b]	GACG	0.3702	0.326	1.00		0.021
	AGCG	**0.2384**	**0.2752**	**1.32 (1.13–1.54)**	**4×10^{-4}**	
	AACG	0.2478	0.255	1.17 (1.00–1.36)	0.044	
	AGTG	0.0864	0.0891	1.16 (0.93–1.45)	0.19	
	AGCT	0.0444	0.0438	1.12 (0.84–1.51)	0.44	
	Rare	0.0128	0.0109	0.98 (0.52–1.85)	0.95	
NBS1[c]	GGTCCGC	0.3705	0.3886	1.00		0.00019
	GGTCCGG	0.2955	0.2925	0.94 (0.82–1.09)	0.44	
	GGTGCAC	0.2786	0.2905	0.99 (0.86–1.15)	0.92	
	GGCGCAC	**0.0225**	**0.0147**	**0.62 (0.39–0.97)**	**0.037**	
	Rare	**0.0329**	**0.0137**	**0.42 (0.26–0.66)**	**2×10^{-4}**	

Data in bold highlight the statically significant results

[a] ATM haplotypes are arranged in the order of rs1800057, rs3218688, rs3218695, rs4987945, rs2230674, rs2234997, rs2235000, rs3218708

[b] *MRE11A* haplotypes are arranged in the order of rs556477, rs601341, rs10831234, rs1009456

[c] *NBS1* haplotypes are arranged in the order of rs769416, rs769420, rs13312840, rs1805794, rs6413508, rs12677527, rs1805787

tri-complex are linked to increased chromosomal instability which leads to cancer [50]. The rs556477 common variant is located in intron 15 of the *MRE11A* gene. The rs556477 MAF is 40% in Caucasians as reported in NCBI's dbSNP database; the same as that observed in our population. The functionality of this SNP is not clear. Using the TFSEARCH webtool (http://www.cbrc.jp/research/db/TFSEARCH.html), we searched for potential transcription factors binding sites at this position. The rs556477 SNP is located in a region that is a potential transcription factor-binding site of activator protein 1 (*AP-1*), which plays a critical role in signal transduction pathways in many cells. A recent study has shown that inhibition of *AP-1* transcription factors suppresses breast cancer growth. Inhibitors that are capable of blocking *AP-1* activation may be promising agents for the treatment and prevention of breast cancer [51]. The reduced risk of breast cancer for carriers of rs556477 SNP found in our study is in contrast with the above finding since it is expected that the creation of an *AP-1* binding site will result in an increased breast cancer risk. However, it must be taken into account that the prediction that rs556477 A to G substitution results in a gain of an *AP-1* binding site is based on in silico analysis and this remains to be proven by in vitro data. Furthermore, the *MRE11A* rs556477 polymorphism may not be causal, but could be in linkage disequilibrium with a true protective variant.

In the current study, we present evidence for an increased breast cancer risk for women carrying the *MRE11A* AGCG (rs556477, rs601341, rs10831234, rs1009456) haplotype. It is noteworthy that in a previous study conducted by our group there was evidence for an increased breast cancer risk for women homozygous for the MRE11A rs601341 A allele [35]. The rs601341 A to G substitution results in potential binding of ubiquitous transcription factor Ying Yang 1 (*YY1*) that has a fundamental role in normal biologic processes such as differentiation, replication and cell proliferation. *YY1* overexpression and/or activation results in uncontrolled cellular proliferation, resistance to apoptotic stimuli and tumorigenesis [52]. Given the intronic position of the two associated SNPs, it is unlikely that these SNPs in and by themselves are disease associated. Rather, in all likelihood, they are in linkage disequilibrium with other variants that cause the associations observed.

Our study has several strengths, including a high participation rate of eligible cases (98%) and a population sample from a homogeneous ethnic background (all participants are Greek Cypriots) thus reducing any potential bias due to population stratification. In addition, our study population (both cases and controls) was from all over the country minimizing potential selection bias.

However, there were limitations in our study, one of which is the possibility of survivor bias. This is one of the

known disadvantages of all retrospective case–control studies. In our study, samples from breast cancer cases were collected between 2004 and 2006 for cases diagnosed between 1999 and 2006. Our study may therefore have excluded a number of women with the most aggressive form of breast cancer, diagnosed between 1999 and 2003. It is possible that this could lead to "survivor bias" if genotypes differ between those who succumb quickly compared with longer-term breast cancer survivors.

The SNP selection for this study was based solely on functionality and their position in genes interacting with BRCA1/2 rather than allele frequency. As a result of this, a number of monomorphic/low-polymorphic SNPs were included in the study. It is noted that this is the first time that these SNPs were studied in the Cypriot population, and their allele frequencies were a priori unknown. Rare SNPs can also contribute to disease risk [53]. However, our study did not have sufficient power to detect such associations, and the possibility that some of the low-polymorphic SNPs studied contribute to breast cancer risk cannot be ruled out.

Another limitation of our study is that we did not consider the possibility of gene–gene interactions or gene–environment interactions. It is possible that the risks observed are the result of interactions, but we have not attempted to assess such effects, since the estimate of an interaction effect will be unreliable because of the small numbers available. Furthermore, we did not account for multiple testing. When multiple comparisons are being made, statistically significant associations may be identified by chance alone. Replication in independent, well-powered studies is the gold standard of bona fide true associations from chance findings. A Cypriot replication set is not available to attempt to replicate the variants identified, and replication will need to be performed in other populations.

In conclusion, this study provides support for the hypothesis that genetic variants in DNA repair genes influence breast cancer risk and provides further evidence for the polygenic model of breast cancer. However, large-scale genetic epidemiologic studies are warranted to further examine and corroborate the associations observed between polymorphisms and breast cancer in multiethnic groups. In addition, elucidation of the functional impact of the breast cancer associated SNPs is needed in order to provide further insights into their mechanistic effects on risk.

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EPIDEMIOLOGY

DNA-repair genetic polymorphisms and risk of breast cancer in Cyprus

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Abstract The DNA repair pathway is known to play a role in the etiology of breast cancer. A number of studies have demonstrated that common germline variants in genes involved in the DNA repair pathway influence breast cancer risk. To assess whether alterations in DNA repair genes contribute to breast cancer, we genotyped 12 single nucleotide polymorphisms (SNPs) in 1,109 Cypriot women with breast cancer and 1,177 age-matched healthy controls. We found significant associations with breast cancer for SNPs in the *BRCA2* and *MRE11A* genes. Carriers of the *BRCA2* rs1799944 variant (991 Asp) were found to have an increased risk of breast cancer (OR = 1.41, 95% CI 1.08–1.83, $P = 0.01$) with $P_{trend} = 0.0076$. Homozygous carriers of the *MRE11A* rs601341 A allele had an increased risk of breast cancer (OR = 1.36, 95% CI 1.08–1.71, $P = 0.009$) with $P_{trend} = 0.0087$. This study suggests that genetic variants in *BRCA2* and *MRE11A* are associated with breast cancer risk.

Keywords Breast cancer · Case-control study · Cyprus · DNA repair genes · Genetic epidemiology · SNP

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Introduction

Breast cancer is the most common malignancy affecting women worldwide and it is the leading female cancer in Cyprus, with approximately 350–400 new cases diagnosed annually [1].

The DNA repair pathway is essential for maintaining genomic stability of mammalian cells. Deficiencies in DNA repair mechanisms lead to high penetrance genetic syndromes such as Fanconi anemia and Bloom syndrome, which have cancer as a predominant phenotype [2]. Ten different genes, involved in pathways critical to genomic integrity, have been implicated in inherited predisposition to breast cancer. Germline mutations in these genes significantly increase breast cancer risk and thus support a major role of the DNA repair pathway in breast carcinogenesis. The most important of these genes are *BRCA1* and *BRCA2* [3]. There is also evidence from in vitro studies that reduced DNA repair capacity is associated with increased breast cancer risk [4, 5].

The known breast cancer susceptibility genes have been estimated to explain only 5% of breast cancer cases, thus it is likely that other breast cancer susceptibility genes exist [6]. Based on the fact that the DNA repair pathway is involved in familial breast cancer it was suggested that single nucleotide polymorphisms (SNPs) in genes involved

in DNA repair may influence DNA repair capacity and, in turn, confer an altered susceptibility to develop breast cancer. Several studies have investigated the role of SNPs in DNA repair genes in relation to breast cancer and have reported associations with breast cancer risk [7–10].

In this study, we hypothesized that genetic variation in the DNA repair pathway may modify susceptibility to breast cancer in Cypriot women. As part of an ongoing study we assessed genetic variation in 12 SNPs in 11 DNA repair-related pathway genes, specifically *BARD1*, *BRCA2*, *ERCC2*, *FANCA*, *MLH1*, *MRE11A*, *MSH2*, *OGG1*, *RAD51*, *RAD52*, and *TP53* and their association with breast cancer in a case-control study of Cypriot women.

Materials and methods

Subjects

The study population was women participating in the *MASTOS* study, a population-based case-control study of breast cancer in Cyprus [11]. Blood samples were collected between 2004 and 2006 from 1,109 female breast cancer patients diagnosed between 40 and 70 years old and 1,177 aged-matched healthy controls. Cases participating in the study were women previously diagnosed with breast cancer between January 1999 and December 2006. In addition to blood samples, a risk factor questionnaire that included extensive demographic, epidemiological, and pathological data was obtained from each participant through a standardized interview. Breast cancer cases were verified by reviewing histological reports. The study was approved by the National Bioethics Committee of Cyprus, and all participants provided signed informed consent.

Genotyping

DNA was isolated from blood samples using standard procedures (phenol-chloroform method). The 12 single nucleotide polymorphisms (SNPs): *BARD1* rs1048108 (Pro24Ser), *BRCA2* rs1799944 (Asn991Asp), *ERCC2* rs13181 (Lys751Gln), *FANCA* rs2239359 (Gly501Ser), *MLH1* rs1799977 (Ile219Val), *MRE11A* rs601341, *MSH2* rs2059520, *OGG1* rs1052134 (Ser326Cys), *RAD51* rs1801320 and rs1801321 (135G > C-5′UTR and 172 G > T-5′UTR), *RAD52* rs11226 (2259 C > T-3′UTR) and *TP53* rs1042522 (Pro72Arg) were genotyped in all subjects participating in the study. All SNPs studied had a minor allele frequency of over 0.05. Genotyping was performed using the Taqman SNP genotyping assays from Applied Biosystems Inc. (ABI). For genotyping SNP rs1801320 the primers and probes described previously by Kuschel et al. were used [8]. Each assay was carried out using 10 ng genomic DNA in a 5 μl reaction using Taqman Universal PCR Master Mix (ABI), forward and reverse primers, and FAM and VIC-labelled probes purchased from Applied Biosystems (ABI Pre-Designed assays). All assays were carried out in 384-well plates. The fluorescence profile was read on an ABI PRISM 7900HT instrument and the results analyzed with Sequence Detection Software (ABI). For quality control, random samples were genotyped in duplicate and had identical genotyping assignments. Genotype call rates ranged from 99% to 100% and duplicate concordance rates were higher than 99%.

Data analysis

We performed a chi square test (χ^2) to assess Hardy–Weinberg equilibrium (HWE) in the control samples. Genotype frequencies were compared across groups using the χ^2 test and the Mantel–Haenzel test for linear trend. The association between breast cancer and each SNP was examined using logistic regression with the SNP genotype tested under models of complete dominance and recessive inheritance. Statistical analysis was carried out using the SPSS v 13 software (SPSS Inc., Chicago, Illinois) and GraphPad InStat v 3.06 (GraphPad Software, San Diego California).

Results

Genotype distributions of controls at each locus were consistent with HWE. However, the *RAD51* genotype frequencies in the controls were not in HWE ($P < 0.05$), but were similar to the frequencies reported by the HapMap Project (www.hapmap.org) [12]. This may be due to hidden population structures that specifically affect RAD51. Neither of the *RAD51* SNPs were associated with breast cancer. Table 1 summarizes the distribution of genotypes among cases and controls, as well as the allele frequencies of the 12 SNPs under study.

The median ages of both the breast cancer cases and the controls were 56 years. The mean age at diagnosis for the breast cancer cases was 51.6 years (standard deviation (SD) ± 9.2) and mean age at ascertainment for the controls was 56.4 years ((SD) ± 9.2).

The associations of the SNPs and breast cancer risk in Cypriot women are shown in Table 2. We found significant associations of the *BRCA2* rs1799944 (Asn991Asp) and *MRE11A* rs601341 variants and breast cancer risk. Carriers of *BRCA2* 991 Asp were found to have an increased risk of breast cancer (OR = 1.41, 95% CI 1.08–1.83, $P = 0.01$) with $P_{trend} = 0.0076$. Homozygous carriers of the *MRE11A* rs601341 A allele had an increased risk of breast cancer (OR = 1.36, 95% CI 1.08–1.71, $P = 0.009$) with $P_{trend} = 0.0087$. A marginal association ($P = 0.05$) was

Table 1 Genotypes and allele frequencies for the 12 SNPs under study

Gene/SNP	Cases	Controls	*P*-value[a]	Gene/SNP	Cases	Controls	*P*-value[a]
MSH2 (rs2059520)				*RAD51* 135G > C (rs1801320)			
A/A	512	562	0.8	G/G	915	952	0.5
A/G	471	489		G/C	193	216	
G/G	108	119		C/C	0	0	
MAF[b]	0.31	0.31		MAF[b]	0.09	0.09	
Hardy–Weinberg[c]		0.41		Hardy–Weinberg[c]		0.0005	
MLH1 Ile219Val (rs1799977)				*RAD51* 172 G > T (rs1801321)			
Ile/Ile	543	568	0.78	G/G	340	400	0.24
Ile/Val	449	497		G/T	522	530	
Val/Val	98	110		T/T	236	236	
MAF[b]	0.3	0.31		MAF[b]	0.45	0.43	
Hardy–Weinberg[c]		0.93		Hardy–Weinberg[c]		0.01	
MRE11A (rs601341)				*BRCA2* Asn991Asp (rs1799944)			
G/G	385	452	0.02	Asn/Asn	945	1058	0.03
G/A	530	566		Asn/Asp	133	108	
A/A	190	156		Asp/Asp	8	4	
MAF[b]	0.41	0.37		MAF[b]	0.07	0.05	
Hardy–Weinberg[c]		0.31		Hardy–Weinberg[c]		0.49	
BARD1 Pro24Ser (rs1048108)				*OGG1* Ser326Cys (rs1052134)			
Pro/Pro	515	514	0.18	Ser/Ser	615	647	0.93
Pro/Ser	445	520		Ser/Cys	422	455	
Ser/Ser	138	138		Cys/Cys	71	72	
MAF[b]	0.33	0.34		MAF[b]	0.25	0.26	
Hardy–Weinberg[c]		0.71		Hardy–Weinberg[c]		0.5	
FANCA Gly501Ser (rs2239359)				*TP53* Pro72Arg (rs1042522)			
Gly/Gly	387	433	0.52	Pro/Pro	555	638	0.08
Gly/Ser	524	543		Pro/Arg	463	438	
Ser/Ser	190	186		Arg/Arg	85	97	
MAF[b]	0.41	0.39		MAF[b]	0.29	0.27	
Hardy–Weinberg[c]		0.47		Hardy–Weinberg[c]		0.08	
ERCC2 Lys751Gln (rs13181)				*RAD52* 2259C > T (rs11226)			
Lys/Lys	331	383	0.06	C/C	561	568	0.4
Lys/Gln	603	585		C/T	448	494	
Gln/Gln	171	208		T/T	92	108	
MAF[b]	0.43	0.43		MAF[b]	0.29	0.3	
Hardy–Weinberg[c]		0.55		Hardy–Weinberg[c]		0.97	

[a] Genotype frequency *P*-value

[b] MAF = minor allele frequency

[c] *P*-value from Chi-square test

observed between *TP53* rs1042522 (Pro72Arg) and risk of breast cancer. No significant associations with breast cancer were observed for the other nine SNPs studied.

Discussion

Breast cancer is a common, polygenic, and heterogeneous disease. Genetic epidemiology data suggest that part of breast cancer etiology can be explained by common, low-penetrance alleles that increase susceptibility to breast cancer risk [13]. DNA repair is essential for maintaining genomic integrity. Deficiencies in the DNA repair pathway lead to genetic instability which in turn may lead to cancer development. Genetic polymorphisms in DNA repair genes may contribute to differential DNA repair capability between individuals [14]. In an attempt to identify low-penentrance breast cancer susceptibility alleles, we

Table 2 Genotype frequencies and risk estimates calculated using the recessive and dominant inheritance models

Gene	SNP	Model	OR	95% CI	*P*-value
MSH2	rs2059520	Dominant	1.05	0.89–1.23	0.6
		Recessive	0.97	0.74–1.28	0.83
					0.76[a]
MLH1	rs1799977	Dominant	0.94	0.8–1.11	0.48
		Recessive	0.96	0.72–1.27	0.76
					0.5[a]
MRE11A	rs601341	Dominant	1.17	0.99–1.39	0.07
		Recessive	1.36	1.08–1.71	0.009
					0.0087[a]
BARD1	rs1048108	Dominant	0.88	0.75–1.04	0.15
		Recessive	1.08	0.84–1.39	0.56
					0.43[a]
FANCA	rs2239359	Dominant	1.1	0.92–1.3	0.3
		Recessive	1.09	0.88–1.37	0.42
					0.25[a]
ERCC2	rs13181	Dominant	1.13	0.95–1.35	0.18
		Recessive	0.85	0.68–1.06	0.16
					0.89[a]
RAD51	rs1801320	Dominant	0.93	0.75–1.15	0.5
		Recessive			
					0.5[a]
RAD51	rs1801321	Dominant	1.16	0.98–1.39	0.09
		Recessive	1.08	0.88–1.32	0.46
					0.13[a]
BRCA2	rs1799944	Dominant	1.41	1.08–1.83	0.01
		Recessive	2.16	0.65–7.2	0.2
					0.0076[a]
OGG1	rs1052134	Dominant	0.98	0.83–1.16	0.85
		Recessive	1.05	0.75–1.47	0.79
					0.96[a]
TP53	rs1042522	Dominant	1.18	1–1.39	0.05
		Recessive	0.93	0.68–1.25	0.62
					0.19[a]
RAD52	rs11226	Dominant	0.89	0.76–1.06	0.19
		Recessive	0.9	0.68–1.21	0.5
					0.19[a]

[a] P_{trend}

investigated the hypothesis that common variation in 11 DNA repair-related pathway genes modifies risk for breast cancer. We genotyped 12 SNPs in a cohort of 2,286 Cypriot women (1,109 breast cancer patients and 1,177 healthy controls). We found that SNPs in *BRCA2* and *MRE11A* may be associated with breast cancer risk.

