Genetic determinants of breast cancer susceptibility in the Byelorussian population

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades

Doktor der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

Natalia Valerjevna Bogdanova

geboren am 22. Oktober 1978 in Minsk (Weissrussland)

2008

Referent: Professor Dr. Jürgen Alves
Korreferent: Professor Dr. Ekkehard Dikomey
Tag der Promotion: 21. August 2008

I dedicate my work to the memory of excellent researcher, outstanding person, my teacher and friend Dr. Sergej Pavlovich Festchenko.

Abstract

Breast cancer is the most common lethal malignancy of women all over the world. Mutations in known breast cancer susceptibility genes including *BRCA1* and *BRCA2* mutations can confer a high life-time risk for breast cancer up to 85%, whereas *CHEK2, ATM* and *NBN* mutations are proposed to be associated with a lower penetrance. The products of these genes are involved in DNA double-strand break signaling and repair, and considerable efforts have been made during the past years to identify the mutational spectra of these genes in familial and sporadic breast cancer. However, the known genes explain only a minor proportion of breast cancer cases whereas epidemiological studies indicate that most breast cancer may be, at least in part, due to an inherited susceptibility. Presently large association studies are being conducted worldwide and have already uncovered some common genetic variants that increase breast cancer risk.

Hereditary and environmental factors may have acted synergistically in many cases to modulate the probability and progression of the disease. For instance, ionizing radiation is for long time being recognized as a potent carcinogen that leads to DNA double strand breaks (DSBs) – the most severe type of DNA damage – and increases breast cancer risk in exposed young women. Variations in the genes involved in the cellular responses towards ionizing radiation are thus under special consideration as possible breast cancer susceptibility alleles. The connection between cellular radiosensitivity and susceptibility towards breast cancer may be relevant in regard of the chronic exposure to radiation of the Byelorussian population after the Chernobyl accident.

The genetic epidemiology of breast cancer has not been investigated in the Republic of Belarus, before, so the presented thesis for the first time reveals the mutational spectrum of breast cancer susceptibility alleles in the Byelorussian population. Presented thesis report seventeen susceptibility alleles at eleven genomic loci in a large case-control series of 1759 breast cancer cases and 1019 population controls. Three mutations in the *BRCA1* gene (5382insC, 4153delA, T300G) and one mutation in the *BRCA2* gene (6174delT) accounted for a total of 79 Byelorussian breast cancer patients (4.5 %) and 9.1% of hereditary breast cancer. In contrast with published data, the *BRCA1**4153delA mutation was clearly associated with an increased breast cancer risk in my study population (OR 4.7, p=0.02). Furthermore, five founder mutations in the *CHEK2* (n=3), *NBN* and *ATM* genes were each associated with breast cancer and together accounted for 147 Byelorussian breast cancer cases (8.4 %). The results on *CHEK2* mutations indicate higher risks for truncating mutations compared with one missense mutation. The functional consequences of *CHEK2* and *NBN* mutations were studied in more detail using EBV (Epstein-Barr virus) immortalised lymphoblastoid cell lines established from selected mutation carriers. A functional impact of p.R215W variant at the NBN protein level revealed that only about one-third of the wild-type level of full-length nibrin could be attributed to the p.R215W allele in cell lines from heterozygous patients with NBS

(Nijmeen Breakage Syndrome) and with breast cancer. A functional assessment of the p.I157T missense variant at the CHEK2 protein level showed no distinctions from wild-type in terms of expression and radiation-induced phosphorylation, indicating a possible downstream defect. Transcript analysis of *CHEK2*dele(9,10) revealed full expression of the deletion product and at low level, i.e. about 5% of the dele(9,10) allele, an additional splice variant with three exons deleted. Immunoblot analyses showed an unexpectedly large reduction of CHEK2 phosphorylation in lymphoblastoid cells from a *CHEK2*dele(9,10)/657del5 compound heterozygous and a similarly marked reduction in cells from a 657del5 homozygous NBS patient possibly reflecting a functional interaction between the proteins NBN and CHEK2.

The study was then extended to investigate the role of common polymorphisms as potential modifiers of breast cancer risk. *XRCC4* is proposed as a candidate breast cancer susceptibility gene in the presented study as its splicing mutation IVS7-1G>A appeared associated with an approximate doubling of breast cancer risk in the Byelorussian population. In addition, common low-penetrance alleles at five genomic loci, including two near the *FGFR2* and *TOX3* genes, two coding variants in *CASP8* and *TGFB1*, and one locus on chromosome 2q35, were investigated. The CASP8 p.D302H substitution appeared protective in familial breast cancer cases, and the rare allele of rs3803662 in *TOX3* was significantly associated with overall breast cancer risk in the Byelorussian case-control series.

When the clinical characteristics and the frequencies of the detected mutations were compared between breast cancer patients stratified by different regions, no markedly heterogenity was found, so that the observed significant differences in the age at diagnosis and family history between regions can not be explained due to founder effects or gene-environment interactions of identified genetic factors.

Taken together, the data indicate that there are at least three classes of breast cancer susceptibility alleles: (i) rare mutations associated with high risks and familial aggregation, (ii) mutations with low to moderate frequencies associated with two- to fourfold increase in risk, and (iii) common single nucleotide variants associated with some 20-50% increases in risk. When all genes analysed in the present study were taken into consideration, over 99% of patients were carriers of at least one known susceptibility allele, and this appears as a strong indication that most if not all breast cancers in the Republic of Belarus arise in patients with some heritable predisposition.This study should provide a valuable basis for further research in the biological relevance of some of the genes, possible gene-gene and gene-environment interactions, and their potential exploitation in the prevention and therapy of breast cancer.

Key words: breast cancer, human genetic, mutation, population.

Zusammenfassung

Brustkrebs ist die häufigste bösartige Tumorerkrankung von Frauen überall auf der Die bisher bekannten für Brustkrebs disponierenden Genveränderungen schließen Mutationen in *BRCA1* und *BRCA2* ein, die eine hohe lebenslange Erkrankungswahrscheinlichkeit für Brustkrebs bis zu 85 % mit sich bringen, oder Mutationen in *CHEK2*, *ATM* und *NBN* mit einer niedrigeren Penetranz. Den Produkten dieser Gene werden zentrale Funktionen in dem Signalnetzwerk der DNA-Doppelstrangbrüche und -Reparatur zugeordnet, und die vorliegende Arbeit ist Teil umfangreicher Bemühungen, die während der letzten Jahre unternommen worden sind, um die Mutationsspektren dieser Gene in sporadischem und familiärem Brustkrebs zu identifizieren. Allerdings erklären die bekannten Gene nur einen geringen Anteil von Brustkrebsfällen, wohingegen epidemiologische Studien anzeigen, dass möglicherweise ein weitaus größerer Teil der Brustkrebsfälle mindestens teilweise durch eine ererbte Prädisposition mitverursacht ist. Derzeit werden weltweit große Assoziationsstudien durchgeführt, und diese haben bereits einige genetischen Varianten mit einem erhöhten Brustkrebsrisiko assoziiert.

Erbliche und umweltbedingte Faktoren könnten auch synergisch in vielen Fällen gewirkt haben, um die Wahrscheinlichkeit und das Fortschreiten der Krankheit zu bestimmen. Zum Beispiel sind ionisierende Strahlen seit langer Zeit als ein starkes Karzinogen bekannt, das zu DNA Doppelstrangbrüchen (DSB) - der stärksten Form des Chromosomenschadens - führt und die Erkrankungswahrscheinlichkeit von Brustkrebs bei betroffenen jungen Frauen vergrößert. Varianten in den Genen, die an der Zellantwort auf ionisierende Strahlung teil nehmen, sind derzeit besonders im Blickfeld als mögliche für Brustkrebs disponierende Allele. Sie waren darüber hinaus in der hier präsentierten Studie vor dem Hintergrund der chronischen Exposition von Teilen der weißrussischen Bevölkerung gegenüber niedrig dosierter Strahlung nach dem Chernobyl Katastrophe von besonderem Interesse.

Da die genetische Epidemiologie des Brustkrebses in der Republik Weißrusslands vorher nicht untersucht worden war, offenbart die präsentierte Arbeit zum ersten Mal das Mutationsspektrum von für Brustkrebs relevanten Genen in der weißrussischen Bevölkerung. Sie zeigt siebzehn Prädispositionsallele an elf genomischen Loci in einer großen Fall-Kontrolle Studie von 1759 Brustkrebspatientinnen und 1019 Bevölkerungskontrollen aus Weißrussland auf. Drei Mutationen im *BRCA1* Gen (5382insC, 4153delA, T300G) und eine im *BRCA2* Gen (6174delT) waren für insgesamt 79 Patienten (4.5 %) und 9.1 % des erblichen (familiären) Brustkrebses verantwortlich. Im Kontrast zu veröffentlichten Daten wurde für die *BRCA1**4153delA Mutation erstmals ein erhöhtes Brustkrebs-Risiko belegt (OR 4.7, p=0.02). Außerdem wurden fünf Mutationen der Gene *CHEK2* (n=3), *NBN* und *ATM* jeweils signifikant mit Brustkrebs assoziiert und waren zusammen für 147 weißrussische Brustkrebs-Fälle (8.4 %) verantwortlich.

Die Ergebnisse der *CHEK2* Mutationen weisen auf höhere Risiken für proteinverkürzende Mutationen (insbesondere IVS2+1G>A und *CHEK2*dele(9,10)) im Vergleich zu einer Aminosäuresubstitution (p.I157T) hin.

Die funktionellen Konsequenzen einiger CHEK2 und NBN Mutationen wurden von mir anhand von EBV (Epstein-Barr Virus) immortalisierten lymphoblastoiden Zelllinien ausgewählter Mutationsträgerinnen genauer untersucht. Ein funktioneller Einfluss von p.R215W-Variante auf NBN Protein Ebene zeigte, dass nur ungefähr ein Drittel des Wildtyps vom gesamten Nibrin synthetisiert werden konnte vom R215W-Allel in Zelllinien von heterozygoten NBS (Nijmeen Breakage Syndrome) Patienten und Brustkrebspatienten. Eine funktionelle Rolle der p.I157T Variante auf CHEK2 Protein Ebene zeigte keinen Unterscheid vom Wildtyp in Bezug auf Expression und strahleniduzierte Phosphorylierung was einen möglichen abwärts gelegenen Defekt anzeigte. Die Transkript-Analyse von CHEK2dele (9,10) offenbarte eine volle Expression des Deletionsproduktes und eines Spliceproduktes das ungefähr 5 % des Allels entsprach und zusätzlich eine Deletion von drei Exons aufwies. Immunoblot-Analysen zeigten die unerwartet große Verminderung der CHEK2 Phosphorylierung in lymphoblastoiden Zellen von einer CHEK2dele(9,10)/657del5 heterozygoten Trägerin und diese Reduzierung wurde auch in Zellen von einem 657del5 homozygoten NBS Patienten, was vielleicht eine funktionelle Interaktion zwischen den Proteinen NBN und CHEK2 widerspiegelt.

Die Untersuchungen wurden dann ausgedehnt auf die Analyse häufiger Polymorphismen und ihrer Rolle in der genetischen Disposition für das Mammakarzinom. Die Untersuchungen ergaben *XRCC4* als potenzielles Kandidatengen für Brustkrebs, da die Spleißmutation IVS7-1G>A etwa doppelt so häufig unter Patientinnen als unter Kontrollpersonen in der weißrussischen Bevölkerung zu finden war. Außerdem wurden, teilweise im Rahmen des internationalen Breast Cancer Association Consortiums, putative Risiko-Allele an fünf genomischen Loci untersucht, davon zwei in bzw. nahe den Genen *FGFR2* und *TOX3* , zwei kodierende Varianten in *CASP8* und *TGFB1*, und ein uncharakterisierter Genort auf Chromosom 2q35. Die Aminosäuresubstitution D302H in CASP8 erschien protektiv bei familiärem Brustkrebs, und das seltene Risikoallel rs3803662 in *TOX3* war mit dem Auftreten von Brustkrebs signifikant assoziiert.

Ebenfalls wurden die genetischen und die verfügbaren klinischen Daten der Patientinnen nach regionalen Gesichtspunkten analysiert. Dabei konnte keine deutliche Heterogenität festgestellt werden, so dass die beobachteten signifikanten Unterschiede im Diagnosealter und der Familienanamnese zwischen den kontaminierten und nicht kontaminierten Gebieten durch Effekte von Gen-Umwelt-Interaktionen von identifizierten genetischen Faktoren nicht erklärt werden konnten.

Zusammengenommen zeigen die Ergebnisse, dass es mindestens drei Klassen von für Brustkrebs disponierenden Allelen gibt: (i) seltene Mutationen, verbunden mit hohem Risiko und familiärerHäufung, (ii) Mutationen mit einer niedrigen oder moderaten Frequenz, verbunden mit zwei - bis vierfach erhöhtem Brustkrebsrisiko, und (iii) Polymorphismen, deren einzelne Nukleotidvariantenmit einer Riskozunahmen von ungefähr 20-50 % assoziiert sind. In der Gesamtschau aller bisher bekannten genetischen Dispositionen wurde deutlich, dass bei mehr als 99% aller Patientinnen in meiner Studie mindestens ein disponierendes Allel zu finden war, so dass vermutlich die große Mehrzahl, wenn nicht sogar alle, Brustkrebsfälle in der weißrussischen Republik unter dem Einfluss einer erblichen Disposition entstanden sind. Diese Arbeit bildet eine wertvolle Grundlage für die weiterführende Erforschung der biologischen Relevanz dieser Dispositionen, ihrer Gen-Gen- sowie Gen-Umwelt-Interaktionen, sowie ihrer Bedeutung für die zukünftige Krebsvorsorge und als potenzieller therapeutische Angriffspunkte.

Sclüsselworte: Brustkrebs, Humangenetik, Mutation, Population.

Table of contents

Abbreviations

A	Adenine
abs.	Absolute
APS	Ammoniumpersulfat
A-T	Ataxia- telangiectasia, Louis-Bar-Syndrome
ATM (gene and protein)	A-T mutated
ATP	Adenosintriphosphate
ATR	ATM and Rad3-related kinase
bidest. H_2O	Bidistilled water
BRCT	BRCA1 carboxyterminal
BSA	Bovine serum albumine
bp	Base pairs
C	Cytosine
°C	Grad Celsius
cDNA	Complementary DNA
DMSO	Dimethyl sulfoxide
DSB	Double strand break(s)
del	Deletion
DEPC	Diethylpyrocarbonate
DNA	Desoxyribonucleic acid
EBV	Epstein-Barr-Virus
EDTA	Ethylendiamintetraacetate
EGTA	Ethylenglycoltetraacetate
EtBr	Ethidium bromide
EtOH	Ethanol
FHA	Fork-Head associated domain
FCS	Fetal calf serum
G	Guanine
g	Gram
GTC	Guanidinium thiocyanate
GTS	Glycine-Tris-SDS buffer
Gy	Gray
h	Hours
H_2O	Water
HPLC	High Performance Liquid Chromatography
HR	Homologous Recombination
IR	Ionizing radiation
Kb	Kilobase
KCl	Kalium (potassium) chloride
kDa	Kilo Dalton
L	Liter
LiCl	Lithium chloride
M	Molar
m-	milli-
mA	Milliampere
min	Minute
$MgCl_2$	Magnesium chloride
ml	Millilitre
$MnCl_2$	Manganese chloride
mM	millimolar
mRNA	messenger RNA
µ-	mikro-
n-	nano-

NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
NaF	Sodium fluoride
$NaHCO_3$	Sodium hydrogencarbonate
NaOAc	Sodium acetate
NBS	Nijmegen Breakage Syndrome
NHEJ	Non-homologous end-joining
OD	Optical density
p-	pico-
PAGE	Polyacrylamide gel electrophoreses
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PEG	Polyethylenglycol
pH	Potential of hydrogen (pondus hydrogenii)
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
rpm	rounds per minute
RT	Reverse transcription / Room temperature
RT-PCR	Reverse transcription followed by PCR
sec	Second
SDS	Sodium dodecylsulfate
Ser	Serine
T	Thymine
TAE	Tris-Acetate-EDTA buffer
TBE	Tris-Borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethylendiamine
Thr	Threonine
TP53/p53	Tumor suppressor gene/ protein, 53 kDa
Tris	Trishydroxyethylamine
U	Unit
UV	Ultraviolet
V	Volt
Vol.	Volume
v/v	Volume per volume
w/v	Weight per volume
W	Watt
WHO	World health organization

All genes/proteins abbreviations were taken from NCBI data base in accord with the last nomenclature update.

1. Introduction

1.1. A primer

"Cancer is a genetic disease of the somatic cells". This statement (Shiloh 2003) assumes an increasing importance (sense) with every new oncogene and tumor-suppressor gene discovery. The way to cancer is complicated and paved with alterations in the sequence and organization of the cell genome that range from single-nucleotide substitutions to chromosomal aberrations. Sequence alterations arise from spontaneous changes, replication errors and damage of DNA (Hoeijmakers, 2001). DNA damage agents can be endogenous – from normal cell metabolism - or exogenous - from the environment. Damaging agents such as ionizing radiation and reactive chemicals are able to induce a plethora of DNA lesions. Some are extremely cytotoxic; others are mutagenic and can affect the production, structure and function of cellular proteins with consequences that range from cell malfunction to malignant transformation. Therefore many mutagens are also carcinogens and there is a high correlation between both effectualities (Davidson et al. 2002). A cell can protect itself from hazards by means of basic cellular response – recognition and repair of damage, using activation of phylogenetically conserved signaling cascades (checkpoint pathways). The activated checkpoint pathways delay cell cycle progression to facilitate DNA repair. In addition, the type and amount of damage might defeat the survival response machinery and in this case programmed cell death (apoptosis) will be triggered. A defect in the genome maintenance mechanisms including DNA repair, apoptosis and cell cycle checkpoint pathways leads to accumulation of genetic changes (mutations and aberrations), genetic instability (one of the hallmarks of cancer cells) and finally to the transformation of a normal cell to a cancer cell and the development from normal tissue to non-malignant and eventually to invasive, malignant tumours.

Breast cancer is the most frequent malignancy in women all over the world, with a cumulative lifetime risk estimated to be 10 to 20% (Claus at al. 1991; Eeles et al. 1994). As the major affliction of women, breast cancer has a rising incidence rate. Molecular analysis of breast cancer tumors suggests that the development of disease includes accumulation of various genetic alterations, activation of oncogenes as well as inactivation of tumour-suppressor genes, and genomic instability (Black, 1994; El-Ashry et al. 1994; Chin et al. 2004). Most breast cancers have been thought to be "sporadic", but some are the result of a familial predisposition, due to mutations in *BRCA1* and *BRCA2* genes (*breast cancer susceptibility genes 1 and 2)*, which account for approximately 30% of patients with a strong family history of breast or ovarian cancer and might be responsible for 3-5% of all breast and ovarian cancers population-wide (Claus et al. 1996, Pharoah et al. 1997, Peto et al. 1999, Anglian Breast Cancer Study Group 2000) Women with mutations in these genes in the general population are at a significantly higher risk of developing breast and/or ovarian cancer, though the risk might also be influenced by nongenetic (environmental) factors (Antoniou et al.

2002). Because mutations in *BRCA1* and *BRCA2* explain only a fraction of familial cases, it was hypothesised that other less penetrant genes might explain the remainder of genetically predisposed breast cancers (Nathanson and Weber 2001, Pharoah et al. 2002). Recent data including those presented in this thesis indicate that mutations in *ATM (ataxia-telangiectasia mutated)*, *CHEK2* (*checkpoint kinase 2)* and *NBN (Nijmegen Breakage Syndrome protein)* are low-penetrance breast cancer susceptibility alleles (The CHEK2 Breast Cancer Case-Control Consortium 2004, Bogdanova et al. 2005, Renwick et al. 2006; Walsh et al. 2006; Cybulski et al. 2006). Furthermore, mutations in the *BRIP1 (BRCA1 interaction partner)* and *PALB2 (for 'partner and localizer of BRCA2')* genes have been associated with breast cancer (Seal et al. 2006, Rahman et al. 2007, Erkko et al. 2007). The protein products of all these genes and a large number of others function in a complex signalling network that is activated in response to DNA damage – the DNA Double Strand Break (DSB) repair pathway. Several studies suggested links between deficient repair of DSBs and genetic predisposition to breast cancer and one major argument is that the products of *BRCA1* and *BRCA2* function in this common biochemical pathway (Parshad and Sanford 2001; Speit and Trenz 2004). In search for further low-penetrance genes that alter susceptibility to breast cancer large association studies, including those presented in this thesis, are presently being conducted worldwide. It is hypothesized that the life-time risk for breast cancer is modulated by variants and maybe their combinations in numerous other genes, several of which may participate in the cellular DNA damage response.

1.2 Factors that influence the lifetime risk of breast cancer

As mentioned above, breast cancer is a leading cause of cancer mortality among women all over the world with the life-time risk about 10%-20%. Incidence rates increase dramatically with age. While the rate of increase in breast cancer incidence is greatest in women under age 50, the majority of cases in Western populations occur after age 50. Women of higher socioeconomic status, married women, or women living in urban versus rural areas have the highest rates (WHO www.who.int/en, Heck and Pamuk 1997, Pukkala and Weiderpass 1999). Risk factors, that influence the development of the disease, could be mainly divided into three large groups:

1. Lifestyle-Related Factors

2. Risk Factors You Cannot Change

3. Environmental Factors.

Lifestyle-related factors include:

Hormonal factors:

Pregnancies/Breast-feeding: Women who have had no children or who had their first child after age 30 are at higher risk of breast cancer (Layde et al. 1989, Ewertz et al. 1990, Kelsey et al. 1993).

Having multiple pregnancies and becoming pregnant at an early age reduces breast cancer risk (Collaborative Group on Hormonal Factors in Breast Cancer 2002). Some studies suggest that breast-feeding may slightly lower breast cancer risk (Reimer 1996, Bedinghaus1997).

- *Use of oral contraceptive?:* It is still not certain whether oral contraceptives might play a part in breast cancer risk but some studies have suggested a possible increase in breast cancer risk at an early age (before age 45) among long-term oral contraceptive users, and those who started taking it at a young age (Marchbanks et al. 2002, Kahlenborn et al 2006, Cerhan 2006, Reid 2007). This is contrary to ovarian cancer risk which is significantly long-term reduced by oral contraceptive use (Collaborative group on epidemiological Studies of Ovarian Cancer Lancet 2008).
- *Hormonal substitution?:* Some studies reveal that long-term use (several years or more) of hormone replacement therapy (HRT) after menopause, particularly estrogens and progesterone in combination may increase risk of breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer 1996, Li et al. 2003).

***Alcohol*:** Use of alcohol has been linked to an increased risk of developing breast cancer and the risk increases with the amount of alcohol consumed (Garfinkel et al. 1988, Smith-Warner et al. 1998, Zhang et al. 2007).

Obesity: Obesity has been found to be a potential breast cancer risk factor, especially for women after menopause (Bernstein et al. 1994, Zheng et al. 1998, Bartsch et al. 1999, Morimoto et al. 2002)

To **Risk Factors You Cannot Change** belongs:

Gender Simply being a woman is the main risk factor for developing breast cancer. Men can develop breast cancer as well, but the risk is about 100 times less than in women (Prechtel K and Prechtel V 1997, Giordano et al 2004, La Pinta et al 2008).

Age: risk of developing breast cancer increases with age (Perkins et al 2007).

Mammographic density: Extent of radiodense tissue on a mammogram (mammographic densities) is significantly associated with increased breast cancer risk (Russo et al. 2001, Boyd et al. 2007, Martin and Boyd 2008).

***Menstrual periods*:** Women who started menstruating at an early age (before age 12) or who went through menopause at a late age (after age 55) may have a slightly higher risk of breast cancer (Trichopoulos et al. 1972, MacMahon et al 1982, Brinton et al. 1988, Collaborative Group on Hormonal Factors in Breast Cancer 1997).

Genetic factors: Mutations in known breast cancer susceptibility genes include *BRCA1* and *BRCA2* conferring a high life-time risk for breast cancer up to 85%, or in *CHEK2, ATM*, *NBS1*, *BRIP1* and *PALB2* mutations with a lower penetrance (see 1.7.)

Environmental factors: Both genetic and environmental factors play a role in a risk of developing the disease and they may act synergistically in many cases to modulate the probability and progression of breast cancer. One environmental factor, for which strong evidence of an association with breast cancer risk exists, is ionizing radiation exposure as outlined in more detail in the next two chapters.

1.3 Inherited predisposition to breast cancer and the environment

Several clinical characteristics may serve as an indicator for a possibly inherited form of breast cancer. In general, these include an unusually early age at onset, about 10 to 20 years earlier than the average age of onset; bilateral breast cancer; the occurrence of a second ipsilateral breast cancer; male breast cancer in the family; the occurrence of additional cancer diagnoses in a single individual or among close relatives; the occurrence of characteristic co-morbidities which are associated with known rare genetic syndromes; and the occurrence of multiple affected family members from one lineage, maternal or paternal. Known genes with mutations of high penetrance include *BRCA1* and *BRCA2*, the *TP53* gene encoding tumour suppressor p53 in the context of Li-Fraumeni syndrome, *PTEN* (also known as *MMAC1* for „mutated in multiple advanced cancers 1“) in the context of Cowden syndrome, *MSH2* and *MLH1* (mismatch repair genes) in context of Muir-Torre syndrome, *BLM* gene in context of Bloom syndrome, or *LKB1* (also known as STK11 - Serine/threonine kinase 11) in the context of Peutz-Jeghers syndrome. Although breast cancer appears to be part of the above-mentioned syndromes, germline mutations of the *PTEN, LKB1, MSH2, MLH1* or *BLM* genes have not been found in breast-cancer-only families or sporadic breast cancer patients, thus far. Moreover these genes explain only a very small proportion of familial cases and there is evidence that additional genetic factors with lower penetrance modulate the life-time risk for breast cancer in the majority of patients (Burke et al. 1999, Nathanson and Weber 2001, de Jong et al. 2002, Pharoah et al. 2002). It was shown that inherited disposition towards breast cancer is complex, and many genetic variants and polymorphisms have been postulated to play a role in this condition (Martin and Weber 2000, de Jong et al. 2002), but for being a *bona fide* breast cancer gene at least two criteria should be fulfilled:

1. mutations in the gene should co-segregate with breast cancer in families, or: mutations in the gene should have been validated as breast cancer susceptibility alleles in powerful association studies,
2. dysfunction of the mutant gene product should be biochemically proven.

Until today there are few genes that meet these criteria: beside *BRCA1/BRCA2* also *ATM*, *NBS1*, *CHEK2* and recently described *PALB2*, *BRIP1* (see 1.7). Remarkably, their products interact with each other in intracellular pathways of radiation-induced cell cycle arrest and DNA repair (see 1.5.1). Some other genes involved in hormonal regulation or biotransformation appeared to be associated with moderate effects on breast cancer risk, but the results of such studies are not always conclusive and further research is required (Dunning et al. 1999, Kristensen et al. 2000, de Jong et al. 2002, Mitrunen et al. 2000, Egan et al. 2004, Gold et al. 2004, Cui et al. 2005, Einarsdottir et al. 2006, Breast Cancer Association Consortium 2006).

The penetrance of a genetic disposition towards breast cancer is age-dependent and may be modulated by several additional genetic, environmental and accidental factors (Nathanson and Weber 2001, Antoniou et al. 2002, Pharoah et al. 2002). A significant increase in breast cancer risk is observed in women with a previous history of exposure to high or multiple doses of ionizing radiation (Hall and Angele 1999, Ronckers et al. 2005). Ionizing radiation is for long time being recognized as a potent carcinogen that leads to the intracellular formation of reactive oxygen species and other radicals which in turn cause single and double strand-breaks in chromosomal DNA (Cox 1994, Leach et al. 2001, Mikkelsen and Wardman 2003). As a consequence, ionizing radiation induces genomic instability in many cell types including breast epithelial cells (Morgan et al. 1996, Ponnaiya et al. 1997). The link between exposition to high doses of radiation and a subsequent development of breast cancer has been shown in numerous epidemiological studies. The evidence is primarily based on investigations of either of two types of cohorts. The first group consists of women with breast cancer and a previous history of radiation exposure: these include, for example, women suffering from the consequences of the atomic bomb (Tokunaga et al. 1987, Ronckers et al. 2005) but also women with a history of therapeutic irradiation in a young age because of tuberculosis, thymic hyperplasia or Hodgkin lymphoma (Hildreth et al. 1989, Hrubec et al. 1989, Bhatia et al. 1996). The second group consists of patients with a rare inherited radiation sensitivity syndrome and their blood relatives: well-known examples are the recessive disorders ataxia-telangiectasia (A-T) and Nijmegen Breakage Syndrome (NBS), both of which are characterized by an extremely high cellular radiation sensitivity (Shiloh 2003, Hall and Angele 1999). As will be outlined below, heterozygous carriers from A-T or NBS families face an increased breast cancer risk (Swift et al. 1987, Seemanova 1990, Swift et al. 1991, Seemanova et al. 2007). Risk of breast cancer from exposure to very low levels of ionizing radiation, such as for example chest X-rays and mammograms, is still controversial. Assessing cancer risk from low-dose radiation presents several obstacles, including the difficulties in measuring lifetime exposure, the large sample series needed to quantify effects, and the appropriateness of linear extrapolation from high to low dose. For increasing power to detect the effects of low-level environmental exposures is

for example to identify genetically susceptible subgroups, or groups with common, low-penetrance susceptibility genes that interact with radiation exposure to increase risk of breast cancer. A few recent studies indicate that carriers of pathogenic alleles in DNA repair and damage recognition genes may have an increased risk of breast cancer following exposure to ionising radiation, even at low doses (Andrieu et al. 2006, Cardis et al. 2007, Broeks et al. 2007). Such observations could have important implications for the protection of patients and their close relatives, but still need to be further substantiated. A possible interaction between genetic susceptibility and ionizing radiation may also impact on the genetic epidemiology of breast cancer in the Republic of Belarus, in regard of the chronic exposure to low-dose radiation of the Byelorussian population after the Chernobyl accident.

1.4 Breast cancer in the Republic of Belarus and ionizing radiation

Among the entire spectrum of malignant neoplasms, breast cancer has a special rank in the morbidity structure of Byelorussian population. In the last 15 years, the morbidity from this pathology in the Byelorussian female population dramatic increased and breast cancer incidence has remained that high until today, with an apparent peak of 29,0 in 1998, over twelve years after the Chernobyl accident (Public Health Ministry of the Republic of Belarus 2002, 2006). The Chernobyl accident took place on 26 of April 1986 and led to the release of more than 10^{19} Becquerel (Bq) of radioisotopes with high levels of fallout over Belarus, Northern Ukraine and part of the Russian Federation. An estimated 70% of the radioactive blow-outs fell out on the territory of Belarus (Figure 1.1). An increase in the incidence of thyroid cancer observed among those exposed in childhood and adolescence in the most contaminated territories of Belarus, Russia and Ukraine has initially been the only scientifically demonstrated radiation-related increase in cancer incidence. This observation provided important information on the risk of thyroid cancer related to ^{131}I and on factors, such as iodine deficiency and stable iodine supplementation, which can modify this risk.

Reports on increases in the incidence of other types of cancer are difficult to interpret because of methodological limitations. As the majority of these studies cover a relatively short time period, it is not possible to fully evaluate the radiological impact of the accident, and it is premature to draw conclusions on the risk of cancers other than that of thyroid. Predictions, based on the experience of other populations exposed to ionizing radiation, suggest that a substantial number of cancers could occur, especially in the most contaminated areas.

During 1988-2002, breast cancer was ranking first among female cancers in Belarus according to its frequency ratio. Although it is not clear how much of the incidences may be connected to irradiation and how much due to increased detection, a comparison of different regions within Belarus indicated that there is an association with the degree of contamination (Public Health

Ministry of the Republic of Belarus 2006, Pukkala et al. 2006). Breast cancer morbidity among female population in Belarus remains on average level in comparison with other countries. Nevertheless breast cancer morbidity increases every year in many industrial developed countries and in regions with negative environmental conditions, including Belarus, and in the last 20 years increased by over 50% from the year 1985 to the year 2005 (Public Health Ministry of the Republic of Belarus 2006). In a recently published study a significant and possibly radiation-related increase in breast cancer was found among women from the regions most contaminated by radiation. This increase was two times higher in comparison with women in less contaminated areas, and was most pronounced among women who were below the age of 45 at the time of the Chernobyl accident (Pukkala et al. 2006).

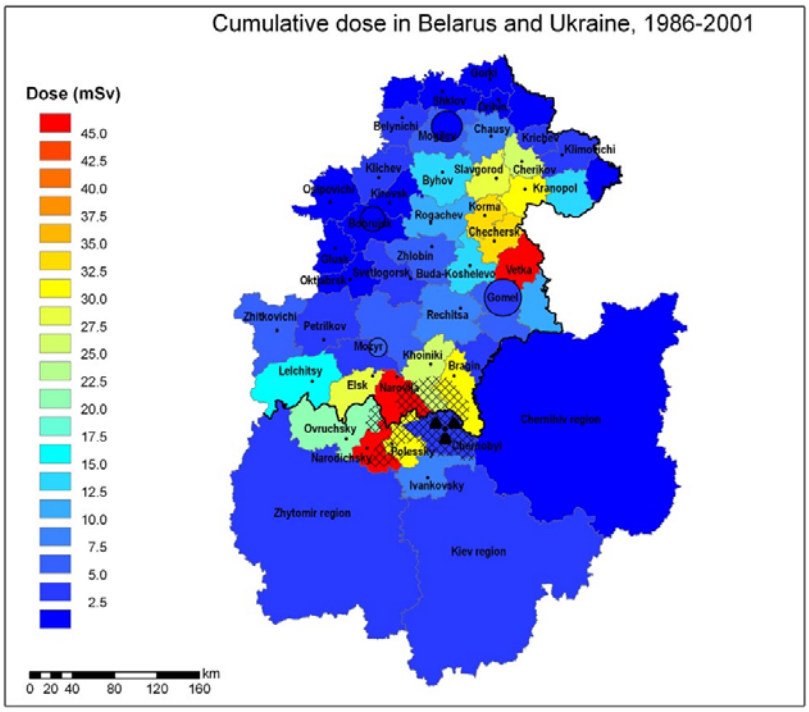


Figure 1.1 **Distribution of cumulative doses in regions of South and Eastern Belarus and in Northern Ukraine.** Uninhabited after accident areas are marked (screen); doses are whole body doses in mSv, lagged by 5 years and cumulated up to 2001 (Pukkala et al, 2006).

It is known that exposure to ionizing radiation leads to a whole spectrum of chromosomal rearrangements that follow double-strand DNA breakage and can give rise to several oncogenic events. Furthermore, an individual's capacity to repair DNA double-strand breaks determines the extent of the chromosomal rearrangements due to unrepaired damage in the exposed cells. It seems very likely, from the recent genetic studies (1.3), that inherited variation in DNA double-strand break repair genes will shape the individual's relative risk towards malignancies after radiation exposure, and one of the malignancies which are most intimately connected with radiation-induced DNA double-strand breaks appears to be breast cancer. As one step to elucidate this further, it is important to determine the mutational spectrum of genetic predispositions towards breast cancer in Byelorussian females.

1.5 Cellular responses to ionizing radiation

1.5.1 Detection and signalling of DNA double strand breaks (DSBs) At the molecular level, the most severe form of radiation-induced damage is DNA double-strand breakage that is under permanent control of the cell repair machinery. Exposure to ionizing radiation activates the complex signalling network including sensors of damage and a large number of downstream mediators and effectors. As a result of this signalling, some mechanisms block cell cycle progression by arrest at defined checkpoints to allow for repair of massive DNA damage or for apoptosis if the damage cannot be repaired (Figure 1.2).

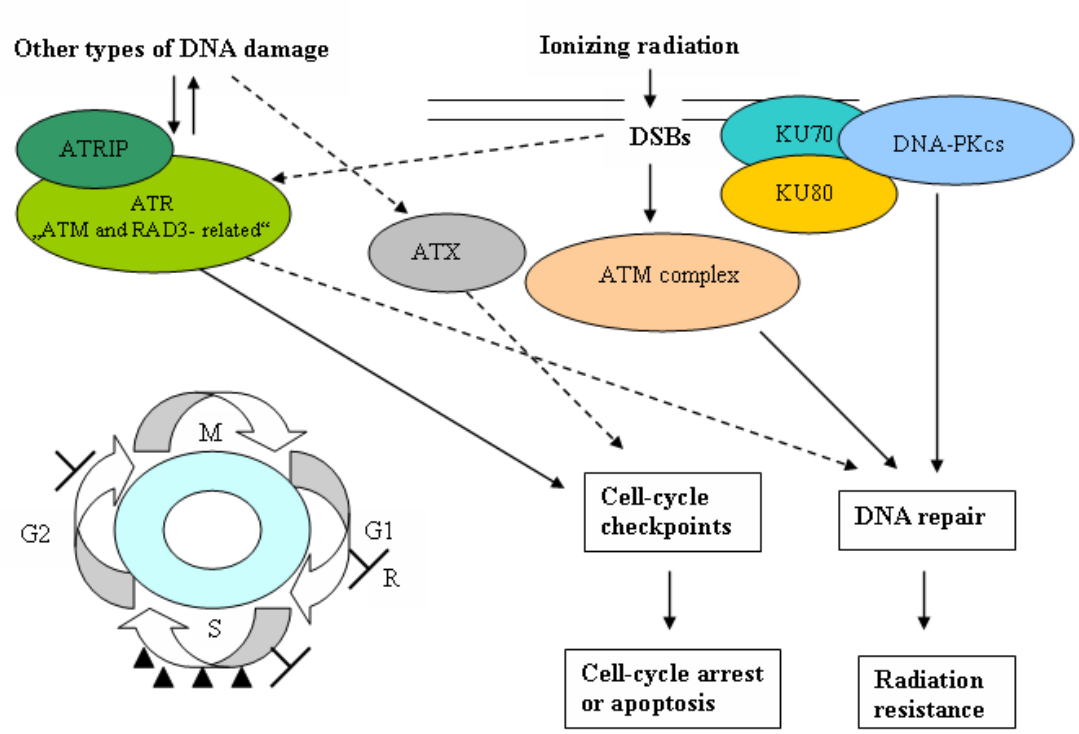


Figure 1.2 ***Cell-cycle checkpoints and DNA repair pathways***. After exposure to ionizing radiation, cell-cycle progression is blocked at defined checkpoints. Checkpoint activation pauses the cell cycle and gives the cell time to repair the damage before continuing to divide. Checkpoint activation is controlled by two master kinases ATM and ATR (although ATX may also take part). ATM responds to DNA DSBs and disruptions in chromatin structure, whereas ATR primarily responds to persistent single-stranded DNA (ssDNA), which commonly occurs at stalled replication forks, but also to DSBs. It was shown that ATR activation is regulated by ATM in a cell-cycle dependent manner in response to DSBs (Jazayeri et al. 2006). These kinases phosphorylate downstream targets in a signal transduction cascade, leading to cell cycle arrest. In G1-phase of cell cycle, arrest occurs before or at so-called "Restriction point" (R). In S-phase, cells are arrested at each point to avoid replication. In phase G2, irradiated cells complete with each other before proceeding into Mitosis (M). **ATRIP**- ATR Interaction Partner; **ATX** or hSMG-1- protein involved in nonsense-mediated mRNA decay (NMD) as part of the mRNA surveillance complex as well as in the DNA DSBs repair; **KU70** or XRCC6 and **KU80** or XRCC5 - „X-ray repair cross-complementing", this complex functions as a single-stranded DNA-dependent ATP-dependent helicase and may be involved in the repair of nonhomologous DNA ends such as that required for double-strand break repair, transposition, and V(D)J recombination (the process that generates diversity in B-cell and T-cell receptors in the vertebrate immune system); **DNA-PKcs** - DNA dependent protein kinase, required for non-homologous end-joining (see below) Modified from Kastan und Lim, 2000.

The cellular response to DNA damage is a very complex process, and it usually starts with the "sensing" or "detection" of the damage, followed by a series of events that include signal transduction and activation of effectors, which execute various cellular functions. DSBs are naturally formed and sealed during physiological DNA processing in replication and it is safe to assume that cellular DSB repair mechanisms maintain continuously at low-level activity. But when DSBs are inflicted on the genome by damaging agents, such as free radicals or ionizing radiation, they rapidly, within minutes, set in motion a DNA-damage response (Jackson, 2002). In this multi-branched signalling network of transducers and effectors, the quick effect is achieved by the operation of many pathways and the transducers are also involved in the assembly of DNA repair complexes at the site of the damage, so DBS repair and signalling are functionally linked. In the case of DSBs, the initial and primary transducer is ATM (although related protein kinases are also involved – ATR, DNA-PKcs, and ATX), which transmits the message via a standard signalling mode: protein phosphorylation. Activated in response to damage is a complex signaling network, including sensor complex MRN (MRE11/RAD50/NBN, see below for the details), and a large number of downstream transducers and effectors including BRCA1, BRCA2, CHEK2, p53 and others, that regulate cell cycle and facilitate repair, or in the event of incomplete repair, apoptosis (Figure 1.3). One hallmark of this response is the activation of cell-cycle checkpoints. Deficiencies in these pathways lead to malfunction of cell cycle, DNA repair and/or apoptosis.

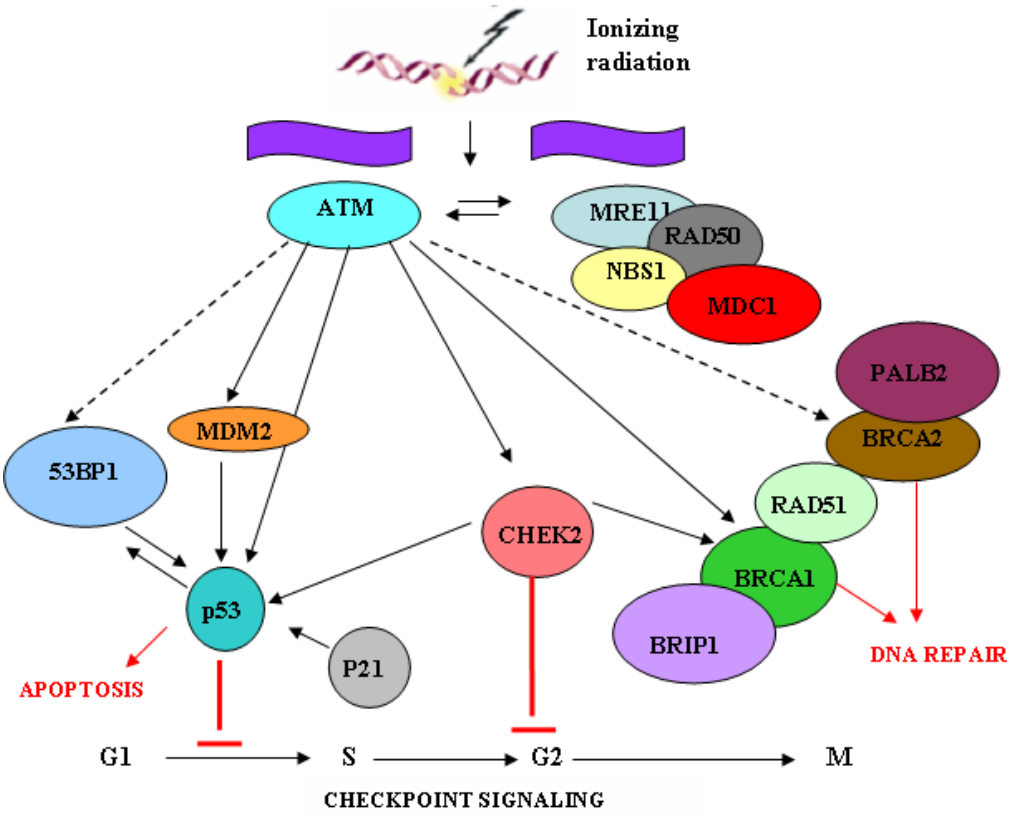


Figure 1.3 **Pathways involved in the response to DNA DSBs.** DSBs in DNA induce transcriptional changes, cell-cycle checkpoints and DNA repair processes. Loss of fidelity in repairing DSBs leads to

chromosomal rearrangements and genomic instability, which are con attributes of cancer cells. **ATM** orchestrates the DSB response by phosphorylating substrates required for the G_1/S, intra S and G_2/M checkpoints. **MRN complex** (**MRE11** - meiotic recombination protein 11- is a nuclear protein with 3' to 5' exonuclease activity and endonuclease activity involved in homologous recombination, telomere length maintenance, and DNA double-strand break repair; **RAD50** is a member of the structural maintenance chromosomes (SMC) protein family, important for DNA double-strand break repair, cell cycle checkpoint activation, telomere maintenance, and meiotic recombination; **NBN** forms a multimeric complex with MRE11/RAD50 nuclease at the C-terminus and recruits or retains them at the vicinity of sites of DNA damage) acts as a break sensor and functions in the activation and propagation of signalling pathway that, in addition, influences recombinational DNA repair through promoting recombination between sister chromatids (Kobayashi et al. 2005). **MDC1**- Mediator of DNA damage checkpoint 1- is required to activate the intra-S phase and G_2/M phase cell cycle checkpoints in response to DNA damage, regulates function of BRCA1 (Lou et al. 2003), is required for CHEK2 activation (Lou et al. 2003a), controls the formation of damage-induced 53BP1 foci (Stewart et al. 2003), interacts with phosphorylated histone H2AX near sites of DNA double-strand breaks and facilitates recruitment of the ATM kinase and MRN complex to DNA damage foci (Lukas et al. 2004), and regulates DNA damage repair by influencing DNA-PK autophosphorylation. **BRCA1** is directly involved in the repair of damaged DNA, is thought to transiently interact with **RAD51 (**homolog of the RecA**,** plays role in homologous pairing and strand transfer of DNA), which in turn is also found to interact with **BRCA2** (Henning and Stuerzbecher, 2003). BRCA2 is shown to regulate both the intracellular localization and DNA-binding ability of RAD51 and required in homologous recombination. **PALB2** (also known as FANCL) interacts with BRCA2, is implicated in its nuclear localization and stability and is required for some functions of BRCA2 in homologous recombination and double-strand break repair at the S phase checkpoint (Simpson et al. 2007). **BRIP1** (also known as BACH1, FANCJ) **–** interacts with BRCA1 and is required for DNA damage-induced checkpoint control during the G_2/M phase of the cell cycle (Yu et al. 2003). **CHEK2** - (also known as Cds1) is activated by ATM in response to DNA damage and phosphorylates cell cycle regulators such as p53, Cdc25 and BRCA1 (Caspari et al. 2000). **TP53** (or p53) - is a tumor suppressor, regulates the cycle of cell division and can bind directly to DNA, plays a critical role in determining whether the DNA will be repaired or the cell will undergo apoptosis if the DNA cannot be repaired (Lacroix 2006). TP53 controls the expression of **p21** (also known as CIP1, WAF1 or CDKN1A - cyclin-dependent kinase inhibitor), which binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a p53-dependent regulator of cell cycle progression at G1; can interact with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor, and plays a regulatory role in S phase DNA replication and DNA damage repair; p21 was also reported to be specifically cleaved by CASP3-like caspases, which thus leads to a dramatic activation of CDK2, and may be instrumental in the execution of apoptosis following caspase activation (Gartel and Radhakrishnan, 2005). **MDM2** (murine double minute oncogene also known as HDM2 for human homologue) is an important negative regulator of the p53 (together with its structural homolog MDM4, also called MDMX). MDM2 functions as an E3 ubiquitin ligase that recognizes the N-terminal trans-activation domain (TAD) of the p53, targeting also itself for degradation by the proteasome and as an inhibitor of p53 transcriptional activation (Momand et al. 2000). **53BP1** - the p53-binding protein is central in both the S and G_2 checkpoints after exposure to ionizing radiation. 53BP1 participates in the organization of nuclear foci and facilitates the phosphorylation of specific substrates by the CHEK2 and ATM (including p53 and SMC1, required for sister chromatid cohesion), interacts with BRCA1 and may take part in DNA repair) (DiTullio et al. 2002).

1.5.2. Mechanisms of DNA double strand break repair

Broken chromosomes can be repaired either by a mechanism similar to homologous recombination (HR) in a high-fidelity repair process between sister chromatids, or by a non-homologous repair mechanism such as Non-homologous End Joining (NHEJ) in a rapid error-prone process that quickly seals the breaks at the expense of creating local microdeletions (Figure 1.4).

Homologous recombination repair (HRR) in S/G_2 cell-cycle phases entails the invasion of an undamaged DNA molecule by a damaged molecule of identical or very similar sequence, followed

by resynthesis of the damaged region using the undamaged molecule as a template. A sister chromatid may be used as the template for repair, or less frequently the paternal and maternal copies of chromosomes may provide the required homology. HRR allows the replacement of damaged regions without loss or alteration of base sequence. In HR, the DNA ends are first resected in the 5′ to 3′ direction by nucleases. The resulting 3′ single-stranded tails then invade the DNA double helix of a homologous, undamaged partner molecule, and are extended by the action of DNA polymerase, which copies information from the partner. Following branch migration, the resulting DNA crossovers (Holliday junctions) are resolved to yield two intact DNA molecules (Figure 1.4).

There are several types of homologous repair: gene conversion, break-induced replication and single-strand annealing (SSA). The SSA pathway takes place when direct repeat sequences flank the two DNA ends and leads to loss of one of the two direct repeats and the intervening DNA. In contrast, NHEJ of two DNA ends in phases G_1/S does not require an undamaged partner and does not rely on extensive homologies between the two recombining ends. In this process, sometimes after limited degradation at the termini, the two ends are ligated together. Consequently, NHEJ is often prone to error, and small sequence deletions are usually introduced.

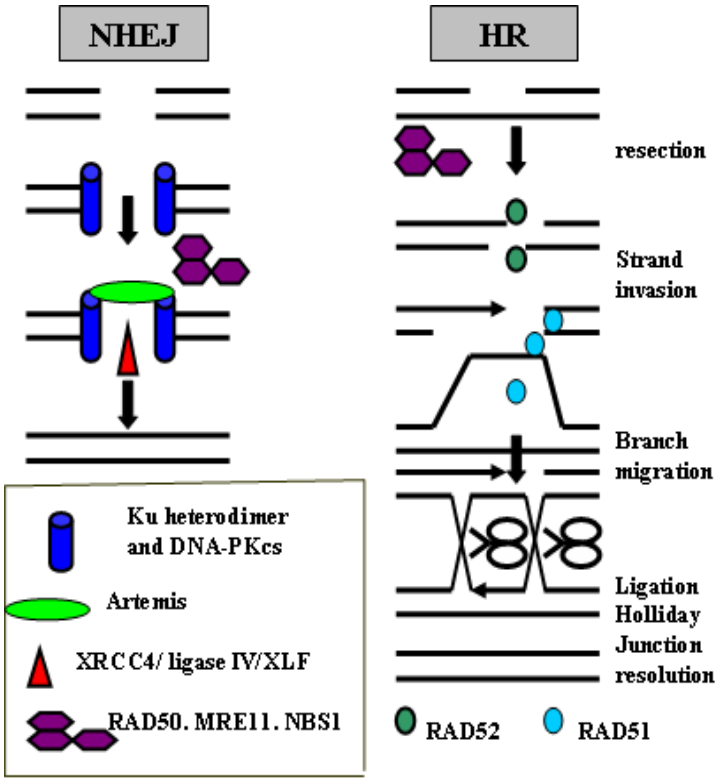


Figure 1.4 **Pathways of DSB repair.** The termini of a DNA DSB introduced by ionising radiation or other means are bound either by the KU heterodimer /DNAPKcs complex or by RAD52. In the NHEJ rejoining pathway, repair is completed by DNA ligase IV/XRCC4/XLF (XRCC4-like factor, also named Cernunnos) in presence of Artemis (also known as DCLRE1C - DNA cross-link repair, apart DNA repair is also involved in V(D)J recombination). DNA strand invasion of the intact sister chromatid, facilitated by RAD51,

initiates repair by homologous recombination. Resection and annealing of short regions of complementary sequence initiates repair by the SSA pathway in which ligation is preceded by the trimming of noncomplementary single-stranded DNA tails. The scheme is based on Rijkers, 1998.

One of the first steps during homology-directed DSB repair (HHR and SSA) is recognition of damage by the protein complex MRN, which is proposed to perform multiple structural and enzymatic functions in DNA end processing and alignment (Valerie et. al. 2003; Stracker et.al, 2004). Following DSB induction, MRN rapidly forms foci at the damaged sites. These foci include additional players in the DSB response, such as RAD51 and BRCA1. During the central step in HHR, RAD51 (supported by the paralogous cofactors XRCC2, XRCC3, as well as by RAD51IP, RAD52, RAD54 and BRCA2) forms nucleoprotein filaments with the 3´ overhanging ssDNA of the resected DSB (this process is probably initiated by RAD51-BRCA2 complex) and catalyses homologous pairing and strand exchange. SSA, a nonconservative mechanism of homology-directed DSB repair, does not depend on RAD51, but requires RAD52, which forms heptameric rings on ssDNA ends and promotes paring before tail removal by the structure-specific endonuclease ERCC1-XPF (XPF also known as ERCC4 - Excision repair cross-complementing rodent repair deficiency) (Valerie et al. 2003). In the NHEJ pathway, that is thought to be the predominant repair mode in mammalian cells, KU70 and KU80 bind the DSB, followed by recruitment and activation of the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), which mediates synapsis and recruits XRCC4, DNA ligase IV and XLF. End processing may involve nuclease activity of protein Artemis, which is activated by DNA-PKcs and ATM and shows an epistatic relationship with MRE11 (Riballo et al. 2004). Despite the disadvantages of its low fidelity, this pathway can act quickly, as required of an emergency mechanism, and, unlike HR, it does not depend on sister DNA molecule, which exist in the cell only after DNA replication.

1.6 Links between DSB repair and breast cancer susceptibility

Initial studies suggested that NHEJ was the predominant mechanism of DSB repair in higher eukaryotes, but it is now established that HR also has a very crucial role. Research in clarifying the enzymology of DNA DSB repair pathways has indicated key roles for these pathways in preventing mutations, chromosomal instability and cancer. DNA repair systems are responsible for maintaining the integrity of genome and have a critical role in protecting against mutations that can lead to cancer. Absent or incorrect repair can initiate carcinogenesis through the activation of oncogenes, the inactivation of tumor-suppressor genes, or loss of heterozygosity (LOH). Repair of damaged DNA involves many proteins performing functions directly at damaged DNA as well as the interaction and interplay with proteins involved in regulation of DNA replication and progression through the cell cycle (Lehmann 1998). Studies have shown that genes directly involved in DNA

repair and the maintenance of genome integrity, or genes indirectly involved in DNA repair through the regulation of the cell cycle, are critical for protecting against the mutations that lead to cancer (Bohr 1995; Mohrenweiser 1998).

At least two major lines of evidence suggest links between deficient repair of DSBs and genetic predisposition to breast cancer. First, studies of the key breast cancer susceptibility genes *BRCA1* and *BRCA2* indicate that their products function in one common biochemical pathway, which plays an important role in DSB repair and chromosome stability (Haber 2000, Valerie et al. 2003, Yoshida and Miki 2004, Gatz and Wiesmüller 2006). Second, increased frequencies of chromatid breaks and gaps after exposure to radiation in G_2-phase of cell cycle have been observed in cultured cells from predisposed individuals, sporadic breast cancer patients and their first degree relatives with two- to threefold higher incidence of cancer (Parshad and Sanford 2001, Patel et. al 1997, Scott 2004).

Several groups have postulated that DSB-initiated cromosomal instability (CIN) is a major motive power for breast cancer progression (Shen et al., 2000, DePinho and Polyak 2004). Support for this hypothesis comes from the observation that CIN, initiated by DSB, leads to genome-wide LOH, which significantly increases in consecutive steps toward tumour progression to later stages (Shen et al. 2000). Interestingly, the fact that in the genome-wide screen the loci of *p53* and *ATM* were lost at the earliest stage indicated that the ATM-p53 signaling pathway involved in DSB repair and checkpoint control (Figure 1.3) is critically important in the suppression of breast tumorigenesis as a barrier against genomic instability before malignant conversion. Moreover it was shown that in clinical speciment from different stages of breast tumours (and other human tumours), the early precursor lesions (but not normal tissue) commonly express markers of an activated DNA damage response – phosphorylated kinases ATM and CHEK2, and p53, which leads to the same hypothesis that the DNA damage response network becomes activated in very early stages of tumorigenesis and defects in this checkpoints might allow cell proliferation, increasing CIN and tumour progression (Bartkova et.al 2005).

DSB repair pathways and checkpoints appear to be particularly important in breast tumorigenesis, and this tissue specificity may partly be explained by the dual role of reproductive hormones – estrogens as a growth stimulation factor and strand break inducing agent. Epidemiological and experimental data indicate that metabolites of estrogens may cause oxidative DNA damage and strand breaks (Yager and Davidson 2006). Hormonal stimulated proliferation in breast epithelium can further lead to replication fork stalling, DSBs and recombinational repair for restart of replication. Deregulated recombinational repair in turn can cause structural chromosomal aberrations, gene amplifications and LOH as a major genotoxic effect of estrogens (Cheng et al. 2005, Liehr 2001).

1.7. Breast cancer predisposition alleles and their impact on disease risk

1.7.1. Diversity of breast cancer susceptibility alleles

Breast cancer is often associated with mutations in different genes in pathways critical to genomic integrity. *BRCA1* and *BRCA2* mutations confer very high risks of breast and ovarian cancer. *PTEN, p53, LKB1 MSH2, MLH1* and *BLM* mutations lead to very high breast cancer risks associated with rare inherited cancer syndromes. Mutations in *CHEK2*, *ATM*, *NBS1*, *BRIP1*, *PALB2* and possibly *RAD50* are associated with an approximately doubling of breast cancer risks (Meijers-Heijboer et al. 2002, Walsh et al. 2006, Gorski et. al 2003, Heikkinen et al. 2006, Seal et. al. 2006, Renwick et al. 2006, Rahman et al. 2007, Erkko et al. 2007, and this work). And several common genetic variants may exist which are associated with less than 1.5-fold increases in breast cancer risk (Cox et al. 2007, Easton et al. 2007, Stacey et al. 2007).

1.7.2. High-risk breast cancer susceptibility alleles

1.7.2.1. BRCA1 and BRCA2

BRCA1 and *BRCA2* are the two major familial breast cancer susceptibility genes, mutations of which are associated with early-onset breast and/or ovarian cancer. Both genes are large and complex and encode products that promote DSB repair. Hundreds of mutations have been found in both genes, with a lot of them being “private” mutations found in only a single family. Most of the mutations are predicted to result in a truncated protein product, thus the deleterious nature of these mutations is easy to interpret. Because missense mutations are rare, their clinical significance is not well known. Mutations in *BRCA1* and *BRCA2* genes account for approximately 30% of families with a strong family history of cancer and might be responsible for 3-5% of all breast and ovarian cancers population-wide. Women with mutations in *BRCA1* and *BRCA2* are at a significantly higher risk of developing breast and/or ovarian cancer, though the risk is also influenced by nongenetic factors (Burke and Austin 2002). Mutation in either of both genes also appears to increase a person’s risk for other type of tumors such as prostate cancer, colon cancer, pancreatic cancer, tumors of Fallopian tube, or melanoma, though the penetrance for these cancers is much lower than for breast or ovarian cancer. Moreover, precancerous lesions (dysplasia) within the Fallopian tube have been linked to *BRCA1* gene mutations, and *BRCA2* mutations confer higher risk for male breast cancer. Founder mutations with a high penetrance have initially been described in Ashkenazim in *BRCA1* (185delAG, 5382insC) and in *BRCA2* (6174delT) (reviewed by Berchuck et al. 1999, Figure 1.5).

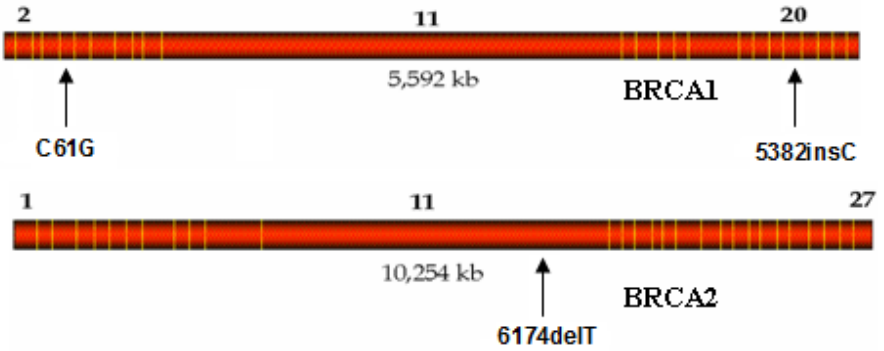


Figure 1.5 **A schematic diagram of *BRCA1* and *BRCA 2* genes showing the sites of founder mutations**.
The number of exons and the length of genes are shown.

In Central and East Europe, among a plethora of different gene alterations, the *BRCA2* deletion 6174delT and the *BRCA1* frameshift insertion 5382insC appear to be frequent, together with the RING finger substitution c.T300G (Cys61Gly) in the *BRCA1* gene (Backe et al. 1999, Gorski et al, 2005a).

1.7.2.2 BRCA1 gene and protein

The *BRCA1* gene is located on chromosome 17q21-12 and constitutes an essential tumour suppressor gene (Figure 1.6) (Hall et al. 1990, Miki et al. 1994) which encodes a 220 kDa nuclear protein functioning in cell cycle control and DNA repair (reviewed by Zhang and Powell, 2005). Being part of a large genomic surveillance and repair complex termed „BASC“ (Wang et al. 2000), BRCA1 interacts with several other proteins that regulate cellular responses to chromosomal breaks and other types of DNA damage, including ATM, CHEK2 and p95/nibrin (product of *NBN* gene). Female-specific effects of *BRCA1* mutations in gynaecological cancers may be partly explained by the ability of the BRCA1 protein to bind and inhibit the estrogen receptor alpha (Fan et al. 1999, Zheng et al. 2001) and/or to control the process of X-chromosome inactivation (Ganesan et al. 2002).

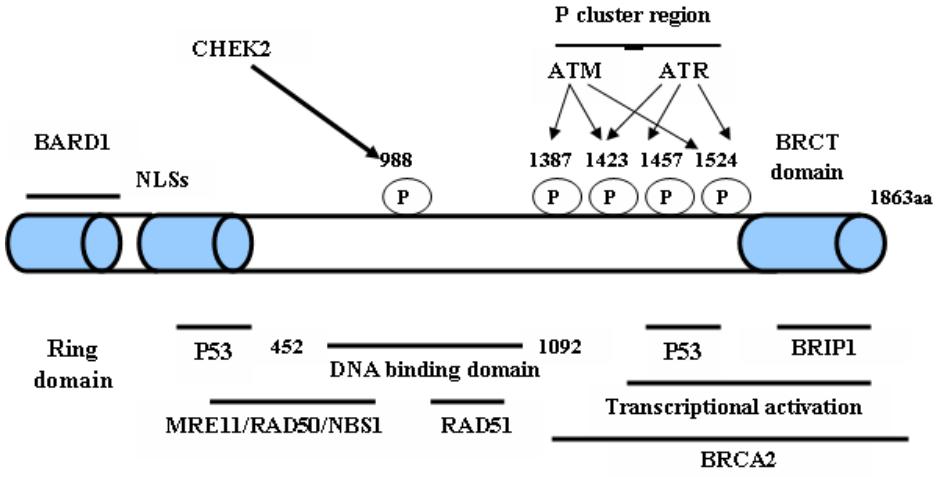


Figure 1.6 **Schematic diagram of BRCA1.** Survey of functionally important sites, including the sites of BRCA1 protein phosphorylation. The **BRCT** domain was defined by its location in the COOH terminus of BRCA1, and it is an important protein interaction domain for many phosphorylated DNA repair proteins (in BRCA1 responsible for interaction with BRIP1). The RING **domain** at the NH_2 terminus of the protein has

the features of an E3 ubiquitin ligase. BRCA1 may function in cooperation with BARD1 as an ubiquitin ligase toward the radiation-activated histone H2AX (Mallery et al. 2002) or towards the progesterone receptor (Poole et al. 2006) and/or the estrogen receptor alpha (Eakin et al. 2007); nuclear localization signals (**NLSs**) at the NH_2 terminus are shown and are necessary for BRCA1 transport into nuclei. The **DNA-binding** domain of BRCA1 has been mapped to a central region of the protein (amino acids 452-1092). There is a CHEK2-dependent phosphorylation site at Ser988 within this domain. A **P cluster region** adjacent to the DNA-binding domain has multiple ATM and ATR target sites of phosphorylation. Further interaction sites with RAD50, RAD51, BRCA2 and p53 are also shown.

