OULU 2008
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ACTA UNIVERSITATIS OULUENSIS

Hannele Erkko

TOPBP1, *CLSPN* AND *PALB2* GENES IN FAMILIAL BREAST CANCER SUSCEPTIBILITY

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TOPBP1, *CLSPN* AND *PALB2* GENES IN FAMILIAL BREAST CANCER SUSCEPTIBILITY

Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium 5 of Oulu University Hospital, on December 19th, 2008, at 12 noon

OULUN YLIOPISTO, OULU 2008

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Acta Univ. Oul. D 996, 2008

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ISBN 978-951-42-8967-5 (Paperback)
ISBN 978-951-42-8968-2 (PDF)
http://herkules.oulu.fi/isbn9789514289682/
ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)
http://herkules.oulu.fi/issn03553221/

Cover design
Raimo Ahonen

OULU UNIVERSITY PRESS
OULU 2008

Erkko, Hannele, *TOPBP1*, *CLSPN* and *PALB2* genes in familial breast cancer susceptibility
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Acta Univ. Oul. D 996, 2008
Oulu, Finland

Abstract

The currently known susceptibility genes account for approximately 25% of familial breast cancer predisposition. Additional factors contributing to the pathogenesis of breast cancer are, therefore, likely to be discovered. Most of the known genes affecting breast cancer predisposition function in the DNA damage response pathway. In this study three genes, *TOPBP1*, *CLSPN* and *PALB2*, involved in this complex process were investigated to reveal potentially pathogenic mutations associated with breast cancer susceptibility.

In the analysis of the *TOPBP1* gene, one novel putative pathogenic alteration was observed. The Arg309Cys variant was found at an elevated frequency among familial cases (19/125) vs. controls (49/697) (p = 0.002; OR 2.4; 95% CI 1.3–4.2). In addition, altogether 18 other germline alterations were observed in this gene, but they all appeared to be harmless polymorphisms.

Investigation of *CLSPN* alterations among familial breast cancer families revealed altogether seven different changes. No clearly pathogenic alterations were observed. However, a potential modifier effect was discovered for the 1195delGlu change. The obtained results suggest that *CLSPN* alterations are unlikely to be significant breast cancer susceptibility alleles.

In the *PALB2* gene, a pathogenic mutation c.1592delT was identified at an elevated frequency among breast cancer patients (0.9%) compared to controls (0.2%) (p = 0.003, OR 3.94, 95% CI 1.5–12.1). Among familial cases the frequency of c.1592delT was even higher (2.7%). This mutation was also functionally deficient. It had a markedly decreased BRCA2-binding affinity and was unable to support homologous recombination or to restore cross link repair in PALB2 knock-down cells. Additionally, this mutation was discovered in a familial prostate cancer family and was found to segregate with the disease, suggesting some association also with prostate cancer.

The penetrance and hazard ratio associated with *PALB2* c.1592delT were determined in unselected breast cancer families. A substantially increased risk of breast cancer (HR 6.1; 95% CI 2.2–17.2; p = 0.01) was discovered resulting in an estimated 40% (95% CI 17–77) breast cancer risk by age 70 years, comparable to that for carriers of *BRCA2* mutations. This markedly increased cancer risk suggests that genetic counselling for carriers is needed and screening for this mutation should be considered.

Keywords: breast neoplasms, CLSPN, Genetic predisposition to disease, mutation, PALB2, penetrance, TOPBP1

Acknowledgements

This study was carried out in the Department of Clinical Genetics, University of Oulu and Oulu University Hospital during 2004–2008. I wish to express my gratitude to all those who participated in this project.

The head of the Department of Clinical Genetics Professor Jaakko Ignatius is thanked for providing excellent research facilities and for the opportunity to conduct research at this department.

My thesis supervisor Professor Robert Winqvist is acknowledged for his guidance and support during this project and for giving me the opportunity to work on such an interesting field of research.

The official referees of this thesis: Professor Aarno Palotie and Docent Minna Nyström are thanked for their invaluable comments to improve this thesis. The language revision of Anna Vuolteenaho is also gratefully acknowledged.

My co-authors and collaborators, Professor Lauri Aaltonen, Dr Daphne W Bell, Dr. James Dowty, Dr Ronny I Drapkin, Dr Mervi Grip, Dr Daniel A Haber, Professor Kaija Holli, Professor John L Hopper, Docent Arja Jukkola-Vuorinen, Professor Anne Kallioniemi, Docent Vesa Kataja, Professor Juha Kere, Professor Veli-Matti Kosma, Dr Guilan Li, Professor David M. Livingston, Docent Arto Mannermaa, Henna Mattila M.Sc., Dr Alexander Miron, Dr Aki Mustonen, Dr Helmut Pospiech, Dr Kaarina Reini, Professor Johanna Schleutker, Dr Qing Sheng, Professor Ylermi Soini, Dr Melissa Southey, Dr Kirsi Syrjäkoski, Professor Juhani E Syväoja and Dr Bing Xia deserve my sincere thanks for their important contribution to the research projects included in this thesis.

My colleagues and some co-authors as well, Katri Pylkäs, Ph.D., Katrin Rapakko, Ph.D., Sanna-Maria Karppinen, M.Sc, Jenni Nikkilä, M.Sc, Szilvia Sólyom, M.Sc Mikko Vuorela, M.Sc and Maria Haanpää, MD are thanked for creating an inspiring and supportive atmosphere in the lab and for great collaboration in various research projects. The technical assistance provided by Helmi Konola and Arja Tapio is also greatly appreciated. In addition, all current and former members of the laboratory of cancer genetics are acknowledged. The staff members at the laboratory of clinical genetics and the department of clinical genetics are also thanked for their assistance and excellent company.

My family and friends, without you I could not have completed this project. First, I want to thank my mother Anna-Maija for always having faith in me and for all her support. My sisters Irene, Elina and Anni and their families are thanked for their encouragement and good times spent together. My friends, especially

Anne, Annika, Pia and Tiina, are thanked for great friendship, invaluable help with scientific matters and also for the funny emails that brightened up some of the not-so-great moments. Finally, my loving thanks to my wonderful fiancé Timo for his unfailing support and love.

I also wish to express my gratitude to all the patients and their family members for participating in this study.

Financial support for this thesis from the Finnish Cultural Foundation, the Maud Kuistila Memorial Foundation, the Cancer Society of Northern Finland, the Emil Aaltonen Foundation, the Finnish Breast Cancer Group, the Finnish Cancer Society, Oulu University Scholarship Foundation, University of Oulu and Oulu University Hospital is most gratefully acknowledged.

Oulu, November 2008 Hannele Erkko

Abbreviations

AML	acute myelogenous leukaemia
AT	ataxia telangiectasia
ATM	ataxia telangiectasia mutated
BARD1	BRCA1-associated RING domain 1 gene
BC	breast cancer
BRCA1/BRCA2	breast cancer gene 1/2
bp	base pair(s)
BRIP1/ *BACH1*	BRCA1-interacting protein 1/BRCA1 C-terminal helicase 1 gene
BRCT	BRCA1 carboxyterminal
C-terminal	carboxyterminal
CHEK1/2	checkpoint kinase 1/2 gene
CI	confidence interval
Claspin	CHEK1 large associated protein
CLSPN	Claspin gene
CSGE	conformation sensitive gel electrophoresis
DCIS	ductal carcinoma *in situ*
DDR	DNA damage response
DSB	double-strand break
DSBR	double-strand break repair
ESE	exonic splicing enhancer
ER	oestrogen receptor
PR	progesterone receptor
FA	Fanconi anaemia
FA-N	Fanconi anaemia subtype N
FANCD1	Fanconi anaemia gene D1 (BRCA2)
FANCN	Fanconi anaemia gene N (*PALB2*)
FANCJ	Fanconi anaemia gene J (*BRIP1*)
Fs	Frame shift
HER2/ERBB2	human epidermal growth factor receptor 2
IDC	infiltrating ductal carcinoma
IHC	immunohistochemistry
ILC	infiltrating lobular carcinoma
HDGC	hereditary diffuse gastric cancer
HR	homologous recombination or hazard ratio (in study IV)

IR	ionizing radiation
kb	kilobase(s)
KD	kilo Dalton
LCIS	lobular carcinoma *in situ*
LCL	lymphoblast cell line
LOH	loss of heterozygosity
MLPA	multiplex ligation-dependant probe amplification
MMC	mitomycin C
MRN	MRE11, RAD50 and NBS1 complex
NBS	non-breast cancer combined
NBS	Nijmegen breakage syndrome
N-terminal	amino-terminal
NHEJ	non-homologous end-joining
NLS	nuclear localization signal
OR	odds ratio
p	short arm of chromosome
p53	tumour protein 53
PAF	population attributable fraction
PALB2	gene for partner and localizer of BRCA2
PJS	Peutz-Jeghers syndrome
q	long arm of chromosome
SCE	sister-chromatid exchange
SNP	single nucleotide polymorphism
STK11	serine/threonine kinase 11
TOPBP1	gene for topoisomerase binding protein 1
TP53	gene for tumour protein 53

List of original articles

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

I Karppinen S-M, Erkko H, Reini K, Pospiech H, Heikkinen K, Rapakko K, Syväoja JE & Winqvist R (2006) Identification of a common polymorphism in the *TopBP1* gene with hereditary susceptibility to breast and ovarian cancer. Eur J Cancer 42: 2647–2652.

II Erkko H, Pylkäs K, Karppinen SM & Winqvist R (2008) Germline alterations in the *CLSPN* gene in breast cancer families. Cancer Lett 261: 93–97.

III Erkko H*, Xia B*, Nikkilä J, Schleutker J, Syrjäkoski K, Mannermaa A, Kallioniemi A, Pylkäs K, Karppinen S-M, Rapakko K, Miron A, Sheng Q, Li Q, Mattila H, Bell DW, Haber DA, Grip M, Reiman M, Jukkola-Vuorinen A, Mustonen A, Kere J, Aaltonen LA, Kosma V-M, Kataja V, Soini Y, Drapkin RI, Livingston DM & Winqvist R (2007) A recurrent mutation in *PALB2* in Finnish cancer families. Nature 446: 316–319.

IV Erkko H*, Dowty JG.*, Nikkilä J, Syrjäkoski K, Mannermaa A, Pylkäs K, Southey MC, Holli K, Kallioniemi A, Jukkola-Vuorinen A, Kataja V, Kosma V-M, Xia B, Livingston DM, Winqvist R & Hopper JL (2008) Penetrance analysis of *PALB2* c.1592delT founder mutation. Clin Cancer Res 14: 4667–4671.

In addition, some unpublished information is discussed.

* Equal contribution

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1 Introduction

Breast cancer is the most common malignancy in females in the Western world. Each year in Finland approximately 4000 women are diagnosed with this disease (Finnish Cancer Registry). The prevalence of breast cancer is increasing which is partly due to better diagnostics but also because of the aging of population and several environmental effects. The five year survival ratio for breast cancer patients is approximately 86% (Gondos *et al.* 2008) but if the disease is diagnosed at a late stage, the prognosis remains poor. Therefore, identifying individuals who are genetically susceptible to develop malignancy is important for improving early diagnostics and treatment.

It is estimated that 5–10% of breast cancer is caused by significant hereditary predisposition (Claus *et al.* 1996). The major susceptibility genes involved in familial breast cancer susceptibility are *BRCA1* and *BRCA2* (Miki *et al.* 1994, Wooster *et al.* 1995). Mutations in these genes account for a great majority of families with a clearly dominant inheritance pattern breast and ovarian cancer (Ford *et al.* 1998). However, these genes are only estimated to account for about 20–25% of the overall hereditary breast cancer risk (Thompson & Easton 2004, Antoniou & Easton 2006). In northern Finland, *BRCA1* and *BRCA2* mutations are only found in 12% of familial breast cancer patients (Huusko *et al.* 1998). Additional susceptibility genes, most of which are involved in certain cancer syndromes, such as *TP53* (mutated in Li-Fraumeni syndrome) and *PTEN* (involved in Cowden syndrome) exist but mutations in these genes are very rare and account for less than 5% of cancer families. The search for additional candidate genes, therefore, remains an important task to be pursued.