For the *BRCA2* 991Asp allele, the additive model showed a significant trend ($P = 0.0076$) towards increased risk of breast cancer with the number of copies of the Asp allele among Cypriot women. It is located in the conserved BRC repeat region of the BRCA2 gene in exon 11 [15]. This variant has been found in many individuals with a family history of breast cancer and has been classified as a variant of no clinical significance in the Breast Cancer Information Core Database (BIC) [16]. On the other hand, in silico prediction methods suggest that this is a non-tolerated amino acid substitution within the limits of confidence in the alignments [17]. Therefore, until functional data become available, the pathogenicity of this variant cannot be excluded, and it may be a variant that increases risk moderately, but is indeed, not highly penetrant. There was no association between the presence of the *BRCA2* 991Asp allele and family history of breast cancer. A moderately strong association of this *BRCA2* polymorphism with malignant melanoma has been reported. The presence of this common *BRCA2* variant was associated with malignant melanoma risk ($P = 0.002$ after Bonferroni correction), in over 9% of the cases studied. The authors suggested that this variant is not a neutral missense mutation and that follow-up studies should be undertaken in melanoma and breast cancer populations to precisely define its pathogenicity [18]. The role of this SNP in breast cancer risk has been investigated in the Multiethnic Cohort study and no association was found [19]. Previous studies that we performed in our population revealed a different spectrum of mutations in the *BRCA1* and *BRCA2* genes compared to other populations [20, 21]. The over-representation of the *BRCA2* Asn991Asp polymorphism in the breast cancer group supports that this variant is associated with an increased breast cancer risk among Cypriot women and it is possible that this association is characteristic only for the Cypriot population.

In the current study, there was also evidence for an increased breast cancer risk for women homozygous for the *MRE11A* rs601341 A allele. The *MRE11A* gene forms a complex with *RAD50* and *NBS1* genes which is involved in the cellular response to DNA double strand breaks. Defects in the members of this tri-complex are linked to increased chromosomal instability which leads to cancer [22]. To our knowledge, the role of rs601341 in breast cancer has not been investigated but a protective effect of this SNP against follicular lymphoma has been reported [23]. rs601341 may be in LD with another variant in the region. Functional studies will need to be performed in the future to identify the actual causal variant.

There are contradictory reports regarding the role of the *TP53* Pro72Arg polymorphism and breast cancer. Our results suggest a marginal increased risk for breast cancer ($P = 0.05$) for carriers of the Pro allele. A meta-analysis conducted by the Breast Cancer Association Consortium concluded that this variant is not associated with breast cancer [24].

Our study has several strengths, including a high participation rate of eligible cases (98%) and a sample from a homogeneous ethnic background (100% of study participants are Greek Cypriots) thus reducing the bias due to population stratification. In addition, our study population (both cases and controls) was from all over the country minimizing potential selection bias. Limitations of this study are that our analysis did not consider the possibility of gene-gene interactions. It is possible that the risks observed are the result of interactions but we have not attempted to assess such effects since the estimate of an interaction effect will be unreliable because of small numbers. We also did not adjust for possible differences in lifestyle factors.

In conclusion our results suggest that genetic variation in the DNA repair pathway is associated with breast cancer risk in Cypriot women. The associations with SNPs rs1799944 and rs601341 should be considered for replication efforts in other larger studies to increase confidence in reported association and to clarify whether the association is only specific for the Cypriot population.

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EPIDEMIOLOGY

Genetic polymorphisms in the DNA repair genes *XRCC1*, *XRCC2* and *XRCC3* and risk of breast cancer in Cyprus

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Abstract Population-based studies have reported significant associations between specific genetic polymorphisms and breast cancer susceptibility. A number of studies have demonstrated that common variants of genes involved in the DNA repair pathway act as low penetrance breast cancer susceptibility alleles. We aimed to investigate the association of single nucleotide polymorphisms (SNPs) in the DNA repair genes *XRCC1*, *XRCC2* and *XRCC3* and breast cancer in *MASTOS*, a population-based case–control study of 1,109 Cypriot women with breast cancer diagnosed between 40 and 70 years and 1,177 age-matched healthy controls. Five coding SNPs were genotyped including rs1799782, rs25489 and rs25487 in *XRCC1*, rs3218536 in *XRCC2* and rs861539 in *XRCC3*. Homozygous *XRCC1* 280His carriers had an increased risk of breast cancer (odds ratio 4.68; 95% CI 1.01–21.7; $P = 0.03$). The *XRCC2* 188His allele was associated with a marginal protective effect for breast cancer (odds ratio 0.79; 95% CI 0.62–1.00; $P = 0.05$). No significant associations were observed between the other three SNPs and breast cancer. This study suggests that genetic variation in SNPs in *XRCC1* and *XRCC2* genes may influence breast cancer susceptibility.

Keywords Breast cancer · Case–Control study · Cyprus · DNA repair · Genetic epidemiology · Polymorphisms · *XRCC1* · *XRCC2* · *XRCC3*

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Introduction

Breast cancer is the most common malignancy affecting women worldwide [1]. In Cyprus, it is the most frequent type of cancer in women, with approximately 350–400 new cases diagnosed annually. It is estimated that about 5% of breast cancer cases are related to mutations in rare but highly penetrant genes, such as *BRCA1* and *BRCA2* [2]. Previous studies that we performed in our population revealed a different spectrum of mutations in the BRCA1/2 genes compared to other populations [3, 4]. It is likely that low penetrant cancer susceptibility genes contribute to a larger proportion of breast cancer cases.

The DNA repair pathway is essential for maintaining genomic stability of mammalian cells. Deficiencies in the DNA repair system are likely to cause chromosomal aberrations which in turn lead to cell malfunctioning, cell death and tumorigenesis [5]. Several studies have demonstrated that polymorphisms in genes responsible for maintaining genomic integrity are modifiers of disease risk [6, 7]. Therefore, single nucleotide polymorphisms (SNPs) of genes involved in DNA repair are good candidates for low penetrance breast cancer susceptibility alleles.

The *XRCC* (X-Ray cross-complementing) genes were initially discovered through their role in DNA damage response caused by ionizing radiation. They are important components of various DNA repair pathways contributing to DNA-damage processing and genetic stability [8]. *XRCC1* gene is an important component of the base excision repair (BER) pathway acting as a scaffold for other BER enzymes [9]. *XRCC2* and *XRCC3* genes are necessary for homologous recombination repair (HRR) and are required for *RAD51* focus formation [10, 11].

The aim of this study was to test the hypothesis that common variants in the *XRCC* genes modify susceptibility to breast cancer. We focused the present investigation on evaluating five potentially functional SNPs in the *XRCC1*, *XRCC2* and *XRCC3* genes.

Materials and methods

Subjects

The study population was women participating in the *MASTOS* (Greek word for breast) study, a population-based case–control study of breast cancer in Cyprus. Blood samples were collected between 2004 and 2006 from 1,109 female breast cancer patients diagnosed between 40 and 70 years old and 1,177 aged-matched healthy controls. Cases participating in the study were women who were previously diagnosed with breast cancer between January 1999 and December 2006. In addition to blood samples, a risk factor questionnaire which included extensive demographic, epidemiological and pathological data was obtained from each participant through a standardized interview. Breast cancer cases were verified by reviewing histological reports. The study was approved by the National Bioethics Committee of Cyprus, and all participants provided signed informed consent.

Genotyping

DNA was isolated from blood samples using standard procedures (phenol–chloroform method). The five single nucleotide polymorphisms (SNPs): *XRCC1* Arg194Trp (rs1799782), *XRCC1* Arg280His (rs25489), *XRCC1* Arg399Gln (rs25487), *XRCC2* Arg188His (rs3218536) and *XRCC3* Thr241Met (rs861539) were genotyped in all subjects participating in the study. We used three PCR-RFLP (restriction fragment length polymorphism) assays for genotyping *XRCC1* 194 and 399 and *XRCC3* 241 SNPs as described previously by Hu et al. [12]. The *XRCC1* 280 SNP was also identified by PCR-RFLP using GTCTGA GGGAGGAGGGTCTG and CAGAGGAGCTGGGGAA GATC primers. Detailed PCR conditions are available on request. Amplified DNA was digested with *Rsa*I enzyme and size separated on a 2% agarose gel. A similar protocol was followed for *XRCC2* 188 SNP using the primer pairs CACCCATCTCTCTGCCTTT and CCTCTCGACGACTG TGTGAT. Amplified DNA was digested with *Sex*AI enzyme and size separated on a 2% agarose gel. Overall success rate for the genotyping assays was 99%. For quality control, random samples were genotyped in duplicate and had identical genotyping assignments. Direct sequencing was also used to confirm these calls.

Data analysis

Using the control samples, a Chi square test (χ^2) was applied to test for Hardy–Weinberg equilibrium (HWE). Genotype frequencies were compared across groups using the χ^2-test and the Mantel–Haenzel test for linear trend. The association between breast cancer and each SNP was examined using logistic regression with the SNP genotype tested under models of complete dominance and recessive inheritance. Because the *XRCC2* 188His alleles are uncommon in this population, individuals with genotypes Arg/His and His/His were combined into one group as 188His allele carriers and were compared with Arg/Arg homozygotes as the reference. Statistical analysis was carried out using the SPSS v 13 software (SPSS Inc., Chicago, IL) and GraphPad InStat v 3.06 (GraphPad Software, San Diego, CA).

Results

The genotype frequencies in the controls were in HWE for the two SNPs in *XRCC1* (codons 194 and 399) and the SNP in *XRCC3* (codon 241). The *XRCC1* 280 SNP and the *XRCC2* 188 SNP significantly deviated from HWE ($P < 0.05$). The deviations observed are likely to be chance results rather than genotyping errors since the homozygotes for these SNPs are very rare in this population. In *XRCC1*, only two controls were homozygous for the 280His allele and only one control was homozygous for the *XRCC2* 188His allele. Table 1 summarizes the distribution of genotypes among cases and controls as well as the allele frequencies of the five SNPs under study.

The median age of both the breast cancer cases and the controls was 56 years. The mean age at diagnosis for the breast cancer cases was 51.6 years (standard deviation (SD) ± 9.2) and mean age at ascertainment for the controls was 56.4 ((SD) ± 9.2).

We found significant associations of the *XRCC1* Arg280His and *XRCC2* Arg188His variants and breast

Table 1 Genotypes and allele frequencies for the five SNPs under study

	Cases	Controls	*P*-value[b]
XRCC1 Arg194Trp			
Arg/Arg	914	973	0.97
Arg/Trp	175	182	
Trp/Trp	8	9	
Arg allele frequency	0.91	0.91	
Trp allele frequency	0.09	0.09	
Hardy–Weinberg[a]		0.88	
XRCC1 Arg280His			
Arg/Arg	923	959	0.05
Arg/His	177	207	
His/His	9	2	
Arg allele frequency	0.91	0.91	
His allele frequency	0.09	0.09	
Hardy–Weinberg[a]		0.01	
XRCC1 Arg399Gln			
Arg/Arg	506	520	0.7
Arg/Gln	479	516	
Gln/Gln	122	140	
Arg allele frequency	0.67	0.66	
Gln allele frequncy	0.33	0.34	
Hardy–Weinberg[a]		0.49	
XRCC2 Arg188His			
Arg/Arg	972	999	0.14
Arg/His	135	177	
His/His	1	1	
Arg allele frequency	0.94	0.92	
His allele frequency	0.06	0.08	
Hardy–Weinberg[a]		0.02	
XRCC3 Thr241Met			
Thr/Thr	312	351	0.75
Thr/Met	560	600	
Met/Met	220	226	
Thr allele frequency	0.54	0.55	
Met allele frequency	0.46	0.45	
Hardy–Weinberg[a]		0.28	

[a] *P*-value from χ^2-test

[b] Genotype frequency *P*-value

cancer risk. Homozygous carriers of *XRCC1* 280His were found to have an increased risk of breast cancer (OR = 4.68, 95% CI 1.01–21.7, $P = 0.03$). Carriers of the *XRCC2* His allele had a decreased risk of breast cancer (OR = 0.79, 95% CI 0.62–1.0, $P = 0.05$). No significant associations with breast cancer were observed for SNPs *XRCC1* Arg194Trp, *XRCC1* Arg399Gln and *XRCC3* Thr241Met (Table 2).

Discussion

Breast cancer is a polygenic disease. The polygenic model of breast cancer suggests that there are multiple low-penetrance alleles, each or in combination, together with environmental interactions that have a small effect on breast cancer risk [13]. DNA repair is essential for maintaining genomic integrity. Deficiencies in the DNA repair pathway lead to genetic instability which in turn may lead to cancer development. Genetic polymorphisms in DNA repair genes may contribute to differential DNA repair capability between individuals [14]. In an attempt to identify low-penentrance breast cancer susceptibility alleles we have examined the hypothesis that common variation in *XRCC1*, *XRCC2* and *XRCC3* modifies risk for breast cancer. We have genotyped five SNPs in a cohort of 2,286 Cypriot women (1,109 breast cancer patients and 1,177 healthy controls). We found that SNPs in *XRCC1* and *XRCC2* may be associated with breast cancer risk.

Comparison of the *XRCC1* Arg280His genotypes between breast cancer cases and healthy controls revealed that the *XRCC1* 280His allele is associated with an increased risk of breast cancer. We observed a 4- to 5-fold increased risk for breast cancer in women homozygous for the His allele compared to those homozygous for the Arg allele. The *XRCC1* codon 280 polymorphism is located in the linker region that separates DNA polymerase β interacting domain from PARP (poly-ADP-ribose polymerase) interacting domain [15]. A recently published functional study, which investigated the role of *XRCC1* variants in altering DNA repair capacity, has shown that relative to the wild-type protein, the 280His variant decreases the DNA repair capacity of mammalian cells exposed to chemical stresses. In the same study, a positive association was observed between the *XRCC1* Arg280His genotype, breast cancer and smoking [16]. In our study three of the nine women who were homozygous for the 280His variant were smokers who started smoking at a young age. It is possible that the additional 280His homozygous carriers were exposed for a long duration to passive smoking and that the combination of the exposure and the *XRCC1* 280His genotype has increased their breast cancer risk. Unfortunately information on passive smoking exposure was not collected and our hypothesis cannot be confirmed.

In the current study, there was also some evidence of a dominant protective effect for the *XRCC2* 188His allele carriers. There are contradictory reports regarding the role of this rare *XRCC2* variant in breast cancer, with some studies showing an association with increased risk of breast cancer whereas others did not. A recent meta-analysis conducted by the Breast Cancer Association Consortium concluded that this variant is not associated with breast cancer [17]. We observed a protective effect of 188His

Table 2 Genotype frequencies and risk estimates calculated using the recessive and dominant inheritance models

Gene	SNP	Genotypes	Cases, *n*	Controls, *n*	OR	95% CI	*P*-value
XRCC1	Arg194Trp	Arg/Arg	914	973	1.00		
		Arg/Trp	175	182	1.02	0.82–1.28	0.84
		Trp/Trp	8	9	0.95	0.36–2.46	0.91
	P_{trend}						0.89
XRCC1	Arg280His	Arg/Arg	923	959	1.00		
		Arg/His	177	207	0.89	0.71–1.11	0.29
		His/His	9	2	4.68	1.01–21.7	0.03
	P_{trend}						0.77
XRCC1	Arg399Gln	Arg/Arg	506	520	1.00		
		Arg/Gln	479	516	0.95	0.8–1.14	0.6
		Gln/Gln	122	140	0.9	0.68–1.18	0.43
	P_{trend}						0.71
XRCC2	Arg188His	Arg/Arg	972	999	1.00		
		Arg/His and His/His	136	178	0.79	0.62–1	0.05
XRCC3	Thr241Met	Thr/Thr	312	351	1.00		
		Thr/Met	560	600	1.05	0.87–1.27	0.62
		Met/Met	220	226	1.1	0.86–1.39	0.46
	P_{trend}						0.45

variant, similar to what was observed in a previous study [18]. In the Polish population, homozygotes for the 188His variant showed a decreased risk for breast cancer, but the association was only borderline significant when data were pooled with those from a U.S. study [18]. It is interesting to note that a moderately strong association of the *XRCC2* Arg188His polymorphism with epithelial ovarian cancer has been reported. Women carrying one His allele had a 20% reduction in risk and those carrying two His alleles had a 50% reduction in risk of epithelial ovarian cancer compared with those homozygous for the Arg allele [19]. It is therefore possible that the rare *XRCC2* 188His allele is associated with a decreased risk of breast cancer but its functional effect must be determined in order to clarify its role in breast cancer.