1.7.2.3 BRCA2 gene and protein

The *BRCA2* gene, mapped and identified on chromosome 13q12 (Wooster et al. 1995), codes for a 390 kDa nuclear protein. The official name of this gene is *"breast cancer 2, early onset."* The *BRCA2* gene was found to be identical to the *XRCC11* ("X-ray cross-complementing 11") gene (Kraakman-van der Zweet et al. 2002). Furthermore, as inherited hypomorphic *BRCA2* mutations in the homozygous state cause certain forms of Fanconi anemia (Fanconi anemia D1, FA-D1), a cancer-prone chromosomal instability recessive syndrome (Howlett et al. 2002), *BRCA2* is also identical to *FANCD1*.

The 3,418 residue BRCA2 gene product does not exhibit significant similarity to any previously known protein (Figure 1.7). Eight 30- 40 residue motifs (Bork et al. 1996) – the so-called BRC repeats – are encoded in exon 11 and conserved between several mammalian species, which suggests they have an essential function (Bignell et al. 1997). In fact, some BRC repeats have been shown to mediate the binding of BRCA2 to RAD51 (Bork et al. 1996, Bignell et al.1997, Wong et al. 1997). Interactions of BRCA2 with RAD51 are fundamental for the maintenance of cell division and chromosome structure.

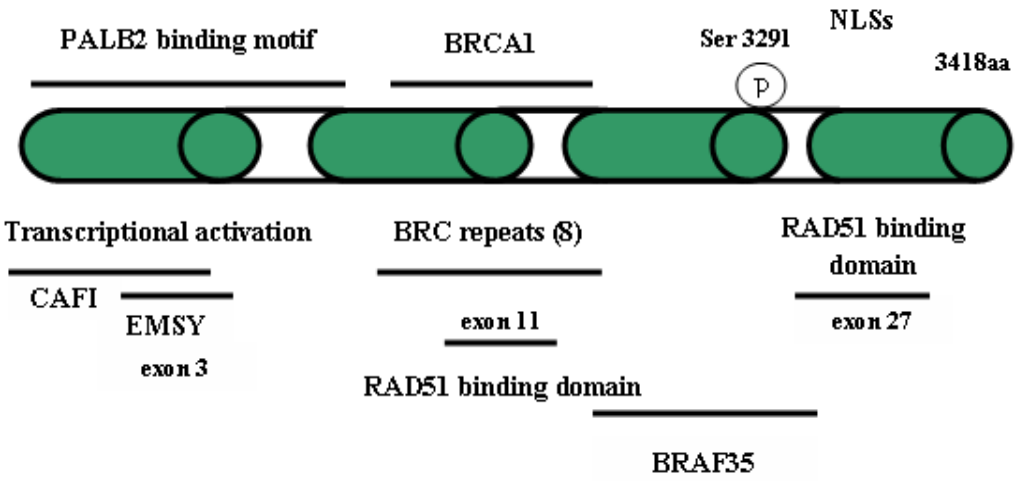


Figure 1.7. **Schematic diagram of BRCA2.** Survey of the functionally important sites, including phosphorylation site at Ser 3291 (by a cyclin-dependent kinase). The **PALB2** binding motif in the highly conserved transactivation region of N-terminus, which also important for interaction with other proteins, such as **CAF1 -** Chromatin assembly factor I, required for the assembly of histone octamers onto newly-replicated DNA or in DNA repair; and with **EMSY –** member of the family of chromatin regulation proteins and in response to DNA damage colocalized with H2AX. Overexpression of the EMSY (the repressor protein for BRCA2) may mimic the effect of BRCA2 inactivation (Raouf et al. 2005). The **BRCA1 binding**

domain has been mapped to a central region of the protein. There is also binding motif for **BRAF35** (BRCA2-associated factor 35, a structural DNA-binding protein) within this region (Marmorstein et al., 2001). The COOH-terminal region has an important role in the tumor-suppressor function of BRCA2, mediates ssDNA binding and the association of the BRCA2-RAD51 complex with sites of DNA damage. BRCA2 has two unrelated **RAD51-binding domains**: a partly degenerate 30-40–amino acid motif (**BRC repeat**), eight copies of which are interspersed throughout the middle 1000 residues of BRCA2 (exon 11), and a distinct RAD51 binding region encoded by exon 27 (**NLSs** region). The BRC repeats of BRCA2 are holding RAD51 in an essentially inactive, monomeric form. After DNA damage RAD51– BRCA2 complexes localize to the DNA break sites. Unknown phosphatases dephosphorylate Ser3291 of BRCA2, what probably activates the COOH-terminal region, which in turn supports the oligomerization of RAD51 on the nucleoprotein filament for processing of homologous recombination (Lord and Ashworth, 2007).

1.7.2.4 Role of BRCA1 and BRCA2 in DBS repair and cell-cycle checkpoints

BRCA1 and *BRCA2* are involved in a multitude of pivotal cellular processes. In particular, both genes contribute to DNA repair and transcriptional regulation in response to DNA damage, required for maintenance of chromosomal stability; they transcriptionally regulate some other genes involved in DNA repair, the cell cycle, and apoptosis. Many of these functions are mediated by a large number of cellular proteins that interact with BRCA1 or BRCA2. The elucidation of the precise molecular functions of BRCA1, BRCA2 and their "partners" is important to improve our understanding of hereditary as well as sporadic mammary carcinogenesis.

Major clues to the role of BRCA1 and BRCA2 proteins in DBS repair have come from assays based on cultured cells. *BRCA1* and *BRCA2* mutant cells exhibit a high degree of spontaneous and induced chromosome aberrations, are sensitive to ionizing radiation and DNA damage agents, and have elevated mutation rates (Jasin, 2002, Kim et al. 2004). Initial evidence suggesting a role of BRCA1 in the repair of damaged DNA was derived from the observation that BRCA1 is hyperphosphorylated at multiple residues by different kinases (ATM, ATR, CHEK2) in response to DNA damage and relocated to sites of replication forks (Wang et al. 2000, Welcsh et al 2000). However, how each type of phosphorylation affects the functions of BRCA1 remains obscure. Subsequent studies demonstrated the involvement of BRCA1 and BRCA2 in complexes that activate the repair of DSBs and initiate HR, linking the maintenance of genomic integrity to tumor suppression. BRCA1 may recruit BRCA2, which facilitates RAD51 filament formation in response to DBSs. BRCA1 is also observed to colocalize with RAD51, which seems to be required for the strand invasion in HR (as described above). Direct interactions between BRCA2-RAD51 and BRCA2-BRCA1 proteins have been reported; however, the association of RAD51 with BRCA1 at the site of DNA damage may be mostly indirect through BRCA2 (Venkitaraman, 2003). BRCA1 has also been found associated with another DNA damage response protein, RAD50, which forms a tight complex with MRE11 and p95/NBS1 (described above). This complex is implicated in both HR and NHEJ (figure 1.4) and BRCA1 apparently functions as a regulator of MRE11/RAD50/NBN (MRN) complex (Wu et al, 2000). Protein foci of MRE11 or RAD50 colocalize with

phosphorylated H2AX and MDC1 foci after DNA damage. BRCA1 can also colocalize with H2AX and is recruited to these sites before RAD50 or RAD51 (Paull et al. 2000), suggesting that BRCA1 may determine the recruitment of kinases responsible for H2AX phosphorylation to DNA lesions before RAD50 and/or RAD51. A study of Foray et al. has revealed that BRCA1 contributes to the ATM-dependent activation of c-Abl - tyrosine kinase, which is ubiquitously expressed and localized in the cytoplasm and nucleus. Nuclear c-Abl is activated by diverse genotoxic agents and induces apoptosis and also implicated as a regulator of transcription and DNA repair (Foray et al. 2002). BRCA1 may also function as a co-activator of p53-mediated gene transcription and appears to be required for a p53-independent S phase block of cell cycle by transactivation of p21 (Somasundaram et al. 1997). In summary, BRCA1 has multiple roles in response to DSBs.

In some contrast, BRCA2 seems to have a single main and more direct function in homologous recombination acting downstream of BRCA1 via its interaction with RAD51. In irradiated cells, BRCA2, co-localizes with phosphorylated H2AX and EMSY, which negatively regulates BRCA2 function in transcriptional activation and is amplified in sporadic breast cancers (Raouf et al. 2005). It was also shown that, after DNA damage, BRCA2 co-localized with BCCIP (BRCA2 and CDKN1/p21 interacting protein), which may be an important cofactor for BRCA2 in tumor suppression via HR (being also co-localized and interacting with RAD51) and a modulator of CDK2 kinase activity via p21 (Lu et al. 2005). Some further evidence suggests that BRCA2 mediates G2/M-phase control by interacting with BRAF35 which binds to branched DNA structures (Marmorstein et al. 2001). Thus, there are now multiple interaction partners of BRCA2 known, the role of which in the DNA damage response and in breast cancer susceptibility remain to be fully clarified.

1.7.3 Breast cancer susceptibility alleles with moderate penetrance and their role in DSB repair

1.7.3.1 Genes harbouring breast cancer susceptibility alleles with moderate penetrance

Although *BRCA1* and *BRCA2* have attracted most attention as high risk factors for inherited breast cancer, these two genes account for only a small proportion of the genetic risk while other more common but less penetrant gene alterations may explain the remainder of genetically predisposed breast cancers. Mutations in *CHEK2*, *ATM*, *NBS1*, *BRIP1*, and *PALB2* have more recently been identified as low-penetrance alleles with an approximately 2-to 3 fold increase in risk.

1.7.3.2 ATM

The *ATM* ("ataxia-telangiectasia mutated") gene has been identified in 1995 as the causative gene in ataxia-telangiectasia (A-T), an autosomal recessive radiation sensitivity syndrome (Savitsky et al.

1995a,b). Hallmarks of this disorder include, aside of severe neurological and immunological symptoms, a marked cancer predisposition (Gatti et al. 1991). While about 1 in 4 children with A-T suffer from leukemia or lymphoma, those patients that survive into late adulthood are at high risk to develop solid carcinomas including breast cancer (Stankovic et al. 1998). Heterozygous carriers of an *ATM* gene mutation, estimated to constitute about 1% of the population, do not develop the clinical symptoms of ataxia or telangiectasia, but they show increased chromosomal radiosensitivity (Tchirkov et al. 1997, Neubauer et al. 2002). The involvement of A-T heterozygosity in breast cancer susceptibility has been suspected for long, beginning as early as in the middle of the 70´s (Swift et al. 1976). Epidemiological studies revealed a two- to sixfold increase in risk for breast cancer among blood relatives of A-T patients compared with spouses (Swift et al. 1987, Swift et al. 1991, Athma et al. 1996, Inskip et al. 1999, Olsen et al. 2001, Thompson et al. 2005). However, it has been difficult to confirm these risk estimates at the population level in diverse case-control studies of familial or unselected breast cancer patients, which raised a considerable debate about the role of *ATM* gene mutations in breast cancer susceptibility (Broeks et al. 2000, Dörk et al. 2001). A recent sequencing study on familial breast cancer cases without *BRCA1/2* mutations (Renwick et al. 2006) has provided strong evidence that truncating *ATM* mutations or, more general, *ATM* mutations which cause A-T in the homozygous state, are breast cancer susceptibility alleles associated with an approximately two- to three-fold increase in risk of the disease, and results in this thesis support this view.

The *ATM* gene encodes a 350 kDa protein that localizes to the nucleus in mitotic cells. The ATM kinase is a key protein in signaling the presence of and responding to DSB (Shiloh 2003, Yang et al 2004). ATM belongs to a conserved family of proteins, most of which possess a serine/threonine kinase activity, and all of these proteins share three motifs: the FAT and FATC domains of unknown functional significance (except that the FAT domain of ATM contains the site of autophosphorylation during ATM activation – serine 1981, see below and figure 1.8) and a domain with a motif typical for phosphatidylinositol 3-kinases (PI3K) – the catalytic site in the active kinases of the family. The mammalian members of this family, which are known at present to be involved in the DNA damage response are DNA-PKcs, ATM, ATR and ATX. ATR and ATX respond to both UV (ultra violet light) damage and DSBs, and ATR also responds to stalled replication forks, whereas ATM and DNA-PKcs respond primarily to DSBs. These pathways are related to each other by sharing a set of substrates (such as CHEK2 and Chk1, the checkpoint kinase 1) and serving the similar purpose at a different time (Brumbaugh et al, 2004; Abraham, 2001). It was shown that ATM and the nuclease activity of MRE11 are required for the processing of DSBs to generate the replication protein A (RPA)-coated ssDNA that is needed for ATR recruitment and the subsequent phosphorylation and activation of Chk1. Efficient ATM-dependent ATR activation

in response to DSBs is restricted to the S and G2 cell cycle phases and requires CDK kinase activity. Thus, in response to DSBs, ATR activation is regulated by ATM in a cell-cycle dependent manner (Jazayeri et al. 2006).

ATM resides in undamaged cells present in inactive dimeric or higher order multimeric form (Bakkenist and Kastan 2004). DNA damage induced by ionizing radiation triggers the auto- or trans-phosphorylation of the serine amino acid residue at position 1981 (Ser^{1981}) in the ATM polypeptide. This leads to the dissociation of inactive ATM complex into catalytically active ATM monomers (Figure 1.8), which in turn activate, in response to chromosome breakage, other oncologically relevant target proteins such as BRCA1, p53, CHEK2, nibrin and others. These signaling events then mediate diverse downstream cellular responses.

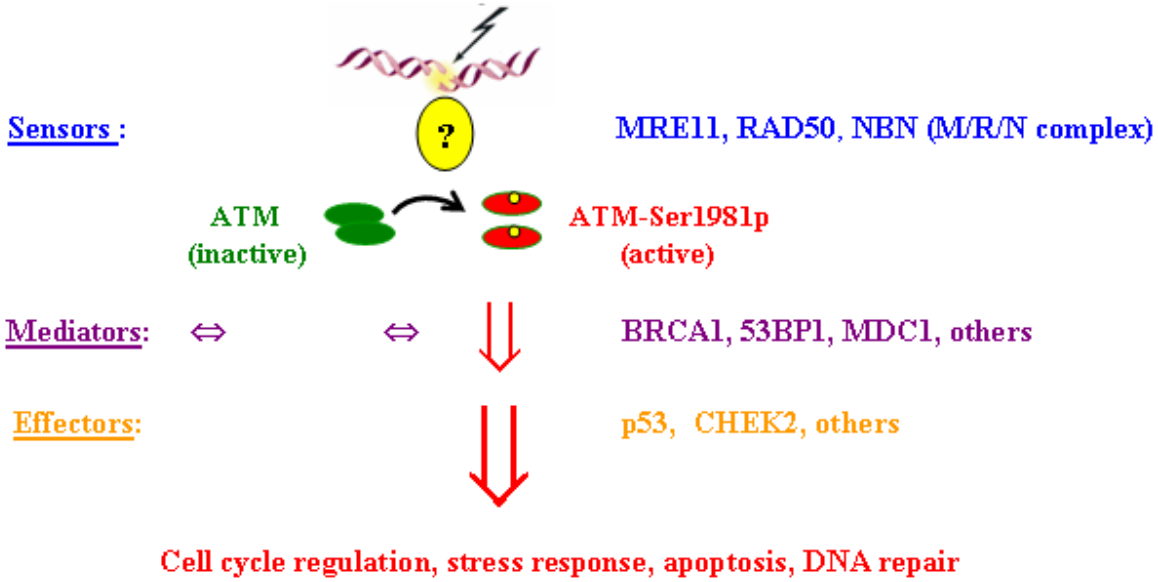


Figure 1.8. **Scheme of ATM protein kinase activation and function.** The ATM protein kinase initiates a complex signal transduction cascade to halt the cell cycle and facilitate repair in response to double-strand DNA breaks (DSBs).

The MRN complex is an essential mediator of ATM recruitment to DSB and activation by DSB (it is both a sensor and effector of ATM activation and signaling in response to DSB). MRE11 is a DNA binding protein, which has 3',5'-exonuclease activity. RAD50 forms homodimers that associate with two MRE11 molecules to form a tetrameric MRE11-RAD50 complex (MR). This complex has a kind of structure that forms bridges between free DNA ends or sister chromatids. Then the p95/nibrin subunit (recruited to the sites of DSB possibly by the direct interaction with phosphorylated histone H2AX) joins to form the MRN complex and, guided by the nibrin carboxyl terminus and possibly by interaction with RAD50, inactive ATM dimers are recruited to DSB sites. Activation of ATM may be triggered by a conformational change in nibrin (reviewed by Abraham and Tibbetts, 2005). MRN may also trigger a conformational change in ATM that

stimulates substrate recruitment. Activated ATM monomers phosphorylate either colocalized substrates or diffuse away from DSBs and phosphorylate other mediators and effectors. ATM targets serine or threonine residues followed by glutamine (S/TQ motifs). The hallmark of ATM's response to DSB is a rapid increase in its kinase activity immediately following DSB formation. ATM-mediated phosphorylation either enhances or represses the activity of its targets, thereby affecting specific processes in which these proteins are involved. Loss of ATM function results in „radioresistant" DNA synthesis, i.e. unhalted progression through S-phase (Painter and Young 1980), and leads to an accumulation of chromosomal aberrations, which in turn can cause malignant cell growth.

1.7.3.3. NBN

Another radiation sensitivity syndrome related to A-T is the Nijmeen Breakage Syndrome (NBS), an autosomal recessive disorder that is predominantly found in populations of Slavic descent including Poland, Czech Republic and Russia. The clinical hallmarks of NBS are microcephaly, growth retardation, immunodeficiency and a high cancer disposition towards leukemias and lymphomas (van der Burgt et al. 1996). Because female NBS patients do not pubertize without hormone substitution and most of them do not survive into adulthood, breast cancer is not a known feature of NBS. However, heterozygous blood relatives, i.e. carriers of one NBS mutation, appear to face an increased cancer risk, including breast cancer (Seemanova 1990, Seemanova et al. 2006, 2007). Most cases of NBS are due to germline mutations in the *NBN* gene (previously termed *NBS1*, Varon et al. 1998). The NBN protein, nibrin, is a 95 kDa nuclear protein, which belongs to the many targets of the ATM kinase (Gatei et al. 2000, Lim et al. 2000, Wu et al. 2000, Zhao et al. 2000). Nibrin is phosphorylated at multiple sites after radiation exposure and is present in radiation-induced foci, together with RAD50 and MRE11 (as part of the MRN complex) and BRCA1, which form at the sites of DNA double strand breaks (Carney et al. 1998, Zhong et al. 1999) (Figure 1.9). Nibrin contains several functional regions: a forkhead-associated (FHA) domain and a "BRCA1 C-terminal" (BRCT) domain at the N-terminus, several SQ motifs (consensus phosphorylation sites by ATM and ATR kinases) in its central region, and MRE11-and ATM-binding sites at the C-terminus. Nibrin is required for several processes protecting chromosomal stability, including sensing DNA double-strand breaks, cell cycle checkpoint regulation and telomere maintenance (Digweed and Sperling, 2004).

In approximately 90% of NBS cases, the *NBN* gene defect could be attributed to the 657del5 mutation, a frameshift deletion (Varon et al 1998). This Slavic founder mutation (Varon et al 1998, 2000) was later suggested in some association studies to be associated with increased breast cancer risk in Polish and Russian populations (Gorski et al. 2003,2005a; Steffen et al. 2004,2006; Buslov

et al. 2005) and work included in this thesis has corroborated this assumption (Bogdanova et al. 2008). *NBN* has since been established as one of breast cancer susceptibility genes with about three-fold increase risk in disease.

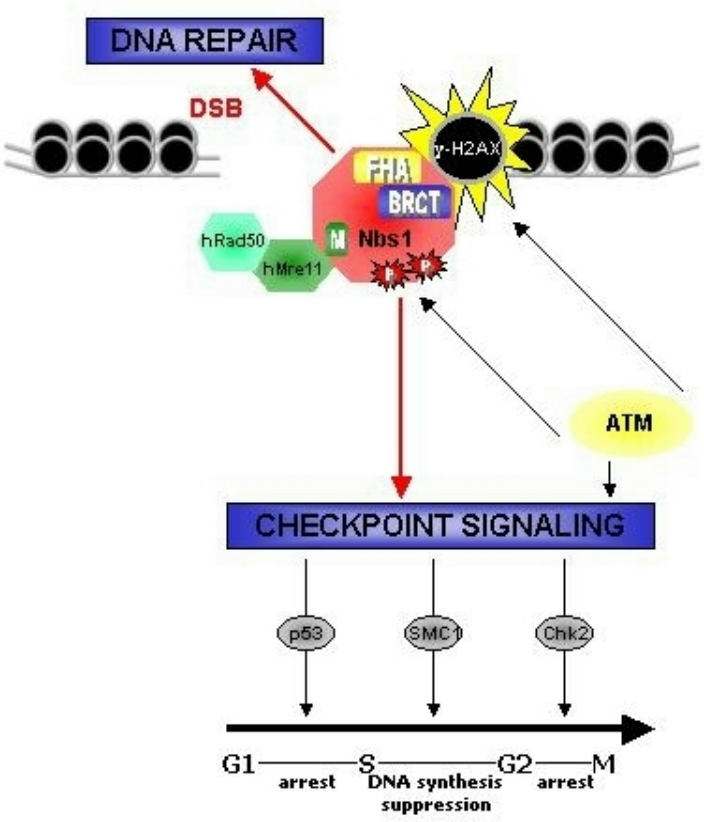


Figure1.9. **DNA damage response involving NBN** (p95/NBS1).Nbs1 acts in the ATM-dependent cell cycle checkpoint activation cascade, possibly as a signal modifier/adaptor in multiple pathways (***Electronic database information: www.nijmegenbreakagesyndrome.net***).NBS1 is recruited to the sites of DSB by some interaction with phosphorylated histone H2AX (γ-H2AX) and subsequently interacts with RAD50 and MRE11 in a multimeric complex that forms foci at sites of DNA damage. This complex has DNA binding and nuclease activity, is essential for normal radiation sensitivity and has a role in lesion processing and repair. At least two different SQ motifs are phosphorylated by ATM in response to DSBs. Intra-S phase checkpoint then appears to be mediated by two parallel routes, one of them involving ATM, NBS1 and SMC1. NBS1 also modulates ATM-mediated phosphorylation of other substrates, such as p53 and CHEK2. More over, NBS1 has been proposed as a p53-independent MDM2 binding protein and links MDM2 to the MRN- regulated DNA repair response (Alt et al 2005).

1.7.3.4 CHEK2

The CHEK2 (previously termed Chk2) protein is a cell cycle regulator originally identified in yeast ("checkpoint kinase 2", also known as Rad53 or Cds1). The *CHEK2* gene encodes the human homolog. The CHEK2 protein is a central mediator of cellular responses to DNA damage (Bartek et al. 2001; Ahn et al. 2004). The Chk2/Rad53/Cds1 family of proteins is characterized by the presence of one or more FHA domains, a Ser/Thr kinase domain, and N-terminal regions rich in Ser-Gln and Thr-Gln (SQ/TQ) amino acid motifs. Ionizing radiation activates the CHEK2 protein via ATM-mediated phosphorylation (Matsuoka et al, 2000; Falck et al, 2001), and activated CHEK2 kinase can subsequently phosphorylate several substrates including Cdc25A (name refers to "**c**ell **d**ivision **c**ycle", controls entry into and progression through S-phase and mitosis), p53, BRCA1 and FoxM1 (forkhead box M1 – transcription factor that regulates expression of cell cycle genes essential for DNA replication and mitosis). The activated Chk2 substrates then mediate cell cycle arrest, apoptosis and the expression of DNA repair enzymes (for example, phosphorylation of

FoxM1 by CHEK2 may increase transcription of XRCC1 and BRCA2) (Bartek and Lukas, 2003; Tan et al. 2007) (Figure 1.10). Furthermore, CHEK2 has also been reported to regulate E2F1 transcription factor activity in response to the DNA damaging agent etoposide (Stevens et al. 2003). On the other hand, E2F1 expression results in an increase in CHEK2 protein levels and may be essential for p53 activation and apoptosis induction (Rogoff et al. 2004). Ectopic expression of E2F1 induces the ATM dependent phosphorylation of CHEK2 and stimulates the kinase activity of CHEK2 (Powers et al. 2004). NBS1 is also required for the induction of CHEK2 phosphorylation induced by E2F1 (Powers et al. 2004). Moreover, CHEK2 may play a critical role in the induction of the pro-apoptotic transcription factor p73 following DNA damage (Urist et al. 2004). The upstream regulation of CHEK2 may also be more complex. A recent study suggests that a mitotic checkpoint kinase TTK participates in the regulation of DNA damage by functioning upstream of CHEK2 and phosphorylating it (Wei et al. 2005). DNA dependent protein kinase is also suggested to be involved in the activation of CHEK2 in response to DNA damage (Li and Stern 2005).

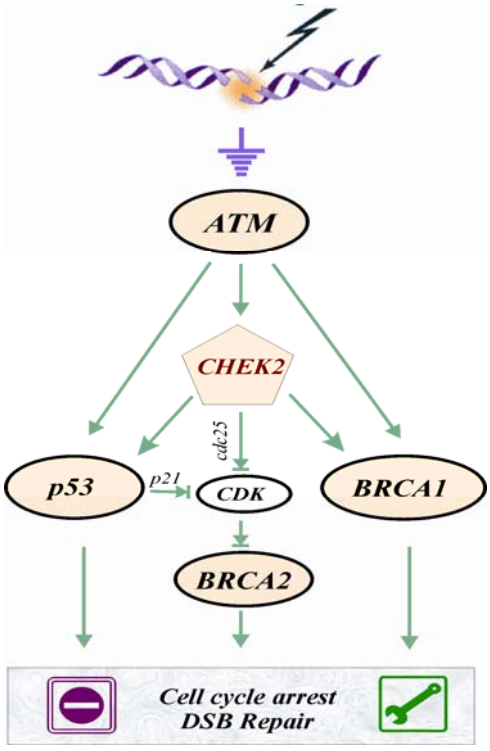


Figure 1.10: **Central role of the CHEK2 protein in the cellular response to ionizing radiation / induced DNA double strand breaks.** This type of damage activates the ATM kinase that phosphorylates CHEK2 on Thr68 and further residues. The activated CHEK2 kinase in turn regulates the activity of other oncoproteins (such as p53, BRCA1 and BRCA2) which, in the healthy state, finally results in cell cycle arrest (preventing entry into S-phase and mitosis) and DNA double strand break repair.

The dimeric CHEK2 protein functions as central mediator of signal transduction pathways induced by DNA damage and shares some of its downstream effectors with Chk1. Through targeting their substrates, Chk1 and CHEK2 regulate fundamental cellular function, and being the critical messengers of the genomic integrity checkpoints, they initiate a secondary wave of phosphorylation events (after ATM and ATR, which initiate a signaling cascade) to extend signaling. CHEK2 phosphorylation of BRCA1 regulates DNA double-strand break repair, and deletion of *CHEK2* potentiates the incidence of mammary carcinomas in *BRCA1* conditional mutant mice (McPherson

et al. 2004). In addition, the activation of CHEK2 by ATM may also regulate PML-dependent apoptosis after gamma irradiation-induced DNA damage (Yang et al. 2002). In vitro, CHEK2 is capable of phosphorylating all members of the Cdc25 family (Matsuoka et al 1998). After DNA damage, CHEK2 participates in the phosphorylation of p53 on Ser20, attenuating the binding of p53 to MDM2 and allowing accumulation and subsequent activation of p21 and G1 arrest (Hirao et al 2000). Noteworthy, inherited mutations of *CHEK2*, like those of *ATM* and *NBN*, confer tumor susceptibility. Initial findings showing germline *CHEK2* mutations in a subset of cancer-prone Li-Fraumeni cases with wild type of p53 (Bell et al 1999a) further underscored the function of cell cycle checkpoints in preventing genetic instability and cancer. A recurrent mutation in the CHEK2 gene (1100delC) was first proposed to be an important cause of breast cancer in 2002 (Meijers-Heijbor et al. 2002, Vahteristo et al. 2002). Since then, numerous studies including this thesis have reported on the prevalence of this mutation and other functionally relevant *CHEK2* mutations in various populations, identifying *CHEK2* as a low-penetrance breast cancer susceptibility allele with approximately two-fold increased risk for breast cancer (The *CHEK2* Breast Cancer Case-Control Consortium 2004, Bogdanova et al. 2005, Gorski et al. 2005a, Cybulski et al. 2006, Walsh et al. 2006, Cybulski et al. 2006, reviewed by Nevanlinna and Bartek 2006).

1.7.3.5 PALB2 and BRIP1: The, Fanconi Anemia pathway and its interaction with DSB repair

PALB2 encodes a recently discovered protein that interacts with BRCA2, is implicated in its nuclear localization and stability and is required for some functions of BRCA2 in homologous recombination and double-strand break repair (Xia et al. 2006). Biallelic *PALB2* mutations are responsible for a subset of Fanconi anemia cases characterized by a phenotype similar to that caused by biallelic *BRCA2* mutations, and constitute a new FA complementation group FA-N (Xia et al. 2007, Reid S et al. 2007). *BRIP1* that interacts with BRCA1 was also found and described as one of the FA genes, biallelic mutations in which are responsible for the Fanconi anemia subtype FA-J (Levran et al. 2005). Most of the FA proteins form a multiprotein E3 ubiquitin ligase, known as FA nuclear core complex that activates FANCD2 (Fanconi anemia, complementation group D2) via monoubiquitination. This ubiquitinylation requires ATM/ATR-dependent phosphorylation in at least two sites: cell cycle checkpoint kinase ATR is required for the efficient monoubiquitination of FANCD2 and also for the function of histone H2AX (Pichierri et al. 2004). H2AX, recently identified as a component of FA network, seems to play a crucial role mediating monoubiquitinylated FANCD2 recruitment to chromatin (Bogliolo et al. 2007). As described above, following DNA damage, ATM phosphorylates H2AX, which forms a megabase length tract marking DNA damaged sites and recruiting other proteins of DNA repair pathways: BRCA1/2,

RAD51, MRE11, NBS1, MDC1, monoubiquitinated FANCD2 and p53 (Fillingham et al. 2006, Tanaka et al. 2006, Bouquet et al. 2006). BRCA1 in turn, helps to mediate the recruitment of FANCD2 by phosphorylated H2AX to damaged chromatin. By contrast, BRCA2, PALB2 and BRIP1 appear to function downstream of the FANCD2 activation step. PALB2 binds to the extreme N terminus of BRCA2 and stabilizes BRCA2 in key nuclear structures, allowing it to function in DNA repair and at the S phase checkpoint and also function as FANCD1 downstream (figure 1.11). Heterozygosity for mutations in *PALB2* and *BRIP1* was found to be associated with increased breast cancer risk (Rahman et al. 2007, Seal et al. 2006), and a *PALB2* founder mutation is associated with breast cancer in Finland (Erkko et al. 2007). Thus, *PALB2* and *BRIP1* appear to be the latest additions to the growing list of genes associated with a moderately, approximately 2-fold increased risk of breast cancer, where they join the *CHEK2* (the first gene of this type described), *NBN* and *ATM* genes.

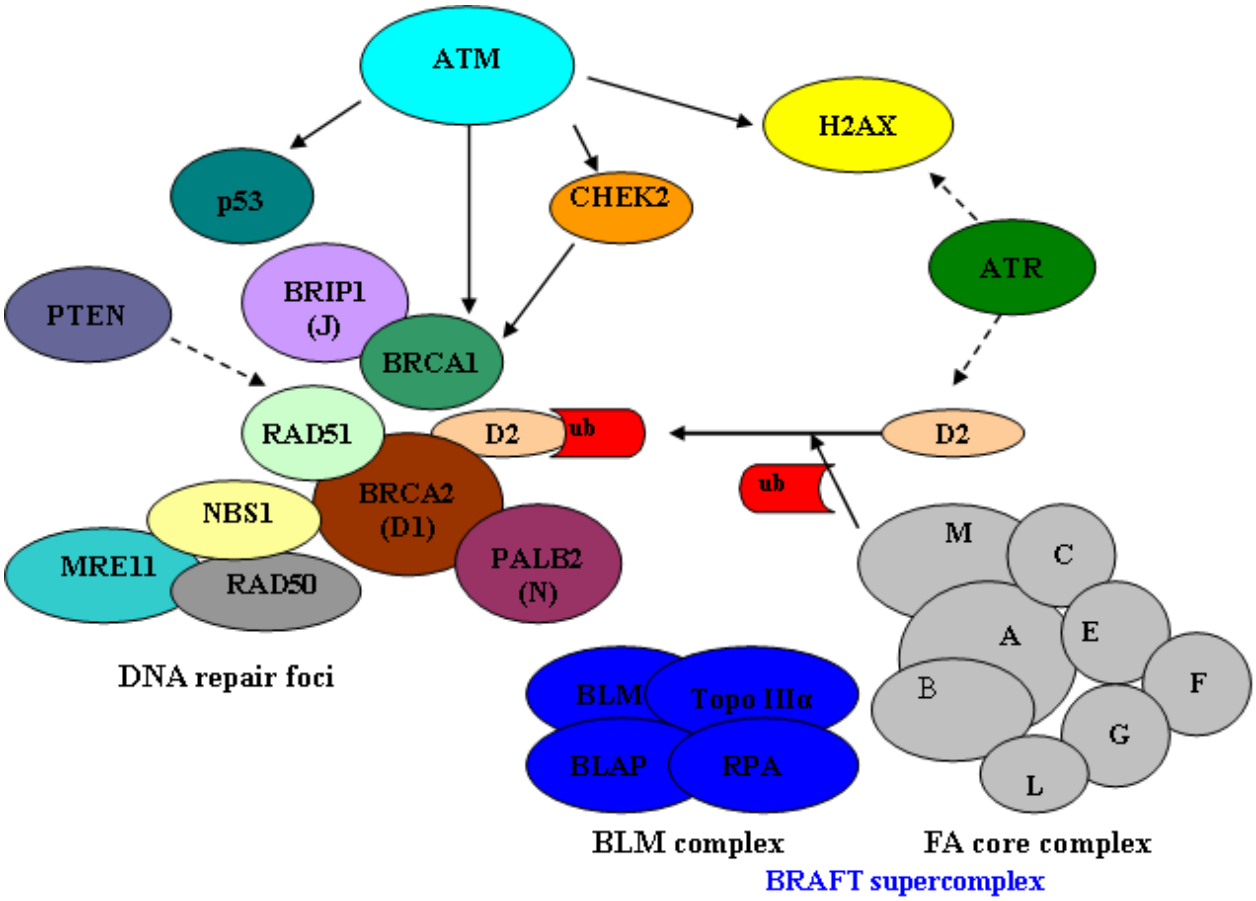


Figure 1.11. **Schematic diagram of the Fanconi anemia–BLM-BRCA pathway and its interaction with BRCA1 pathway.** The Fanconi anemia core complex consists of at least eight Fanconi anemia proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) and is essential for the monoubiquitination and activation of FANCD2 ('D2' in the figure) after DNA damage. Activated FANCD2 is translocated to DNA repair foci, where it colocalizes with other DNA damage response proteins, including BRCA1, BRCA2 and RAD51, and participates in homology-directed repair. DNA damage activates ATM and CHEK2, which in turn activate p53 and BRCA1 by phosphorylation. PTEN binds to the RAD51 promoter and may regulate its transcription (Shen et al., 2007). H2AX has been reported to be essential for

the recruitment of repair/DNA damage response proteins to the site of DNA damage or replication break sites. H2AX is functionally connected to the FA/BRCA pathway to resolve stalled replication forks thus preventing chromosome instability. Once the DNA lesions are repaired, no signal for ATR activation is present and the FA pathway is inactivated. BRCA1 mediates FANCD2 foci formation and colocalizes with FANCD2 at damaged sites, but it does not interact with FANCD2 directly and is dispensable for FANCD2 monoubiquitination. The range of chromosomal abnormalities in FA cells closely resemble those of Bloom syndrome (Joenje et al. 2001), a genetic disease that also features genomic instability and cancer predisposition. In fact, the FA core complex, was purified as part of a larger multiprotein complex with BLM (Meetei et al. 2003, Ciccia et al. 2007, Ling et al. 2007), termed BRAFT (for BLM, replication protein A (RPA), FA and topoisomerase IIIα). BLM is a 3′-to-5′ DNA helicase that can resolve many DNA structures. Topoisomerase IIIα (Topo IIIα) is a type I topoisomerase that works with the BLM helicase to resolve recombination intermediates, such as double-Holliday junctions. The BLM-associated protein BLAP binds double-Holliday junctions and promotes loading of Topo IIIα onto the DNA. Replication protein A (RPA) binds ssDNA and participates in replication, repair and activation of ATM and ATR (scheme based on Rahman et al. 2007)

1.7.4 Additional breast cancer candidate genes involved in DNA repair

The functional analyses of hitherto identified breast cancer genes demonstrate an intriguing connection between the repair of radiation-induced DNA double-strand breaks and breast cancer risk. In fact, disturbances of chromosome break repair appear to play a major role in the development of breast cancer, comparable with the role of mismatch repair in colon cancer or UV-induced damage repair in skin cancer. Why dysfunction of chromosome break repair is of such particular importance for breast tumors is still unknown. However, these findings show the way for the identification of additional breast cancer predisposition alleles since known genes involved in the same pathways whose role is to preserve genomic integrity. Clearly other genes in this pathway are worth to be subject of intense genomic analysis. Furthermore, there may be thus far unrecognized members of this pathway, mutations in which may also be associated with breast cancer.

Some candidates can be assumed among proteins that bind to BRCA1 or BRCA2. One of the partners of BRCA1, the protein **BARD1** („BRCA1-associated RING domain“), is thought to mediate a radiation-induced inhibition of mRNA polyadenylation (Kleiman und Manley 2001) and may be involved in sporadic or hereditary breast cancer (Thai et al. 1998, Karppinen et al. 2006). But more recent studies have not provided evidence for breast cancer susceptibility alleles in *BARD1* (Jakubowska et al. 2008). Another interaction partner of both the BRCA1 and BRCA2 proteins is **RAD51**, a protein essential for homologous recombination. A single-nucleotide polymorphism (SNP) in the 5' untranslated region (UTR) of RAD51, 135G-->C, has been suggested as a possible modifier of breast cancer risk in *BRCA1* and *BRCA2* mutation carriers (Antoniou et al. 2007). Because the RAD51 protein interacts with **RAD52** in homologous recombination, common truncated variants of *RAD52* may also be candidate modifiers (Bell et al. 1999b). Another binding partner of BRCA1 is the **RAD50** protein which is a part of the nuclear MRN repair complex. The *MRE11* and *RAD50* genes both underlie rare hereditary radiation sensitivity syndromes, which

resemble ataxia-telangiectasia and Nijmegen Breakage syndrome, respectively (Stewart et al. 1999, Waltes 2002). It is possible, therefore, that variations in these both genes may be associated with radiation sensitivity and breast cancer risk, and a protein-truncating allele of *RAD50* identified in northern Finland appeared to confer an approximate 4-fold increased risk of familial breast cancer in this population (Heikkinen et al. 2006). Finally, proteins involved in the upstream regulation of these gene products could be strong candidates for breast cancer susceptibility. For example, **ATR**, that is inducible by replication blocks and has several target proteins in common with the homologous ATM kinase, such as p53 or BRCA1. ATR appears to act as a maintenance kinase in ionizing radiation- induced signaling. However, a study of ATR sequence variants has not revealed alterations associated with breast cancer (Heikkinen et al. 2005). Another regulator of BRCA1 is **53BP1**, a p53-binding protein that modulates the radiation-induced phosphorylation of BRCA1 and CHEK2, and appears to be essential for the recruitment of BRCA1 into radiation-induced repair foci (DiTullio et al. 2002). **MDC1** also regulates BRCA1 functions (Lou et al 2003), is required for response of DNA damage, and facilitates recruitment of the ATM and MRN complex to DNA damage foci (Stucki and Jackson 2005). Further proteins involved in DNA double strand break repair include the XRCC proteins, one of which is the XRCC11/BRCA2 protein. The ***XRCC* genes** have initially been identified by complementation of radiation sensitivity of CHO cells and are regarded as suppressors of radiation-induced chromosome aberrations. The XRCC4 protein (together with **DNA ligase IV**), as well as XRCC6/KU70, XRCC5/KU80, for example, act to repair DNA double strand breaks in the "non-homologous end joining" pathway (see 1.4.1) and thereby prevent oncogenic translocations. Several common gene variants of *XRCC1*, *XRCC2*, *XRCC3*, *XRCC4*, *XRCC5*, *XRCC6* and *LIG4*/DNA-Ligase IV were identified as potential modifiers of breast cancer risk (Price et al. 1997, Lunn et al. 1999, Goode et al. 2002, Kuschel et al. 2002, Rafii et al. 2002, Fu et al. 2003), though a large international Consortium study has refuted some of the proposed associations (Breast Cancer Association Consortium 2006). As the ***XRCC9*** gene has been uncovered to be the gene underlying one form of Fanconi anemia – FANCG (de Winter et al. 1998), *BRCA2* was identified as the gene responsible for Fanconi anemia type D, i.e. the FANCD1 gene (Howlett et al. 2002), *PALB2* as FANCN, and finally the FA pathway is connected with the BRCA1 pathway via H2AX, some speculations have been raised that other Fanconi anemia proteins may also be candidates for breast cancer susceptibility.

1.7.5 Polymorphic variants and breast cancer susceptibility

Since the identification of *BRCA1* and *BRCA2*, researches tried to identify additional high-penetrance breast cancer genes („*BRCA3*") or genetic modifiers by using traditional linkage studies, but except for the identification of the *CHEK2* gene, they unfortunately have failed. These

observations have led to the conclusion that breast cancer susceptibility may be largely 'polygenic' and the progress in identifying the relevant loci has been slow. As linkage studies lack power to detect alleles with moderate effects on risk, large case-control association studies were required. Such studies have confirmed the role of mutations in the candidate DNA repair genes *ATM*, *NBS*, *CHEK2*, *BRIP1* and *PALB2* that confer an approximately twofold risk of breast cancer. These genes share two important features in their impact on breast cancer:

1. a single deleterious mutation in any one is sufficient to significantly increase breast cancer risk;
2. there are many deleterious mutations, and each variant is individually rare in population (reviewed by Walsh and King 2007).

It is still the majority of familial breast cancer cases, however, which remains unexplained by any of these genes. Advances in association studies have been furthered by the recent progress in the discovery of single nucleotide polymorphisms (SNPs); their vast density throughout the genome, ease of genotyping and moderate cost contribute greatly to their utility. Association testing is efficient when the SNPs being analyzed represent the entire genetic variation of the gene. Evidence has been obtained that nearby SNPs are organized into regions of high linkage disequilibrium (LD) separated by short segments of very low LD. Regions of high LD contain redundant information and can be reduced to smaller subsets of tagging- SNPs (tSNPs), such that tSNPs identify all common haplotypes within the region of high LD. Technological advances have provided possibility to investigate hundreds of thousands of SNPs in one go, that giving a basis for identifying moderate risk alleles without prior knowledge of position or function. However, because recombination tends to occur at distinct 'hot-spots', the majority of common genetic variants can be evaluated for association using a few hundred thousand SNPs as tags for all the other variants. Recently, common missense variants in two genes, ***CASP8*** (caspase 8, an important initiator of apoptosis and is activated by external death signals and in response to DNA damage) and ***TGFB1*** (transforming growth factor beta 1, controls proliferation, differentiation, and other cell functions), have been shown to be associated with breast cancer risk through a sufficiently powered multicenter analyses, including our group (Cox et al. 2007). This study demonstrated the importance of large-scale analysis, because individual studies often have not enough statistical power to identify common variants conferring modest increases in the risk of breast cancer. Towards this goal, to facilitate such collaborative studies in breast cancer, the Breast Cancer Association Consortium (BCAC) was established in April 2005. The consortium currently includes over 25 international collaborating research groups, with a potential combined sample size of more than 30,000 cases and 30,000 controls. A recent study of the BCAC revealed novel independent breast cancer susceptibility loci that were identified through a whole-genome scan and contain plausible

candidate genes (***FGFR2*** - fibroblast growth factor receptor 2, influence mitogenesis and differentiation; ***TNRC9*** – also known as *TOX3* high mobility group box family member 3, binds to DNA, regulates transcription; ***MAP3K1*** - mitogen-activated protein kinase, involved in cellular response to a number of mitogenic and metabolic stimuli, including insulin and many growth factors; and ***LSP1*** - lymphocyte-specific protein 1, an intracellular F-actin binding protein). Tagging SNPs at these and two further loci: 2q35 qnd 8q24, exhibited strong and consistent evidence of association with breast cancer (Easton et al. 2007, Stacey et al. 2007); more details can be found in the results section of this thesis. To date it is not known how these genes interact with each other or with lifestyle factors, each of which may increase the risk (Easton et al. 2007). But these reports indicate that many additional common susceptibility alleles may be identifiable by this approach.

1.8 Aim of the project

The main aim of this thesis was to examine the role of heritable genetic factors in the development of breast cancer, with a particular focus on the Byelorussian population and their potential chronic exposure to low-dose ionizing radiation after the Chernobyl accident. Towards this goal, the prevalence of founder mutations in major DNA double-strand break repair genes and the prevalence of common polymorphic variants identified as breast cancer susceptibility alleles should be comparatively investigated in a case-control series of some 1000 breast cancer patients and 1000 population controls from Hannover Medical School and in a similarly sized case-control series to be established from different regions in the Republic of Belarus. This study should delineate the geographic distribution of candidate mutations and provide risk estimates for the identified susceptibility alleles. Where appropriate, some of the mutations should further be characterized by functional assessment of the radiation-induced DNA damage response in patient lymphoblastoid cell lines. Furthermore, clinical evaluation of patient data should uncover genotype-phenotype correlations with regard to age at diagnosis, bilaterality, family history and tumour characteristics in mutation carriers. Altogether, the results should for the first time reveal the mutational distribution of breast cancer susceptibility genes in the Byelorussian population and provide an initial data set to increase our insights into the radiobiology of breast cancer.

2. Materials

2.1 Chemicals and reagents

Acrylamide, 40% / Bisacrylamide 19:1	Biorad-Laboratories, München
Acrylamid, 40%	Biorad-Laboratories, München
Agarose	Invitrogen Life Technologies,
Agarose-1000	Invitrogen Life Technologies
Aprotinin	Serva, Feinbiochemika, Heidelberg
Ammonium persulfate (APS)	Biorad-Laboratories, München
β-Glycerophosphat	Merck, Darmstadt
Bisacrylamide, 2%	Biorad-Laboratories, München
Boric Acid	Gibco_BRL, Eggenstein
Bromphenolblue	Sigma Chemie, Steinheim
Coomassie Brilliant Blue G250	Serva, Feinbiochemika, Heidelberg
Chloroform	J.T.Baker, Deventer, Niederlande
DEPC (diethylpyrocarbonat)	Sigma Chemie, Steinheim
DTT (dithiothreitol)	Sigma Chemie, Steinheim
EDTA	Sigma Chemie, Steinheim
EGTA	Serva, Feinbiochemika, Heidelberg
Ethidium bromide	Sigma Chemie, Steinheim
Ethanol, abs. 99,8%	Merck, Darmstadt
Formaldehyde, 37%	Merck, Darmstadt
Formamide	Merck, Darmstadt
Glycerin, 87%	Merck, Darmstadt
Glycerin, abs.	Merck, Darmstadt
Glycine	Merck, Darmstadt
Guanidiniumthiocyanat	Fluka Feinchemikalien, Neu-Ulm
HPLC-grade water	J.T.Baker, Deventer, Niederlande
Isopropanol	Merck, Darmstadt
Potassium chloride	Merck, Darmstadt
Leupeptin	Serva, Feinbiochemika, Heidelberg
Magnesium chloride	Merck, Darmstadt
Mercaptoethanol	Sigma Chemie, Steinheim
3-Methacryloxypropyltrimethoxysilane	Merck, Darmstadt
Methanol	Merck, Darmstadt
Sodium acetate	Merck, Darmstadt

Sodium carbonate, water free	Merck, Darmstadt
Sodium chloride	Merck, Darmstadt
Sodium fluoride	Sigma Chemie, Steinheim
Sodium hydrogencarbonate	Merck, Darmstadt
Sodium thiosulfate	Sigma Chemie, Steinheim
Sodium metavanadate	Sigma Chemie, Steinheim
Nonidet P-40	Sigma Chemie, Steinheim
Phenol	Merck, Darmstadt
Phenol/ Chloroform/ Isoamyl alcohol	ICN Biomedicals, Eschwege
Polyethylenglycol 8000	Sigma Chemie, Steinheim
Phenylmethanesulphanylflouride (PMSF)	Serva, Feinbiochemika, Heidelberg
SDS (Sodium Dodecyl Sulphate)	Serva, Feinbiochemika, Heidelberg
Seakem-Agarose	Biozym, Hess. Oldendorf
NNN'N'Tetrqmethylethan-1,2-diamin (TEMED)	Serva, Feinbiochemika, Heidelberg
Tris	Merck, Darmstadt
Tris-HCl	Merck, Darmstadt
Triton	Sigma Chemie, Deisenhofen
Tween 20	Sigma Chemie, Deisenhofen
Xylencyanol FF	Sigma Chemie, Deisenhofen

All chemicals not mentioned above will be outlined in certain paragraphs.

2.2 Enzymes, biological substances

Ampicillin	Invitrogen BV, Groningen, Niederlande
Desoxyribonucleoside triphosphates (dNTPs)	Boehringer, Mannheim Biochemika
Low-fat milk powder	Marvel, UK
Oligodesoxyribonucleotides (primers)	GE Healthcare, Freiburg
	Invitrogen, Eggenstein
	Eurogentec, Seraing, Belgium

The synthetic oligonucleotide primers used either for PCR or sequencing of gDNA and/or cDNA are listed in attachment 1.

Proteinase K	Merck, Darmstadt
Restriction endonucleases and implements	New England Biolabs, Schwalbach
Taq DNA-Polymerase and implements	Promega, Mancheim
	Qiagen, Hilden

Antibodies:

Mouse-anti-human β-Actin	A5441 (1:3000), Sigma, St.Louis, USA
Rabbit-anti-human Nibrin	hp95/Nibrin/NBS total (1:5000); Novus Biologicals, Littleton
pRabbit -anti-human Nibrin	Phospho-p95/NBS (Ser343), (1:500); Cell Signaling, New England Biolabs GmbH Frankfurt am Main
Rabbit-anti-human CHEK2	CHEK2 total (1:1000); Cell Signaling, New England Biolabs GmbH Frankfurt am Main
pRabbit-anti-human CHEK2	pCHEK2 (Ser19),(1:1000); Cell Signaling, New England Biolabs GmbH Frankfurt am Main
pRabbit-anti-human CHEK2	pCHEK2 (Ser33/35), (1:1000); Cell Signaling, New England Biolabs GmbH Frankfurt am Main
pRabbit-anti-human CHEK2	pCHEK2 Thr68 (1:500); Cell Signaling, New England Biolabs GmbH Frankfurt am Main

DNA molecular weight ladders:

1 kb DNA Ladder	Invitrogen/Gibco BRL, Eggenstein
100 bp DNA Ladder	Invitrogen/Gibco BRL, Eggenstein

Protein molecular weight ladders:

Rainbow Coloured Protein Molecular Weight Marker (14,3 – 220 kDa)	GE Healthcare, Freiburg

Cell culture medium and implements:

Cyclosporin A	Sigma Chemie, Deisenhofen
DMSO	Sigma Chemie, Deisenhofen
Fetal calf serum (FCS)	Seromed (Biochrom), Berlin

Hygromycin	Boehringer, Mannheim Biochemika
Cryocontainer	Greiner, Frickenhausen; Sarstedt, Nümbrecht
Penicillin-Streptomycin (500x)	Boehringer, Mannheim Biochemika
RPMI 1640 with L-Glutamine	Invitrogen/Gibco-BRL, Eggenstein
Sterile filter (Minisart 0,45 und 0,20µm)	Sartorius, Göttingen
MEBM medium and supplements for HMEC (growth factors and reagents)	Lonza, Belgium
Trypsin reagents	Lonza, Belgium
Cell culture flask	Nunc, Wiesbaden

2.3 Kits

ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit With AmpliTaq®DNA Polymerase, FS	Applied Biosystems, Weiterstadt
310 Genetic Analyzer Buffer with EDTA	Applied Biosystems, Weiterstadt
3100 Avant POP-6™	Applied Biosystems, Weiterstadt
First Strand cDNA Synthesis Kit	GE Healthcare, Freiburg
Protein Assay Dye Reagent Concentrate	Biorad-Laboratories, München

Western Blot Chemiluminescence Reagents

"PIERCE" – Super Signal West Dura Extended Duration Substrate	Pierce/ Perbio Sciences, USA

2.4 Materials and equipment

2.4.1 Films

Hyperfilm ECL	GE Healthcare, UK

2.4.2 Equipment

ABI PRISM 3100 Avant Genetic Analyzer	Applied Biosystems, Darmstadt
Capillary, 36 cm	Applied Biosystems, Darmstadt
Printer	Hewlett Packard Deskjet 6122
Computer	DELL GX 270
Monitor	17in FLTAT 1703 FRGRAY

ABI PRISM 7000 Sequence Detection System	Applied Biosystems, Darmstadt
Printer	Hewlett Packard Deskjet 9900xi
Computer	DELL LATITUDE C810 LAPTOP
Autoclav LVSA 50/70	Zirbus, Bad Grund
Autoradiography cassette	GE Healthcare, UK
Electrophoresis chamber	
horizontal: Horizon 58/11.14/20.25/	Gibco-BRL, Eggenstein
vertical: PEQLAB TwinEx ws	PeqLab, Erlangen
Incubators:	
MEMMERT, Model 400	MEMMERT, Schwabach
‚Hera safe'	Heraeus Sepatech, Osterode
O_2/CO_2 Incubator Sanyo MCO-20AIC	via Landgraf Laborsysteme, Langenhagen
Centrifuges:	
Beckmann J2.21	
(Rotors JA14 und JA20)	Beckmann, München
Beckmann L5-6	
(Rotors SW28, SW50.1)	Beckmann, München
Eppendorf centrifuge 5415C	Eppendorf, Hamburg
Eppendorf centrifuge 5415D	Eppendorf, Hamburg
Eppendorf centrifuge 5810R	Eppendorf, Hamburg
Mini Spin	Eppendorf, Hamburg
Gel documentation system	Biostep GmbH, Jahnsdorf
Camera PIPER FK 751 12IQ-IR	
Computer Belnea	
Printer Mitsubishi P91D	
Transilluminator (312 nm) UV light	
GenAmp® PCR system 2700	Applied Biosystems, Darmstadt
Microwave	Panasonic
pH-Meter	Jürgens, Hannover
(Bio) Photometer	Eppendorf, Hamburg

Pipettes	
Easy pet	Eppendorf, Hamburg
Reference	Eppendorf, Hamburg
Research	Eppendorf, Hamburg
Research Pro	Eppendorf, Hamburg
Serological pipettes	Sarstedt, Nümbrecht
PTC-200 Thermal Cycler MJ Research	Biozym, Hess. Oldendorf
Power Suppliers	
Biometra Standart Power Pack P25	
Biometra Whatman PS 304	Biometra, Göttingen
Scales	Sartorius, Göttingen
	Stuart Scientific über Dunn, Asbach
Shake incubator	Jürgens, Hannover
Tank Transfer Unit	
with power supply EPS 2A200	GE Healthcare, Freiburg
Thermo mixer Comfort	Eppendorf, Hamburg
Thermo mixer HLC	Landgraf Laborsystems, Langenhagen
Thermo mixer 5436	Eppendorf, Hamburg
Thermostat Plus	Eppendorf, Hamburg
Platform Shaker	Stuart Scientific, United Kingdom
UV-Transilluminator	Bachofer Laboratoriumsgeräte
Varioklav	H+P Labortechnik, Oberschleissheim
Vortex Genie-Mixer	Jürgens, Hannover
Water bath	GFL, Burgwedel
7500 Fast-Real Time PCR System	Applied Biosystems, Darmstadt
Printer	Epson Colour 900
Computer	DELL LATITUDE D510 LAPTOP

2.4.3 Small materials

Capillary tips 200µl	Biozym Scientific, Hess. Oldendorf
Crystal tips	Eppendorf, Hamburg
Falcon-tubes	Sarstedt, Nümbrecht
Fast Optical 96-well Reaction Plates	Applied Biosystems, Darmstadt
Filter paper	Whatman, Maidstone
Gloves	Safeskin Corp., San Diego, USA

	Ansell GmbH, München
Kleenex	Kimberly-Clark

Membrane

Hybond™-C extra (Nitrocellulose)	GE Healthcare, Freiburg
Optical Adhesive Films	Applied Biosystems, Darmstadt
Parafilm	American National Can, Greenwich

Pipette tips

1000µl (blue) and 200µl (yellow)	Sarstedt, Nümbrecht
Pincers	Jürgens, Hannover
Reaction tubes (1.5ml and 0.5ml)	Sarstedt, Nümbrecht;
Scalpel	Jürgens, Hannover
Sealing Tapes, optically clear	Sarstedt, Nümbrecht
96-well Multiply®-PCR Plates	Sarstedt, Nümbrecht

2.5 Solutions, medium and buffers

loading buffer (6 x)

0.25 %	Bromphenolblue	
0.25 %	Xylencyanol FF	
100 %	Formamid	

10 x TBE	0.9 M	Tris-HCl	108 g Tris-HCl
	0.9 M	Boric Acid	54 g Boric Acid
	0.02 M	EDTA	7.2 g EDTA
			pH 8.3; ad 1L
10 x PBS	1.4 M	NaCl	80 g
	27 mM	KCl	2 g
	90 mM	Na_2HPO_4	14,4 g
	15 mM	KH_2PO_4	2,4 g
		water	pH 7.4; ad 1L
10 x GTS	1.9 M	Glycine	144 g Glycine

	0.25 M	Tris	30 g Tris
	1 %	SDS	10 g SDS
		water	ad 1L
10 x Carbonate-Puffer	0.1 M	$NaHCO_3$	8.4 g $NaHCO_3$
(Transfer Buffer)	30 mM	Na_2CO_3	3.18 g Na_2CO_3
		water	ad 1L

Blocking-Buffer	1 x	PBS	
	5 %	(w/v)	low-fat milk powder
	0.05 %	(w/v)	Tween 20

Wash-Buffer			
(PBS-T)	1 x	PBS	
	0.05 %	(w/v)	Tween 20

Cell culture medium for lymphoblastoid cells

RPMI-Medium 1640	500 ml
Add:	
Penicillin	50000 U
Streptomycin	50 mg
FCS	10-20 % (v/v)

Cell culture medium for HMEC (Human mammary epithelial) cells

MEBM-Medium (basal medium)		
Mammary Epithelial Basal Medium		
(no growth factors)	500ml	
Add (growth supplements):		
BPE (pitnitary extract)	2ml	MEGM (growth medium)
hEGF (human epidermal growth factor)	0.5ml	
Hydrocortisone	0.5ml	
GA-1000 (gentamycin/amphotericin)	0.5ml	
Insulin	0.5ml	

Reagent pack for trypsinization of HMEC

100ml	Trypsin/EDTA
100ml	HEPES-BSS (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline)

100ml TNS (trypsin neutralizing solution)

2.6 Sterilization of solutions and equipments

All solutions, which were not heat sensitive, were sterilized at 121°C, 105 Pa for 20 min in an autoclav (Zirbus, Bad Grund). Heat sensitive solutions were filtered through a disposable sterile filter (0.2 to 0.45 µm pore size, Millipore, Schwalbach,). Plastic ware was autoclaved, as described above.

2.7 Patients and controls

For the purpose of this study, DNA samples from breast cancer patients and cancer-free control individuals were subjected to molecular genetic analysis, and lymphoblastoid cell lines (LCLs) were established from selected patients for further analyses at the RNA or protein level (LCLs were initially established from peripheral B-lymphocytes through EBV immortalization by Britta Wieland and placed at my disposal for further culturing and studies) Genomic DNA (gDNA) was extracted from peripheral lymphocytes of 5 ml EDTA blood samples and additional gDNA samples were obtained in some cases from established LCLs.

The scope of this project does not include treatment or therapeutic experiments on human individuals. The experiments with patient samples and research using patient data comply with the recommendations of the World Medical Council. All genetic tests were performed after thorough information about the research project has been provided and written informed consent of the respective individual has been obtained. Informed consent of the probands was approved by the Ethics Commission of the State Organization “Institute for Hereditary Diseases”, Ministry of Health, Republic of Belarus, and the project received additional approval from the Ethics Commission at Hannover Medical School (No. 3221). All individuals who wish to be informed in more detail were offered a genetic counseling session before, during or after the end of a mutation screening. Genetic counseling inform about the connection between ionizing radiation and breast cancer, familial risks, sensitivity and specificity of the genetic testing, and options concerning a possible positive test result.

A total of 1759 breast cancer patients were recruited during the course of this thesis who were unselected by age or family history and were diagnosed in the Republic of Belarus during the years 1998-2007 at the Byelorussian Institute for Oncology and Medical Radiology “Aleksandrov N.N.” (Minsk) or at one of five Regional Oncological Centers (Brest, Vitebsk, Grodno, Gomel, Mogilev). The median age of the breast cancer patients was 48 years. The controls were 1019 cancer-free volunteers from the same population who had been ascertained at the Institute for Inherited Diseases in Minsk, Belarus. The median age of the control individuals was 46 years. According

whole body doses accumulated after the Chernobyl accident in the study regions (Pukkala et al, 2006), the investigated cohorts could be divided into five subgroups: **0** not contaminated; **I** <5mSv; **II** 5-19.9 mSv; **III** 20-39.9 mSv and **IV** >40 mSv (table 2.1, figure 2.1). Roughly half of both the cases and the controls from contaminated regions originated from each of the two centers in the Gomel and Mogilev oblasts (table 2.1, figure 2.1). The median age at onset of breast cancer was 48 years for all patients but a marked heterogeneity was noted when patients were stratified by region of origin. The difference in the age at diagnosis for patients in non-contaminated regions – 50 years versus the age at diagnosis in contaminated regions – 44 years, was highly significant: $p< 0.00001$, median test). According dividing into subgroups median age at diagnosis in contaminated regions was 42 years for subgroup **I**, 46 for **II**, 44 for **III** and 43 for **IV** respectively. 298 (17%) of all patients reported at least one first-degree relative affected with breast cancer, 64 patients (3.6%) had a bilateral breast cancer. Of the bilaterally affected cases, 15/64 (23%) reported a family history of the disease, 35/64 (55%) patients were from contaminated areas. 20 patients (1.1%), with 12/20 from contaminated areas, beside breast cancer had also ovarian carcinoma and 14 (0.8%), with 9/14 from contaminated regions, had a relative affected with ovarian carcinoma (figure 2.2 and 2.3).