Since the discovery of *BRCA1* and *BRCA2*, several attempts were made to discover similar genes through linkage studies but these studies have not been successfully replicated and have failed to identify causative mutations. It has, therefore, been suggested that the remaining unexplained breast cancer predisposition could be attributed to the polygenic model. This theory proposes that most familial cancers are due to combinations of mutations acting together multiplicatively while the effect of a single mutation can be quite small. These types of mutations are estimated to have lower penetrance and probably only affect a small proportion of cancer families (Pharoah *et al.* 2002, Antoniou & Easton 2003).

Most of the known breast cancer susceptibility genes are involved in the DNA damage response pathway. Therefore, other genes involved in this process

represent excellent candidates for identifying further cancer predisposing alleles. This approach has gained some success with the identification of, for example, mutations in the *CHK2* and *ATM* genes (Meijers-Heijboer *et al.* 2002, Pylkäs *et al.* 2007). Genes involved in e.g. cell cycle regulation, carcinogen and hormone metabolism or the immune system have also been investigated as potential candidates relating to breast cancer predisposition (Dunning *et al.* 1999). Identifying patients with a defective DNA damage response is also important because of newly developed potential cancer treatments which particularly target tumour cells with defects in e.g. Fanconi anemia-BRCA pathway.

The aim of the current study was to identify further susceptibility genes by searching for pathogenic mutations in the *TOPBP1*, *CLSPN* and *PALB2* genes. These candidate genes were chosen based on their involvement in the DNA damage response and their interaction with known susceptibility genes. The coding regions and exon-intron boundaries of these genes were screened for mutations in breast families exhibiting properties of familial predisposition to malignancy. The prevalence of the observed variants was also studied in controls in order to determine potential pathogenicity. Bioinformatic analyses were performed to assess the potential functional significance of the changes observed. In addition, the cancer-related *PALB2* c.1592delT mutation was further examined to determine a more precise hazard ratio and penetrance associated with this mutation in unselected breast cancer families. Prior to commencing this study, mutations in *PALB2*, *CLSPN* and *TOPBP1* had never been studied in relation to hereditary breast cancer predisposition.

2 Review of the literature

2.1 Concept of cancer

Cancer is a genetic disease caused by multiple mutations occurring in somatic cells. These mutations usually affect genes involved in cell differentiation and proliferation. The hallmarks of cancer cells are self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, avoidance of apoptosis, unlimited replicative potential, capacity for angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg 2000).

It has been estimated that cancer development requires at least 3–7 consecutive mutations (Miller 1980, Weinberg 1989, Vogelstein & Kinzler 1993). Considering that the estimated rate of mutations in normal cells is approximately 1.4×10^{-10} mutations per base pair per cell generation and assuming that the DNA repair machinery is functioning properly, the probability of acquiring this many mutations in a single cell seems very low (Loeb 1991). However, it has been suggested that certain mutations may affect genomic stability and thus increase the mutation rate (Loeb 1991, Loeb 1994). Mutations may also result in increased cell proliferation, which would give the mutated cell a growth advantage compared to corresponding normal cells (Nowell 1976).

Genes involved in tumourigenesis are usually divided into proto-oncogenes and tumour suppressors. Proto-oncogenes are involved in stimulating cell proliferation and differentiation. Their protein products are mainly involved in signal transduction pathways and include growth factors, growth factor receptors, signal transducers and transcription factors. Proto-oncogenes can be activated to become dominantly acting oncogenes through a variety of mechanisms, including point mutations within the gene and amplification of the entire gene and the activation results in growth advantage for cells (Koorstra *et al.* 2008).

Tumour suppressor genes are considered to be genes that normally control cell division and apoptosis or are involved in the repair of DNA damage. The inactivation of a tumour suppressor gene can lead to cancer development. The classic Knudson "two-hit" model derived from studying familial retinoblastoma suggests that both alleles of a tumour suppressor need to be inactivated for cancer to develop (Knudson 1971). In cases with familial predisposition to cancer, the first hit is an inherited mutation and the second hit occurs by somatic loss of the wild-type allele in the tumour. In the case of retinoblastoma, somatic loss of the

RB1 gene is microscopically visible in chromosome preparations (Knudson 1971). Inactivation of the wild-type allele can also occur through hypermethylation of the promoter region of the gene. All tumour suppressor genes do not behave like classic tumour suppressors, e.g. LOH (loss of heterozygosity) is not commonly observed in many genes, suggesting that alternate mechanisms of tumourigenesis exist. Suggested pathways include e.g. haploinsufficiency and dominant-negative effect of certain mutations. Haploinsufficiency means that the single functional allele does not produce sufficient amounts of the protein in question (Wilkie 1994). Dominant-negative mutations on the other hand produce stable proteins that are functionally defective and interfere with the function of the non-mutated protein (Payne & Kemp 2005).

Tumour suppressors are further divided into gatekeepers and caretakers. Caretakers function to protect the genome against damage and mutations, while gatekeeper genes induce cell death or cell cycle arrest of cells in which damage has occurred (Kinzler & Vogelstein 1997).

2.2 Characteristics of breast cancer

Breast cancer is the most common female malignancy in the Western world. On the basis of tumour histology, breast cancers can be divided into various subtypes. The most common of these are ductal (about 75%) and lobular (15%) carcinomas. Rare subtypes accounting for about 10% of breast cancers include e.g. medullary, mucinous, papillary and tubular carcinomas and Paget's disease (Li *et al.* 2003, Li *et al.* 2005).

Based on the mRNA expression patterns, five distinct subtypes of breast cancer can be defined: Luminal A, Luminal B, ERBB2-overexpressing (human epidermal growth factor receptor 2, also called HER2 or NEU), basal-like, and normal-like (Perou *et al.* 2000). These subtypes are associated with different clinical outcomes and treatment options (Sorlie *et al.* 2001). Approximately 85% of breast tumours belong to luminal subtypes A and B. These are oestrogen receptor and progesterone receptor positive and express cytokeratin 18. Basal-like tumours comprise about 3–15% of breast cancers and are oestrogen receptor, progesterone receptor and HER2 negative (Sorlie *et al.* 2003) but positive for basal cytokeratins, epidermal growth factor receptor (EGFR) or KIT protein (Nielsen *et al.* 2004, Kim *et al.* 2006). These different expression-based subtypes of breast cancer may actually be different diseases which merely occur in the same anatomical location.

2.3 The genetics of familial breast cancer

Breast cancer is a common and heterogenic disease. The majority of breast cancers occur in individuals without a significant familial history of cancer. However, having a first-degree relative diagnosed with breast cancer incurs an approximately twofold increased breast cancer risk (Pharoah *et al.* 1997). It is estimated that approximately 5–10% of breast cancer cases have a familial background (Claus *et al.* 1996). Some studies have even suggested that up to 30% of all breast cancers could be due to heritable factors (Lichtenstein *et al.* 2000). The major susceptibility genes are *BRCA1* (chapter 2.3.1) and *BRCA2* (chapter 2.3.2) (Miki *et al.* 1994, Wooster *et al.* 1995). Mutations in *BRCA1* and *BRCA2* are responsible for a great majority of families exhibiting a dominant disease inheritance pattern for both breast and ovarian cancers (Ford *et al.* 1998). In families with less breast cancer cases and in the absence of ovarian or male breast cancer, the contribution of these genes is much less significant. Other high-risk genes include e.g. *TP53*, *PTEN*, *STK11*/*LKB1* and *CDH1,* although the cancer risk associated with mutations in these genes is less well defined than those of *BRCA1* and *BRCA2* (Turnbull & Rahman 2008). Mutations in all the currently identified susceptibility genes account for about 20–25% of familial breast cancer (Thompson & Easton 2004, Antoniou & Easton 2006).

The polygenic theory of breast cancer susceptibility suggests that the residual unexplained familial cancer risk may be due to several variants. Each variant alone may only cause a small and often undetectable increase in cancer risk but the combined effect of several variants could be sufficient for tumourigenesis. These effects are also likely to be influenced by environmental factors (Pharoah *et al.* 2002, Antoniou & Easton 2003, Antoniou & Easton 2006).

2.3.1 BRCA1 gene

By performing a genetic linkage analysis in 23 early-onset breast cancer families, the *BRCA1* gene was first localized to chromosome 17q21 (Hall *et al.* 1990) and was subsequently cloned (Miki *et al.* 1994). *BRCA1* consists of 22 coding exons and encodes a protein of 1863 amino acids. Highly conserved functional motifs of BRCA1 include the N-terminal ring finger domain which is involved in interaction with BARD1 and two C-terminal BRCT (BRCA carboxy terminal) domains, which are found in many DNA damage repair proteins and are involved

in numerous protein-protein interactions with binding partners such as BRIP1, Abraxas (ABRA1) and CtIP (Yu *et al.* 1998, Cantor *et al.* 2001, Wang *et al.* 2007).

BRCA1 functions in the maintenance of genomic integrity and in homologous recombination mediated DNA repair. Other functions of this protein include e.g. cell cycle checkpoint control, chromatin remodelling, DNA decatenation and ubiquitylation (Narod & Foulkes 2004, Ashworth 2005, Lou *et al.* 2005, Gudmundsdottir & Ashworth 2006, Turner & Reis-Filho 2006). BRCA1 also regulates the transcription of some genes, such as the oestrogen receptor (Narod & Foulkes 2004, Turner & Reis-Filho 2006).

2.3.2 BRCA2 gene

The *BRCA2* gene was first localized by analysing 15 high-risk breast cancer families that did not show linkage to the *BRCA1* locus on chromosome 17q21. This analysis mapped *BRCA2* to chromosome 13q12–q13 (Wooster *et al.* 1994, Wooster *et al.* 1995). *BRCA2* mutations confer a high risk of breast cancer but compared to *BRCA1* mutations, they do not incur an equally elevated risk of ovarian cancer (Wooster *et al.* 1994, Wooster *et al.* 1995). Other cancer types associated with *BRCA2* mutations include male breast and prostate cancer. The *BRCA2* gene has 27 exons and gives rise to a protein of 3418 amino acids (Tavtigian *et al.* 1996).

BRCA2 functions primarily in homologous recombination (HR) and HR-based DNA double-strand break repair (DSBR) (Shivji & Venkitaraman 2004) and has additional functions in cell growth and DNA damage checkpoint control (Xia *et al.* 2006). BRCA2 also has transcriptional and co-transcriptional activities (Milner *et al.* 1997, Shin & Verma 2003). In addition, BRCA2 is a Fanconi anaemia (FA) protein FA-D1 (Howlett *et al.* 2002).

Cancer risk for carriers of BRCA1/2 mutation

Pathogenic mutations in *BRCA1* and *BRCA2* have been reported with highly variable frequencies in different populations. Hundreds of truncating mutations have been reported. In addition, there are several pathogenic missense mutations as well as a large proportion of variants whose clinical significance is currently uncertain. The classification of particularly missense changes as pathogenic or neutral can be difficult, and as knowledge of these genes increases, more pathogenic missense mutations may be discovered in both *BRCA1* and *BRCA2*.

Estimates of the cancer risk caused by BRCA1/2 mutations are highly variable depending on the population studied and/or the mutation in question. The risk may also be dependent on modifier genes or alleles that can enhance or reduce the cancer risk in certain families. A large population-based study determined that for *BRCA1* mutations the risk would be 65% (95% CI 44–78%) for breast cancer and 39% (18–54%) for ovarian cancer. For *BRCA2*, the corresponding figures were estimated to be 45% (31–56%) for breast cancer and 11% (2.4–19%) for ovarian cancer (Antoniou *et al.* 2003). Much higher estimates have also been presented, but these may be subject to ascertainment bias because the approximations are mainly derived from studying families with an extremely elevated familial cancer risk, such as people attending genetic counselling to determine their cancer risk.