Our data did not support a significant association of risk of breast cancer with the *XRCC1* Arg188His, Arg399Gln and *XRCC3* Thr241Met SNPs. These results are comparable with those reported from the Breast Cancer Association Consortium where variants *XRCC1* Arg399Gln and *XRCC3* Thr241Met did not modify breast cancer risk [17]. In addition, the *XRCC1* Arg194Trp variant has been studied extensively and no association with breast cancer has been observed [20].

Conflicting evidence for association may be due to population-specific differences. One example is the *XRCC1* Arg399Gln SNP which was associated with an increased risk of lung cancer among Asians but not among Caucasians [21]. The SNPs evaluated in the present study have been studied extensively in many population datasets and conflicting results have been reported [17]. The Greek Cypriots appear to be a genetic isolate, as shown by the results obtained from our study of the genetics of familial breast cancer [3]. It is possible that the associations observed with the *XRCC1* 280 and *XRCC2* 188 SNPs and breast cancer are characteristic mainly for the Cypriot population.

Our study has several strengths, including a high participation rate of eligible cases (98%) and a sample from a homogeneous ethnic background (100% of study participants are Greek Cypriots) thus reducing the bias due to population stratification. In addition, our study population (both cases and controls) was from all over the country minimizing potential selection bias. Limitations of this study are that our analysis did not consider the possibility of gene–gene interactions. It is possible that the risks observed are the result of interactions but we have not attempted to assess such effects since the estimate of an interaction effect will be unreliable because of small numbers. For this type of analysis a very large sample size is essential. We also did not adjust for possible differences in lifestyle factors nor account for multiple testing.

In conclusion our results suggest that genetic variation in the DNA repair pathway is associated with breast cancer risk in Cypriot women. Taking into account the important roles of *XRCC1* and *XRCC2* genes in BER and HRR pathways, the small but statistically significant differences in breast cancer risk in Cypriot women associated with the *XRCC1* 280His and the *XRCC2* 188His variants may indicate a true association.

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A4. **M Loizidou**, Y Marcou, E Kakouri, V Anastasiadou, R Newbold, A Hadjisavvas, K Kyriacou. Contribution of BRCA1 and BRCA2 germline mutations to the incidence of early-onset breast cancer in Cyprus. **Clin Genet.** 2007 Feb;71(2):165-70.

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Short Report

Contribution of BRCA1 and BRCA2 germline mutations to the incidence of early-onset breast cancer in Cyprus

Loizidou M, Marcou Y, Anastasiadou V, Newbold R, Hadjisavvas A, Kyriacou K. Contribution of BRCA1 and BRCA2 germline mutations to the incidence of early-onset breast cancer in Cyprus.
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In Cyprus, the prevalence of breast cancer associated with BRCA1 and BRCA2 mutations in young women is unknown. In this study, we present the results of mutational analysis of the BRCA1 and BRCA2 genes in 26 Cypriot women diagnosed with breast cancer by the age of 40. The entire coding regions, including splice sites, of the BRCA1 and BRCA2 genes were sequenced using cycle sequencing. We identified four pathogenic mutations: two in BRCA1 [c.1840A>T (K614X), c.5310delG (5429delG)] and two in BRCA2 [c.3531-3534delCAGC (3758del4), c.8755delG (8984delG)] in six of 26 unrelated patients. The BRCA2 mutation c.3531-3534delCAGC (3758del4) is novel and the BRCA1 mutation c.1840A>T (K614X) is reported for the first time in Cypriot patients. The BRCA2 Cypriot founder mutation c.8755delG (8984delG) was detected in three unrelated patients. Additionally, we identified one novel BRCA1 missense mutation, two novel polymorphisms and three novel intronic variants of which BRCA1 c.4185+3A>G (IVS12+3A>G) may be pathogenic. Of the six BRCA1/2 mutation carriers, only four had a family history. These results show that the prevalence of BRCA1 and BRCA2 mutations in Cypriot women diagnosed with early-onset breast cancer is high. We conclude that Cypriot women with early-onset breast cancer should be offered BRCA1/2 testing irrespective of their family history.

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Breast cancer is the commonest malignancy, which affects women worldwide. The incidence rates of breast cancer are increasing annually and it is estimated that by the year 2010 there will be 1.5 million new cases worldwide (1). In Cyprus, breast cancer is the most frequent type of cancer in women, with approximately 350 new cases diagnosed every year (2), with about 20–30 of these, occurring in patients younger than 40 years of age.

Around 5–10% of breast cancer is due to genetic predisposition, and two susceptibility genes, namely, BRCA1 (3) (MIM 113705) and BRCA2 (4) (MIM 600185) have been discovered, which substantially increase the risk of breast/ovarian cancer. Data from studies of these genes reveal that women who carry a pathogenic mutation tend to develop breast cancer at a young age (5, 6).

It is currently accepted that a variable proportion of early-onset breast cancer cases is associated with mutations in these two tumour suppressor genes (7), and that the proportion may be higher in populations harbouring founder mutations. The contribution of the BRCA1 and BRCA2 mutations to the population incidence of early-onset breast cancer ranges from 20% in populations with strong founder effects, such as Ashkenazi Jews (8, 9) or Icelandic populations (10, 11) to between 5% and 10% among less isolated populations (12–16).

In a previous study, results from our department revealed a different spectrum of BRCA mutations in Cypriot families compared to other Mediterranean countries. Recently, we have also described a founder mutation in the BRCA2 gene in Cypriot families (17). The aim of this study was to evaluate the frequency and distribution of mutations in the BRCA1 and BRCA2 genes, in a cohort of Cypriot women with early-onset breast cancer.

Materials and methods

Twenty-six consecutive cases of women diagnosed with breast cancer, before the age of 40, between the years 2003 and 2004, participated in this study. These Cypriot patients were selected on the basis of a diagnosis of early-onset breast cancer, under age 40, irrespective of their family history. Participating patients received genetic counselling and information about the aims of the study; all 26 agreed to undergo genetic testing, gave an informed consent and provided blood samples. Information on family history of cancer, with emphasis on breast or ovarian cancer incidence, was also obtained and pedigrees were constructed. Cancer diagnoses for patients and their affected relatives were verified by reviewing histological reports. In addition, a control group that consisted of 50 DNA samples from 50, age-matched, unrelated healthy Cypriot women, with no history of breast or ovarian cancer, was recruited. This was used to estimate the frequency of the detected BRCA1 and BRCA2 variants in the general population.

Mutation detection

Genomic DNA was isolated from peripheral-blood lymphocytes using standard extraction protocols. Polymerase chain reaction (PCR) was used to amplify the entire coding sequence and intron–exon junctions of the BRCA1 and BRCA2 genes. Following PCR amplification, sequencing was carried out using the ABI PRISM di-Deoxy Terminator Cycle sequencing kit on an ABI 9700 thermal cycler and an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The PCR products were sequenced using the same primers as the ones used for PCR amplification. When a mutation was identified, a new PCR product using a second DNA sample was sequenced so as to confirm the result.

In order to detect large genomic rearrangements in the BRCA1 and BRCA2 genes, multiplex ligation-dependent probe amplification (MLPA, MRC Holland, Amsterdam, The Netherlands) using the P087 and P045 kits was carried out following the manufacturer's protocols. Fragment analysis was carried out on an ABI 310 Genetic Analyzer using ROX-500 as a size standard.

To evaluate potential alternative splicing effects, three in silico sequence analysis tools, namely NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html), SpliceSiteFinder (http://violin.genet.sickkids.on.ca/~ali/splicesitefinder.html) and NetGene (http://www.cbs.dtu.dk/services/NetGene2/) were used.

Results

Our results show that of the 26 women diagnosed with early-onset breast cancer, six had pathogenic mutations: two in BRCA1 and four in BRCA2. In total, mutation analysis of the two genes revealed the presence of 20 variants in the BRCA1 gene and 26 variants in the BRCA2 gene. In describing the individual variants, we applied the mutation nomenclature guidelines of the HGVS (Human Genome Variation Society) (http://www.hgvs.org/rec.html) and the nomenclature used in BIC database (18), which appears in brackets.

The 20 variants identified in the BRCA1 gene include two truncating mutations, six missense mutations, five polymorphisms and seven intronic variants (Table 1). The two truncating mutations are a nonsense mutation c.1840A>T (1959A>T), at codon 614 in exon 11, a lysine to a STOP (K614X) and a frameshift mutation at position c.5310delG (5429delG) at codon 1770 in exon 21, which introduces a STOP 22 amino acids further down (p.Gly1770fs23). These mutations were detected in two unrelated patients. The six missense mutations are c.1067A>G (Q356R), c.1984C>T (H662Y), c.2612C>T (P871L), c.3113A>G (E1038G), c.3348A>G (L1183K) and c.4837A>G (S1613G). It is noted that missense mutation c.1984C>T (H662Y), polymorphism c.1482A>G (Q494Q) and intronic variants c.126-23C>A (IVS5-23C>A) and c.4185+3A>G (IVS12+3A>G) are novel. The three in silico sequence analysis tools used, predicted that the intronic variant c.4185+3A>G (IVS12+3A>G) may cause aberrant splicing. Details of the results of the analysis of the 20 BRCA1 variants from 50 DNAs of the healthy group and the frequency of the variants in the patient group are also displayed in Table 1.

The 26 BRCA2 variants include two truncating mutations, seven missense mutations, six

Table 1. Details of the 20 variants detected in the BRCA1 gene in the patient group and in the control group

Exon	Sequence variant	Amino acid variant	Mutation type	Mutation effect	Frequency in the patient group (%)	Frequency in the control group (%)
Truncating mutations						
11	c.1840A>T (1959A>T)	p.Lys614X	N	N	2	0
21	c.5310delG (5429delG)	p.Gly1770fsx23	F	F	2	0
Missense mutations						
11	c.1067A>G (1186A>G)	p.Gln356Arg (Q356R)	M	P	15	5
11	**c.1984C>T (2103C>T)**	**p.His662TTyr (H662Y)**	**M**	**UV**	**2**	**0**
11	c.2612C>T (2731C>T)	p.Pro871Leu (P871L)	M	P	29	50
11	c.3113A>G (3232A>G)	p.Glu1038Gly (E1038G)	M	P	37	41
11	c.3348A>G (3467A>G)	p.Leu1183Lys (L1183K)	M	P	31	42
16	c.4837A>G (4956A>G)	p.Ser1613Gly (S1613G)	M	P	37	41
Polymorphisms						
9	c.591C>T (710C>T)	Synonymous (Cys197Cys)	P	P	2	0
11	**c.1482A>G (1601A>G)**	**Synonymous (Gln494Gln)**	**P**	**P**	**2**	**0**
11	c.2082C>T (2201C>T)	Synonymous (Ser694Ser)	P	P	37	39
11	c.2311 T>C (2430T>C)	Synonymous (Leu771Leu)	P	P	37	34
13	c.4308T>C (4427T>C)	Synonymous (Ser1436Ser)	P	P	37	47
Intronic variants						
5	**c.126-23C>A (IVS5-23C>A)**		**UV**	**UV**	**2**	**0**
8	c.442-34T>C (IVS7-34T>C)		UV	P	60	100
9	c.548-57delT (IVS8-57delT)		UV	P	31	29
12	**c.4185+3A>G (IVS12+3A>G)**		**UV**	**UV**	**2**	**0**
17	c.4987-68A>G (IVS16-68A>G)		UV	P	31	31
17	c.4987-94A>G (IVS16-94A>G)		UV	P	31	31
18	c.5152+66G>A (IVS18+66G>A)		UV	P	37	29

F, frameshift; N, nonsense; UV, unclassified variant; M, missense; P, polymorphism.
Entries in bold are novel variants.
Mutation nomenclature is according to GenBank accession number U14680 (BRCA1) with numbering starting at the A of the first ATG. The nomenclature as used in the BIC database (18) is given in parentheses.

polymorphisms and 11 intronic variants (Table 2). The two truncating mutations include a novel frameshift c.3531-3534delCAGC (p.Asp1177fsx19) in exon 11 which introduces a STOP codon (1196X). This mutation was detected in one patient. The second frameshift mutation is c.8755delG (8984delG) in exon 22 which introduces a STOP codon 7 amino acids further down (p.Gly2919fsx8). This mutation was detected in three unrelated patients. The seven missense mutations are c.865A>C (N289H), c.1114C>A (H372N), c.1889C>T (T630I), c.2971A>G (N991D), c.4258G>T (D1420Y), c.5744C>T (T1915M) and c.7544C>T (T2515I). It is noted that polymorphism c.7140T>C (H2380H) and intronic variant c.681+43A>G (IVS8+43A>G) are novel. Details of the results of the analysis of the 26 BRCA2 variants from 50 DNAs of the healthy group and the frequency of the variants in the patient group are also displayed in Table 2.

The analysis of BRCA1 and BRCA2 genes with MLPA resulted in no detection of gene rearrangements or duplications within the group of 26 DNA samples evaluated in this study.

Out of the 26 patients recruited, 15 women reported a family history of at least one breast or ovarian cancer, whereas 11 women had a negative history. Furthermore, of the six BRCA1/2 mutation carriers detected, two had a negative family history (two of 11, approximately 20%). A summary of the family history of breast and ovarian cancer for the six carriers of the pathogenic mutations, as well for the one case carrying the variant c.4185+3A>G (IVS12+3A>G) is presented in Table 3.

Discussion

This is the first study focusing on the contribution of BRCA1 and BRCA2 germline mutations to the incidence of early-onset breast cancer in Cypriot patients diagnosed under age 40, irrespective of their family history.

We have analysed a total of 26 DNA samples from women diagnosed with breast cancer at age below 40, and detected two pathogenic mutations in BRCA1 affecting two patients and two pathogenic mutations in BRCA2 affecting four patients. All characterized disease-associated mutations, were truncating mutations causing premature stop codons and were detected in six unrelated patients.

Table 2. Details of the 26 variants detected in the BRCA2 gene in the patient group and in the control group

Exon	Sequence variant	Amino acid variant	Mutation type	Mutation effect	Frequency in the patient group (%)	Frequency in the control group (%)
Truncating mutations						
11	**c.3531-3534delCAGC (3758del4)**	**p.Asp1177fsx19**	**F**	**F**	**2**	**0**
22	c.8755delG (8984delG)	p.Gly2919fsx8	F	F	6	0
Missense mutations						
10	c.865A>C (1093A>C)	p.Asn289His (N289H)	M	P	12	11
10	c.1114C>A (1342C>A)	p.His372Asn (H372N)	M	P	21	22
10	c.1889C>T (2117C>T)	p.Thr630Ile (T630I)	M	UV	2	0
11	c.2971A>G (3199A>G)	p.Asn991Asp (N991D)	M	P	12	7
11	c.4258G>T (4486G>T)	p.Asp1420Tyr (D1420Y)	M	P	2	2
11	c.5744C>T (5972C>T)	p.Thr1915Met (T1915M)	M	P	2	0
15	c.7544C>T (7772C>T)	p.Thr2515Ile (T2515I)	M	P	2	0
Polymorphisms						
10	c.1365A>G (1593A>G)	Synonymous (Ser455Ser)	P	P	12	10
11	c.2229T.C (2457T>C)	Synonymous (His743His)	P	P	8	10
11	c.3396A>G (3624A>G)	Synonymous (Lys1132Lys)	P	P	27	26
11	c.3807C>T (4035C>T)	Synonymous (Val1269Val)	P	P	54	57
14	**c.7140T>C (7368T>C)**	**Synonymous (His2380His)**	**P**	**P**	**2**	**1**
14	c.7242A>G (7470A>G)	Synonymous (Ser2414Sser)	P	P	23	20
Intronic variants						
2	c.1-203G>A (203G>A)		P	P	23	25
4	c.425+36A>G (IVS4+36A>G)		UV	UV	2	0
8	**c.681+43A>G (IVS8+43A>G)**		**UV**	**UV**	**2**	**0**
8	c.681+56C>T (IVS8+56C>T)		UV	P	12	17
10	c.1909+12delT (IVS10+12delT)		UV	P	100	100
11	c.1910-51G>T (IVS11-51G>T)		UV	P	2	11
11	c.6841+80del4 (IVS11+80del4)		UV	P	29	51
14	c.7435+53C>T (IVS14+53C>T)		UV	P	2	11
17	c.7618-14T>C (IVS16-14T>C)		UV	P	46	51
25	c.9118-16T>C (IVS24-16T>C)		UV	UV	2	1
27	c.10333A>G (IVS27+76A>G)		UV	UV	2	1

F, frameshift; N, nonsense; UV, unclassified variant; M, missense; P, polymorphism.
Entries in bold are novel variants.
Mutation nomenclature is according to GenBank accession number U43746 (BRCA2) with numbering starting at the A of the first ATG. The nomenclature as used in the BIC database (18) is given in parentheses.

As described in the materials and methods section, the patients were consecutive cases diagnosed with early-onset breast cancer, by age 40, between the years 2003 and 2004. This number represents the annual expected incidence of this group of cases, as indicated by the accumulated data from the Cyprus Cancer Registry (2). Of the 26 recruited patients, 15 reported a positive family history of at least one incidence of breast or ovarian cancer. In this group, four patients were found to carry BRCA1/2 pathogenic mutations. The remaining 11 patients had no relevant family history and it is of interest that two of these were found to carry BRCA1/2 pathogenic mutations. In addition, in this group of 11 patients, a possible splice-site variant c.4185+3A>G (IVS12+3A/G) was detected in one patient raising the number of pathogenic carriers to a possible three (three of 11, approximately 30%).