Table 2.1 **Study groups**

Series	**Total (n)**	**Series**	**Total (n)**
Cases	**1759**	**Controls**	**1019**
Familial	298		
Not contaminated areas (0)	**976**	**Not contaminated areas (0)**	**604**
Familial**	**132**/976		
Minsk	292	Minsk	246
Brest	256	Brest	146
Grodno	228	Grodno	104
Vitebsk	200	Vitebsk	108
Contaminated areas* (I-IV)	**783**	**Contaminated areas* (I-IV)**	**415**
Familial**	**166**/783		
Gomel	416	Gomel	207
Mogilev	367	Mogilev	208

Cases from contaminated areas (n)				**Controls from contaminated areas (n)**			
I	**II**	**III**	**IV**	**I**	**II**	**III**	**IV**
269/783	**298**/783	**175**/783	**41**/783	**180**/415	**197**/415	**31**/415	**7**/415

*****regions contaminated with long-lived radionuclides after Chernobyl accident

****** subset of cases with at least one first-degree relative affected with breast cancer

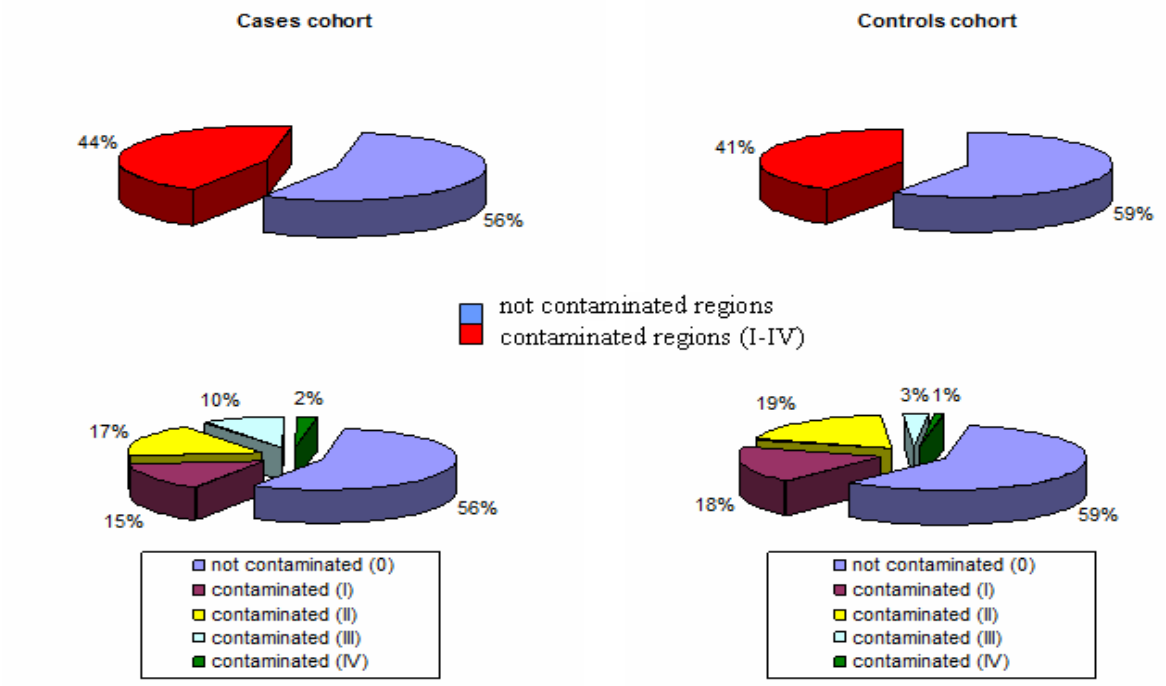


Figure 2.1 **Diagrams showing distribution of studied subjects in different regions.** First panel: proportion of cases and controls from contaminated and non-contaminated areas; second panel: proportional distribution of cases and controls according division into subgroups.

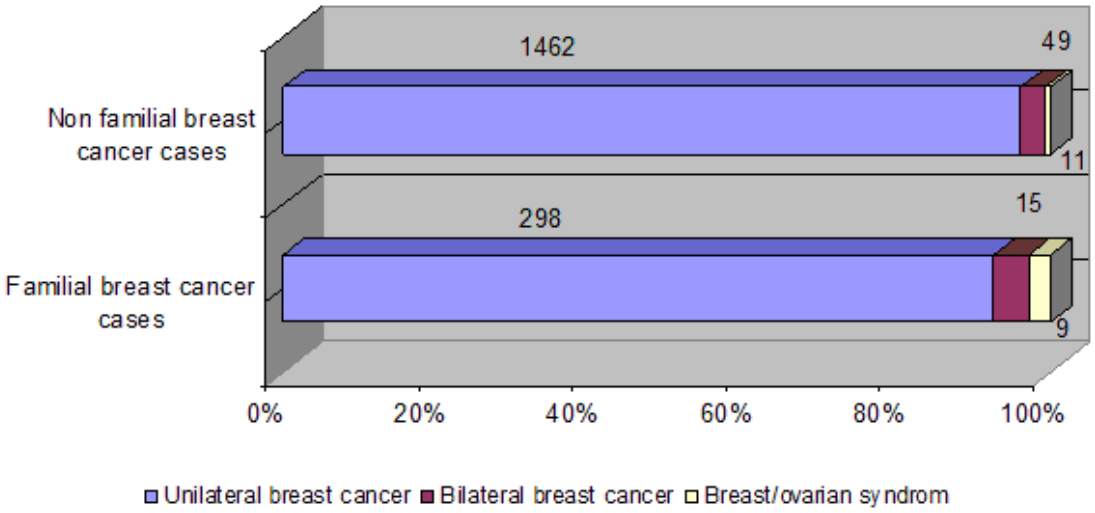


Figure 2.2 **Distribution of multisite cancer in the studied subjects, stratified by family history of the disease.** Numbers on diagram provide total numbers of cases with unilateral, bilateral and breast/ovarian disease.

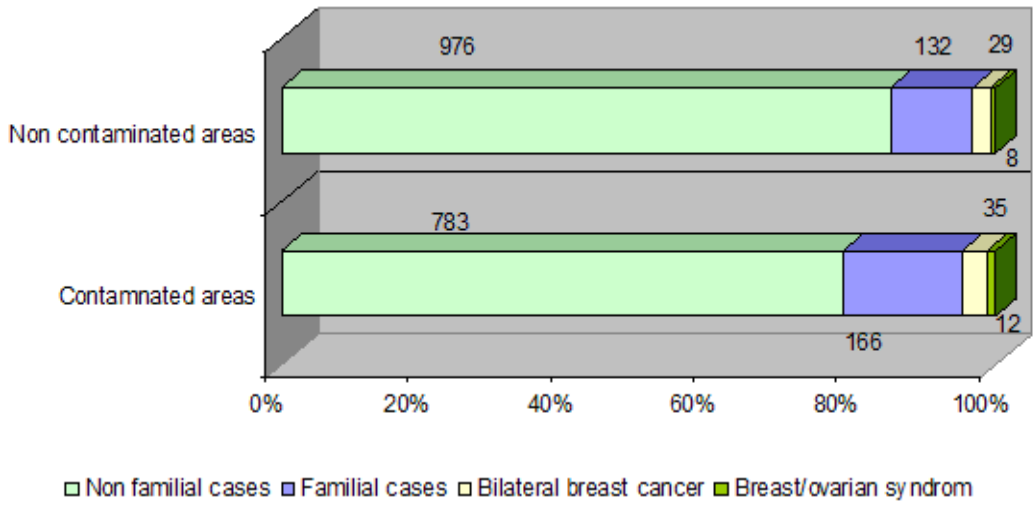


Figure 2.3 **Distribution of familial and multisite cancer among the studied patients, stratified by region.** Numbers on diagram refer to the total numbers of cases in each category.

3. Methods

3.1 Extraction of genomic DNA

3.1.1 Isolation of peripheral lymphocytes from whole EDTA blood

To isolate lymphocytes, 5 ml patient's blood (in 0.1 mM EDTA tubes) was mixed with 10 ml erythrocyte lysis buffer and centrifuged (3000 rpm, 15 minutes, 4°C). Sediment was washed twice with 5 ml of erythrocyte lysis buffer, centrifuged (3000 rpm, 15 minutes, 4°C), transferred in new autoclaved 1.5 ml tubes and resuspended in 400µl proteinase K reaction mix with subsequent proteolysis at 56°C over night.

Erythrocyte lysis buffer (usually fresh, autoclaved, stored at 4°C):

155 mM ammonium chloride,

10 mM KHCO3,

0.1 mM EDTA, pH 7.4

proteinase K reaction mix (per sample):

190µl	sterile HPLC grade water
150µl	proteinase K (10 mg / ml)
40µl	10 x STE
20µl	10 % SDS

10 x STE buffer:

0.5 M	Tris-HCl
1 M	NaCl
0.01 M	EDTA

Ad 1 L with bidistilled water (pH 7,5)

3.1.2 Phenol-chloroform extraction and ethanol precipitation of gDNA

Protein impurities were removed by vigorous shaking of samples after proteinase K reaction with an equal volume (400µ) of phenol/chloroform/isoamyl alcohol mixture (25:24:1, with TE buffer brought to pH 8.0). The emulsion was then centrifuged for 10 min, 13000 rmp, at 4°C, and the upper aqueous phase was collected in new autoclaved tubes, mixed once again with an equal volume of phenol/chloroform/isoamyl alcohol, centrifuged for 10 min, 13000 rpm, at 4°C, and again the upper aqueous phase was transferred into new autoclaved tubes, then mixed with an equal volume of pure chloroform and centrifuged (10 min, at 4°C, 13000 rpm). Finally, the upper aqueous phase was collected for precipitation. Nucleic acids were precipitated by addition of 3M sodium acetate (pH 4.8) and 3 volumes of absolute ethanol (100 %). The mixture was then carefully swayed, incubated for 30 min at 4°C to complete DNA precipitation and centrifuged (10 min, 4°C,

13000 rpm). The pellet was washed with 70% ethanol (to remove residual salts) and centrifuged (10 min, room temperature, 13000 rmp). After washing, the supernatant was aspirated and the pellet was dried at 50°C for ~5 min. The dried pellet was resolved in 1 x TE buffer and the sample was stored at 4°C.

10 x TE-Buffer:
0.1 M Tris-HCl
0.01 M Na_2EDTA
Ad 1 L with bidistilled water (pH 8.0)

3.1.3 Isolation of gDNA from cell lines

For the goals of this study, gDNA was also isolated from selected established cell lines. The main step is also proteolysis using proteinase K following phenol-chloroform extraction (see sections 3.1.1 and 3.1.2). Cells with medium were transferred into a 15 ml Falcon tubes, centrifuged at 3000 rpm, washed 2 times with ice-cold 1 x PBS (sterile phosphate balanced saline) and for the last washing step transferred into new autoclaved 1.5 ml tubes, finally I added 400µl of proteinase K reaction mix and proceeded as described in 3.1.2.

3.1.4 Purification of DNA

3.1.4.1 Sodium acetate- ethanol precipitation

Sodium acetate- ethanol precipitation was also used to remove nucleoside triphosphates and buffer components from PCR products or sequencing reactions. To the samples was added 1/10 volume of 3 M NaAc and 3 volume of absolute ethanol with subsequent vortexing, incubation for 1 hour at RT or over night at 4°C and centrifugation for 30 min at 13000 rpm (at 4°C). The pellet was washed with 70% ethanol, centrifuged 30 min at 13000 rpm (4°C). After washing and drying, the pellet was dissolved in 20 µl of 95% formamide (for a subsequent sequencing reaction), or for other purposes in 1 x TE, bidestilled water or special buffer.

3.1.4.2 Polyethylenglycol precipitation

Before sequencing reactions, PCR products were purified by adding 1 volume of polyethylenglycol (PEG) solution with subsequent vortexing, incubation for 10 min at RT, and centrifugation at 13000 rpm for 10 min at RT. The supernatant was aspirated and the pellet was washed with 100µl of absolute ethanol. After centrifugation with 13000rpm for 10 min at RT, the supernatant was aspirated and the pellet was dried at 50°C for 5-7 min. The dried pellet was resolved in 10-20µl sterile HPLC grade water.

PEG solution:

262 g	Polyethylenglycol 8000
1.2 g	Magnesiumchloride hexahydrate
4.2 g	Sodium acetate

Ad 1000 ml bidistilled water

3.2 Polymerase chain reaction (PCR)

3.2.1 Principle of method

Polymerase chain reaction (PCR) is a technology to amplify selected pieces of DNA. The invention of PCR is ascribed to Kary Mullis who, at the time he considered PCR in 1983, was working in Emeryville, California for Cetus, one of the first biotechnology companies. There, he was charged with making short chains of DNA for other scientists. Mullis has written that he conceived of PCR while cruising along the Pacific Coast Highway 1 at one night in his car (Mullis 1990). He was playing in his mind with a new way of analyzing changes (mutations) in DNA when he realized that he had instead invented a method of amplifying any DNA region. Mullis has said that before his trip was over, he was already savoring the prospects of a Nobel Prize. He indeed shared the Nobel Prize in Chemistry with Michael Smith in 1993. As Mullis has written in the Scientific American: "Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat." (Mullis 1990).

At present PCR is one of the most important techniques in the field of molecular biology. It is a very sensitive and powerful technique (Saiki et al., 1988) that is widely used for the exponential amplification of specific DNA sequences *in vitro* by using sequence-specific synthetic oligonucleotides (primers). The general principle of PCR starts from a pair of oligonucleotide primers that are designed so that a forward or sense primer directs the synthesis of DNA on one strand in a 5´-3´direction, and a reverse or antisense primer on the complementary strand *vice versa*. From the second cycle on, the daughter strands can themselves serve as templates for PCR products with restricted lengths defined by both primers. During the several extension cycles of PCR, the *Taq* DNA polymerase (a heat stable polymerase) (Chien et al. 1976) catalyses the synthesis of new DNA strands complementary to the template DNA from the 5' to 3' direction by a primer extension reaction, resulting in the exponential production of the selected DNA region flanked by the two primers. It allows the rapid and unlimited amplification of a specific nucleic acid sequence that may be present at very low concentration in any sample.

3.2.2 PCR amplification of DNA

A mix for PCR was prepared with final concentrations of 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.0 U heat stable DNA polymerase (*Taq* DNA polymerase from *Thermus aquaticus*), 0.5µM each primer, 1 x specific amplification buffer, recommended by manufactures of enzyme, 50 ng genomic DNA and sterile HPLC-grade water up to 15-25 µl end volume in either0.5 ml PCR tubes or 96-well plates. Amplification was performed in a peltier-controlled thermocycler (mostly PTC-200 from Biozym/ MJ Research) where an initial denaturation of 15 min (for HotStar Polymerase) or 5 min (for GoTaq Polymerase) was followed by an orderof 33 to 40 cycles of denaturation-annealing-extension steps, depending on the primer pair. Denaturation and extension steps were performed for 1 min. Denaturation temperature was 95°C, extension temperature 72°C. Annealing temperature was adjusted according to primer composition and was selected for each primer pair in a primer optimization procedure.

initial denaturation	95°C	5/15 min	
annealing	54-68°C	1 min	33-40cycles
elongation	72°C	1 min	
denaturation	94°C	1 min	
final elongation	72°C	5 min	

Primers were designed using published data or using reference sequences obtained from the NCBI Genbank. Primers were generally chosen to be 20-25 nucleotides long, to consist of similar proportions of A/T and G/C nucleotides, to avoid repetitive sequences and to end with a G or C.

The PCR volume and the corresponding input of template were dependent on the purpose of amplification and subsequent experimental procedures. For ARMS-PCR and for PCR followed by restriction enzyme reactions, we used 50-100 ng of genomic DNA as template in a total volume of 15µl; for multiplex PCR 100-150 ng genomic DNA in a total volume of 20µl, and for PCR followed by sequencing 150-200 ng in a total volume of 25µl. Each PCR assay was done including so called “water control” (no template control) for check of PCR quality.

3.3 Enzymatic cleavage of DNA using restriction endonucleases

3.3.1 Restriction endonucleases

Restriction enzymes are proteins (more specifically, endonucleases) that are produced by bacteria or archaea as part of their restriction/ modification system. Restriction endonucleases cleave double-stranded DNA at sequence-specific sites. Most of them are "type II" restriction endonucleases that act as homodimers to recognize palindromic, i.e. complementary symmetric, sequences of 4-8 bp (Pingoud and Jeltsch 2001). Hundredsof restriction endonucleases have been found, from many different species. The term *restriction* comes from the fact that these enzymes were discovered in *E. coli* strains that appeared to be restricting the infection by certain bacteriophages. Restriction enzymes therefore are believed to be part of a mechanism evolved by bacteria to resist viral attack and to help in the removal of viral sequences, in concert with the protection of own DNA by corresponding sequence-specific methyltransferases (the restriction/ modification system). Thus, in the living bacterial cell, restriction enzymes destroy the DNA of certain bacteriophages, thus placing a "restriction" on the number of viral strains that can cause infection; while the bacterium's own DNA is protected from cleavage by methyl (CH_3) groups. The enzyme makes two phosphodiester bond incisions, one through each of the sugar-phosphate backbones of the double helix without damaging the bases, resulting in fragments with either blunt or sticky ends depending on the enzyme.

3.3.2 Procedure

Restriction enzyme cleavage reactions were performed by incubating double-stranded PCR products with an appropriate amount of restriction enzyme in its respective buffer (optionally BSA was added) as recommended by the supplier, and at the optimum temperature for the specific enzyme. Usually, 4 µl PCR products were incubated without further purification with 1 µl 10x buffer and 1.5 U of the respective enzyme in a total volume of 10 µl. These reactions were usually incubated over night to insure complete cleavage. Enzymes used for this thesis are summarized in table 3.1.

Table 3.1 **Restriction enzymes applied in this study.**

Enzyme	Recognition site	Optimal temperature (°C)
*Ava*II	5'-G$^{\nabla}$GWCC-3' *	37°
*Dde*I	5'-C$^{\nabla}$TNAG-3'	37°
*Mse*I	5'-T$^{\nabla}$TAA-3'	37°
*Pst*I	5'-CTGCA$^{\nabla}$G-3'	37°
*Rsa*I	5'-GT$^{\nabla}$AC-3'	37°
*ScrF*I	5'-CC$^{\nabla}$NGG-3' *	37°
*Tsp509*I	5'-$^{\nabla}$AATT-3'	65°

***** underlined single letters code: **N –** any nucleotide, **W –** A or T. $^{\nabla}$ cleavage site

3.4 DNA Sequencing

3.4.1 Principle of the method

DNA sequencing is the process of determining the order of nucleotides in a given DNA fragment. Currently, most DNA sequencing is performed using the chain termination method developed by Frederick Sanger (Sanger et al 1977). This technique uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates. In chain-termination sequencing (Sanger sequencing), the extension is initiated at a chosen site on the template single stranded DNA by using a short oligonucleotide 'primer' complementary to the template at that region. The oligonucleotide primer is extended using a DNA polymerase. Included with the primer and DNA polymerase are the four desoxyribonucleosidetriphosphates, along with a low concentration of at least one chain terminating nucleosidetriphosphate (most commonly a **di-**desoxyribonucleoside triphosphate labelled with a separate fluorescent dye). Limited incorporation of the chain terminating nucleotide (which fluoresces at a different wavelength) by the DNA polymerase at different positions in repeated cycles results in a series of related DNA fragments that are terminated only at positions where that particular nucleotide is used. The end-labelled fragments are then size-separated by electrophoresis either in a slab gel or in a narrow glass tube (capillary) filled with a viscous polymer.

3.4.2 Sequencing procedure

During presented thesis, non-radioactive sequencing reactions were performed with the Sanger method using a Big Dye Terminator Cycle Sequencing-Kit v. 1.1 (Applied Biosystems, Darmstadt). The sequencing reaction was carried in a total volume of 10µl, usually containing 2µl of purified PCR products, 1µl of sequencing primer (5µM) and 1.5 µl BigDye reaction mix (contains reaction buffer, dNTPs, four differentially labelled ddNTPs and polymerase). It was important to set up the mix with fluorescence-free HPLC grade water (Baker). Elongation and chain termination takes

place during the following program in a thermocycler: 5 min denaturing followed by 25 cycles as follows: 95°C for 30 sec, denaturing; 50°C for 15 sec, annealing; 60°C for 4 min, elongation.
After the sequencing reaction in the thermocycler, the reaction products were precipitated with sodium acetate and 100 % ethanol as described in section 3.1.5.1. After washing and drying, pellet was dissolved in 20 µl of 95% formamide, incubated either at RT (1 hour) for processing on the same day or at 4°C (over night) in darkness. Finally, samples were denatured at 95°C for 3 min, immediately placed on ice and then transferred into 96-well plates to be loaded into the sequencing analyzer, mainly the ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems). Electrophoresis run parameters were defined using the Data Collection Software and the raw fluorescence data were evaluated and further processed by using the Sequencing Analysis Software Package (Applied Biosystems).

3.5 RNA analyses

3.5.1 Precautionary measures

Precautions were used while isolating and handling RNA in order to prevent degradation. All used solutions, glassware and plastics were treated with 0.1% DEPC water and sterilized to ensure the inactivation of unwanted nucleic acids and proteins including RNase, which is an extremely stable enzyme requiring no cofactors to exert its effect of RNA digestion and degradation. Everything was handled only while wearing gloves. RNA was always dissolved in autoclaved DEPC water and stored at –80°C or kept on ice while handling.

DEPC water:

0.1 % DEPC was shaken vigorously to dissolve the DEPC (dieethylpyrocarbonate). The required plastic ware was incubated with DEPC water for 1 hour under a fume and finally autoclaved to inactivate residual DEPC.

3.5.2 Isolation of total RNA from cultured cells

Suspension cells were collected by centrifugation at 3000 rpm for 5 min, and the pellet was rinsed once with 1xPBS. Adherent cells were trypsinizated from the culture dishes, collected in a tube and similarly washed with 1xPBS. The cell pellet could be stored at –20°C up to 2 weeks before the RNA was isolated. Total RNA from cell pellets was extracted using TRI Reagent® (AB Gene, Göttingen). This phenol-based reagent contains a guanidine thiocyanate in a monophasic solution, including some combination of denaturants and RNase inhibitors, and was used in a single-step disruption/separation procedure described first by Chomczynski and Sacchi (1987). Cell samples were homogenized in the TRI Reagent, then 0.2 ml of chloroform was added, and the samples were

vortexed and incubated on ice for 5 min. After centrifugation at 13000 rpm for 15 min at 4°, the mixture separated into three phases (aqueous phase, narrow interphase, organic phase). The colourless upper aqueous phase was transferred into a new tube. The RNA was then precipitated from the aqueous phase by adding 500 µl of isopropanol, followed by vortexing and spinning at 13000 rpm for 5 min. Finally, the pellet was washed with 85% ethanol and dissolved in 15-30 µl autoclaved DEPC water. The entire procedure could be completed in no more than one hour to produce high yields of intact RNA from several samples for subsequent use. The amount and purity of RNA was estimated using photometry at 230, 260 and 280 nm (Biophotometer, Eppendorf), as described in 3.6, and 2 µl of the RNA solution were analysed on a 1% agarose gel to evaluate the 28S and 18S rRNA bands as markers for the integrity of the isolated RNA. The RNA solution was then stored at -80°C.

3.5.3 Reverse transcription and subsequent PCR (RT-PCR)

RT-PCR is a technique, which generates cDNA fragments from RNA templates and thereafter amplifies the cDNA by PCR. 8 µl RNA dilution containing approximately 1 µg of total RNA was used as a template for the first strand cDNA synthesis. The procedure employed random hexamer priming and M-MuLV Reverse Transcriptase purchased from Amersham/GE Healthcare and was performed according the protocol of the manufacturer without any modifications. In brief, the RNA sample was denatured for 10 min at 68°C and placed on ice. 7 µl Enzyme mix containing the reverse transcriptase, dithiothreitol and dNTPs was added, and the sample was incubated for 1 hour at 37°C, followed by a final denaturation at 95° for 10 min. An aliquot of the synthesized cDNA was directly used without further purification for subsequent PCR amplifications.

3.6 Determination of nucleic acids concentration

The concentration of isolated nucleic acids was determined photometrically by measuring their optical density (OD) at 260 and 280 nm (Biophotometer, Eppendorf, Hamburg). 2µl of samples were added to 98 µl sterile HPLC water (1:50 dilution). The concentration was determined based on the fact that absorption of 1 at 260nm is roughly equivalent to a double stranded DNA concentration of 50µg/ml or an RNA concentration of 40µg/ml. For example, with DNA the formula was applied: Absorption (OD_{260}) × 50 µg/ml × Dilution Factor (50) ≈ Sample Concentration µg/ml. The purity of nucleic acids in solution can be estimated by the relative extinction at 260 nm and 280 nm ($OD_{260/280nm}$). Protein contaminations result in a ratio of < 1.7., the ratio between the absorptions at 260 nm and 280 nm for DNA should be in the range from 1.7 to 2.0 for an acceptable purity of nucleic acids (Maniatis et al. 1982).

3.7 Agarose gel electrophoresis

3.7.1 Agarose gel electrophoresis of DNA and RNA

Electrophoresis is a technique by which a mixture of charged macromolecules, especially nucleic acids or proteins, can be separated in an electric field according to their electrophoretic mobility which is directly proportional to the macromolecule's charge to mass ratio.

Nucleic acids can be separated according to their lengths by gel electrophoresis through agarose gels, with the separation range being dependent on the agarose concentration (in the presented work were used 1-3% w/v agarose in TBE). The 1-3 g of agarose was dissolved in 100 ml 1 x TBE buffer, boiled in the microwave, then cooled down to about 50-80°C before adding ethidium bromide (10 mg/ml, final concentration of 0.1 µg/ml) or Gel-Red (1,000 fold in DMSO, final dilution 1:50,000), thoroughly mixed by gentle swirling and then poured into an electrophoresis tray with appropriate comb(s). After solidification of the agarose, the tray was transferred to a horizontal electrophoresis chamber and the gel was covered with 1xTBE buffer. The 4-8 µl of sample were mixed with 2-4 µl of 6x loading buffer, applied onto the gel, and run at 100-150 V at RT. To determine the size of the nucleic acids fragments on agarose gels, molecular weight ladders were loaded in parallel. After gel electrophoresis, the DNA was visualized on a UV transilluminator.

3.8 Allelic discrimination assays

3.8.1 Principle of the method

Allelic discrimination is a process by which two variants of a nucleic acid sequence are detected and discriminated in a chosen sample, and this method is now widely used for single-nucleotide polymorphism (SNP) detection and screening. In presented thesis, allelic discrimination was achieved by using fluorogenic 5' nuclease assays in a probe technology that exploits the 5´-3´ nuclease activity of AmpliTaq Gold® DNA Polymerase to allow direct detection of the PCR product by the release of a fluorescent reporter as a result of PCR. Fluorogenic probes are constructed for two possible allelic sequences and, after mixing of the primers, reagents and DNA samples, the PCR is run on a thermal cycler. During the PCR, the fluorogenic probes anneal specifically to their complementary sequence between the forward and reverse primer sites on the generated PCR product. During the extension step, the DNA polymerase cleaves those probes that are perfectly hybridized to the matching allelic sequence(s) present in each sample. Because the fluorescence signals are generated in significant amounts only if the target sequences for the probes are amplified during PCR, non-specific amplification is not detected. Furthermore, the double-labelled probes require the cleavage by the polymerase as the fluorescence from the 5´-reporter dye (such as FAM or VIC) is quenched via Förster transfer (fluorescence resonance energy transfer, FRET) by a 3´-quencher dye (such as BHQ- Black Hole Quencher). The cleavage of each perfectly

matched probe separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter, what is detected during the PCR amplification (figure 3.1). Imperfectly matched probes are not cleaved, thereby allowing for allele-specific signals, and by quantifying and comparing the fluorescence signals (using the software from manufactures), it is possible to determine the allelic distribution in each sample.

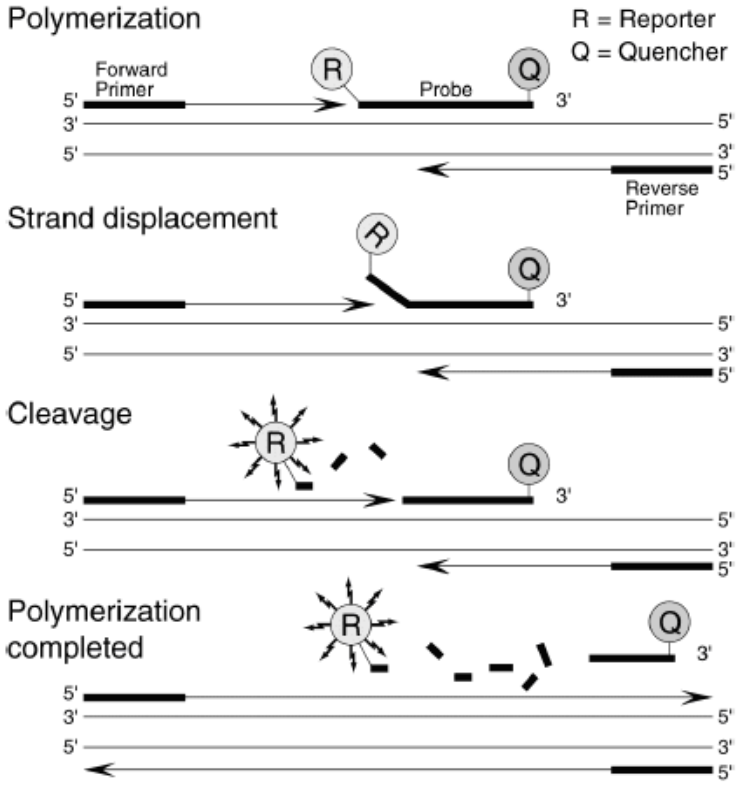


Figure 3.1 **The fork-like-structure-dependent, polymerisation -associated, 5´–3´ nuclease activity of AmpliTaq Gold DNA Polymerase during one extension phase of PCR.** Both the dual-labelled probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorescent reporter with the quencher prevents the reporter from fluorescing. During the PCR extension step, the polymerase extends the primer. When the enzyme reaches the dual-labelled probe, its 5'–3' exonuclease activity cleaves the fluorescent reporter from the probe (if the probe matches). The fluorescent signal from the free reporter is measured. (Scheme is taken from TaqMan Allelic Discrimination protocol, Applied Biosystems).

3.8.2 Procedure

Allelic discrimination assays were performed in 96-well plates. Initially, the plate was prepared by supplying an aliquot of DNA sample to each well and adding a PCR master mix with total volume of 7µl. Then background fluorescence was measured (pre-read step), followed by the PCR amplification. PCR amplification curves could be inspected in real time on the SDS7000 or the 7500 FAST Sequence Detection System platforms (Applied Biosystems). After PCR, fluorescence was measured again (post-read step) and the initial background fluorescence was subtracted. The results of each allelic discrimination run were analyzed using the 7500 Fast System Software, graphics software from the supplier (Applied Biosystems). During the analysis, the software (7500 Fast System Software, Applied Biosystems) automatically applies transformations to the raw data and determines the spectral contribution of each dye in the unknown sample.

PCR master mix (per well):

1.5µl (50-100ng) DNA sample

plus either

-for predesigned assays:

2.8µl 2x TaqMan Genotyping Master Mix (containing dNTPs, AmpliTaq Gold DNA polymerase, $MaCl_2$), and

0.07µl or 0.14µl 80x or 40x SNP Genotyping Assay Mix, respectively (containing probes labelled with VIC und FAM dyes, constructed for two possible alleles; and primers for the region of interest)

or

-for self-designed assays

2.8µl 2x TaqMan Genotyping Master Mix (containing dNTPs, AmpliTaq Gold DNA polymerase, $MaCl_2$), and

0.0667µl each primer (15µM)

0.045 µl each fluorogenic probe

Add 7µl sterile HPLC grade water

Pre- and post-read steps were done at 60°C during 1 min.

Routine PCR conditions were the following:

initial denaturation	95°C	15 min	
denaturation	95°C	15 sec	40/60 cycles
annealing	60-65°C	30 sec	
elongation	72°C	30 sec	

The quality of genotyping was controlled in several ways:
- every plate included a no template control for PCR specificity,
- for each assay, the replication of one plate was performed to reproduce obtained results,
- results for some TaqMan assays were interinstitutionally controlled for by using a “Coriell plate”-DNA collection from CEPH families with known genotypes, and data were compared between members of the Breast Cancer Association Consortium.

- results for some TaqMan assays were controlled by using restriction enzyme cleavage or direct sequencing of selected samples with certain genotype, which were 100% concordant if compare both techniques.

3.9 Reverse transcription and real time PCR analysis (qRT-PCR) using SYBR Green I

3.9.1 Principle of the method

Real-time polymerase chain reaction, also called "quantitative" real time polymerase chain reaction (qRT-PCR) or "kinetic" polymerase chain reaction, is a technique based on PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (either as absolute number of copies or as relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample. The procedure follows the general principle of PCR as described in 3.2.; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. Relative concentrations of DNA present during the exponential phase of the reaction are determined by plotting fluorescence against cycle number on a logarithmic scale (so that an exponential increase will give a straight line). A threshold for detection of fluorescence above background can be automatically determined or manually defined. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold, C_t. Since the quantity of DNA approximately doubles every cycle during the exponential phase, relative amounts of DNA can be calculated, e.g. a sample whose C_t is 3 cycles lower ("earlier") than another's, might have had about $2^3 = 8$ times more template. Absolute amounts of RNA or DNA can be determined by comparing the results to a standard curve produced by RT-PCR of serial dilutions of a known amount of RNA or DNA. In order to more accurately quantify relative gene expression, the measured amount of RNA from the gene of interest is divided by the amount of RNA from a housekeeping gene measured in the same sample, to normalize for possible variation in the amount and quality of RNA between different samples. This normalization permits accurate comparison of expression of the gene of interest between different samples, provided that the expression of the reference (housekeeping) gene used in the normalization is very similar across all the samples. Furthermore, the addition of an independent dye (such as ROX) into the PCR master mix controls for pipetting errors during the handling of different samples.

SYBR Green I is a DNA-binding dye which binds to all double-stranded DNA, causing enhanced fluorescence of the dye. The intensity of the signal is thus proportional to the amount of dsDNA present in the reaction. Therefore, in each step of the PCR reaction, the signal intensity increases as the amount of product increases. Thus it provides a very simple and reliable method to monitor the accumulating PCR products in a real time course. Another advantage of this technique is that no

modification in oligonucleotide primers are required which facilitates primer design/synthesis and more important it lowers the running cost of PCR reaction. However, careful optimization of the reaction conditions for each primer set is required as non-specific by-products would also be detected.

3.9.2 Procedure

In this thesis, the expression of genes of interest was quantitated using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the 2x POWER SYBR Green PCR Master Mix (Qiagen). Briefly, according to manufactures instructions, the Master-Mix for reactions was prepared by combining the following components on ice:

10 µl 2x SYBR Green Mix
2 µl Primer mix (forward and reverse, 5µM/µl)
2 µl cDNA template
6 µl HPLC water

The cDNA samples for standard curves were prepared in serial dilutions: 1; 1:10; 1:100; 1:1000. All samples were amplified in quadruplicates. The program for the real time PCR was created consisted of the following steps:

initial denaturation	95°C	15 min	
denaturation	95°C	30 sec	40 cycles
annealing/elongation	63°C	1 min	

Data analysis was performed using the ABI PRISM 7000 SDS (Applied Biosystems) software with the standard curve method, and the expression of the investigated gene was normalized to cyclophylin mRNA expression. Results and standard deviations were illustrated by box plots prepared with Microsoft Office Excel.

3.10 Lymphoblastoid cell culture

Peripheral B-Lymphocytes have a limited life-span of about 5-7 days. A simple routine use procedure to overcome this limitation exploits the efficient immortalisation ("transformation") of peripheral B-lymphocytes by Epstein-Barr virus (EBV, a human lymphotropic herpes virus) and thus the establishment of permanent lymphoblastoid cell lines (LCL). Converted *ex vivo* to continuously dividing, efficiently immortalized lymphoblastoid cells, B-lymphocytes from selected patients can then be used for a plethora of assays, including isolation and investigation of DNA or RNA, harvesting of proteins for functional investigations, or flow cytometry and immunohistochemistry.

Peripheral blood lymphocytes from selected Byelorussian breast cancer patients or control persons were isolated by a Ficoll gradient, and Britta Wieland from our group kindly established EBV-infected cell lines for this project. All cell lines were cultured at 37°C and 95% humidity in the presence of 5% CO_2 in a SANYO incubator. Lymphoblastoid cell lines were maintained in RPMI 1640 medium (Invitrogen) supplemented with 15% heat-inactivated fetal calf serum (BIOCHROM AG Seromed®, Berlin), 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate and 2 mM glutamine (Invitrogen, Karlsruhe). Cell culture was fed every 2-3 days.

For certain functional assays cells were irradiated. One day before irradiation cells were fed with fresh medium, and the cultures were brought to the same volume (usually 10 ml of cell culture). Dependent on the assay planned, cells were irradiated with 6 or with 10 Gy using a Mevatron Mx2 irradiator (Siemens). At least one flask was left unirradiated as a control. After irradiation, cells were incubated in 5% CO_2 at 37 °C for 30 min, 6 hours or 24 hours, according goals of experiment, followed by centrifugation and extraction of RNA, DNA or protein.

For long-term storage, confluent cells were collected by centrifugation at 1000 rpm for 5 min at 4°C, twice washed with sterile ice cold PBS and resuspended in freezing solution (2 ml freezing solution for cells from a confluent 10 ml culture). The cell suspension was transferred to appropriate cryovials and these were placed into a cryocontainer with isopropanol and kept for 24 hours at –80°C. Thereafter, the cells were transferred to liquid nitrogen for long-term storage. For thawing, cryotubes were placed at 37°C in a water bath. Immediately after thawing, the cell suspension was transferred into a 15 ml vial containing 5 ml regular growth medium and centrifuged at 1000 rpm for 5 min at room temperature. The cell pellet was resuspended in growth medium and transferred into a T25 flask or culture dish.

Freezing solution:

20 % fetal calf serum
10% glycerin
} in regular growth medium

3.11 Human mammary epithelial cell culture (HMEC)

Normal human mammary epithelial cells for experimental applications were obtained from Lonza (Belgium) cryopreserved in the seventh passage and were cultivated till passage 12. Cells were cultured at 37°C and 95% humidity in the presence of 5% CO_2 in an incubator (Sanyo), using MEBM medium (Lonza) exclusively supplemented with growth factors and reagents (store at -20°C) provided by Lonza and using the protocol recommended by manufacturer. Cell culture was fed every 2-3 days until cells were 60-80% confluent with many mitotic figures through the flask before subculturing.

For certain functional assays (see chapter 4.5), the cells were irradiated with 5 Gy using a Mevatron Mx2 irradiator (Siemens). One day before irradiation cells were fed with fresh medium. At least one flask was left unirradiated as a control. After irradiation, cells were incubated in 5% CO_2 at 37 °C for up to 24 hours, according the goals of experiment. HMEC are adhesive cells and were collected for experiments by trypsinization (also was used for subculturing, according manufactures prescriptions). Briefly, for the collection of cells after irradiation the medium was aspirated, cells were rinsed with 5 ml of HEPES-BSS to neutralize the complex proteins in growth medium which may inactivate trypsin. Subsequent 3 ml of trypsin/EDTA were used to remove cells from culture flasks surface, and the trypsinization process was microscopically examined (takes about 3-6 min) until 90% of cells were rounded up. This was followed by neutralization of trypsin with 3 ml of TNS and adding fresh warm medium for further culturing of the cells, or by centrifugation and extraction of RNA (see chapter 3.5.1).

Freezing solution:

10 % DMSO in regular growth medium.

3.12 Protein extraction and analyses

3.12.1 Isolation of total protein from lymphoblastoid cell lines

Lymphoblastoid cells were cultured at 37°C in a humidified incubator with 5% CO_2 (as described above), and 5-10 ml of cell culture containing about 2 x 10^7 cells were treated with irradiation before isolation of total proteins. Cells were transferred to a separate 15 ml Falcon tubes, centrifuged at 1000 rpm, washed twice with ice-cold sterile PBS and after the last wash step were transferred into new autoclaved eppendorf tubes, centrifuged at 3000 rpm 5 min and finally were suspended in 50-100µl (2-3 fold volumes of pellet) of lysis buffer. The samples were homogenized by pipetting, incubated for 30 min on ice (mixed gently every 10 min) and centrifuged for 15 min at 13000 rpm. The supernatant was taken as the whole cell lysate, which was either directly used for Western blotting after Bradford determination of protein concentration or stored at -80°C.

Lysis buffer (store at 4°C):

50 mM	Tris-Cl (pH 7.4)
150 mM	NaCl
0,1 %	NP-40 (v/v)
10 %	glycerol (v/v)
25 mM	Na-β-glycerophosphate
2mM	EGTA (tock solution 20mM)
2mM	EDTA (stock solution 0.5M)

Directly before use, proteinase inhibitors were added from concentrated stock solutions to the following final concentrations:

1 mM PMSF, stock solution 100 mM in ethanol

10 mM Na_3VO_4, stock solution 2 M in water

5 µM Leupeptin, stock solution 5 mM in water

20 mM NaF, stock solution 1M in water

8 µg/ml Aprotinin, stock solution 1.6 mg/ml in water

3.12.2 Determination of protein concentration

Protein concentration in cell lysates was determined with the Bio-Rad protein assay, a modified Bradford assay (Bio-Rad, Munich), following the manufacturer´s recommendations. Briefly, 1 ml Bio-Rad protein assay solution was mixed with sample (2 µl) and the absorbance was measured at 595 nm in a Bio-Photometer (Eppendorf). For the standard (calibration) curve, 0, 2, 4, 6, 8 and 10µl of 1mg/ml BSA were added to 1 ml Bio-Rad reagent and were measured in parallel. Sample concentration was estimated as OD/2α, where α is OD of 1 µl BSA in 1 ml Bio-Rad solution (Bradford 1976).

3.12.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

3.12.3.1 Gel preparation

Proteins can be separated largely on the basis of their relative molecular mass by electrophoresis in SDS-polyacrylamide gels under denaturing conditions. For SDS-polyacrylamide gel electrophoresis, I used the vertical electrophoresis system from PeqLab to cast the gel and perform the electrophoresis. For the analysis of proteins with molecular weights between 60-100 kDa (1 Da approximately equals $1{,}66 \times 10^{-27}$ kg), 7.5 % SDS-polyacrylamide gels were employed.

Inner and outer glass plates were assembled in a casting tray.

For the **separation gel**

0.1 %	SDS (w/v)
400 mM	Tris-Cl (pH 8,8)
0.1 %	APS (w/v)
0.08 %	TEMED (v/v)

and acrylamide / bisacrylamide (19:1) up to the desired concentration (7.5%) were mixed and adjusted with water to a final volume of 10 ml (APS and TEMED were added last to initiate polymerization). The gel solution was quickly poured into the vertical gel chamber and carefully overlayed with 70% ethanol. After complete polymerization of the separation gel, the ethanol was poured off and the 5% stacking gel was poured onto the separation gel.

Stacking gel:

5 %	Acrylamide: bisacrylamide (v/v)
0.1 %	SDS (w/v)
130 mM	Tris-Cl (pH 6,8)
0.1 %	APS (w/v)
0.1 %	TEMED (v/v)

A comb was inserted into the stacking gel to form the sample slots and the gel was allowed to polymerize. After polymerization, the comb was removed; the gel chamber was then disconnected from the casting tray and inserted into electrophoresis chamber.

3.12.3.2 Electrophoresis

Protein samples (50 - 80 µg) were mixed with loading buffer at a ratio of 5:1, boiled for 5 min at 95°C and chilled on ice. Samples were briefly centrifuged and loaded onto the gel. For molecular weight determination, a protein molecular weight marker was loaded parallel to the samples onto a separate lane (BioRad Precision Protein Standards, Munich). The gel was run initially at 80 V until the samples entered the separation gel, then for 2-3 hours at 100-120 V constant voltage at 4°C, until the bromphenol blue had nearly reached the bottom of the gel.

Loading buffer (before use, can be stored at –20°C):

40µl	10 % SDS
25µl	0.5 M Tris (pH 6.8)
10µl	2-β-mercaptoethanol
4µl	bromphenol blue (1 mg / ml bidistilled water)
3µl	glycerol

Running buffer:

1 x GTS – buffer

3.12.4 Western blotting

Electrophoretically separated proteins were subsequently blotted onto nitrocellulose membranes (Hybond-C, GE Healthcare) in carbonate containing transfer buffer using a Protean 3 TE 62 blotting chamber (GE Healthcare) following manufacturer's instructions. After the electrophoresis system was disassembled, the stacking gel was cut off and the separation gel was assembled with the nitrocellulose membrane, which was cut to the size of the gel, and equilibrated in Transfer buffer (1x carbonate buffer) for 10 min. Three sheets of Whatman filter paper (Maidstone) were cut and soaked in the transfer buffer. The gel with the membrane was placed subsequently onto the pre-soaked filter papers. Another three sheets of presoaked filter paper were applied to complete the "sandwich", and the whole was placed into the blotting chamber. The gel/membrane assembly was held securely between the two halves of the blot module ensuring complete contact without bubbles of all components. The blot module was filled with transfer buffer and run at 4°C by 35 V for 150 min.

After blotting was completed, successful transfer was proven by staining the membrane with 0.2% Ponceau Red (Sigma, Steinheim) for 3 min at RT. The membrane was destained by washing 10-15 min with distilled water before the immunoreactions were performed.

3.12.5 Immunological detection of membrane-bound proteins

Proteins can be detected on the membrane with antibodies, which bind to a specific region of the protein (epitope). All antibodies used were diluted according to manufacturer's instructions in PBST-5% low-fat milk powder (w/v). The incubations and washing steps were performed on a shaker. The membrane was first blocked in PBST-5% low-fat milk powder (w/v) for at least 1 hour at room temperature or overnight at 4°C followed by an incubation step with a primary antibody for 2 hours at room temperature or overnight at 4°C. This incubation was followed by three washes with PBST (15 min each) to remove unbound antibody and then the membrane was incubated with the appropriate secondary antibody diluted in PBST-5% low-fat milk. After incubation with the secondary antibody conjugated to horseradish peroxidase for 1 hour at RT, the membrane was washed as before. The membrane was rinsed with water to replace PBST and incubated in the dark for 5 min with developing solution, which either was made freshly in the dark by mixing self-prepared ECL solutions 1 and 2 (1:1) or was mixed from the components of the SuperSignal West Dura Extended Duration Substrate (PIERCE/ Perbio Sciences). In general, the self-prepared ECL solutions were sufficient for highly expressed proteins such as β-actin whereas the extended-

duration substrate was used for proteins closer to the detection limit. Afterwards, the membrane was placed bubble free in a transparent plastic bag and exposed to an X-ray film (Amersham/ GE Healthcare). Oxidation of luminol by horseradish peroxidase in the presence of hydrogen peroxide leads to emission of photons, which can be detected by a light sensitive film. Depending on signal strength the exposure ranged from 5 sec to 30 min, after which no further improvement could be achieved. In general, at least three different exposure times were chosen per blot. After protein detection, the membrane was stored wet at 4°C for its eventual reuse. For the detection of other proteins/antigens, the membrane was washed several times with PBST over night and the procedure was repeated with another antibody.

ECL Solution 1:
2.5 mM luminol
400 µM p-coumaric acid
100 mM Tris·Cl pH 8.5

ECL Solution 2:
0.02 % H_2O_2 (v/v)
100 mM Tris·Cl
Store ECL solution 1 and 2 at 4°C

Stock solutions (store at –20°C):
250 mM Luminol in DMSO
90 mM p-coumaric acid in DMSO

3.13. Statistical methods

Statistical analyses were conducted using Statistix7.0 (Statistix for Windows Analytical Software) for calculating of odds ratios with 95% confidence intervals, the Yates' corrected chi-square and p-values in 2-by-2 tables. Statistix7.0 software was also used for median tests to determine the differences in the median age between groups.

Crude and adjusted Mantel-Haenszel odds ratios were calculated in 2x2 stratified analyses using EpiCalc v1.02 Software Package (Gilman J, Myatt M 1998, Brixton Books). The methods used to calculate the Mantel-Haenszel summary odds ratios and respective confidence intervals are described in:Rothman KJ. Modern Epidemiology. (1986) *Little brown and Company*, ISBN 0-316-75776-4, pp. 177-236.

4. Results

4.1. Screening for founder *BRCA1* and *BRCA2* mutations

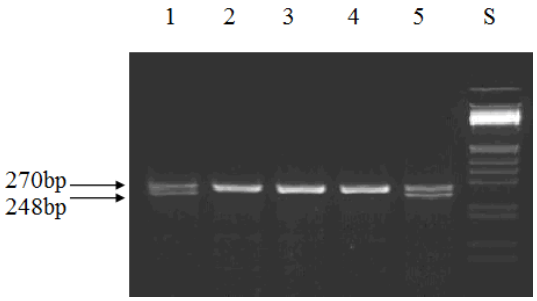
4.1.1. Rationale

Although plenty of different mutations have already been identified in the two genes *BRCA1* and *BRCA2*, it is still controversial how different mutations contribute to breast, ovarian and other cancers. Clearly some mutations are responsible for the very high risks identified in some families, but others have been reported with lower risks and many unclassified variants are likely to be within the normal polymorphic variations found in many genes and may not be relevant to cancer. It is also likely that different mutations may determine different types of cancers with different pathologies and different outcomes. Moreover the same mutations may behave differently in different populations of women depending on gene-gene and gene-environment interactions.

BRCA1/BRCA2 mutation detection is complex because of the large size of both genes and the absence of mutational hot spots. Three mutations in *BRCA1* (5382insC, 4153delA and p.C61G) and the frameshift mutation 6174delT in *BRCA2* gene were investigated in this study in the whole series of Byelorussian breast cancer patients and controls. These mutations were chosen in regard that they are common in Caucasians and mainly prevalent in Central Europe, Poland, Russia and Baltic countries. Belarus is geographically placed between these countries and its population also belongs to Caucasians. Thus, the obtained results for the first time should provide knowledge about these known mutations and their frequency in the Byelorussian population.

4.1.2 BRCA1 5382insC

For screening of 5382insC in the *BRCA1* gene, a simple restriction enzyme based PCR assay was used that had previously been established in our group (Britta Skawran, Diploma thesis). A genomic region surrounding the mutation site was amplified by 35 cycles of PCR using GoTaq –Polymerase (Promega) and 60ºC annealing (for primers see attachment 1). Subsequently, the PCR products were digested at 37°C over night with *Dde*I (New England BioLabs). Restriction digest products were separated on 3% Agarose-1000 and visualized on the UV transilluminator after ethidium bromide staining (figure 4.1 A). In the presence of the mutation, the 270 bp PCR product was cleaved by *Dde*I to fragments of 248 and 22 bp (to small to be distinguished from primers), whereas wild-type remained uncut. All samples positive for the 5382insC were verified by direct sequencing (figure 4.1 B).



A

B

Figure 4.1 **Identification and confirmations of the 5382insC mutation by screening of PCR products, using *DdeI* and direct sequence.** Panel **A –** fragment of 3% agarose-1000 gel; S – size marker; lanes 1 and 5 heterozygous carriers of the mutation; lanes 2-4 wild-type controls. Panel **B –** sequences fragments of *BRCA1* exon 20 by wild-type control and 5382insC heterozygous carrier, anti-sense strands, with ▼ designed start of frameshift.

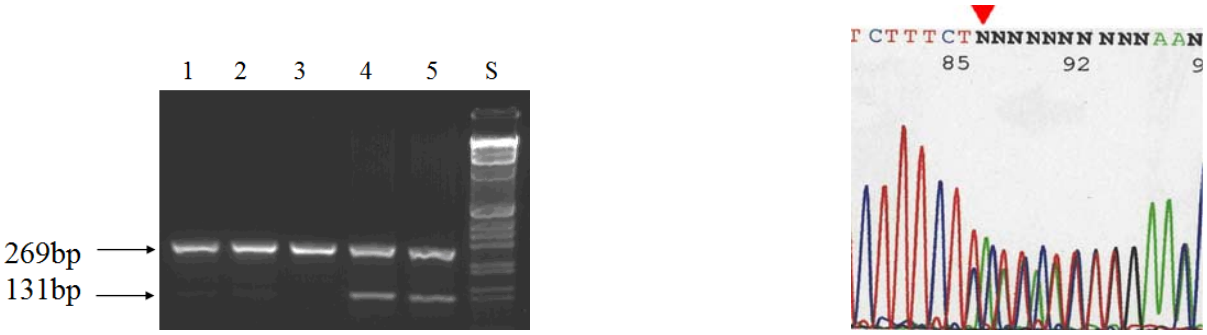
Genotyping was successfully completed for 100% of cases. In the breast cancer cases series, the insertion was found in 44 cases (2.5%), and 18 mutation carriers reported a family history of breast cancer (at least one first-degree relative also affected with breast cancer), two patients had bilateral disease and one patient also had an ovarian carcinoma. 22/44 cases were from contaminated areas with 12/22 from areas with more than 5 mSv whole body cumulative dose (subgroups **II-IV**, table 2.1), and 7 of these had a first-degree relative with breast cancer (table 4.1). The approximately two-fold increase in familial cases in the highly contaminated areas was not statistically significant (p=0.28). Thirty-four patients with 5382insC were diagnosed below the age 50, the median age at diagnosis among mutation carriers was 42.5 years versus 48 in non-carriers (median test p=0.001). The age at diagnosis seemed lower in carriers from contaminated regions (median 41.5) compared with 45.5 years in non-contaminated oblasts, but again the difference was not significant (median test p=0.55). Frequencies of the 5382insC allele in contaminated and non-contaminated regions are presented in table 4.1, and the ages at diagnosis for carries and non carriers in different regions are shown in table 4.2. The 5382insC allele was also found in 1 out of 1019 control individuals, this carrier was from a non-contaminated region. In summary, this study confirmed a high prevalence of the 5382insC mutation in ~1/40 Byelorussian breast cancer patients and a highly significant association with breast cancer (OR: 26.0, 95%CI: 3.6-189.9, p<0.00001).

4.1.3 BRCA1 4153delA

For screening of 4153delA in *BRCA1* gene, an allele-specific PCR (ARMS, Newton et al. 1989) was performed using a mutation-specific forward primer so that PCR products were obtained only in the presence of the 4153delA allele (for primer sequences see attachment 1). Thirty-five cycles of

PCR amplification were performed using HotStar - Taq-Polymerase in betain containing buffer ("Q-Solution", Qiagen) and 58°C annealing. PCR was followed by 2% agarose gel electrophoresis, and the gel was stained with GelRed. In the presence of the 4153delA mutation, a 131 bp PCR product was obtained. A 269 bp fragment of the *ATM* gene (exon 42) was used as an internal control for amplification (figure 4.2 A). All positive samples were verified by direct sequencing of the *BRCA1* gene fragment, using primers flanking the mutation (figure 4.2 B).

Genotyping was successfully for 100% of cases. The 4153delA allele was found in 16 breast cancer cases (0.9%) all were heterozygous for the mutation. Six of them reported a family history of breast cancer, none of the carriers had bilateral disease and/or ovarian carcinoma but one patient has a relative affected with ovarian carcinoma.



A **B**

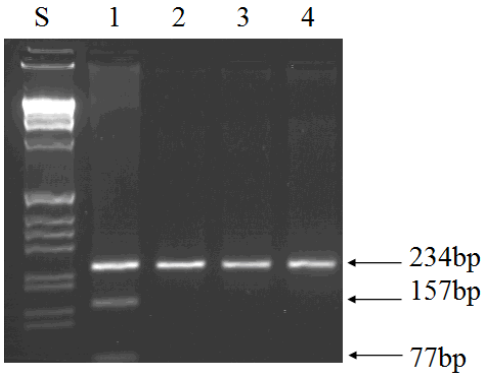
Figure 4.2 **Identification and verification of 4153delA mutation.** Panel **A –** fragment of 2% agarose gel with allele-specific PCR fragment of *BRCA1* gene and *ATM* gene serves as an internal control: S – size marker, line 4,5 heterozygous carrier of p.C61G mutation, lanes 1-3 wild type controls. Panel **B -** sequencing of PCR products from patient a heterozygous carrier of 4153delA (anti-sense strand with ▼designating beginning of frameshift).

Ten carriers of the 4153delA mutation were from contaminated areas, with 6 out of 10 from areas with more than 5 mSv whole body cumulative dose (subgroups **II-IV**, table 2.1), and four reported first-degree relatives with breast cancer (table 4.1). Thirteen were diagnosed below the age 50, median age in carriers was 41.5 years versus 48 in non-carriers (median test p=0.05). The median age in mutation carriers from contaminated areas was 38 years compared with 46.5 years in carriers from non-contaminated areas, a marginally significant difference (median test p=0.04). Frequencies of the 4153delA allele in contaminated and non-contaminated regions are presented in table 4.1, and differences in the age at diagnosis for carries and non carriers in different regions are shown in table 4.2. The 4153delA allele was also found in 2 out of 1019 control individuals (one carrier from contaminated and another one from non-contaminated regions). In summary, this study revealed a high prevalence of the 4153delA mutation observed in ~1/110 Byelorussian breast cancer patients, and a significant association with breast cancer (OR: 4.7, 95%CI: 1.1-20.3, p=0.04). As will be

discussed later, the association of 4153delA with breast cancer has been a matter of debate in previous studies.

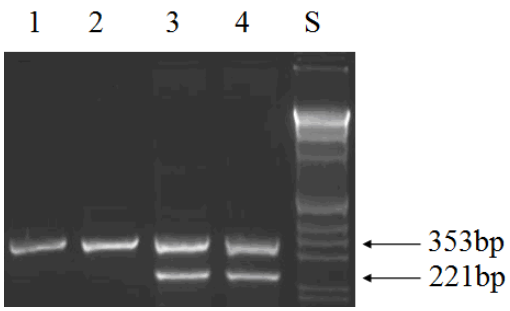
4.1.4 BRCA1 p.C61G

The mutation p.C61G (also known as nucleotide substitution T300G) generates a novel restriction enzyme site in exon 5 of the *BRCA1* gene, and in our initial series of breast cancer cases, this mutation was detected after digesting amplified DNA with the restriction enzyme *Ava*II. Thirty-five cycles of PCR with 59°C annealing temperature for primers flanking mutation site (attachment 1) was performed using GoTaq - Polymerase. PCR products were incubated overnight with *Ava*II at 37ºC followed electrophoresis in a 2% agarose gel, stained with ethidium bromide. In the presence of the mutation 234 bp PCR product was cleaved to 157 bp and 77 bp products, while wild type remains uncut (figure 4.3 A). In the following, the majority of samples from breast cancer patients were screened for the p.C61G mutation using a more rapid and economic ARMS assay. A mutation-specific forward primer was created so that PCR products were obtained only in the presence of the p.C61G mutation (for primers sequence see attachment 1). Thirty-five cycles of PCR amplification were performed using HotStar - *Taq*-Polymerase at 62°C annealing. Subsequently, PCR products were separated on 2% agarose gels which were stained with ethidium bromide. In the presence of the mutation, a 221 bp PCR product was obtained. As an internal amplification control, a 353 bp fragment of the *MDC1* gene was used (figure 4.3 B). A subset of some 200 samples was screened using both techniques for quality control and comparison of the results, which were 100% concordant. All positive samples were verified by direct sequencing of exon 5 in *BRCA1* gene. Sequencing reaction was performed using the reverse primer (figure 4.3 C). PCR for following sequence reaction was performed by using GoTaq-Polymerase, 35 amplification cycles at 58° annealing temperature (primer sequences used for this approach see attachment 1).

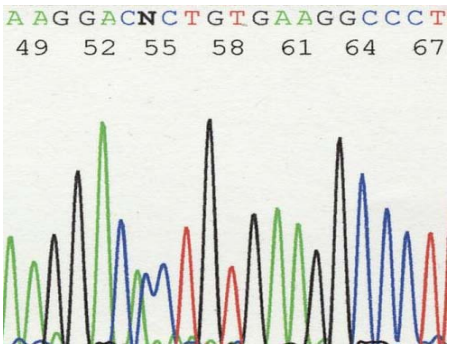


A

Figure 4.3 **Screening for C61G mutation in *BRCA1* gene.** Panel **A –** fragment of 2% agarose gel with *Ava*II enzymatic digestion; S – size marker, line 1 heterozygous carrier of C61G mutation, lanes 2-4 wild type controls. Panel **B** - ARMAS PCR, fragment of *MDC1* gene serves as an internal control: S, size marker, lanes 3,4 mutation carriers, lines 1,2 wild-type controls. Panel **C** - sequencing of PCR products from patient a heterozygous carrier of C61G (anti-sense with **N** designating mutation).



B



C

Genotyping was successfully completed for 100% of cases. The p.C61G allele was obtained in 15 breast cancer cases (0.85%), 3 of them reported family history of breast cancer (all from non-contaminated regions), and none of the carriers had bilateral disease and/or ovarian carcinoma. 6/15 cases were from contaminated areas with 4 out of 6 from areas with more than 5 mSv whole body cumulative dose (subgroups **II-IV**, table 2.1), and none reported first-degree relatives with breast cancer (table 4.1). Thirteen patients were diagnosed below the age 50, median age at diagnosis in carriers was 46.5 years versus 48 in non-carriers (median test p=0.39).The median age in mutation carriers from contaminated areas was 43 years compared with 48 years in non-contaminated areas (median test p=0.28). Frequencies of the p.C61G allele in contaminated and non-contaminated regions are presented in table 4.1, and the ages at diagnosis for carries and non carriers in different regions are shown in table 4.2. The p.C61G allele was also found in 1/1019 controls (carrier was from contaminated area). In summary, this study confirmed a relatively high prevalence of the p.C61G missense mutation present in ~1/110 Byelorussian breast cancer patients, and a significant association with breast cancer (OR: 8.8, 95%CI: 1.2-66.4, p=0.02).

4.1.5. Proportion of all studied BRCA1 mutations in breast cancer patients

Taken together, the three founder *BRCA1* mutations 5382insC, 4153delA and p.C61G were identified in 75/1759 (4.3%) of unselected breast cancer patients from Belarus. Frequencies of all investigated *BRCA1* mutations stratified by region are summarized in table 4.1.