2.3.3 TP53 (Li-Fraumeni syndrome)

The *TP53* gene is located on chromosome 17p13.1. Its protein product, p53, is involved in many cellular pathways that regulate the cell cycle, apoptosis and DNA repair. *TP53* is considered to act as guardian of the genome, and inactivation of this gene results in suppression of the mechanisms that prevent DNA damage. Germline mutations in this gene are extremely rare, with less than 400 families reported worldwide. In contrast, somatic mutations in *TP53* are quite common and are observed in 20–60% of breast cancers. (Malkin *et al.* 1990, Pluquet & Hainaut 2001, de Jong *et al.* 2002).

Li-Fraumeni syndrome (LFS) is characterized by several primary malignancies in children and adolescents. Cancer types associated with this syndrome include soft tissue sarcomas, osteosarcomas, breast cancer, brain tumours, leukaemia and adrenocortical tumours. Mutations in the *TP53* gene account for 70% of families with classic LFS, the criteria of which are: one patient with sarcoma < 45 years with a first-degree relative diagnosed with any cancer < 45 years and an additional first- or second-degree relative with cancer < 45 years or sarcoma at any age. LFS displays an autosomal dominant disease inheritance pattern. The likelihood of a *TP53* mutation carrier developing any malignancy is 50% by age 30 and almost 90% by age 70. The risk of breast cancer, one of the most frequent cancers is *TP53* mutation carriers, is 28–56% by age 45 (Malkin *et al.* 1990, Birch *et al.* 1994, Frebourg *et al.* 1995, Varley *et al.* 1997, Evans *et al.* 2002, Oldenburg *et al.* 2007). *TP53* mutations are less common in families not meeting the stringent criteria of LFS and are rarely detected in

families solely on the basis of increased breast or ovarian cancer susceptibility (Rapakko *et al.* 2001, Evans *et al.* 2002).

2.3.4 PTEN (Cowden syndrome)

Cowden syndrome (CS) is a rare autosomal dominant disorder characterized by multiple hamartomas of the skin, breast, thyroid, endometrial, gastrointestinal tract or the central nervous system and susceptibility to breast, uterine and thyroid cancer. The prevalence of this disorder is estimated to be around 1/300,000. The *PTEN* gene was originally identified through a linkage genome scan analysing 12 families. Approximately 80% of CS patients have mutations in this gene, which is located in chromosome 10q23.3. (Mallory 1995, Nelen *et al.* 1996, Liaw *et al.* 1997, Lindor & Greene 1998, Schweitzer *et al.* 1999). Particularly, truncating mutations of *PTEN* predispose to malignancy (Marsh *et al.* 1998). The risk of breast cancer is estimated to be 25–50% and most CS-related breast cancer occurs after age 30–35 years (Eng 1998). Mutations in *PTEN* are also linked to male breast cancer susceptibility (Fackenthal *et al.* 2001). *PTEN* mutations have not been observed in breast cancer families without the CS features (Chen *et al.* 1998, Carroll *et al.* 1999).

2.3.5 LKB1/STK11 (Peutz-Jeghers syndrome)

LKB1/STK11 is a tumour suppressor gene located in chromosome 19p13.3. This gene was identified by comparative genomic hybridization and targeted linkage analysis. Germline mutations of *LKB1/STK11* cause Peutz-Jeghers syndrome (PJS), which is an autosomal dominant disorder characterized by hamartomatous polyps of the gastrointestinal tract and by melanine pigmentation of the lips, buccal mucosa, fingers and toes. Elevated risks of breast, ovarian, gastrointestinal, pancreatic, uterine, cervical, testicular and lung cancer are associated with this disorder. (Wada *et al.* 1987, Giardiello *et al.* 1987, Spigelman *et al.* 1989, Boardman *et al.* 1998, Westerman *et al.* 1999). The risk of any malignancy for mutation carriers is about 50% and that of breast cancer 29–54% (Giardiello *et al.* 2000, Lim *et al.* 2003). Somatic mutations in *LKB1* and LOH in chromosome 19p13 are considered to be rare in sporadic patients (Bignell *et al.* 1998).

2.3.6 CDH1 (Hereditary diffuse gastric cancer-syndrome)

The *E-cadherin* gene (*CDH1*) is located in chromosome 16q22.1. The CDH1 protein is involved in cell-cell adhesion molecules and the maintenance of cell differentiation and architecture of epithelial cells. The gene was originally identified through linkage analysis in New Zealand Maori families with hereditary diffuse gastric cancer. Sequencing analysis revealed three separate truncating mutations in three families affected by gastric cancer (Guilford *et al.* 1998).

CDH1 mutations exhibit a dominant pattern of inheritance with incomplete penetrance. Female carriers are estimated to have 83% risk of diffuse gastric cancer by age 80. The lifetime risk of breast cancer is estimated to be 20–40% (Berx *et al.* 1996, Pharoah *et al.* 2001, Chan & Wong 2001, Graziano *et al.* 2003). *E-cadherin* mutations are particularly associated with lobular breast cancer (Suriano *et al.* 2005). LOH is frequently observed in tumours of germline mutation carriers, implicating this gene as a tumour suppressor (Berx *et al.* 1996). Somatic mutations of this gene have also been frequently observed in infiltrating and *in situ* lobular breast cancers, but less often in other histopathological subtypes (Berx *et al.* 1996, Sarrio *et al.* 2003, Mastracci *et al.* 2005).

2.3.7 Intermediate risk susceptibility genes

Intermediate risk genes involve genes with lower penetrance compared to *BRCA1* and *BRCA2* mutations. The risks usually range from two- to four-fold compared to non-carriers. There may be markedly varied risks between genes and between different mutations of the same gene. Several genes of this type have been described, some of which are briefly discussed: *ATM, CHEK2* and *BRIP1/BACH1/FANCJ*. Other genes in this category are e.g. *RAD50* and *NBS1* (Walsh & King 2007).

ATM

The *ATM* gene is located in chromosome 11q23. This protein is involved in DNA double-strand break sensing and signalling (Bakkenist & Kastan 2003). Biallelic mutation carriers suffer from ataxia telangiectasia (AT). This disorder is characterized by cerebellar degeneration (ataxia), dilated blood vessels in the skin and eyes (telangiectasia), immunodeficiency, chromosomal instability and

increased sensitivity to radiation. An increased cancer predisposition is also typical of this disorder. Most common cancer types are lymphomas and leukaemias. The estimated incidence of AT varies from 1/40,000 to 1/100,000 and mutation carrier frequency is estimated to range from 1/100 to 1/200. The breast cancer risk of monoallelic female mutation carriers is estimated to be elevated as well (Pippard *et al.* 1988, Swift *et al.* 1990, Easton 1994). The relative risk of BC associated with *ATM* mutations has been estimated to range from 1.3 to 13 (Hall 2005, Thompson *et al.* 2005, Renwick *et al.* 2006). The differences in risk estimates were thought to be due to different types of mutations. Some missense mutations encode stable but functionally deficient proteins which could compete with the normal protein and result in a dominant-negative phenotype. The truncating mutations, however, often produce an unstable protein which is degraded, and individuals retain 50% of wild-type ATM activity resulting in an almost normal phenotype (Gatti *et al.* 1999, Meyn 1999). The results of studies of ATM mutations have been contradictory, with some studies reporting relatively high risks while others observed no significantly increased risks (Tamimi *et al.* 2004, Renwick *et al.* 2006, Pylkäs *et al.* 2007).

CHEK2

The *CHEK2* gene maps to chromosome 22q12.1. This protein functions as G2 (gap2) checkpoint kinase and is involved in DNA repair. In response to ionizing radiation ATM phosphorylates CHEK2, which results in activation of other important cell cycle proteins such as BRCA1 and TP53. The *CHEK2* 1100delC mutation was originally identified in an individual with LFS but no *TP53* mutation (Bell *et al.* 1999). *CHEK2* 1100delC was the first lower penetrance allele associated with breast cancer susceptibility (Meijers-Heijboer *et al.* 2002). This mutation eliminates the kinase activity of CHEK2. *CHEK2* 1100delC is more frequent among *BRCA1/BRCA2*-mutation negative familial breast cancer patients, and the frequency is estimated to range from 4.9% to 11.4% (Meijers-Heijboer *et al.* 2002, Vahteristo *et al.* 2002, Oldenburg *et al.* 2003). This variant is also found at a lower frequency in unselected breast cancer cases and in controls. A recent meta-analysis estimated that the breast cancer risk associated with 1100delC would be about 37% (Weischer *et al.* 2008).

BRIP1/BACH /FANCJ

The *BACH1/BRIP1* gene (BRCA1-interacting protein 1) is located in chromosome 17q22–q24. The protein product of this gene functions as a DEAH helicase, which co-operates with BRCA1. *BRIP1* is also involved in certain DNA damage repair functions of BRCA1. BRIP1 interacts directly with the BRCA1 C-terminal BRCT repeats (Peng *et al.* 2006).

Involvement of *BRIP1* mutations in breast cancer susceptibility has been investigated by several studies (Cantor *et al.* 2001, Luo *et al.* 2002, Karppinen *et al.* 2003), most of which have not found a significant contribution to breast cancer predisposition. However, a recent study identified five different truncating variants in this gene in familial breast cancer patients and estimated that they confer an approximately two-fold increased relative risk to breast cancer (Seal *et al.* 2006). Interestingly, *BACH1/BRIP1* is also *FANCJ*; the gene mutated in Fanconi anaemia subtype J (Levitus *et al.* 2005, Levran *et al.* 2005) and is, therefore, associated with the FA syndrome, which typically includes congenital malformations, cancer predisposition and sensitivity to DNA crosslinking agents (Joenje & Patel 2001).

2.3.8 Common low-risk variants

A new class of susceptibility alleles has been identified, mainly through genome-wide association studies using tag SNPs and to some extent also via analysis of candidate genes. To be confirmed, these kinds of variants need to genotyped in thousands of cases and controls, and identification nearly always requires large multicentre co-operation in order to produce enough samples for this kind of analysis. Therefore, currently only a few variants with statistically undeniable results have been obtained and verified in different populations (Stratton & Rahman 2008).

Low risk variants are associated with ORs ranging from 1.07 to 1.26 for carriers of one risk allele. However, due to their high prevalence (ranging from 28 to 87%) in populations they are estimated to contribute to a significant proportion of familial breast cancer. Variants described by Easton *et al.* (2007) and Cox *et al.* (2007) are thought to be responsible for as much as 3.9% of familial breast cancer predisposition in European populations. Surprisingly, and in contrast to the susceptibility alleles associated with high and moderate risk, the risk-associated

variant may even be more common than the non-risk allele (Stratton & Rahman 2008).

One problem in identifying low-risk variants is that it is often difficult to determine the actual causal variant due to large chromosomal regions of high linkage disequilibrium. Significant associations have even been made to genomic regions that do not harbour any known protein encoding genes. These types of associations have been found in chromosomes 2q and 8q (Easton *et al.* 2007, Hunter *et al.* 2007, Stacey *et al.* 2007). The association in 8q is in the same location as susceptibility variants for prostate and colorectal cancer (Amundadottir *et al.* 2006, Gudmundsson *et al.* 2007, Haiman *et al.* 2007a, Haiman *et al.* 2007b, Tomlinson *et al.* 2007, Yeager *et al.* 2007, Zanke *et al.* 2007). Therefore, the association seems more likely to be contributory than coincidental. The most often recognized low-risk variants are near the following genes: *CASP8*, *FGFR2*, *TNCR9*, *MAP3K1* and *LSP1* (Cox *et al.* 2007, Easton *et al.* 2007, Frank *et al.* 2007, Hunter *et al.* 2007, Stacey *et al.* 2007). However, a recent report suggests that there may be different variants affecting breast cancer susceptibility in different populations. A genome-wide association study conducted in the Ashkenazi Jewish population confirmed the association with *FGFR2* locus. In addition, they identified a novel association with chromosome 6q22.33 with an OR of 1.41. Genes in this region include *ECHDC1* and *RNF146. ECHDC1* is involved in fatty acid oxidation in the mitochondria, and *RNF146* is likely to be an ubiquitin protein ligase (Gold *et al.* 2008).