Table 3. Details of the BRCA1 and BRCA2 pathogenic mutation carriers

Gene	Exon	Mutation	Amino acid variant	Age at diagnosis	BC/OV (family history)
BRCA1	11	c.1840A>T (1959A>T)	p.Lys614X	40	1 MBC
BRCA1	21	c.5310delG (5429delG)	p.Gly1710fs23	33	–
BRCA1	12	c.4185+3A>G (IVS12+3A>G)		27	–
BRCA2	11	c.3531-3534delCAGC (3758del4)	p.Asp1177fsx19	32	3 BC, 1 OC
BRCA2	22	c.8755delG (8984delG)	p.Gly2919fs8	33	–
BRCA2	22	c.8755delG (8984delG)	p.Gly2919fs8	34	3 BC
BRCA2	22	c.8755delG (8984delG)	p.Gly2919fs8	30	9 BC

BC, breast cancer; M, male; OC, ovarian cancer.

The detailed results of the present study are displayed in Tables 1 and 2, which show all the variants detected in the BRCA1 and BRCA2 genes, respectively. It is noted that five out of the six missense mutations identified in the BRCA1 gene are polymorphisms since they were also present in more than 5% of the control group. Similarly, three out of the seven missense mutations identified in the BRCA2 are polymorphisms. In addition, missense mutations c.4258G>T (D1420Y), c.5744C>T (T1915M) and c.7544C>T (T2515I) have been previously reported as polymorphisms (19–21).

The unclassified variant c.4185+3A>G (IVS12+3A>G) in the BRCA1 gene identified in this study is of particular interest. We have applied three different theoretical splicing and skipping prediction methods, in an attempt to predict aberrant splicing based on the DNA sequence. All three softwares used predicted that this intron variant might result in deleterious alterations at the mRNA level. Unfortunately, an RNA sample from this patient, which would allow us to assess the transcript that is produced by this variant allele, is not available. The novel missense mutation c.1984C>T (H662Y) in the BRCA1 gene may be also pathogenic. The amino acid 662 is located in the DNA-binding region of the BRCA1 gene, and it contributes to the DNA-repair-related functions of the BRCA1 (22). Mutation c.1984C>T (H662Y) alters the amino acid histidine from a basic polar, positively charged molecule, to the aromatic, non-polar amino acid tyrosine. It is well known that the stoichiometry and the charges of the amino acids play a role in the conformation and the function of proteins. Therefore, based on the above, we suggest that missense mutation c.1984C>T (H662Y) in the BRCA1 gene may lead to the synthesis of a dysfunctional BRCA1 protein which in turn predisposes to the breast cancer phenotype.

In terms of tumour pathology, infiltrating ductal carcinoma was the predominant tumour type in both BRCA1/2 mutation carriers and non-carriers. It is noted that two of the infiltrating ductal carcinomas were comedo type and were diagnosed in women with truncating BRCA1/2 mutations.

Previous population-specific studies revealed that the contribution of BRCA1 and BRCA2 mutations to the incidence of early-onset breast cancer ranges between 5% and 10% (12–16). In contrast, our findings show that 23% (six of 26) of Cypriot early-onset breast cancer cases are associated with a germline mutation in either the BRCA1 or the BRCA2 genes. This figure of 23% is higher than most studies but compares favourably with data from two other ethnic populations, for which a higher proportion of BRCA-associated early-onset breast cancers have been reported. Data show that the prevalence rates can be as high as 30% for Ashkenazi Jews (9) and around 25% for Icelanders (10, 11). This high percentage is a result of the presence of founder mutations in these ethnic populations. More specifically, three ancestral mutations [BRCA1 c.68_69delAG (185delAG), c.5266insC (5382insC) and BRCA2 c.5946delT (6174delT)] appear in about 2% of Ashkenazi Jews (23), while about 0.5% of Icelanders carry the c.771delTCAAA (999del5) mutation in the BRCA2 gene (10, 11). The high prevalence rates of BRCA1 and BRCA2 mutations in our cohort of patients may also be explained by the presence of Cypriot founder mutation c.8755delG (8984delG) in the BRCA2 gene (17). Although the effect of this founder mutation is not as striking as the case of the Icelanders or Ashkenazi Jews, our results show that it makes a substantial contribution to the incidence of early-onset breast cancer in the Cypriot population. In addition, it is likely that the contribution of these genes to early-onset breast cancer might be even higher given that certain unclassified variants might be also causative.

In our group of patients, the highest proportion of mutations was reported in the BRCA2 gene, 15% (four of 26 positive) *vs* 7.7% (two of 26 positive), in the BRCA1 gene. These results indicate that BRCA2 mutations make a greater contribution to the breast cancer phenotype, in young Cypriot women, compared with BRCA1 mutations. This is in agreement with our previous data on familial breast/ovarian cancer studies in Cypriot families (17, 24). It appears that in most other populations studied, mutations in the two genes make approximately equal contributions to early-onset breast cancer (12, 13, 14, 16) with the exception of Iceland where BRCA2 accounts for most of the early-onset breast cancer cases, and the Ashkenazi Jews where BRCA1 accounts for the majority of cases.

In the present study, BRCA1/2 pathogenic mutations were detected in four of 15 patients with a family history, as well as in three of 11 patients without a family history. Our findings support a strong correlation between the early-onset breast cancer phenotype and BRCA1/2 gene analysis, since the prevalence of BRCA1/2 mutations in young Cypriot patients is relatively high. Based on these results, we recommend that BRCA1/2 screening should be offered to patients with a diagnosis of early-onset breast cancer irrespective of their family history.

Acknowledgement

We thank the participating patients for their willingness to co-operate in the study. The contract grant sponsor is Cyprus Research Promotion Foundation (PENEK 0603/12).

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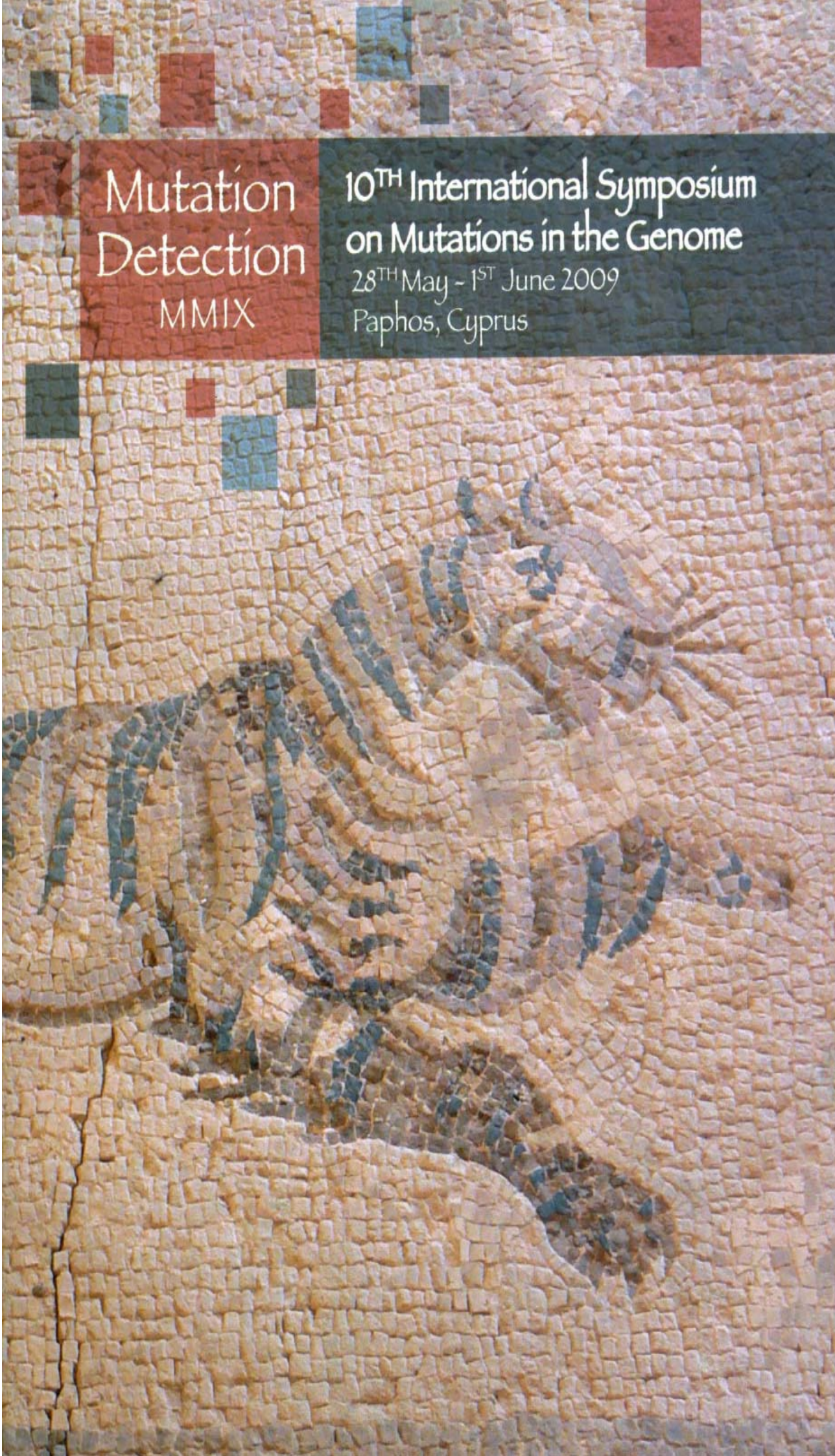
B1. **M. Loizidou**, M. Cariolou, E. Bashiardes, A. Hadjisavvas, K. Kyriacou, "MALDI-TOF assisted SNP genotyping for finding associations with breast cancer risk in the Cypriot population", 10th International Symposium on Mutation in the Genome, Paphos, Cyprus, 28 May -1 June 2009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28TH May - 1ST June 2009
Paphos, Cyprus

of CTX. Upon navigation of the literature, difficulties were encountered to estimate the relative frequency of each mutation, identify evidence of pathogenicity for some variants and evaluate potential genotype-phenotype correlations. In many instances nomenclature recommendations for human gene mutations were not followed or the reference sequence used was not indicated. A curated database of gene variations in CYP27A1 is under development, which will help both to the diagnosis and the study of the functional basis of the disease. Using the Leiden Open Variation Database (LOVD) as the data management framework provides an adequate and reliable approach for the storage of most commonly used informative descriptors, allowing the adaptation of a reference repository structure to the CTX data specific needs.

Lynch Syndrome in South American Families

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Lynch Syndrome (LS) is caused by germline mutations in the DNA mismatch repair (MMR) genes, mostly MLH1 and MSH2. As part of a larger study to characterize LS, 120 families from Argentina, Uruguay and Brazil were identified by applying the Amsterdam or Bethesda criteria. They were submitted to genetic counseling and peripheral blood collection. All exons and flanking intronic regions of MLH1 and MSH2 genes were analyzed by direct sequencing.

By the time, DNA testing revealed 4 intronic alterations (three in intron 15 and one in intron 12), 13 synonymous alterations (in exon 3, 6, 10, 16, 18 and 19), 26 missense mutations (exons 4,12 and 16) and fifteen clinically important mutations of MLH1 gene. For MSH2 gene, 2 pathogenic alterations were found in exon 13 and 35 missense alteration. All identified mutations were compared with previously reported mutations in the mutation database for MLH1, maintained by the Human Gene Mutation Database http://www.hgmd.org and International Society for Gastrointestinal Hereditary Tumors http://www.insight-group.org).

Mutations were identified in 55.9% of patients that belonged into Amsterdam families and 32.5% that fullfield Bethesda criteria had mutations in MLH1 or MSH2.

The following characteristics were associated to a higher frequency of mutations: probands with sincronic/metachronic tumors (75.0% vs 35.9%); probands with proximal tumors (64% vs 24.4%) and family history of endometrial cancer (77.8% vs 38.2%).

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MALDI-TOF assisted SNP genotyping for finding associations with breast cancer risk in the Cypriot population

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Breast cancer is the most common malignancy affecting women worldwide and it is the leading female cancer in Cyprus, with approximately 400 new cases diagnosed annually. Inability to correctly repair DNA damage is known to play a role in the development of breast cancer. Single nucleotide polymorphisms (SNPs) of DNA repair genes have been identified which modify the DNA repair capacity, which in turn may affect the risk of developing breast cancer. To assess whether alterations in DNA repair genes contribute to breast cancer, we genotyped 62 SNPs in 29 genes in 1,109 Cypriot women with breast cancer and 1,177 age-matched healthy controls. SNPs were genotyped using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry technology. This methodology is one of the cheapest and most error free technologies for high throughput SNP typing. It uses samples arrayed in 384 well plates and allows custom genotyping of SNPs within candidate genes or genomic intervals. For our study, two multiplex assays, a 34-plex and a 28-plex were designed using the Sequenom MassARRAY Assay Design software. SNPs were genotyped using Sequenom iPLEX chemistry on a MALDI-TOF Compact Mass Spectrometer (Sequenom Inc., San Diego, CA, USA).Five SNPs were associated with breast cancer. SNPs rs13312840 and rs769416 in the NBS1 gene were associated with a decrease in breast cancer risk (OR TT v TC/CC = 0.58; 95% CI, 0.37 to 0.92; p = 0.019 and OR GG vs GT/TT = 0.23, 95% CI 0.06–0.85, p = 0.017 respectively). The variant allele of MRE11A rs556477 was also associated with a reduced risk of developing the disease (OR AA vs AG/GG = 0.76; 95% CI, 0.64 to 0.91; p = 0.0022). MUS81 rs545500 and PBOV1 rs6927706 SNPs were associated with an increased risk of developing breast cancer (OR GG vs GC/CC = 1.21, 95% CI, 1.02 to

1.45; p = 0.031; OR AA vs AG/GG = 1.53, 95% CI, 1.07 to 2.18; p = 0.019, respectively). Finally, haplotype-based tests identified significant associations between specific haplotypes in MRE11A and NBS1 genes and breast cancer risk. This study provides support for the hypothesis that genetic variants in DNA repair genes influence breast cancer risk and provides further evidence for the polygenic model of breast cancer. However, large-scale genetic epidemiologic studies are warranted to further examine and corroborate the associations observed between polymorphisms and breast cancer in multi-ethnic groups.

Company Lecture Fluidigm

Integrated Fluidic Circuits for Next-Generation Genomics

Leila Smith, Director, Business Development

Fluidigm Corporation, Europe

Fluidigm's novel, ultrasensitive and highly versatile microfluidic platform is a next generation technology for driving forward the expansion of genome science. Integrated fluidic circuits (IFCs) enable PCR to be used across much larger sets of samples at low cost while low input volume requirements allow studies of rare samples and single cells. We describe IFC technology and show examples of use for targeted resequencing, copy number variation, and single cell gene expression.

- A barrier to full utilization of second generation sequencers is lack of cost effective and practical sample preparation methods. We developed the 48.48 AccessArray™ for target enrichment and sample multiplexing. This IFC performs 2,304 PCR reactions in parallel (48 assays x 48 samples) and quantitatively pools the PCR products for further processing. Specific target enrichment is achieved by PCR, while tagged PCR primers enable sample multiplexing. Blunt-end ligation and fragmentation steps can be eliminated when PCR primers are tailed with universal sequencing primers.

- Generation 2 sequencing tools and CNV-oriented microarray tools have accelerated discovery of human genome variation, but this creates an accute need for more accurate tools for genome wide association studies and validation of complex genotypes. We demonstrate both accurate discrimination of high copy number variants and cost effectiveness for case-cohorts studies where the number of loci is small by the number of samples is large.

- Metagenomics and cancer genomics create the need to understand genomic variation and gene expression at the single-cell level, yet these studies remain difficult and expensive to do. Low sample input requirements and in-chip reactions make microfluidics the ideal platform. We introduce single-cell gene expression and single-cell genotyping methods for inexpensive, reproducible studies of population of single cells.

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Session 7 Methods III & Variants I

New Developments in Melting Curve Analysis

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Separation of the two strands of DNA with heat (melting) is a fundamental property of DNA that is conveniently monitored with fluorescence. Conventional melting is performed after PCR on any real-time instrument to monitor product purity (dsDNA dyes) and sequence (hybridization probes). Recent advances include high-resolution instruments and saturating DNA dyes that distinguish many different species without fluorescent probes that require covalent labeling. For example, mutation scanning (identifying heterozygotes) by melting is closed-tube and has similar or superior sensitivity and specificity compared to methods that require physical separation, such as dHPLC. Single base variants and small insertions or deletions can be genotyped without probes. More complex regions can be typed with unlabeled hybridization probes or snapback primers. Mutation scanning and genotyping with one or more unlabeled probes can be performed at the same time in the same tube, often eliminating the need for resequencing in genetic analysis. Highly polymorphic regions (such as HLA) can be melted to establish sequence identity for transplantation or identity matching. High-resolution DNA melting is homogeneous, closed-tube, rapid (1-10 min), non-

B2. **M. Loizidou**, T. Michael, Y. Marcou, M. Daniel, E. Kakouri, P. Papadopoulos, S. Malas, K. Kyriacou, A. Hadjisavvas, "DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women", European Human Genetics Conference 2008, Barcelona, Spain, 31 May - 2 June 2008

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for BRCA2 gene. We found 21 different sequence variants in BRCA1 (2 novel) and 36 variants in BRCA2 gene (7 novel).
We analyzed the distribution and occurrence of sequence variants in BRCA1 and BRCA2 genes on a healthy population of women in Croatia in an attempt to distinguish non-tumorigenic from tumorigenic changes in genomic sequences of BRCA1 and BRCA2 genes. This may contribute to easier distinction of potentially dangerous from harmless changes in patients with family history of breast cancer.