Table 4.1 **Frequencies of *BRCA1* mutations 5382insC, p.C61G and 4153delA in contaminated and non-contaminated regions**

Cohort	Total (n)	5382insC		*p***	p.C61G		*p***	4153delA		*p***	Any *BRCA1* mutation		*p***
		(n)	(%)		(n)	(%)		(n)	(%)		(n)	(%)	
cases [a]	783	22	2.8		6	0.77		10	1.28		38	4.85	
familial cases [a]*	166	7	4.2		0	-		4	2.41		11	6.63	
cases [b]	976	22	2.25	0.55	9	0.92	0.93	6	0.62	0.23	37	3.80	0.33
familial cases [b]*	132	11	8.3	0.14	3	2.27	0.17	2	1.52	0.89	16	12.12	0.15

* subset of patients with at least one first-degree relative also affected with breast cancer

** compared in cases between contaminated and non-contaminated regions in two-by-two tables
[a] cases from contaminated regions
[b] cases from non-contaminated regions

The median ages at diagnosis for carriers of all three studied *BRCA1* variants stratified by region are presented in table 4.2. In total, the diagnosis of breast cancer was made significantly earlier in the identified *BRCA1* mutation carriers compared with non-carriers. Because we also noted a significantly lower age at diagnosis in contaminated versus non-contaminated areas, the mutation carrier group was further stratified by geographic region. A lower age at diagnosis for *BRCA1* carriers was observed in both geographic groups, but the effect of the *BRCA1* mutation seemed to be less significant in contaminated areas (Table 4.2.).

Table 4.2 ***BRCA1*** **mutation prevalence stratified by median age at diagnosis**

Median age at diagnosis / Median test *(*p* value)		**age**	***p****
contaminated regions	**non-carriers**	**44**	
	5382insC	41.5	0.26
	p.C61G	43	0.65
	4153delA	38	0.20
combined carriers versus non carriers in contaminated regions		40.5/44	0.082
non-contaminated regions	**non-carriers**	**50**	
	5382insC	45.5	0.01
	p.C61G	48	0.02
	4153delA	46.5	0.10
combined carriers versus non carriers in non-contaminated regions		46/50	0.0001
Total	carriers versus non carriers	44/48	0.0001

*carriers versus non-carriers

*4.1.6 BRCA2** 6174delT

A screening of the 6174delT frameshift deletion in the *BRCA2* gene was initially performed using cleavage with *Alu*I and *Pflm*I, and later by an ARMS-assay in subsequent samples using a mutation-specific forward primer. In the first DNA series from breast cancer patients thirty-five cycles of PCR with 58°C annealing temperature for primers flanking mutation site (for primer sequence see attachment 1) was performed using HotStar - Polymerase. PCR products were subjected incubated overnight by 37ºC with *Alu*I and *Pflm*I followed by electrophoresis in a 3% agarose gel, which was stained with ethidium bromide. PCR product of 438 bp was cleaved to 310 bp and 128 bp products by *Alu*I, and cleavage fragment of 128 bp, containing site for *Pflm*I was cut in 107 bp and 21 bp in wild type sequence and remains uncut in the presence of the mutation (figure 4.4 A). Subsequent samples from breast cancer patient were screened for the deletion using ARMS. A mutation-specific forward primer was created so that PCR products were obtained only in the presence of the

6174delT allele (in attachment 1 presented the sequences of used primers). Thirty-five cycles of PCR amplification were performed using HotStar - Taq-Polymerase at 64° annealing temperature. Subsequently, the PCR products were separated on a 2% agarose gel, stained with GelRed. In the presence of the mutation, a 189 bp PCR product was obtained. As an internal control for amplification, a 436 bp fragment of the *ATM* gene (exon 52) was used (figure 4.4 B). Subsets of the samples (n=230) were analysed using both techniques to compare the results, which were 100% concordant. All positive samples were verified by direct sequencing of a fragment flanking 6174delT mutation in *BRCA2* gene. Sequencing reaction was performed using the forward primer (figure 4.4 C). PCR for following sequence reaction was performed by using GoTaq-Polymerase, 35 amplification cycles at 58°C annealing temperature (primers sequences used for this approach see attachment 1).

Genotyping was successfully completed for 100% of cases. The 6174delT mutation was found in 4/1759 breast cancer cases (0.23%), all of them heterozygotes. One of them reported a family history of breast cancer (from contaminated region), and none of the carriers had bilateral disease and/or ovarian carcinoma. 3/4 cases were from contaminated areas (2 out of 3 from areas with more than 5 mSv whole body cumulative dose - subgroups **II-IV**, table 2.1) and none reported first-degree relatives with breast cancer.

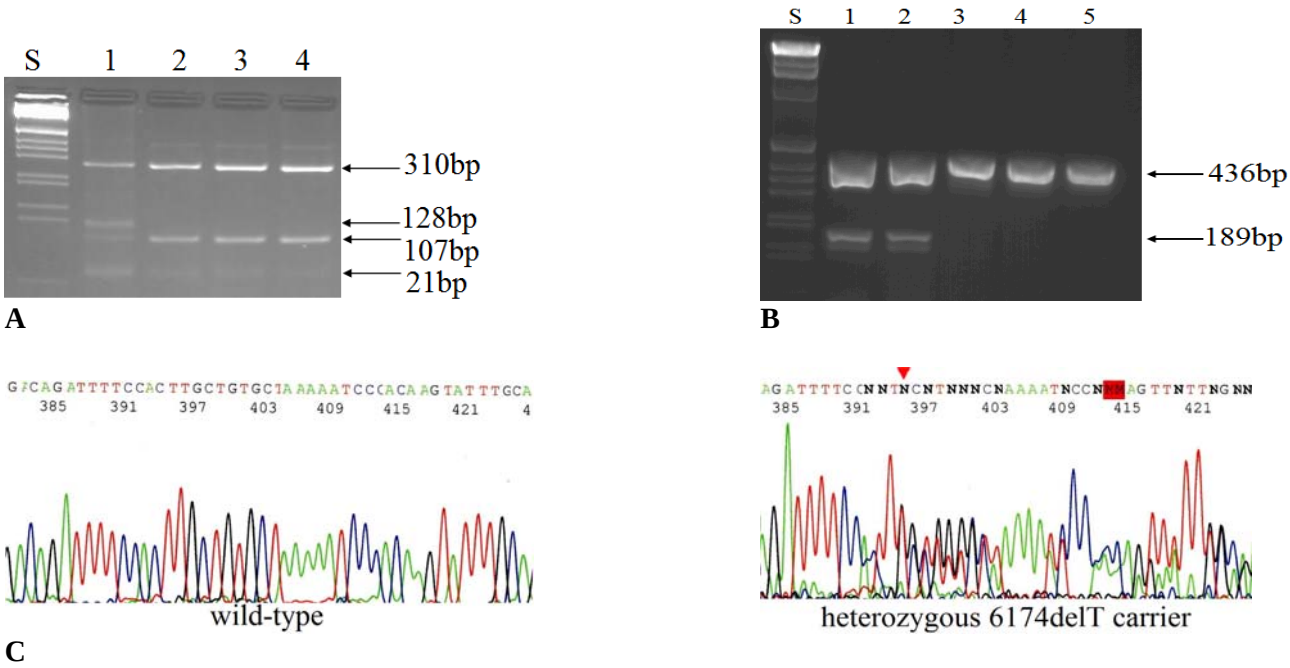
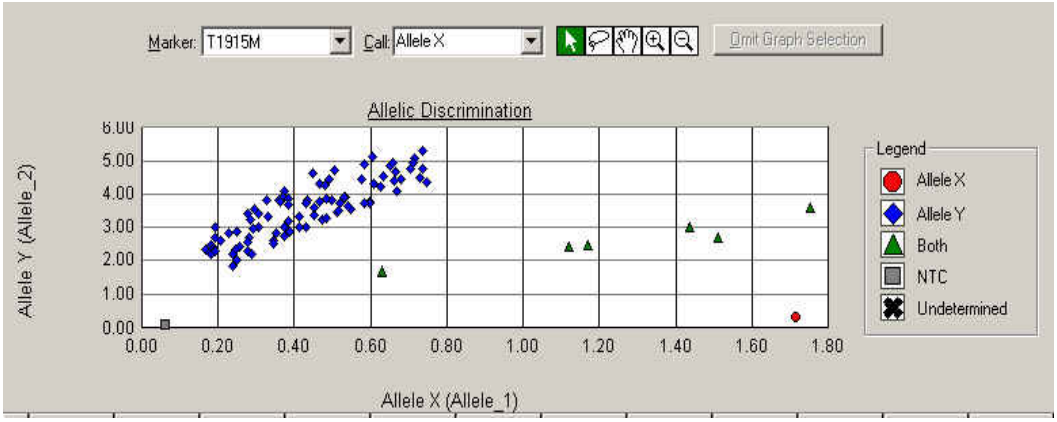


Figure 4.4 **Screening for 6174delT mutation in *BRCA2* gene.** Panel **A –** fragment of 3% agarose gel with *Alu*I and *Pfl*mI enzymatic digestion; S – size marker, line 1 heterozygous carrier of mutation, lanes 2-4 wild type controls. Panel **B** - ARMS assay, *ATM* (exon 51) serves as an internal control: S, size marker, lanes 1,2 deletion carriers, lines 3-5 wild-type controls. Panel **C** - sequencing of PCR products from patient a heterozygous carrier of 6174delT and wild type control (sense with ▼designating mutation).

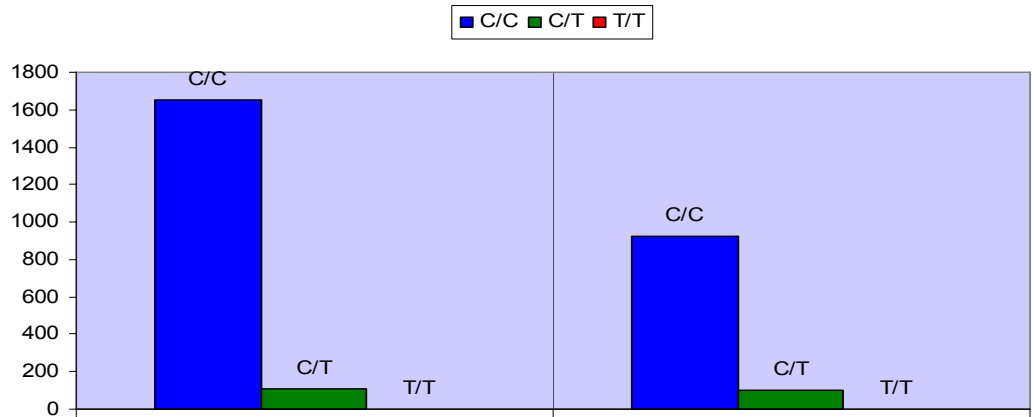
Three patients were diagnosed below the age 50, median age at diagnosis in carriers was 44 years versus 48 in non-carriers (median test p=0.58). Because the low frequency of 6174delT allele in the case series, controls were not tested.

4.1.7 BRCA2 missense variant C5972T (T1915M)

While *BRCA2* mutation 6174delT belongs to the class of protein-truncating mutations, as well as *BRCA1* mutations 5382insC and 4153delA, which are deleterious and increase the risk of breast cancer over a lifetime up to 80%, there are additional substitutions known in the *BRCA2* gene the consequences of which are more difficult to predict. Missense variants are often difficult to classify as mutation apart from the few cases where the amino acid substitution clearly affects protein function (as for example for the RING finger substitution p.C61G in *BRCA1*). A pathogenic missense substitution would be expected at a greater allele frequency in breast cancer cases than in population controls. The *BRCA2* C5972T (p.T1915M) substitution that changes the amino acid sequence at codon 1915 from threonine to methionine and was reported as predisposing to early onset breast cancer in Polish population (Gorski et al. 2005b). This variant was present in approximately 6% of Polish population and was associated with significant increase in risk for breast cancer patient diagnosed before age 40 (OR=1.4; p=0.04) and the effect was most pronounced in woman with ductal carcinoma *in situ* (DCIS). In order to investigate the effect of T1915M allele in the Byelorussian population, the frequencies of this missense variant were determined in case and control cohorts using TaqMan technique with newly designed probes (Allelic Discrimination assay, see 3.8). The assay was run in 96-well plates on a 7500FAST Real-time PCR Thermocycler (Applied Biosystems) with forty amplification cycles and annealing/hybridisation temperatures of exon-specific primers and fluorescence-labelled probes (see attachment 2) at 62°C. Homozygous and heterozygous genotypes were evaluated from their relative fluorescence using the 7500 FAST System SDS Software. Distributions of genotypes (C/C – wild-type; C/T – heterozygous carrier and T/T – homozygous carrier of rare variant) among cases and controls and a representative assay are shown on figure 4.5



A



	cases	controls
C/C	1653	920
C/T	105	98
T/T	1	1

B

Figure 4.5 **C5972T genotypes distribution among studied cases and controls.** Panel **A –** example of 5´-nuclease allelic discrimination assays. Clustering of three genotypes: rare homozygotes (TT, red) are represented by fluorescence along the x-axis, common homozygotes (CC, blue) are clustering along the y-axis, and heterozygotes (CT, green) show fluorescence emission from both dyes. NTC - no template control. Panel **B** – distribution of all three genotypes among cases and controls in studied population (numbers – amount of individuals who are carriers of certain genotype).

Genotyping was successfully completed in 100% of cases and controls. The p.T1915M variant was present in 6.0% of investigated cases and in 9.7% of controls (OR: 0.60, 95%CI: 0.45-0.79, p=0.0004), which would be in line with a protective effect for this allele (table 4.3). In the study two probands (one case and one control) were homozygous for the rare variant (T/T), both were from non-contaminated regions. There was little difference in carrier frequencies if stratified by region albeit the allelic effect seemed less significant in contaminated areas (OR: 0.56, 95%CI: 0.4-0.8, p=0.004 for non-contaminated areas and OR: 0.64, 95%CI: 0.4-1.0, p=0.06 for contaminated areas). There was no clear association between this variant and early onset breast cancer (for patients diagnosed at age 40 years or below) (OR: 1.1, 95%CI: 0.71-1.73, p=0.74, table 4.4) or familial breast cancer (OR: 1.4, 95%CI: 0.9-2.2, p=0.23)**.**

Among the heterozygous carriers of the p.T1915M variant were also four carriers of 5382insC allele in *BRCA1*, one carrier of 4153delA variant in *BRCA1* and one carrier of 6174delT in *BRCA2*. The concurrence of p.T1915M with *BRCA1* mutations was in the expected range (OR 1.05, p=0.99) Seven patients with p.T1915M had bilateral disease, two breast/ovarian cancer and one reported relative affected with ovarian cancer.

Table 4.3. **Genotype frequency distribution for the p.T1915M variant in breast cancer patients and control individuals**.

Genotype/ (n) of carriers	C/C		C/T		T/T	
	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Population controls (whole cohort) (N=1019)	**920**	90.30	**98**	9.61	**1**	0.09
Population controls from non-contaminated regions (N=604)	**545**	90.23	**58**	9.60	**1**	0.17
Population controls from contaminated regions (N=415)	**375**	90.36	**40**	9.64	**0**	-
Breast cancer patients (whole cohort) (N=1759)	**1653**	93.97	**105**	5.97	**1**	0.06
Breast cancer patients from non-contaminated regions (N=976)	**920**	94.26	**55**	5.64	**1**	0.10
Breast cancer patients from contaminated regions (N=783)	**733**	93.60	**50**	6.40	**0**	-

Table 4.4. **Frequencies of the T1915M variant in breast cancer patients stratified by age at diagnosis**.

Genotype	C/C		C/T		T/T	
Group	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Cases at or below age 40 (n=433)	**405**	93.53	**28**	6.47	**0**	-
Cases over age 40 (n=1326)	**1248**	94.10	**77**	5.83	**1**	0.07

4.2 Search for the A-T nonsense mutation p.E1978X and for *ATM* missense variants p.S49C, p.S707P, p.L1420F, p.P1054R and p.F858L in breast cancer cases and population controls

4.2.1. Rationale

Studies based on relatives of A-T patients had shown that heterozygous carriers of *ATM* variants are clinically unaffected but are at an increased risk of cancer, especially the female obligate heterozygotes who have a two- to seven-fold increased risk of breast cancer (Swift et al. 1987, Pippard et al. 1988, Borresen et al. 1990, Swift et al. 1991, Easton 1994, Athma et al. 1996, Olsen et al. 2001, Thompson et al. 2005). In contrast, mutation screening of *ATM* in breast cancer case-control sets outside A-T families had produced mixed results and had suggested allelic heterogeneity of *ATM* and that only a specific class of variants might contribute to breast cancer risk in heterozygotes (FitzGerald et al. 1997, Teraoka et al. 2001, Gatti et al. 1999, Chenevix-Trench et al. 2002). More recently, during the course of this thesis, it has been shown in a UK study using a

familial breast cancer case-control population that, as a whole, A-T causing biallelic *ATM* mutations were breast cancer susceptibility alleles in monoallelic carriers outside AT families, and the combined *ATM* mutation prevalence and contribution to breast cancer incidence was similar to *CHEK2* 1100delC; both conferring an estimated two-fold increased risk of breast cancer (Renwick et al. 2006). A potential association of certain missense variants such as c.7271T>G or p.Ser49Cys, has been reported in other large case control studies (Bernstein et al. 2006, Stredrick et al. 2006). In a study from the Breast Cancer Association Consortium, the Ser49Cys substitution was not significantly associated with overall breast cancer risk, but a modest association was not excluded either, and this SNP appeared to increase the risk of PR positive breast cancer (Cox et al. 2007). Results from association studies of other common polymorphisms (SNPs) in *ATM* and risk of breast cancer have been controversial so far. Because of our relatively large sample size, we decided to investigate the effect of *ATM* gene alterations on breast cancer in more detail.

4.2.2 ATM truncation mutation p.E1978X (c.5932G>T)

The nonsense mutation E1978X leads to a premature termination codon but also to the skipping of exon 42 and a downstream frameshift, thereby resulting in truncated proteins. This mutation has been found in low abundance in European A-T cohorts and was reported in 44% of Russian A-T patients (Birrell et al. 2005). The relatively high prevalence of the c.5932G>T variant in Russians compared with other ethnic groups suggested that this founder-effect mutation may be of Russian origin and prompted us to investigate its frequency in Belarus.

E1978X allele creates an *Mse*I restriction endonuclease site that we used to rapidly screen the samples from Byelorussian breast cancer patients and population controls for the presence of the mutation. Thirty-five cycles of PCR with 58°C annealing temperature for primers flanking mutation site (attachment 1) was performed using HotStar - Polymerase. PCR products were incubated overnight at 37ºC with *Mse*I followed by electrophoresis in a 2% agarose gel, stained with ethidium bromide. There are several cleavage sites for *MseI* in amplified fragment and c5932G>T variant generates a novel one. PCR product of 269 bp was cleaved into fragments of 208 bp, 34 bp and 27 bp. In the presence of the mutation 208 bp cleavage product was cut into 167 bp and 41 bp fragments, while wild type 208 bp product remains uncut (figure 4.6 A fragments of 41 bp, 34 bp and 27 bp were to small to be distinguished from primers). All positive samples were verified by direct sequencing of *ATM* exon 42 (the same primers were used as for mutational screening). Thirty-five cycles of PCR were performed using GoTaq-Polymerase by 58° annealing temperature. Sequencing reaction was performed using the forward primer (figure 4.6 B).

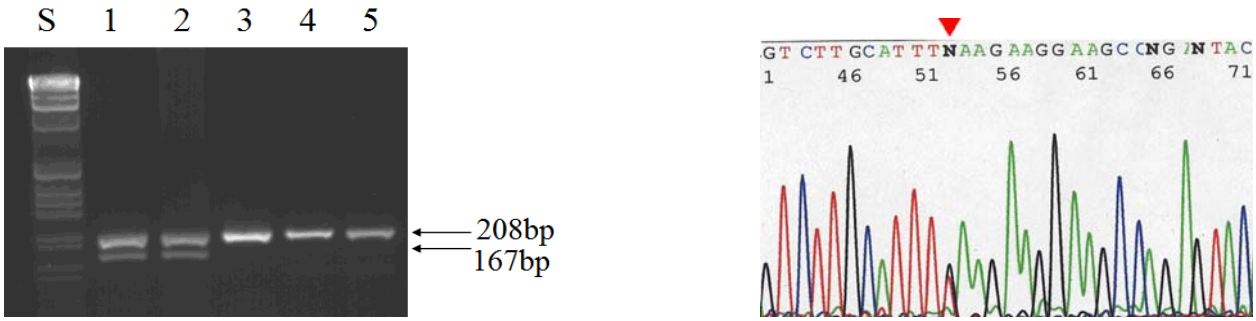


Figure 4.6 **Screening for the p.E1978X mutation in the *ATM* gene.** Panel **A –** fragment of 2% agarose gel with *Mse*I enzymatic digestion; S – size marker, line 1,2 heterozygous carrier of mutation, lanes 3-5 wild type controls. Panel **B** - sequencing of PCR products from patient a heterozygous carrier of p.E1978X (sense with ▼designating mutation).

Genotyping was successfully completed for 100% of cases and controls. The p.E1978X allele was observed in 9 breast cancer cases (0.5%). One of them reported a family history of breast cancer (from contaminated region), and none of the carriers had bilateral disease and/or ovarian carcinoma. 6 cases were from contaminated areas (3 out of 6 from areas with more than 5 mSv whole body cumulative dose - subgroups **II-IV**, table 2.1). Seven patients were diagnosed below the age 50, median age at diagnosis in carriers was 43 years versus 48 in non-carriers (median test p=0.10), median age in mutation carriers from contaminated areas was 42.5 years compared with 47 years in non-contaminated regions. The p.E1978X allele was also found in 1/1019 controls (carrier was from contaminated area). Interestingly, two patients were also carriers of the p.C61G mutation in the *BRCA1* gene and one was a carrier of the *CHEK2*dele(9,10) allele that will be described later. In summary, this study identified an about five-fold higher prevalence of the p.E1978X mutation in Byelorussian breast cancer patients compared with controls, though the difference did not reach statistical significance (OR: 5.2, 95%CI: 0.76-41.4, p=0.15). As will be discussed later, additional work in Russian and Ukrainian breast cancer series was performed to corroborate these results.

4.2.3 ATM variants S49C, S707P, L1420F, P1054R and F858L

4.2.3.1 Rationale and methodology

Unlike the nonsense mutation E1978X, the missense substitutions S49C, S707P, L1420F, P1054R and F858L do not represent classical A-T mutations, and their effect on ATM function still remain to be clarified. However, there was some prior evidence for an association of each of these variants with breast cancer (Dork et al. 2001, Teraoka et al. 2001, Bretsky et al. 2003, Tamimi et al. 2004, Buchholz et al. 2004, Lee et al. 2005, Stredrick et al. 2006). These missense variants were genotyped during the course of this thesis as part of a Breast Cancer Association Consortium study using a 5´-nuclease allelic discrimination assay with mutation-specific fluorescence-labelled MGB probes from Applied Biosystems (Foster City, CA, U.S.A see attachment 2). The assays were run in

96-well plates on a 7500FAST Real-time PCR Thermocycler (Applied Biosystems) with annealing/hybridisation temperatures at 60/62°C, in general forty cycles of amplification were used (see attachment 2). Homozygous and heterozygous genotypes were evaluated from their relative fluorescence using the 7500 FAST System SDS Software. The call rate was above 98% for all SNPs (see below for details).

4.2.3.2 S49C

In case of the genotyping for the Ser49Cys missense alteration (rs1800054), the call rate was 99.7% for cases and 99.9% for controls. Distributions of genotypes (C/C – wild-type; C/G – heterozygous carrier; G/G – homozygous carrier of rare variant) among cases and controls are shown in Table 4.5.

The p.S49C (rs1800054) variant was present in 1.7% of investigated cases and in 2.0% of controls (allele carrier OR: 0.80, 95%CI: 0.46-1.41, p=0.53) (table 4.5). One proband (control) was homozygous for the rare variant (G/G). There was no detectable difference in carrier frequencies when stratified by region (OR: 0.78, 95%CI: 0.35-1.74, p=0.69 for non-contaminated areas, and OR: 0.89, 95%CI: 0.39-2.05, p=0.95 for contaminated areas). Median age at diagnosis among p.S49C carriers was 44 years versus 48 in not carriers (p=0.29).

Table 4.5. **S49C allele distribution in cohorts of breast cancer patients and controls**.

Genotype/ (n) of carriers	**C/C**		**C/G**		**G/G**	
	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Population controls (whole cohort) (N=1018)	**998**	98.0	**19**	1.9	**1**	0.1
Population controls from non-contaminated regions (N=603)	**592**	98.2	**10**	1.7	**1**	0.2
Population controls from contaminated regions (N=415)	**406**	97.8	**9**	2.2	**0**	-
Breast cancer patients (whole cohort) (N=1753)	**1724**	98.4	**29**	1.7	**0**	-
Breast cancer patients from non-contaminated regions (N=976)	**962**	98.6	**14**	1.4	**0**	-
Breast cancer patients from contaminated regions (N=777)	**762**	98.1	**15**	1.9	**0**	-

The S49C allele appeared to be present in Byelorussian cases and population controls at a similar frequency. There was also no association between this variant and familial breast cancer (OR: 0.78,

95%CI: 0.27-2.25, p=0.83). Among the breast cancer patients heterozygous for p.S49C , there were four carriers of the 5382insC allele in *BRCA1*, one carrier of the 4153delA variant in *BRCA1* and one carrier of I157T in *CHEK2*; the increased prevalence of p.S49C among *BRCA1* mutation carriers was significant (5/75 compared with 29/1753, OR 4.2, 95%CI 1.6-11.3, p= 0.002). None of the patients carrying p.S49C had bilateral disease or breast/ovarian cancer, and four reported a first-degree relative affected with breast cancer.

4.2.3.3 S707P

The Ser707Pro variant is caused by a nucleotide substitution c.2119T/C in exon 15 of *ATM* (rs4986761). Genotyping was successful in 99.7% of cases and controls. Distribution of genotypes (T/T – wild-type; T/C – heterozygous carrier; C/C – homozygous carrier of rare variant were not present in Byelorussian population) among cases and controls is shown on table 4.6. The p.S707P (rs4986761) variant was present in 1.5% of investigated cases and in 2.2% of controls (OR: 0.71, 95%CI: 0.40-1.25, p=0.29) (table 4.6). There was no difference in carrier frequencies when stratified by region (OR: 0.66, 95%CI: 0.31-1.37, p=0.35 for non-contaminated areas and OR: 0.80, 95%CI: 0.32-1.96, p=0.79 for contaminated areas). Median age at diagnosis among S707P carriers was 51 years versus 48 in non-carriers (p=0.21).

Table 4.6. **S707P allele distribution in cohorts of breast cancer patients and controls**.

Genotype/ (n) of carriers	**T/T**		**T/C**	
	Total (n)	(%)	Total (n)	(%)
Population controls (whole cohort) (N=1016)	**994**	97.8	**22**	2.2
Population controls from non-contaminated regions (N=602)	**588**	97.7	**14**	2.3
Population controls from contaminated regions (N=414)	**406**	98.1	**8**	1.9
Breast cancer patients (whole cohort) (N=1754)	**1727**	98.5	**27**	1.5
Breast cancer patients from non-contaminated regions (N=976)	**961**	98.5	**15**	1.5
Breast cancer patients from contaminated regions (N=778)	**766**	98.5	**12**	1.5

The S707P allele appeared to be present in Byelorussian population controls at similar frequency as in cases. There was no detectable association between this variant and familial breast cancer (OR: 1.73, 95%CI: 0.72-4.12, p=0.32). Among the patients heterozygous for p.S707P were also three carriers of I157T in *CHEK2* (see chapter 4.4. about *CHEK2* mutations); three patients had bilateral disease, one breast/ovarian cancer, and seven reported first-degree relatives affected with breast cancer.

4.2.3.4 L1420F

The Leu1420Phe substitution is caused by a nucleotide substitution c.4258C/T in exon 31 of *ATM* (rs1800058). The call rate was 99.1% for cases and 99.8% for controls. Distributions of genotypes (C/C – wild-type; C/T – heterozygous carrier; homozygotes rare variant were not present in Byelorussian population) among cases and controls are shown in Table 4.7.

The p.L1420F (rs1800058) variant was present in 4.8% of investigated cases and in 5.8% of controls (OR: 0.82, 95%CI: 0.58-1.15, p=0.29) (table 4.7). There was no significant difference in carrier frequencies when stratified by region (OR: 0.85, 95%CI: 0.53-1.37, p=0.59 for non-contaminated areas and OR: 0.76, 95%CI: 0.46-1.27, p=0.36 for contaminated areas). Median age at diagnosis among p.L1420F carriers was 47 years versus 48 in non-carriers (p=0.21).

Table 4.7. **L1420F allele distribution in cohorts of breast cancer patients and controls**.

Genotype/ (n) of carriers	C/C		C/T	
	Total (n)	(%)	Total (n)	(%)
Population controls (whole cohort) (N=1017)	**959**	94.2	**58**	5.8
Population controls from non-contaminated regions (N=602)	**571**	94.9	**31**	5.2
Population controls from contaminated regions (N=415)	**388**	93.5	**27**	6.5
Breast cancer patients (whole cohort) (N=1743)	**1661**	95.2	**82**	4.8
Breast cancer patients from non-contaminated regions (N=971)	**928**	95.6	**43**	4.4
Breast cancer patients from contaminated regions (N=772)	**733**	95.0	**39**	5.1

The L1420F allele appears to be present in Byelorussian population controls at similar frequencies as in cases. There was no significant association between this variant and familial breast cancer (OR: 1.49, 95%CI: 0.88-2.54, p=0.18). Among breast cancer heterozygous carriers of L1420F variant, there was one carrier of 4153delA variant in *BRCA1*, three carriers of *CHEK2*dele(9,10) mutation, and seven carriers of I157T in *CHEK2* (see chapter 4.4. about *CHEK2* mutations); two patients had bilateral disease one breast/ovarian cancer and one reported a first-degree relative affected with ovarian cancer.

4.2.3.5 P1054R

The missense substitution Pro1054Arg is caused by a nucleotide substitution c.3061C/G (rs1800057) in exon 24 of *ATM*. Genotyping was successful in 99.9% of case and 100% of controls. Distribution of genotypes (C/C – wild-type; C/G – heterozygous carrier; G/G – homozygous carrier of rare variant) among cases and controls is shown in Table 4.8.

The p.P1054R allele was present in 2.8% of investigated cases and in 3.5% of controls (OR: 0.80, 95%CI: 0.52-1.24, p=0.37) (table 4.8). Carrier Odds Ratios were slightly but non-significantly different when stratified by region (OR: 1.09, 95%CI: 0.58-2.02, p=0.92 for non-contaminated areas and OR: 0.57, 95%CI: 0.31-1.06, p=0.10 for contaminated areas), but in a case-only comparison between regions no difference was found (OR: 0.98, 95%CI: 0.55-1.73, p=1.00). Median age at diagnosis among P1054R carriers was not different for carriers and non-carriers: 47.5 years versus 48 years (p=0.86).

Table 4.8. **P1054R allele distribution in cohorts of breast cancer patients and controls**.

Genotype/ (n) of carriers	**C/C**		**C/G**		**G/G**	
	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Population controls (whole cohort) (N=1019)	**983**	96.5	**36**	3.5	**0**	-
Population controls from non-contaminated regions (N=604)	**588**	97.4	**16**	2.7	**0**	-
Population controls from contaminated regions (N=415)	**395**	95.2	**20**	4.8	**0**	-
Breast cancer patients (whole cohort) (N=1758)	**1708**	97.2	**49**	2.8	**1**	0.1
Breast cancer patients from non-contaminated regions (N=976)	**948**	97.1	**28**	2.9	**0**	-
Breast cancer patients from contaminated regions (N=782)	**760**	97.2	**22**	2.7	**1**	0.1

There was no significant association between this variant and familial breast cancer (OR: 0.79, 95%CI: 0.35-1.78, p=0.71). Among patients heterozygous for the p.P1054R variant, there were also one carrier of the 4153delA allele and one carrier of 5382insC in *BRCA1*, two carriers of I157T and one of IVS1+2G>A in *CHEK2*; two patients had bilateral disease, one breast/ovarian cancer and seven reported a first-degree relative affected with breast cancer.

4.2.3.6 F858L

p.F858L, a nucleotide substitution 2578T/C in exon 18 (rs1800056) of *ATM* gene, occurs in strong linkage disequilibrium with p.P1054R on the same allele and was screened only in individuals who are carriers of the p.P1054R variant. The call rate was 100%. Distributions of genotypes (T/T – wild-type; T/C – heterozygous carrier; C/C – homozygous carrier of rare variant were not present in Byelorussian population) among cases and controls are shown in Table 4.9.

The p.F858L allele was present in 62% of the investigated cases harboring p.P1054R (corresponding to 1.8% in the whole case series) and in 44.4% of the investigated controls with p.P1054R (corresponding to 1.6% in the whole control series) (OR: 2.04, 95%CI: 0.85-4.87, p=0.16) (table 4.9). There was no significant difference in carrier frequencies if cases were stratified

by region (OR: 1.71, 95%CI: 0.50-5.92, p=0.59 for non-contaminated areas and OR: 2.62, 95%CI: 0.74-9.21, p=0.23 for contaminated areas). Median age at diagnosis among p.F858L carriers was 47 years versus 50 in non-carriers (p=0.38) and 48 in the whole cohort (p=0.40).

No association was found between this variant and familial breast cancer (OR: 1.43, 95%CI: 0.61-3.36, p=0.56). Among breast cancer patients heterozygous for p.F858L were one carrier of 4153delA and one carrier of 5382insC in *BRCA1*, one carrier of IVS1+2G>A in *CHEK2*; two patients had bilateral disease, one breast/ovarian cancer and seven reported first-degree relative affected with breast cancer.

Table 4.9. **Carrier frequencies in breast cancer patients and controls for the p.F858L variant**.

Genotype/ (n) of carriers	T/T		T/C	
	Total (n)	(%)*	Total (n)	(%)*
Population controls (N=36 p.P1054R carriers / N=1019 whole cohort)	**20/36**	55.6/2.0	**16/36**	44.4/1.6
Population controls from non-contaminated regions (N=16 p.P1054R carriers / N=604 whole cohort)	**9/16**	56.3/1.5	**7/16**	43.8/1.2
Population controls from contaminated regions (N=20 p.P1054R carriers / N=415 whole cohort)	**11/20**	55.0/2.7	**9/20**	45.0/2.2
Breast cancer patients (N=50 p.P1054R carriers / N=1758 whole cohort)	**19/50**	38.0/1.1	**31/50**	62.0/1.8
Breast cancer patients from non-contaminated regions (N=28 p.P1054R carriers / N=976 whole cohort)	**12/28**	42.9/1.2	**16/28**	57.1/1.6
Breast cancer patients from contaminated regions (N=22 p.P1054R carriers / N=782 whole cohort)	**7/22**	31.8/0.9	**15/22**	68.2/1.9

* percentages in screened samples/whole cohort

4.3 Search for 657del5, R215W and I171V alleles in the *NBN* gene

4.3.1 Rationale

Breast cancer is not observed in NBS patients who fail to mature through puberty and usually die early in life, but blood relatives of NBS patients have been reported to be at a generally increased risk for lymphoid and epithelial malignancies (Seemanova et al. 2007). Association studies of *NBN* gene alterations in breast cancer series have not generally supported the suggestion that *NBN* mutations may contribute to breast cancer susceptibility, but during the course of this thesis, more recent investigations in the Polish and Russian populations had provided suggestive evidence that the founder mutation c.657del5 could be associated with an increased breast cancer risk (Gorski et al 2003, 2005; Steffen et al 2004, 2006; Buslov et al 2005). The p.R215W mutation has been suggested to increase the risk for colorectal cancer, while its impact on breast cancer has remained uncertain (Steffen et al 2004, 2006). Recent investigations in the Polish population raised the hypothesis that the missense substitution p.I171V could be associated with a nine-fold increased breast cancer risk (Roznowski at al 2008).

A splice site mutation in intron 11 has been associated with gastric and colorectal cancer risk in a Japanese study population but was not found in breast cancer cases (Ebi et al. 2007). To clarify the role of *NBN* in breast cancer, we chose to investigate the *NBN* gene alterations previously known from European populations for their prevalence in Byelorussian cases and controls and for their potential functional impact on the cellular radiation response.

4.3.2 Mutation analysis of 657del5 and R215W

Mutation analysis of c.657del5 in *NBN* was performed by an ARMS assay using a mutation-specific forward primer so that a 391bp PCR product was obtained only in the presence of the c.657del5 mutation (for primer sequence see attachment 1). A 353 bp fragment of the *MDC1* gene was used as an internal control for amplification (figure 4.7 A). All positive samples were verified by direct sequencing of exon 6 in the *NBN* gene using the reverse primer (see attachment 1) (figure 4.7 B).

For p.R215W screening, PCR products were screened by restriction enzyme analysis using a mutagenic forward primer (for primers sequence see attachment 1) to create a site for allele-specific restriction enzyme cleavage. PCR products were incubated by 37°C overnight with *Rsa*I. Restriction enzyme reaction products were separated on a 3% agarose gel and were evaluated by ethidium bromide staining.

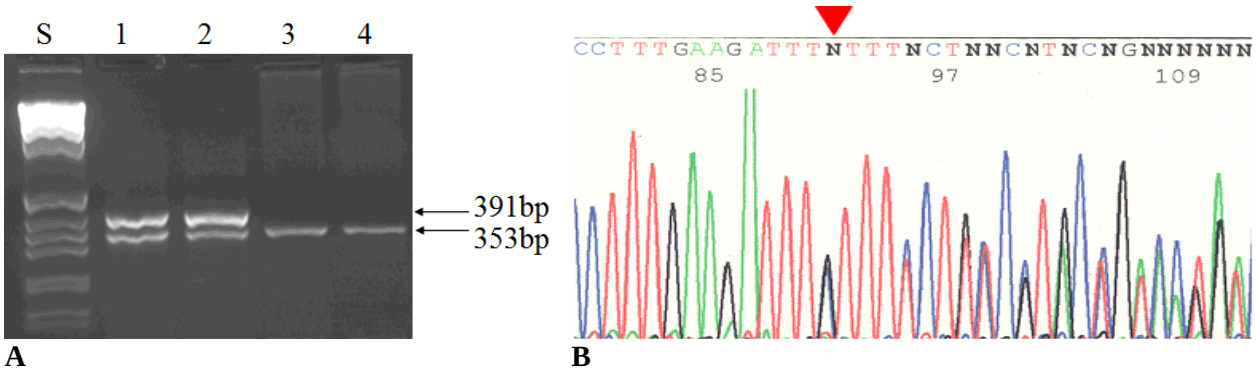
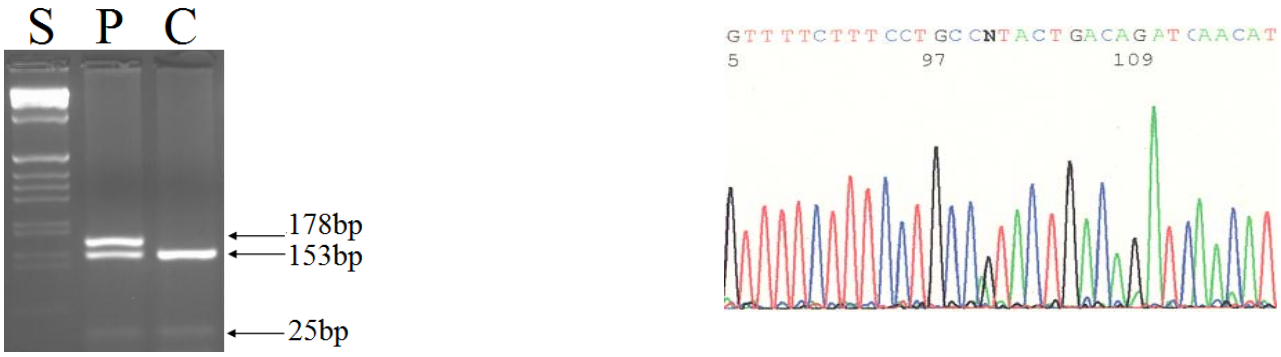


Figure 4.7 **Screening for c.657del5.** Panel **A** - ARMS PCR, *MDC1* served as an internal control: S, size marker, lanes 1,2 deletion carriers, lanes 3-4 wild-type controls. Panel **B** - sequencing of PCR product from a heterozygous carrier of c.657del5 (anti-sense strand with ▼designating the deletion start).

In the presence of the p.R215W mutation, the 178 bp wild-type PCR product was cleaved by *Rsa*I to fragments of 153 and 25 bp, whereas the mutant product remained uncut (figure 4.8 A). Any positive samples were verified by direct sequencing (figure 4.8 B). For this purpose, exon 6 of the *NBN* gene were amplified using primers flanking R215W (see attachment 1) and sequencing reaction was performed using forward primer.



A **B**

Figure 4.8 **Screening for the p.R215W allele in NBS1 gene.** Panel **A** – Fragment of 3% gel with *Rsa*I digestion: S, size marker, lanes P – p.R215W substitution carriers, C – wild-type control. Panel **B –** sequencing of PCR product from patient a heterozygous carrier of p.R215W (sense with **N** designating the substitution).

Genotyping was successfully completed in 100% of cases and controls. The c.657del5 mutation accounted for 16 cases (0.91%) and was found in only one of 1014 population controls (females were excluded as controls for NBN mutational screening if they had a child with a neurodegenerative disorder) (OR: 9.3, 95%CI: 1.2-70.2, p=0.02) (table 4.10). Eight of the 16 patients carrying the c.657del5 allele had been diagnosed below age 50, and 1 patient reported a first-degree family history of breast cancer. Three patients were from the contaminated regions (one from the subgroup **II** and two from the subgroup **I**) and none of them had relatives affected with breast cancer. One patient had bilateral disease. Median age at diagnosis in deletion carriers was not significantly different from non-carriers: 49.5 years compared with 48 years, although carriers from contaminated areas had median age at diagnosis 46 years (all three carriers were diagnosed below age 50) compared with 50 in non-contaminated regions, but this was not statistically significant (p=0.57, table 4.11). One patient was also carrier of the splicing mutation IVS2+1G>A in *CHEK2*.

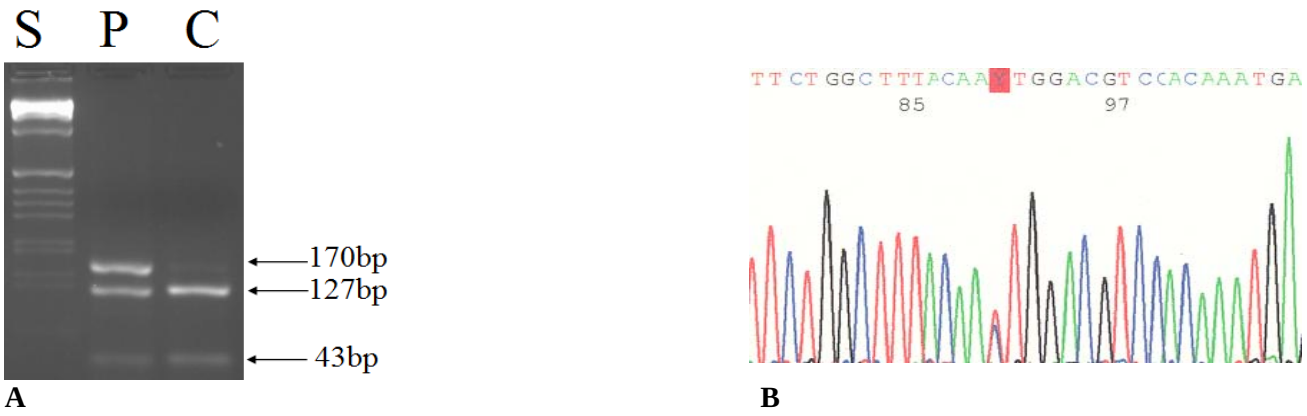
The p.R215W substitution was found in 9 cases (0.5%). Four of the 9 patients carrying the p.R215W substitution had been diagnosed below age 50, and 2 patients reported a first-degree family history of breast cancer, none of the patients reported bilateral disease or ovarian carcinoma. The p.R215W missense substitution was also observed in 5 population controls (p=1.00) (table 4.10). The median age at onset of breast cancer was not significantly different between p.R215W carriers and non carriers (50 years compared with 48 years, p=0.70). One patient was also carrier of the p.C61G mutation in *BRCA1*.

4.3.3 Missense variant p.I171V in NBN

Simple PCR assay was used followed by restriction enzyme analysis for I171V screening. PCR amplification of 35 cycles was performed using HotStar –Taq DNA Polymerase. PCR products then were incubated overnight at 37°C with *Tsp*509I, subsequently restriction enzyme reaction products were separated on a 3% agarose gel and were evaluated after ethidium bromide staining. In the

presence of the p.I171V substitution, the 170 bp wild-type product was cleaved to fragments of 127 and 43 bp, whereas mutant product remained uncut (figure 4.9 A). Any positive samples were verified by direct sequencing (figure 4.9 B). For this purpose, exon 5 of *NBN* was amplified and the forward primer was used for the sequencing reaction (for primer sequence see attachment 1).

Genotyping was successfully completed in 100% of cases and controls. The p.I171V substitution accounted for 22/1759 cases (1.3%). Two of the p.I171V heterozygous patients reported a first-degree family history of breast cancer (both from contaminated areas), a proportion not higher than among non-carriers (OR 0.5, 95%CI 0.1-2.1, p=0.48). None of the carriers had bilateral disease. Thirteen of the 22 heterozygous patients had been diagnosed at or below age 50. The median age at onset of breast cancer was not significantly different between p.I171V carriers and non carriers (49.5 years in carriers compared with 48 years in non-carriers) and was not different in contaminated and non-contaminated regions (50 and 49 years respectively). Nine patients of the 22 were from contaminated regions and all of them from the subgroups **II-IV**. Importantly, the p.I171V allele was also found at a polymorphic frequency in 19/1014 (1.9%) of control individuals. The reported association with breast cancer could thus not be confirmed in our Byelorussian case-control series (OR: 0.66, 95%CI: 0.36-1.23, p = 0.25) (table 4.10).



A **B**

Figure 4.9 **Identification and confirmation of *NBN* gene variant p.I171V.** Panel **A –** fragment of 3% agarose gel: S, size marker; P, heterozygous patient; C – wild-type control; and panel **B –** sequencing of PCR products from heterozygous patient (sense strand with Y designating the mutated position).

Table 4.10 **Frequency of *NBN* alleles c.657del5, p.R215W and p.I171V in cases and controls**

Cohort	Total (n)	NBS1*657del5		*p***	R215W		*p***	I171V		*p***
		(n)	(%)		(n)	(%)		(n)	(%)	
controls [a]	413	0	-		1	0.24		6	1.45	
cases [a]	783	3	0.38	0.13	1	0.13	1.00	9	1.15	0.86
familial cases [a]*	166	0	-		1	0.60	0.48	2	1.20	1.00
controls [b]	602	1	0.17		4	0.66		13	2.16	
cases [b]	976	13	1.33	0.03	8	0.82	0.96	13	1.33	0.29
familial cases [b]*	132	1	0.76	0.28	1	0.76	1.00	0	-	
Controls	**1014**	**1**	**0.09**		**5**	**0.49**		**19**	**1.87**	
Cases	**1759**	**16**	**0.90**	**0.02**	**9**	**0.51**	**1.00**	**22**	**1.25**	**0.25**
Familial	**298**	**1**	**0.34**	**0.75**	**2**	**0.67**	**1.00**	**2**	**0.67**	**0.48**

* subset of patients with at least one first-degree relative also affected with breast cancer
** compared with population controls
[a] cases and controls from contaminated regions
[b] cases and controls from non-contaminated regions

*Table 4.11 **NBN*** **mutation prevalence and median age at diagnosis**

Median age at diagnosis / Median test *(*p* value)		age	*p**
contaminated regions	**non-carriers**	**44**	
	c.657del5	46	0.57
	p.R215W	Only one patient diagnosed at 23	
	p.I171V	50	0.32
non-contaminated regions	**non-carriers**	**50**	
	c.657del5	50	0.74
	p.R215W	50	0.99
	p.I171V	49	0.80

*carries versus non-carriers

4.3.4 Effect of the p.R215W missense substitution and the c.657del5 allele on protein function

The effect of the p.R215W substitution and NBS1*657del5 was further studied in lymphoblastoid cell lines (LCLs) that were established from the patients heterozygous for these variants. LCLs were established by Epstein Barr virus (EBV) immortalisation and the expression of NBS1 protein was determined by immunoblot analyses of lymphoblastoid cell protein extracts. To determine radiation-induced Nbs1(Ser343) phosphorylation, cell extracts were prepared 30 min after irradiation of the LCLs with 6 Gy. Whole cell extract was loaded at 50µg/lane on 7.5% SDS-PAGE gels. After transfer the membranes were probed with either a 1:5000 dilution of anti-Nbs1 (Oncogene Research) or a 1:500 dilution of p95/Nbs1 (Ser-343) antibody (New England Biolabs), respectively, and the same blot was subsequently probed with a monoclonal antibody raised against β-actin (1:5000, Sigma) to control for loading. Signal ratios Nbs1/ β-actin were densitometrically determined and the relative expression level of Nbs1 was estimated for each of LCLs from patients harbouring a *NBN* mutation relative to the mean of LCLs from healthy individual (figure 4.10).

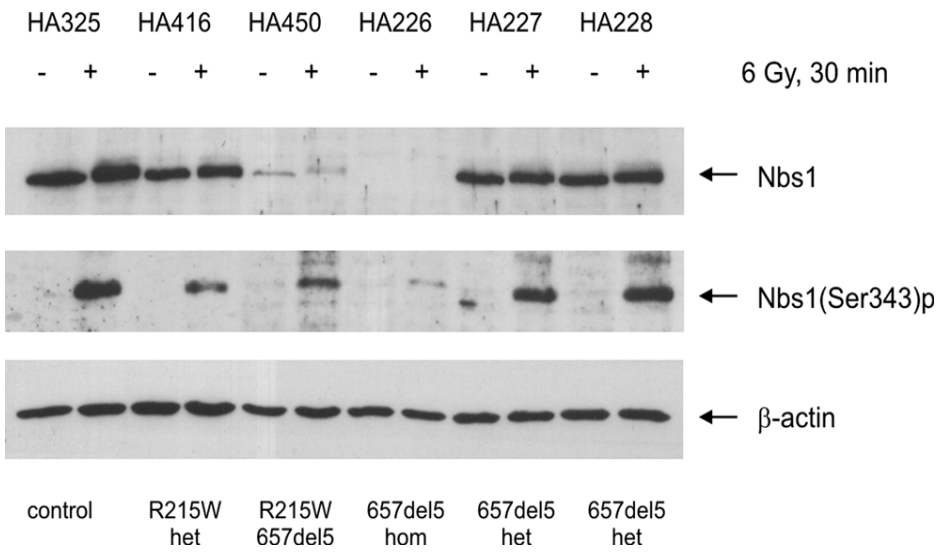


Figure 4.10 Expression **of nibrin (NBS1, also termed NBN or p95) in lymphoblastoid cells**. Identification of NBS1 protein (upper panel) and of radiation induced phosphorylation at NBS1(Ser343) (middle panel) by immunoblot analysis of total cell extracts from six different LCLs without or with previous exposure to 6 Gy irradiation. β-actin is shown as a loading control (lower panel). From left to right: HA325, healthy control individual; HA416, breast cancer patient heterozygous for p.R215W; HA450, NBS patient compound heterozygous p.R215W/c.657del5; HA226, NBS patient homozygous c.657del5; HA227 and HA228, NBS carriers heterozygous for c.657del5.

Predictably, in the c.657del5 homozygous LCL no full-length Nbs1 protein was observed, consistent with previous data on cell lines from NBS patients. Nbs1 protein levels were ~15% in the cells from the NBS patient with the 657del5/R215W genotype, ~70% in cells from the breast cancer patient with the wildtype/R215W genotype, and ~70% in cells from NBS heterozygotes with the wild-type/ 657del5 genotype (figure 4.10, upper panel).

By using a phosphospecific antibody to assess radiation-induced phosphorylation of Nbs1 at Ser-343, the results paralleled those for total Nbs1 protein yielding: some 35% of Nbs1(p-Ser343) immunoreactivity in the cells from the NBS patient with the 657del5/R215W genotype, about 60% in cells from the breast cancer patient with the wildtype/R215W genotype, and about 80% in the wildtype/657del5 heterozygotes (figure 4.10, middle panel). The results obtained for the p.R215W heterozygous breast cancer patient were thus in the similar range as those observed for c.657del5 heterozygotes and were consistent with only about one-third of the wild-type amount of full-length nibrin from the p.R215W allele.

4.4. *CHEK2* mutations 1100delC, I157T, IVS2+1G>A and dele9,10(5kb)

4.4.1 Background and rationale

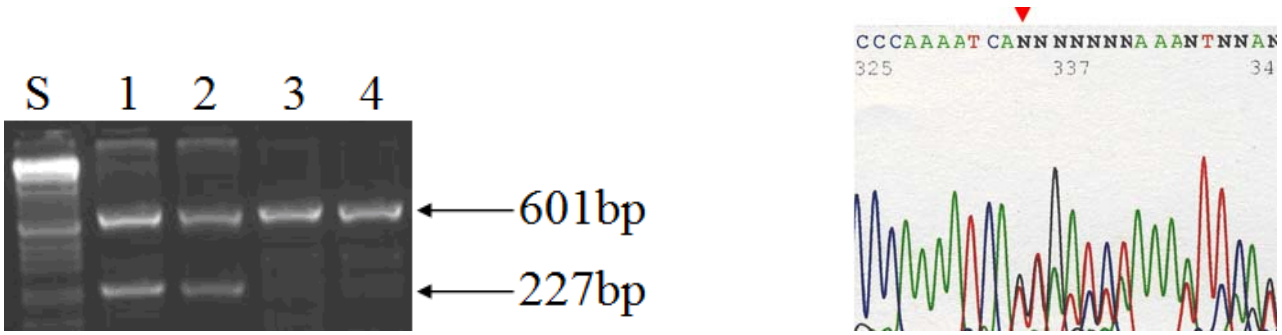
Germ-line mutations in the cell-cycle checkpoint kinase *CHEK2* have been associated with breast cancer. In particular, a frameshift mutation in the *CHEK2* gene, 1100delC, was identified as a low-penetrance breast cancer susceptibility allele (Meijers-Heijboer et al 2003, Vahteristo et al 2002) and

heterozygous carriers of the mutation have a two-to threefold increased risk for breast cancer (CHEK2–Breast Cancer Case-Control Consortium 2004, Weischer et al 2007). Some studies furthermore indicated that a heterozygous carrier status for this *CHEK2* mutation may be associated with a worse prognosis (de Bock et al 2004, Meyer et al 2007, Schmidt et al 2007). Other *CHEK2* mutations were less extensively characterized. During the course of this project, two research groups reported suggestive evidence for an increased breast cancer susceptibility associated with two other *CHEK2* mutations, the splicing mutation IVS2+1G>A and the missense substitution I157T (Kilpivaara et al 2004, Cybulski et al 2004, Gorski et al 2005). Since then, a large deletion in *CHEK2* including the exons 9 and 10, *CHEK2*dele9,10(5kb), has been identified in breast cancer patients of Czech and Slovak origin (Walsh et al 2006) and was subsequently found in breast cancer patients from Poland (Cybulski et al 2007). We therefore decided to test whether these mutations associated with breast cancer risk in Belarus.

4.4.2 Screening for 1100delC mutations

Because several copies of exon 10 of *CHEK2* are dispersed in pseudogenes throughout the human genome, the analysis of this gene region is challenging and for screening of the 1100delC frameshift mutation in *CHEK2* an ARMS assay was established using a reverse primer specific for the expressed sequence of the *CHEK2* gene and a forward primer specific for the *CHEK2**1100delC allele. A genomic region within mutation site was amplified by 33 cycles of PCR using HotStar – Polymerase (Quagen) by 61ºC annealing temperature (primers used for this approach see attachment 1). Subsequently, PCR products were separated on 2% agarose gel and visualized in UV after GelRed staining (figure 4.11 A). PCR product of 227 bp was obtained only in the presence of the *CHEK2**1100delC mutation. A 601 fragment of the *ATM* gene (exon 9) was used as an internal control for amplification. All samples positive for 1100delC were sequenced to verify the presence of the mutation (figure 4.11 B, for primers sequences see attachment 1).

Genotyping was successfully completed for 99.9% of cases and 99.6% of controls. The 1100delC mutation accounted for 9/1757 cases and was found in 3 of 1015 population controls (OR: 1.74, 95%CI: 0.47-6.43, p=0.59) (table 4.12). The increase of 1100delC carriers in the breast cancer series was not significant in the total series, but approached some effect for breast cancer patients in non-contaminated regions (8/976 vs. 0/600, p=0.06).



A **B**

Figure 4.11. **Screening for *CHEK2**1100delC.** Panel **A –** detection of the frameshift mutation 1100delC by allele-specific PCR in two carriers (lanes 1,2) but not in wild-type controls (lanes 3,4), S – size marker. Panel **B** – identification of the *CHEK2**1100delC mutation in a heterozygous patient by direct sequencing of the anti-sense strand; an ▼marks the start of the frameshift.

Three of the 9 patients carrying the 1100delC allele had been diagnosed below age 50. Median age at diagnosis among carriers of mutation was 54 years versus 48 in non carriers (p=0.05). Only one patients was from a contaminated region (from subgroup **III**), and none had relatives affected with breast cancer ore bilateral disease. One patient (from contaminated region) was also a carrier of both the p.C61G mutation in *BRCA1* and the p.R215W substitution in *NBN*.

Table 4.12 Frequency of *CHEK2* mutation 1100delC in cases and controls

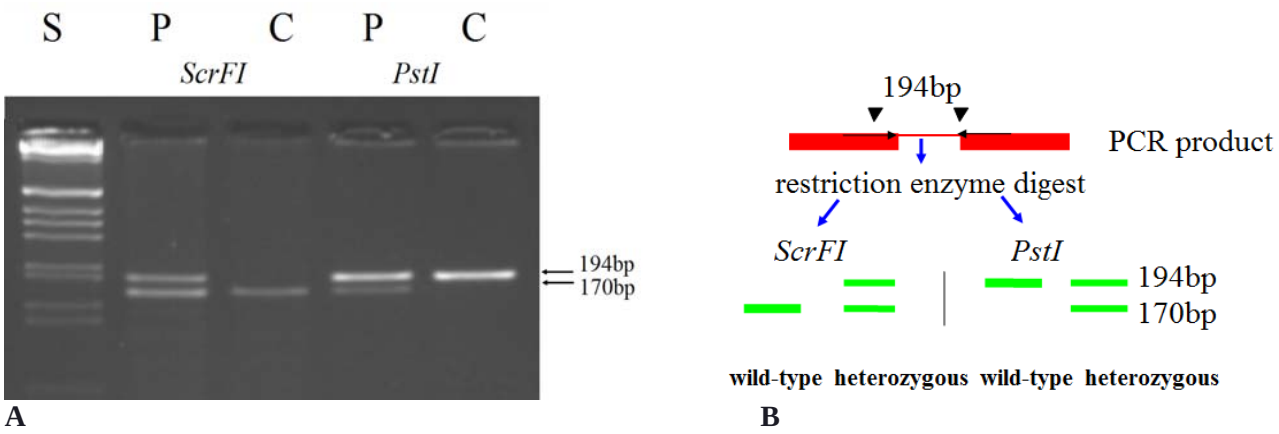
Cohort	Total (n)	*CHEK2**1100delC				
		(n)	(%)	OR**	95%CI**	p**
controls [a]	600	0	-	n.a	n.a	0.06
cases [a]	976	8	0.8			
familial cases [a]*	0	-	-	-	-	-
controls [b]	415	3	0.7	0.18	0.02-1.70	0.24
cases [b]	781	1	0.1			
familial cases [b]*	0	-	-	-	-	-
Total controls	1015	3	0.3	1.74	0.47-6.43	0.59
Total cases	1757	9	0.5			
Total familial cases *	298	0	-	-	-	-

* subset of patients with at least one first-degree relative also affected with breast cancer
** compared between cases and controls in two-by-two tables
[a] cases and controls from non-contaminated regions
[b] cases and controls from contaminated regions
n.a. not applicable due to dividing by null

4.4.3 Mutation analysis of the IVS2+1G>A and p.I157T mutations

A genomic region covering both the IVS2+1G>A and p.I157T mutations in intron 2 and exon 3 of the *CHEK2* gene was amplified by PCR using mutagenic primers to allow for a subsequent restriction-enzyme screening of these two mutations (attachment 1). Thirty-six cycles of PCR amplification were performed with annealing at 60°C using HotStar – Taq Polymerase. PCR

products were separately digested over night by 37°C with either *ScrF*I or *Pst*I to identify carriers of IVS2+1G>A or p.I157T, respectively. Reaction products were separated on a 3% agarose gel and were evaluated after ethidium bromide staining. In the presence of the p.I157T mutation, the 194 bp product was cleaved by *Pst*I to fragments of 20 and 170 bp, whereas wild-type product remained uncut. In case of the IVS2+1G>A mutation, the wild-type product was cleaved by *ScrF*I and the mutant PCR product remained uncut (figure 4.12 A, B). All positive samples were confirmed by direct sequencing of PCR products (figure 4.12 C) using the intronic primers (attachment 1). Sequencing reactions were performed using the forward primer.



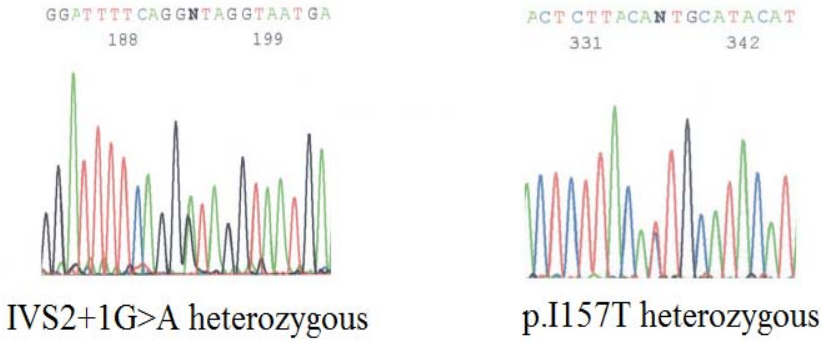


Figure 4.12 **Identification and confirmation of *CHEK2* gene mutations IVS2+1G>A and I157T.** Screening of PCR products using restriction enzymes *ScrF*I and *Pst*I. Upper figure **A**: S, size marker; P, heterozygous patient; C, wild-type control. Panel **B**: scheme of restriction enzyme digest. Panel **C** - subsequent direct sequencing of PCR products from heterozygous patients (sense strand with **N** designating the mutated position).

Genotyping was successfully completed in 99.6% of cases (7 DNA samples gave no PCR product) and in 100% of controls. As shown in table 4.13, the prevalence of IVS2+1G>A was 0.9% (16/1752) among the patients. One of the patients reported a first-degree relative affected with breast cancer, one was also carrier of I171V allele in *NBN* and the second one was also carrier of *NBN**657del5 allele. Five carriers of IVS2+1G>A were from contaminated regions (2 patients from subgroup **I**, two from subgroup **II** and one from subgroup **III**) Six patients heterozygous by IVS2+1G>A were diagnosed at or below age 50. The IVS2+1G>A mutation was not found in any of the 1019 control individuals. The absence of IVS2+1G>A in controls did not allow for a reliable

calculation of odds ratios, however, the association of IVS2+1G>A with breast cancer was highly significant (p=0.005). The missense substitution p.I157T was identified in 4.9% (86/1752) of the cases and 2.3% (23/1019) of the population controls (OR= 1.5, 95% CI 0.9-2.5, p=0.001) (table 4.13). 23.3% out of 86 patients with I157T reported a first-degree family history of breast cancer, compared with 17% out of 1752 in the total series (p=0.18). Five p.I157T carriers had a bilateral breast cancer, two patients beside breast cancer reported ovarian carcinoma, and one had a relative affected with ovarian carcinoma. Forty-two carriers (49%) were from contaminated regions (13/42 from subgroup **I**, 17/42 from subgroup **II**, 11/42 from subgroup **III** and one from **IV**) and 13 of them reported family history of breast cancer. Two of the 86 patients, carriers of p.I157T were homozygous for the mutation and one of them had bilateral disease. Two others were compound heterozygotes for p.I157T and IVS2+1G>A, or p.I157T and *CHEK2*dele(9,10), respectively, the latter one had bilateral disease. One patient heterozygous for p.I157T was also carrier of the p.C61G allele in *BRCA1*, another one was also carrier of 4153delA mutation in *BRCA1* and two others carriers of the p.I171V variant in *NBN*. Fifteen controls (65%) were from contaminated regions and 8 of them from the areas with more than 5 mSv whole body cumulative dose - subgroups **II-IV**. Two controls were homozygous for p.I157T (one from contaminated region – subgroup **I** and another one from non-contaminated).