Interestingly, many of the novel low-penetrance alleles do not seem to be linked to the DNA repair pathway. For example, CASP8 is a protease involved in apoptosis and FGFR2 is a fibroblast growth factor receptor. MAP3K1, on the other hand, is the mitogen-activated kinase 1 and likely contributes to growth signalling. LSP1 encodes the lymphocyte specific protein 1, which is an F-actin binding protein. TNCR9 (also called TOX9) is suggested to be a transcription factor (Easton *et al.* 2007, Hunter *et al.* 2007, Stacey *et al.* 2007). The lack of involvement in the DNA damage response pathway is surprising considering the significant association of high and intermediate penetrance genes in this pathway. However, the abovementioned low penetrance genes are not necessarily the causal genes in cancer susceptibility. The association observed may be linked to other genes located in the same chromosomal region. As the knowledge of the human genome increases, the mechanism by which these variants contribute to breast cancer predisposition is likely to be elucidated in more detail. For example, recently the intronic SNPs in the *FGFR2* gene have been shown to affect

expression levels of the gene and may provide an explanation for its role in cancer susceptibility (Meyer *et al.* 2008).

2.3.9 Searching for novel candidate genes

It is considered unlikely that further high-risk genes like *BRCA1* and *BRCA2* will be identified. Novel high-risk genes could still exist, but the risk alleles are not likely to be as common across populations as the *BRCA1* and *BRCA2* mutations. Therefore, due to genetic heterogeneity identification of these genes will be more difficult.

Further genome-wide association studies might reveal novel breast cancer predisposing genes. It has even been suggested that a substantial proportion of the currently unexplained cancer susceptibility could result from combinations of many low-risk variants. This also includes many of the non-truncating variants in the previously identified susceptibility genes. These variants are often difficult to classify as pathogenic or neutral due to lacking functional analyses because the exact functions of most genes are not completely understood at present. These variants can often only be analysed using bioinformatic methods or by comparing their frequencies between cases and controls. The latter strategy is often not optimal for low-frequency variants, which are probably only observed in a small number of families. Furthermore, segregation studies are also often not successful since penetrance is likely to be incomplete and cancer phenocopies may exist in the studied families.

Moderate risk variants do not always produce families with a high number of breast cancer cases. Therefore, identifying these types of changes through linkage analysis seems difficult. In addition, variants of this kind are so rare that their identification through genome-wide association studies is unlikely and large sample sizes are required to improve the likelihood of detecting lower penetrance genes. The likelihood of identifying such a gene can be somewhat enhanced by the use of familial instead of unselected breast cancer cases (Antoniou *et al.* 2003, Houlston & Peto 2003). In addition, genetically isolated populations are more likely to harbour founder mutations with higher frequency than mixed populations (Antoniou & Easton 2003).

Most moderate risk variants, such as *RAD50* and *CHEK2*, have been identified via analysis of candidate genes involved in the DNA damage-response pathway (Meijers-Heijboer *et al.* 2002, Heikkinen *et al.* 2003). Genes co-

operating in this pathway, therefore, represent good potential candidates as breast cancer susceptibility alleles.

2.4 The DNA damage response pathway

A functional DNA damage response pathway is absolutely essential for cell viability. Several types of DNA damage can occur in cells. These include single- and double-strand breaks, DNA-protein cross-links and damaged bases. Diverse pathways function to repair damages of different types. In mammalian cells at least four main pathways of DNA damage repair (DDR) exist: nucleotide excision repair (NER), base excision repair (BER), homologous recombination (HR) and non-homologous end joining (NHEJ) (Hoeijmakers 2001). All of these mechanisms are also likely to co-operate at least to some extent in the repair of damaged DNA.

NER is the repair system responsible for removing a variety of large, helix-distorting lesions and adducts induced by environmental agents or metabolic products (Wood 1997, de Boer & Hoeijmakers 2000). BER repairs mainly single-strand breaks and small DNA damage caused by oxidation processes (Memisoglu & Samson 2000).

DNA double-strand breaks (DSB) are the most serious type of DNA damage. Exogenous sources of DSBs include ionizing radiation, chemical agents, UV light and cancer chemotherapeutics. DSBs can also be produced by internal processes, such as reactive oxygen species generated by cellular metabolism, collapsed replication forks, V(D)J recombination, nucleases, and in cellular division. If unrepaired, a DSB can result in cell death or chromosomal alterations such as translocations, inversions or duplications or chromosome loss (Shrivastav *et al.* 2008). DSB lesions are repaired by two main pathways: HR and NHEJ, and defects in these pathways cause genomic instability and promote tumourigenesis (Thompson & Schild 2002, Valerie & Povirk 2003, Shrivastav *et al.* 2008).

High-fidelity HR is the prominent repair mechanism in S and G2 phases of the cell cycle when DNA has replicated and the undamaged strand can be used as a template for resynthesis. The more error-prone NHEJ is used as a repair mechanism mainly in the G1 phase when no template is available for HR (Rothkamm *et al.* 2003). The choice of repair mechanism can also be dependent on type of DSB. Frank DSBs produced by IR or nucleases can be repaired by either mechanism. Collapsed replication forks on the other hand produce one-ended DSBs, which are almost entirely repaired by HR (Rothstein *et al.* 2000).

The NHEJ protein complex consists mainly of the DNA binding KU70/KU80 heterodimer, the phosphoinositide kinase-related kinase (PIKK) family member DNA-PK, and the XRCC4/Ligase IV DNA ligase (Lieber *et al.* 2003). DNA-PK and ligase IV act together and ensure the re-joining of broken non-compatible DNA ends. The KU70/KU80 recognizes the break and acts as a mediator in recruiting the DNA-PK catalytic subunit (DNA-PKcs), which is a kinase essential for NHEJ to function effectively (Lees-Miller & Meek 2003, Bassing & Alt 2004, Burma & Chen 2004).

HR uses the sister chromatid as template DNA to achieve accurate repair (Lieber *et al.* 2003, West 2003, Helleday *et al.* 2007). Initiation of HR includes DSB processing by the MRE11-RAD50-NBS1 complex (MRN) in collaboration with CtIP to generate 3' single-stranded DNA that becomes bound by RAD51 to facilitate homology search and strand invasion (Limbo *et al.* 2007, Sartori *et al.* 2007, Takeda *et al.* 2007). The RAD51 recombinase functions with a series of other factors including the RAD51 paralogues, RAD52 and RAD54 proteins in order to endorse strand invasion and subsequent recombinational repair (West 2003, Thacker & Zdzienicka 2004, Wyman *et al.* 2004, Heyer *et al.* 2006, Helleday *et al.* 2007). Several members of Fanconi anaemia pathway are also linked to proper functioning of HR (Wang 2007). A simplified version of the components of NHEJ and HR and other proteins of the DNA damage response is presented in Figure 1.

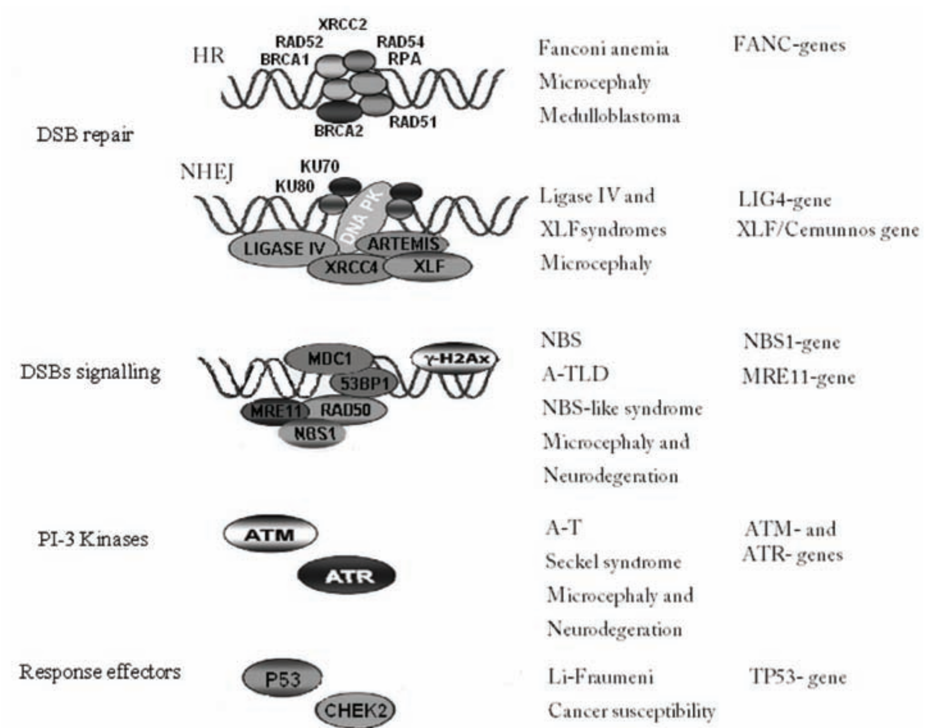

Fig. 1. A simplified representation of the DNA double-strand break response and examples of human syndromes associated with defects in certain parts of this pathway and the causative genes involved when known. Modified from Frappart & McKinnon 2008. A-TLD, ataxia telangiectasia-like disorder.

2.4.1 Involvement of the Fanconi anaemia-BRCA pathway in the repair of damaged DNA

An undisturbed Fanconi anaemia pathway is essential for HR to function properly. The Fanconi anaemia pathway is activated by ATR in response to replication stress and functions during the S-phase of the cell cycle (Andreassen *et al.* 2004, Pichierri & Rosselli 2004, Wang 2007). The FA core complex consists of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCM, FANCL, FAAP100 (Fanconi anaemia-associated polypeptide with a molecular weight of 100 KD) and FAAP24 (Fanconi anaemia-associated polypeptide with a molecular weight of 24 KD). Several members of the core complex are hyperphosphorylated in

response to DNA damage and could serve as signal transducers enabling ATR to regulate the core complex (Wang 2007).

The FA core complex is essential for monoubiquitylation of the FA-ID-complex and deficiencies in any of the core complex proteins result in faults in this process. The FA-ID complex is formed by FANCD2 and FANCI (Garcia-Higuera *et al.* 1999, Garcia-Higuera *et al.* 2001, Meetei *et al.* 2003, Meetei *et al.* 2005). The amount of monoubiquitylated FANCD2 is increased during replication and when DNA damage is encountered (Garcia-Higuera *et al.* 2001, Taniguchi *et al.* 2002). The importance of monoubiquitylation of FANCD2 might be the result of the increased association of FANCD2 with chromatin when ubiquitylated, while the non-ubiquitylated FANCD2 is enriched in soluble cell extract (Meetei *et al.* 2004, Wang *et al.* 2004). The downstream proteins of the Fanconi anaemia pathway include FANCD1 (BRCA2), FANCJ (BRIP1) and FANCN (PALB2) (Figure 2). Defects in the downstream components do not affect the monoubiquitylation of FANCD2 but nevertheless cause FA. FANCD1 and FANCN function mainly in the homologous recombination pathway. FANCJ has been suggested to be downstream mediator of BRCA1 function, but its exact role remains elusive. Several proteins interact with the FA pathway, including BRCA1, CHEK1, TOPBP1, BLM, NBS1 and γH2AX (Wang 2007) and they co-operate to repair damaged DNA.