P07.020
DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women

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Breast cancer is the most common malignancy which affects women worldwide. In an attempt to identify genetic variants which modify breast cancer risk we are contacting a case-control genetic epidemiology study using a cohort of 2286 Cypriot women (1109 breast cancer patients and 1177 age-matched healthy controls). In the present study we genotyped 11 single nucleotide polymorphisms (SNPs) in BRCA2, ERCC2, FANCA, MLH1, MRE11A, MSH2, OGG1, p53, RAD51 and RAD52 genes which are all involved in the DNA repair pathway. The prevalence of the 11 SNPs was compared between cases and controls. Genotype frequencies were compared across groups using the chi square test and the Mantel-Haenzel test for linear trend. The association between breast cancer and each SNP was examined using logistic regression with the SNP genotype tested under models of complete dominance and recessive inheritance. Three SNPs showed significant associations with breast cancer. For the most significant SNPs, the estimated ORs were 0.74 (95%CI 0.59-0.93) and 1.41 (95%CI 1.08-1.83) under a dominant inheritance model, with a combined Ptrend 0.0087 and 0.0076 respectively. These results suggest that a proportion of the SNPs under study are modifying breast cancer risk. Large numbers of samples will be needed to verify our results in other populations. We are currently expanding our analysis to include a greater number of SNPs and to evaluate potential underlying gene-gene or gene-environment interactions, in order to advance our knowledge on the effect of genetic polymorphisms on breast cancer susceptibility in Cypriot women.

P07.021
Variants in the vitamin D receptor gene and breast cancer

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Breast cancer is the most commonly occurring cancer among women, constituting 23% of all cancers. 5-10% of all breast cancers are caused by germ-line mutations in *BRCA1* and *BRCA2*. Multiple low-risk genes with variants common in the general population are thought to produce a mild susceptibility risk to sporadic breast cancer.
The vitamin D receptor (VDR) gene is a key mediator in the vitamin D pathway, and has been of long interest in breast cancer aetiology, since vitamin D exposure has been reported to reduce breast cancer risk. In the present study we have explored the implication of VDR in sporadic breast cancer, in the Spanish population since previous studies have been done in different populations than South European ones.
A total of 576 healthy controls from the Spanish population and 576 consecutive and non-related sporadic breast cancer cases, collected from different hospitals in Spain, were used in this study. Genotyping studies were carried out over four SNPs within the *VDR* gene, located on exons, in the putative promoter region or in untranslated regions. Genotyping was performed using TaqMan.
We detected associations for two of the selected SNPs: rs10735810 with OR=1.49 (95% C.I. 1.01-2.21; p=0.045), and rs731236 with OR=0.72 (95% C.I. 0.51-1.02; p=0.064). We also studied both haplotype and diplotype using PHASE v2.0, and detected associations with disease that were considered with the genotype results. Additionally, VDR proliferation parameters such as tumor differentiation grade and tumor aggressiveness will be discussed.

P07.022
Mutations in CARD15 and smoking confer susceptibility to Crohn´s disease in the Danish population

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INTRODUCTION: Three mutations in the CAspase Recruitment Domain gene (CARD15) predispose to Crohn´s disease (CD) in Caucasian populations.
The frequencies of the three most common CARD15 mutations differ greatly between ethnic groups. Heterogeneity even exists between the European countries.
AIMS & METHODS: The aim of this study was to investigate the mutation frequency in patients with inflammatory bowel disease and healthy controls in Denmark. Genotyping of the three CARD15 mutations were performed in 388 patients with Crohn´s disease, 565 patients with ulcerative colitis and 796 healthy controls using Real-Time PCR. A comparison of allele and genotype frequencies in the three groups was made. A possible additive effect of smoking on CARD15 mutations was also examined.
RESULTS: CARD15 mutations were significantly more common in CD patients compared with healthy controls (21% vs. 10%; P<0.001). A gene-dosage effect was observed ($OR_{adj.smoking}$ 22.2; P<0.001 for two CARD15 mutations vs. $OR_{adj.smoking}$ 1.8; P=0.01 for one CARD15 mutation). The 1007insC protein truncating mutation was the major contributing mutation. Among the Danish CD patients ileal involvement was more common among patients having CARD15 mutations ($OR_{adj.smoking}$ 3.6; P<0.001). Smoking was independently associated with CD (OR 1.8; P<0.001), and no additive effect of smoking on CARD15 genotypes was found.
CONCLUSION: In the Danish population CARD15 mutations were associated with Crohn´s disease. The CARD15 mutation frequency was in agreement with the lower frequency found in Northern European countries. Smoking was found to be a risk factor for developing CD.

P07.023
Cultural practice of consanguineous marriages in Morocco : prospective study in Rabat city

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The practice of consanguineous marriages has been widespread for hundreds of years, and is still very common, especially among various Middle Eastern, Asian and African populations. Their health consequences are primarily linked to the increased risk of congenital malformations and autosomal recessive disease.
The present study set out to determine the frequency of consanguineous marriages, identify and analyze the factors associated (bivariate analysis) with a randomly selected sample of 270 mothers postpartum in maternity of the Souissi Hospital in Rabat city (north-west of Morocco), between November 2004 and June 2005. All information was based on structured face-to-face interviews.
The Results showed that 21% of marriages were consanguineous (95% CI 12-28%), among which 70% were between first cousins. According to these results, several variables are associated with the choice of this type of marriage, namely women's education, their age at first marriage, place of residence, as well as type of childhood residence (rural or urban).

P07.024
Matrimonial choice in the Souss Massa Draa region in Morocco

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The study of geographical endogamy has relevance for indications on the genetic variability of the population, given that related parents have more chances to carry the same alleles, favouring homozygosity in their children.
The objective of this study was to evaluate geographical endogamy in the population of the region of Souss Massa Draa in south Morocco, as an indicator of the degree of genetic isolation of the population.
A prospective study was carried out between October 2005 and April 2007 on 190 randomly picked families of Souss Massa Draa. The choice of the mate was evaluated according to the geographical origin

B3. **M. Loizidou**, Y. Marcou, T. Michael, M. Daniel, P. Papadopoulos, S. Malas, K. Kyriacou, A. Hadjisavvas. "DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women", American Association for Cancer Research International Conference "Advances in Cancer Research: From the Laboratory to the Clinic", Dead Sea, Jordan, 16-19 March 2008.

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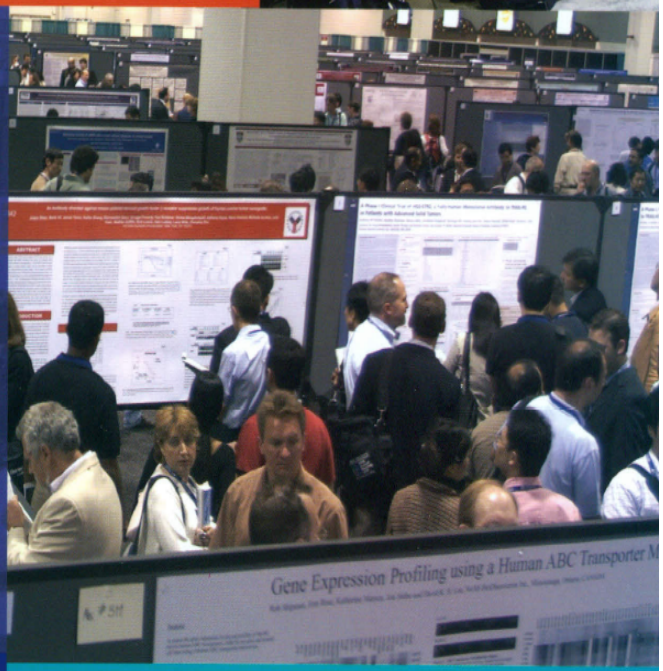


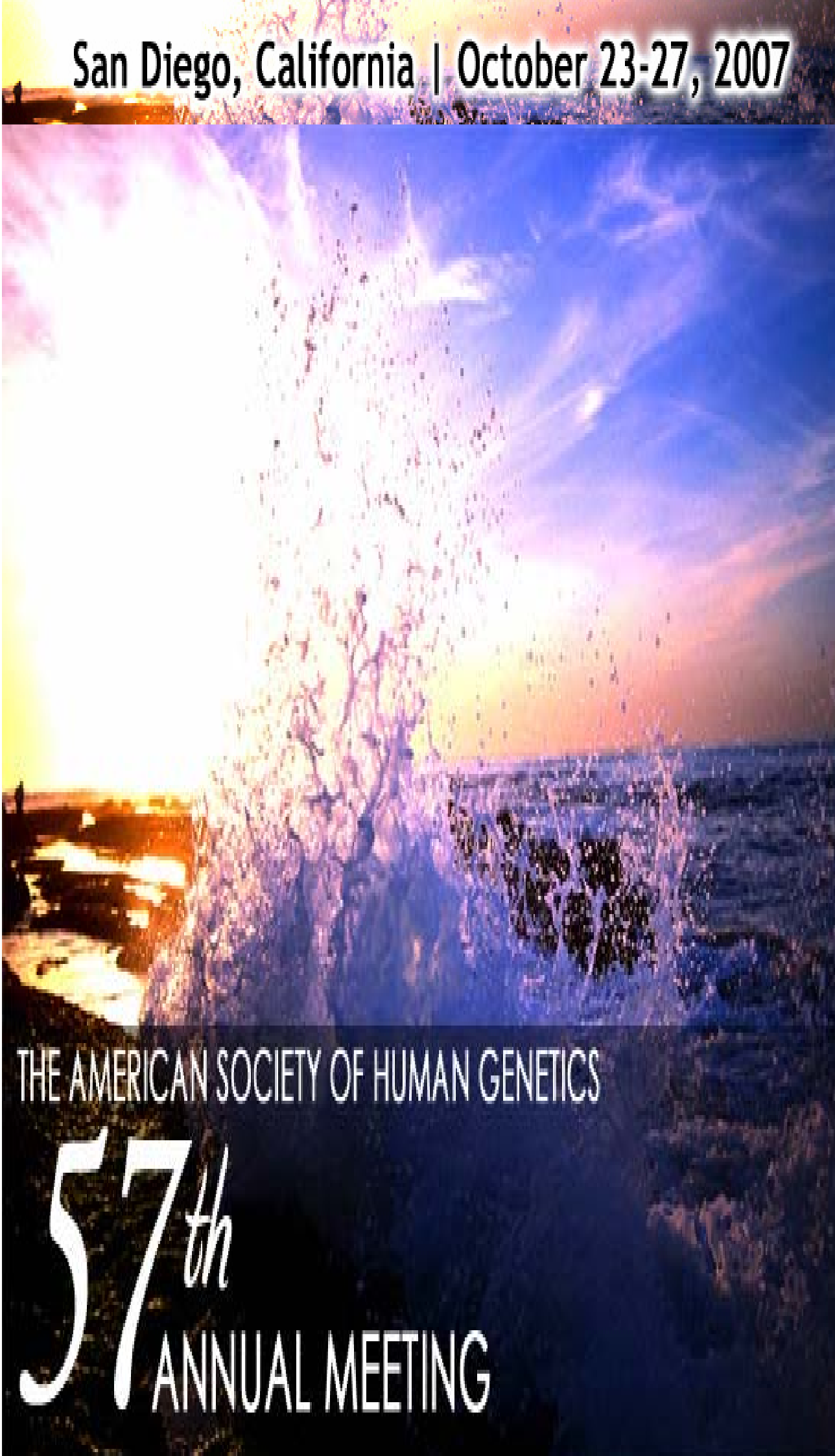
Program and Proceedings

DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women

Maria Loizidou[1], Yiola Marcou[2], Thalia Michael[1], Maria Daniel[2], Panayiotis Papadopoulos[2], Simon Malas[3], Kyriacos Kyriacou[1], Andreas Hadjisavvas[1]. [1]The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus; [2]Bank of Cyprus Oncology Centre, Nicosia, Cyprus; [3]Limassol General Hospital, Limassol, Cyprus.

Breast cancer is the commonest malignancy which affects women worldwide. Genetic factors are important in breast cancer but less than 20% are attributable to the inheritance of mutations in susceptibility genes such as BRCA1 and BRCA2. The polygenic model of breast cancer suggests that there are multiple low-penetrance alleles, each or a combination of which, have a small effect on breast cancer risk. The DNA repair pathway is essential for maintaining genomic stability of mammalian cells. Deficiencies in the DNA repair system are likely to cause chromosomal aberrations which in turn lead to cell malfunctioning, cell death and tumorigenesis. Several studies have demonstrated that polymorphisms in genes responsible for maintaining genomic integrity are modifiers of disease risk. Therefore, single nucleotide polymorphisms (SNPs) of genes involved in DNA repair are good candidates for low penetrance breast cancer susceptibility alleles. The aim of this study was to test the hypothesis that common SNPs in genes involved the DNA repair pathway may be risk factors for breast cancer. In an attempt to identify genetic variants which modify breast cancer risk we are contacting a case-control genetic epidemiology study using a cohort of 2286 Cypriot women (1109 breast cancer patients and 1177 age-matched healthy controls). In the present study we genotyped 11 single nucleotide polymorphisms (SNPs) in BRCA2, ERCC2, FANCA, MLH1, MRE11A, MSH2, OGG1, p53, RAD51 and RAD52 genes which are all involved in the DNA repair pathway. Genotyping was contacted using Taqman® probes (Applied Biosystems) following manufacturers instructions. The prevalence of the 11 SNPs was compared between cases and controls. Genotype frequencies were compared across groups using the chi square test and the Mantel-Haenzel test for linear trend. The association between breast cancer and each SNP was examined using logistic regression with the SNP genotype tested under models of complete dominance and recessive inheritance. Three SNPs showed significant associations with breast cancer. For the most significant SNPs, the estimated ORs were 0.74 (95%CI 0.59-0.93) and 1.41 (95%CI 1.08-1.83) under a dominant inheritance model, with a combined Ptrend 0.0087 and 0.0076 respectively. These results suggest that a proportion of the SNPs under study are modifying breast cancer risk. Large numbers of samples will be needed to verify our results in other populations. We are currently expanding our analysis to include a greater number of SNPs and to evaluate potential underlying gene-gene or gene-environment interactions, in order to advance our knowledge on the effect of genetic polymorphisms on breast cancer susceptibility in Cypriot women. This information can then be used to assist clinicians and their patients in more accurately assessing their breast cancer risks and in designing effective preventative strategies.

B6. A. Hadjisavvas, **M. Loizidou**, S. Malas, Y. Marcou, K. Kyriacou, "DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women", American Society of Human Genetics Annual Meeting, San Diego, California, USA, 23-27 October 2007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DNA-repair genetic polymorphisms and breast cancer risk among Cypriot women. *A. Hadjisavvas[1], M. Loizidou[1], S. Malas[3], Y. Marcou[2], K. Kyriacou[1].* 1) Department of EM/ Molecular Pathology, The Cyprus Institute of Neurology & Genetics, Nicosia, Cyprus; 2) Bank of Cyprus Oncology Centre, Nicosia, Cyprus; 3) Department of Oncology, Limassol General Hospital, Limassol, Cyprus.

Genetic factors are important in breast cancer but less than 20% are attributable to the inheritance of mutations in genes such as BRCA1 and BRCA2. The polygenic model of breast cancer suggests that there are multiple low-penetrance alleles which have a small effect on breast cancer risk. In an attempt to identify genetic variants which modify breast cancer risk we are contacting a case-control genetic epidemiology study using a cohort of 2286 Cypriot women (1109 patients and 1177 healthy controls). In the present study we genotyped 6 single nucleotide polymorphisms (SNPs) in genes which are involved in the DNA repair pathway: BRCA2 N991D, OGG1 S326C, RAD51 135G/C and 172G/C and p53 P72R. The prevalence of the 6 SNPs was compared between cases and controls. Odds ratios were generated from 2x2 tables, and statistical significance was assessed using the Pearson Chi-Square test.BRCA2 N991D was found at a significantly higher frequency in the population-based series of breast cancer patients (142/1086, 12.9%, odds ratio [OR] =1.42, 95% confidence interval [CI]= 1.09-1.85, p=0.01) than among population controls (112/1177, 9.5%). Furthermore, a marginally significant association between the p53 P72R variant and breast cancer was observed ([OR] (PP vs. PR+RR) =1.18, 95% CI (1.0-1.39), p=0.05). In addition, our results show that the effect of RAD51 135 C allele may be protective indicating that women who harbour this allele have a reduced risk of breast cancer compared with women who carry the G allele. These results suggest that a proportion of the SNPs under study are modifying breast cancer risk, but the effects of individual SNPs are likely to be small. Large numbers of samples will be needed to verify our results in other populations. We are currently expanding our analysis to include a greater number of SNPs and to evaluate potential underlying gene-gene or gene-environment interactions, in order to advance our knowledge on the effect of genetic polymorphisms on breast cancer susceptibility.

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Identification of men with a genetic predisposition to prostate cncer: targeted screening in BRCA1 and BRCA2 mutation carriers and controls. The 'IMPACT' study: pilot data. *A. Mitra[1], E. Bancroft[2], R. Eeles[1,2].* 1) Cancer Genetics, Institute of Cancer Research, London, UK; 2) Cancer Genetics, The Royal Marsden Hospital NHS Foundation Trust, London, UK.