Table 4.13 **Frequency of *CHEK2* mutations IVS2+1G>A and p.I157T in cases and controls**
* subset of patients with at least one first-degree relative also affected with breast cancer

Cohort	Total (n)	IVS2+1G>A					p.I157T				
		(n)	(%)	OR**	95%CI**	*p***	(n)	(%)	OR**	95%CI**	*p***
controls [a]	415	0	-				15	3.6			
cases [a]	780	5	0.6	n.a.	n.a.	0.24	42	5.4	1.52	0.83-2.77	0.22
familial cases [a]*	166	0	-	-	-	-	13	7.8	1.70	0.88-3.38	0.17
controls [b]	604	0	-				8	1.3			
cases [b]	972	11	1.1	n.a.	n.a.	1.0	44	4.5	3.50	1.65-7.5	0.001
familial cases [b]*	132	1	0.8	n.a.	n.a.	0.02	7	5.3	1.26	0.55-2.89	0.75
Controls	**1019**	**0**	**-**				**23**	**2.3**			
Cases	**1752**	**16**	**0.9**	**n.a.**	**n.a.**	**0.005**	**86**	**4.9**	**2.2**	**1.4-3.6**	**0.001**
Familial	**298**	**1**	**0.3**	**0.32**	**0.04-2.45**	**0.41**	**20**	**6.7**	**1.5**	**0.9-2.5**	**0.15**

** compared with population controls
[a] cases and controls from contaminated regions
[b] cases and controls from non-contaminated regions
n.a. not applicable through dividing by zero

The median age at diagnosis among breast cancer patients was not different between carriers and non-carriers neither for the p.I157T mutation (48.5 compared with 48, p=0.4) nor for IVS2+1G>A mutation (53 years in carriers compared with 48 in non-carriers, p=0.4) as well as between carriers in contaminated and non-contaminated areas for both mutations (for IVS2+1G>A p=0.09 and for p.I157T p=0.06) and between carriers and non-carriers in different regions; generally carriers of I157T and IVS2+1G>A tended to have a higher age at diagnosis in comparison with non carriers and whole breast cancer patients cohort (table 4.14).

Table 4.14 ***CHEK2*** **IVS2+1G>A and p.I157T by median age at diagnosis**

Median age at diagnosis / Median test *(*p* value)		**age**	***p****
contaminated regions	**non-carriers**	**44**	
	IVS2+1G>A	44	0.99
	I157T	45.5	0.75
combined carriers versus non carriers in contaminated regions (both loci)		45/44	0.77
non-contaminated regions	**non-carriers**	**50**	
	IVS2+1G>A	57.5	0.12
	I157T	50	0.69
combined carriers versus non carriers in non-contaminated regions (both loci)		51/50	0.54
Total	carriers versus non carriers (both loci)	49/48	0.31

4.4.4 Screening for CHEK2dele9,10(5kb)

The large *CHEK2* deletion of about 5.8 kb spanning the exons 9 and 10, initially described as a Czech founder mutation, reportedly accounts for 0.9% of breast cancer patients in Poland (see above 4.4.0). Screening for the presence of the *CHEK2*dele(9,10) mutation was performed using a previously established allele-specific duplex polymerase chain reaction assay (Cybulski et al 2007), and was subsequently confirmed in positive patients by long-range polymerase chain reaction (Walsh et al 2006). Allele specific duplex PCR in mutation-negative cases amplified only two PCR fragments of 379 and 522 bp from the wild-type allele. The forward primer of the first pair flanking the intron 8 breakpoint and the reverse primer of the second pair flanking the intron 10 breakpoint amplified an additional PCR product of 450 bp in mutation- positive cases (figure 4.13 A). In all duplex PCR-positive samples, the presence of the deletion was confirmed by long-range PCR using one primer pair flanking the deletion (attachment 1), so that PCR product of 1.8kb was yielded only in the presence of deletion, and PCR products were subsequent loaded on 1% agarose gel and visualised after GelRed staining (figure 4.13 B).

Figure 4.13 **Identification and confirmation of *CHEK2*dele(9,10) mutation.** Panel **A** – fragment of 2% agarose gel with allele-specific duplex polymerase chain reaction assay: lane 1 – heterozygous carrier of *CHEK2*dele(9,10), lanes 2,3 – wild-type control. Panel **B** – verification of the mutation by long-range PCR in carriers (lanes 1,2) and in wild-type control (lane 3).

Genotyping was successfully completed in 99.7% of cases and in 99.9% of controls. As shown in table 4.15, the prevalence of the *CHEK2*dele(9,10) mutation was 1.1% (20/1749) among the patients. Four patients reported a first-degree relative affected with breast cancer, one of them reported bilateral breast cancer and was also carrier of I157T allele, and none of the patients had breast and/or ovarian cancers. Eight mutation carriers were from contaminated regions (4 patients from subgroup **I**, one from subgroup **II** and three from subgroup **III**). Twelve patients heterozygous for *CHEK2*dele(9,10) were diagnosed below age 50. The median age at diagnosis was not different between carriers and non-carriers for *CHEK2*dele(9,10) mutation (44.5 compared with 48, p=0.53) as well as between carriers in contaminated and non-contaminated regions p=0.07, although the median age at diagnosis among mutation carriers in contaminated areas is 42.5 years and in non-contaminated 51. Between carriers and non-carriers when stratified by region also no difference was found (in non-contaminated 51 in carriers versus 50 in non-carriers, p=0.98; in contaminated 42.5 compared with 44, p=0.15). The *CHEK2*dele(9,10) mutation was further found in one of the 1018 control individuals, and this was the same person who also carried the c.657del5 in the *NBN* gene. The association of *CHEK2*dele(9,10) with breast cancer was highly significant (OR= 11.8, 95% CI 1.6-87.8, p=0.005) (table4.15).

Table 4.15 **Frequency of *CHEK2*dele(9,10) in cases and controls**

Cohort	Total (n)	*CHEK2*dele(9,10)				
		(n)	(%)	OR**	95%CI**	p**
controls [a]	415	0	-	n.a.	n.a.	0.09
cases [a]	777	8	1.0			
familial cases [a]*	166	3	1.8	2.23	0.53 – 9.43	0.49
controls [b]	603	1	0.1	7.53	0.98 – 58.02	0.05
cases [b]	972	12	1.2			
familial cases [b]*	132	1	0.8	0.57	0.07 – 4.49	0.91
Total controls	1018	1	0.1	11.76	1.58 – 87.8	0.005
Total cases	1749	20	1.1			
Total familial cases *	298	4	1.3	1.22	0.40 – 3.68	0.96

* subset of patients with at least one first-degree relative also affected with breast cancer
** compared between cases and controls in two-by-two tables
[a] cases and controls from contaminated regions
[b] cases and controls from non-contaminated regions
n.a. not applicable due to dividing by zero

When all four *CHEK2* mutations were combined, the presence of any *CHEK2* mutation was strongly associated with breast cancer (OR= 2.97, 95% CI 1.95-4.53, p<0.00001), and this increase in risk was mainly driven by the relatively few mutation carriers among controls from non-contaminated regions (table 4.16).

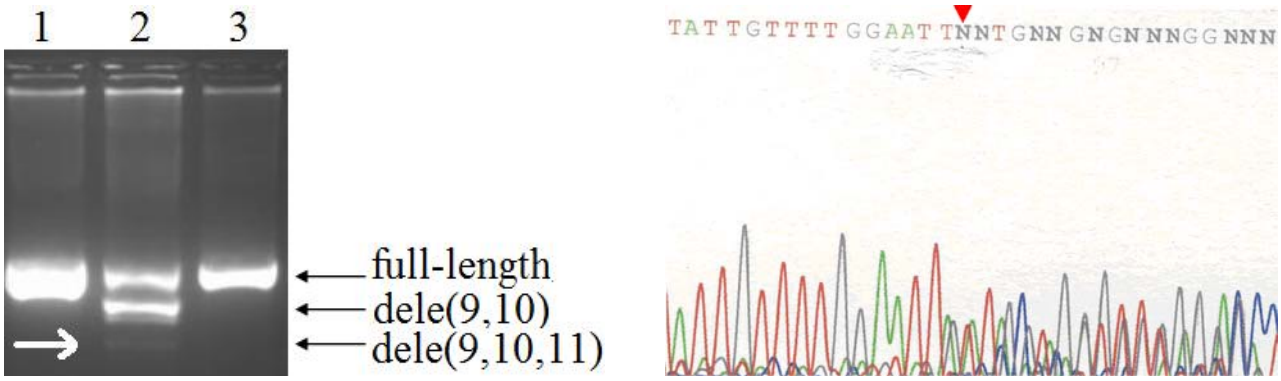
Table 4.16 **Combined frequencies of *CHEK2* mutations 1100delC, IVS+1G>A, p.I157T and *CHEK2*dele(9,10) in cases and controls**

Cohort	Total (n)	1100delC, IVS+1G>A, I157T and *CHEK2*dele(9,10) combined				
		(n)	(%)	OR**	95%CI**	p**
controls [a]	415	18	4.3	1.71	0.99 – 2.94	0.07
cases [a]	780	56	7.2			
familial cases [a]*	166	16	9.6	1.57	0.85 – 2.90	0.19
controls [b]	604	9	1.5	5.53	2.75 – 11.12	<0.00001
cases [b]	972	75	7.7			
familial cases [b]*	132	9	6.8	0.90	0.48 – 2.04	1.0
Total controls	1019	27	2.7	2.97	1.95 – 4.53	<0.00001
Total cases	1752	131	7.5			
Total familial cases *	298	25	3.4	1.28	0.81 – 2.03	0.35

* subset of patients with at least one first-degree relative also affected with breast cancer
** compared between cases and controls in two-by-two tables
[a] cases and controls from contaminated regions
[b] cases and controls from non-contaminated regions

4.4.5. Transcript analysis of the CHEK2dele(9,10) mutation

While the effect of the *CHEK2* mutations 1100del C and IVS2+1G>A have been investigated by previous investigators, the consequences of the *CHEK2*dele(9,10) allele are largely unknown. In order to assess the consequence of the *CHEK2*dele(9,10) allele at the transcript level, total RNA was isolated from LCLs established from individual who was a heterozygous carrier of the *CHEK2*dele(9,10)/*NBN**657del5 genotype. The RNA was reverse transcribed, and a region of the cDNA spanning the exons 6-14 was amplified with exonic primers (see table 4.17). Subsequently, RT-PCR products were separated on a 2% agarose gel, stained with GelRed (figure 4.14, A), for amplification control. Direct sequencing was performed using forward primer (figure 4.14, B).



A **B**

Figure 4.14. **RT-PCR analysis of *CHEK2* transcripts from total RNA of a *CHEK2*dele(9,10) carrier.** Panel **A** – Fragment of 2% gel with *PCR* products spanning exon 6 -14 of the *CHEK2* mRNA transcript, lines 1,3: wild-type control individual, lane 2: PCR product from the *CHEK2*dele(9,10) heterozygous carrier. A white arrow designates a faint smaller band indicative of aberrant splicing. Panel **B –** direct sequencing of RT-PCR product from the patient carrying *CHEK2*dele(9,10) (sense strand with ▼ designating the start of a frameshift in part of the products).

Table 4.17 **Primer used for sequencing of *CHEK2*dele(9,10) allele**

Primer name	Sequence	Annealing t°	Predicted product size
CHEK2-6F	5'-CCCAGCTCTCAATGTTGAAACAG-3'	59°C	888 bp
CHEK2-14R	5'-GATGACAGAGTGAAAGAAGGTAC-3'		

The analysis of RT-PCR products after agarose gel electrophoresis confirmed the presence of a smaller deletion product in approximately the same amount as the product of wild-type length, and there was no evidence for a nonsense-mediated decay of *CHEK2*mRNA harbouring the deletion. In addition, some smaller splice product was seen at a low level in the sample with *CHEK2*dele(9,10) (figure 4.14, A). When this minor band was excised from the gel, reamplified and also sequenced as described before; the direct sequencing of this small product showed a deletion not only of exons 9 and 10 but also the downstream exon 11, consistent with an alternative splicing event. The exon 11 skipping which was observed exclusively in the patient sample, restores the reading frame in the context of the *CHEK2*dele(9,10) deletion, however the shortened product was observed at about 5% of wild-type and has lost 117 codons encoding a large part of the CHEK2 kinase domain.

4.4.6 Effect of p.I157T and CHEK2dele(9,10) on protein level and radiation-induced protein phosphorylation

The physiologic impact of p.I157T mutation is controversial, this substitution was relatively common in healthy control individuals and was proposed as a polymorphism (Vahteristo et al. 2001), but on the other hand it is located in the conserved FHA domain. Biochemical data from other study suggest that this mutation may be deleterious (Li et al. 2002) and, while it does not

result in a size change of CHEK2, may affect associations of CHEK2 with certain substrate proteins (as for example with TP53, BRCA1 or Cdc25C).

In order to study the physiological impact of the p.I157T mutation and of the *CHEK2*dele(9,10)/*NBN**657del5 carrier status, LCLs were established from breast cancer patients homozygous for I157T and from the heterozygous carrier of the *CHEK2*dele(9,10)/*NBN**657del5 mutations. Expression of CHEK2 protein was determined by immunoblot analyses of lymphoblastoid cell protein extracts. To determine radiation-induced CHEK2 phosphorylation, cell extracts were prepared 30 minutes after irradiation of the LCLs with 6 Gy. Whole cell extract was loaded at 50µg/lane on 7.5% SDS-PAGE gels. After transfer the membranes were probed with either a 1:1000 dilution of anti-CHEK2 (Cell Signaling) or anti-pCHEK2 (dilution 1:000 of Ser-19, dilution 1:500 of Thr-68, dilution 1:500 of Ser-33/35) antibody (Cell Signaling), respectively. The same blot was subsequently probed with a monoclonal antibody raised against β-actin (1:5000, Sigma) to control for loading. After incubation with secondary antibody, signals were detected using enhanced chemiluminescence (Pierce/ Perbio Science) for CHEK2/pCHEK2 and normal luminol chemiluminescence for β-actin followed by autoradiography. Signal ratios CHEK2/β-actin were densitometrically determined and compared between the cell lines. The relative expression level of CHEK2 was estimated for each of the LCLs from patients harbouring a *CHEK2* mutation relative to the mean of LCLs from two healthy individuals (figures 4.15, 4.16).

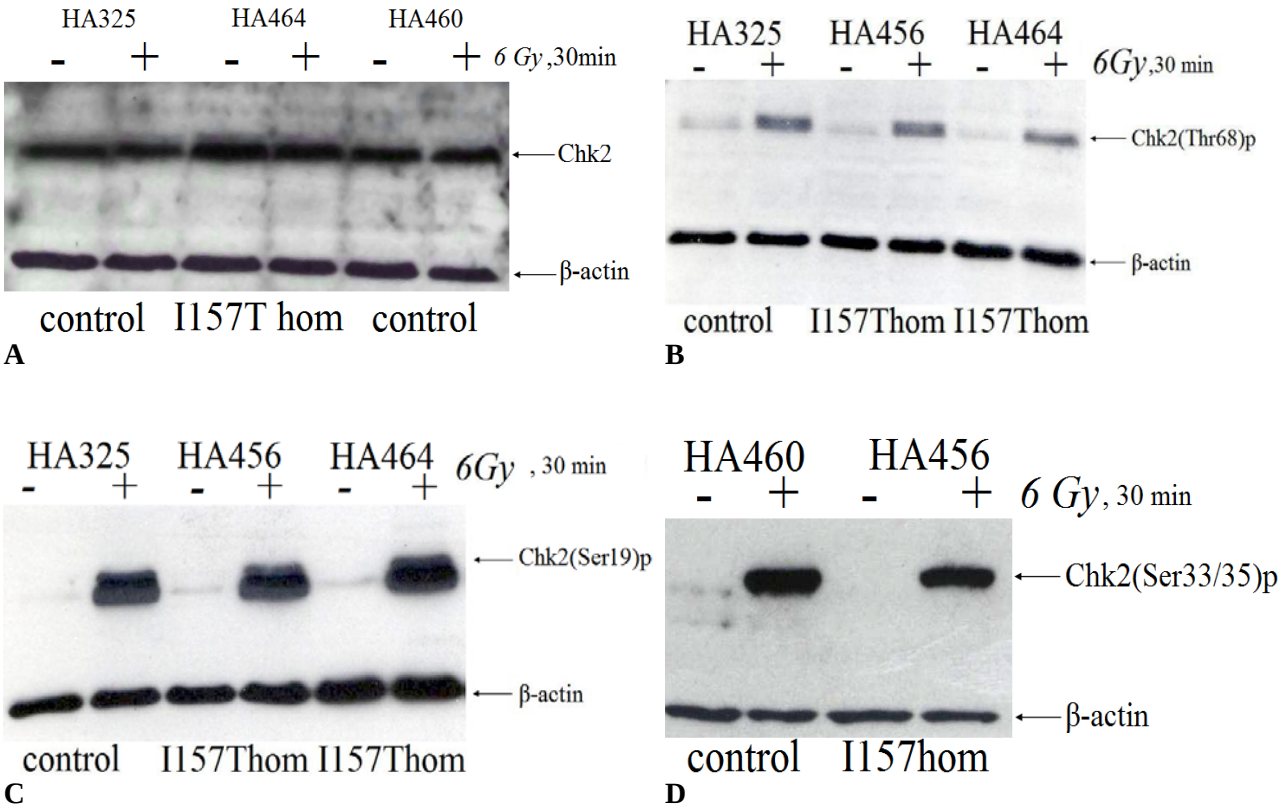
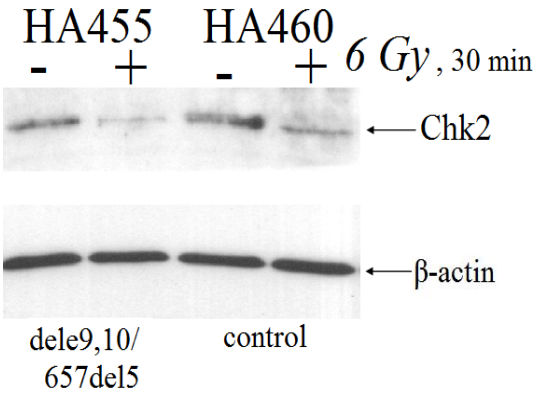


Figure 4.15 **Expression of CHEK2 protein in lymphoblastoid cells**. Identification of CHEK2 protein (panel **A**) and of radiation-induced phosphorylation at CHEK2 Thr-68 (panel **B**), Ser-19 (panel **C**), or Ser-

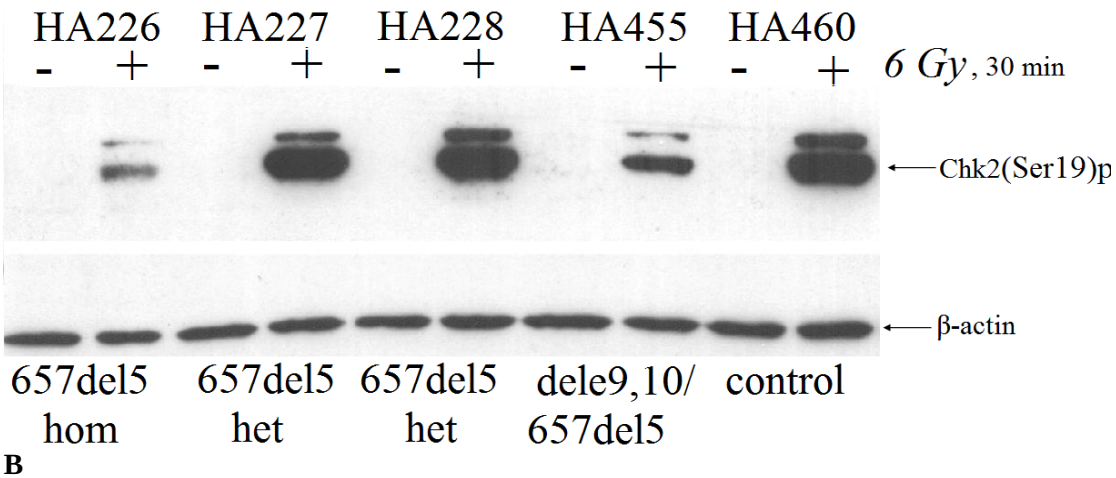
33/35 (panel **D**) by immunoblot analysis of total cell extracts from four different LCLs without or with previous exposure to 6 Gy irradiation. β-actin is shown as a loading control. HA325 and HA460: healthy control individuals; HA464 and HA456: breast cancer patients homozygous for p.I157T.

In the p.I157T homozygous LCLs, full-length CHEK2 protein was observed at a similar level as in control LCLs (figure 4.15, panel A). By using a phosphospecific antibody to assess radiation-induced phosphorylation of CHEK2 at Thr-68, Ser19 and Ser33/35, the results paralleled those for total CHEK2 protein, yielding no detectable difference between p.I157T homozygous carriers and control LCLs regarding Ser19 and Thr68 phosphorylation and only a slight, if any, decrease in Ser33/35 phosphorylation. Thus, CHEK2 protein levels and radiation-induced CHEK2 phosphorylation were in the same range for wild-type controls and p.I157T homozygotes (figure 4.15, panels B, C, D).

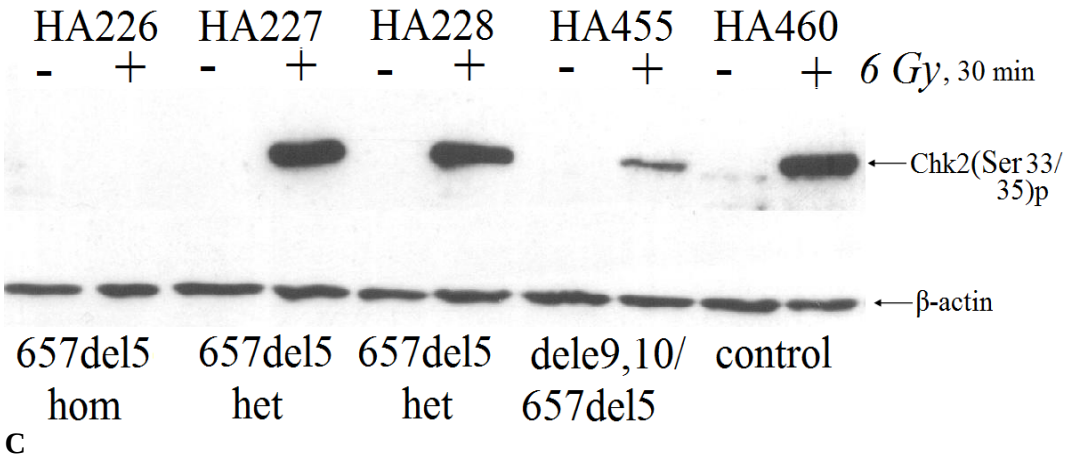


A

Figure 4.16 **Expression and radiation-induced phosphorylation of CHEK2 in lymphoblastoid cells with different *CHEK2* and *NBN* mutational status**. Total CHEK2 protein (panel A) and specific radiation induced phosphorylation at CHEK2 Ser-19 (panel B) and Ser-33/35 (panel C) by immunoblot analysis of total cell extracts from different LCLs without or with previous exposure to 6 Gy of irradiation. β-actin is shown as a loading control. HA226, NBS patient homozygous for 657del5; HA227 and HA228, NBS carriers heterozygous for 657del5; HA455 breast cancer patient carrier of *CHEK2*(dele9,10)/NBS*657del5 genotype; HA460 healthy control individual.



B



C

In the *CHEK2*dele(9,10)/*NBN**657del5 carrier LCL, full-length CHEK2 protein was observed, but protein level was reduced to approximately 50% in comparison with LCLs from wild-type genotype and there was no apparent phosphorylation shift after irradiation with 6 Gy (figure 4.16, panel A).

By using a phosphospecific antibody to assess radiation-induced phosphorylation of CHEK2 at Ser-19 and Ser-33/35, the obtained results showed some 30% of CHEK2 (p-Ser19 and p-Ser33/35) immunoreactivity in the cells from the dele(9,10)/657del5 compound heterozygous patient. We observed also a dramatic reduction to about 20% in cells from the NBS patient with the 657del5/657del5 genotype, whereas both NBS parents heterozygous for 657del5 mutation had apparent phospho-protein levels in the same range as for wild-type control individuals (figure 4.16, panels B, C). Altogether, the data from LCLs suggest that the dele9,10/657del5 genotype may produce some 50% of the wild-type amount of full-length CHEK2 protein but may allow for only about one-third of phosphorylated CHEK2 what in turn may markedly affect CHEK2 protein function.

4.5. Analysis of *BRCA2* mRNA levels using real-time PCR

In a recently published study, CHEK2 dependent phosphorylation of FoxM1 protein on Ser-361 caused increased stabilization of the latter with a corresponding increase of *XRCC1* and *BRCA2* gene transcription in mouse embryonic fibroblasts and in osteosarcoma U2OS cells (Tan et al. 2007). We wondered whether such an increase in BRCA2 mRNA levels could be an effect of CHEK2 in human mammary epithelial cells, and whether it could be useful to assess the effect of *CHEK2* mutations.

Total RNA was isolated from irradiated and non-irradiated HMEC and from LCLs from wild-type control individuals and breast cancer patient homozygous for the p.I157T variant in the *CHEK2* gene. Complementary DNA was prepared by reverse transcription using random hexamer primers (see chapter 3.5). The expression of *BRCA2* mRNA transcripts was analysed by polymerase chain reaction using SYBR Green as fluorescent marker as described in chapter 3.9. The primers used for

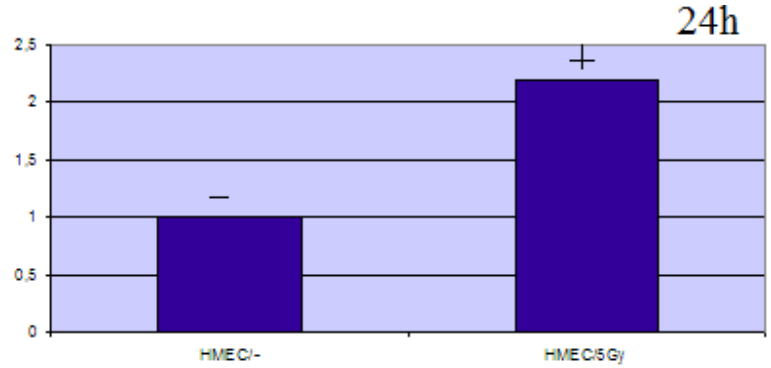
this approach were the same as in the published article (see table 4.18), and the expression level of investigated transcripts was normalised to cyclophilin mRNA expression, a house-keeping gene to compensate for possible differences in sample amounts and/or quality. The efficiency of the primer pairs (table 4.20) was verified by the standard curve method using serial dilutions. For estimating the relative expression level of the gene of interest (*BRCA2*) a semiquantitative method was used, which is based on calculating of DCt value, defined as the difference in the Ct (threshold cycle) values of target (*BRCA2*) and reference genes (cyclophilin).

Analysis of mRNA levels from non-irradiated and irradiated (5 Gy) HMEC was assessed 24 hours after treatment by RT-PCR and revealed evidence for an about 2 fold increase in the expression of *BRCA2* transcripts after irradiation (figure 4.17, A). However, when lymphoblastoid cells (LCL) from two different control individuals were irradiated with 6Gy and harvested after 24 hours, the real-time PCR analysis from LCLs did not show an increase of *BRCA2* expression and as the results from irradiated cells were almost in the same range as from non-irradiated, no significant difference was found (figure 4.17, B, three independent experiments). Hence, this assay could not be applied to investigate the effect of the I157T mutation under the conditions chosen.

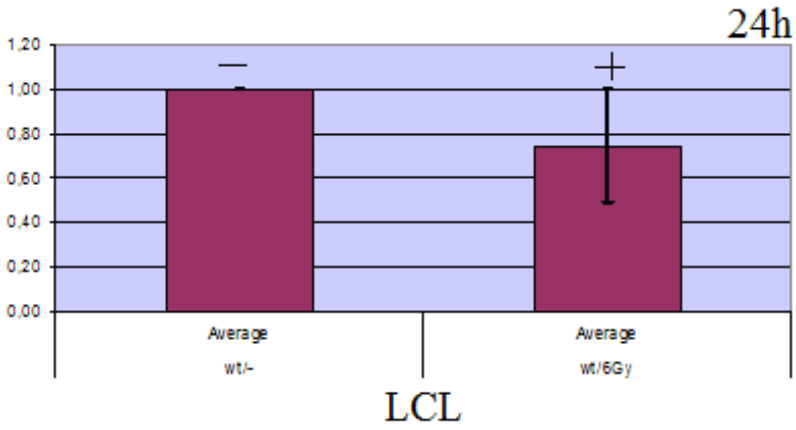
Table 4.18 ***Primers*** **used for QRT**

Primer name	Sequence	Annealing t°
BRCA2-S	5'-GCCTTGGATTTCTTGAGTAGACTGC-3'	63°C
BRCA2-AS	5'-GTGTTTCGTATTTGGTGCCACAAC-3'	
hCYC-S	5'-GCAGACAAGGTCCCAAAGACAG-3'	63°C
hCYC-AS	5'-CACCCTGACACATAAACCCTGG-3'	

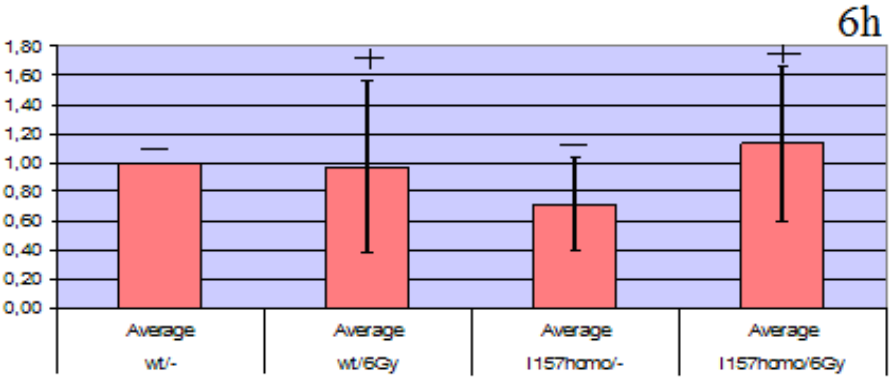
Tan and co-authors showed the increased expression of BRCA2 on protein and mRNA levels at 6 and 18 hours after IR-treatment. In order to determine whether the chosen incubation time influences the detection and quantification of *BRCA2* transcript expression in LCLs, real time PCR was also performed at 6 hours after treatment with 6Gy. This experiment was done three times with two LCLs, from a wild-type control individual and from a breast cancer patient homozygous for the I157T variant in the *CHEK2* gene. Again, the results did not show any significant increase of *BRCA2* expression, and were almost in the same range as after 24 hours, although a less than ~ 1.6 fold induction of *BRCA2* mRNA could not be excluded in the 6 hrs experiment (figure 4.17, C).



A



B



C

Figure 4.17 **Real time PCR for mRNA levels in HMECs and LCLs.** Expression of BRCA2 mRNA without and after exposure to IR. Panel **A** – HMEC cells untreated *HMEC/-* or exposed to IR (5Gy), harvested at 24 hours after treatment. Panel **B** – QRT-PCR RNA analysis from LCL cells untreated *wt/-* or treated with 6Gy, collected at 24 hours after IR exposure. Panel **C** – mRNA levels of BRCA2 in LCLs from wild-type control: *wt* and breast cancer patient homozygous for I157T, harvested 6 hours after exposure to 6Gy or from unexposed. As "–" designated not irradiated cells as"+" – irradiated.

Because only lymphoid cells and not mammary epithelial cells were available from *CHEK2* mutation carriers, this assay was not applicable for the analysis of mutation-specific effects. It is possible that the observed differences are cell-type specific, and they may relate to the total expression levels of BRCA2. In general, the expression of *BRCA2* transcripts in lymphoblastoid cells was very low, and when calculated from the standard curves, accounted for less than 1% compared with the housekeeping gene expression (cyclophilin).

4.6. Missense substitutions p.Q559R, p.E672Q and p.G998E in the *PALB2* gene

PALB2 ("partner and localizer of BRCA2") is one of the most recently discovered Fanconi anemia genes (Reid et al. 2007). Biallelic *PALB2* germline mutations are responsible for a subset of Fanconi anemia (FA-N) cases characterized by a phenotype similar to that caused by biallelic

BRCA2 mutations (Reid et al. 2007, Xia et al. 2007). During the course of the thesis, monoallelic *PALB2* mutations were also found in individuals with breast cancer from familial breast cancer pedigrees that were negative for *BRCA1* and *BRCA2* mutations. The breast cancer population attributable fraction of *PALB2* mutations in UK population was estimated to be 0.23% and the percentage of familial relative risk due to *PALB2* to be 0.24 %. (Rahman et al. 2007). A truncating mutation, being a possible founder mutation, in *PALB2* was also detected in familial and sporadic breast cancer cases and in one prostate cancer family in the Finnish population (Erkko et al. 2007). *PALB2* mutations showed an incomplete segregation in affected relatives and were estimated to confer a 2 to 3 fold increase in breast cancer risk, and it was suggested that the risks of breast cancer associated with *PALB2* mutations may be age-dependent.

In parallel work of our group, three missense variants p.Q559R, p.E672Q and p.G998E initially described by Rahman (Rahman et al. 2007) were detected to be in linkage disequilibrium across exons 5-9 of the *PALB2* gene, and a haplotypic combination of all three substitutions showed evidence for an association with increased breast cancer risk, with some evidence of an early age of breast cancer onset in triple carriers versus non- carriers (M. Blaut, MD thesis in preparation). In order to validate these findings, we also genotyped the three variants in Byelorussian cases and population controls by using self-established 5´-nuclease allelic discrimination assays with FAM and Yakima-Yellow labelled probes (Eurogentec,primers and probes see attachment 2). The assays were run in 96-well plates on a 7500FAST Real-time PCR Thermocycler (Applied Biosystems) with annealing/hybridisation temperatures at 60°/62°/65°C for p.Q559R, p.E672Q and p.G998E respectively and forty cycles of amplification. Homozygous and heterozygous genotypes were evaluated from their relative fluorescence using the 7500 FAST System SDS Software. A representative assay is shown in figure 4.18. The call rate was above 99% for all SNPs (see below for details).

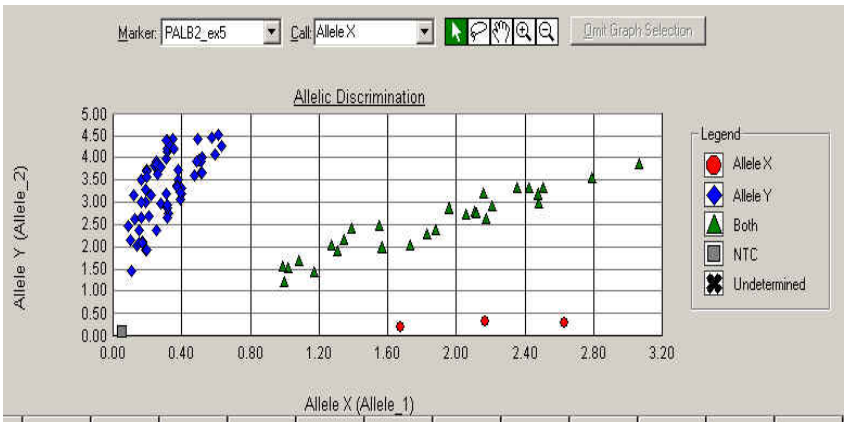


Figure 4.18 **5´-nuclease allelic discrimination assay for *PALB2* variant p.E627Q.** Clustering of three genotypes: common homozygotes (blue) are represented by fluorescence along the y-axis, rare homozygotes (red) are represented by fluorescence along x-axis and heterozygotes (green) show fluorescence emission from both dyes and cluster between axes x and y. NTC - no template control.

Genotyping was successfully for 99.9% of cases and 100% of controls for SNP c.1676A>G (p.Q559R). This variant was found in 372/1757 breast cancer cases (21.2%) and in 196/1019

(19.2%) of controls (table 4.19; OR 1.11, 95% CI=0.93-1.33, p=0.25). There was also no difference found if stratified by region: OR 1.18, 95% CI=0.94-1.49, p=0.17 for non-contaminated regions and OR 1.04, 95% CI=0.78-1.37, p=0.85 for contaminated regions, neither was an association found with familial breast cancer (OR 1.03, 95% CI=0.76-1.40, p=0.90). Median age at diagnosis for carriers and non-carriers was 48 years, and no genotype-specific differences became apparent if stratified by region (in non-contaminated areas for carriers and non carriers 50 years and for contaminated regions 45.5 years for carriers versus 44 in not carriers). In the second stage, all carriers of the p.Q559R allele in cases and controls were screened for the presence of the c.2014G>C (p.E672Q) variant. Further, all samples carrying the p.E672Q substitution were analysed for the presence of the c.2933G>A (G998E). No significant differences were detected in allele frequencies between cases and controls with any of these SNPs though a c.2933A/A homozygous variant was detected only in breast cancer cases (OR 1.10 95% CI=0.77-1.56, p=0.67 for p.E672Q and OR 1.10 95% CI=0.65-1.52, p=1.00 for p.G998E). The p.E672Q allele was present in 28.5% of investigated cases (representing 6.0% in the whole case series) and in 26.5% of investigated controls (representing 5.1% in the whole control series) (table 4.19). There was no significant difference in carrier frequencies if stratified by region (OR: 1.33, 95%CI: 0.83-2.14, p=0.28 for non-contaminated areas and OR: 0.83, 95%CI: 0.48-1.42, p=0.59 for contaminated areas). Median age at diagnosis among p.E672Q carriers was 50.5 years versus 48 years in non-carriers (p=0.36) and no difference was found in the median age at diagnosis among carriers and non-carriers if stratified by region: 52 years versus 49.5 (p=0.16) for non-contaminated areas and 43 years versus 46 (p=0.71) for contaminated regions.

Table 4.19 **Frequency of *PALB2* alleles p.Q559R, p.E672Q and p.G998E in cases and controls**

Series		**Cases (N=1757)**		**Controls (N=1019)**	
Locus/genotype		contaminated areas (n)	non-contaminated areas (n)	contaminated areas (n)	non-contaminated areas (n)
p.Q559R (c.1676A>G)	G/G	8	13	2	9
	A/G	144	208	78	107
	A/A	631	754	335	488
		Cases (N=372)		**Controls (N=196)**	
p.E672Q (c.2014G>C)	C/C	1	2	1	0
	G/C	39	64	23	28
	G/G	112	154	55	88
		Cases (N=106)		**Controls (N=52)**	
p.G998E (c.2933G>A)	A/A	2	0	0	0
	G/A	27	37	20	16
	G/G	24	16	9	7

The p.G998E variant was present in 18.2% of investigated cases (3.9% in the whole case series) and in 18.4% of investigated controls (3.5% in the whole control series) (table 4.19). There was no significant difference in risk if stratified by region (OR: 1.25, 95%CI: 0.68-2.29, p=0.58 for non-contaminated areas and OR: 0.79, 95%CI: 0.43-1.44, p=0.53 for contaminated areas), nor in the distribution of cases among different regions: OR: 1.22, 95%CI: 0.74-2.07, p=0.51. Median age at diagnosis among p.G998E carriers was 52 years versus 48 in not carriers (p=0.25) and no difference was found in the median age at diagnosis between carriers and non-carriers when stratified by region: 55.5 years versus 50 (p=0.096) for non-contaminated areas and 44.5 years versus 45.5 (p=1.00) for contaminated regions. Also, no significant difference was found in regard of the median age at diagnosis between carriers from different regions: 44.5 years in contaminated regions versus 55.5 in non-contaminated (p=0.076). For both loci was no association detected between variant carrier status and familial breast cancer (OR: 0.87, 95%CI: 0.50-1.50, p=0.70 for p.E672Q; OR: 0.80, 95%CI: 0.39-1.63, p=0.66 for p.G998E).

4.7 Splice variant IVS7-1G>A in the *XRCC4*

XRCC4 is a non-homologous end-joining protein employed in DNA double strand break repair and in V(D)J recombination and, as an important caretaker of the overall genome stability, is thought to play an important role in the human carcinogenesis. Some non-coding variants of *XRCC4* were reported to be associated with breast cancer susceptibility (Allen-Brady et al. 2006, Chiu et al. 2008). The IVS7-1G>A *XRCC4* variant was reported to be significantly associated with bladder cancer risk (Figueroa et al. 2007), and in primary work of our group showed evidence for an excess of rare homozygotes in German breast cancer cases compared with controls (K. Gerriets, MD thesis submitted). This mutation was found to abolish the conserved acceptor site of the last intron of *XRCC4* gene and results in the exclusive activation of cryptic acceptor site six nucleotides downstream in lymphoblastoid cells from carriers of the mutation. The aberrant splicing leads to the loss of two amino acids and the substitution of the third in the variant protein. Lymphoblastoid cells from homozygous mutation carriers show grossly normal levels and phosphorylation of the shorter XRCC4 protein (K. Gerriets, MD thesis submitted) but may exhibit an increased frequency of micronuclei after irradiation (Janet Hall, pers. comm.).

For screening the IVS7-1G>A variant, a simple restriction enzyme based PCR assay was used. A genomic region covering the splice acceptor site was amplified by 35 cycles of PCR using GoTaq – Polymerase at 60ºC annealing temperature (primers used for this approach see attachment 1). Subsequently, PCR products were cleaved at 65°C for 8 hours with *Tsp*509I (New England BioLabs). Reaction products were separated on 2% agarose and visualized by UV after ethidium bromide staining (figure 4.19 A). In the presence of the A/A genotype, the 247bp PCR product was

cleaved by *Tsp*509I to fragments of 162bp and 85bp, whereas wild-type samples remained uncut. Some samples carrying IVS7-1G>A were directly sequenced to verify the presence of the mutation (figure 4.19 B, C, D). For this purpose the same primers were used as for PCR.

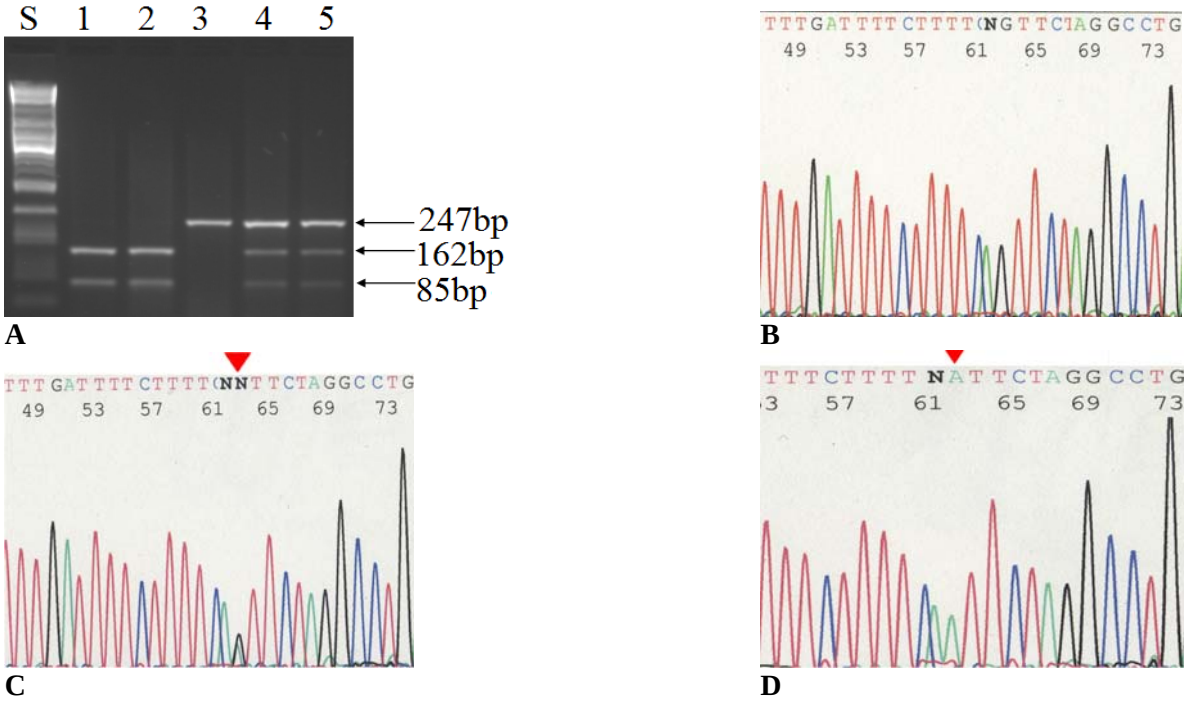


Figure 4.19 **Screening for the IVS7-1G>A splice site mutation in the *XRCC4* gene.** Panel **A** –2% agarose gel with *Tsp509*I cleavage products; S – size marker, lanes 4,5 heterozygous carriers of mutation (G/A), lanes 1,2 homozygous carriers of mutation (A/A) and lane 3 wild type control. Panel **B**: sequencing of PCR product from control individual. Panels **C** and **D** sequencing of PCR products from heterozygous and homozygous carriers of IVS7-1G>A respectively (sense strand with ▼ designating of mutation).

Genotyping was successfully completed for 100% of cases and controls. The IVS7-1G>A variant presented with an allele frequency of 23.4% in cases and 19.8% in controls, with a higher prevalence of the A/A genotype in carriers among cases: 34 (8.3%) out of 411 carriers in comparison with 7 (3.5%) out of 202 carriers among controls (OR: 1.27, 95%CI: 1.06-1.51, p=0.01). If stratified by region, a significant difference between cases and controls was obtained only for non-contaminated regions: OR: 1.35, 95%CI: 1.07-1.71, p=0.01, for contaminated OR: 1.16, 95%CI: 0.89-1.51, p=0.29 (table 4.20). There was also no association found between this variant and familial breast cancer (OR: 0.80, 95%CI: 0.60-1.06, p=0.14) (table 4.20).

Median age at diagnosis among heterozygous mutation carriers and non-carriers was not different – 48 years in both groups, but among carriers of rare allele in homozygous state median age was 51.5 years though this was not a significant difference (p=0.07). If stratified by region it was the same tendency that rare homozygotes had a slightly later age at diagnosis although statistical significance was not obtained (for non-contaminated regions: median age at diagnosis among G/G genotype

carriers - 50, among G/A -50, among A/A- 52.5 years; for contaminated regions: median age at diagnosis among G/G genotype carriers - 44, G/A- 45 and A/A – 46.5 years).

Table 4.20 **Frequency of *XRCC4* mutation IVS7-1G>A in cases and controls**

Series	Total (n)	IVS7-1G>A				
		G/G	G/A	A/A	OR, 95%CI**	p**
controls [a]	415	326	85	4	1.16, 95%CI: 0.89-1.51	0.29
cases [a]	783	597	172	14		
familial cases [a]*	166	128	34	4	0.94,95%CI:0.63-1.41	0.85
controls [b]	604	491	110	3	1.35,95%CI:1.07-1.71	0.01
cases [b]	976	751	205	20		
familial cases [b]*	132	100	27	5	1.10, 95%CI: 0.70-1.66	0.81
Total controls	1019	817	195	7	1.27, 95%CI: 1.06-1.51	0.01
Total cases	1759	1348	377	34		
Total familial cases *	298	228	61	9	0.80, 95%CI: 0.60-1.06	0.14

* subset of patients with at least one first-degree relative also affected with breast cancer
** compared between cases and controls in two-by-two tables
[a] cases and controls from contaminated regions
[b] cases and controls from non-contaminated regions

Among carriers of IVS7-1G>A were 58 patients who also harboured previously identified breast cancer risk alleles: 9 carriers of 5382insC, 6 carriers of p.C61G and 4 carriers of 4153delA in the *BRCA1* gene; furthermore 20 carriers of p.I157T (inclusive 2 homozygotes), 5 carries of IVS1+2G>A and 5 carriers of the *CHEK2*dele(9,10) allele in the *CHEK2* gene; 3 carriers of the *NBN**657del5 allele, one carrier of the p.R215W allele in the *NBN* gene and 5 carriers of the p.E1978X allele in the *ATM* gene. These risk alleles co-occurred with IVS7-1A at a marginally lower frequency than with the more common IVS7-1G allele (OR 0.73; 95%CI 0.53-1.00, p=0.05). Thus, if carriers of previously identified mutations in the genes *BRCA1*, *BRCA2*, *CHEK2*, *ATM* and *NBN* were excluded, the observed association of IVS7-1G>A with breast cancer became even stronger (OR 2.14, 95%CI: 1.82-2.53, p<0.0001). Ninety patients carrying IVS7-1G>A had bilateral disease, three patients had breast/ovarian cancer syndrome and five reported first degree relatives affected with ovarian cancer.

4.8 Low-penetrance alleles p.D302H in *CASP8* and p.L10P in *TGFB1*

4.8.1 Rationale and methodology

During the course of this thesis, there had been indications that the D302H polymorphism (rs1045485) in *CASP8,* the gene encoding caspase-8, which results in an aspartic acid to histidine substitution at codon 302, could reduce breast cancer risk what was recently confirmed by a study of the Breast Cancer Association Consortium, a collaboration of 25 international research groups in

which we had the chance to take part (Cox et al. 2007). Weaker evidence was found in the same study for a *TGFB1* (Transforming growth factor-beta) L10P variant. The Leu10Pro polymorphism resides in the signal peptide sequence of TGFβ and the peptide with Proline at residue 10 reportedly causes a 2.8 fold increase in secretion compared with the Leucine isoform (Dunning et al. 2003). The analysis of the Leu10Pro variant by the Breast Cancer Association Consortium showed a significant dose dependent association of the Pro allele with increased risk of invasive breast cancer, and it was estimated to account for approximately 0.2 % of the excess familial risk of breast cancer in populations of European ancestry (Cox et al. 2007). Therefore we decided to validate these results in the case-control series from Belarus.

Both variants D302H and L10P were genotyped by using 5´-nuclease allelic discrimination assays with mutation-specific FAM- or VIC-labelled probes and TaqMan Universal PCR Master Mix from Applied Biosystems (Foster City, U.S.A., primers and probes see attachment 2). The assays were run in 96-well plates on a 7500FAST Real-time PCR Thermocycler (Applied Biosystems) with annealing/hybridisation temperatures at 60°, forty cycles for L10P and sixty cycles of amplification for D302H respectively. Homozygous and heterozygous genotypes were evaluated from their relative fluorescence using the 7500 FAST System SDS Software. Examples are shown in figures 4.20, and 4.21. The call rate for investigated Byelorussian series of cases and controls was above 98% for both SNPs (see below for details).

*4.8.2 CASP8*D302H*

In genotyping the *CASP8**D302H substitution among Byelorussian cases and controls, the call rate was 99.0% for cases and 98.0% for controls. A representative assay is shown on figure 4.20.

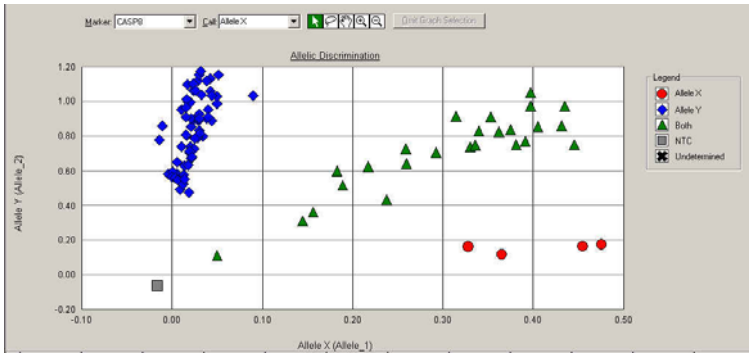


Figure 4.20 ***D302H*** **genotype distribution among studied cases and controls.** Clustering of three genotypes: common homozygotes (GG, blue) are represented by fluorescence along the y-axis, rare homozygotes (CC, red) along x-axis, and heterozygotes (CG, green) show fluorescence emission from both dyes and cluster between axes x and y. NTC - no template control.

D302H variant was found in 396/1742 breast cancer cases (22.7%) and in 251/999 (25.1%) of controls (table 4.21). The association of this SNP with breast cancer was tested by comparing the allele frequencies between breast cancer cases and controls, and the difference was not significant in regard of the whole series (OR 0.87, 95% CI=0.74-1.02, p=0.09), though the odds ratio appeared

consistent with the previously proposed protective effect (Cox et al. 2007). There was also no difference found if stratified by region: OR 0.89, 95% CI=0.72-1.10, p=0.32 for non-contaminated regions and OR 0.84, 95% CI=0.65-1.08, p=0.19 for contaminated regions. However, a significant association was found for D302H in familial breast cancer (40/295 versus 312/1477, carrier OR: 0.57, 95% CI=0.40-0.81, p=0.002) (table 4.21). The median age at diagnosis for carriers was 47 years and for non carrier 48 years (p=0.065), this was also not different if stratified by region (in non-contaminated areas for carriers 49 and non carriers 50 years and for contaminated regions 43 years for carriers versus 44 in not carriers).

Table 4.21. **D302H genotype distribution in breast cancer patients and controls**.

Series/Genotype/ (n) of carriers	**G/G**		**G/C**		**C/C**	
	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Population controls (whole series) (N=999)	**748**	74.9	**227**	22.7	**24**	2.4
Population controls from non-contaminated regions (N=591)	**447**	75.6	**126**	21.3	**18**	3.1
Population controls from contaminated regions (N=408)	**301**	73.8	**101**	24.8	**6**	1.5
Breast cancer patients (whole series) (N=1742)	**1346**	77.3	**368**	21.1	**28**	1.6
Familial cases *(N=295)	**255**	86.4	**38**	12.9	**2**	0.7
Non Familial cases (N=1447)	**1135**	78.4	**288**	19.9	**24**	1.7
Breast cancer patients from non-contaminated regions (N=968)	**745**	77.0	**206**	21.3	**17**	1.8
Breast cancer patients from contaminated regions (N=774)	**601**	77.7	**162**	20.9	**11**	1.4

* subset of patients with at least one first-degree relative also affected with breast cancer, the distribution of genotypes in familial versus non familial cases was significantly different by Chi-square test (p=0.007)

Among breast cancer patients, carriers of D302H variant were also seven carriers of 5382insC, five carriers of 4153delA and six carriers of p.C61G in *BRCA1*, four carriers of *CHEK2*dele(9,10), one of IVS1+2G>A and eighteen carriers of p.I157T in *CHEK2*; if these were excluded, the association of the D302H variant with breast cancer became no difference: OR 0.89, 95%CI 0.73-1.07, p=0.23. Fifteen patients had bilateral disease, seven breast/ovarian cancer and one reported a relative affected with ovarian cancer.

4.8.3 p.L10P in TGFB1

Genotyping was successful in 99.6% for cases and 99.5% for controls. It was noted that the assay for this variant which is embedded in a highly GC-rich portion of *TGFB1*, only worked with the improved TaqMan Genotyping PCR Master Mix provided by the company (Applied Biosystems), whereas TaqMan Universal PCR Master Mix had to be supplemented with additional polymerase

and a higher initial denaturation had to be performed to achieve satisfactory results and a reliable discrimination. A representative assay is shown on figure 4.21.

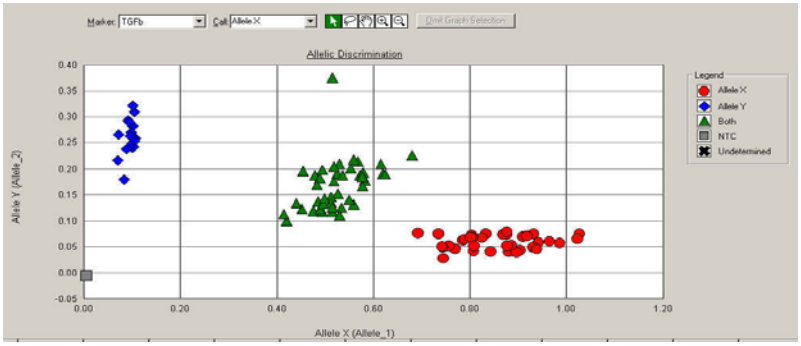


Figure 4.21 ***L10P*** **genotypes distribution among studied cases and controls.** Clustering of three genotypes: common homozygotes (CC, red) are represented by fluorescence along the x-axis, rare homozygotes (TT, blue) along y-axis and heterozygotes (CT, green) show fluorescence emission from both dyes and intermediate clustering. NTC - no template control.

The L10P variant was found in 1142/1751 breast cancer cases (65.2%) and in 666/1014 (65.7%) of controls (table 4.22) so that the proposed association of this SNP with breast cancer could not be detected (OR 1.02, 95% CI=0.91-1.14, p=0.75). There was a slight difference between cases if stratified by region: OR 1.14, 95% CI=0.99-1.32, p=0.08 for non-contaminated regions and OR 0.88, 95% CI=0.74-1.04, p=0.15 for contaminated regions, and this difference was significant in a case-only comparison of different regions in a way that cases from contaminated areas were less likely to harbour the TGFB1*L10P substitution: OR 0.82, 95% CI=0.94-0.78, p=0.006. Median age at diagnosis for carriers and for non-carriers was 48 years; it was also not different if stratified by region (in non-contaminated areas for both carriers and non-carriers 50 years, and for contaminated regions 44 years for carriers versus 44.5 in non-carriers).

Table 4.22. **L10P genotype distribution in breast cancer patients and controls**.

Series/Genotype/ (n) of carriers	**C/C**		**C/T**		**T/T**	
	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Population controls (whole series) (N=1014)	**348**	34.3	**507**	50.0	**159**	15.7
Population controls from non-contaminated regions (N=601)	**210**	34.9	**301**	50.1	**90**	15.0
Population controls from contaminated regions (N=413)	**138**	33.4	**206**	49.9	**69**	16.7
Breast cancer patients (whole series) (N=1751)	**609**	34.8	**843**	48.1	**299**	17.1
Breast cancer patients from non-contaminated regions (N=974)	**323**	33.2	**460**	47.2	**191**	19.6
Breast cancer patients from contaminated regions (N=777)	**286**	36.8	**383**	49.3	**108**	13.9

Rare homozygosity for the variant L10P was present in more cases than controls although this difference was not significant. Among breast cancer carriers of rare variant were also seven carriers

of 5382insC, one carrier of 4153delA and six carriers of p.C61G in *BRCA1*, three carriers of 6174delT in *BRCA2*, three carriers of *CHEK2*dele(9,10), one of IVS1+2G>A and nine carriers of p.I157T in *CHEK2*; if these patients with previously detected higher-risk and moderate risk alleles were excluded, the odds ratio was not really changed and became 1.0 and it was also not significant. Eleven patients had bilateral disease, four breast/ovarian cancer and four reported a relative affected with ovarian cancer.

4.9 Genetic variants rs2981582 in *FGFR2*, rs3803662 at the *TOX3* locus and rs13387042 on chromosome 2q35

4.9.1 Rationale and methodology

The three new loci within the *FGFR2* gene on chromosome 10q26, adjacent to the *TOX3/TNRC9* gene on chromosome 16q12, and on chromosome 2q35 have been found during the course of this thesis in large genome-wide searches by the Breast Cancer Association Consortium and others to be associated with an increased risk of breast cancer (Easton et al. 2007, Hunter et al. 2007, Stacey et al. 2007). For each of these SNPs, the minor allele in Europeans was associated with an increased risk of breast cancer in a dose-dependent manner, with a higher risk of breast cancer in homozygous than in heterozygous carriers. Associations with family history and bilaterality are to be expected for susceptibility loci and there was evidence of an association with family history of breast cancer for rs2981582 and rs3803662, as the susceptibility allele was commoner in women with a first-degree relative with the disease than in those without, and rs2981582 was also associated with bilaterality (Easton et al. 2007).

Genotyping for all three loci was done in the frame of a Breast Cancer Association Consortium study using a 5´-nuclease allelic discrimination technique as described in 4.6. The methods were run with annealing/hybridisation temperatures at 60° for the *TOX3* and 2q35 loci, at 62° for *FGFR2* and forty cycles. Representative assays are shown in figures 4.22, 4.23 and 4.24. The call rate was above 99% for all SNPs (see below for details).

4.9.2 FGFR2 (rs2981582)

In case of the *FGFR2* locus, the call rate was 100% for Byelorussian cases and controls. Distributions of genotypes (C/C – wild-type; C/T – heterozygous carrier; T/T – homozygous carrier of rare variant) among cases and controls are shown in Table 4.23 and on figure 4.22.

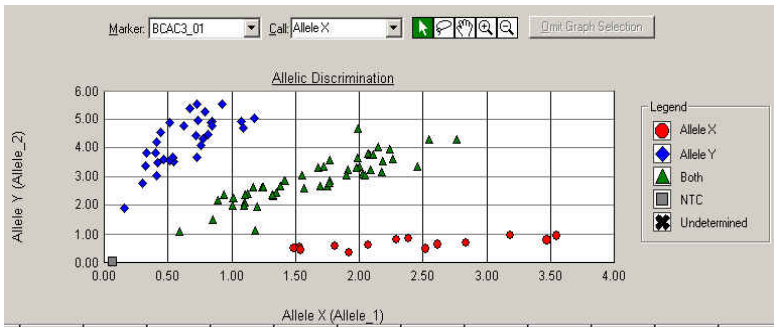


Figure 4.22 **SNP rs 2981582 genotype distribution among studied cases and controls.**. Clustering of three genotypes: common homozygotes (CC, blue) are represented by fluorescence along the y-axis, rare homozygotes (TT, red) – along x-axis and heterozygotes (CT, green) show fluorescence emission from both dyes and intermediate clustering between axes x and y. NTC - no template control.