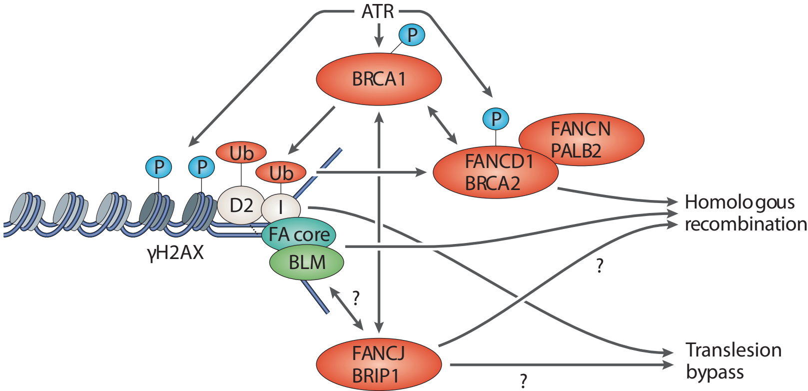

Fig. 2. The involvement of the FA-BRCA pathway in response to DNA damage. Adapted by permission from MacMillan Publishers Ltd: [Nature reviews Genetics] Weidong Wang: Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Copyright (2007).

2.5 BRCA1-interacting genes as candidates for involvement in familial breast cancer susceptibility

BRCA1 mutations account for a significant proportion of familial breast cancer cases. Genes functioning in similar tasks or interacting directly with BRCA1 are considered to be good candidates to identify novel predisposing genes. Some BRCA1- interacting genes such as *BRIP1* (chapter 2.3.7) have been already implicated in the pathogenesis of familial breast cancer (Seal *et al.* 2006).

2.5.1 TOPBP1 has structural and functional similarities with BRCA1

TOPBP1 (topoisomerase binding protein 1) gene maps to chromosome 3q22.1 and encodes a 1,522-amino-acid protein comprising 29 exons (Mäkiniemi *et al.* 2001). The protein structure is depicted in Figure 3. TOPBP1 was originally identified through its association with DNA topoisomerase IIβ (Yamane *et al.* 1997). TOPBP1 has eight phosphoprotein binding BRCT domains that have been found in many proteins involved in DNA repair and cell cycle regulation (Callebaut & Mornon 1997, Yamane *et al.* 1997). In the C-terminal part of TOPBP1, the two BRCT domains display considerable similarity to the corresponding region of BRCA1 (Yamane *et al.* 1997, Mäkiniemi *et al.* 2001).

In addition to the sequence homology, TOPBP1 also shares many other features with BRCA1. The expression of both proteins is highest in S-phase cells, and in response to DNA damage or replication blocks both TOPBP1 and BRCA1 are phosphorylated by ATM and co-localize to sites of DNA damage at IR-induced nuclear foci or arrested replication forks, respectively (Cortez *et al.* 1999, Mäkiniemi *et al.* 2001, Yamane *et al.* 2002). The localization patterns of TOPBP1 and BRCA1 also have similarities during late mitosis as well as in meiotic prophase I (Reini *et al.* 2004). Furthermore, the two proteins have been shown to possess overlapping functions in G2/M checkpoint regulation and to be required for activation of CHEK1, a downstream substrate of ATR, in response to DNA damage (Yamane *et al.* 2003). In intact cells, TOPBP1 associates with ubiquitin ligase hHYD, and ubiquitination leads to degradation of the protein through the proteosome pathway (Honda *et al.* 2002). IR-induced phosphorylation inhibits TOPBP1 ubiquitination, stabilising the protein, and stimulates its co-localization with BRCA1 and several other molecules critical for DSB DNA repair, including the immediate DNA damage marker H2AX, PML, RAD50, ATM, RAD9, BLM, NBS1 and 53BP1 (Honda *et al.* 2002, Yamane *et al.* 2002, Xu *et al.* 2003).

Expression studies have shown that TOPBP1 is required for normal cell survival, since down-regulation of TOPBP1 results in reduced cell viability due to increased apoptosis (Yamane *et al.* 2002).

TOPBP1 has homologues in yeast (*Schizosaccharomyces pombe* Rad4/Cut5 and *Saccharomyces cerevisiae* Dpb11 proteins) and fly (*Drosophila melanogaster* Mus101 protein), which are involved in DNA damage and replication checkpoint pathways (Yamamoto *et al.* 2000, Mäkiniemi *et al.* 2001, Yamane *et al.* 2002).

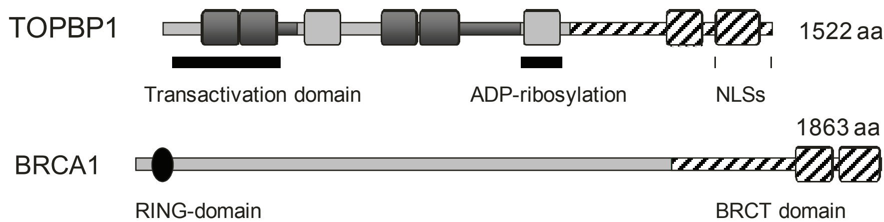

Fig. 3. TOPBP1 protein structure and comparison with BRCA1. The major functional domains of both proteins are marked. BRCT domains are boxed. Stripes refer to the BRCT domain at the C-terminus shared by TOPBP1 and BRCA1; black squares represent homology between TOPBP1 and Cut5. NLS, nuclear localization signal. Figure modified from Figure 1 in study I.

2.5.2 Claspin interacts with CHEK1 and BRCA1

Claspin (CHEK1 large associated protein) was originally identified in *Xenopus* as a CHEK1 interacting protein (Kumagai & Dunphy 2000). Most of the initial studies on claspin function were performed in *Xenopus* egg extracts, but more recent investigations also suggest a similar role for the human homologue (Chini & Chen 2003, Lin *et al.* 2004, Clarke & Clarke 2005, Chini & Chen 2006, Brondello *et al.* 2007).

The human *CLSPN* gene is located in chromosome 1p34 and consists of 25 exons. The structure of the protein product is depicted in Figure 4. This ring-shaped molecule has a C-terminal CHEK1-binding domain (CKBD) and an N-terminal DNA binding domain (DBD) capable of binding to branched DNA structures (Chini & Chen 2004, Sar *et al.* 2004). In addition, claspin has several SQ/TQ (serine-glutamine/threonine-glutamine) motifs that are potential substrates for kinases such as ATM and ATR (Chini & Chen 2004).

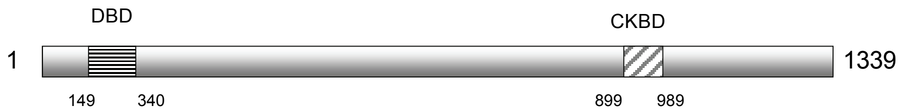

Fig. 4. Claspin protein structure showing the major functional domains. Figure modified from Figure 1 in study II.

Claspin is a cell cycle-regulated protein with peak expression at S/G_2 phase. Down-regulation of claspin promotes the premature chromatin condensation induced by DNA damaging agents and inhibits the UV-induced reduction of DNA synthesis and results in decreased cell survival (Chini & Chen 2003). The expression of claspin is upregulated in cancer cell lines and tumour specimens and may prove to be a sensitive marker for tumour proliferation. Current observations indicate that claspin, depending on the circumstances, is involved in both tumour suppressing and cell proliferation promoting functions (Chini & Chen 2003, Tsimaratou *et al.* 2007).

During S-phase claspin is bound to and interacts with chromatin. Claspin may also function as a sensor of DNA replication and is assumed to have a role in the replication checkpoint by associating directly with replication forks (Sar *et al.* 2004, Clarke & Clarke 2005, Brondello *et al.* 2007). Recently, claspin was shown to be critical for normal replication fork progression and may, therefore, affect genome stability (Petermann *et al.* 2008). Claspin is also present in a protein complex containing PCNA, an essential component of the DNA replication machinery, and this complex is released upon DNA replication arrest (Brondello *et al.* 2007). In response to replication stress CHEK1 activation and phosphorylation are initiated by many proteins, including claspin, ATR and RAD9 (Chini & Chen 2003). Interestingly, CHEK1 is also proposed to promote the phosphorylation of CKBD in claspin, maintaining its cellular stability. This suggests a complex relationship between claspin and CHEK1 (Chini *et al.* 2006) although some recent results do not support this association and suggest that a kinase distinct from CHEK1 is involved in claspin phosphorylation (Bennett *et al.* 2008).

Claspin, ATR and the RAD9-RAD1-HUS1 complex also control CDC25A, another significant protein in the S-phase checkpoint function (Sorensen *et al.* 2004). In addition, claspin has been linked to the nucleotide excision repair pathway (Praetorius-Ibba *et al.* 2007). Upon DNA damage, a complex with BRCA1 and claspin is formed. This results in localization of both these proteins

to cell nuclei and contributes to the activation of CHEK1 (Lin *et al.* 2004). Recent evidence also links claspin to the FA pathway because claspin function has been shown to be required for FANCD2 monoubiquitylation (Guervilly *et al.* 2008).

2.6 BRCA2-interacting protein PALB2

2.6.1 Identification

PALB2 (partner and localizer of BRCA2) was first identified through its interaction with BRCA2. Endogenous BRCA2 was immunoprecipitated with a collection of BRCA2 antibodies. Using BRCA2 antibody OP95, a small number of proteins were discovered in whole Hela cell extracts. The major proteins identified were BRCA2 and RAD51. In addition, a novel protein was precipitated and identified as FLJ21816/LOC79728. This protein was later named PALB2 (Xia *et al.* 2006).

Endogenous BRCA2 and PALB2 were shown to interact with each other in the nucleus and the majority of these complexes were associated with nuclear matrix and/or chromatin. PALB2 was also shown to co-localize with BRCA2 and to a lesser extent also with BRCA1 in nuclear foci in S-phase and in DNA damage-induced foci. This suggests that also PALB2 participates in the DNA damage response and homologous recombination. Nuclear BRCA2 abundance and localization appears to be dependent upon PALB2, but PALB2 nuclear abundance and matrix/chromatin association were not significantly affected by RNAi-mediated depletion of BRCA2. Certain missense variants in exon three of *BRCA2* failed to bind PALB2 and were dysfunctional in HR/DSBR. PALB2, therefore, seems to enable certain tumour suppressor and DNA repair functions of BRCA2 (Xia *et al.* 2006).

2.6.2 Structure and function

The putative PALB2 open reading frame consists of 1,186 residues corresponding to a predicted protein size of about 130 KD. The *PALB2* gene consists of 13 exons and the gene maps to chromosome 16p12.1 (Xia *et al.* 2006). The PALB2 protein structure is depicted in Figure 5. This protein has four WD40-repeats located in the C-terminus (Xia *et al.* 2006). WD40-repeats are motifs that function in a

variety of cellular processes such as control of cell division, RNA processing and transcriptional regulation (Williams *et al.* 1991, Hoey *et al.* 1993, Feldman *et al.* 1997). In the N-terminus of PALB2, there are sequence homologies with a segment of prefoldin and LC3 (light chain 3) of microtubule-associated protein MAP1 and a putative coiled-coil domain. The vast majority of PALB2, however, appears to be unique (Xia *et al.* 2006).

The nuclear accumulation of RAD51 is also partially regulated by PALB2. Currently no other binding partners of PALB2 have been reported (Xia *et al.* 2006), although an association with BRCA1 has been suggested (Greenberg 2008). Proteins acting in or with the FA pathway (chapter 2.8.3) also represent potential binding partners for PALB2.

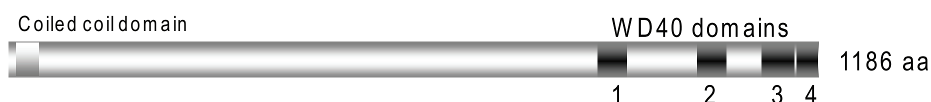

Fig. 5. Schematic diagram of PALB2 protein structure. The black boxes represent the WD40 domains and the grey box corresponds to the putative coiled coil-domain at the N-terminal part. Figure modified from Figure 1a in study III.

2.6.3 PALB2/FANCN as a Fanconi anaemia gene

Fanconi anaemia (FA) is a recessively inherited syndrome characterized by bone marrow failure, various developmental abnormalities and a high incidence of malignancies (Joenje & Patel 2001). Currently, 13 complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, M, N) have been described for this highly heterogeneous syndrome. The different complementation groups have been defined by somatic cell fusion studies and mutation screening. Each of these complementation groups is assigned to a certain gene. Most of these genes reside in the autosomes, but *FANCB* is located in the X chromosome (Ameziane *et al.* 2008).