Introduction The relative risk of prostate cancer (PC) in BRCA1 and BRCA2 carriers under the age of 65 years may be as high as 1.85 and between 7.33 and 23 fold respectively. IMPACT, the largest international prospective screening study of men with a known genetic predisposition to PC, aims to assess the role of targeted PSA screening and to determine the incidence and pathology of PC in this group. Methods 500 BRCA1 carriers and 350 BRCA2 carriers aged 40-69 will be recruited over 5 years. 850 controls will be recruited from men who are predictive test negative for a known familial mutation. Annual serum PSA, free:total PSA, testosterone and sex hormone binding globulin is taken. Prostate biopsy is offered if PSA is above 3ng/ml. The pilot study is recruiting in 8 UK cancer genetics centres and 2 international centres. Results 70 men (27 BRCA2, 19 BRCA1 and 24 controls) have been recruited to the study so far. Uptake rates have varied between centres but range from 76% to 94%. 4 men have had a PSA above 3ng/ml. A BRCA1 carrier and a control group man have been diagnosed with PC (Gleason score 3+4, stage T2b, PSA 3.8ng/ml and Gleason score 3+3, stage T2b, PSA 4.3ng/ml respectively). A 69 year old BRCA2 mutation carrier, PSA 6.7, has been found to have benign prostatic hypertrophy (BPH). A 69 year old control with a PSA of 7.2ng/ml had BPH only. Conclusions One of the limiting factors of the ERSPC and PLCO studies is the low recruitment rate in the target populations. In European countries that randomise men into a screening and control group, uptake rates are 25-46%. In those countries that randomise only to a screening arm the recruitment rate is 64%. Surveys suggest that as few as 3% of eligible men participate in the PLCO study. It appears that men who are at an increased genetic risk of developing PC are more likely to enter a PC screening study. This has implications for future targeted screening as new increased risk genotypes are identified by genome wide association studies.

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Replication of a Genome-Wide Mapping Case-Control Study in Esophageal Cancer. *D. Ng[1], N. Hu[1], Y. Hu[2], C. Giffen[3], Z.Z. Tang[4], X.Y. Han[4], H.H. Yang[2], M.P. Lee[2], A.M. Goldstein[1], P.R. Taylor[1].* 1) Genetic Epidemiology Branch, DCEG/NCI/NIH/DHHS, Bethesda, MD, USA; 2) Laboratory of Population Genetics, CCR/NCI/NIH/DHHS, Bethesda, MD, USA; 3) Information Management Systems, Silver Spring, MD, USA; 4) Shanxi Cancer Hospital, Taiyuan, Shanxi, PRC.

Background: Previously, we applied the Affymetrix mapping 10K SNP array in a pilot case-control study to determine differences in genotypes between esophageal squamous cell carcinoma (ESCC) cases and controls from a high-risk area in China and identified 38 SNPs in or near one of 33 genes. The present study attempted to replicate the results of these 38 gene-related SNPs in a new sample of cases and controls. Methods: A subset of 300 ESCC cases and 300 matched controls from a larger case-control study conducted in Shanxi Province, China was selected for the present study. A series of multiplex oligonucleotide ligation assays to genotype these 38 target SNPs were developed and applied to germline DNA from study subjects. Assays were validated by direct sequencing of eight SNPs in 12 pairs of cases and controls, and Hardy-Weinberg equilibrium was examined in control samples. General linear models were used to derive odds ratios (ORs) for dominant, recessive, and additive modes of transmission adjusted for baseline risk factors and one or more SNPs. Factor analysis was used to predict individual risk of ESCC. Results: Among 36 evaluable SNPs, four were significant in one or more analyses, including SNPs in EPHB1, PIK3C3, SLC9A9, and PGLYRP2. Risks were significantly increased for subjects with the T/T genotype in SNPs in EPHB1, PIK3C3, and SLC9A9, and for subjects with the A/A genotype in the PGLYRP2 SNP. The best factor analysis models accurately classified case/control status in approximately half of the subjects. Conclusions: Four of 38 previously identified gene-related SNPs remained significant in this replication study. While EPHB1, a receptor protein tyrosine kinase previously associated with colorectal cancer, merits particular consideration as a candidate tumor suppressor gene for ESCC, further exploration of all four genes in ESCC is recommended.

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Increased risk of stomach and nervous system cancers in Finnish prostate cancer families. *S. Pakkanen[1], M. Matikainen[2], E. Pukkala[3], P. Koivisto[1], T. Tammela[2], J. Schleutker[1].* 1) Laboratory of Cancer Genetics, Institution of Medical Technology, University of Tampere and Tampere University Hospital, Finland; 2) Department of Urology, Tampere University Hospital and Medical School, University of Tampere, Finland; 3) Finnish Cancer Registry, Helsinki, Finland.

Clinical features of families with prostate cancer (PCa) and other malignancies associated with this disease are not well known. A family with PCa is characterized as two or more PCa cases among first degree relatives. The aim of this study was to assess weather primary tumors other than prostate carcinoma aggregate in Finnish families with PCa or weather this disease can be considered site specific. Based on the national population based Finnish Cancer Registry (FCR), we calculated standardized incidence ratios (SIR) for 5546 members of 202 Finnish families with PCa with confirmed genealogy, either using the first diagnosed PCa among brothers as a single index or multiple indexes. Information of family members were confirmed from population registry and cancer data from hospital records and Finnish Cancer Registry respectively. The total number of cancers (all sites) among males was 552 (SIR 1.79) in single index group, 234 (SIR 0.94) in multiple index group and among females 205 (SIR 0.98). The number of PCa cases was 373 (SIR 6.73) in single index group and 71 (SIR 1.21) in multiple index group. The sisters of the index person had more stomach cancer than expected (SIR 2.12, 95% confidence interval 1.02-3.90) the mothers of the indexes had increased number of central nervous cancers in the age group of 60-69 years (SIR 19.4, 2.35-70.08) when compared general population. Spouses had no increased risk to any cancer, suggesting that special environmental risk factors can be excluded. In most of the families with excess numbers of prostate cancer the disease appears to be site specific. However in a subgroup of families, a suggestive tendency towards gastric and central nervous cancers was detected. Further analysis is warranted to carry out multivariate analysis based on selected clinical and family characteristics, possibly enabling separation of families with sporadic cases to a different cohort.

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Association between invasive ovarian cancer and alleles involved with breast cancer and prostate cancer susceptibility. *H. Song[1], S. Ramus[2], S.K. Kjaer[3], R.A. DiCioccio[4], L. Quaye[2], E. Hogdall[3], A.S. Whittemore[5], D.E. Easton[6], C.L. Pearce[7], G. Chenevix-Trench[8], S.A. Gayther[2], P. Pharoah[1].* 1) Department of Oncology, University of Cambridge, UK; 2) Translational Research Laboratories, University College London, London, UK; 3) Danish Cancer Society, Copenhagen, Denmark; 4) Roswell Park Cancer Institute, Buffalo, NY, USA; 5) Stanford University School of Medicine, Stanford, USA; 6) Genetic Epidemiology Unit, University of Cambridge, UK; 7) University of Southern California, Keck School of Medicine, Los Angeles, CA, USA; 8) The Queensland Institute of Medical Research, Post Office Royal Brisbane Hospital, Australia.

Background: Several alleles have recently been identified by genome-wide association study in hormone related cancers (breast and prostate cancer). The aim of this study was to test these alleles for association with invasive ovarian cancer.

Methods: Eleven breast cancer associated SNPs and 2 prostate associated SNPs were genotyped in approximately 2400 invasive ovarian cancer cases and 4100 controls from 6 studies (from Australia, UK, Denmark and USA). Genotype frequencies in cases and controls were compared using a likelihood ratio test in a logistic regression model stratified by studies.

Results: Three of 13 SNPs showed a weak association with ovarian cancer: carriers of the minor allele of rs2107425 (*H19*) were at reduced risk (per-allele odds Ratio (OR)=0.92[0.85-0.99], P-trend=0.03); and the minor allele of rs7313833 (*PTHLH*) was associated with increased risk (per-allele OR=1.09[1.01-1.18], P-trend= 0.02). In analyses restricted to serous ovarian cancer, carriers of the minor allele of rs4954956 (*NXPH2*) were associated with increased risks (per allele OR=1.12[1.01-1.24], P-trend=0.03).

Conclusions: The 5 most significant SNPs from breast cancer study and 2 SNPs from prostate cancer study were not associated with ovarian cancer. However, 3 of the remaining 6 showed association with ovarian cancer which warrant confirmation in independent studies.

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Investigation of mismatch repair protein expression in ovarian tumors. *J. Wey[1], D. Boulware[1], N. Valkov[2], S. Livingston[2], S. Nicosia[2], J-H. Lee[1], R. Sutphen[1], J. Schildkraut[3], S. Narod[4], T. Sellers[1], T. Pal[1].* 1) Moffitt Cancer Center, Tampa, FL; 2) Univ of S FL, Tampa, FL; 3) Duke, NC; 4) Univ of Toronto, Canada.

Background: The frequency of mismatch repair (MMR) deficiency in epithelial ovarian cancer (EOC) has ranged from 2-17%. Limited data exist regarding representative sampling from paraffin-embedded EOC tissue blocks utilized for construction of tissue microarrays (TMA) in preparation for immunohistochemistry(IHC). Methods: EOC tumor blocks from 59 cases were investigated by IHC for expression of hMLH1, hMSH2, and hMSH6. TMAs were created using three replicate 1 mm cores sampled from the center of a donor tissue block. Loss of expression of at least one protein was observed in an unexpectedly high number of cases, prompting creation of full sections of the donor blocks, which revealed lack of expression in the central portion, but positive expression in the periphery. Follow-up analyses for cases initially lacking expression were performed by obtaining cores from the periphery of up to 5 additional donor tissue blocks (triplicate cores per block). A linear mixed model for each protein was used to investigate differences between results from the initial donor block and follow-up blocks. Results: Loss of expression of at least one protein was revealed for 17 of the 59 (29%) cases. Follow-up analyses of the 17 cases that initially showed loss of protein expression revealed loss of expression in only 6 cases (10%). For each protein, statistically significant differences ($p<0.05$) were detected between the initial donor block and the majority of the follow-up blocks. Conclusions: When performing IHC analyses for loss of MMR protein expression in ovarian carcinomas, it is important to preferentially sample from the periphery of tumor blocks where exposure to tissue fixatives is optimal. This may reduce the likelihood of tissue fixation as the cause of the lack of protein expression.

Authors present at boards in Exhibit Hall E: Wednesday, 4:30 PM–6:30 PM (Session I: W posters); Thursday, 4:30 PM–6:30 PM (Session II: T posters); Friday, 10:30 AM–12:30 PM (Session III: F posters)

B7. A. Hadjisavvas, **M. Loizidou**, T. Michael, Y. Marcou, E. Kakouri, S. Malas, K. Kyriacou, "Searching for molecular targets for breast cancer in genes involved in the DNA-repair pathway among Cypriot women", Molecular targets for cancer prevention diagnosis and treatment, Limassol, Cyprus, 7-10 October 2007

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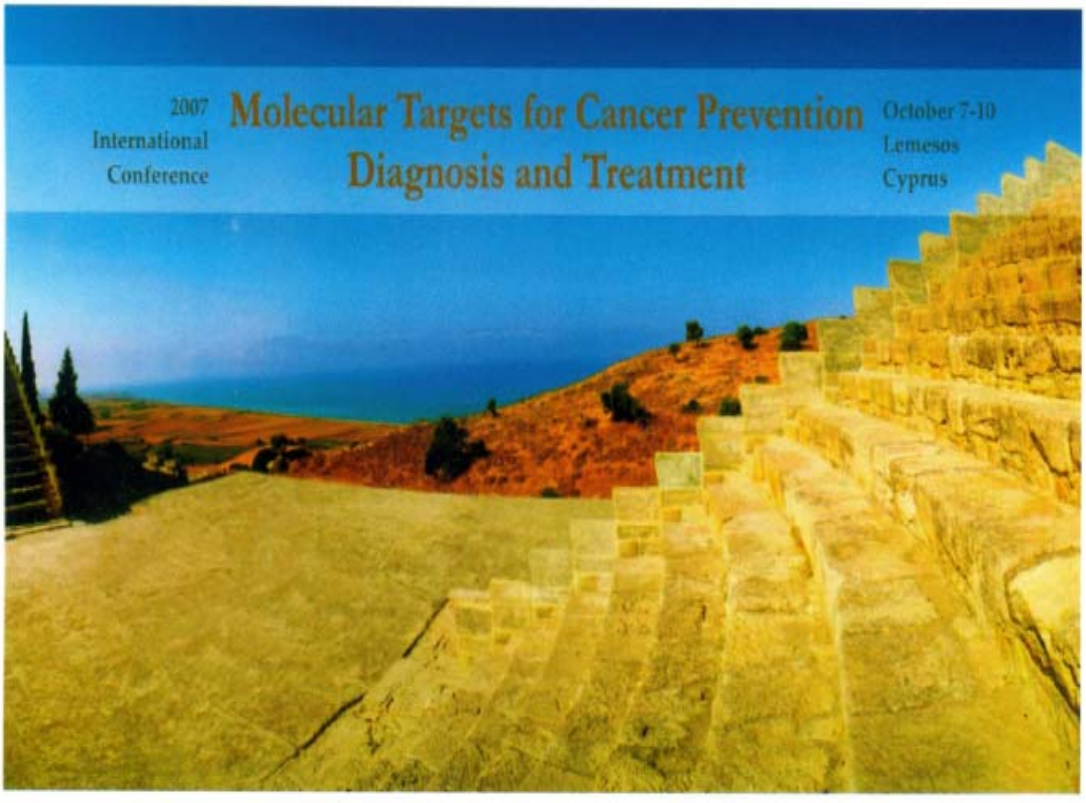

PROGRAM AND PROCEEDINGS

P5. Searching for molecular targets for breast cancer in genes involved in the DNA-repair pathway among Cypriot women.

A.Hadjisavvas[1], M. Loizidou[1], T. Michael[1], Y. Marcou[2], E. Kakouri[2], S. Malas[3], K.Kyriacou[1]

1. *The Cyprus Institute of Neurology and Genetics*
2. *The Bank of Cyprus Oncology Center, Nicosia, Cyprus*
3. *Department of Oncology, Limassol General Hospital, Cyprus*

Background

Breast cancer is the commonest malignancy which affects women worldwide. Genetic factors are important in breast cancer but less than 20% are attributable to the inheritance of mutations in susceptibility genes such as BRCA1 and BRCA2. The polygenic model of breast cancer suggests that there are multiple low-penetrance alleles, each or in combination, together with environmental interactions have a small effect on breast cancer risk. In the future, a combination of the presence of low-risk variants together with other breast cancer risk factors may have the power to predict an individual's risk of breast cancer and may become clinically important. In an attempt to identify genetic variants which modify breast cancer risk we are contacting a case-control genetic epidemiology study using a cohort of 2286 Cypriot women (1109 breast cancer patients and 1177 age-matched healthy controls)

Methods

In the present study we genotyped 11 single nucleotide polymorphisms (SNPs) in genes which are involved in the DNA repair pathway: BRCA2 N991D (rs1799944), OGG1 S326C (rs1052133), RAD51 135G/C (rs1801320) and 172G/C (rs1801321), p53 P72R (rs1042522), XRCC1 Arg194Trp (rs1799782), Arg280His (rs25489) and Arg399Gln (rs25487), XRCC2 Arg188His (rs3218536) and XRCC3 Thr241Met (rs861539). Genotyping was contacted using either Taqman® probes (Applied Biosystems) following the manufacturer's instructions or by PCR followed by restriction digestions. The prevalence of the 11 SNPs was compared between cases and controls. Odds ratios were generated from 2x2 tables, and statistical significance was assessed using the Pearson Chi-Square test.

Results

BRCA2 N991D was found at a significantly higher frequency in the population-based series of breast cancer patients (142/1086, 12.9%, odds ratio [OR] =1.42, 95% confidence interval [CI]= 1.09–1.85, p=0.01) than among population controls (112/1177, 9.5%). Homozygotes at XRCC1 R280H were found to be associated with an increased risk of breast cancer ([OR] (HH vs. RR+RH) =4.77, 95% CI (1.03–22.13), p=0.03). Furthermore, a marginally significant association between the p53 P72R variant and breast cancer was observed ([OR] (PP vs. PR+RR) =1.18, 95% CI (1.0–1.39), p=0.05). In addition, our results show that the effect of RAD51 135 C allele may be protective indicating that women who harbour this allele have a reduced risk of breast cancer compared with women who carry the G allele.

Conclusions

These results suggest that a proportion of the SNPs under study are modifying breast cancer risk, but the effects of individual SNPs are likely to be small. Large numbers of samples will be needed to verify our results in other populations. We are currently expanding our analysis to include a greater number of SNPs and to evaluate potential underlying gene-gene or gene-environment interactions, in order to advance our knowledge on the effect of genetic polymorphisms on breast cancer susceptibility in Cypriot women.

B9. A. Hadjisavvas, **M. Loizidou**, M. Daniel, E. Kakouri, S. Malas, Y. Markou, K. Kyriacou. "A Preliminary Study on X-ray Repair Cross Complementing (XRCC) Gene Polymorphisms as Possible Biomarkers of Breast Cancer Susceptibility among Cypriot Women", American Society of Human Genetics Annual Meeting, New Orleans, Louisiana, USA, 9-13 October 2006.

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Knockdown of estrogen receptor alpha gene expression by siRNA in human breast cancer cell line. *M. El-Zawahri[1], Y. Luqmany[2], A. Al-Azmi[1].* 1) Dept Biological Sci, Fac Sci, Kuwait Univ,; 2) Det Pharmaceutical Chemistry, Fac Farmacy, Kuwait Univ, Kuwait.