Heterozygosity or homozygosity for the rare allele if the *FGFR* rs2981582 polymorphism was present in 61.3% of investigated cases and in 60.7% of controls (per allele OR: 1.07, 95%CI: 0.96-1.20, p=0.25) (table 4.23). Homozygosity for the rare allele T of rs2981582 was found more often in breast cancer cases (15.4%) compared with controls (12.9%) but this difference was not significant (carrier OR 1.22, 95%CI: 0.96-1.55, p=0.12). There was no significant difference in allele frequencies if stratified by region (OR: 1.09, 95%CI: 0.94-1.26, p=0.29 for non-contaminated areas and OR: 1.04, 95%CI: 0.88-1.24, p=0.67 for contaminated areas). Median age at diagnosis among carriers was 47 years versus 48 in non-carriers and the difference between the groups was borderline significant (p=0.055). Homozygote carriers of rare allele have median age at diagnosis of 48 years, what was not differing from non carriers. If stratified by region, we observed no difference in median age at diagnosis for contaminated regions: carriers – 44 years and not carriers – 43.5, but a significant difference was found in non-contaminated regions with carriers diagnosed at a lower age than non-carriers (49 years among carriers versus 51 in non-carriers, p=0.003)

Table 4.23. **Distribution of rs2981582 SNP genotypes in breast cancer patients and controls**

Series/Genotype/ (n) of carriers	**C/C**		**C/T**		**T/T**	
	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Population controls (whole series) (N=1019)	**401**	39.4	**487**	47.8	**131**	12.9
Population controls from non-contaminated regions (N=604)	**242**	40.1	**289**	47.9	**73**	12.1
Population controls from contaminated regions (N=415)	**159**	38.3	**198**	47.7	**58**	14.0
Breast cancer patients (whole series) (N=1759)	**681**	38.7	**807**	45.9	**271**	15.4
Breast cancer patients from non-contaminated regions (N=976)	**385**	39.5	**441**	45.2	**150**	15.4
Breast cancer patients from contaminated regions (N=783)	**296**	37.8	**366**	46.7	**121**	15.5

There was no significant association between this variant and familial breast cancer (OR: 0.88, 95%CI: 0.68-1.13, p=0.35). Among breast cancer patients who were rare homozygous carriers of

rs2981582 were also three carriers of 4153delA, seven carrier of 5382insC and three carriers of p.C61G in *BRCA1*, one carrier of 6174delT in *BRCA2*, three carriers of *CHEK2*dele(9,10), thirteen carriers of p.I157T and six carriers of IVS1+2G>A in *CHEK2*, one carrier of the *NBN**657del5 allele and one of p.E1978X in *ATM*; if these patients with previously detected high- or moderate-risk mutations were excluded, the odds ratios were 1.06 and significance was also not achieved (p=0.65). Eight patients had bilateral disease, four breast/ovarian cancer, fifty-six reported first-degree relatives affected with breast cancer and five with ovarian cancer.

4.9.3 TOX3 (rs3803662)

Genotyping for rs3803662 at the *TOX3/TNRC9* locus was successfully completed in 99.7% for cases and 99.2% in controls. Distributions of genotypes (C/C – wild-type; C/T – heterozygous carrier; T/T – homozygous carrier of rare variant) among cases and controls are shown on figure 4.23 and in Table 4.24.

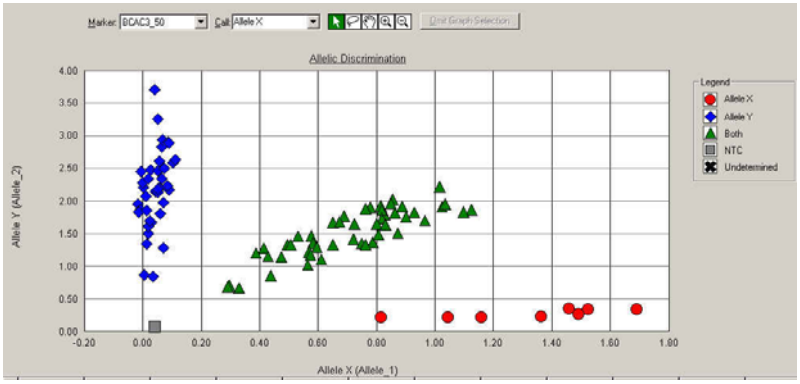


Figure 4.23 **SNP rs3803662 genotype distribution among studied cases and controls.** Clustering of three genotypes: common homozygotes (CC, blue) are represented by fluorescence along the y-axis, rare homozygotes (TT, red) – along x-axis and heterozygotes (CT, green) show fluorescence emission from both dyes and intermediate clustering. NTC - no template control.

The rare allele of *TOX3* rs3803662 was present in 50.4% of investigated cases and in 45.8% of controls (table 4.24). This SNP was tested by comparing the allele frequencies between breast cancer cases and controls and a borderline significant difference was found towards an increased frequency of the rare allele among cases (per-allele OR: 1.15, 95%CI: 1.02-1.30, p=0.03). Homozygosity for the rare allele T-rs3803662 also was present more often in breast cancer cases (9.8%) compared with controls (8.7%). The observed difference in allele frequencies appeared to be confined to non-contaminated areas where significance was borderline (OR: 1.17, 95%CI: 1.00-1.37, p=0.058), while for contaminated areas no difference was observed (OR: 1.13, 95%CI: 0.93-1.36, p=0.24). Median age at diagnosis among carriers and non-carriers was 48 years (p=0.94). If stratified by region, there was also no difference in median age at diagnosis, which for contaminated regions was 44 years for both carriers and non-carriers, in non-contaminated regions 50 years among both carriers and non-carriers.

The rs3803662 variant was not significantly associated with familial breast cancer (OR: 1.23, 95%CI: 0.96-1.58, p=0.12). Among breast cancer patients who were rare homozygous carriers of the rs2981582 variant, six were also carriers of 5382insC, one carrier of p.C61G in *BRCA1*, one carrier of 6174delT in *BRCA2*, five carriers of *CHEK2*dele(9,10), eleven carriers of p.I157T and one carrier of IVS1+2G>A in *CHEK2*, and four were carriers of the *NBN**657del5 allele; the apparently 2.6 fold increased occurrence of *NBN**657del5 carriers among *TOX3* rare homozygotes did not reach statistical significance (p=0.08). When the above-mentioned patients with previously detected high- or moderate-risk mutations were excluded, the odds ratios were 1.09, but statistical significance was not achieved (p=0.20). Four patients had bilateral disease, thirty-eight reported first degree relatives also affected with breast cancer and one patient had breast/ovarian cancer.

Table 4.24. **Distribution of rs3803662 SNP genotypes in breast cancer patients and controls**

Series/Genotype/ (n) of carriers	C/C		C/T		T/T	
	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Population controls (whole Series) (N=1011)	**548**	54.2	**375**	37.1	**88**	8.7
Population controls from non-contaminated regions (N=596)	**317**	53.2	**228**	38.3	**51**	8.6
Population controls from contaminated regions (N=415)	**231**	55.7	**147**	35.4	**37**	8.9
Breast cancer patients (whole Series) (N=1754)	**870**	49.6	**713**	40.7	**171**	9.8
Breast cancer patients from non-contaminated regions (N=973)	**470**	48.3	**404**	41.5	**99**	10.2
Breast cancer patients from contaminated regions (N=781)	**400**	51.2	**309**	39.6	**72**	9.2

4.9.4 Chromosomal locus 2q35 (rs13387042)

Genotyping for rs13387042 on 2q35, a locus not associated with any gene, was successfully completed in 100% of the cases and 99.8% of the controls. Distributions of genotypes (G/G – wild-type; G/A – heterozygous carrier; A/A – homozygous carrier of rare variant) among cases and controls are presented in table 4.25 and are illustrated in figure 4.24.

The rare allele of the rs13387042 polymorphism on chromosome 2q35 was present in 66.7% of investigated cases and in 67.6% of controls (table 4.25). Aassociation of this SNP with breast cancer was tested by comparing the allele frequencies between breast cancer cases and controls but there was no significant difference found (per-allele OR: 1.00, 95%CI: 0.89-1.11, p=0.90).

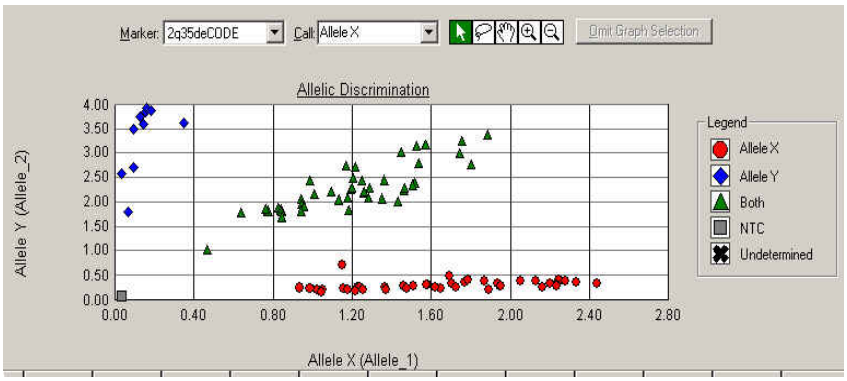


Figure 4.24 **SNP rs13387042 genotype distribution among studied cases and controls.** Clustering of three genotypes: common homozygotes (GG, red) are represented by fluorescence along the x-axis, rare homozygotes (AA, blue) – along y-axis and heterozygotes (GA, green) show fluorescence emission from both dyes and intermediate clustering between axes x and y. NTC - no template control.

Homozygosity for the rare allele A of rs13387042 was present slightly more often in breast cancer cases (19.7%) than in controls (19.3%). There was little difference in allele frequencies if stratified by region (for non-contaminated areas OR: 1.04, 95%CI: 0.90-1.20, p=0.61 and for contaminated areas OR: 0.93, 95%CI: 0.79-1.10, p=0.43), and comparison of carrier frequencies stratified by region among cases also showed no significant difference: OR: 0.93, 95%CI: 0.81-1.06, p=0.30. Median age at diagnosis among carriers and non- carriers was 48 years (p=0.68). If stratified by region, there was also no difference in median age at diagnosis: for contaminated regions 44 years for carriers and not carriers, in non-contaminated regions 50 years among carriers and not carriers. Nevertheless some difference was found in the median age at diagnosis for homozygous carriers of rare A-rs13387042 genotype in comparison with heterozygotes: 45 years versus 49 (p=0.008, median test), and this tendency was more pronounced if stratified by region: in contaminated regions median age for rare homozygous was 41 years versus 45 in heterozygous (p=0.0005), whereas in non-contaminated significance was not achieved (49 versus 50, p=0.18).

Table 4.25. **Distribution of rs13387042 SNP genotypes in breast cancer patients and controls**

Series/Genotype/ (n) of carriers	**G/G**		**G/A**		**A/A**	
	Total (n)	(%)	Total (n)	(%)	Total (n)	(%)
Population controls (whole series) (N=1017)	**330**	32.5	**491**	48.3	**196**	19.3
Population controls from non-contaminated regions (N=602)	**202**	33.6	**282**	46.8	**118**	19.6
Population controls from contaminated regions (N=415)	**128**	30.8	**209**	50.4	**78**	18.8
Breast cancer patients (whole series) (N=1759)	**585**	33.3	**828**	47.1	**346**	19.7
Breast cancer patients from non-contaminated regions (N=976)	**316**	32.4	**461**	47.2	**199**	20.4
Breast cancer patients from contaminated regions (N=783)	**269**	34.4	**367**	46.9	**147**	18.8

The rs13387042 variant was not found to be associated with familial breast cancer (carrier OR: 0.94, 95%CI: 0.72-1.22, p=0.66). Among breast cancer patients who were rare homozygous carriers of rs13387042, there were also seven carriers of 5382insC, three carriers of p.C61G and one carrier of 4153delA in *BRCA1*, one carrier of 6174delT in *BRCA2*, four carriers of *CHEK2*dele(9,10), eleven carriers of p.I157T and three carriers of IVS1+2G>A in *CHEK2*, and three carriers of *NBN**657del5 allele. When the above-mentioned patients with previously detected high- or moderate-risk mutations were excluded, the odds ratios were 0.95 and this effect was not significant with p=0.36. Thirteen patients had bilateral disease, four patients reported breast/ovarian cancer, sixty-eight reported first degree relatives affected with breast cancer and three had relatives with ovarian carcinoma.

In summary, the described variants were not significantly associated with familial breast cancer with the exception of *CASP8* (see chapter 4.8.1), and only the SNP adjacent to *TOX3* was found to be associated overall with breast cancer in Byelorussian population. Summarized results for the tested SNPs are presented in table 4.26.

Table 4.26. **Summary of results from investigated SNPs**

Locus/SNP ID	Carrier OR, (95% CI), p value	Per-allele OR, (95% CI), p value
CASP8, rs1045485	OR: 0.88, (0.73-1.05), p=0.17	OR 0.87, (0.74-1.02), p=0.09
TGFb, rs1982073	OR: 0.98, (0.83-1.15), p=0.84	OR 1.02, (0.91-1.14), p=0.75
FGFR2, rs2981582	OR: 1.03, (0.88-1.20), p=0.77	OR: 1.07, (0.96-1.20), p=0.25
TOX3, rs3803662	OR: 1.20, (1.03-1.40), p=0.02	OR: 1.15, (1.02-1.30), p=0.03
2q35, rs13387042	OR: 0.96, (0.82-1.14), p=0.69	OR: 1.00, (0.89-1.11), p=0.90

For some loci, a significant difference was found in the median age at diagnosis between carriers and non-carriers, which was particularly strong for homozygous carriers of rare A-rs13387042 genotype. In general, the results for all tested polymorphic variants were in line with published data, but as a single study the power appeared as too low to prove the statistical association for some of these with breast cancer in the Byelorussian population. This shows the importance of large-scale studies and motivates our participation in the international Breast Cancer Association Consortium when the goal is to identify common variants conferring low increases in the risk for breast cancer.

4.10. Resume

It was predicted that most breast cancers are sporadic, and only some are the result of inherited predisposition, due to mutations in *BRCA1* and *BRCA2* genes. Mutations in both genes account for approximately 30% of families with a strong family history of cancer and might be responsible for 3-5% of all breast and ovarian cancers population-wide (figure 4.25). In the studied Byelorussian

population, four founder *BRCA1* and *BRCA2* mutations accounted for 4.5% of unselected breast cancer patient and 9.1% of hereditary breast cancer. Further previously described mutations in other genes predisposing to breast cancer with moderate penetrance include *ATM, NBN, CHEK2, PALB2* and *BRIP1*. These genes might explain next ~ 15-20% of all familial cases and up to 10% of all breast cancers population-wide. In Byelorussian investigated series, mutations in the "moderate penetrance genes" *ATM, NBN, CHEK2* were responsible for additional 9.3% of unselected breast cancer cases and 9.4% of familial breast cancers. Importantly, this study confirmed the association of a single truncating *ATM* mutation with breast cancer, the matter of a long-standing debate. Nevertheless the most breast cancers are still to be clarified.

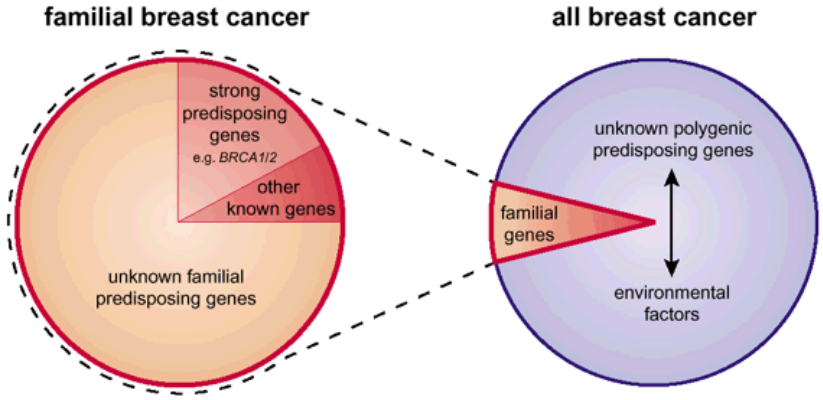


Figure 4.25 **Part of familial breast cancers among all breast cancers population-wide and impact of known high and moderate susceptibility genes on disease risk.** (Figure is taken from Balmain et al. 2003.)

During the course of the work, it was hypothesized that variants and maybe their combinations in numerous other genes may modulate the life-time risk for breast cancer as low-penetrance alleles. World-wide studies were performed to identify new genetic risk factors, and some studies using latest technologies in very large multi-center and multi-ethnic cohorts for genome-wide association searches were successful to identify new genetic loci predisposing to breast cancer (the most strongly associated variants are described in the present thesis). Taking in account the accumulation of knowledge about the role of low- penetrance alleles in their contribution to breast cancer risk and the results provided by the present work, it turned out that almost all breast cancer patients in the present study (99.95%) carry as minimum one breast cancer susceptibility allele (figure 4.26).

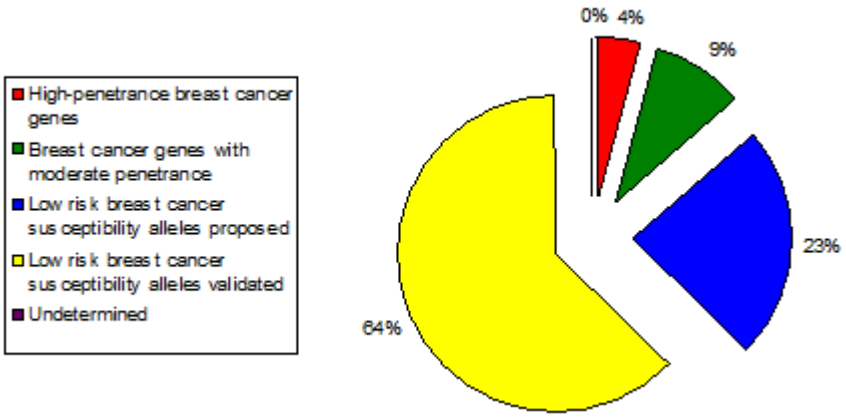


Figure 4.26 **Distribution of breast cancer predisposing alleles in the investigated Byelorussian breast cancer patients.** Red: *BRCA1*, *BRCA2*, green: *ATM*, *NBN*, *CHEK2*, blue: *XRCC4*, yellow: *CASP8*, *TGFB1*, *FGFR2*, *TOX3*, 2q35.

5. Discussion

5.1 High-risk susceptibility alleles in Byelorussian breast cancer patients and controls

5.1.1 Founder mutations in BRCA1 and BRCA2 and breast cancer risk

BRCA1 and *BRCA2* are considered as gatekeeper tumour suppressor genes that are involved in the prevention of multiple tumour types but seem to be particulary important in the breast epithelium. BRCA1 and BRCA2 participate in the biological response to DNA damage, which involves the activation of cell cycle checkpoints and the recruitment of the machinery for DNA repair (see chapter 1.7.2). Germline mutations in *BRCA1/2* may account for about 5-10% of all breast cancer patients overall in population (Nicolletto et al. 2001). *BRCA1/2* mutation carriers also run a greater risk of developing breast cancer before menopause and a significantly higher risk of developing contralateral breast cancer (Nicolleto et al. 2001). Of the plenty *BRCA1* and *BRCA2* mutations, many are unique, but there are also numerous examples of founder mutations, which have been reported in genetically isolated populations, such as Ashkenazim, Icelandic, Greenlandic Inuits and others. Founder mutations have also been noted in Slavic countries, including a small sample of West Byelorussian breast/ovarian cancer families (Oszurek et al. 2001). However, the relative contribution of such mutations at the population level in Belarus, their impact on the background of low-level radiation exposure, and – in case of 4153delA – their overall risk for breast cancer had not been determined.

During the course of the thesis, three founder *BRCA1* mutations 5382insC, 4153delA and p.C61G overall were identified in 75/1759 (4.3%) of unselected breast cancer patients from six oncological centers in Belarus. The 5382insC mutation accounted for the majority - 2.5% among all studied breast cancer patients and 58.7% of all investigated mutations. Two other *BRCA1* mutations were less common, so 4153delA was observed in 16/1759 (0.9%) of breast cancer patients (32.2% of all identified mutations) and p.C61G in 15/1759 (0.9%). The *BRCA2**6174delT mutation was found in 4/1759 breast cancer cases (0.2%). As expected for known familial breast cancer susceptibility alleles, the three *BRCA1* mutations were more common in the familial versus non-familial cases (9.1% versus 3.3%). Among the few 6174delT carriers in *BRCA2* was only one, who reported family history of breast cancer, and none of the carriers had bilateral disease and/or ovarian carcinoma. Although *BRCA1* and *BRCA2* carriers have increased risk to develop contralateral breast cancer and cancers at other sites, so ovarian cancer; only two out of 64 patients with bilateral disease and one patient out of 20 with breast/ovarian cancers were carriers of 5382insC, and only one carrier of 4153delA reported relative affected with ovarian cancer, indicating that other mutations may exist which confer increased bilateral breast cancer and ovarian cancer risk. *BRCA1* and *BRCA2* mutation carriers are known to have a younger age at breast cancer diagnosis, what was also found in presented study. Patients diagnosed at or below age 50 (1048/1759) carried any

screened *BRCA1* mutation more often (61/1048) than patients diagnosed over 50 (14/711, OR: 3.1, 95%CI: 1.7-5.5, p=0.0001). A similar trend was noted, though not significant, for *BRCA2* allele 6174delT: three patients out of four were diagnosed below the age 50, median age at diagnosis in carriers 44 years versus 48 in non-carriers (p=0.58).

To get more insight into the breast cancer risk conferred by each of these mutations, here approached as odds ratios, the distributions of all three *BRCA1* mutations (5382insC, p.C61G and 4153delA) were studied not only in cases but also in a control series. The highest odds ratio was associated with truncating mutation 5382insC (OR=26), while it was found to be lower for missense mutation p.C61G (OR=8.8) or the other truncating mutation 4153delA (OR=4.7); however, the confidence limits around these estimates are wide (see chapter 4). Nevertheless, all three *BRCA1* mutations were significantly associated with breast cancer, including the 4153delA mutation which had previously been discussed to predispose to ovarian cancer only (see below).

All of the above-described *BRCA1* and *BRCA2* mutations are believed to be deleterious: p.C61G is a missense mutation located in the RING finger domain of the BRCA1 protein which is required for its function as an E3 ubiquitin ligase (Daniel 2002, Venkitaraman 2002); 5382insC is a frame shift mutation that truncates the protein within the BRCT domain of the BRCA1 protein, lacking 34 amino acids, an important protein interaction domain for many phosphorylated DNA repair proteins (Lu and Arrick, 2000, Yu et al. 2003); 4153delA is a frame shift mutation, which leads to a premature stop codon in exon 11. The *BRCA2**6174delT mutation also results in a truncated protein, leading to a stop codon in exon 11 of *BRCA2*) and belongs to founder mutations although some researches classify 6174delT as low risk allele (Nathanson et al. 2001, Satagopan et al. 2001) due to its reduced penetrance. Indeed, this variant had a low frequency in Byelorussian breast cancer cases, thus controls cohort was not tested as this study was not powerful enough to give a good estimate for its disease predisposing effect in the Byelorussian population. Concerning mutations in *BRCA1*, the obtained data suggest that the breast cancer risk may vary somewhat between studied mutations, but the presence of any is significantly associated with an increase in risk for the disease. And these observations are worthy of particular discussion in regard of 4153delA mutation.

There is some evidence that, for unknown reasons, 4153delA patients may have been referred more frequently with ovarian cancer than with breast cancer (Gorski et al. 2000, 2004, 2005a; Gronwald et al. 2005), thus the studies which are based only on breast cancer cases may lead to an underestimation of the medical importance of 4153delA. From a biological perspective, there is little reason why the frameshift mutation 4153delA should not be associated with breast cancer like other *BRCA1* frameshift mutations. However, in selected breast cancer cases from Russia (bilateral disease/unilateral with early onset and/or familial history of breast cancer), 4153delA mutation was

found in three (1%) of studied patients (3/302) (Sokolenko et al. 2007) and the same group found no selectivity of this variant toward ovarian cancer versus breast cancer (Krylova et al. 2006). Although this study was small, it may support our observations that the reported only about two-fold, if any, increase in breast cancer risk (Gorski et al., 2005a) may be an underestimation due to a relatively low frequency of carriers among breast cancer cases in Polish population. In the Byelorussian population, the 4153delA allele was associated with a clearly increased risk of breast cancer in the range of a moderate-penetrance mutation with an OR of 4.7. Although the confidence limits in our study include the ratios in previous reports (95%CI: 1.1-20.3, p=0.04),it is the first study to unequivocally show the association of 4153delA with breast cancer.

In summary, the obtained data show that founder *BRCA1* and *BRCA2* mutations present at a significant frequency in the Byelorussian population, accounting for 4.5% of unselected breast cancer patients and 9.1% of "hereditary" (familial) breast cancer, what provides an important basis and perspective for the breast cancer genetic counselling in Belarus.

5.1.2 Missense variant T1915M in BRCA2: association with breast cancer?

The *BRCA2**T1915M allele is an unclassified missense variant with the amino acid substitution at codon 1915 from threonine to methionine. It is located outside of the DNA-binding domain and affects a less conserved residue of unknown functional significance. This variant was reported as predisposing to early onset breast cancer in the Polish population (Gorski et al. 2005b). Homozygosity for the Met-isoform was present in approximately 6% of Polish population (OR: 1.1, p=0.7) and reportedly was associated with a significant increase in risk for breast cancer diagnosed before age 40 (OR=1.4; p=0.04), and the effect was most pronounced in woman with ductal carcinoma *in situ* (DCIS) OR=2.8, p<0.0001. Allele T1915M was described previously, but initially was no association with breast cancer found (OR: 0.36 95%CI 0.11-1.19, Healey et al. 2000). In earlier work of our group also no significant association of T1915M allele with breast cancer and specially with early onset had been obtained in a series of German hospital-based cases and population controls. Rather, a trend towards a protective direction was found, although not significant. These findings are supported by the data set from the Byelorussian series, where the T1915M variant was significantly associated with breast cancer as a protective allele (OR: 0.60, 95%CI: 0.45-0.79, p=0.0004). The potential increase in risk for carriers was significant in the Polish study for breast cancer in women with both copies of rare variant (OR: 4.7, p=0.02). In our studies, homozygous patients for the Met allele were very rare, and was therefore not possible for us to evaluate the risk for homozygous carriers. Furthermore, it is not excluded that T1915M allele could exert some predisposing effect in combination with other alleles (Johnson et al. 2007). In summary: the C5972T allele appears to be overrepresented in Byelorussian population controls rather than in

breast cancer patients, indicating that it does not increase breast cancer risk under a simple dominant or co-dominant model. Furthermore one patient was compound heterozygous carrying C5972T/6174delT genotype and displayed no Fanconi anemia phenotype, suggesting that C5972T allele do not truncated protein function. While the observations from published studies and those presented here are controversial so far, further research is required and may be worthwhile to elucidate a possible protective effect of the T1915M variant.

5.2 *ATM* gene alterations and breast cancer susceptibility

5.2.1 Classical mutations, causing A-T

The ATM kinase is a regulatory component of the cell-cycle check point and DSB repair machinery that senses DNA damage and mediates diverse downstream cellular responses (see chapter 7.1.3).

Studies based on relatives of A-T patients have shown that heterozygous carriers of *ATM* variants are clinically not affected but face an increased risk of cancer, especially the female carriers who reportedly have an up to seven-fold increased risk of breast cancer (Swift et al. 1987, Pippard et al. 1988, Swift et al. 1991, Easton 1994, Athma et al. 1996, Olsen et al. 2001, Thompson et al. 2005). Mutation screening of *ATM* in breast cancer case controls sets outside A-T families, in contrast, presented mixed and controversely debated results, and it was suggested that only a specific class of dominant-negative variants contribute to breast cancer risk in heterozygotes (FitzGerald et al. 1997, Broeks et al. 2000, Teraoka et al. 2001, Gatti et al. 1999, Chenevix-Trench et al. 2002). But during the course of this thesis, it has been convincingly shown in a UK study using a familial breast cancer case-control population that a large diversity of A-T causing biallelic *ATM* mutations might act as breast cancer susceptibility alleles in monoallelic carriers outside A-T families, and the combined *ATM* mutation prevalence and contribution to breast cancer incidence was similar to *CHEK** 1100delC allele, both conferring an estimated twofold risk of breast cancer (Renwick et al. 2006). If this were true, it would be predicted that any A-T causing mutation common enough to be effectively screened in a population, should be found enriched among breast cancer patients.

Among European A-T family cohorts, the A-T mutation E1978X was present at a relatively high prevalence in 44% of Russian A-T patients suggesting that this mutation may be of Russian origin (Birrell et al. 2005). This mutation is known as functionally deleterious in leading to the skipping of exon 42 and/or a premature truncation, and was found in Byelorussian breast cancer cases and controls (presented in thesis) at a frequency lower than 1% with OR: 5.2, 95%CI: 0.7-41.4, p=0.15. Median age at diagnosis in carriers was 43 years versus 48 in non-carriers, although the difference was not significant (p=0.10). One potentially interesting fact was that 2 out of nine E1978X carriers were also carriers of the p.C61G mutation in *BRCA1* and this was about 27-fold more than expected from the combined carrier frequencies for both loci (95%CI 2.4-296.1, p=0.003). Possible

explanations include some interaction of both loci, what is supported by the fact that the protein products ATM and BRCA1 interact in the same signalling pathway, or a finding by chance what could be supported by the observation that significance was lost if the carrier status for any *BRCA1* mutation and E1978X was combined: OR 5.2, 95%CI 0.5-57.7. However, it should be noted that one of the further tested *ATM* variants, p.S49C, also appeared to associate with *BRCA1* mutations (see chapter 5.2.2 below). Subsequently, and beyond the results presented in this thesis, the E1978X mutation was investigated in an additional set of Byelorussian breast cancer patients and in smaller case-control sets from two other Slavic populations (Russian and Ukrainian) what leads to a combined Mantel-Haenzel OR of 7.4, corroborating the association of the E1978X mutation with breast cancer (p=0.04, 95%CI: 1.00-55.5) for all studied populations. Thus, the findings in this thesis revealed that single A-T causing mutations, though rare at the population level, can make a significant contribution to breast cancer. One parallel study from Finland provides similar evidence that a frameshift insertion known from A-T families, 6903insA, in *ATM* is associated with breast cancer in Finnish patients (Pylkäs et al. 2007). It may be effective also in other populations if cancer case-control series were investigated for specific founder mutations identified in A-T families, similar to the E1978X in Slavic populations.

5.2.2 ATM missense variants

Prior evidence had suggested an association of some *ATM* missense variants (p.S49C, p.S707P, p.L1420F, p.P1054R, p.F858L) with breast cancer (Dörk et al. 2001, Teraoka et al. 2001, Bretsky et al. 2003, Tamimi et al. 2004, Buchholz et al. 2004, Lee et al. 2005, Stredrick et al. 2006). However, in a UK study using familial breast cancer cases and controls, Renwick and co-authors have concluded that polymorphic variants in *ATM* gene contribute very less to the identification of women at substantial risk of breast cancer (Renwick et al. 2006). The identification of moderate to low risks requires very large sample sizes, and so the role of *ATM* missense variants is currently pursued in the frame of the Breast Cancer Association Consortium. In the first Breast Cancer Association Consortium study, the p.Ser49Cys substitution was not significantly associated with overall breast cancer risk, but a modest association was not excluded either, and this SNP appeared to influence the risk of PR positive breast cancer (Cox et al. 2007).

In my thesis, the investigated SNPs in *ATM* (p.S49C, p.S707P, p.L1420F, p.P1054R, p.F858L) are polymorphisms with carrier frequencies between 1-5%, and overall were found in 12.6% of unselected breast cancer patients and in 15.0% of controls. The p.L1420F substitution accounted for the majority – 4.8% among all studied breast cancer patients and 5.8% of all investigated cases. Second ranked p.P1054R in 2.8% of breast cancer patients and 3.5% of controls. Only one variant – p.F858L was found to be slightly but non-significantly more frequent in breast cancer cases than in

controls 1.8% versus 1.6%. There were no differences in median age at diagnosis for carriers and not carriers of certain allele with the possible exception of the p.S49C variant (44 years in carriers versus 48 in not carriers), but the difference was not significant. None of the screened SNPs showed significant differences in the prevalence of substitutions by region, and no association was found with familial and bilateral breast cancer. There was some evidence of an increased prevalence of the p.S49C allele among *BRCA1* mutation carriers, and this finding was nominally significant (5/75 compared with 29/1753, OR 4.2, 95%CI 1.6-11.3, p= 0.002). A similar tendency had been observed for p.E1978X (see above), however, no other of the tested *ATM* missense substitutions accumulated in *BRCA1* mutation carriers.

The impact of *ATM* polymorphisms on protein function still has to be clarified but some variants were described to have possible functional effect on in vitro cellular radiosensitivity. The functional consequences of p.S707P, p.L1420F, p.P1054R and p.F858L have been studied in lymphoblastoid cell lines established from breast cancer patients who carried these polymorphisms, by assaying micronuclei formation and comparison with wildtype cell lines (Gutierrez-Enriquez et al. 2004). The cell lines from variant carriers showed higher mean levels of micronuclei formation after ionising radiation exposure to that found in control cell lines. In particular, the linked variants p.P1054R and p.F858L had a higher level of micronuclei after radiation treatment when compared to the wildtype cell lines or remaining cell lines with other *ATM* substitutions (Gutierrez-Enriquez et al. 2004). As an intrinsic weakness of such studies, the authors did not exclude that increasing micronuclei levels observed in their study could be related to the presence of other undetected variants in the *ATM* gene or in other genes participating in the cellular response to ionising radiation. In summary: the association between common polymorphisms (SNPs) in *ATM* and risk of breast cancer is controversial so far, although large case control studies were conducted. The results from this thesis can exclude only major risks associated with these variants. Possibly, polymorphic variants in *ATM* gene do not contribute to breast cancer risk or act in synergy with other alleles which may also contribute only very small risks and in combination can cause wide risk variations which may also be population-dependent.

5.3 *NBN* gene mutations and risk of breast cancer

Nibrin (product of *NBN* gene) is required for several processes protecting chromosomal stability, including sensing DNA double-strand breaks, cell cycle checkpoint regulation and telomere maintenance (see chapter 1.7.3). Mutations of the *NBN* gene are responsible for the majority of patients with Nijmegen Breakage Syndrome (NBS), a radiation sensitivity disorder (van der Burgt et al. 1996, Digweed et al. 2004). The major NBS mutation is a five-basepair-deletion of the *NBN* gene, 657del5, which predominantly occurs in populations of Slavic descent (Varon et al. 1998,

2000). Another *NBN* gene mutation, p.R215W, has been described in two severely affected NBS siblings who were compound heterozygous for c.657del5/p.R215W (Seemanova et al. 2006).

The role of *NBN* gene mutations in breast cancer susceptibility was less clear. Breast cancer is not observed in NBS patients whose clinical features include failure of puberty and markedly reduced survival. Blood relatives of NBS patients have been reported to be at a generally increased risk for lymphoid and epithelial malignancies (Seemanova 1996, Seemanova et al. 2006). Initial association studies of *NBN* gene alterations in breast cancer series have not generally supported the suggestion that *NBN* may contribute to breast cancer susceptibility (Carlomagno et al. 1999, Kuschel et al. 2002, Forsti et al. 2004, Millikan et al. 2005, Zhang et al. 2005) , but more recent investigations in the Polish and Russian populations provided evidence that the founder mutation 657del5 could be associated with an increased breast cancer risk (Gorski et al. 2003, 2005a; Steffen et al. 2004, 2006; Buslov et al. 2005). The mutation p.R215W has been suggested to increase the risk for colorectal cancer, while its impact on breast cancer has remained uncertain (Steffen et al. 2004, 2006).

In the study presented here, the 657del5 mutation accounted for 16/1759 breast cancer patients and was found in only one of 1014 Byelorussian population controls (females were excluded as controls for *NBN* mutational screening if they had breast cancer or had a child with a neurodegenerative disorder) (OR: 9.3, 95%CI: 1.2-70.2, p=0.02). The p.R215W substitution was found in 9/1759 cases, but R215W was also observed in 5 population controls (OR: 1.04, 95%CI: 0.35-3.11, p=1.00). The data observed thus seemed to provide support for an association of the 657del5 mutation with breast cancer (p = 0.02), whereas such an effect was not detected for R215W (p = 1.00). In another series of 1076 German breast cancer cases and 1017 female population controls, that was analysed in parallel by our group, the 657del5 mutation was very rare and was detected only in 1 patient who had familial breast cancer, and in none of the controls. The R215W substitution accounted for 9 German cases (0.8 %) compared with 2 population controls (0.2 %; OR= 4.3, 95%CI 0.9 – 19.8, p = 0.09) (Bogdanova et al. 2008). In a further analysis of these data sets, no significant increase of R215W in familial, bilateral or premenopausal cases in patient series from Germany and Belarus was found. When both studies were combined, the relative risks for breast cancer associated with R215W appeared smaller than those for 657del5 in Byelorussian and German populations (657del5 combined OR: 10.1, 95%CI 1.4-75.3 versus R215W combined OR: 1.8, 95%CI 0.8-4.3). Stratified analysis for both NBS mutations yielded a Mantel-Haenszel odds ratio of 2.9 (95%CI 1.4-6.3) in all breast cancer cases (p=0.006). Altogether, the obtained results are in line with previous reports suggesting that the 657del5 mutation and perhaps also the R215W mutation confer an increased breast cancer risk (Gorski et al. 2003, 2005a; Steffen et al. 2004, 2006; Buslov et al. 2005). Because the potential contribution of the 657del5 allele is limited by its very low frequency in populations of non-Slavic descent, evidence has come mainly from studies in

Eastern Europe, thus far. The results from the Byelorussian series support these initial findings and provide the strongest evidence to date that *NBN**657del5 is a breast cancer susceptibility allele. Some previous studies that have failed to detect an increased risk for *NBN* gene alterations either were limited by focussing on the rare 657del5 allele in non-Slavic populations (Carlomango et al. 1999) or screened for common *NBN* gene alterations that are not NBS-causing variants and may be functionally neutral (Kuschel et al. 2002, Forsti et al. 2004, Millikan et al. 2005, Zhang et al. 2005). The biological and clinical impact of the two investigated mutations is indicated by their previous identification in NBS patients and by findings of reduced NBN protein levels in cell lines from these patients as well as from one patient with breast cancer in this study (see chapter 4.3). The 657del5 mutation results in a frameshift and subsequent loss of NBN protein, although some residual activity of a shorter NBN isoform can result from internal reinitiation of translation (Maser et al. 2001). The results obtained for nibrin protein levels in lymphoblastoid cells from the R215W heterozygous breast cancer patient in the presented study were in the same range as those observed for 657del5 heterozygotes, and levels were strongly reduced in a p.R215W/c.657del5 compound heterozygous cell line, suggesting that the R215W allele may produce only about one-third of the wild-type amount of full-length nibrin. The R215W mutation, located in the C-terminus of the one BRCT domain, was recently reported to impair the binding of histone γ-H2AX to NBN protein after induction of DNA damage that in turn leads to a delay in DNA-DSB rejoining. In this assay, the R215W mutation may be acting with a dominant-negative effect (what could explain the severe phenotype observed in 657del5/R215W NBS patients) (di Masi et al. 2008). Authors found by analysis of the three-dimensional model of the NBN BRCT domains (figure 5.1) evidence that the phosphorylated tail of γ-H2AX binds through molecular contacts provided by both BRCT domains in NBN. The molecular determinants of γ-H2AX recognition are evolutionary conserved in NBN and required electrostatic interaction between several key residues in the γ-H2AX tail and both BRCT domains in NBN (K160 residue of the first BRCT domain and the phosphate group of the γ-H2AX; Y142 residue and phosphorylated S139 of the histone, and a hydrophobic patch made up by residues contributed by both BRCT domains -I159, L238, and L312; and also a hydrogen bond between mentioned Y142 histone tail residue and the Q234 residue of the second NBN BRCT domain) (figure 5.1). The R215 residue, would be located at the C-terminus of the first NBN BRCT domain in a region which connects two BRCT domains and is probably important for the relative orientation of the NBN BRCT domains and therefore for γ-H2AX recognition. In immunoprecipitation experiments the authors found that mutated NBS1 is unable to bind γ-H2AX shortly after IR, and the immunofluorescence analysis indicated that NBN-R215W does not form foci up to 6 hours after IR treatment, although some co-localization with γ-H2AX occurred at 24 h after IR (di Masi et al. 2008). Interestingly, there were also significantly higher levels of unrepaired

damage in NBS/R215W cells, as compared to normal and NBS fibroblasts (di Masi et al. 2008). All these data would be in line with the view that R215W is a biologically – and clinically -relevant mutation. As a caveat, the molecular model illustrated in Figure 5.1 has to be proven by biochemical analyses of the NBN structure.

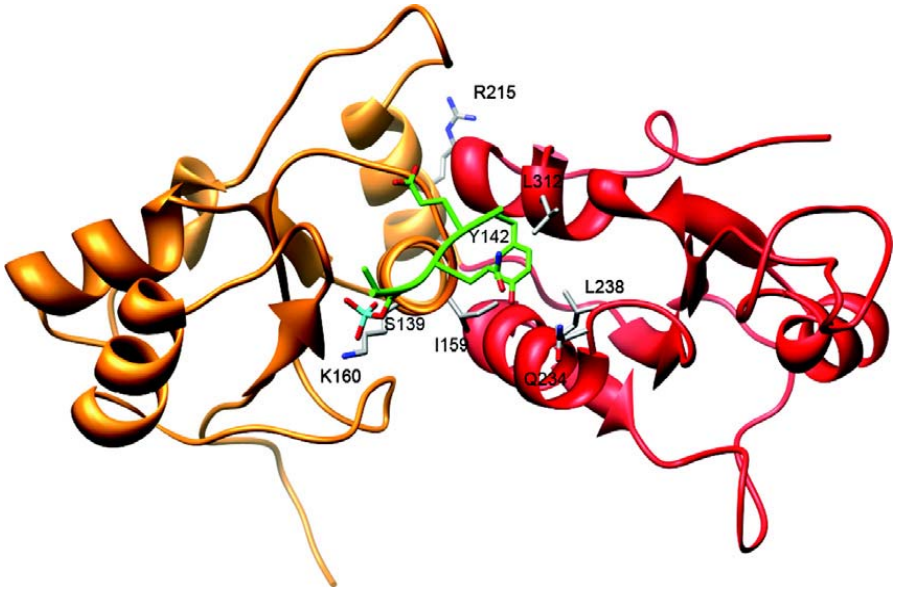


Figure 5.1. **Molecular model of the tandem BRCT domains in NBN.** The phosphorylated tail of the γ-H2AX histone is shown in green. NBN´s first BRCT domain is coloured in orange and the second in red (taken from di Masi et al. 2008).

Although the relative risk for breast cancer associated with p.R215W appears to be small, and in our studies was confined to a German case-control set and not seen in Belarus, taking in attention the possible functional relevance of the R215W substitution, the potential role of this variant should not be neglected at the present time, and large multi-center studies might be required to formally show whether the R215W substitution is associated with cancer.

In concern of missense substitution p.I171V, another variant within the BRCT domain of the NBN protein, the relevance of its screening had been suggested by a previous study that reported an about nine-fold increase in general breast cancer risk and an about six-fold increased proportion of familial breast cancer in Polish carriers of the I171V substitution (Roznowski et al. 2008). Association of the I171V with breast cancer was therefore tested in two case-control series from Belarus and Germany. Both studies are large, and each one should have had an over 99% power to detect the reported nine-fold increase in risk and 80% power to detect a 2-3 fold increased risk. Nevertheless, no association was observed for I171V allele with breast cancer in the series under investigation (for Byelorussian population OR: 0.7, 95%CI 0.4-1.2, p=0.25; for German population: OR: 1.4, 95%CI 0.5-3.7, p=0.7; combined OR: 0.83, 95%CI 0.49-1.39, p=0.56). Thus, from the results in this thesis we can exclude a nine-fold increase in risk and the data were more consistent with a very small, if any, allelic effect. The difference in the carrier frequencies among controls between the Byelorussian (1.8%) or the German (0.7%) series, and the previously published Polish study (0.2%) was notable. The Polish study used 500 anonymous newborns as population-based

controls while the Byelorussian study recruited adult healthy volunteers and the German study ascertained random blood donors as hospital-based controls. Because a higher prevalence of NBS-causing variants would be expected in a newborn series rather than in an adult population, the difference remains unexplained. Another possible explanation for the discrepancy between our results and the Polish breast cancer study (except of the too low frequency of I171V in the Polish control series) is that I171V could be in linkage disequilibrium with a yet undefined modifier variant that selectively occurs in the Polish population and is not present in Belarus or Germany. A third possibility would be that I171V could confer a very minor risk compatible with all studies, in the range of OR 1.1-1.3. For such small risks, very large multi-center studies might be required to formally show whether the p.I171V substitution is associated with breast cancer and the functional of this missense variant is also not clear.

In summary: the data obtained for missense variant p.I171V did not support an association with breast cancer in two large case-control series from Germany and Belarus. Further work is warranted to clarify its reported impact in other malignancies (Mosor et al. 2006, Ziotkowska et al. 2007, Nowak et al. 2008). The potential role of this variant should be treated with caution since p.I171V occurs at a highly conserved residue within the BRCT domain of nibrin, and although the described I171V homozygosity did not result in a NBS phenotype, it was associated with aplastic anaemia and reportedly contributed to genomic instability (Shimada et al. 2004). The c.657del5 truncating mutation was clearly associated with an increased breast cancer risk in this study, and the p.R215W substitution may represent a cancer susceptibility allele with lower penetrance. It seems justified to add the *NBN* gene to the growing list of genes involved in DNA double-strand break repair which if mutated confer a 2-4 fold increased risk for breast cancer (Walsh and King 2007). However, not all *NBN* gene alterations are of equal importance for breast cancer risk, and each substitution has to be interpreted individually.

5.4 Prevalence of studied *CHEK2* variants and their risk for breast cancer

The CHEK2 protein is a central mediator of cellular responses to DNA damage which regulates the activities of other oncoproteins (such as p53, BRCA1 and BRCA2) that, in the healthy state, finally result in cell cycle arrest (preventing entry into S-phase and mitosis) (see chapter 1.7.3). The first germline *CHEK2* mutations were reported as early as in 1999 in both sporadic and hereditary human cancers. The initial findings suggested that *CHEK2* is a tumour suppressor gene conferring predisposition to sarcoma, breast cancer and brain tumours, and provided a link to the central role of p53 inactivation in human cancer (Bell et al. 1999a). More recent studies have confirmed that germ-line mutations in the *CHEK2* gene are associated with breast cancer (Nevanlinna and Bartek 2006). In particular, a frame-shift mutation in the *CHEK2* gene, 1100delC, was identified as a low-

penetrance breast cancer susceptibility allele (Vahteristo et al. 2002, Meijers-Heijboer et al. 2003) and heterozygous carriers of the mutation have a two-to threefold increased risk for breast cancer (CHEK2–Breast Cancer Case-Control Consortium 2004, Weischer et al. 2007). Rare homozygotes of the *CHEK2* 1100delC mutation do not appear to have additional symptoms apart from cancer proneness (van Puijenbroek et al. 2005).

The 1100delC mutation was here found in 9/1757 of breast cancer patients and in 2/1015 of population controls in the Byelorussian series (OR: 2.6, 95%CI: 0.6-12.0, p=0.34), and although the association of this variant and breast cancer was not significant in this study, the results are in line with the two- to threefold increased risk reported in several, though not all, previous breast cancer studies. It was also reported that exposure to ionising radiation may be associated with disease in *CHEK2**1100delC heterozygotes (Bernstein et al. 2006), therefore it was interesting to investigate the presence of 1100delC in contaminated regions of Belarus compared with non-contaminated. However, results stratified by region showed no significant difference though carrier frequency in non-contaminated areas tended to be higher with 0.51% in comparison with contaminated 0.25% (OR 0.26, 95%CI 0.02-2.93, p=0.58). Thus, further studies examining the joint roles of *CHEK2**1100delC carrier status and radiation exposure are needed. It was also reported, that the patients carrying 1100delC variant appear to be at an increased risk for contralateral breast cancer (Broeks et al. 2004), and first-degree relatives of bilateral cases have an estimated three-fold increase in breast cancer risk (Johnson et al. 2005). However, in the Byelorussian series of breast cancer cases none of the nine 1100delC carriers had bilateral disease or reported first-degree relatives affected with breast cancer.

The role of variants in *CHEK2* other than 1100delC was less clear for breast cancer risk. Early studies have led to the conclusion that other *CHEK2* mutations do not make a major contribution to breast cancer susceptibility (Allinen et al. 2001, Schutte et al. 2003, Dufault et al. 2004). However, more recent investigations suggested that the missense substitution p.I157T as well as the splicing mutation IVS2+1G>A might confer an elevated risk of prostate cancer (Seppälä et al. 2003, Cybulski et al. 2004), and the common p.I157T mutation might be associated with increased breast cancer risk (Kilpivaara et al. 2004). A first part of results presented in my thesis has been published in parallel to this work (Bogdanova et al. 2005) showing evidence for an increased breast cancer susceptibility associated with both the splicing mutation IVS2+1G>A and the missense substitution p.I157T in Byelorussian and German populations. Thereafter, a large deletion of 5395 bp in *CHEK2* including the exons 9 and 10, *CHEK2*dele9,10(5kb), has been identified in breast cancer patients of Czech and Slovak origin (Walsh et al. 2006) and was subsequently found in breast cancer patients from Poland, Germany and Belarus (Bogdanova et al. 2007, Cybulski et al. 2007). The possible risks conferred by the IVS2+1G>A, p.I157T and *CHEK2*dele9,10(5kb) mutations had not been

extensively studied. Therefore, we aimed to corroborate the initial findings in additional case-control series from Belarus what has lead to my observation that these three *CHEK2* mutations are strongly associated with breast cancer in Byelorussian population, though with some differences in their prevalence and mutation-specific risk estimates.

Previous data including our published findings suggested that the IVS2+1G>A splicing mutation might be a predisposing *CHEK2* mutation in breast cancer patients from Eastern Europe (Bogdanova et al. 2005, Gorski et al. 2005, Cybulski et al. 2007), and one group before us has reported a statistically significant association, thus far (Cybulski et al. 2007). Our previous findings from the Byelorussian population were replicated in a study of additional patients and population controls with a combined frequency for the IVS2+1G>A of 0.9% (16/1752) among the Byelorussian patients. The IVS2+1G>A mutation was not found in any of the 1019 Byelorussian control individuals, indicating that this mutation is indeed associated with breast cancer (p=0.005). The missense substitution p.I157T had been suggested as a common breast cancer susceptibility allele in patients from Eastern Europe (Kilpivaara et al. 2004, Bogdanova et al. 2005, Gorski et al. 2005) though with considerable variation between studies. In the present thesis, the I157T variant was identified in 4.9% (86/1752) of the cases and 2.3% (23/1019) of the population controls (OR= 2.2, 95% CI 1.4-3.6, p=0.001). 23% out of 86 patients with I157T reported a first-degree family history of breast cancer, compared with 17% out of 1752 in the total series, suggesting only a subtle trend towards familial breast cancer (p=0.18). This is consistent with observations for the 1100delC mutation which demonstrated only a weak association with familial cancer and partial segregation of this allele in families (CHEK2–Breast Cancer Case-Control Consortium 2004). Initial studies had failed to establish an association of I157T and IVS2+1G>A genetic variants with breast cancer may be because heterogeneous and smaller cohorts (Allinen et al. 2001) a very low frequency of the I157T allele (Schutte et al. 2003) or a sampling bias toward multiple case families who were negative for BRCA1 and BRCA2 gene mutations (Schutte et al. 2003, Dufault et al. 2004). Our results and results reported by another group (Cybulski et al 2005a, 2007) now clearly demonstrate a role for the *CHEK2* mutations I157T and IVS2+1G>A in inherited breast cancer susceptibility.

We subsequently investigated the frequency of the *CHEK2*dele9,10(5kb) mutation, a large deletion of some 5395 bp that eliminate two exons encoding for part of the kinase domain. The *CHEK2*dele9,10(5kb) allele among Byelorussia patients was also significantly higher than among controls (OR 11.8, 95% CI 1.6-87.8, p<0.005). Thus, there is a clear association of the deletion with breast cancer in Byelorussian population and the obtained data are in line with other findings (Walsh et al. 2006, Cybulski et al. 2007).

When the data sets for all mutations were combined, the median age at diagnosis was not significant different between carriers and non-carriers for any *CHEK2* mutation. Apart from the large deletion,

CHEK2 mutation carriers tended to show a slightly higher age at diagnosis in comparison with non-carriers, and for 1100delC carriers the difference approached borderline significance with p=0.05 (54 years in carriers versus 48 years in non-carriers). This may be partly due to the relatively high prevalence of *BRCA1* mutations in the Byelorussian case series which are associated with significantly lower age at diagnosis; there were only three patients carrying both, *CHEK2* and *BRCA1* mutations. When stratified by family history, the frequency of *CHEK2* mutations in patients with at least one first-degree relative affected by breast cancer was not significantly higher (with the possible exception for IVS2+1G>A mutation stratified by region, see chapter 4.4). Similarly, in other published studies to date, no clear trend in risk with the strength of the family history for those *CHEK2* mutations was found (Kilpivaara et al. 2004, Cybulski et al 2005, 2007, Walsh et al. 2006), indicating a reduced penetrance of these mutations. In contrast with another studies (Broeks et al. 2004, Johnson et al. 2005), we also did not detect an association with bilateral disease. The low-penetrance susceptibility associated with the investigated *CHEK2* mutations is thus apparent in women unselected for family history or bilateral disease and may be explained by an interaction of *CHEK2* mutations with susceptibility alleles in other genes to increase the inherited predisposition to breast cancer (Oldenburg et al. 2003, Kilpivaara et al. 2004, CHEK2–Breast Cancer Case-Control Consortium 2004).

In summary: the presence of a *CHEK2* mutation was associated with increased breast cancer risk in the Byelorussian population, and this association was statistically significant for three out of four mutations tested (p=0.34 for 1100delC, p=0.005 for IVS+1G>A, p=0.001 for I157T and p=0.005 for *CHEK2*dele(9,10), respectively). The odds ratios were 11.8 (95%CI 1.6-87.8) for variant *CHEK2*dele(9,10), 2.6 for 1100delC (95%CI 0.6-12.0) and 2.2 for the missense variant I157T (95%CI 1.4-3.6), while for IVS+1G>A splicing variant an OR could not reliably be calculated due to its absence in over 1000 controls. All the data would be consistent with a previously estimated two- to three-fold increase in risk. Although the I157T missense substitution appeared to confer a lower risk than the truncating mutations, its association with breast cancer was significant (p= 0.001). If any *CHEK2* mutation carrier frequencies were combined, the calculated OR for all four variants corroborated the strong association of such mutations with breast cancer (OR= 3.0, 95% CI 2.0--4.6, p<0.00001). Similar data were obtained in a replication study of these *CHEK2* mutations in breast cancer patients from Russia (M. Bermisheva, pers. comm.). The results extend our knowledge about *CHEK2* mutations and help to better define the population frequency and the magnitude of risk associated with these common inherited breast cancer susceptibility alleles. It is important to note that *CHEK2* mutations meanwhile also have been associated with other epithelial cancers, such as prostate or colon carcinomas (Cybulski et al. 2004, Kilpivaara et al. 2006), and thus the mutations studied in this thesis might represent more general cancer susceptibility alleles.

*5.4.1 Physiological impact of p.I157T homozygosity and CHEK2 dele(9,10)/NBS1*657del5 carrier status on CHEK2 protein function*

Following ionizing radiation-induced DNA damage, CHEK2 is rapidly phosphorylated at multiple sites in the SQ/TQ-rich region by ATM (Matsuoka et al 2000) and becomes localized to nuclear foci (Ward et al. 2001). ATM phosphorylation of Thr68 appears to be a prerequisite for subsequent autophosphorylation at Thr383 and Thr387 in the activation loop of the kinase domain. This activation mechanism is evolutionarily conserved and necessary for the subsequent CHEK2 dependent activation of substrates involved in cellular response to DNA damage. However, there are additional ATM phosphorylation sites on CHEK2, including those on Ser19, Ser33 and Ser35, that have emerged as similarly important regulatory sites (Buscemi et al. 2006).

The physiologic impact of the IVS2+1G>A mutation has been clarified by observations that this genetic variant results in an aberrantly spliced *CHEK2* mRNA encoding a truncated protein (Dong et al. 2003). The 1100delC allele also leads to the production of a shorter, non functional CHEK2 protein that is truncated within its kinase domain. The p.I157T mutation in the FHA domain of the CHEK2 protein occurs at polymorphic frequency in some populations (Vahteristo et al. 2001), but biochemical data from a few studies suggest that this variant may be deleterious (Falk et al. 2001, Li et all. 2002) and, though it does not result in apparent changes in the size of CHEK2, may affect associations of CHEK2 with substrate proteins. We aimed to address this in the first cell lines that we have been able to establish from patients homozygous for the p.I157T substitution. Immunoblot analyses showed full-length CHEK2 protein and no difference in radiation-induced CHEK2 phosphorylation (at Thr-68, Ser19 and Ser33/35 sites) in comparison with wild-type lymphoblastoid cell lines (see chapter 4.4). This indicated that there is no effect in the expression level or regulation of the p.I157T CHEK2 protein but it does not exclude effects at the level of protein interaction and downstream pathways. As a possible functional test, we chose conditions from a recently published study of Tan with co-authors. In this work, the authors reported the CHEK2 dependent phosphorylation of FoxM1 protein on Ser-361 what caused increased stabilization of the latter with a corresponding increase of *XRCC1* and *BRCA2* gene transcription in mouse embryonic fibroblasts and osteosarcoma U2OS cells (Tan et al. 2007). However, although my initial experiments were consistent with such a radiation-induced increase of *BRCA2* expression in mammary epithelial cells, we failed to show any changes in *BRCA2* expression after irradiation in lymphoblastoid cells from either a homozygous carrier of p.I157T or control individuals (see chapter 4.5). Expression of *BRCA2* transcripts in lymphoblastoid cells was very poor, less than 1% from housekeeping gene expression (cyclophilin) in contrast with the other experiment done on HMECs where results revealed increased in about 2 fold expression of *BRCA2* transcripts at 24 hours after treatment with IR. The reasons for the failure to confirm the published data in LCLs may be tissue/cell specific

expression of *BRCA2*, particularly low *BRCA2* expression in LCLs which was almost at the limit of detection, and therefore an unfavourable signal-to-noise ratio in the RT-PCR studies. Nevertheless it is reasonable to speculate that the p.I157T mutant may be defective in protein function, because this variant is located in the FHA domain of CHEK2, which may be involved in protein-phosphoprotein interactions. Some of these interactions may be essential for transmitting DNA damage signals to CHEK2, and any alteration of CHEK2 association with upstream signaling proteins could lead to the failure of CHEK2 activation following DNA damage, as for example in the case of R145W allele that is also located in FHA domain (figure 5.2). CHEK2 activation appears to require the FHA domain and is defective by the R145W mutation, which retains some kinase activity *in vitro* but is incapable of being activated following gamma radiation *in vivo*, most likely because it is not phosphorylated at Thr-68 by ATM kinase. However, this activation process was found to be not affected in I157T (Wu et al. 2001), consistent with my findings in the p.I157T homozygous cell lines. CHEK2 phosphorylation following DNA damage in ATM-dependent manner, results in an altered electrophoretic mobility (phosphorylation shift) which was shown to be abolished in the R145W mutant (Wu et al. 2001) but not in I157T (Wu et al. 2001, and chapter 4.4.5 in this thesis).

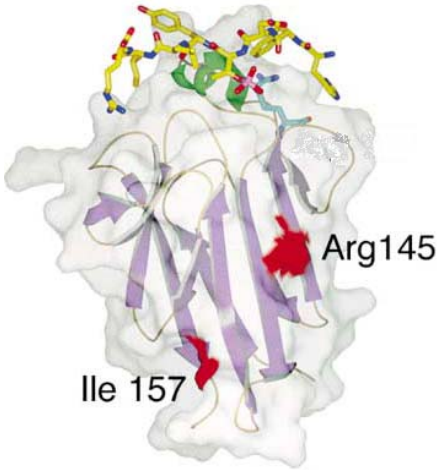


Figure 5.2. **Structural model of the FHA domain in CHEK2.** Both Arg145 and Ile157 are located on the more conserved face of the FHA domain sandwich, and they are remote from the site of phosphopeptide binding. (taken from Li et al. 2002).

In addition, the FHA domain may also mediate transmitting signals from CHEK2 to downstream effectors such as TP53, BRCA1, and Cdc25C. The R145W variant and, interestingly, also the I157T mutant FHA domains were found to fail in detectably binding to BRCA1 in experiments with hydroxyurea induced DNA damage (Li et al. 2002). Thus it was suggested that Ile157 possibly forms part of an accessory hydrophobic surface that acts to stabilize an overall interaction in which specificity is provided through the phospho-dependent binding site (Li et al. 2002, see Fig. 5.2). The importance of the accessory surface may not be limited only to CHEK2-BRCA1 interactions

since CHEK2 activates also TP53 and Cdc25C. But these hypotheses still remain to be experimentally proven.

Concerning the physiological impact of *CHEK2*dele(9,10), my analysis of lymphoblastoid cDNA from a heterozygous carrier showed the presence of a smaller deletion product in approximately the same amount as the product of wild-type length, and there was no evidence for a nonsense-mediated decay of *CHEK2*mRNA harbouring the deletion. In addition, some smaller splice transcript was seen at a low level in the sample with *CHEK2*dele(9,10) which by direct sequencing exhibits a deletion of 3 exons (including also exon 11). This transcript was present in a relative proportion of less than 5% of the wildtype. This skipping of exon 11 restores the reading frame in the context of the *CHEK2*dele(9,10) deletion, but protein which may then be synthesised will lack of 117 amino acids of the CHEK2 kinase domain and should have about 35 kDa. Such protein product was not detected on my western blots what could be because of its very low level of expression. I also failed to detect a truncated protein corresponding to the normally spliced *CHEK2*dele(9,10) mutation, although antibodies against amino-terminal epitopes were used. As this transcript was not subject to nonsense-mediated decay (NMD) and was observed at a relative level similar to wildtype, the shortened protein of about 49 kDa might have been expected. However, it has been reported that truncated proteins are often unstable, as recently exemplified for the CHEK2*1100delC mutant after inhibition of NMD (Anczukow et al. 2008), and this instability may be due to a translational repression of transcripts that escape NMD, such as the *CHEK2*dele(9,10) mutation (You et al. 2007).

At the protein level, CHEK2 was observed in full-length in LCLs from the proband with *CHEK2*dele(9,10) heterozygous genotype but the protein level was approximately reduced by half in comparison with LCLs of wild-type genotype -as might have been expected for a heterozygote –, and moreover the phosphorylation shift was not detected (see chapter 4.4). The latter was unexpected and pointed to a role for the *NBN**657del5 mutation that had also been identified in the same individual. Radiation-induced CHEK2 phosphorylation (at Ser19 and Ser33/35 sites) in LCLs from dele(9,10)/657del5 compound heterozygote indeed showed a reduction of about 60%-70% of pCHEK2 protein in comparison with wild-type, which in turn may markedly affect CHEK2 protein stability and function. Remarkably was the finding that cells from NBS obligate heterozygtes for the 657del5 mutation had phospho-protein levels in the same range as for wild-type controls, but cells from a homozygous NBS patient with the 657del5/657del5 genotype had a dramatic reduction (to about 10% of wild-type) of phospho-protein levels. These observations suggest a possible interaction between CHEK2 and NBN, i.e. full-length NBN is required for the full activation of CHEK2, in particular its phosphorylation at Ser19 and Ser33/35. Cells with mutations in both genes may be functionally more compromised, and correspondingly the respective patients may be at an

increased risk of cancer. However, the results of presented study suggest that the number of such patients will be relatively low.

In summary, the two investigated CHEK2 mutations differed in their effects at the functional level. The p.I157T substitution exhibited by immunoblot analyses no distinction from wild-type in the size of CHEK2 protein or its radiation induced phosphorylation, but since the analysis of *BRCA2* expression in LCLs did not prove to be a reliable assay of CHEK2 function, further studies of the cell lines from I157T homozygotes and control probands will be needed to clarify the possible impact of this missense variant on protein function. The results obtained for a *CHEK2*dele(9,10)/657del5 compound heterozygote demonstrated an allele-specific suppression of the deletion product at the protein but not at the mRNA level, and showed a surplus reduction in the radiation-induced CHEK2 protein phosphorylation, what may be explained by a possible interaction between NBN and CHEK2 mutations.