Biallelic truncating mutations in *FANCN/PALB2* have been shown to cause a novel subtype of Fanconi anaemia (Reid *et al.* 2007, Xia *et al.* 2007). Fanconi anaemia subtype N (FA-N) patients are prone to bone marrow failure and childhood solid tumours such as Wilms tumour and medulloblastoma and are, therefore, phenotypically quite similar to *BRCA2/FANCD1* mutation carrying FA-D1 patients (Howlett *et al.* 2002, Reid *et al.* 2007, Xia *et al.* 2007). Patients with *BRCA2/FANCD1* and *FANCN/PALB2* mutations both differ from the

classical phenotype of FA due to the high risk of childhood solid tumours and are associated with a more severe course of disease and relatively early onset of haematological malignancies (Reid *et al.* 2007, Xia *et al.* 2007). For example, a FA-N individual was diagnosed with three separate malignancies (Wilms tumour, AML and medulloblastoma) within one year from birth (Reid *et al.* 2007). The similarity of phenotypes of FA-D1 and FA-N individuals could reflect the close functional relationship that exists between BRCA2 and PALB2. In addition to the phenotypes associated with FA-D1 and FA-N, patients belonging to these complementation groups also display typical FA features including growth retardation, various congenital malformations and cellular sensitivity to interstrand crosslinking agents, e.g. mitomycin C (MMC) (Howlett *et al.* 2002, Reid *et al.* 2007, Xia *et al.* 2007). Several different mutations have been described in biallelic FA-N carriers. These mutations and the clinical features associated with them are summarized in Table 1.

Table 1. *PALB2* mutations identified in Fanconi anaemia patients and their clinical manifestations. Data from Reid *et al.* (2007) and Xia *et al.* (2007).

Ethnic origin	1. Mutation		2. Mutation		Clinical features	
	Nucleotide change	Effect on protein	Nucleotide change	Effect on RNA/protein	Cancer (age at diagnosis)	Other features
Albanian, Moroccan	395delT	V132fs	3113+5G>C	r.2835_3113del279/ A946fs	Medulloblastoma (3.5 y)	GR, radial ray hypoplasia, absent right kidney
German	757_758delCT	L253fs	3294_3298 delGACGA	K1098fs	WT (0.9 y), AML (0.9 y), medulloblastoma (1 y)	Severe GR, hypoplastic thumbs, left pelvic kidney, anal atresia, microcephaly, congenital cataract, microphthalmia
Hispanic, North American	2257C >T	R753X	3549C>A	Y1183X	WT (1 y)	Severe IUGR, postnatal GR, microcephaly, microphthalmia, skin hyperpigmentation
German	2393_2394insCT	T799fs	3350+4A>G	r.3350insGCAG/ F1118fs	Medulloblastoma (4 y)	GR, microcephaly, microphthalmia, bifurcated anus
North American, African ancestry	2521delA	T841fs	3323delA	Y1108fs	WT (1.5 y), medulloblastoma (1.5 y)	GR, microcephaly, skin hyper- and hypopigmentation, horseshoe kidney, gonadal dysgenesis
British	2962C>T	Q988X	3549C >G	Y1183X	Medulloblastoma (2.3 y)	GR, microcephaly, hypoplastic thumb
North American	3116delA	N1039fs	3549C >G	Y1183X	Neuroblastoma (0.7 y), AML (2 y)	GR, microcephaly, VSD, ASD, thumb and radial anomalies, skin hyperpigmention
Not reported	1802T>A	Y551X	Breakpoints not reported	Deletion of exons 1–10	aplastic anaemia, kaposiform hemangioendothelioma	GR, microcephaly, hypertelorism, short neck, heart defect, hypoplastic thumb, imperforate anus, ectopic right kidney, café-au lait spots, epicanthus

WT, Wilms tumour, GR, growth retardation, AML, acute myelogenous leukaemia; IUGR, intrauterine growth retardation; ASD, atrial septal defect, VSD, ventricular septal defect.

3 Aims of the study

The currently identified breast cancer susceptibility genes only account for about 25% of familial breast cancer patients. Therefore, additional predisposing genes remain to be discovered. The majority of potential candidate genes have been proposed to be involved in the DNA damage response. Genes involved in this pathway, therefore, represent potential candidates for breast cancer susceptibility. In this investigation the *TOPBP1*, *CLSPN* and *PALB2* DNA damage response genes were evaluated as putative candidates. The specific aim of each study was to:

1. Identify mutations in the *TOPBP1* gene and to evaluate their potential involvement in breast cancer susceptibility.
2. Study the *CLSPN* gene in relation to breast cancer predisposition by ascertaining the mutations in the exons and exon-intron boundaries of this gene.
3. Screen the *PALB2* gene for germline mutations and to determine the functional consequences of the observed changes in relation to female and male breast, prostate and colorectal cancer.
4. Determine a more precise penetrance and hazard ratio associated with the *PALB2* c.1592delT founder mutation in unselected breast cancer families.

4 Materials and methods

4.1 Subjects

4.1.1 Studies I, II and III

Patients from 113–126 breast and/or ovarian cancer families were included in the mutation screening of the *TOPBP1*, *CLSPN* and *PALB2* genes. In these genes mutations were screened in the coding regions and the exon-intron flanking sequences. The families were geographically derived from the Northern Ostrobothnia Health Care District. Inclusion criteria for the high-risk families were as follows: 1) three or more breast and/or ovarian cancer cases in first- and second-degree relatives or 2) two cases of breast and/or ovarian cancer in first- and second-degree relatives, of which at least one with early age of disease onset (< 35 years), bilateral disease or multiple primary tumours. The remaining families were considered moderate risk families with two cases of breast and/or ovarian cancer in the first- and second-degree relatives. A summary of the families in each study is given in Table 2. All the high-risk families were previously screened for germline mutations in *BRCA1, BRCA2*, *CHK2* and *TP53* (Huusko *et al.* 1998, Huusko *et al.* 1999, Allinen *et al.* 2001, Rapakko *et al.* 2001). In studies I and II, ten families were included who had been identified as carriers of pathogenic mutations in either *BRCA1* or *BRCA2,* and an additional three families had *TP53* mutations. The families have also been previously screened for mutations in, for example, *RAD50*, *MRE11*, *NBS1*, *ATM*, *BRIP1/BACH1* and *BARD1* genes (Heikkinen *et al.* 2003, Karppinen *et al.* 2003, Karppinen *et al.* 2004, Heikkinen *et al.* 2005).

Table 2. A summary of the families included in studies I, II and III.

Family risk category	Number of families		
	Study I	Study II	Study III
High risk families	75	75	65
Moderate risk families	50	50	48
Total number of families	125	125	113

4.1.2 Study III

In addition to the previously mentioned familial breast cancer cases (n = 113), in study III also unselected breast cancer cases were studied. The role of *PALB2* mutations in prostate cancer, male breast cancer and colorectal cancer was investigated as well.

The unselected breast cancer patients studied were recruited without selection for or against family history of cancer and consisted of all available, consecutively diagnosed breast cancer cases at three university hospitals in Finland [Oulu (n = 534), Tampere (n = 888) and Kuopio (n = 496)]. The blood samples collected in Oulu were from patients diagnosed with breast cancer during two different periods: 1) between 1988 and 1994, and 2) between 2000 and 2004. The samples from Tampere were from patients diagnosed between 1) 1986 and 1994, and 2) 1997 and 2000. The specimens from Kuopio were collected between 1990 and 1995. The average age at breast cancer diagnosis was 57.7 (ranging from 23 to 95).

Unselected Finnish prostate cancer patients (n = 475) were diagnosed with cancer during 2000–2001. The mean age at diagnosis was 69.5 years (ranging from 45 to 93 years). Familial prostate cancer patients (n = 164), collected from all of Finland, were identified as having at least two affected family members (80 families had three or more affected members). The colorectal cancer material (familial cases, n = 188; unselected cases, n = 288) was collected at Helsinki University Hospital between 1994 and 2002. Inclusion criteria for the familial colorectal cases were malignancy in two or more first-degree relatives. The mean age at cancer diagnosis was 66.1 years (ranging from 27 to 90 years). All of the familial colorectal cancer cases had previously been studied for microsatellite instability (MSI) and all positive individuals were screened and had tested negative for mutations in the *MLH1* and *MSH2* genes. Male breast cancer samples were collected from all available patients diagnosed in Finland between 1967 and 1996. The mean age at diagnosis was 65.6 years (range 30 to 94 years).

The control samples used (n = 2501; 70.6% = 1765 females and 29.4% = 736 males) were from consecutive anonymous (only gender, age and place of birth were known) Finnish Red-Cross blood donors originating from the same geographical region as the studied cancer cases. All control individuals were cancer-free at the time of donation of the blood sample. There was no follow-up on donor health status. The age of the blood donors varied between 18 and 65, the average age being 42 years.

4.1.3 Study IV

A total of 17 *PALB2* c.1592delT carriers with breast cancer identified in an unselected cohort (see description in chapter 4.1.2) in study III and their family members were further analysed to determine the increased risk (hazard ratio, HR) and the age-specific cumulative risk (penetrance) associated with this mutation.

4.2 DNA extraction (I, II, III)

DNA was extracted from blood lymphocytes using standard phenol-chloroform method or the Puregene D-50K purification kit (Gentra, Minneapolis, MN, USA).

4.3 Mutation detection methods

4.3.1 Conformation sensitive gel electrophoresis (I, II, III)

Mutation screening of the candidate genes was done using conformation sensitive gel electrophoresis (CSGE). This method was used to scan the coding regions and exon-intron boundaries of candidate genes. The assay is based on the use of denaturing solvents that emphasize the conformational changes produced by mismatches in double-stranded DNA. This results in different migration patterns between homo- and heteroduplexes (Ganguly *et al.* 1993, Körkko *et al.* 1998). In studies I-III, all coding regions and flanking intronic sequences were amplified by PCR using AmpliTaqGold^{TM} polymerase (Applied Biosystems, Foster City, CA, USA). After PCR the products were denatured at 98°C for 5 min and then cooled to 68°C for 30 min to facilitate the formation of heteroduplexes. CSGE was performed in gels prepared with mild denaturing solvents for 20–26 hours. Ethidium bromide staining was used. Bands were detected with UV light and the gels were photographed.

4.3.2 Direct sequencing (I, II, III)

Samples with band shifts detected with CSGE were reamplified and the sequencing analysis was performed on a Li-Cor IR^2 4200-S DNA Analysis system (Li-Cor Inc., Lincoln, USA) using the SequiTherm EXEL^{TM}II DNA Sequencing Kit-LC (Epicentre Technologies, Madison, WI, USA). Sequencing was also used

as a primary method to detect mutations in sequences not suitable for analysis with CSGE, such as those containing poly-T-repeats or GC-rich segments.

4.3.3 Restriction enzyme digestion (I)

In order to detect variant homozygotes for the *TOPBP1* Arg309Cys alteration not distinguished from each other with CSGE, PCR followed by restriction fragment analysis was utilized. The identification of wild-type (C/C) and variant (T/T) homozygotes and Arg309Cys heterozygotes at position 1010 was based on amplification of a 383-bp DNA fragment digested with *Tat*I (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions.