The role of estrogen receptor alpha (ERα) in breast cancer has been highlighted by numerous studies. Consequently, inhibition of ERα is one of the major strategies for prevention and treatment of breast cancer. However, failure to overcome development of endocrine resistance, arising despite continued expression of tumor ERα, limits this approach. The aim of this study is to establish a breast cancer cell line containing a permanent source of small interference RNA (siRNA) which specifically inhibits the production of ERα protein, to produce a model system to investigate loss-of-function of ERα. Three siRNA constructs (PI-III) targeting different sequences of human ERα, and a scrambled sequence were cloned into the pRNA-U6.1/Neo GenScript vector. MCF7 breast cancer cells were transformed with 2 or 4ug of each plasmid (6 and 24h exposure), using lipofectamin or Xtreme reagent conjugates. Transformants were rescued by growth in G418 selection medium. ERα mRNA levels were determined by Real Time RT-PCR of extracted RNA, and ERα protein by Western blotting; normalization was achieved by simultaneous analysis of β-actin. Presence of plasmid DNA in transformants was verified with primers targeting various regions of the vector. Stably transfected cells maintaining antibiotic resistance over several passages were established by continuous culture. Linearized anti-ERα-siRNA construct PII most effectively down-regulated ERα mRNA (as evidenced by Real-Time RT-PCR analysis) and protein in these cells as compared to G418 resistant transformants containing scrambled siRNA; complete knockdown was not observed. Whereas 4μg produced more transformants, 24h exposure did not increase transformation efficiency. In conclusion, Our three anti-ERα-siRNA vector constructs silenced its target mRNA specifically and we have successfully established a long term culture of MCF7 breast cancer cells that exhibit decreased expression of ERα. This is hoped to provide a model system in which to study aspects of endocrine resistance. (Supported by Kuwait University Grant YS 01/04).

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A Preliminary Study on X-ray Repair Cross Complementing (XRCC) Gene Polymorphisms as Possible Biomarkers of Breast Cancer Susceptibility among Cypriot Women. *A. Hadjisavvas[1], M. Loizidou[1], M. Daniel[2], E. Kakouri[2], S. Malas[3], Y. Marcou[2], K. Kyriacou[1].* 1) EM and Molecular pathology, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus; 2) Bank of Cyprus Oncology Centre, Nicosia, Cyprus; 3) Department of Oncology, Limassol General Hospital, Limassol, Cyprus.

Population-based molecular epidemiology studies on genetic variations of genes have shown associations between specific genetic polymorphisms and breast cancer susceptibility. A number of studies have demonstrated that common variants of genes involved in the DNA Double-Strand Break pathway can act as low penetrance breast cancer susceptibility alleles. This preliminary molecular epidemiology study which is carried out for the first time on a Cypriot cohort of patients/controls, aims to investigate the role of polymorphisms in DNA repair genes XRCC1, XRCC2 and XRCC3 in breast cancer. We analysed the XRCC1 Arg194Trp, Arg280His and Arg399Gln variants as well as the XRCC2 Arg188His and XRCC3 Thr241Met gene polymorphisms. To identify these polymorphisms PCR and restriction enzyme digest were performed on DNA obtained from 500 Cypriot breast cancer patients age between 40-70 and 600 age-matched normal controls. Extensive demographic, epidemiological and pathological data were also recorded. Using the Chi-square analysis, we identified a statistically significant (p= 0.05) difference between the case group and the control group for the XRCC1 Arg280His polymorphism. Evaluation of potential underlying gene-gene or gene-environment interactions, which might magnify the effect of the variants under study, will require even larger sample sizes. We are currently expanding our analysis to include a greater number of subjects to determine the importance of these initial findings and improve our knowledge on the effect of XRCC gene polymorphisms, on breast cancer susceptibility in Cypriot women.

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Analysis of the clinical relevance of intron variants in BRCA1 and BRCA2. *M.P.G. Vreeswijk[1], J.N. Kraan[1], H.v.d. Klift[1], C.J.van Asperen[1], G.R. Vink[1], C.J. Cornelisse[2], P. Devilee[1,2].* 1) Dept. Human and Clinical Genetics, Center for Human and Clinical Genetics, LUMC, Leiden, Netherlands; 2) Dept. Pathology, LUMC, Leiden, the Netherlands.

Germ-line mutations in BRCA1 and BRCA2 confer a high risk to breast and/or ovarian cancer. Whereas mutations causing frameshifts and premature stop codons are unambiguously defined as pathogenic, an increasing number of variants has been identified which cannot be readily distinguished as either disease-associated mutations or benign polymorphisms. These socalled unclassified variants include variants that are located in the intronic sequences of BRCA1 and BRCA2. The purpose of this study was to identify the effect of six of these variants on RNA splicing in order to differentiate between pathogenic or neutral variants.

For the IVS2-6 T>A variant in BRCA1, sequence analysis of cDNA from fibroblasts derived from a carrier of the variant showed the introduction of four bases from intron 2, leading to an out-of-frame fusion of BRCA1 exon 2 and 3.

A coding polymorphism in exon 16 was used to show monoallelic BRCA1 expression in a carrier of the IVS16+5 G>T variant. This was confirmed with the use of a primer specific for the wildtype transcript. Inhibition of nonsense mediated decay (NMD) revealed an additional fragment and sequence analysis showed the insertion of 65 nucleotides from intron 16.

RNA analysis from a carrier of an intronic variant in BRCA2 (IVS21+5 G>A) revealed the absence of transcript from the variant allele. Analysis after inhibition of NMD showed an additional transcript, containing an insertion of 46 nucleotides from intron 21.

Since these intronic variants (IVS2-6 T>A; IVS16+5 G>T in BRCA1 and IVS21+5 G>A in BRCA2) result in aberrant splicing of the mRNA transcript, they are considered to be clinically significant. Two other variants in BRCA1 (113 G>A; IVS4-15delTTTC) and one variant in BRCA2 (IVS2-7 T>A) did not show any effect on RNA splicing and can therefore be considered to be neutral alterations.

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SNP association study of esophageal squamous cell carcinoma in a high-risk population from China. *D. Ng[1], N. Hu[1], X.Y. Han[2], C. Giffen[3], Z.Z. Tang[2], A.M. Goldstein[1], M.P. Lee[4], P.R. Taylor[1].* 1) Genetic Epidemiology Branch, DCEG, NCI, NIH, DHHS, Bethesda, MD; 2) Shanxi Cancer Hospital, Taiyuan, Shanxi, PR China; 3) Information Management Services, Inc., Silver Spring, MD; 4) Laboratory of Population Genetics, CCR, NCI, NIH, DHHS, Bethesda, MD.

Esophageal squamous cell carcinoma (ESCC) incidence and mortality rates in north-central China are the highest in the world; rates in this region exceed the average Chinese rate by 10-fold and the US Caucasian rate by 100-fold. Geographic variation of ESCC rates in China suggests that environmental and/or lifestyle factors are major contributors to the etiology of esophageal cancer. In high-risk regions such as Shanxi Province, there is a strong tendency towards familial aggregation suggesting an etiologic role for genetic susceptibility and/or gene-environment interactions. To identify susceptibility genes that predispose to ESCC, we completed a pilot case-control whole genome SNP association study using the Affymetrix 10K SNP array in 50 ESCC patients and 50 matched controls from Shanxi Province. Using the generalized linear model (GLM) with adjustments for potential confounders and multiple comparisons, we identified 37 SNPs associated with disease assuming a recessive mode of inheritance, 48 SNPs assuming a dominant mode and 53 SNPs in a continuous mode. When the 37 SNPs identified from the GLM recessive mode were used in a principal component analysis, the first principal component correctly predicted 46 of 50 cases and 47 of 50 controls. To correct for 10,264 separate analyses, we used a Bonferroni-adjusted significance level of $P < 4.87187 \times 10\text{-}6$ to select combined SNPs identified from the GLMs of all three modes of transmission. Thirty-eight SNPs were identified that met the Bonferroni-adjusted significance level and were located in or near genes. To further validate these SNPs for genetic susceptibility/risk assessment, we performed a second study in which 300 new ESCC cases and their matched controls were genotyped using a multiplex oligonucleotide ligation assay to examine the 38 SNPs identified from the pilot case-control study.

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A genome-wide scan of non-synonymous SNPs in a phase III clinical trial identifies variants influencing outcome in chronic lymphocytic leukemia. *G.S. Sellick[1], R. Wade[2], M.F. Rudd[1], S. Richards[2], D. Catovsky[3], R.S. Houlston[1].* 1) Section of Cancer Genetics, Institute of Cancer Research, Sutton, Surrey, UK; 2) Clinical Trial Service Unit, University of Oxford, Oxford, UK; 3) Section of Haemato-Oncology, Institute of Cancer Research, Sutton, Surrey, UK.

B-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disease with a variable clinical course. Staging systems are useful for predicting survival and treatment requirements, however, even for patients with same-stage disease there is variability in clinical outcome. Germline sequence variation may influence disease prognosis. We undertook a genome-wide scan of non-synonymous SNPs (nsSNPs) in 425 CLL patients participating in a phase III trial (UK LRF CLL4) established to compare the efficacy of fludarabine, chlorambucil, and fludarabine and cyclophosphamide as a first-line treatment for patients with Binet stage B, C and A-progressive disease. The 990 nsSNPs in 870 genes were selected for their relevance to cancer biology and were strongly biased towards those likely to be functionally deleterious. Genetic data was linked to individual patient outcome and response to chemotherapy. The effect of genotype on progression free survival (PFS) and overall survival (OS) was assessed and the relationship between nsSNPs and PFS and OS was evaluated. Our study shows that germline variation influences CLL survival and identifies a number of variants that provide insight into the biological determinants of prognosis.

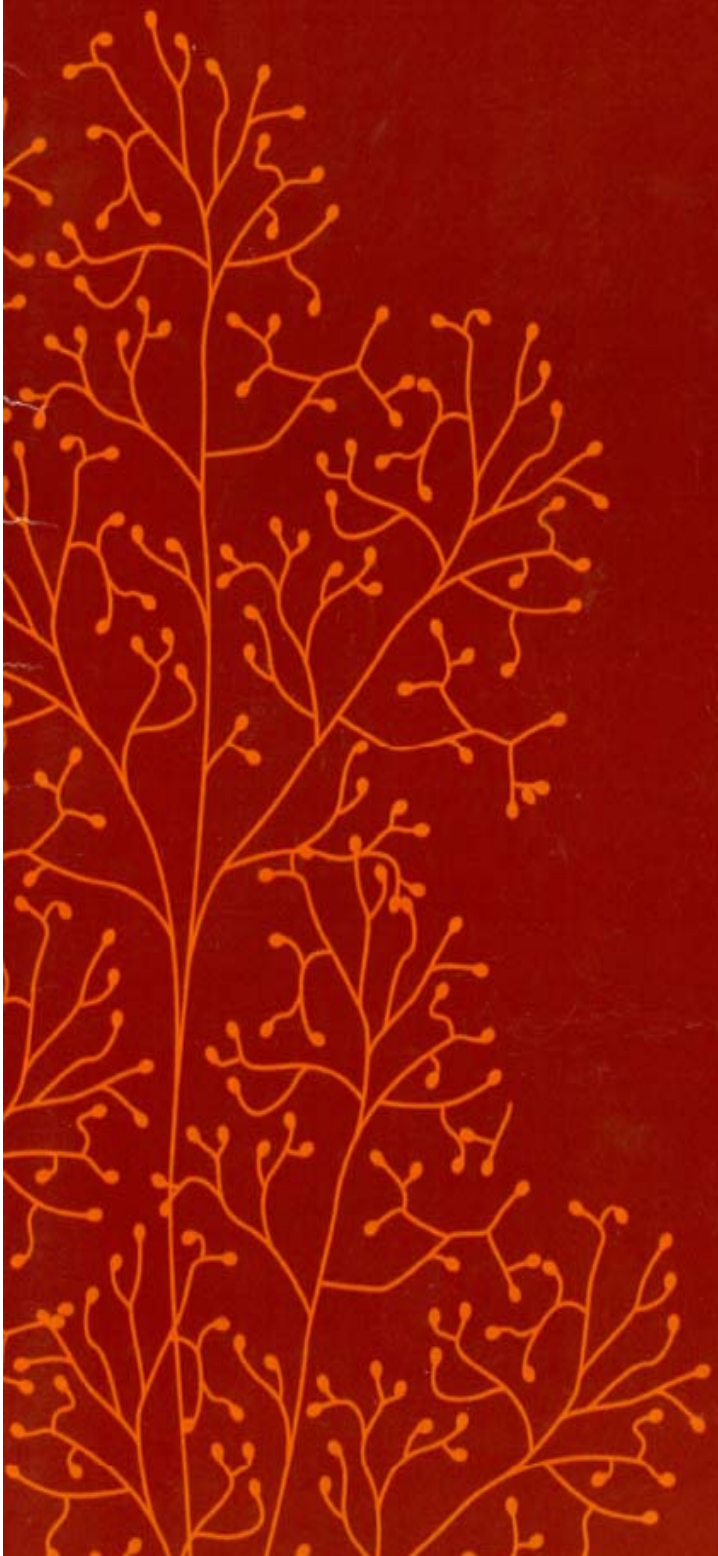
427/C

The Development of Genomic SELEX for the Identification of Direct Transcriptional Targets of Pax3, FKHR and the Oncogenic Fusion Protein Pax3-FKHR. *A. Sidhu, K.E. Johanson, R.J. Scioneaux, A.D. Hollenbach.* Department of Genetics, LSU Health Sciences Center , New Orleans, LA.

Alveolar rhabdomyosarcoma (ARMS, MIM 268220) is a soft tissue tumour, arising primarily in the trunk and extremities of adolescents and young adults. The four year overall survival rate is less than 30% for ARMS, with a lower incidence of survival in patients with metastases and bone marrow involvement. ARMS is an aggressive malignancy most commonly characterized by a t(2;13) (q35;q14) chromosomal translocation, which results in the fusion of two transcription factors important for myogenesis, Pax3 and FKHR. The resulting oncogenic fusion protein, Pax3-FKHR, is a potent transcriptional activator and functions as a dominant acting oncogene. Thus, screening for target genes of Pax3, Pax3-FKHR, and FKHR would be of great significance in determining the molecular basis of this disease and its clinical outcome. Therefore, the long term goal of this work is to identify the direct transcriptional targets of Pax3 and FKHR and to determine how gene expression differs in the presence of Pax3-FKHR. We will address this goal with the development of a Genomic SELEX technique, through a modification of the standard SELEX, in order to identify promoter elements directly bound and regulated by Pax3 and FKHR. We will then confirm, identify, and evaluate the promoter elements that are bound by Pax3 and FKHR. Lastly, we will determine how Pax3-FKHR alters the expression of the identified genes in the physiologically relevant mouse primary myoblasts. The identification of physiologically relevant genes whose expression is altered by the presence of Pax3-FKHR will allow us to elucidate the cellular pathways that contribute to ARMS tumor progression. The knowledge of these aberrantly expressed genes will provide us with critical information that will allow the development of novel therapies for the treatment of ARMS.

B10. **M. Loizidou**, Y. Markou, D. Papamichael , M. Televantos, G. Kalakoutis, K. Kyriacou, A. Hadjisavvas "Contribution of BRCA1 and BRCA2 mutations to the incidence of breast and ovarian cancer in young Cypriot women", EMBO Molecular Medicine Conference: Mammary Gland Development and Breast Cancer Progression, Dublin, Ireland, 6-8 June 2006

EMBO
Molecular Medicine Conference



6-8 June 2006
Dublin | Ireland

A25

Contribution of BRCA1 and BRCA2 Germ-Line Mutations to the Incidence of Breast and Ovarian Cancer in Young Cypriot Women.

Maria Loizidou[1], Yiola Marcou[2], Demetris Papamichael[2], Marios Televantos[3], Gabriel Kalakoutis[3], Kyriacos Kyriacou[1] and Andreas Hadjisavvas[1]. Department of Electron Microscope/Molecular Pathology[1], The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus;Bank of Cyprus Oncology Center[2], Nicosia, Cyprus;Makarios III Hospital[3], Nicosia, Cyprus.

Breast cancer is the commonest malignancy that affects women worldwide and around 5% to 10% of all breast and ovarian cancers are due to genetic predisposition. Women, who carry a pathogenic mutation in BRCA genes, manifest breast cancer at a younger age. We carried out mutational analysis of the BRCA1 and BRCA2 genes in 25 Cypriot women diagnosed with breast cancer before the age of 40 and 20 women diagnosed with ovarian cancer before the age of 60. Patients were selected solely because they were diagnosed with breast or ovarian cancer at a young age. A total of 5 pathogenic mutations were identified in 7 unrelated probands; two in BRCA1 and three in BRCA2. Two out of the five mutations are novel. The BRCA2 8984delG mutation was detected in three non-related breast cancer patients. This study has revealed that one in five Cypriot early onset breast cancer cases (20%) are associated with a germ-line mutation in either the BRCA1 or the BRCA2 gene, which is high in comparison to the expected 5-10%, recorded in other populations. This may be a result of the presence of the already identified Cypriot founder mutation in the BRCA2 gene. Regarding the ovarian cancer patients, 2 out of 20 (10%) carried a pathogenic BRCA mutation, as expected. These results confirm that in the Cypriot population the BRCA2, rather than the BRCA1 gene appears to play a more significant role in the breast cancer phenotype .Our results indicate that BRCA1/2 testing could be offered to all the early onset breast cancer patients irrespective of their family history.

C1. Association studies for discovering new breast cancer genes / the MASTOS study. University of Nicosia, Department of Life and Health Sciences, Nicosia, Cyprus, 12 December 2008.