5.5 Missense substitution in *PALB2* and relevance for breast cancer

PALB2 (partner and localizer of BRCA2) gene encodes a recently discovered protein that binds to and colocalizes with the BRCA2 protein in nuclear foci and likely permits the stable intranuclear localization and accumulation of BRCA2 (see chapter 1.7.3). Thus, the protein is important in the regulation of homologous recombinational repair (chapter 1.5.2). Biallelic *PALB2* germline mutations are responsible for a subset of Fanconi anemia (FA-N) with similar phenotype like that caused by biallelic *BRCA2* mutations (Reid et al. 2007, Xia et al. 2007). Monoallelic *PALB2* mutations were also found in individuals with breast cancer from familial breast cancer pedigrees that were negative for *BRCA1* and *BRCA2* mutations. A truncating mutation, possibly a founder mutation, in *PALB2* was also detected in familial and sporadic breast cancer cases and in one prostate cancer family in the Finnish population (Erkko et al. 2007). *PALB2* mutations showed an incomplete segregation in affected relatives and were estimated to confer a 2 to 3 fold increase in breast cancer risk, and it was suggested that the risks of breast cancer associated with *PALB2* mutations may be age- dependent (Xia et al. 2007, Rahman et al. 2007). Three investigated missense *PALB2* variants Q559R, E672Q and G998E (originally described by Rahman et al. 2007) were in parallel work of our group detected to be in linkage disequilibrium, and the combination of all three rare genotypes appeared associated with increased breast cancer risk with some evidence of early age of breast cancer in carriers *versus* non carriers (MA Blaut, MD thesis in preparation). These three variants were genotyped in Byelorussian cases and population controls and the association with breast cancer was not confirmed (see chapter 4.6). Median age at diagnosis for carriers versus non carrier was also not significantly different neither for certain genotypes nor if

stratified by region, so was no evidence obtained for an association with early onset breast cancer. For all three variants was no association detected between carrier status and familial breast cancer.

While the relative risk conferred by inactivating (truncating) mutations for *PALB2* was estimated to be about 2-3fold increased, it was also proposed that polymorphic variants in this gene contribute little, if anything, to the identification of woman at substantial risk (Rahman et al. 2007). This is supported by the results obtained in Byelorussian cases and controls. Another possible explanation of different results obtained in German and Byelorussian populations is that the polymorphic variants could confer a very minor risk that escaped detection in the Belarus study and large multi-center studies are required to find an association and estimate the risk. It is also possible that some *PALB2* variants modify the risk of *BRCA2* mutations, but the number of *BRCA2* mutation carriers in the present study was much too small to address this question. The investigated *PALB2* variants p.E672Q and p.G998E have been suggested to be related to a possibly altered protein function by bioinformatic criteria, although any functional assays remain to be done (Rahman et al. 2007). In summary: because only few studies in this field exist so far and somewhat controversial results were obtained from Byelorussian and German populations, further research is necessary to show whether the studied substitutions in *PALB2* associated with minor risks of breast cancer and which of them may have functionally relevance.

5.6 *XRCC4* variant IVS7-1G>A and its impact on breast cancer risk

XRCC4 encodes protein that functions together with DNA ligase IV in the repair of DNA double strand breaks by non-homologous end joining (see chapters 1.5.2 and 1.7.4). The transition of Guanine to Adenine in the last nucleotide of intron 7 abolishes the consensus acceptor site and results in the utilisation of an internal alternative splice site. The aberrant splicing changes three amino acids in the mutant protein: deletion of arginine and serine (at position 298 and 299, respectively) and substitution of asparagine to lysine at position 300. In primary work of our group (K. Gerriets, MD thesis submitted), carrier frequencies were not different between cases and controls in German collective, but it was indicated significant excess of homozygous among breast cancer cases. In the Byelorussian breast cancer case- control series, the IVS7-1G>A variant was found at a frequency of 23.4% in cases and 19.8% in controls (OR: 1.19, 95%CI: 1.04-1.37, p=0.01) with about twice as many homozygotes in the case group. If stratified by region a significant difference between cases and controls was obtained only for non-contaminated regions (see chapter 4.7). This trend is similar to the observations for other SNPs and will be discussed later.

Allen-Brady with co-authors reported a possible role of *XRCC4* haplovariants in association with breast cancer risk and suggests that it could be age- dependent (Allen-Brady et al. 2006). In a study

of German patients, some evidence was indeed found of a lower age at diagnosis for carrier of the uncommon IVS7-1A/A genotype, but in the Byelorussian population carriers of the same genotype tended to be older than heterozygous carriers or non-carriers, although a significant difference was not achieved (p=0.07). During the course of the thesis, the IVS7-1G>A *XRCC4* variant was also found to be significantly associated with bladder cancer risk (Figueroa et al. 2007), and in that study there was no evidence found for an interaction with age either.

It was some evidence in Byelorussian study that some IVS7-1G>A allele carriers also harboured previously identified *BRCA1* or *BRCA2* founder mutations (19/411, 4.6%), this proportion was the same as in non-carriers (OR: 1.00, 95%CI 0.13-7.5, p=1.0). However if carriers of previously identified mutations not only in high penetrance genes *BRCA1*/2 but also in the *CHEK2*, *ATM* and *NBN* were excluded, the observed association of IVS7-1G>A with breast cancer became even stronger (OR 2.1, 95%CI: 1.8-2.5, p<0.0001), suggesting that IVS7-1G>A constitutes an independent risk factor.

There are only few studies about the possible role of the IVS7-1G>A splice variant in the *XRCC4* gene and cancer risk, so far, and results from German and Byelorussian populations were not entirely consistent, although both point to a risk increase in carriers of this splice mutation. These findings need to be replicated in other populations, particularly since homozygote variants were rare in the hitherto published studies (Wu et al. 2006, Figueroa et al. 2007). The NCBI SNP databank suggests that the mutant splice site allele (A) may be particularly common in Asians, suggesting that case-control studies in these populations will have a high statistical power, and preliminary findings in populations from Russia support this observation (M. Bermisheva, pers. communication).

Previous work of our group (K. Gerriets, MD thesis submitted) addressing the functional relevance of the IVS7-1G>A variant had shown by immonoblot analysis that IVS7-1A/A allele produces full-length XRCC4 protein and becomes normally phosphorylated after exposure to ionising radiation in an ATM-dependent manner. These findings could be supported by the fact that XRCC4 is activated/phosphorylated on distinct residues which are not changed by splice mutation IVS7-1G>A. Thus, the splice isoform is at least partially active and the effect of the IVS7-1G>A mutation, if any, appears to be more subtle. In summary: obtained results provide evidence for an association between IVS7-1G>A genetic variant in *XRCC4* gene and breast cancer in Byelorussian population, thus *XRCC4* can be proposed as one of the low penetrance genes predisposing to breast cancer. However, future replication studies are required to confirm these findings and more thorough analysis are needed to elucidate the impact of the shortened isoform on protein function.

5.7 Low-penetrance loci, identified to be associated with breast cancer

Breast cancer has a strong inherited component but a large proportion arise in a genetically susceptible minority from underlying genetic factors which are little known. During the course of the thesis, evidence has been accumulating that part of the heritable disposition is due to common low-penetrance susceptibility alleles. Recently conducted genome-wide association studies have reported common polymorphisms that are strongly associated with breast cancer risk (Cox et al. 2007, Easton et al. 2007, Hunter et al. 2007, Stacey et al. 2007, Gold et al. 2008). Two of these studies were conducted by Breast Cancer Association Consortium, established in 2005 to facilitate such collaborative studies in breast cancer and currently comprising over 25 international research groups including our team (Easton et al. 2007). The genotyping of SNPs chosen by this consortium and data analysis was first done in a case-control series from the German population (HaBCS), and the most interesting findings were further investigated in a case-control series from the Byelorussian population (HMBCS), as part of the thesis presented here. Variants D302H in *CASP8* (rs1045485), L10P in *TGFB1* (rs1982073), and the three non-coding variants rs2981582 in *FGFR2*, rs3803662 at *TOX3/TNRC9* and rs13387042 on chromosome 2q35 which were most significantly associated with breast cancer in the large genome-wide studies, were chosen to be screened in the series of Byelorussian cases and controls. Caspase-8 is an important initiator of apoptosis and is activated in response to DNA damage as well as external death signals (Ding et al. 2000, Hengartner 2000). Transforming growth factor-b (TGFb) is a polypeptide cytokine that has a role in regulating cell growth, differentiation and migration. It regulates normal mammary gland development and function by activating the TGFb signaling pathway, and it also modulates the ATM pathway (Kirshner et al. 2006, Wiegmann et al. 2007). There is a dual role of action in which the TGFb signaling suppresses tumour initiation but can also promote tumour progression and metastasis when antiproliferative effect of the TGFb signaling pathway has been overridden by other oncogenic mutations (Bierie and Moses 2006, Derynck et al. 2001).

The D302H polymorphism in CASP8 results in a substitution of an aspartic acid to histidine and in our consortium study appeared to reduce breast cancer risk (Cox et al. 2007), but the functional consequences of this substitution are not yet known, and further experiments are required to establish whether D302H itself causative, or another variant in strong linkage disequilibrium with it. Weaker evidence for breast cancer association was found in the same study for the TGFbeta1 L10P variant. The Leu10Pro polymorphism resides in the signal peptide sequence of TGFbeta which has been associated with increased levels of protein and mRNA in individuals with the rare Pro allele; the peptide with Pro at residue 10 causes a 2.8 fold increase in secretion compared with the Leu form (Dunning et al. 2003). The association study of the Leu10Pro variant showed a significant dose dependent association of the Pro allele with increased risk of invasive breast cancer and it was

estimated to account for approximately 0.2 % of the excess familial risk of breast cancer in populations of European ancestry (Cox et al. 2007). Both SNPs, in *CASP8* and in *TGFB1*, gave no significant association with breast cancer in our case-control series from the Byelorussian population (and neither in German cases and controls if data was calculated from single study), neither over all nor stratified by region, but the D302H substitution was significantly associated with familial breast cancer in the Byelorussian population as a protective allele (see chapter 4.8). Thus, the data are in the same direction as published in the frame of the BCAC (Cox et al. 2007).

Locus rs2981582 in intron 1 of *FGFR2* – the gene encoding fibroblast growth factor receptor 2 which influence mitogenesis and differentiation - and locus rs3803662 close to *TOX3*, also known as *TNRC9* encoding a high mobility group box family member 3 which may bind to DNA and regulate transcription, were found to be most significantly associated with breast cancer in the subsequent genome-wide study of the Breast Cancer Association Consortium (Easton et al. 2007) and were confirmed in independent research (Hunter et al. 2007, Stacey et al. 2007) where also as an additional locus rs13387042 on chromosome 2q35 was found to be associated with breast cancer risk. For the LD block containing rs13387042, not any known genes or human RNAs was found. Moving proximally (left) outside the LD block, the nearest known genes are TNP1 – transition protein 1 (during histone to protamine replacement) (181 kb proximal), IGFBP5 (345 kb proximal) and IGFBP2 (376 kb proximal) – insulin-like growth factor binding proteins 5 and 2. Moving distally outside the LD block, the nearest known gene is TNS1 (tensil 1), a protein that localises to focal adhesions, crosslinks actin filament and contains a Scr homology 2 (SH2) domain, which is often found in molecules involved in signal transduction (761 kb distal). Further analysis in the LD blocks containing these genes did not show any signals that could account for the observed association with rs13387042 (Stacey et al. 2007).

All three non-coding variants were found to be not significantly associated with familial breast cancer in presented thesis and only SNP in *TOX3* was confirmed to be associated over all in Byelorussian population with breast cancer. However, for the other two loci (*FGFR2* and 2q35), a significant difference was found in median age at diagnosis between carriers and non- carriers, which was particularly pronounced for homozygous carriers of rare A-rs13387042 genotype. Thus, the obtained results in this thesis are in the same line with the published data, but as a single study there was insufficient power to prove the general statistical association of these low-penetrance susceptibility alleles with breast cancer.

To date it is not known how the described loci interact with each other or with lifestyle factors that may modulate the risk, or which functional consequences have some of the found variants and, in several cases, even the disease-causing variant itself remains to be defined. However, the published reports emphasize the importance of large-scale studies when the goal is to identify common

variants conferring modest increases in the risk of breast cancer. The most recent study of the Breast Cancer Association Consortium furthermore shows that some of the common variants may also play a role in the further progression of breast carcinomas as is exemplified by the strong association of the *FGFR2* risk allele with ER positive tumours (Garcia-Closas et al. 2008). In other recent study was found that described SNP withing intron 2 of the *FGFR2* gene (rs2981582) alter the binding of transcription factor C/EBPβ what in turn cause an increase in *FGFR2* gene expression (Meyer et al. 2008).

Recently a new genome-wide association study using cases and controls from a genetically isolated population of Ashkenazi Jews identified another new locus potentially associated with breast cancer risk (Gold et al. 2008). This study reported the association of breast cancer with the *RNF146/ ECHDC1* region at 6q22.33 chromosome. *RNF146* encodes ring finger protein 146, also called dactylidin, aprotein that is differentially expressed in neurodegenerative diseases, is ubiquitously expressed with cytoplasmic localization, and possibly functions as a ubiquitin protein ligase (E3). Protein degradation through the ubiquitin proteasome system regulates such processes as cell cycle, apoptosis, transcription, protein trafficking, signaling, DNA replication and repair, and angiogenesis. Defects in this pathway have been well documented in breast cancer (Mani and Gelmann 2005) and as well known examples *BRCA1*, *BRCA2*, *BARD1*, and *MDM2* ubiquitin ligases, deregulation of which found in subsets of human breast cancers (Chen et al. 2006). Although *RNF146* is little studied in cancer, it belongs to a class of genes including *BRCA1*, *BRCA2*, and *BARD1* suggesting that it could play a role in tumorigenesis of breast or other malignancies. On the other hand, *ECHDC1* encoding enoyl-CoA hydratase domain containing 1 may function in fatty acid metabolism and mitochondrial fatty acid oxidation. It has been also little studied in breast cancer. It is established that inhibition of fatty acid oxidation can induce apoptosis in breast cancer cell lines, and this effect was increased 300-fold in *TP53*-silenced cell lines (Zhou et al. 2003, Menendez and Lupu 2005). Thus, it could also play some role in breast tumorigenesis. However, it could not be determined whether the breast cancer association reported in the study of Gold and co-authors (2008) was arising from the *ECHDC1* gene, *RNF146* gene, or another locus in linkage disequilibrium. Future genetic association studies in divergent populations could prove helpful to define the responsible gene more precisely.

In summary, recent technological advances have provided unanticipated possibilities to analyse hundreds of thousands of SNPs in association studies, thus providing a basis for identifying low risk alleles without prior knowledge of position or function In the tradition of early founders of modern biology and genetics, for instance Gregor Mendel or Charles Darwin, the identification and careful description of such associations will lay the ground for a better understanding of biological and biochemical mechanisms and finally pave the way for future cancer therapies.

5.8 Regional differences in Byelorussian investigated series

One distinctive feature of the Byelorussian case-control series investigated in this thesis is the fact that a relatively large proportion of individuals has been chronically exposed to low-dose ionising radiation as a long-term consequence of the Chernobyl accident over 20 years ago. As outlined in the introduction, ionising radiation is a risk factor for breast cancer. The radiation exposure could affect the relative frequency of mutation carriers in different ways. One possibility is that the penetrance of mutations (for example in damage repair genes) is increased by gene-radiation interaction which then may result in a relative increase of these mutations in the case group of environmentally exposed. Another possibility is that the environmental exposure may act independently as an additional risk factor which then may increase the total number of diseased non-mutation carriers thereby decreasing the relative proportion of mutation carriers among cases. We aimed to investigate this further by comparing the carrier frequencies in both contaminated and non-contaminated regions.

Both the case and control series were divided in two subgroups, according to the estimated whole body doses accumulated after the Chernobyl accident in the respective study regions (see chapter 2.7): 1**)** non-contaminated (**0**); 2) contaminated regions (with subdividing ranking from very low contamination with 5mSv to high contamination with doses >40 mSv, **I-IV**). This regional division was based on the data published by Pukkala et al. (2006). The first findings about regional differences in the case-control series came from the comparison of median ages at onset of breast cancer which for non-contaminated regions was 50 years and for contaminated regions 44 years. This difference was statistically highly significant with $p < 0.00001$, indicating that the patient groups stratified by these criteria are indeed different. The next observation was concerning the proportion of patients with a family history of breast cancer. This group was termed “familial cases” and included the subset of patients with at least one first-degree relative affected with breast cancer. Regional discrepancy was exhibited as a higher proportion of familial cases in contaminated versus non-contaminated areas and was statistically significant with OR 1.72 (95%CI: 1.34-2.21, $p<0.00001$). Both facts, the younger age at diagnosis and a stronger family history in contaminated regions could theoretically be explained by a higher prevalence of mutations in breast cancer susceptibility genes *BRCA1/2* in these regions (founder effect), because both genes are two major predisposing to familial and early-onset breast and/or ovarian cancers. However carrier frequencies for the mutations tested were not significantly different between both regions: OR 1.36, 95%CI: 0.87-2.14, $p=0.22$, and these most common mutations were very rare among the controls in both regions (attachment 3). Thus, founder effects for *BRCA1* and *BRCA2* mutations are very unlikely to explain the regional differences. The same tendency was seen for breast cancer susceptibility alleles with moderate penetrance (*ATM, NBN, CHEK2*): combined mutations in these genes were slightly

less frequent in cases from contaminated regions than in non-contaminated regions (8.4% versus 9.9%) although statistical significance was not achieved (OR 0.83, 95%CI 0.60-1.16, p=0.32). In the control group, there seemed to be more combined carriers of high-penetrance and moderate risk alleles in contaminated regions (4.8% versus 2.7%) but this observation was also not statistically significant (OR 1.86, 95%CI 0.95-3.64, p=0.1). Even though the products of these genes are involved in DNA damage pathways: as the potentially higher carrier frequency in contaminated areas was not reflected in the case group, there was no evidence for a gene-radiation interaction in these patients. For the studied low-penetrance alleles there was mainly the same allelic distribution between regions (attachment 3), without detectable difference with the interesting exception of *TGFB1* where the L10P variant was less frequent in cases from contaminated regions than in non-contaminated (per-allele OR in cases: 0.82, 95%CI: 0.72-0.94, p=0.006) whereas there was no difference in controls (OR 1.06, 95%CI 0.88-1.26, p=0.59), so that the association of this variant with breast cancer was confined to non-contaminated regions.

It was also aimed to investigate possible gene-gene interactions between those 11 loci which were shown in other studies and by us to be associated with breast cancer (see attachment 4). An analysis done for two-way SNP interactions in the series of breast cancer cases showed some decrease in the observed carrier frequencies for pI157T/ 2q35 rs13387042 combined carriers (attachment 4) in comparison with the expected carrier rate, and this association was borderline significant if heterozygotes and homozygotes for the rare alleles were combined (Chi-square test: p=0.04) (table 5.1). By contrast, in the series of Byelorussian control individuals there was no significant difference between observed and expected carrier frequencies for these combined genotypes (table 5.2).. The potential gene-gene interaction for pI157T/rs13387042 combined carriers in breast cancer cases was not differing between regions (Chi-square test: p=0.79).

Table 5.1 **Combined carrier frequencies for p.I157T and rs13387042 among Byelorussian breast cancer patients.**

Gene/locus	**2q35 (rs13387042)**			
	Genotype	Homozygotes for common allele	Heterozygotes	Homozygotes for rare allele
CHEK2	WT/WT	504 (551)	859 (785)	307(334)
	pI157T/WT	44 (29)	32 (41)	10 (17.6)
	pI157T/pI157T	1 (0.6)	1 (0.8)	0 (0.4)

In black shown observed number of carriers, and in blue shown expected carrier numbers.

Table 5.2 **Combined carrier frequencies for p.I157T and rs13387042 among Byelorussian control individuals**

Gene/locus	**2q35 (rs13387042)**			
CHEK2	Genotype	Homozygous for common allele	Heterozygous	Homozygous for rare allele
	WT/WT	324 (320)	479 (479)	191 (199.7)
	pI157T/WT	6 (6.5)	10 (10)	5 (4.1)
	pI157T/pI157T	0 (0.65)	2 (1)	0 (0.4)

In black shown observed number of carriers, and in blue shown expected carrier numbers.

While these two potentially interacting variants were found to be associated with breast cancer, the functional importance of such an interaction remains uncertain particularly due to the unknown gene for locus rs13387042 on chromosome 2q35 and no knowledge to date if this SNP causative or some other.

Thus, in general, the distribution of the mutations was not much different between regions and the current observations can not explain the observed heterogeneity in the age at diagnosis and familial history in regions in terms of genetic factors. If anything, it appeared that the currently known risk alleles tended to be less prevalent in cases from contaminated regions and, if this were to be confirmed, it could reflect an independent action of additional risk factors in these areas. Thus, it is not excluded that there is a role of environmental factors in breast cancer development, what may be relevant for investigated Byelorussian population in regard of chronic exposure to ionizing radiation after the Chernobyl accident, but this study does not allow to draw conclusions about a possible synergistic role of both genetic and environmental factors. In particular, the present results do not support the hypothesis that such environmental factors act more effectively on genetically predisposed persons what may lead to regional differences due to gene-gene and gene-environment interaction. However, as a note of caution, we should keep in mind that we have no information about biodosimetry data of the patients to reconstruct individual doses, beyond their geographic region of living. While the ground contamination in the geographic area of origin has been well-documented (Pukkala et al. 2006), and we observed significant differences between patient groups stratified by region in terms of age and family history of breast cancer, the geographic origin might be considered as an important factor but it is not the only factor determining the individual dose. This limits our study in its present design but allows for future projects to examine the gene-radiation relationship in more detail. For example, the present results can provide a basis to follow up the blood relatives in the respective Byelorussian families who are carriers of a defined mutation, and to determine their risks in future cohort studies similar to those that have recently been initiated

in the European Union with the aim to identify risks associated with medical diagnostic radiation in *BRCA1* mutation carriers (Andrieu et al. 2006).

6. Conclusions

The objective of this thesis was to investigate the contribution of founder mutations in major DNA double-strand break repair genes and the prevalence of common polymorphic variants as breast cancer susceptibility alleles in case-control sets from the Republic of Belarus, and to characterize some of the identified variants by functional assessment of the radiation-induced DNA damage response in patient lymphoblastoid cell lines.

These studies demonstrated that:

- Four germline mutations in the *BRCA1* and *BRCA2* genes accounted for 4.5% of unselected Byelorussian breast cancer patient and 9.1% of hereditary breast cancers. Breast cancer risk appeared heterogeneous for the studied *BRCA1* mutations, but the presence of any, including 4153delA was significantly associated with an increase in risk for the disease.
- Single truncating *ATM* mutations can contribute to breast cancer outside A-T families, so a significant association of the p.E1978X nonsense mutation with breast cancer in three combined populations of Slavic descent could be shown.
- The c.657del5 truncating *NBN* mutation was clearly associated with an increased breast cancer risk in this study, and the p.R215W substitution of nibrin may also represent a cancer susceptibility allele, although large multi-center studies might be required to formally show whether the p.R215W substitution associates with cancer.
- *CHEK2* mutations were strongly associated with the breast cancer the radiation response in cell lines suggested an interaction with mutations in NBN.
- A significant association was observed between the IVS7-1G>A splice variant in the repair gene *XRCC4* and breast cancer risk in the Byelorussian population. This variant may be proposed as a low penetrance breast cancer allele, but the findings need to be confirmed in other populations.
- Additional low-penetrance alleles investigated in this study were not overall significant associated with breast cancer in Byelorussian population, with the exception of a variant located close to the *TOX3/TNRC9* gene. Another variant, p.D302H in the gene encoding caspase-8, was underrepresented in cases with familial breast cancer.
- Possible gene-gene interactions were identified between variants in *ATM* and *BRCA1*, and between variants in *CHEK2* and the 2q35 locus. However, since these interactions were not significant under conditions of multiple testing, further research is needed to validate these findings.
- The frequency distribution of the mutations across different regions of Belarus was not markedly heterogeneous, so that the observed significant differences in the age at diagnosis

and family history between regions can not be explained due to founder effects or gene-environment interactions of hitherto identified genetic factors.

As the genetic epidemiology of breast cancer has not been investigated in the Republic of Belarus, before, the presented study for the first time reveals the spectrum of breast cancer susceptibility alleles in the Byelorussian population. These data may serve as a basis for subsequent studies in several aspects. The impact of some gene variants on the respective protein function has still to be clarified. So, a functional assessment of the p.I157T missense variant at the CHEK2 protein level revealed no distinctions from wild-type in terms of expression and radiation-induced phosphorylation, indicating a possible downstream defect. More analysis also required to elucidate the impact of the shortened XRCC4 isoform due to the IVS7-1G>A splice mutation on differential protein functions. The functional interaction between the NBN and CHEK2 proteins as deduced from the immonoblot findings need to be tested in other model systems. Also observed heterogeneity in the age at diagnosis and familial history between geographic regions across Belarus should be investigated in more details, probably by prospective studies of the blood relatives in the respective Byelorussian families who are carriers of a defined mutation. Some more precise estimates of the individual radiation dose could be helpful for the determination of risks possibly associated with gene-environmental interaction.

Altogether the results obtained in this study imply that virtually all breast cancer patients in Belarus are genetically predisposed and carry at least one of the identified high-, moderate- or low-penetrance breast cancer susceptibility alleles. It may be also effective for other Slavic populations to investigate the mutations described in the presented thesis.

At the present stage, the results of this study should provide an important and valuable basis for deeper research into the biological relevance of some of the genes, their impact on prognosis and mortality, possible gene-gene and gene-environment interactions, and their potential exploitation in the future prevention and therapy of breast cancer.

7. References

Abraham RT. (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases *Genes and Dev.* **15**: 2177-2196

Abraham RT and Tibbetts RS (2005) Guiding ATM to broken DNA. *Science* **308**: 510-511.
Ahn J, Urist M, Prives C. (2004) The Chk2 protein kinase. *DNA Repair* **3**:1039–1047.

Allen-Brady K, Cannon-Albright LA Neuhausen SL and Nicola J. Camp NJ. (2006) A Role for XRCC4 in Age at Diagnosis and Breast Cancer Risk *Cancer Epidemiol Biomarkers Prev* 15(7): 1306-1310.

Allinen M, Huusko P, Mäntyniemi S, Launonen V, Winqvist R. (2001) Mutation analysis of the CHK2 gene in families with hereditary breast cancer. *Br J Cancer.* **85(2)**: 209-212.

Alt JR, Bouska A, Fernandez MR et al. (2005) Mdm2 Binds to Nbs1 at Sites of DNA Damage and Regulates Double Strand Break Repair The Journal of Biological Chemistry **280(19)**: 18771–18781.

Anczukow O, Ware MD, Buisson M, Zetoune AB et al. (2008) Does the nonsense-mediated mRNA decay mechanism prevent the synthesis of truncated BRCA1, CHK2 and p53 proteins. *Human Mutation* **29(1)**: 65-73.

Andrieu N, Easton DF, Chang-Claude J, Rookus MA, Brohet R, Cardis E, Antoniou AC et al. (2006) Effect of chest X-rays on the risk of breast cancer among BRCA1/2 mutation carriers in the international BRCA1/2 carrier cohort study: a report from the EMBRACE, GENEPSO, GEO-HEBON, and IBCCS Collaborators Group. *J Clin Oncol.* **24(21)**: 3361-3366.

Anglian Breast Cancer Study Group. (2000) Prevalence and penetrance of BRCA1 and BRCA2 mutations in a population based series of breast cancer cases. *Br J Cancer* **83**: 3-18.

Antoniou A, Pharoah PDP, McMullan G, et al. (2002) A comprehensive model for familial breast cancer incorporating BRCA1, BRCA2 and other genes. *Br J Cancer* **86**: 76-83

Antoniou AC, Sinilnikova OM, Simard J, Léoné M, et al. (2007) RAD51 135G-->C modifies breast cancer risk among BRCA2 mutation carriers: results from a combined analysis of 19 studies. *Am. J Hum. Genet*. **81**: 1186–1200.

Athma P, Rappaport R, Swift M (1996) Molecular genotyping shows that ataxia-telangiectasia heterozygotes are predisposed to breast cancer. *Cancer Genetics and Cytogenetics* **92**: 130-134.

Backe J, Hofferbert S, Skawran B, Dörk T, et al. (1999) Frequency of the BRCA1 5382insC mutation in German breast cancer patients. *Gynecologic Oncology* **72**: 402-406.

Bakkenist CJ., Kastan MB (2004) Initiating Cellular Stress Responses. *Cell* **118**: 9-17.

Balmain A, Gray J, Ponder B. (2003) The genetics and genomics of cancer. *Nature Genetics* **33**: 238-244.

Bartek J, Falck J, Lukas J. (2001) CHK2 kinase: a busy messenger. *Nat Rev Mol Cell Biol* **2**:877-86.

Bartek J, Lukas J. (2003) Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* **3**:421-429.

Bartkova I, Horejski Z, Koed H et al. (2005) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* **343**: 865-870.

Bartsch H, Nair J, Owen RW. (1999) Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis* **20**: 2209-2218.

Bedinghaus J. M. (1997). Care of the breast and support of breast-feeding. *Primary Care* **24**: 147-160.

Bell DW, Varley JM, Szydlo TE, Kang DH et al. **(**1999a) Heterozygous germ line h*CHK2* mutations in Li-Fraumeni syndrome. *Science* **286:** 2528–2531.

Bell DW, Wahrer DC, Kang DH, MacMahon et al. (1999b) Common nonsense mutations in RAD52. *Cancer Research* **15**:3883-3888.

Berchuck A, Schildkraut JM, Marks JR and Futreal PA. (1999). Managing hereditary ovarian cancer risk. *Cancer* **86**: 2517-2524.

Bernstein L, Henderson BE, Hanisch R, Sullivan-Halley J, Ross RK. (1994) Physical exercise and reduced risk of breast cancer in young women. *J Natl Cancer Inst* **86**:1403-1408.

Bernstein JL, Teraoka S, Southey MC et al. (2006) Populationbased estimates of breast cancer risks associated with ATM gene variants c.7271T>G, and c.10666T>G (IVS106T>G) from the

Bhatia S, Robison LL, Oberlin O, Greenberg M, Bunin G, Fossati-Bellani F, Meadows AT (1996) Breast cancer and other second neoplasms after childhood Hodgkin´s disease. *New England Jounal of Medicine* **334**: 745-751

Bierie B, Moses HL. (2006) Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat. Rev. Cancer* **6(7)**: 506-520.

Bignell G, Micklem G, Stratton M R, Ashworth A and Wooster R. (1997) The BRC repeats are conserved in mammalian BRCA2 proteins. *Hum Mol. Genet*. **6**: 53-58.

Birrell GW, Kneebone K, Nefedov M, Nefedova E, Jartsev MN et al. (2005) ATM Mutations, haplotype analysis, and immunological status of Russian patients with ataxia telangiectasia. *Hum. Mutat.* **25(6)**: 593.

Black DM. (1994) The genetics of breast cancer. *Eur J Cancer* **13**: 1957-1961.

Bliziotis IA. (2008) Ovarian cancer and oral contraceptives. *Lancet* **371**: 1663-1663 I.

Bogdanova N, Enssen-Dubrowinskaja N, Feshchenko S, Lazjuk et al. (2005) Association of two mutations in the CHEK2 gene with breast cancer. *Int J Cancer* **116**: 263–266.

Bogdanova N, Feshchenko S, Cybulski C, Dörk T. (2007) CHEK2 mutation and hereditary breast cancer. *J Clin Oncol.* **25(19)**: e26.

Bogdanova N, Schürmann P, Waltes R, Feshchenko S, Zalutsky IV, Bremer M, Dörk T. (2007) NBS1 variant I171V and breast cancer risk. *Breast Cancer Res Treat.* Published online 2007 Nov 30. [Epub ahead of print].

Bogdanova N, Feshchenko S, Schürmann P, Waltes R, Wieland B, Hillemanns P, Rogov YI, Dammann O, Bremer M, Karstens JH, Sohn C, Varon R, Dörk T. (2008) Nijmegen Breakage Syndrome mutations and risk of breast cancer. *Int J Cancer.* **122(4)**: 802-806.

Bogliolo M, Lyakhovich A, Callen E, Castella M et al. (2007) Histone H2AX and Fanconi anemia FANCD2 function in the same pathway to maintain chromosome stability. *EMBO J* **26**:1340-1351.

Bohr VA, Anson RM (1995) DNA damage, mutation and fine structure DNA repair in aging. *Mutat Res* **338**: 25-34.

Bork P, Blomberg N and Nilges M. (1996) Internal repeats in the BRCA2 protein sequence. *Nat. Genet.* **13**: 22-23.

Borresen A-L, Andersen TI, Garber J, Barbier-Piraux N, Thorlacius S, Eyfjord J, Ottestad L, Smith-Sorensen B, Hovig E, Malkin D, Friend SH (1992) Screening for germ line TP53 mutations in breast cancer patients. *Cancer Research* **52**: 3234-3236.

Bouquet F, Muller C, Salles B. (2006) The loss of gH2AX signal is a marker of DNA double strand breaks repair only at low levels of dna damage. *Cell Cycle* **5**:1116-1122.

Boyd NF, Guo H, Martin LJ, Sun L, Stone J et al. (2007) Mammographic Density and the Risk and Detection of Breast Cancer *NEJM* **356**: 227-236.

Breast Cancer Association Consortium. (2006) Commonly studied singlenucleotide polymorphisms and breast cancer: Results from the Breast Cancer Association Consortium. *J Natl Cancer Inst* **98**:1382-1396.

Breast Cancer Family Registry. *Hum Mut* **27**: 1122-1128.

Bretsky P, Haiman CA, Gilad S, Yahalom J, Grossman A, Paglin S et al. (2003) The relationship between twenty missense *ATM* variants and breast cancer risk: the Multiethnic Cohort. Cancer *Epidemiol Biomarkers Prev* **12**: 733–738.

Brinton LA, Schaiere C, Hoover RN, et al. (1988) Menstrual factors and risk of breast cancer. *Cancer Invest* **6**:145–154.

Broeks A, Urbanus JHM, Floore AN, Dahler EC, Klijn JGM, Rutgers EJT, Devilee P, Russell NS, van Leeuwen FE, van´t Veer L (2000) ATM-heterozygous germline mutations contribute to breast cancer-susceptibility. *American Journal of Human Genetics* **66**: 494-500.

Broeks A, de Witte L, Nooijen A, Huseinovich A et al. (2004) Excess risk for contralateral breast cancer in CHEK2*1100delC germline mutation carriers. *Breast Cancer Res. and Treat.* **83**: 91-93.

Broeks A, Braaf LM, Huseinovic A, Nooijen A, Urbanus J, Hogervorst FB, Schmidt MK et al. (2007) Identification of women with an increased risk of developing radiation-induced breast cancer: a case only study. *Breast Cancer Res.* **9**: R26.

Brumbaugh K, Otterness D, Geisen C, Oliveira V et al. (2004) The mRNA Surveillance Protein hSMG-1 Functions in Genotoxic Stress Response Pathways in Mammalian Cells. *Molecular Cell* **14(5)**: 585-598.

Buchholz TA, Weil MM, Ashorn CL, Strom EA, Sigurdson A, et al. (2004) A Ser49Cys variant in the ataxia telangiectasia, mutated, gene that is more common in patients with breast carcinoma compared with population controls. *Cancer* **100**: 1345–1351.

Burke W, Press N, Pinsky L (1999) BRCA1 and BRCA2: a small part of the puzzle. *Journal of the National Cancer Institute* **91**:904-905.

Burke W, Austin AM. (2002) Risk in Context: Calculating the Penetrance of BRCA1 and BRCA2 Mutations. *Journal of the National Cancer Institute* **16**: 1185-1187.

Buscemi G, Carlessi L, Zannini L, Lisanti S et al. (2006) DNA damage-induced cell cycle regulation and function of novel Chk2 phosphoresidues. *Molecular and Cellular Biology* **26(21)**: 7832-7845.

Buslov KB, Iyevleva AG, Chekmariova EV, Suspitsin EN, Togo AV et al. (2005) NBS1 657del5 mutation may contribute only to a limited fraction of breast cancer cases in Russia. *Int J Cancer* **114**:585–589.

Cardis E, Hall J and Tavtigian SV. (2007) Identification of women with an increased risk of developing radiation-induced breast cancer. *Breast Cancer Research* **9**:106.

Carlomagno F, Chang-Claude J, Dunning AM, Ponder BA. (1999) Determination of the frequency of the common 657Del5 Nijmegen breakage syndrome mutation in the German population: no association with risk of breast cancer. *Genes Chromosomes Cancer.* **25(4)**: 393-395.

Carney JP, Maser RS, Olivares H, Davis EM, Le Beau M, Yates III. JR, Hays L, Morgan WF, Petrini JHJ (1998) The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell* **93**: 477-486.

Caspari T (2000) How to activate p53. *Curr. Biol.* **10**: R315-7.

Cerhan JR. (2006) Oral contraceptive use and breast cancer risk: Current status. *Mayo Clinic Proceedings* **81(10)**:1287–1289.

CHEK2–Breast Cancer Case-Control Consortium (2004) CHEK2*1100delC and susceptibility to breast cancer: a collaborative analysis involving 10,860 breast cancer cases and 9,065 controls from 10 studies. *Am J Hum Genet* **74**:1175–1182.

Chen C, Seth AK, Aplin AE. (2006) Genetic and expression aberrations of E3 ubiquitin ligases in human breast cancer. *Mol Cancer Res* **4**: 695–707.

Chenevix-Trench G, Spurdle AB, Gatei M et al. (2002) Dominant negative ATM mutations in breast cancer families. *J Natl Cancer Inst* **94**: 205-215.

Cheng TC, Cen DT, Huang CS, Fu YP et al. (2005) Breast cancer risk, associated with genotype polymorphisms of the catechol estrogen-metabolizing genes: a multigenic study on cancer susceptibility, *Int. J. Cancer* **113**: 345-353.

Chien, A, Edgar, DB and Trela, JM. (1976) Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus. *J. Bacteriol*. **127**:1550–1557.

Chin S-F, Vias M, Naderi A, Iyer N, Roberts I, Kranjac T, Pinder S et al. (2004) Profiling breast cancers by array-CGH *Proc Amer Assoc Cancer Res* **45(1)**: 235.

Chiu CF, Wang HC, Wang CH, Wang CL, Lin CC, Shen CY, Chiang SY, Bau DT. (2008) A new single nucleotide polymorphism in XRCC4 gene is associated with breast cancer susceptibility in Taiwanese patients. *Anticancer Res*. 28(1A): 267-270.

Chomczynski P and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**: 156-159.

Ciccia A, Lig Ch, Coulthhard R, Yan Zh et al. (2007) Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. *Mol. Cell* **25**: 331–343.

Claus EB, Risch N., Thompson WD. (1991) Genetic analysis of breast cancer in the cancer and steroid hormone study. *Am J Hum Genet* **48**: 232-42.

Claus EB, Schildkraut JM, Thompson WD, Risch NJ. (1996) The genetic attributable risk of breast and ovarian cancer. *Cancer* **77**: 18-24.

Collaborative Group on Hormonal Factors in Breast Cancer. (1996) Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53297 women with breast cancer and 100239 women without breast cancer from 54 epidemiologic studies. *Lancet* **347**:1713–1727.

Collaborative Group on Hormonal Factors in Breast Cancer. (1997) Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52 705 women with breast cancer and 108 411 women without breast cancer. *Lancet* **350**: 1047-1059.

Collaborative Group on Hormonal Factors in Breast Cancer (2002) Breast cancer and breastfeeding: collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50302 women with breast cancer and 96973 women without the disease. *Lancet* **360**:187–195.

Collaborative group on epidemiological Studies of Ovarian Cancer. (2008) Ovarian cancer and oral contraceptives: collaborative reanalysis of data from 45 epidemiological studies including 23257 women with ovarian cancer and 87303 controls. *Lancet* **371**: 303-314.

Cox R (1994) Molecular mechanisms of radiation oncogenesis. *International Journal of Radiation Biology* **65**: 57-64

Cox A, Dunning AM, Garcia-Closas M, Balasubramanian S, Reed MW, Pooley KA, Scollen S, Baynes C, Ponder BA, Chanock S, Lissowska J, Brinton L, Peplonska B, Southey MC et al. (2007) A common coding variant in CASP8 is associated with breast cancer risk. *Nat Genet.* **39(3)**: 352-358.

Cui Y, Shu XO, Cai Q, et al. (2005) Association of breast cancer risk with a common functional polymorphism (Asp327Asn) in the sex hormone binding globulin gene. *Cancer Epidemiol Biomrkers Prev* **14**: 1096-1101.

Cybulski C, Gorski B, Huzarski T, Masojc B, Mierzejewski M, Debniak T et al. (2004). CHEK2 is a multiorgan cancer susceptibility gene. *Am J Hum Genet* 75:1131-1135.

Cybulski C, Wokolorczyk D, Huzarski T, Byrski T et al. (2006) A deletion in *CHEK2* of 5,395 bp predisposes to breast cancer in Poland. *Breast Cancer Res Treat*: 119-122.

Cybulski C, Wokolorczyk D, Huzarski T, Byrski T, Gronwald J, Gorski B et al. (2007) A deletion in CHEK2 of 5,395 bp predisposes to breast cancer in Poland. *Breast Cancer Res Treat* 102: 119-122.

Daniel DC. (2002) Highlight: BRCA1 and BRCA2 proteins in breast cancer. *Microscopy research and technique* **59**: 68-83.

Davidson JF, Guo HH and Loeb LA. (2002) Endogenous mutagenesis and cancer. *Mutat. Res*. **509**: 17-21.

de Bock GH, Schutte M, Krol-Warmerdam EM, Seynaeve C, Blom J, Brekelmans CT et al. (2004) Tumour characteristics and prognosis of breast cancer patients carrying the germline CHEK2*1100delC variant. *J Med Genet* 41: 731-735.

de Jong MM, Nolte IM, te Meerman GJ et al. (2002) Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility. *Journal of Medical Genetics* **39**: 225-242.

de Winter JP, Waisfisz Q, Rooimans MA et al. (1998) The Fanconi anaemia group G gene FANCG is identical with XRCC9. *Nature Genetics* **20**: 281-283.

DePincho RA, Polyak K. (2004) Cancer chromosomes in crisis. *Nat. Genet*. **36**: 932-934.

Derynck R, Akhurst RJ, Balmain A. (2001) TGF-b signaling in tumor suppression and cancer progression. *Nature Genetics* **29**:117-129.

di Masi A, Viganotti M, Ponticelli F, Ascenzi P, Tanzarella C, Antoccia A. (2008) The R215W mutation in NBS1 impairs c-H2AX binding and affects DNA repair: Molecular bases for the severe phenotype of 657del5/R215W Nijmegen breakage syndrome patients. *Biochem. Biophys. Res. Commun*. doi:10.1016/j.bbrc.2008.02.129.

Digweed M, Sperling K (2004) Nijmegen breakage syndrome: clinical manifestation of defective response to DNA double-strand breaks. *DNA Repair* **3(8-9)**: 1207-1217.

DiTullio RA Jr, Mochan TA, Venere M, Bartkova J et al. (2002) 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nature Cell Biology* **4**: 998–1002.

Dong X, Wang L, Taniguchi K, Wang X, Cunningham JM, McDonnell SK et al. (2003) Mutations in CHEK2 associated with prostate cancer risk. *Am J Hum Genet* 72:270–280.

Dörk T, Bendix R, Bremer M, Rades D et al. (2001). Spectrum of ATM gene mutations in a hospital-based series of unselected breast cancer patients. *Cancer Research* **61**: 7608-7615.
Dufault MR, Betz B, Wappenschmidt B, Hofmann W, Bandick K, Golla A, Pietschmann A, Nestle-Krämling C, Rhiem K et al. (2004) Limited relevance of the CHEK2 gene in hereditary breast cancer. *Int J Cancer*. **110(3)**: 320-325.

Dunning AM, Healey CS, Pharoah PD, Teare MD et al. (1999) A systematic review of genetic polymorphisms and breast cancer risk. *Cancer Epidemiology, Biomarkers and Prevention* **8**: 843-854.

Dunning AM, Ellis PD, McBride S, et al. (2003) A transforming growth factorß1 signal peptide variant increases secretion in vitro and is associated with increased incidence of invasive breast cancer. *Cancer Res* **63**: 2610-2615.

Eakin CM, Maccoss MJ, Finney GL, Klevit RE. (2007) Estrogen receptor alpha is a putative substrate for the BRCA1 ubiquitin ligase. *Proc Natl Acad Sci U S A.* **104(14)**: 5794-5799.

Easton DF. (1994) Cancer risks in AT heterozygotes. *Int J Radiat Biol* **66(6Suppl)**: S177-S182.

Easton DF, Pooley KA, Dunning AM, Pharoah PDP, Thompson D, Ballinger DG et al. (2007) A genome-wide association study identifies novel breast cancer susceptibility loci. *Nature* **447**: 1087-1093.

Ebi H, Matsuo K, Sugito N, Suzuki M, Osada H, Tajima K, Ueda R, Takahashi T. (2007) Novel NBS1 heterozygous germ line mutation causing MRE11-binding domain loss predisposes to common types of cancer. *Cancer Res.* **67(23)**:11158-11165.

Eeles RA, Stratton MR, Goldgar DE, Easton DF. (1994) The genetics of familial breast cancer and their practical implications. *Eur J Cancer* **9**: 1383-1390.

Egan KM, Cai Q, Shu XO, et al. (2004) Genetic polymorphisms in GSTM1, GSTP1, and GSTT1 and the risk for breast cancer: results from the Shanghai Breast Cancer Study and metaanalysis. *Cancer Epidemiol Biomarkers Prev* **13**: 197-204.

Einarsdottir K, Rudqvist RT, Humphreys K, et al. (2005) CYP17 polymorphism in relation to breast cancer risk: a case control study. *Breast Cancer Res* **7**:R890-R896.

El-Ashry D, Lippman ME. (1994) Molecular biology of breast carcinoma. *World J Surg* **18**: 12-20.

Electronic database information: www.nijmegenbreakagesyndrome.net
www.who.int/en

Erkko H, Xia B, Nikkila J, Schleutker J,Syrjakoski K, Mannermaa A et al. (2007) A recurrent mutation in PALB2 in Finnish cancer families. *Nature* **446**: 316–319.

Ewertz M, Duffy SW, Adami HO et al. (1990) Age at parity and risk of breast cancer: a meta-analysis of 8 studies from the Nordic countries. *Int J Cancer* **46**:597-603.

Falck J, Mailand N, Syljuåsen RG, Bartek J, Lukas J. (2001) The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature* **410**: 842-847.

Fan S, Wang JA, Yuan R, Ma Y, Meng Q, et al. (1999) BRCA1 inhibition of estrogen receptor signaling in transfected cells. *Science* **284**: 1354-1356.

Figueroa JD, Malats N, Rothman N, Real FX et al. (2007) Evaluation of genetic variation in the double-strand break repair pathway and bladder cancer risk. *Carcinogenesis* 28(8):1788-1793.

Fillingham J, Keogh MC, Krogan NJ. (2006) Gamma H2AX and its role in DNA double-strand break repair. *Biochem Cell Biol* **84**:568-577.

FitzGerald MG, Bean JM, Hedge SR, et al. (1997) Heterozygous ATM mutations do not contribute to early onset of breast cancer. *Nat Genet* **15**: 207-210.

Foray N, Marot D, Randrianarison V, Venezia ND, et al. (2002) Constitutive association of BRCA1 and c-Abl and its ATM-dependent disruption after irradiation. *Mol Cell Biol* **22**: 4020–4032.

Forsti A, Angelini S, Festa F et al (2004) Single nucleotide polymorphisms in breast cancer. *Oncol Rep* **11**:917–922.

Fu Y-P, Yu J-Ch, Cheng T-Ch, Lou MA et al. (2003) Breast Cancer Risk Associated with Genotypic Polymorphism of the Nonhomologous End-Joining Genes: A Multigenic Study on Cancer Susceptibility. *Cancer Research* **63**: 2440–2446.

Ganesan S, Ganesan S, Silver DP, Greenberg RA et al. (2002) BRCA1 supports XIST RNA concentration on the inactive X chromosome. *Cell* **111**: 393-405.

Garcia-Closas M, Hall P, Nevanlinna H, Pooley K, Morrison J, Richesson DA, Bojesen SE, Nordestgaard BG, Axelsson CK, Arias JI, Milne RL, Ribas G et al. (2008) Heterogeneity of breast cancer associations with five susceptibility Loci by clinical and pathological characteristics. *PLoS Genet.* **4(4)**: e1000054.

Garfinkel L, Boffetta P, Stellman SD. (1988) Alcohol and breast cancer: a cohort study. *Prev Med* **17**:686-693.

Gartel L, Radhakrishnan SK. (2005) Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Research* **65**: 3980-3985.

Gatei M, Young D, Cerosaletti KM, Desai-Mehta A, Spring K, Kozlov S, Lavin MF, Gatti RA, Concannon P, Khanna KK (2000) ATM-dependent phosphorylation of nibrin in response to radiation exposure. *Nature Genetics* **25**: 115-119.

Gatti RA, Boder E, Vinters HV, Sparks RS, Norman A, Lange K (1991) Ataxia-telangiectasia: An interdisciplinary approach to pathogenesis. *Medicine* **70**:99-117.

Gatti RA, Tward A, Concannon P. (1999) Cancer risk in ATM heterozygotes: a model of phenotypic and mechanistic differences between missense and truncating mutations. *Mol Genet metab* **68**: 419-423.

Gatz SA, Wiesmüller L. (2006) p53 in recombination and repair. *Cell Death Differ* **13**: 1003-1016.

Giguere Y, Dewailly E, Brisson J, Ayotte P et al. (2001) Short polyglutamine tracts in the androgen receptor are protective against breast cancer in the general population. *Cancer Research* **61**: 5869-5874.

Giordano, Sharon H; Cohen DS, Buzdar AU, Perkins G, Hortobagyi GN (2004). Breast carcinoma in men. *Cancer* **101(1)**: 51-57.

Gold B, Kalush F, Bergeron J, et al. (2004) Estrogen receptor genotypes and haplotypes associated with breast cancer risk. *Cancer Res* **64**:889-900.

Gold B, Kirchhoff T, Stefanov S, Lautenberger J, Viale A, Garber J, Friedman E, Narod S et al. (2008) Genome-wide association study provides evidence for a breast cancer risk locus at 6q22.33. *PNAS* **105(11)**: 4340-4345.

Goode EL, Dunning AM, Kuschel B, Healey CS et al. (2002) Effect of germ-line genetic variation on breast cancer survival in a population-based study. *Cancer Research* **62**: 3052-3057.

Goode EL, Ulrich CM, Potter JD. (2002) Polymorphisms in DNA repair genes and associations with cancer risk. *Cancer Epidemiol Biomarkers Prev.* **11(12)**: 1513-1530.

Gorski B, Byrski T, Huzarski T, Jakubowska A et al. (2000) Founder mutations in the BRCA1 gene in Polish families with breast-ovarian cancer. *Am J Hum Genet*. 66(6): 1963-1968.

Gorski B, Debniak T, Masojc B, Mierzejewski M et al. (2003) Germline 657del5 mutation in the NBS1 gene in breast cancer patients. *Int J Cancer* **106**: 379-381.

Gorski B, Cybulski C, Huzarski T, Byrski T, Gronwald J, Jakubowska A et al. (2005a) Breast cancer predisposing alleles in Poland. *Breast Cancer Res Treat* **92**:19–24.

Gorski B, Narod SA and Lubinski J. (2005b) A common missense variant in BRCA2 predisposed to early onset breast cancer. *Breast Cancer Res.*7: 1023-1027.

Goss PE, Sierra S (1998) Current perspectives on radiation-induced breast cancer. *Journal of Clinical Oncology* **16**: 338-347

Green Brody J, Rudel RA, Michels KB et al. (2007) Environmental pollutants, diet, physical activity, body size, and breast cancer. *Cancer* **109**: 26-27.

Gronwald J, Elsakov P, Gorski B et al. (2005) High incidence of 4153delA BRCA1 gene mutations in Lithuanian breast and breast-ovarian cancer families. *Breast Cancer Res Treat* 94: 111–113.

Gutiérrez-Enríquez S, Fernet M, Dörk T, Bremer M, Lauge A, Stoppa-Lyonnet D, Moullan N, Angèle S, Hall J. (2004) Functional consequences of ATM sequence variants for chromosomal radiosensitivity. *Genes Chromosomes Cancer.* **40(2)**: 109-119.

Haber JE. (2000) Partners and pathways repairing a double-strand break. *Trends Genet.* **16**: 259–264.

Hall J, Lee M, Newman B, Morrow J et al. (1990) Linkage of early onset familial breast cancer to chromosome 17q21. *Science* **250**: 1684-1689.

Hall J, Angele S (1999) Radiation, DNA damage and cancer. *Molecular Medicine Today* **5**: 157-164.

Healey CS, Dunning AM, Teare MD, Chase D et al. (2000) A common variant in BRCA2 associated with both breast cancer risk and prenatal viability. *Nature Genetics* 26: 362-364.

Heck KE, Pamuk ER. (1997) Explaining the relation between education and postmenopausal breast cancer. *Am J Epidemiol* **145**:366-372.

Heikkinen K, Mansikka V, Karppinen SM, Rapakko K, Winqvist R. (2005) Mutation analysis of the ATR gene in breast and ovarian cancer families. *Breast Cancer Res.* **7(4)**: R495-501.

Heikkinen K, Rapakko K, Karppinen S-M et al. (2006) RAD50 and NBS1 are breast cancer susceptibility genes associated with genomic instability. *Carcinogenesis* **27**: 1593–1599.

Henning W, Stürzbecher H-W. (2003) Homologous recombination and cell cycle checkpoints: Rad51 in tumour progression and therapy resistance *Toxicology* **193(1-2)**:91-109.

Hildreth NG, Shore RE, Dvoretsky PM (1989) The risk of breast cancer after irradiation of the thymus in infancy. *New England Journal of Medicine* **321**: 1281-1284

Hirao A. Kong YY, Matsuoka S, Wakeham A et al. (2000) DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* **287:**1824–1827.

Hoeijmakers JH. (2001) Genome maintenance mechanisms for preventing cancer. *Nature* **411**:366-374.

Howlett NG, Taniguchi T, Olson S, Cox B et al. (2002) Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* **297**: 606-609

Hrubec Z, Boice JD, Monson RR, Rosenstein M (1989) Breast cancer after multiple chest fluoroscopies: second follow-up of Massachusetts women with tuberculosis. *Cancer Research* **49**: 229-234

Hughes-Davies L, Huntsman D, Ruas M et al. (2003) EMSY Links the BRCA2 Pathway to Sporadic Breast and Ovarian Cancer. *Cell* **115**: 523–535.

Hunter DJ, Kraft P, Jacobs KB, Cox DG, Yeager M, Hankinson SE, et al. (2007) A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. *Nat Genet* **39(7)**:870-874.

Inskip HM, Kinlen LJ, Taylor AMR, Arlett CF (1999) Risk of breast cancer and other cancers in heterozygotes for ataxia-telangiectasia. *British Journal of Cancer* **79**: 1304-1307.

Jackson SP. (2002) Sensing and repairing DNA double-strand breaks. *Cancerogenesis* **23**: 687-696.

Jakubowska A, Cybulski C, Szymańska A, Huzarski T, Byrski T, et al. (2008) BARD1 and breast cancer in Poland. *Breast Cancer Res Treat.* **107(1)**: 119-122.

Jang J, Xu ZhP, Huang Y, Hamrich HE et al. (2004) ATM and ATR: Sensing DNA damage. *World J Gastroenterol* **10(2)**: 155-160.

Jasin M. (2002) Homologous repair of DNA damage and tumorigenesis: the BRCA connection. *Oncogene* **21**: 8981-8993.

Jazayeri A, Falck J, Lukas C, Bartek J, Smith GC, Lukas J and Jackson SP. (2006) ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat. Cell Biol.* **8**: 37–45.

Jekimovs CR; Chen X, Arnold J, Gatei M, et al. (2005) Low frequency of CHEK2 1100delC allele in Australian multiple-case breast cancer families: functional analysis in heterozygous individuals. *British J of Cancer* **92**: 784-790.

Joenje H, Patel KJ. (2001) The emerging genetic and molecular basis of fanconi anaemia. *Nature Rev.Genet.* **2**: 446–459.

Johnson N, Fletcher O, Naceur-Lombardelli C et al. (2005) Interaction between CHEK2*1100delC and other low-penetrance breast-cancer susceptibility genes: a family study. *Lancet* **366**: 1554-1557.

Johnson N, Fletcher O, Palles C, Rudd M, et al. (2007) Counting potentially functional variants in BRCA1, BRCA2 and ATM predict breast cancer susceptibility. *Hum. Mol. Genet.* 16: 1051-1057.

Kahlenborn C, Mudugino F, Potter DM, Severs WB. (2006) Oral contraceptive use as a risk factor for premenopausal breast cancer: a meta-analysis. *Mayo Clinic Proceedings* **81(10)**: 290–302.

Karppinen S-M, Heikkinen K, Rapakko K, Winqvist R. (2006) Mutation screening of the *BARD1* gene: evidence for involvement of the Cys557Ser allele in hereditary susceptibility to breast cancer. *Journal of Medical Genetics* **41**: e114.

Kastan MB and Lim DS (2000) The many substrates and functions of ATM. *Nat Rev Mol Cell Biol* **1**: 179-186.

Kelsey JL, Gammon MD, John EM. (1993) Reproductive factors and breast cancer. *Epidemiol Rev* **15**: 36-47.

Kilpivaara O, Vahteristo P, Falck J, Syrjakoski K, Eerola H, Easton D et al. (2004) CHEK2 variant I157T may be associated with increased breast cancer risk. *Int J Cancer* 111: 543-547.

Kilpivaara O, Alhopuro P, Vahteristo P, Aaltonen LA, Nevanlinna H. (2006) CHEK2 I157T associates with familial and sporadic colorectal cancer. *J Med Genet.* **43(7)**: e34.

Kim MK, Zitzmann S, Westermann F et al. (2004) Increased rates of spontaneous sister chromatid exchange in lymphocytes of BRCA2+/- carriers of familial breast cancer clusters. *Cancer Lett.***210**: 85-94.

Kirshner J, Jobling MF, Pajares MJ, Ravani SA, Glick A, Lavin M, Koslov S, Shiloh Y, Barcellos-Hoff MH. (2006) Inhibition of TGFb1 signaling attenuates ATM activity in response to genotoxic stress. *Cancer Research 66*:10861-10868.

Kleiman FE, Manley JL (2001) The BARD1-CstF50 interaction links mRNA 3′-end formation to DNA damage and tumor suppression. *Cell* **104**: 743-753.

Kobayashi J, Antoccia A, Tauchi H et al. (2005) NBS1 and its functional role in the DNA damage response.*DNA Repair (Amst.)* **3**: 855-861.

Kraakman-van der Zweet M, Overkamp WJ, van Lange RE et al. (2002) Brca2 (XRCC11) deficiency results in radioresistant DNA synthesis and a higher frequency of spontaneous deletions. *Molecular and Cellular Biology* **22(2)**: 669-679.

Kristensen VN, Borresen-Dale AL (2000) Molecular epidemiology of breast cancer: genetic variation in steroid hormone metabolism. *Mutation Research* **462**: 323-333.

Krontiris DG, Devlin B, Karp DD, Robert NJ, Risch N (1993) An association between the risk of cancer and mutations in the HRAS1 minisatellite locus. *New England Journal of Medicine* **329**: 517-523.

Krylova NYu, Lobeiko OS, Sokolenko AP, Iyevleva AG, Rozanov ME, Mitiushkina NV et al. (2006) BRCA1 4153delA founder mutation in Russian ovarian cancer patients. *Hereditary Cancer in Clinical Practice* **4(4)**: 193-196.

Kuschel B, Auranen A, McBride S et al (2002) Variants in DNA double-strand break repair genes and breast cancer susceptibility. *Hum Mol Genet* **11**:1399–1407.

La Pinta M, Fabi A, Ascarelli A, Ponzani T, Di Carlo V, Scicchitano F, Saputo S, Russillo M, Andrich R. (2008) Male breast cancer: 6-year experience. *Minerva Chir*. **63(2)**:71-78.

Lacroix M (2006). p53 and breast cancer, an update. *Endocr Relat Cancer* **13(2)**: 293-325.

Lavigne JA, Goodman JE, Fonong T, Odwin S et al. (2001) The Effects of Catechol-*O*-Methyltransferase Inhibition on Estrogen Metabolite and Oxidative DNA Damage Levels in Estradiol-treated MCF-7 Cells. *Cancer Research* 61: 7488–7494.

Layde PM, Webster LA, Baughman AL, Wingo PA, Rubin GL, Ory HW. The independent associations of parity, age at first full term pregnancy, and duration of breastfeeding with the risk of breast cancer. (1989) Cancer and Steroid Hormone Study Group. *J Clin Epidemiol* **42**: 963-973.

Lee KM, Choi JY, Park SK, et al. (2005) Genetic polymorphisms of ataxia telangiectasia mutated and breast cancer. *Cancer Epidemiol Biomarkers Prev* **14**: 821-825.

Lehmann AR (1998) Dual functions of DNA repair genes: molecular, cellular, and clinical implications. *Bioessays* **20**: 146-55.

Levran O, Attwooll C, Henry RT et al. (2005) The BRCA1interacting helicase BRIP1 is deficient in Fanconi anemia. *Nat Genet* **37**: 931-933.

Levy-Lahad E and Friedman E. (2007) Cancer risks among BRCA1 and BRCA2 mutation carriers *British Journal of Cancer* **96**: 11-15.

Li Ch-I.;Malone KE; Porter PL; Weiss NS; Tang M-T et al. (2003) Relationship between long durations and different regimens of hormone therapy and risk of breast cancer.*Obstetrical and Gynecological Survey* **58(11)**: 733-735.

Li J, Williams BL, Haire LF, Goldberg M, Wilker E, Durocher D et al. (2002) Structural and functional versatility of the FHA domain in DNA-damage signaling by the tumor suppressor kinase Chk2. *Mol Cell* 9:1045–1054.

Li J, Stern DF. (2005) Regulation of CHEK2 by DNAdependent protein kinase. *J Biol Chem* **280**: 12041-12050.

Liehr JG. (2001) Genotoxicity of the steroidal estrogens estrone and estradiol: possible mechanism of uterine and mammary cancer development. *Hum. Reprod, Update* **7**: 273-281.

Lim DS, Kim ST, Xu B, Maser RS, Lin J, Petrini JH, Kastan MB (2000) ATM phosphorylates p95/nbs 1 in an S-phase checkpoint pathway. *Nature* **404**: 613-617.

Ling C Ishiai M, Ali AM, Medhurst AL et al. (2007) FAAP100 is essential for activation of the Fanconi anemia-associated DNA damage response pathway. *EMBO J.* **26**: 2104–2114.

Lord Ch. and Ashworth A. (2007) RAD51, BRCA2 and DNA repair: a partial resolution. *Nature Structural and Molecular Biology* **14**: 461-462.

Lou Zh, Christiano C, Chini S, Minter-Dykhouse K and Chen J (2003). Mediator of DNA damage Checkpoint Protein 1 regulates BRCA1 localization and phosphorylation in DNA damage Checkpoint Control. *The Journal of Biological Chemistry* **278(16)**: 13599–13602.