4.4 Cell culture and Western blot analysis (I)

Lymphoblastoid cell lines (LCLs) were derived from four TOPBP1 Arg309Cys carriers and two wild-type individuals diagnosed with breast cancer. Cells were cultured in RPMI 1640 medium supplemented with 20% foetal bovine serum, 1% L-glutamine and gentamycin (10 mg/ml) at 37°C/5% CO_2. Cells were centrifuged, washed twice with PBS and the pellet was suspended with 2.5 x Laemmli buffer and sonicated briefly. The protein concentration was measured using the RC DC protein assay kit (Bio-Rad, Hercules, CA, USA). 25 μg of protein was separated by SDS-PAGE, after which the gels were stained with Sypro Orange (Bio-Rad, Hercules, CA, USA) to verify equal loading and electroblotted onto PVDF membranes (Millipore, Billerica, MA, USA). The rabbit polyclonal α-TOPBP1.2 antibody has been described previously (Mäkiniemi *et al.* 2001) and peroxidase-conjugated goat α-rabbit was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Secondary antibody was used at dilutions of 1:10,000 to 1:15,000. Signals were detected by chemiluminescence, using the SuperSignal West Femto detection kit (Pierce, Rockford, IL, USA). ImageQuant 5.2 (Amersham Lifesciences, Toronto, ON, Canada) was used for quantification of densitometrically scanned Western blot signals. Analyses were performed in triplicate.

4.5 Loss of heterozygosity analysis and laser capture microdissection (III)

To test whether loss of heterozygosity had occurred in tumours of individuals heterozygous for the *PALB2* c.1592delT mutation, genomic DNA extracted from formalin-fixed, paraffin-embedded tumour sections from six patients was analysed. Before DNA extraction, laser-capture microdissection was performed to isolate pure cancer cell populations from tumours from five patients. *PALB2* gene segments (about 200 base pairs) surrounding the c.1592delT mutation were amplified by polymerase chain reaction with multiple primer pairs and sequenced.

4.6 Immunohistochemistry (III)

Formalin-fixed, paraffin-embedded tissue sections (4 mM) were de-paraffinized and rehydrated in graded alcohols. Heat-induced epitope retrieval was performed with a digital pressure cooker in citrate buffer (pH 6.0) prior to application of the appropriate polyclonal (or monoclonal) antibody. The primary antibody was detected using the Envision + system (K4011, DAKOCytomation, Carpinteria, CA, USA) that employs horseradish peroxidase-labelled polymer conjugated to goat anti-rabbit immunoglobulin antibodies. The immune complexes were identified using a peroxidase reaction with 3, 3'-diaminobenzidine-plus as chromogen. Slides were counterstained with Mayer's haematoxylin.

Antibodies against ER (monoclonal, clone 1D5), PR (monoclonal, clone PgR636) and HER-2 (polyclonal) were all purchased from DAKO and used at 1:100, 1:200 and 1:1,000, respectively. Anti-p53 (monoclonal, clone DO-1) was purchased from Immunotech and used at 1:1,200.

4.7 Homologous recombination double-strand break repair function and mitomycin C sensitivity testing (III)

Fanconi anaemia cells are sensitive to DNA cross-linking agents such as MMC. To test whether *PALB2* c.1592delT is a true loss-of-function allele, several functional studies were performed to assess whether the mutant allele can overcome the mitomycin sensitivity induced by RNAi-mediated depletion of PALB2. The DR-U2OS HR reporter cell line and the knockdown-and-rescue assay were performed as described (Xia *et al.* 2006). To generate EUFA1341 (FANCN) fibroblasts stably expressing wild-type and mutant PALB2, cells were

infected with amphotropic pOZC-PALB2, pOZC-PALB2-1592delT, or pOZC-PALB2-3433G>C retroviruses (previously described in Xia *et al.* 2006). Each encodes both a desired PALB2 protein and an interleukin-2 (IL2) receptor. After 72 h, cells were selected using paramagnetic M-450 Dynabeads (Dynal Biotech) coupled to anti-IL2 receptor antibody (Upstate, Millipore, Billerica, MA, USA).

To assay for MMC sensitivity, cells were seeded in 96-well plates at 1,000 cells (in 100 µl) per well. Four hours later, 50 µl of MMC-containing medium was added to achieve the desired MMC concentrations. Cells were incubated with the drug for 5 days, and their survival rates were measured using a CellTiter Glo kit (Promega, Madison, WI, USA) following the manufacturer's instructions. This method of determining the number of viable cells in culture is based on quantitation of the ATP present, which is an indicator of metabolically active cells.

4.8 Plasmid vectors and protein analysis (III)

Monoclonal anti-BRCA2 (Ab-1, #OP95) and anti-FLAG (M2) were purchased from Calbiochem (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The retroviral PALB2 cDNA vectors called pOZN-PALB2 and pOZC-PALB2 have been described previously (Xia *et al.* 2006). The mutations, c.1592delT (Leu531Fs) and 3433G>C (G1145R), were introduced into these vectors by site-directed mutagenesis using the QuikChange method (Stratagene, La Jolla, CA, USA). Transient transfections were carried out in 6-well plates using FuGENE 6 (Roche, Basel, Switzerland) for 40 hr. Whole cell lysates were prepared in a high salt- and detergent-containing buffer (NETN420).

Immunoprecipitation to test for the BRCA2 binding capacity of PALB2 Leu531Fs was performed for 3 hr at 4ºC with anti-FLAG M2-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) according to the protocol described in Xia *et al.* 2006. Tris-Glycine (4–12%) gels from Invitrogen were used for protein separation.

4.9 Statistical methods (I–IV)

4.9.1 Comparison of allele frequencies and the effect of mutations on the age of disease onset (I–III)

Carrier frequencies were compared using Pearson's χ^2 or Fisher's exact test. All the p-values were 2-sided. The Mann-Whitney U-test for non-parametric

variables was used to compare mean ages of disease onset between *PALB2* c.1592delT mutation carriers and non-carriers. All tests were performed with the SPSS version 12.0 for Windows.

4.9.2 Estimation of hazard ratio and age-specific penetrance (IV)

The hazard ratio (HR), indicating the ratio of sex- and age-specific cancer incidence for carriers to that for non-carriers, was estimated for breast cancer (BC) and for all non-breast cancers combined (NBC). The hazard ratios were estimated by a modified segregation analysis fitted under maximum likelihood theory. The likelihood function was based on the Elston-Stewart formulation so phenotypes were assumed to be conditionally independent given genotypes (Elston & Stewart 1971). Hardy-Weinberg equilibrium was also assumed. To adjust for ascertainment, the likelihood for each pedigree was conditioned on the ascertainment criteria, namely that the proband was both a carrier of *PALB2* c.1592delT and a BC case. Under the assumption that all pedigrees were independent, the sum of the conditional log likelihoods was maximized over the parameter space using the statistical package MENDEL (Lange *et al.* 1988).

4.10 Bioinformatic analyses (I, II, III)

4.10.1 PolyPhen analysis (II, III)

All the missense alterations discovered were tested for possible pathogenicity by using PolyPhen software (Ramensky *et al.* 2002). (Available at http://genetics. bwh.harvard.edu/cgi-bin/pph/polyphen.cgi).

4.10.2 NNSplice analysis (I, II, III)

Potential splicing effects of the alterations discovered were investigated using the NNSplice software (Reese *et al.* 1997). (Available at http://www.fruitfly.org/ seq_tools/splice.html).

4.10.3 ESEfinder analysis (I, II, III)

ESEfinder 2.0 software (Cartegni *et al.* 2003, Smith *et al.* 2006), (available at http://rulai.chsl.edu/tools/ESE/), was used to determine if the exonic variants were located in ESE (exonic splicing enhancer) sequences and could therefore affect ESE function.

4.11 Ethical issues (I–IV)

Approval to perform the study was obtained form the Ethical board of the Northern Osthrobothnia Health Care District and the Finnish Ministry of Social Affairs and Health, and for studies III and IV also from the ethical boards of the other participating university hospitals (Helsinki, Kuopio and Tampere). All the patients and their family members gave their informed consent to take part in a study to identify novel cancer susceptibility genes. Family members were only contacted with permission from the proband. No information on the results of the mutation analyses was given to patients or their family members.

5 Results

5.1 Mutation screening of the *TOPBP1* gene (I)

Altogether 19 germline alterations were observed in the mutation analysis of the *TOPBP1* gene. Ten of the observed alterations occurred in protein coding regions; three of them were novel, while the rest have been previously reported in the single nucleotide polymorphism (SNP) database. Four novel and five previously known variants were observed in the introns.

Of the novel exonic alterations, two were missense (Arg309Cys, Arg1013Gln) and one resulted in a synonymous change (Ser1417Ser). Arg1013Gln was observed at similar frequencies both in cases and controls, while the frequency for Arg309Cys was found to be significantly elevated in the familial cancer cases and was consequently considered as a possible disease-associated alteration. The novel A to G transition at position 4336 was not observed in controls, but it resulted in a synonymous change (Ser1417Ser) and had no effect on predicted splicing consensus sequences or ESE motifs, and is therefore unlikely to be a pathogenic alteration. All intronic changes as well as all exonic variants previously reported in the SNP database, were observed at similar frequencies in both cases and controls and none of them had any effect on splice site consensus sequences.

Therefore, the only potentially pathogenic alteration discovered was Arg309Cys in exon eight. To further investigate the prevalence of this alteration it was genotyped by restriction enzyme digestion in blood DNA samples from 697 healthy controls and 187 sporadic breast cancer cases. These results are given in Table 3. In the familial material, one T/T homozygous individual diagnosed with breast cancer at age 45 was observed. The only additional sample from the same family was from a paternal aunt, who had breast cancer at age 52 and tested heterozygous for the alteration. No T/T homozygotes were found among the controls or sporadic breast cancer cases. An incomplete segregation pattern was observed in *TOPBP1* Arg309Cys carrier families with both healthy carriers and non-carriers with breast cancer.

Table 3. The observed frequencies of *TOPBP1* Arg309Cys.

Change	Familial cases (*p*-value, OR; 95% CI)	Sporadic cases (*p*-value, OR; 95% CI)	Controls	Family history of breast cancer (*p*-value)	Family history of breast-ovarian cancer (*p*-value)
Arg309Cys	15.2%,19/125 (*p* = 0.002; OR 2.4; 95% CI 1.3–4.2)	9.1%,17/187 (*p* = 0.341; OR 1.3; 95% CI 0.7–2.4)	7.0%, 49/697	18.2 6/33 (*p* = 0.578[a])	14.1%, 13/92 (*p* = 0.578[b])

[a]compared to cases with family history of breast-ovarian cancer, [b]compared to cases with family history of only breast cancer.

5.2 *CLSPN* alterations and breast cancer predisposition (II)

In the *CLSPN* gene altogether seven different changes were discovered in 125 breast or breast-ovarian cancer families studied. Of these, three resulted in amino acid substitutions. All of the exonic changes, however, located outside the main known functional motifs (figure 4 in chapter 2.7). In order to evaluate possible pathogenicity of the observed variants, their frequencies were compared between patients and healthy population control individuals.

Of the observed exonic changes, 17G>A (Gly6Asp) and 3839C>T (Ser1280Leu) have previously been described in sequence databases, whereas the third one, 3583–3585delGAA (1195delGlu), represents a novel variant. Gly6Asp was observed in 9% (11/125) of the patients and 4% (13/300) of the controls ($p = 0.07$). Ser1280Leu was seen in 10% (12/125) of the patients and 5% (16/300) of the controls ($p = 0.1$). A neutral effect was predicted for the Gly6Asp change, whereas Ser1280Leu could potentially have harmful consequences on protein function based on the analysis using the PolyPhen software. Splicing was not shown to be affected by either of these variants. Both sequence changes were located in predicted ESE element binding sites: SC35 (Gly6Asp) and SRp40 (Ser1280Leu) and could potentially affect ESE functions.

The novel change in exon 22 leading to the deletion of glutamic acid 1195 located in a repeat sequence containing five glutamic acid residues and falls within the predicted ESE sequence for SF2/ASF. Altogether 10% of the patients studied (12/125) carried the 1195delGlu alteration compared to 5% (14/300) in the controls ($p = 0.05$). The 1195delGlu change occurred in a haplotype with the Ser1280Leu alteration. Curiously, 56% (7/12) of the 1195delGlu/Ser1280Leu haplotype carriers also displayed some previously detected susceptibility gene

mutations, although the association was not statistically significant. Sequence comparisons between different species demonstrated the 1195delGlu variant also in some other mammalian genomes (for example dog and rat). All three exonic variant alleles displayed incomplete segregation with the cancer phenotype, as both healthy mutation carriers and non-carrier breast cancer cases were observed among the studied families.