UNIVERSITY OF NICOSIA
ΠΑΝΕΠΙΣΤΗΜΙΟ ΛΕΥΚΩΣΙΑΣ

The Department of Life and Health Sciences of the School of Sciences, will like to invite you to a talk on:

"Association studies for discovering new Breast Cancer Genes / the MASTOS study"

By Ms. Maria Loizidou
The Cyprus Institute of Neurology and Genetics

In room A101 (1st floor, Main Bld.) at 14:00 hours
On Friday, 12th of December, 2008

ABSTRACT: Breast cancer, the most common malignancy that affects women worldwide, has an estimated annual incidence of about one million cases.[1] The family history of breast cancer constitutes one of the most important and well-established risk factors with first degree relatives of patients having a two-fold elevated risk compared to the general population.[2] In the 1990's it was identified that inherited germline mutations in the two breast cancer susceptibility genes BRCA1 and BRCA2.[3,4] confer a strong lifetime risk of developing breast and ovarian cancer.[5] In addition to these genes, other high-risk breast cancer susceptibility genes such as PTEN and TP53 were discovered[6,7] while a number of genes including CHEK2, TGFβ1 and ATM comprise the "low to moderate-risk" breast cancer susceptibility group.[8,9,10] Together, it is estimated that 5-10% of all breast cancers are caused by mutations in the above genes.[11] However, although, to date, linkage analysis has failed to identify other major breast cancer genes it is believed that a substantial proportion, as high as 30%, of the total breast cancer incidence can be attributed to genetic factors.[12] It is therefore highly likely that breast cancer is polygenic and that susceptibility is caused by a number of gene loci which individually or in combination, together with environmental factors contribute to the development of breast cancer.[13]

Towards identifying genetic variants that modify breast cancer risk in the Cypriot population we are conducting a case-control genetic epidemiology study using 1109 breast cancer patients and 1177 age-matched healthy controls. One of our aims is to investigate the contribution of single nucleotide polymorphisms (SNPs) in DNA repair and related genes to breast cancer risk in the Cypriot population. The results of this study will be communicated in this presentation.

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3) Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 1994;266:66–71.
4) Wooster R, Bignell G, Lancaster J, et al. Identification of the breast cancer susceptibility gene BRCA2. Nature 1995;378:789–92.
5) Antoniou A, Pharoah PDP, Narod S, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet 2003;72:1117–30.
6) Malkin D, Li F, Strong L, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 1990;250:1233–8.
7) Liaw D, Marsh DJ, Li J, et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 1997;16:64–7.
8) The CHEK2 Breast Cancer Case-Control Consortium CHEK2*1100delC and susceptibility to breast cancer: a collaborative analysis involving 10,860 breast cancer cases and 9,065 controls from ten studies. Am J Hum Genet 2004;74:1175–82.
9) Dunning AM, Ellis PD, Mcbride S, et al. A transforming growth factorbeta1 signal peptide variant increases secretion in vitro and is associated with increased incidence of invasive breast cancer. Cancer Res 2003;63:2610–5.
10) Renwick A, Thompson D, Seal S, et al. ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. Nat Genet 2006;38:873–5.
11) Antoniou AC, Pharoah PDP, McMullan G, et al. A comprehensive model for familial breast cancer incorporating BRCA1, BRCA2 and other genes. Br J Cancer 2002;86:76–83.
12) Lichtenstein P, Holm NV, Verkasalo PK, et al. Environmental and heritable factors in the causation of cancer–analyses of cohorts of twins from Sweden, Denmark, and Finland. N Engl J Med 2000;343:78– 85.
13) Pharoah PDP, Antoniou A, Bobrow M, et al. Polygenic susceptibility to breast cancer and implications for prevention. Nat Genet 2002;31:33–6.

C2. DNA repair genetic polymorphisms and the risk of breast cancer in Cyprus. 16th Postgraduate Congress in Clinical Oncology, Crete, Greece, 12-15 November 2008.

12-15 Νοεμβρίου 2008 • Terra Maris - Χερσόνησος Ηράκλειο Κρήτης

16ο Μετεκπαιδευτικό Συνέδριο Κλινικής Ογκολογίας

ΠΡΟΓΡΑΜΜΑ

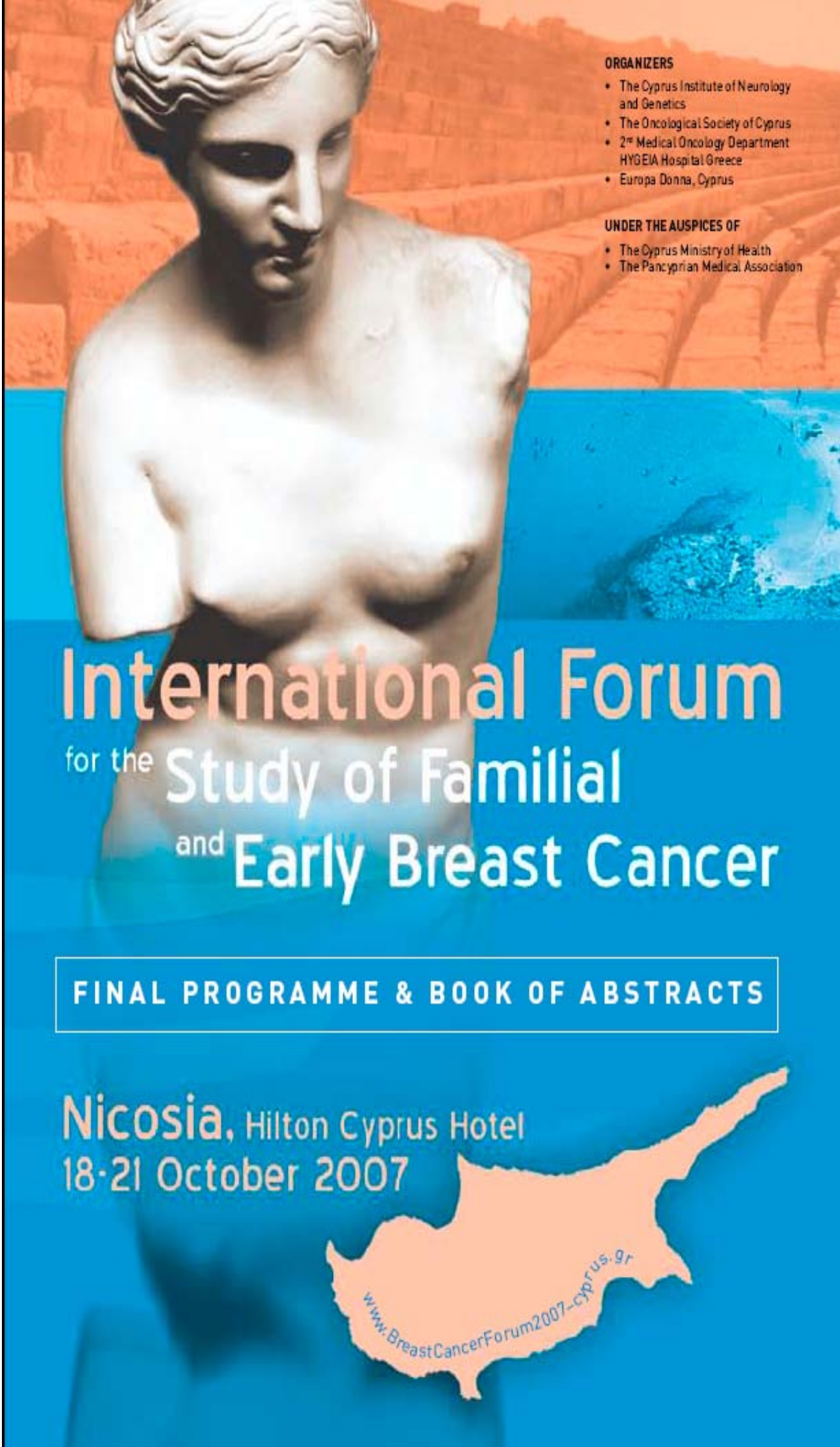
ΝΙΚΟΣ ΜΟΣΧΟΣ «Γι' αυτό...» |

"Τι είναι ο άνθρωπος πριν το ωραίο ξυπνήσει μέσα του την αυθόρμητη χαρά για τα πράγματα της ζωής, και πριν η γαλήνη της μορφής απαλύνει την άγρια ζωή."

"Friedrich Schiller"

18.00-20.00	**Ερευνητικό Τραπέζι: Ερευνητικές δραστηριότητες στην Ελλάδα** **Συντονισής: Δ. ΜΑΥΡΟΥΔΗΣ**
Χ. Παπαδάκη	Φαρμακογενωμική μελέτη στο ΜΜΚΚΠ
Γ. Νασιούλας	Ερευνητικά προγράμματα στον κληρονομούμενο καρκίνο
Μ. Λοϊζίδου	Πολυμορφισμοί σε γονίδια που εμπλέκονται στην επιδιόρθωση του DNA και κίνδυνος ανάπτυξης καρκίνου του μαστού στη Κύπρο.
Ειρ. Μπιζιώτα	Μελέτη κυτταρικής και μοριακής δράσης της vinorelbine και του ενεργού μεταβολίτη της 4-O-deacetyl-vinorelbine σε ενδοθηλιακά κύτταρα, σε ένα in vitro μοντέλο προσομοίωσης μετρονομικής θεραπείας
Π. Τουπλικιώτη	Notch και αγγειογένεση στον καρκίνο του μαστού
Λ. Κοντοβίνης	Βιολογικοί δείκτες στη θεραπεία με sunitinib του καρκίνου του νεφρού
Π. Βορκάς	Ανάπτυξη και εφαρμογές μεθόδων ανάλυσης μεταλλάξεων μέσω υψηλής διακριτικότητας καμπυλών τήξης DNA.
Σ. Μπαλκουρανίδου	Μελέτη δεικτών μεθυλίωσης DNA στον μη-μικροκυτταρικό καρκίνο του πνεύμονα NSCLC
Ε. Βασάλου	Έκφραση γονιδίων που εμπλέκονται στην επιδιόρθωση του DNA στον ΜΜΚΠ. Σύγκριση επιπέδων mRNA και πρωτεΐνης

C3. Association studies for discovering new breast cancer genes: do they exist? International forum for the study of Familial and Early Breast Cancer, Nicosia, Cyprus, 18-21 October 2007.



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- The Oncological Society of Cyprus
- 2nd Medical Oncology Department HYGEIA Hospital Greece
- Europa Donna, Cyprus

UNDER THE AUSPICES OF

- The Cyprus Ministry of Health
- The Pancyprian Medical Association

International Forum

for the Study of Familial and Early Breast Cancer

FINAL PROGRAMME & BOOK OF ABSTRACTS

Nicosia, Hilton Cyprus Hotel
18-21 October 2007

www.BreastCancerForum2007-cyprus.gr

SCIENTIFIC PROGRAMME

Thursday, October 18th, 2007

16:00 - 18:00 REGISTRATION

18:00 - 20:00 SESSION I

MOLECULAR BIOLOGY / GENETICS

Chairpersons: K. Kyriacou (CY) - G. Nasioulas (GR)

18:00 - 18:30 World wide Incidence of BRCA1 and BRCA2
G. Nasioulas (GR)

18:30 - 19:00 Spectrum of BRCA mutations in Cypriot families
A. Hadjisavvas (CY)

19:00 - 19:30 Spectrum of BRCA mutations in Greek families
K. Yiannoukakos (GR)

19:30 - 20:00 Association studies for discovering new Breast Cancer Genes: do they exist?
M. Loizidou (CY)

20:00 - 21:00 OPENING CEREMONY

Chairmen: P. Kosmidis (GR) - K. Kyriacou (CY)

20:00 - 20:30 Welcome Addresses

20:30 - 20:50 Kyprida Aphroditi "The great Goddess of Cyprus"
T. Loizidou (CY)
President Association of Cypriot Women in Tourism

20:50 - 21:20: Opening Lecture

EU policy for breast cancer
A. Adamou (CY)
Member of The European Community Parliament

21:20 - 23:00 *Welcome Reception*

Friday, October 19th, 2007

09:00 - 12:00 SESSION II

SCREENING / PATHOLOGY / MANAGEMENT

Chairpersons: S. Papadopoulos (GR) - S. Kyriakides (CY)

09:00 - 09:30 Population screening for breast cancer
V. Partassides (CY)

09:30 - 10:00 Pathology of mammographically detected breast lesions; new challenges
F. Iacovou (CY)

10:00 - 10:30 Genetic counseling
V. Anastasiadou (CY)

10:30 - 11:00 The concept of a breast clinic
W. Gatzemeier (IT)

11:00 - 11:30 Patient's needs
S. Kyriakides (CY)

11:30 - 11:45 Presentation by Pancyprian Association of Cancer Patients and Friends
B. Pitsillides (CY)

11:45 - 12:00 Presentation by Anticancer Society
E. Karatzia (CY)

12:00 - 12:30 Coffee Break

SESSION I
MOLECULAR BIOLOGY / GENETICS

>ASSOCIATION STUDIES FOR DISCOVERING NEW BREAST CANCER GENES: DO THEY EXIST?

Maria Loizidou

Breast cancer is the most common malignancy which affects women worldwide, with an estimated annual incidence of about one million cases.1 When evaluating breast cancer risk factors, family history of breast cancer constitutes one of the most important established risk factors with first degree relatives of patients having a two-fold elevated risk compared to the general population.2 In the 1990's two breast cancer susceptibility genes were cloned; BRCA1 and BRCA2.3,4 Inherited germline mutations in the BRCA1 and BRCA2 genes confer a strong lifetime risk of developing breast and ovarian cancer.5 In addition to BRCA1 and BRCA2 genes, other high-risk breast cancer susceptibility genes such as PTEN and TP53 were discovered6,7 while a number of genes including CHEK2, TGFB1 and ATM comprise the "low to moderate-risk" breast cancer susceptibility group.8,9,10 Together, it is estimated that 5-10% of all breast cancers are caused by mutations in the above breast cancer susceptibility genes.11 However, it is believed that a substantial proportion, as high as 30%, of the total breast cancer incidence can be attributed to genetic factors.12 To date, linkage analysis has failed to identify other major breast cancer genes and much of the observed familial clustering remains unexplained. It is therefore still highly likely that there are more breast cancer susceptibility genes to be found. The failure to localize further breast cancer genes has led to the proposal that breast cancer is polygenic and that susceptibility is caused by a number of loci, each or in combination, together with environmental interactions effect on breast cancer.13 The search for finding novel breast cancer susceptibility loci has nowdays shifted towards the conduction of genome-wide association (GWA) studies in large population-based series of breast cancer cases and controls. Chip technology allows the simultaneous analysis of hundreds of thousands of single nucleotide polymorphisms (SNPs). The aim of a case-control association study is to identify differential distribution of the genetic marker under study between the two groups (patients and controls) denoting an association of the genetic variant with the disease. The implementation of association studies has led to the identification of common variants that contribute to breast cancer which could not have been detected by linkage analysis. Examples include variants in the CHEK2, ATM, BRIP1 and PALB2 genes which confer an approximately twofold risk of breast cancer.8,10,14,15 Recently a large collaborative GWA on breast cancer has identified five novel breast cancer susceptibility loci demonstrating that there are still more breast cancer susceptibility alleles to be found each conferring a small risk of disease.16 In the future, a combination of the presence of low-risk variants together with other breast cancer risk factors may have the power to predict an individuals risk of breast cancer and may become clinically important. The next decade is very important as it is anticipated that many more breast cancer susceptibility loci will be identified, which will explain a larger proportion of the genetic susceptibility to breast cancer.

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4. AWARDS

EUROPEAN HUMAN GENETICS CONFERENCE 2009
Vienna, Austria, May 23 - 26, 2009

2009-05-2

CERTIFICATE OF ATTENDANCE

CONFIRMATION OF PAYMENT

This is to confirm that

Prof./Dr./Ms./Mr. Loizidou Maria

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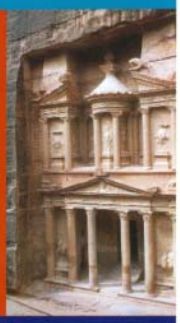

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9th MARIANNA LORDOS SYMPOSIUM
29th February - 2nd March 2008
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POSTER AWARD

This is to certify that

Maria Loizidou

was awarded the prize for the poster presentation

"Genetic polymorphisms in the DNA repair genes and risk of breast cancer in Cyprus"

organised by the

Marianna Lordos Cancer Memorial Fund

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All participants are provided with a maintenance allowance during their training program in the United States. The allowance covers lodging, meals, and miscellaneous expenses. Each participant's maintenance is calculated using strict guidelines set by the United States Agency for International Development. The established guidelines are intended to provide an adequate allowance to cover basic living expenses while staying in the U. S. **THE MAINTENANCE IS NOT INTENDED TO COVER NON-PROGRAM RELATED EXPENSES** (sightseeing tours, gifts, theater trips, entertainment, clothing, etc.).

The maintenance allowance for each participant is different. The amount of maintenance you will receive depends on several factors. These are:

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Ms. M. Loizidou
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Cyprus

Date	**Your reference**	**Direct phone number**
15 May 2006		+31 (0)10 463 8480
Subject	**Our reference**	**Contact**
Nihes/ESP Fellowship 2006		Ms Sytske Flores s.flores@erasmusmc.nl

Dear Ms. Loizidou,

It is my pleasure to inform you that you have been selected for a Nihes/ESP Fellowship for participation in the Erasmus Summer Programme 2006 taking place from 7 to 25 August 2006 in Rotterdam, the Netherlands. You have been admitted to 'Research Training in Genetic Epidemiology'. Congratulations!

The fellowship covers the three-week tuition fee including course materials, except book, and lunch on weekdays (€ 2.500), and accommodation in the Erasmus International House of the Erasmus University Rotterdam (€ 250 instead of € 515). All other costs are at your expenses.

A confirmation form and an accommodation reservation form have been attached, which I kindly ask you to sign and return by fax **before Wednesday 24 May 2006**. The fax number is: +31 10 408 9382. Please note that your room is available to you the entire month of August. You are welcome to arrive prior to the Erasmus Summer Programme and leave on the 30th (at the latest). Of course, subsistence costs during these additional days are at your expenses.

As soon as we have received your confirmation and accommodation request, we will send you further details about your participation in the Erasmus Summer Programme 2006. Please make sure that the amount due for accommodation is the appropriate bank account before June 15th (see the confirmation form for details).

The Nihes Staff and I look forward to welcoming you to Rotterdam this summer.

With kind regards,

Albert Hofman, MD PhD
Scientific Director

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