Lou Zh, Minter-Dykhouse K, Wu X and Chen J. (2003a) MDC1 is coupled to activated CHK2 in mammalian DANN damage response pathways. *Nature* **421**: 957-961(a).

Lu H, Guo X, Meng X et al. (2005) The BRCA2-Interacting Protein BCCIP Functions in RAD51 and BRCA2 Focus Formation and Homologous Recombinational Repair *Mol. and Cell. Biol.*: 1949–1957.

Lu M and Arrick BA. (2000) Transactivation of the p21 promoter by BRCA1 splice variants in mammary epithelial cells: evidence for both common and distinct activities of wildtype and mutant forms. *Oncogene* **19**: 6351-6360.

Lukas K, Melander F, Stucki M, Falck J et al. (2004) Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention *The EMBO Journal* **23**: 2674–2683.

Lunn RM, Langlois RG, Hsieh LL, Thompson CL, Bell DA (1999) XRCC1 polymorphisms: Effects on aflatoxin B1-DNA adducts and glycophorin A variant frequency. *Cancer Research* **59**: 2557-2561.

MacMahon B, Trichopoulos D, Brown J, et al. (1982) Age at menarche, urine estrogens and breast cancer risk. *Int J Cancer* **30**:427-431.

Mallery DL, Vandenberg CJ, Hiom K (2002) Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *EMBO Journal* **21**: 6755-6762.

Mani A, Gelmann EP. (2005) The ubiquitin-proteasome pathway and its role in cancer. *J Clin Oncol* **23**: 4776–4789.

Maniatis, T, Fritsch EF, Sambrook J. (1982) Molecular Cloning (A Laboratory Manual. *Cold Spring Harbor Laboratory, Cold Spring Harbor*. New York.

Marchbanks PA, McDonald JA, Wilson HG, Folger SG, Mandel MG, Daling JR, et al. (2002) Oral contraceptives and the risk of breast cancer. *N Engl J Med* **346**: 2025–2032.

Marmorstein LY, Kinev AV, Chan GK, Daniel A. et al. (2001). A human BRCA2 complex containing a structural DNA binding component influences cell cycle progression. *Cell* **104 (2)**: 247-257.

Martin A-M, Weber BL (2000) Genetic and hormonal risk factors in breast cancer. *Journal of the National Cancer Institute* **92**: 1126-1135.

Martin LJ and Boyd NF. (2008) Mammographic density. Potential mechanisms of breast cancer risk associated with mammographic density: hypotheses based on epidemiological evidence. *Breast Cancer Res*. **10(1)**: 201.

Matsuoka S, M. Huang M and Elledge SJ. (1998) Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science* **282**:1893–1897.

Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge S. (2000) Ataxiatelangiectasia mutated phosphorylates Chk2 in vivo and in vitro. *Proc Natl Acad Sci* **97**:10389-10394.

McPherson JP, Lemmers B, Hirao A, Hakem A, Abraham J, et al. (2004) Collaboration of Brca1 and Chk2 in tumorigenesis. *Genes Dev* **18**:1144–1153.

Meetei AR, Sechi S, Wallisch M et al. (2003) A multiprotein nuclear complex connects Fanconi anemia and bloom syndrome. *Mol. Cell. Biol.* **23**: 3417–3426.

Meijers-Heijboer H, van den Ouweland A, Klijn J et al. (2002) Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat Genet* **31(1)**:55-59.

Meijers-Heijboer H, Wijnen J, Vasen H, Wasielewski M, Wagner A et al. (2003) The CHEK2 1100delC mutation identifies families with a hereditary breast and colorectal cancer phenotype. *Am J Hum Genet* **72**:1308-1314.

Menendez JA, Lupu R (2005) RNA interference-mediated silencing of the p53 tumorsuppressor protein drastically increases apoptosis after inhibition of endogenous fatty acid metabolism in breast cancer cells. *Int J Mol Med* **15**:33–40.

Meyer A, Dörk T, Sohn C, Karstens JH, Bremer M. (2007) Breast cancer in patients carrying a germ-line *CHEK2* mutation: outcome after breast conserving surgery and adjuvant radiotherapy. *Radiother Oncol* **82**: 349-353.

Meyer KB, Maia A-T, O'Reilly M et al. (2008) Allele-Specific Up-Regulation of FGFR2 Increases Susceptibility to Breast Cancer. *PLoS Biology* **6(5)**: e108.

Miki Y, Swensen J, Shattuck-Eidens D et al. (1994) A strong candidate for the breast ovarian cancer susceptibility gene BRCA1. *Science* **266***:* 66-71.

Mikkelsen, R.B. and Wardman, P., 2003, Biological chemistry of reactive oxygen and nitrogen and ionizing radiation-induced signal transduction mechanisms. Oncogene **22**: 5734-5754.

Millikan RC, Player JS, Decotret AR et al (2005) Polymorphisms in DNArepair genes,medical exposure to ionizing radiation, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **14**:2326–2334.

Mitrunen K, Jourenkova N, Kataja V, et al. (2000) Steroid metabolism gene CYP17 polymorphism and the development of breast cancer. *Cancer Epidemiol Biomarkers Prev* **9**:1343-1348.

Mohrenweiser HW, Jones IM (1998) Variation in DNA repair is a factor in cancer susceptibility: a paradigm for the promises and perils of individual and population risk estimation? *Mutat Res* **400**: 15-24.

Momand J, Wu HH and Dasgupta G. (2000). MDM2--master regulator of the p53 tumor suppressor protein. *Gene* **242**: 15-29.

Morgan WF, Day JP, Kaplan MI, McGhee EM, Limoli CL. (1996) Genomic instability induced by ionizing radiation. *Radiat Res.* **146(3)**: 247-258.

Morimoto LM, White E, Chen Z, Chlebowski RT, Hays J, Kuller L, et al. (2002) Obesity, body size, and risk of postmenopausal breast cancer: the Women's Health Initiative (United States). *Cancer Causes Control* **13(8)**:741–751.

Mosor M, Ziotkowska I, Pernak-Schwarz M et al. (2006) Association of the heterozygous germline I171V mutation of NBS1 gene with childhood acute lymphoblastic leukemia. *Leukemia*, **20**: 1454-1456.

Mullis KB. (1990) Target amplification for DNA analysis by the polymerase chain reaction. *Ann Biol Clin (Paris)*. **48(8)**:579-582.

Nathanson KL, Weber BL (2001) Other breast cancer susceptibility genes: searching for more holy grail. *Human Molecular Genetics* **10**: 715-720.

Nathanson KL, Wooster R, Weber BL (2001) Breast cancer genetics: what we know and what we need. *Nature Medicine* **7(5)**:552-556.

Neubauer S, Arutyunyan R, Stumm M, Dörk T, Bendix R, Bremer M, Varon R, Sauer R and Gebhart E. (2002) Radiosensitivity of Ataxia Telangiectasia and Nijmegen Breakage Syndrome homozygotes and heterozygotes as determined by three-color FISH chromosome painting. *Radiation Research* **157(3)**: 312-321.

Nevanlinna H, Bartek J. (2006) The CHEK2 gene and inherited breast cancer susceptibility. *Oncogene* **25**: 5912-5919.

Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, and Markham AF. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* **17**: 2503–2516.

Nicoletto MO, Donach M, De Nicolo A, Artioli G, Banna G, Monfardini S. (2001) BRCA-1 and BRCA-2 mutations as prognostic factors in clinical practice and genetic counselling. *Cancer Treat Rev.* **27(5)**: 295-304.

Nowak J, Mosor M, Ziotkowska I, Wierbicka M et al. (2008) Heterozygous carriers of the I171V mutation of the NBS1 gene have a significantly increased risk of solid malignant tumours. *European J of Cancer* **44**: 627-630.

Olayioye MA (2001). Update on HER-2 as a target for cancer therapy: intracellular signaling pathways of ErbB2/HER-2 and family members. *Breast Cancer Res* **3(6)**: 385-389.

Olsen JH, Hahnemann MJ, Borresen-Dale A-L, Brondum-Nielsen K, Hammarström L, Kleinerman R, Kääriäinen H et al. (2001) Cancer in patients with ataxia-telangiectasia and in their relatives in the Nordic countries. *Journal of the National Cancer Institute* **93**: 121-127.

Oszurek O, Gorski B, Gronwald J, Prosolow Z, Uglanica K, Murinow A, Bobko I et al. (2001) Founder mutations in the BRCA1 gene in west Belarusian breast-ovarian cancer families. *Clin Genet.* **60(6)**: 470-471.

Painter RB, Young BR. (1980) Radiosensitivity in Ataxia-Telangiectasia: A New Explanation. *Proc. Natl. Acad. Sci. USA* **77(12)**: 7315-7317.

Parshad R, Sanford KK. (2001) Radiation-induced chromatid breaks and deficient DNA repair in cancer predisposition. *Crit. Rev. Oncol. Hematol.* **37**: 87-96.

Patel RK, Trivedi AH, Arora DC, Bhatavdekar JM, Patel DD. (1997) DNA repair proficiency in breast cancer patients and their first-degree relatives. *Int. J. Cancer* **73**: 20-24.

Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. (2000) A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* **10**: 886-895.

Perkins E, Small BJ, Balducci L, Extermann M, Robb C, Haley WE. (2007) Individual differences in well-being in older breast cancer survivors. *Critical Reviews in Oncology and Hematology*. **62(1)**:74-83.

Peto J, Collins N, Barfoot R, et al. (1999) Prevalence of BRCA1 and BRCA2 gene mutations in patients with early onset breast cancer. *J Natl Cancer Inst* **91**:943-949.

Pharoah PDP, Day NE, Duffy S, Easton DF, Ponder BAJ. (1997) Family history and the risk of breast cancer: a systematic review and metaanalysis. *Int J Cancer* **71**:800-809.

Pharoah PDP, Antoniou A, Bobrow M, Zimmern RL, Easton DF, Ponder BAJ (2002) Polygenic susceptibility to breast cancer and implications for prevention. *Nature Genetics* **31**: 33-36.

Phelan CM, Rebbeck TR, Weber BL, Devilee P, Ruttledge MH, Lynch HT et al. (1996) Ovarian cancer risk in BRCA1 carriers is modified by the HRAS1 variable number of tandem repeat (VNTR) locus. *Nature Genetics* **12**: 309-311.

Pichierri P, Rosselli F. (2004) The DNA crosslink-induced S-phase checkpoint depends on ATR-CHK1 and ATR-NBS1-FANCD2 pathways. *EMBO J* **23**:1178-1187.

Pingoud A and Jeltsch A. (2001) Structure and function of type II restriction endonucleases. *Nucleic Acids Research.* **29**: 3705-3727.

Pippard EC, Hall AJ, Barker DJP, Bridges BA. (1988) Cancer inhomozygotes and heterozygotes of ataxiatelangiectasia and xeroderma pigmentosum in Britain. *Cancer Res* **48**: 2929-2932.

Ponnaiya B, Cornforth MN, Ullrich RL. (1997) Radiation-induced chromosomal instability in BALB/c and C57BL/6 mice: the difference is as clear as black and white. *Radiat Res.* **147(2)**:121-125.

Poole AJ, Li Y, Kim Y, Lin SC, Lee WH, Lee EY. (2006) Prevention of Brca1-mediated mammary tumorigenesis in mice by a progesterone antagonist. *Science.* **314(5804)**: 1467-1470.

Powers JT, Hong SK, Mayhew CN, Rogers PM, Knudsen ES, Johnson DG. (2004) E2F1 uses the ATM signalling pathway to induce p53 and Chk2 phosphorylation and apoptosis. *Mol Cancer Res* **2**: 203-214.

Prechtel K, Prechtel V. (1997) Breast carcinoma in the man. Current results from the viewpoint of clinic and pathology *Pathologie* **18(1)**:45-52.

Price EA, Bourne SL, Radbourne R, Lawton PA, Lamerdin J, Thompson LH, Arrand JE (1997) Rare microsatellite polymorphisms in the DNA repair genes XRCC1, XRCC3 and XRCC5 associated with cancer in patients of varying radiosensitivity. *Somat Cell Mol Genet* **23**:237-247.

Public Health Ministry of the Republic of Belarus. (2002) Division of methodology and analysis of medical statistics. *Public health in the Republic of Belarus. Minsk*.

Public Health Ministry of the Republic of Belarus. (2006) Division of methodology and analysis of medical statistics. *Public health in the Republic of Belarus. Minsk*.

Pukkala E, Weiderpass E. (1999) Time trends in socioeconomic differences in incidence rates of cancers of the breast and female genital organs (Finland, 1971-1995). *Int J Cancer* **81**:56-61.

Pukkala E, Kesminiene A, Poliakov S et al. (2006) Breast cancer in Belarus and Ukraine after the Chernobyl accident. *Int. J. Cancer* **3**: 651 – 658.

Pylkäs K, Tommiska J, Syrjäkoski K, Kere J, Gatei M et al. (2007) Evaluation of the role of Finish ataxia-telangiectasia in hereditary predisposition to breast cancer. *Carcinogenesis* **28**: 1040-1045.

Rafii S, O´Regan P, Xinarianos G, Azmy I, Stephenson T, Reed M, Meuth M, Thacker J, Cox A (2002) A potential role for the XRCC2 R188H polymorphic site in DNA-damage repair and breast cancer. *Human Molecular Genetics* **11**: 1433-1438.

Rahman N, Seal S, Thompson D, Kelly P, Renwick A et al. (2007) PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat. Genet*. **39**: 165-167.
Raouf A, Brown L, Vrcelj N, To K, Kwok W, et al. (2005) Genomic Instability of Human Mammary Epithelial Cells Overexpressing a Truncated Form of EMSY. *J. Natl. Cancer Inst.*: **97**: 1302-1306.

Rebbeck TR, Kantoff PW, Krithivas K, Neuhausen S, Blackwood MA et al. (1999) Modification of BRCA1-associated breast cancer risk by the polymorphic androgen-receptor CAG repeat. *American Journal of Human Genetics* **64**: 1371-1377.

Reid RL. (2007) Hormonal Contraception and Breast Cancer: Keeping Perspective. *J Obstet Gynaecol Can* **29(3)**:207–209.

Reid S, Schindler D, Hanenberg H, Barker K et al. (2007) Biallelic mutations in PALB2 cause Fanconi anemia subtype FA-N and predispose to childhood cancer. *Nat. Genet*. **39**: 162-164.

Reimer D. E. (1996). Effect of breast-feeding on risk of breast cancer. *Canadian Medical Association Journal* **155**: 861.

Renwick A, Thompson D, Sheila S, Kelly P et al. (2006) ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles *Nature Genetics* **38**:873-875.

Riballo E, Kühne M, Rief N, Doherty A, Smith G, Recio M, Reis C, Dahm K, Fricke A, Krempler A. (2004) A Pathway of Double-Strand Break Rejoining Dependent upon ATM, Artemis, and Proteins Locating to γ-H2AX Foci. *Molecular Cell* **16(5)**: 715-724.

Riballo E, Kuhne M, Rief N, Doherty A et al. (2004) A pathway of double-strand break rejoining dependent upon ATM, Artemis and proteins locating to damma-H2AX foci. *Mol. Cell* **16**: 715-724.

Rijkers T, Van Den Ouweland J, Morolli B, Rolink AG, Baarends WM, Van Sloun PP, Lohman PH, Pastink A (1998) Targeted inactivation of mouse RAD52 reduces homologous recombination but not resistance to ionizing radiation. *Mol Cell Biol* **18**: 6423-6429.

Rogoff HA, Pickering MT, Frame FM et al. (2004) Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2. *Mol Cell Biol* **24**: 2968-2977.

Ronckers CM, Erdmann ChA, Land ChA. (2005) Radiation and breast cancer: a review of current evidence *Breast Cancer Res* **7**: 21-32.

Roznowski K, Januszkiewicz-Lewandowska D, Mosor M et al (2008) I171V germline mutation in the NBS1 gene significantly increases risk of breast cancer. *Breast Cancer Res Treat.* 110: 343-348.

Russo J, Hu YF, Silva ID, Russo IH. (2001) Cancer risk related to mammary gland structure and development. *Microsc Res Tech* **52**:204–223.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, and Erlich HA. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase *Science* 487-491.

Sanger F, Nicklen S and Coulson AR. (1977) DNA Sequencing with Chain-Terminating Inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**: 5463-5467.

Satagopan JM, Oftit K, Foulkes W et al. (2001) The lifetime risk of breast cancer in Ashkenazi Jewish carriers of BRCA1 and BRCA2 mutations. *Cancer Epidimiol. Biomarkers Prev*, **10**: 467-473.

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L et al. (1995a) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* **268**: 1749-1753.

Savitsky K, Sfez S, Tagle DA, Ziv Y, Sartiel A, Collins FS, Shiloh Y, Rotman G (1995b) The complete sequence of the coding region of the ATM gene reveals similarity to cell cycle regulators in different species. *Human Molecular Genetics* **4**:2025-2032.

Schmidt MK, Tollenaar R, de Kemp SR, Broeks A, Cornelisse CJ, Smit VT et al. (2007) Breast cancer survival and tumor characteristics in premenopausal women carrying the CHEK2*1100delC germline mutation. *J Clin Oncol* 25: 64-69.

Schutte M, Seal S, Barfoot R, Meijers-Heijboer H, Wasielewski M, Evans DG, Eccles D, Meijers C et al. (2003) Variants in CHEK2 other than 1100delC do not make a major contribution to breast cancer susceptibility. *Am J Hum Genet.* **72(4)**: 1023-1028.

Scott D. (2004) Chromosomal radiosensitivity and low penetrance predisposition to cancer. *Cytogenet Genome Res.* **104(1-4)**: 365-370.

Seal Sh, Thompson D, Renwick A, Elliott A et al. (2006) Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. *Nat. Genet.* **38**: 1239–1241.

Seemanova E (1990) Increased risk for malignant neoplasms in heterozygotes for a syndrome of microcephaly, normal intelligence, growth retardation, remarkable facies, immunodeficiency and chromosomal instability. *Mutat. Res.* **238**:321-324.

Seemanova E, Pelz J, Varon R, Jarolim P, Sperling K (2001) Cancer risk in NBS heterozygotes. *European Journal of Human Genetics* **9(S1)**: 106.

Seemanova E, Jarolim P, Seeman P, Varon R, Sperling K. (2006) Increased risk of malignancies in heterozygotes in families of patients with Nijmegen breakage syndrome. *Cas Lek Cesk* 145:138–143.

Seemanova E, Jarolim P, Seeman P, Varon R, Digweed M, Swift M, Sperling K. (2007) Cancer risk of heterozygotes with the NBN Founder Mutation. *JNCI* **99(24)**:1875-1880.

Seppälä EH, Ikonen T, Mononen N, Autio V, Rökman A, Matikainen MP, Tammela TL, Schleutker J. (2003) CHEK2 variants associate with hereditary prostate cancer. *Br J Cancer.* **89(10)**: 1966-1970.

Shen CY, Yu JC, Lo YL et al. (2000) Genome wide search for loss of heterozygosity using laser capture microdissected tissue of breast carcinoma: an implication for mutator phenotype and breast cancer pathogenesis. *Cancer Res.* **60**:3884-3892.

Shen WH, Adayabalam SB, Wang J, Wu H et al. (2007) Essential Role for Nuclear PTEN in Maintaining Chromosomal Integrity. *Cell* **128**: 157-170.

Shiloh Y (1997) Ataxia-telangiectasia and Nijmegen Breakage Syndrome: Related disorders but genes apart. *Annual Reviews of Genetics* **31**: 635-662

Shiloh Y. (2003) ATM and related protein kinases: safeguarding genome integrity (review). *Nature.* **13**: 155-168.

Shimada H, Shimizu K, Mimaki S, Sakiyama T, Mori T et al. (2004) First case of aplastic anemia in a Japanese child with a homozygous missence mutation in the NBS1 gene (I171V) associated with genomic instability. *Hum. Genet.* **115**: 372-376.

Simpson S. (2007) PALB2-new breast-cancer susceptibility gene. *Lancet Oncol*. **8(2)**:105.

Smith-Warner SA, Spiegelman D, Yaun SS, et al. (1998) Alcohol and breast cancer in women: a pooled analysis of cohort studies. *JAMA* **279**:535-540.

Sokolenko AP, Rozanov ME, Mitiushkina NV, Sherina NYu et al. (2007) Founder mutations in early-onset, familial and bilateral breast cancer patients from Russia. *Familial Cancer* **6**: 281-286.

Somasundaram K, Zhang H, Zeng YX, Houvras Y et al. (1997) Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. *Nature* **389**: 187–190.

Speit G, Trenz K. (2004) Chromasomal mutagen sensivity associated with mutations in BRCA genes. *Cytogenet. Genome Res.* **104**: 325-332.

Stacey SN, Manolescu A, Sulem P, Rafnar T, Gudmundsson J, Gudjonsson SA, et al. (2007) Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer. *Nat Genet* **39(7):**865-869.

Stankovic T, Kidd AMJ, Sutcliffe A, GMcGuire M, Robinson P, Weber P et al. (1998) ATM mutations and phenotypes in ataxia-telangiectasia families in the British Isles: expression of mutant ATM and the risk of leukemia, lymphoma, and breast cancer. *Am J Hum Genet* **62**: 334–345.

Steck PA, Pershouse MA, Jasser SA, Yung WKA et al. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nature Genetics* **15**: 356-362.

Steffen J, Nowakowska D, Niwinska A, Gzapczak D et al. (2006) Germline mutation 657del5 of the NBS1 gene contribute significantly to the incidence of brast cancer in Central Poland. *Int J Cancer* **119**:472-475

Steffen J, Varon R, Mosor M, Maneva G, Maurer M et al. (2004) Increased cancer risk of heterozygotes with NBS1 germline mutations in Poland. *Int J Cancer* **111**:67-71.

Stevens C, Smith L, La Thangue NB. (2003) Chk2 activates E2F1 in response to DNA damage. *Nature Cell Biol* **5**: 401-409.

Stewart GS, Maser RS, Stankovic T, Bressan DA, Kaplan MI et al. (1999) The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell* **99**: 577-587.

Stewart GS, Wang B, Bignell CR et al. (2003) MDC1 is a mediator of the mammalian DNA damage checkpoint. *Nature* **421**: 961-966.

Stracker TH, Theunissen JW, Morales M, Petrini JH. (2004) The MRE11 complex and the metabolism of chromosome breakes: the importance of communication and holding things together, *DNA Repair (Amst)* **3**: 845-854.

Stredrick DL, GarciaClosas M, Pineda MA, et al. (2006) The ATM missense mutation p.Ser49Cys (c.146C>G) and the risk of breast cancer. *Hum Mut* **27**: 538-544.

Stucki M, Jackson SP. (2005) MDC1/NFBD1: a key regulator of the DNA damage response in higher eukaryotes. *DNA Repair (Amst.)* **3(8-9)**: 953-957.

Swift M, Sholman L, Perry M, Chase C. (1976) Malignant neoplasms in the families of patients with ataxia-telangiectasia. *Cancer Res* **36**: 209–215.

Swift M, Reitnauer PJ, Morrell D, Chase CL (1987) Breast and other cancers in families with ataxia-telangiectasia. *New England Journal of Medicine* **316**: 1289-1294.

Swift M, Morrel D, Massey RB, Chase CL (1991) Incidence of cancer in 161 families affected by ataxia-telangiectasia. *New England Journal of Medicine* **325**:1831-1836.

Tamimi RM, Hankinson SE, Spiegelman D, Kraft P, Colditz GA, Hunter DJ. (2004) Common ataxia-telangiectasia mutated haplotypes and risk of breast cancer: a nested case-control study. *Breast Cancer Res* **6**: R416–422.

Tan Y, Raychaughuri P and Costa RH. (2007) Chk2 mediates stabilisation of FoxM1 transcription factor to stimulate expression of DNA repair genes. *Molecular and Cellular Biology* **27**: 1007-1016.

Tanaka T, Halicka HD, Huang X, Traganos F, Darzynkiewicz Z. (2006) Constitutive histone H2AX phosphorylation and ATM activation, the reporters of DNA damage by endogenous oxidants. *Cell Cycle* **5**: 1940-1945.

Tchirkov, A,. Bay JO, Pernin D, Bignon YJ, Rio P et al. (1997) Detection of heterozygous carriers of the ataxia-telangiectasia (ATM) gene by G2 phase chromosomal radiosensitivity of peripheral blood lymphocytes. *Hum. Genet.* **101**: 312–316.

Teraoka SN, Malone KE, Doody DR, Suter NM, Ostrander EA, Daling JR, Concannon P. (2001) Increased frequency of *ATM* mutations in breast carcinoma patients with early onset disease and positive family history. *Cancer* **92**:479–487.

Thai TH, Du F, Tsan JT, Jin Y, Phung A et al. (1998) Mutations in the BRCA1-associated RING domain (BARD1) gene in primary breast, ovarian and uterine cancers. *Human Molecular Genetics* **7**: 195-202.

Thompson D, Duedal S, Kirner J et al. (2005) Cancer risks and mortality in heterozygous ATM carriers. *J Natl Cancer Inst* **97**: 813-822.

Tokunaga M, Land CE, Yamamoto T (1987) Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950-80. *Radiation Research* **112**: 243-272.

Trichopoulos D, MacMahon B, Cole P. (1972) Menopause and breast cancer risk. *J Natl Cancer Inst* **48**: 605-613.

Urist M, Tanaka T, Poyurovsky MV, Prives C. (2004) p73 induction after DNA damage is regulated by checkpoint kinases Chk1 and Chk2. *Genes Devel* **18**: 3041-3054.

Vahteristo P, Tamminen A, Karvinen P, Eerola H, Eklund C et al. (2001) p53, CHK2, and CHK1 genes in Finnish families with Li-Fraumeni syndrome: further evidence of CHK2 in inherited cancer predisposition. *Cancer Res* 61:5718–5722.

Vahteristo P, Bartkova J, Eerola H, Syrjakoski K, Ojala S et al. (2002) A CHEK2 genetic variant contributing to a substantial fraction of familial breast cancer. *Am J Hum Genet* 71: 432-438.

Valerie K, Povirk LF. (2003) Regulation and Mechanisms of mammalian double-strand break repair. *Oncogene*: 5792-5812.

van der Burgt I, Chrzanowska KH, Smeets D and Weemaes C (1996) Nijmegen breakage syndrome. *Journal of Medical Genetics* **33**: 153-156.

van Puijenbroek M, van Asperen Ch, Van Mil A, Devilee P et al. (2005) Homozygosity for a CHEK2*1100delC mitation identified in familial colorectal cancer does not lead to a severe clinical phenotype. *J Pathology* **206**: 198-204.

Varon R,Vissinga C, Platzer M, Cerosaletti KM, Chrzanowska KH et al. (1998) Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell* **93**:467–476.

Varon R, Seemanova E, Chrzanowska K, Hnateyko O, Piekutowska-Abramczuk D et al. (2000) Clinical ascertainment of Nijmegen breakage syndrome (NBS) and prevalence of the major mutation, 657del5, in three Slav populations. *Eur J Hum Genet* **8**:900–902.

Venkitaraman AR. (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108**: 171-182.

Venkitaraman AR. (2003) A growing network of cancer-susceptibility genes. *N Engl J Med* **348**: 1917-1919.

Walsh T, Casadei S, Coats KH, Swisher E et al. (2006) Spectrum of Mutations in BRCA1, BRCA2, CHEK2, and TP53 in Families at High Risk of Breast Cancer. *JAMA* **295**:1379-1388.

Waltes R. (2002) Molekulare Ursachen von Strahlensensibilitätssyndromen und ihre Rolle beim Mammakarzinom. *Von dem Fachbereich Chemie der Universität Hannover zur Erlangung des grades Dr.rer.nat.* Dissertation.

Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ, Qin J. (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev* **14**: 927-939.

Ward IM, Wu X, Chen J. (2001) Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. *J Biol Chem.***276(51)**: 47755-47758.

Wei JH, Chou YF, Ou YH, et al. (2005) TTK/hMps1 participates in the regulation of DNA damage checkpoint response by phosphorylating CHK2 on threonine 68. *J Biol Chem* **208**: 7748-7757.

Weischer M, Bojesen SE, Tybjærg-Hansen A, Axelsson CK, Nordestgaard BG. (2007) Increased Risk of Breast Cancer Associated With CHEK2*1100delC. *J Clin Oncol* **25**: 57-63.

Welcsh PL, Owens KN, King MC. (2000) Insights into the functions of BRCA1 and BRCA2. *Trends Genet* **16**:69-74.

Wiegman EM, Blaese MA, Loeffler H, Coppes RP, Rodemann HP. (2007) TGFbeta-1 dependent fast stimulation of ATM and p53 phosphorylation following exposure to ionizing radiation does not involve TGFbeta-receptor I signalling. *Radiotherapy and Oncology* **83**:289-295.

Wong AKC, Pero R, Ormonde PA, Tavtigian SV and Bartel PL. (1997) Rad51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene BRCA2. *J. Biol. Chem.* **272**: 31941–31944.

Wooster R, Bignell G, Lancaster J. Swift S et al. (1995) Identification of the breast cancer susceptibility gene BRCA2. *Nature* **378**: 789-791.

Wu X, Gu J, Grossman HB, Amos ChI, Etzel C, Huang M et al. (2006) Bladder cancer predisposition: a multigenic approach to DNA-repair and cell-cycle-control genes. *The American Journal of human Genetic* **78**: 464-479.

Wu X, Petrini JH, Heine WF, Weaver DT, Livingston DM, Chen J. (2000) Independence of R/M/N focus formation and the presence of intact BRCA1. *Science* **289**: 11.

Wu X, Webster SR, Chen J. (2001) Characterization of tumor-associated Chk2 mutations. *J Biol Chem* **276**: 2971–2974.

Xia B, Sheng Q, Nakanishi K, Ohashi A, Wu J et al. (2006) Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol. Cell* **22**: 719-729.

Xia B, Dorsman JC, Ameziane N, de Vries Y et al. (2007) Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. *Nat. Genet*. **39**: 159-161.

Yager JD and. Davidson NE. (2006) Estrogen Carcinogenesis in Breast Cancer. *N Engl J Med* **354**: 270-282.

Yang J, Xu ZP, Huang Y, Hamrick HE, Duerksen-Hughes PJ, Yu YN. (2004) ATM and ATR: sensing DNA damage. *World J Gastroenterol.* **10(2)**: 155-160.

Yang S, Kuo C, Bisi JE, Kim MK (2002) PML-dependent apoptosis after DNA damage is regulated by the checkpoint kinase hCds1/Chk2. *Nature Cell Biology* **4**: 865-870

Yoshida K, Miki Y. (2004) Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci*. **95**: 866-871.

You KT, Li LSh, Lim N-G, Kang HJ, Koh KH et al. (2007) Selective translational repression of truncated proteins from frameshift mutation-derived mRNAs in tumors. *PLos Biology* **5**: 1098-1109.

Yu X, Chini CC, He M, Mer G and Chen J. (2003) The BRCT domain is a phospho-protein binding domain. *Science* **302**: 639–642.

Zhang J, Powell SN. (2005) The role of BRCA1 tumor suppressor in DNA double-strand break repair. *Mol. Cancer Res*. **3**: 531-539.

Zhang L, Zhang Z, Yan W (2005) Single nucleotide polymorphisms for DNA repair genes in breast cancer patients. *Clin Chim Acta* **359**:150–155.

Zhang ShM, Lee I-M, Manson JE et al. (2007) Alcohol Consumption and Breast Cancer Risk in the Women's Health Study. *Am. J. Epidemiol*. **165**: 667-676.

Zhao S, Weng Y-C, Yuan S-SF, Lin Y-T, Hsu H-C, Lin S-CJ, Gerbino E, Song M, Zdzienicka MZ, Gatti RA, Shay JW, Ziv Y, Shiloh Y, Lee EY-HP (2000) Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature* **405**: 473-477.

Zheng L, Annab LA, Afshari CA, Lee W-H, Boyer TG (2001) BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor. *Proceedings of the National Academy of Sciences (USA)* **98**: 9587-9592.

Zheng W, Gustafson DR, Sinha R, et al. (1998) Well done meat intake and the risk of breast cancer. *J Natl Cancer Inst* **90**: 1724-1729.

Zhong Q, Chen C-F, Li S, Chen Y, Wang C-C, Xiao J, Chen P-L, Sharp ZD, Lee W-H (1999) Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. *Science* **285**:747-750.

Zhou W, Simpson PJ, McFadden JM, Townsend CA et al. (2003) Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. *Cancer Res* **63**:7330–7337.

Ziotkowska I, Mosor M, Wierzbicka M et al. (2007) Increased risk of larynx cancer in heterozygous carriers of I171V mutation of the NBS gene. *Cancer Sci* **98**: 1701-1705.

Acknowledgements

This study was carried out in the Gynaecology Research Unit at Hannover Medical School during the years 2004-2008.

I am deeply indebted to Dr. Thilo Dörk-Bousset, for introducing me to the research of breast cancer genetics, for inviting me to Germany and making this project possible. It has been a privilege to interact intellectually and scientifically with him during working at the project.

I express my sincere thanks to Professor Dr. Peter Hillemanns and Professor Dr. Johann H. Karstens for their support and providing me the opportunity as well as the facilities to complete my work.

I owe a great dept of gratitude to all the people who worked with me for their friendship, help and support: Britta Wieland, Peter Schürmann, Natalia Dubrowinskaja, Dr. Regina Waltes, Katrin Gerriets, Scarlet A. Reinke, Paria Yamini, Sandra Beußel, Eleftheria Mavridou, Dr. Andreas Meyer and Prof. Dr. Michael Bremer.

I would also like to thank Professor Dr. Christof Sohn for the support and possibility to begin my research work in the Gynaecology Research Unit of Hannover Medical School under his headship.

I am especially grateful to Dr. Konstantin Glebov for the help, support and advice I have received whenever I needed it.

I thank Magda Anna Blaut, Lina Köpplin, Irina Coinac for their friendship and continued support.

I am grateful to Dr. Lina Malinoyskaya, Dr. Sergej Bokut, Dr Yuri Rogov and Dr. Dzhaba Gemrikilashvilli for supporting me, especially during the final phase of the project.

I owe my greatest gratitude to my main supervisor and first reviewer Professor Dr. Jürgen Alves for his enthusiasm, encouragement and support.

I wish to sincerely thank the second official reviewer Professor Dr. Ekkehard Dikomey for the valuable time which he will spend to read this thesis.

Special thanks belong to Professor Dr. Olaf Damman for friendliness and teaching the secrets of statistical analyses.

I owe my sincere gratitude to the personnel of all Oncological Centres and patients for the participation in the study.

Specially, I want to acknowledge Elena Gacucova for technical assistance during whole my work.

I also thank the visiting researcher Dr. Marina Bermisheva for friendly support and critical discussions.

I wish to express sincere thanks to Dr. Nurzhan Turmanov and also to family John (Manfred, Silvia and Stefanie) for the friendship and support during the years.

I also wish to thank my all dear friends for their support, encouragement, relaxing companionship and all the joyful moments that we have shared.

I owe my deepest gratitude to my parents Nina Byshko and Valery Bogdanov, for endless caring, love and support. Thank you for being there.

My most loving thanks belong to my fiancé Michael John for his love, care and support. You have brought so much joy and wonderful things into my life.

Hannover, June 2008

Natalia Bogdanova.

Attachment 1 **Sequences of primers used in the thesis for identification and verification of mutations**

Gene	Locus	Primer	Primer sequence	Size of PCR product	Use[1]
BRCA1	exon 5 (C61G)	BR5i5	5′- CTCTTAAGGGCAGTTGTGAG- 3′	234 bp	1,3
		BR5i3	5′- TTCCTACTGTGGTTGCTTCC- 3′		
		C61ARMS	5′- CCAGAAGAAAGGGCCTTCACTGG- 3′	221 bp	2
		C61R	5′- CCTGTATAAGGCAGATGTCC- 3′		
	exon 11 (4153delA)	4153ARMS	5′- GGAATTGGTTTCAGATGATCAG- 3′	131 bp	2
		BR11R	5′- CACTTCTATAAATAGACTGGG- 3′		
		BS5B	5′- CTACTAGGCATAGCACCGTTGC- 3′	423 bp	3
		BR11R	5′- CACTTCTATAAATAGACTGGG- 3′		
	exon 20 (5382insC)	BS10	5′- CCAAAGCGAGCAAGAGAATCTC- 3′*	270 bp	1,3
		BS9	5′- GGGAATCCAAATTACACAGC- 3′		
BRCA2	exon 11 (6174delT)	6174delT	5′- GTGGGATTTTTAGCCCAGCAAG- 3′*	438 bp	1
		BS20	5′- CTGAGTTTACACAGTGCTCTGGG- 3′		
		6174ARMS	5′- GTGGGATTTTTAGCACAGCTAGG- 3′	189 bp	2
		6174delR	5′- GTTCTGGAGTACGTATAGCAG- 3′		
		BS19A	5′- CTTCATAAGTCAGTCTCATCTG- 3′	837 bp	3
		BS20	5′- CTGAGTTTACACAGTGCTCTGGG- 3′		
ATM	exon 9	ATM8F	5′- CTTTCAGCATACCACTTCATAAC- 3′	601 bp	6
		ATM8R	5′- CATAAGTAGCTCCTAGAGGGAAC-3′		
	exon 42 (E1978X)	ATM41F	5′- TGTATTCAGGAGCTTCCAAATAG- 3′	269 bp	1,3,6
		ATM41R	5′- GCTTAGTCCAGTAAGTAAATTCAG- 3′		
	exon 52	ATM51FF	5′- CTTAGGAAGGTGTGTGAATTGCACAG -3′	436 bp	6
		ATM51RR	5′- CAAGCACAGGGTAGAATATTGGGCTG -3′		
NBN	exon 5 (I171V)	NBS171FF	5’-GATGTAAACAGCCTCTTTGTAG-3’	170 bp	1
		NBS171RR	5’-TCAACTGCTTTCAGGAGTTCAG-3’*		

		NBS171F	5’-TCCTGAAAGCAGTTGAGTCC-3’	133 bp	3
		NBS171R	5’-ACAAGCATTAAAGAGGGAGTTAAC-3’		
	exon 6 (657del5)	657ARMS	5’-CAGGACGGCAGGAAAGAAATCT- 3’	391 bp	2
		PAR16	5’-GGTACACAGAACATATTCAACTG -3’		
		R215RSA	5’-AAGTAAAAATGTTGATCTGTCAGTA-3’*	420 bp	3
		PAR16	5’-GGTACACAGAACATATTCAACTG -3’		
	exon 6 (R215W)	R215RSA	5’-AAGTAAAAATGTTGATCTGTCAGTA-3’*	178 bp	1
		NBS6i-3	5’-TGAAATACGTTAACAACTACTG-3’		
		R215RSA	5’-AAGTAAAAATGTTGATCTGTCAGTA-3’*	420 bp	3
		PAR16	5’-GGTACACAGAACATATTCAACTG -3’		
MDC1	exon 9	KIA9-2F	5´- GTCACTTCTGAGCCCACATA- 3´	351 bp	6
		KIA9-4R	5´- AGGGACAGTTGATTCAGGGT- 3´		
CHEK2	exons 2-3 (I157T and IVS2+1G>A)	CHEK2ARTF	5’-GCAAGAAACACTTTCGGATTTTCCGG-3’	194 bp	1
		CHEK2ARTR	5’-CCACTGTGATCTTCTATGTCTGCA-3’*		
		CHEK2F	5’-CCTTCTTAGGCTATTTTCCTAC-3’	580 bp	3
		CHEK3R	5’-AACCATATTCTGTAAGGACAGG-3’		
	exons 9-10 (dele(9,10))	CHLdel2F	5’-TGTAATGAGCTGAGATTGTGC-3’	379 bp	4
		CHLc2R	5’-CAGAAATGAGACAGGAAGTT-3’		
		CHLdelR	5’-GTCTCAAACTTGGCTGCG-3’	522 bp	4
		CHLcF	5’-CTCTGTTGTGTACAAGTGAC-3’		
		CHEK2delF	5’-GAACCACTATTTACATAAC-3’	1.8 Kbp	5
		CHEK2delR	5’-GTCTCAAACTTGGCTGCG-3’		
	exon 10 (1100delC)	CHEK2As2	5’-CCCTTTTGTACTGAACTTTTAGATGAT-3’	227 bp	2
		CHEK210R	5’-ATCACCTCCTACCAGTCTGTGC-3’		
		CHEKBsp	5´- CCCTTTTGTACTGAATTTTAGAGTA- 3´	380 bp	3
		CHEK10RR	5´- GGCATGGTGGTGTGCATC- 3´		

XRCC4	exon 8 (IVS7-1G>A)	XRCC4-8F	5'-CTGTCATTTCACTTATGTGTCTC-3'	247 bp	1,3
		XRCC4-8R	5'-CTACAAGTGATCTGATACAAAAG-3'		

[1] approach for which primers were used:

1 – routine PCR followed by restriction enzyme cleavage
2 – ARMS-PCR
3 – PCR followed by direct sequencing
4 – allele-specific duplex PCR
5 – mutation specific long range PCR
6 – as internal control for ARMS assay
* mismatch primer with modified nucleotide position underlined

Attachment 2 **Summary of TaqMan assays used in the thesis for SNP genotyping**

Locus	SNP ID	Assay ID or Primer sequence (5'→3')	Assay ID or Probe sequence (5'→3')	Annealing/ hybridization t°	Number of cycles
T1915M (*BRCA2*)	rs4987117	**F**: 5'-GGATGATTCAGAGGATATTCTTCATAACTC-3' **R**: 5'-GTTGTAAAATTTCTTCACTCTGAATGTCAG-3'	**Probe1**: 5'-AATGTAGCACGCATTCACATAAG-3'*[1] **Probe2**: 5'-AATGTAGCATGCATTCACATAAG-3'*[2]	62°	40
S49C (*ATM*)	rs1800054	C_2283262_20_Applied Biosystems	C_2283262_20_Applied Biosystems	60°	40
S707P (*ATM*)	rs4986761	C_45273748_10 _Applied Biosystems	C_45273748_10 _Applied Biosystems	60°	40
L1420F (*ATM*)	rs1800058	C_45273752_10_ Applied Biosystems	C_45273752_10_ Applied Biosystems	60°	40
P1054R (*ATM*)	rs1800057	C_45273750_10_ Applied Biosystems	C_45273750_10_ Applied Biosystems	60°	40
F858L (*ATM*)	rs1800056	C_2283286_20_ Applied Biosystems	C_2283286_20_ Applied Biosystems	60°	40
Q559R (*PALB2*)	rs152451	C_ _ _2392113_10_Applied Biosystems	C_ _ _2392113_10_Applied Biosystems	60°	36/40
E672Q (*PALB2*)	rs45532440	**F**: 5'-GTTTGGCCTTTTGGGATGTG-3' **R**: 5'-GAGAGAGACATCTTAAAGAGGGAAGCT-3'	**Probe1**: 5'-AAGGTCCTCTTCTAAGTCCTCC-3'*[1] **Probe2**: 5'-AAGGTCCTCTTGTAAGTCCTCC-3'*[2]	62°	40
G998E (*PALB2*)	rs45551636	**F**: 5'-ACCTGTGATAAAATCATTCTTCATCTAATAGT-3' **R**: 5'-GACCCTTTCTGATCAACAAGTAGAAGT-3'	**Probe1**: 5'-CTTACCCTCCATCTTCTG-3'*[1] **Probe2**: 5'-CTTACCCTTCATCTTCTGCA-3'*[2]	65°	40
D302H (*CASP8*)	rs1045485	**F**: 5'-GCTTTGACCACGACCTTTGAAG-3' **R**: 5'-GTTACTGTGGTCCATGAGTTGGTAGAT-3'	**Probe1 (302H)**: 5'-CAAGCCCCACCATGACTGCACA-3'**[3] **Probe2 (D302)**: 5'-CAAGCCCCACGATGACTGCACA-3'**[4]	62°	36/40
L10P (*TGFb*)	rs1982073	C_22272997_10_ Applied Biosystems	C_22272997_10_ Applied Biosystems	61°	40
FGFR2	rs2981582	C_2917302_10_ Applied Biosystems	C_2917302_10_ Applied Biosystems	63°	40
TOX3	rs3803662	C_25968567_10_ Applied Biosystems	C_25968567_10_ Applied Biosystems	60°	40
2q35	rs13387042	C_32048042_10_ Applied Biosystems	C_32048042_10_ Applied Biosystems	60°	40

*- Probes design and labelling was done via Fa. Eurogentec (red: LNA, labelling: [1] - 5'-FAM, 3'-BHQ; [2]- 5'-YY, 3'-BHQ)
**- Probes design was done on Cambrige University, labelled via Fa. Applied Biosystems ([3] - 5'- VIC, 3'- TAMRA; [4]- 5'-FAM, 3'-TAMRA)

Attachment 3. **Summarized Odds Ratios of investigated variants stratified by region**

Gene	Locus	Contaminated regions			Non contaminated regions		
		OR*per allele	**95%CI***	**p***	**OR***per allele	**95%CI***	**p***
BRCA1	5382insC	n.a	n.a	0.0013	13.91	1.87-103.43	0.0016
	C61G	3.20	0.38-26.65	0.46	n.a	n.a	0.043
	4153delA	5.36	0.68-41.98	0.14	3.73	0.45-31.06	0.36
BRCA2	6174delT	-**	-**	-**	-**	-**	-**
ATM	E1978X	3.20	0.38-26.65	0.46	n.a	n.a	0.44
NBN	657del5	n.a	n.a	0.13	8.11	1.06-62.18	0.034
	R215W	0.53	0.03-8.45	1.00	1.24	0.37-4.12	0.96
CHEK2	1100delC	0.18	0.02-1.70	0.24	n.a	n.a	0.06
	I157T	1.52	0.83-2.77	0.22	3.50	1.65-7.5	0.001
	IVS1+2G>A	n.a.	n.a.	0.24	n.a.	n.a.	1.0
	dele(9,10)	n.a.	n.a.	0.09	7.53	0.98 – 58.02	0.05
XRCC4	IVS7-1G>A	1.16	0.89-1.51	0.29	1.35	1.07-1.71	0.01
CASP8	D302H	0.84	0.65-1.08	0.19	0.89	0.72-1.10	0.32
TGFB1	L10P	0.88	0.74-1.04	0.15	1.14	0.99-1.32	0.08
FGFR2	rs2981582	1.04	0.88-1.24	0.67	1.09	0.94-1.26	0.29
TOX3	rs38003662	1.13	0.93-1.36	0.24	1.17	1.00-1.37	0.058
?/2q35	rs13387042	0.93	0.79-1.10	0.43	1.04	0.90-1.20	0.61

* - compared between cases and controls in two-by-two tables

n.a. - not applicable due to dividing by null

** - can not be calculated because of mutational screening performance only in cases series

Attachment 4 **Combined carrier frequencies for investigated variants in high-penetrance loci (*BRCA1/2*), moderate risk loci (*ATM, NBN, CHEK2*) and low-penetrance loci (*XRCC4, CASP8, TGFB1, FGFR2, TOX3*, 2q35) in Byelorussian case series**

Gen	*BRCA1*			*BRCA2*	*NBN*		*CHEK2*				*ATM*	*XRCC4*	*CASP8*	*TGFB1*	*FGFR2*	*TOX3*	?/2q35
Locus	**5382 insC**	**p.C61G**	**4153 delA**	**6174 delT**	**657 del5**	**p.R215W**	**1100 delC**	**p.I157T**	**IVS1+2 G>A**	**dele (9,10)**	**p.E1978X**	**IVS7-1 G>A**	**p.D302H**	**p.L10P**	**rs2981582**	**rs38003662**	**rs13387042**
5382insC																	
p.C61G	0 (0.4)																
4153delA	0 (0.4)	0 (0.1)															
6174delT	0 (0.1)	0 (0.03)	0 (0.04)														
657del5	0 (0.4)	0 (0.1)	0 (0.2)	0 (0.04)													
p.R215W	0 (0.2)	1 (0.1)*	0 (0.1)	0 (0.02)	0 (0.1)												
1100delC	0 (0.2)	1 (0.1)	0 (0.1)	0 (0.02)	0 (0.1)	1 (0.05)*											
p.I157T	0 (2.2)	1 (0.75)	1 (0.8)	0 (0.2)	0 (0.8)	0 (0.45)	0 (0.45)										
IVS1+2G>A	0 (0.4)	0 (0.1)	0 (0.15)	0 (0.04)	1 (0.15)	0 (0.1)	0 (0.1)	1 (0.8)									
dele(9,10)	0 (0.5)	0 (0.2)	0 (0.2)	0 (0.05)	0 (0.2)	0 (0.1)	0 (0.1)	1 (1)	0 (0.2)								
p.E1978X	0 (0.2)	2 (0.1)	0 (0.1)	0 (0.02)	0 (0.1)	0 (0.05)	0 (0.5)	0 (0.45)	0 (0.1)	1 (0.1)							
IVS7-1G>A	9 (10.3)	6 (3.5)	4 (3.8)	0 (1)	3 (3.8)	1 (2)	1 (2)	20 (20.6)	5 (3.8)	5 (4.7)	5 (2)						
p.D302H	9 (10.1)	5 (3.4)	5 (3.7)	0 (0.9)	2 (3.7)	1 (2)	1 (2)	18 (20)	1 (3.7)	6 (4.6)	0 (2)	96 (95)					
p.L10P	30 (28.7)	11 (10)	9 (10.4)	3 (2.6)	12 (10.4)	8 (5.9)	7 (6)	57 (57.4)	7 (10)	15 (13)	8 (5.9)	261 (268)	273 (264)				
rs2981582	18 (27)	8 (9.2)	13 (9.8)	3 (2.5)	12 (9.8)	4 (5.5)	5 (5.5)	54 (54)	10 (9.8)	14 (12)	4 (5.5)	248 (252)	244 (248)	692 (703)			
rs38003662	22 (22)	9 (7.5)	8 (8)	3 (2.0)	11 (8)	4 (4.5)	6 (4.5)	45 (44)	7 (8)	15 (10)	7 (4.5)	224 (209)	193 (202)	592 (573.6)	522 (539)		
rs13387042	35 (29)	7 (10)	8 (10.6)	3 (2.7)	12 (10.6)	5 (6)	5 (6)	42 (58.4)	10 (10.6)	16 (13)	3 (6)	276 (273)	257 (268.6)	769 (762)	707 (716)	589 (584)	

*- patient carriers additional CHEK2*1100delC.

In black shown obtained number of carriers and blue shows expected carrier numbers.

CURRICULUM VITAE

Personal data

Name: Natalia Bogdanova
Date of birth: 22 October 1978
Place of Birth: Minsk, Belarus
Nationality: Byelorussian

Educational background

1985-1995	Secondary school with profound study of English Nr. 154, Minsk, Belarus
1995-2000	International Sakharov Environmental University, Faculty of Radiobiology, Minsk, Belarus.
08/2000	Diploma in Radiobiology and Environmental Medicine
2000-2003	Post–graduate study in International Sakharov Environmental University, Minsk, Belarus
Since 10/2003	PhD student at the Gottfried Wilhelm Leibniz University Hannover

Work experience

2000–2001	Laboratory assistant, Byelorussian Medical Academia of post–diploma education, Department of Hematology, Minsk, Belarus
2001–2002	Junior researcher of Department Hereditary Diseases Prevention, Byelorussian Institute for Hereditary Diseases Minsk, Belarus
2002-09/2003	Researcher of Department Hereditary Diseases Prevention, Byelorussian Institute for Hereditary Diseases Minsk, Belarus
Since 10/2003	as PhD student at Gynaecology Research Unit, Medical School Hannover

Fellowships

09/2002-12/2002	DAAD Research Scholarship for PhD students at Gynaecology Research Unit, Medical School Hannover

Publications

Publications generated within the scope of the thesis:

Bendix-Waltes R, Gerriets K, Beussel S, Bogdanova N, El-Harith A E-H, Gutierrez Enriquez S, Hall J, Bremer M, Dörk T **A frequent splicing mutation of *XRCC4*: implications for breast cancer?** Medizinische Genetik 2003; 3:294.

Natalia Bogdanova , Natalia Enßen-Dubrowinskaja , Sergei Feshchenko , Gennady I. Lazjuk , Yuri I. Rogov , Olaf Dammann , Michael Bremer , Johann H. Karstens , Christof Sohn , Thilo Dörk **Association of two mutations in the *CHEK2* gene with breast cancer** Int. J. Cancer, 2005,116: 263–266

Natalia Bogdanova, Sergei Feshchenko, Cezary Cybulski, Thilo Dörk ***CHEK2* Mutation and Hereditary Breast Cancer** *Journal of Clinical Oncology*, 2007, 25/19: pp. 26e, doi:10.1200/JCO.2007.114223

Natalia Bogdanova, Peter Schürmann, Regina Waltes, Sergei Feshchenko, Iosif Viktorovich Zalutsky, Michael Bremer. Thilo Dörk ***NBS1* variant I171V and breast cancer risk** *Breast Cancer Res Treat*, 2007 doi: 10.1007/s10549-007-98204

Angela Cox, Alison M Dunning, Montserrat Garcia-Closas, Sabapathy Balasubramanian, Malcolm W R Reed, Karen A Pooley, Serena Scollen, Caroline Baynes, Bruce A J Ponder, Stephen Chanock, Jolanta Lissowska, Louise Brinton, Beata Peplonska, Melissa C Southey, John L Hopper, Margaret R E McCredie, Graham G Giles, Olivia Fletcher, Nichola Johnson, Isabel dos Santos Silva, Lorna Gibson, Stig E Bojesen, Børge G Nordestgaard, Christen K Axelsson, Diana Torres, Ute Hamann, Christina Justenhoven, Hiltrud Brauch, Jenny Chang-Claude, Silke Kropp, Angela Risch, Shan Wang-Gohrke, Peter Schürmann, Natalia Bogdanova, Thilo Dörk, Rainer Fagerholm, Kirsimari Aaltonen, Carl Blomqvist, Heli Nevanlinna, Sheila Seal, Anthony Renwick, Michael R Stratton, Nazneen Rahman, Suleeporn Sangrajrang, David Hughes, Fabrice Odefrey, Paul Brennan, Amanda B Spurdle, Georgia Chenevix-Trench, The Kathleen Cunningham Foundation Consortium for Research into Familial Breast Cancer, Jonathan Beesley, Arto Mannermaa, Jaana Hartikainen, Vesa Kataja, Veli-Matti Kosma, Fergus J Couch, Janet E Olson, Ellen L Goode, Annegien Broeks, Marjanka K Schmidt, Frans B L Hogervorst, Laura J Van't Veer, Daehee Kang, Keun-Young Yoo, Dong-Young Noh, Sei-Hyun Ahn, Sara Wedrén, Per Hall, Yen-Ling Low, Jianjun Liu, Roger L Milne, Gloria Ribas, Anna Gonzalez-Neir, Javier Benitez, Alice J Sigurdson, Denise L Stredrick, Bruce H Alexander, Jeffery P Struewing, Paul D P Pharoah & Douglas F Easton, on behalf of the Breast Cancer Association Consortium **A common coding variant in *CASP8* is associated with breast cancer risk** *Nature Genetics,* 2007, 39: 352 – 358

Douglas F. Easton, Karen A. Pooley, Alison M. Dunning, Paul D. P. Pharoah, Deborah Thompson, Dennis G. Ballinger, Jeffery P. Struewing, Jonathan Morrison, Helen Field, Robert Luben, Nicholas Wareham, Shahana Ahmed, Catherine S. Healey, Richard Bowman, the SEARCH collaborators, Kerstin B. Meyer, Christopher A. Haiman, Laurence K. Kolone, Brian E. Henderson, Loic Le Marchand, Paul Brennan, Suleeporn Sangrajrang, Valerie Gaborieau, Fabrice Odefrey, Chen-Yang Shen, Pei-Ei Wu, Hui-Chun Wang, Diana Eccles, D. Gareth Evans, Julian Peto, Olivia Fletcher, Nichola Johnson, Sheila Seal, Michael R. Stratton, Nazneen Rahman,Georgia Chenevix-Trench, Stig E. Bojesen, Børge G. Nordestgaard, Christen K. Axelsson, Montserrat Garcia-Closas, Louise Brinton, Stephen Chanock, Jolanta Lissowska, Beata Peplonska, Heli Nevanlinna, Rainer

Fagerholm, Hannaleena Eerola, Daehee Kang, Keun-Young Yoo, Dong-Young Noh, Sei-Hyun Ahn, David J. Hunter, Susan E. Hankinson, David G. Cox, Per Hall, Sara Wedren, Jianjun Liu, Yen-Ling Low, Natalia Bogdanova, Peter Schu¨rmann, Thilo Dörk, Rob A. E. M. Tollenaar, Catharina E. Jacobi, Peter Devilee, Jan G. M. Klijn, Alice J. Sigurdson, Michele M. Doody, Bruce H. Alexander, Jinghui Zhang, Angela Cox, Ian W. Brock, Gordon MacPherson, Malcolm W. R. Reed, Fergus J. Couch, Ellen L. Goode, Janet E. Olson, Hanne Meijers-Heijboer, Ans van den Ouweland, Andre´ Uitterlinden, Fernando Rivadeneira, Roger L. Milne, Gloria Ribas, Anna Gonzalez-Neira, Javier Benitez, John L. Hopper, Margaret McCredie, Melissa Southey, Graham G. Giles, Chris Schroen, Christina Justenhoven, Hiltrud Brauch, Ute Hamann, Yon-Dschun Ko, Amanda B. Spurdle, Jonathan Beesley, Xiaoqing Chen, kConFab, AOCS Management Group, Arto Mannermaa, Veli-Matti Kosma, Vesa Kataja, Jaana Hartikainen, Nicholas E. Day5, David R. Cox3 & Bruce A. J. Ponder **Genome-wide association study identifies novel breast cancer susceptibility loci** *Nature*, 2007, 447: 1087-1093

Natalia Bogdanova , Sergei Feshchenko , Peter Schürmann , Regina Waltes , Britta Wieland , Peter Hillemanns , Yuri I. Rogov , Olaf Dammann , Michael Bremer , Johann H. Karstens , Christof Sohn , Raymonda Varon , Thilo Dörk **Nijmegen Breakage Syndrome mutations and risk of breast cancer** *Int. J. Cancer*, 2008, 122: 802–806

Scarlett Reincke, Lina Govbakh, Bettina Wilhelm, Haiyan Jin, Natalia Bogdanova, Michael Bremer, Johann H Karstens, and Thilo Dörk. **Mutation analysis of the *MDM4* gene in German breast cancer patients** *BMC Cancer* 2008, 8:52 doi: 10.1186/1471-2407-8-52 PMCID: PMC 2259322

Montserrat Garcia-Closas, Per Hall, Heli Nevanlinna, Karen Pooley, Jonathan Morrison, Douglas A. Richesson, Stig E. Bojesen, Børge G. Nordestgaard, Christen K. Axelsson, Jose I. Arias, Roger L. Milne, Gloria Ribas, Anna González-Neira, Javier Benítez, Pilar Zamora, Hiltrud Brauch, Christina Justenhoven, Ute Hamann, Yon-Dschun Ko, Thomas Bruening, Susanne Haas, Thilo Dörk, Peter Schürmann, Peter Hillemanns, Natalia Bogdanova, Michael Bremer, Johann Hinrich Karstens, Rainer Fagerholm, Kirsimari Aaltonen, Kristiina Aittomäki, Karl von Smitten, Carl Blomqvist, Arto Mannermaa, Matti Uusitupa, Matti Eskelinen, Maria Tengström, Veli-Matti Kosma, Vesa Kataja, Georgia Chenevix-Trench, Amanda B. Spurdle, Jonathan Beesley, Xiaoqing Chen, Australian Ovarian Cancer Management Group, The Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer, Peter Devilee, Christi J. van Asperen, Catharina E. Jacobi, Rob A. E. M. Tollenaar, Petra E.A. Huijts, Jan G. M. Klijn, Jenny Chang-Claude, Silke Kropp, Tracy Slanger, Dieter Flesch-Janys, Elke Mutschelknauss, Ramona Salazar, Shan Wang-Gohrke, Fergus Couch, Ellen L. Goode, Janet E. Olson, Celine Vachon, Zachary S. Fredericksen, Graham G. Giles, Laura Baglietto, Gianluca Severi, John L. Hopper, Dallas R. English, Melissa C. Southey, Christopher A. Haiman, Brian E. Henderson, Laurence N. Kolonel, Loic Le Marchand, Daniel O. Stram, David J. Hunter, Susan E. Hankinson, David G. Cox, Rulla Tamimi, Peter Kraft, Mark E. Sherman, Stephen J. Chanock, Jolanta Lissowska, Louise A. Brinton, Beata Peplonska, Jan G. M. Klijn, Maartje J. Hooning, Han Meijers-Heijboer, J. Margriet Collee, Ans van den Ouweland, Andre G. Uitterlinden, Jianjun Liu, Low Yen Lin, Li Yuqing, Keith Humphreys, Kamila Czene, Angela Cox, Sabapathy P. Balasubramanian, Simon S. Cross, Malcolm W. R. Reed, Fiona Blows, Kristy Driver, Alison Dunning, Jonathan Tyrer, Bruce A. J. Ponder, Suleeporn Sangrajrang, Paul Brennan, James McKay, Fabrice Odefrey, Valerie Gabrieau, Alice Sigurdson, Michele Doody, Jeffrey P. Struewing, Bruce Alexander, Douglas F. Easton, and Paul D. Pharoah **Heterogeneity of Breast Cancer Associations with Five Susceptibility Loci by Clinical and Pathological Characteristics** *PLoS Genet* 2008, 4: e1000054. doi: 10.1371/journal.pgen.1000054, PMCID: PMC 2291027

Contribution to congresses:

Bogdanova N, Enßen-Dubrowinskaja N, Festchenko S, Lazijuk GI , Rogov YI, Dammann O, Bremer M, Karstens JH, Sohn Ch, Dörk T

Association of two mutations in the CHEK2 gene with breast cancer

European Human Genetics Conference, Prague, Czech Republic, 2005

Bogdanova N, Festchenko S, Sohn Ch, Dörk T

Differential contribution of NBS1 gene alterations to breast cancer susceptibility

17. Jahrestagung Deutsche Gesellschaft für Humangenetik e.V., Heidelberg, Germany, 2006

Presentations as invited speaker:

2006	7th Balkan Meeting on Human Genetics, Skopje, Republic of Macedonia *Last advances in the identification of breast cancer susceptibility alleles*

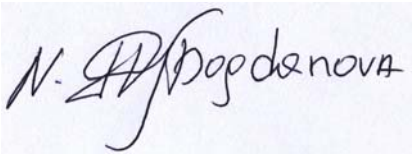
Meine akademischen Lehrer waren die Damen und Herren in Minsk:

Baikov, Bartkevich, Batyan, Bokut, Chistik, Chudakov, Figurin, Goncharova, Gritsai, Gurachevskiy, Gutko, Homenko, Kapitula, Karpovich, Kiselev, Kovaleva Kovkova, Lapko, Lavrinenko, Lobanok, Loiko, Lutsko, Melnov, Merkulova, Milyutin, Mishatkina, Miskevich, Mosse, Naumenko, Novikova, Obolonkin, Piven, Potapnev, Romanovskaya, Rogov, Savanovich, Sautkina, Seliavko, Shavlov, Sheiko, Sholuh, Sychev, Tolstaya, Yurin und Zafranskaya.

Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation mit dem Titel „Genetic determinants of breast cancer susceptibility in the Byelorussian population“ selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.
Die Dissertation wurde nicht schon als Diplom oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 16. Juni 2008



Natalia Bogdanova