Four intronic alterations in *CLSPN* were observed. The variant adjacent to exon 9 (IVS9+28insT) was found in 10% of cases (12/125) and 5% (15/300) of controls ($p = 0.08$). No effect on splicing was predicted. One of the index cases carrying the IVS3-8delT alteration was diagnosed with breast cancer at age 49. Her sister, who had uterine cancer at age 61, was also a carrier. The other sister of the index case had breast cancer at age 51, but tested negative for this alteration. Thyroid cancer, lymphoma and intestinal cancer cases were also observed in the family. Unfortunately, no additional DNA samples were available for analysis. IVS3-8delT is located near the 5' end of exon three, so it could theoretically have an effect on correct splicing. However, no evidence for a possible splicing defect was provided by analysis using NNSplice software. No alteration carriers were identified among the 300 control samples analysed ($p = 0.3$). The two other intronic alterations, IVS10+16G>A and IVS6+11C>T, were present in cases and controls at similar frequencies. According to the NNSplice software, these changes had no effect on splicing.

5.3 Involvement of the *PALB2* c.1592delT mutation in hereditary cancer susceptibility (III)

A total of six different exonic variant alleles were identified in affected index individuals from 113 families (Table 1 of study III). Four of these changes were also detected at similar frequencies in the control population, suggesting that they are not cancer-associated. This view was supported by the results obtained from computer simulations using PolyPhen, ESEfinder and NNSplice software. In contrast, one alteration, c.1592delT was detected in three (2.7%) index individuals, but in only six (0.2%) of 2,501 controls ($p = 0.005$; OR 11.3; 95% CI 1.8–57.8), therefore suggesting a significant disease association. This alteration results in a frame-shift after Leu 531, with the new reading frame progressing for 28 codons before termination. Another alteration, 3433G>C in exon 13, was detected in one index individual but in none of the 971 controls. In

addition, three sequence alterations were detected in introns but none of them seemed disease-related.

Potentially pathogenic alterations c.1592delT and 3433G>C were tested functionally in order to determine whether their BRCA2-binding capacity is altered, to see if the mutations are deficient in HR and if they can complement the MMC sensitivity of PALB2-depleted cells. The c.1592delT mutation resulted in a truncated protein (PALB2-L531Fs), which had a markedly decreased BRCA2-binding affinity without affecting endogenous BRCA2 abundance upon transient overexpression. Consistent with these observations and the functional importance of BRCA2–PALB2 complex formation, PALB2-L531Fs failed to support HR in PALB2-knockdown cells or to restore crosslink repair in PALB2-deficient cells. Thus, c.1592delT is a genuine loss-of-function mutation. In contrast, PALB2-G1145R seemed to be fully capable of BRCA2 binding and to be functional in these two assays.

Subsequently, the *PALB2* c.1592delT mutation was sought in germline DNAs of unselected female breast cancer cases, unselected male breast cancers, colorectal cancers and prostate cancer cases, all from Finland. The results are presented in Table 4.

Table 4. Prevalence of *PALB2* c.1592delT mutation carriers in various cancer types and controls. Information in this table has been previously published as a supplementary Table 1 in study III.

Cancer type	Cases	Controls	p-value (OR; 95% CI)
Familial female breast cancer	2.7% (3/113)	0.2% (6/2501)	0.005* (OR 11.3; CI 1.8–57.8)
Unselected female breast cancer	0.9% (18/1918)	0.2% (6/2501)	0.003* (OR 3.94; CI 1.5–12.1)
Unselected male breast cancer	– (0/141)	– (0/475[2])	NA
Familial prostate cancer	0.6% (1/164)	– (0/475[2])	NS (NA)
Unselected prostate cancer	– (0/475)	– (0/475[2])	NA
Familial colorectal cancer	– (0/188)	0.2% (6/2501)	NS (NA)
Unselected colorectal cancer	– (0/288)	0.2% (6/2501)	NS (NA)

NS not significant, NA not available, OR odds ratio, CI confidence interval, a male controls.

Of a total of 22 (21 breast cancer cases, one prostate cancer case) identified unrelated cancer patients heterozygous for *PALB2* c.1592delT, 16 were tested for possible co-segregation of known Finnish *BRCA1* and *BRCA2* mutations but none was detected. The average age of disease onset for c.1592delT mutation-positive individuals was 52.9 years (variation 39–73 years), which seems slightly younger than the average of the remaining affected individuals in the unselected breast cancer group (57.8 years, variation 23–95 years; $p = 0.17$) but older than those with Finnish *BRCA1* (46 years, variation 32–57 years) and *BRCA2* (48 years, variation 45–67 years) mutations (Sarantaus *et al.* 2000). The mutation was also observed in six controls (0.2%; 6/2501), suggesting incomplete penetrance. However, most control participants heterozygous for the mutation were relatively young (five females aged from 27 to 51 years and one male aged 28 years), compared with the above-noted average age of disease onset for affected c.1592delT carriers. The actual penetrance might therefore be higher than observed in study III. For the 18 unselected mutation-positive breast cancer patients, available records were analysed for evidence of a positive family history, and at least half of these families were found to have an apparently heritable disease history. In addition to breast cancer, all families studied displayed other types of cancer, including colorectal, stomach, endometrial, ovarian and pancreatic cancers and leukaemia. Segregation analysis of the truncation allele with regard to cancer incidence was attempted in three of the families with breast cancer studied but was not sufficiently informative because of a lack of DNA samples from suitable family members. For the remaining families, the analysis was restricted only to the affected index individual who initially displayed the c.1592delT allele. Segregation was also studied in the family of the mutation-positive patient with prostate cancer. Other than the male individual, who died early at 52 years of age, all male carriers developed prostate cancer by the age of 76 years, indicating high penetrance of the mutation in the two generations of this family that were studied.

Tumour properties of *PALB2* c.1592delT mutation carriers were evaluated by obtaining the pathology records and by using immunohistochemistry analysis. This was not possible for all patients due to incomplete information or unavailability of tumour sample. Also due to missing information on tumour properties, the clinical characteristics could not be statistically evaluated. The obtained results are presented in Table 5. LOH analysis was performed by direct sequencing. No evidence of LOH was observed in the breast tumour samples analysed.

Table 5. Clinical characteristics of *PALB2* c.1592delT mutation carriers.

Patient ID	Cancer type	Age at diagnosis (years)	Grade	IHC
764	IDC	73	3	ER+,PR+
766	IDC	54	3	ER- PR-
33	IDC	54	3	ER-, PR-
290	IDC	41	1	ER-, PR-
360	IDC	41	3	N/A
2011	IDC	48	3	ER+,PR+
RI-132B	ILC	61	N/A	ER+,PR+
BR-0267	IDC	64	3	ER+,PR+, Her2-
BR-0225	IDC	61	3	ER+,PR+, Her2+
BR-0344	ILC	56	3	ER- PR-, Her2+
96-782	IDC	63	2	ER+,PR+, Her2-
97-670	IDC	39	N/A	N/A
BR-0426	ILC	51	2	N/A
BR-0134	IDC	62	3	ER- PR-, Her2-
BR-0051	IDC/DCIS	49	N/A	ER+,PR+, Her2-
96-458	ILC	49	2	N/A

N/A not available

5.4 Estimating the clinical significance of *PALB2* c.1592delT (IV)

Based on the 10 families and the seven singletons for whom no family history was recorded, the *PALB2* c.1592delT mutation was estimated to be associated with a 14.3-fold [95% CI 6.6–31.2] increased risk of BC ($p = 0.0001$) and a 2.6-fold (95% CI 1.3–5.1) increased risk of NBC, as a group ($p = 0.03$). When the augmented data set (explained in more detail in the methods section of study IV) was used the estimated HR for BC became 6.1 (95% CI: 2.2–17.2; $p = 0.01$) and the HR for NBC became 1.4 (95% CI: 0.6–3.2; $p = 0.5$). The corresponding age-specific cumulative risks are shown in Figure 6, indicating an estimated BC risk of 40% (95% CI 17–77) to age 70 years. The HR for BC decreased with age by 4.2% per year (95% CI 0.2–8.1), decreasing from 7.5-fold at age 30 years to 2.0-fold at age 60 yrs ($p = 0.06$). These estimates were fairly insensitive both to small changes in the c.1592delT allele frequency and to restrictions of the families to relatives of various degrees of relatedness to the proband.

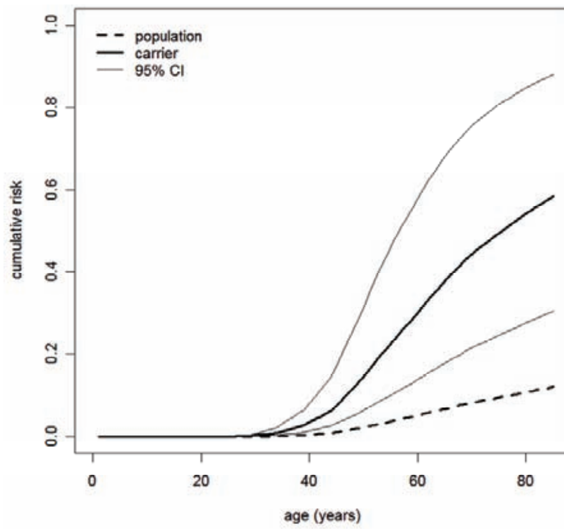

Fig. 6. Estimated age-specific cumulative risks for *PALB2* c.1592delT carriers identified in an unselected cohort of breast cancer cases in Finland. Figure modified from Figure 2 in study IV.

6 Discussion

6.1 *TOPBP1* gene and breast cancer susceptibility (I)

A significant fraction of familial breast and/or ovarian cancer cannot be explained by mutations in the currently known susceptibility genes. *TOPBP1* encodes a checkpoint protein that is required for DNA damage and replication processes and has both structural and functional similarities with BRCA1. Analysis of the *TOPBP1* gene revealed altogether nineteen variants. The novel Arg309Cys substitution was observed at significantly higher frequency in familial breast cancer patients compared to healthy controls. This suggested approximately a 2.4-fold increased disease risk for carriers. This variant was the only putative pathogenic alteration discovered in study I. The variant was not present in ten index cases from *BRCA1/BRCA2* mutation-positive families, but due to the low number of samples tested a possible co-existence of these changes cannot be ruled out. Furthermore, the prevalence of the Arg309Cys variant did not appear to be elevated among breast cancer patients without known family history of the disease.

None of the observed exon region variants resulted in translational frameshift or nonsense codons, and all missense variants located outside of the BRCT domains. Although the novel missense alteration, Arg1013Gln, as well as the previously known alterations in exons 14, 18, 19, 25 and 27 were shown to have an effect on ESE motifs as predicted by the ESEfinder program, equal frequencies in cases and controls indicated that they are likely to be harmless polymorphisms. On the other hand, the novel Arg309Cys alteration caused by C>T transition at nucleotide 1010 was located within a consensus splicing sequence and predicted to affect the consensus acceptor site. However, comparison of TOPBP1 protein levels in wild-type and Arg309Cys heterozygous lymphoblastoid cell lines showed normal TOPBP1 expression also in Arg309Cys carriers, indicating that the variant allele had no effect on TOPBP1 protein levels. The Western blot analysis also failed to show any aberrant-sized protein products. These observations suggest that the variant would not have any effect at the mRNA level either. However, the existence of unstable RNA species not translated to protein cannot be ruled out by these investigations and should be further studied. At the protein level, comparison between several species showed that the Arg309 residue is conserved in both dog and frog, and also falls within a region that shows

conservation in *TOPBP1* homologues from yeast to human. In fact, the replacement by a cysteine residue could have an harmful impact on polypeptide structure and function, since arginine and cysteine are biochemically profoundly different. Both amino acids are hydrophilic, but while arginine has a guanidinium group as its side chain and is positively charged, cysteine is uncharged and carries a very reactive sulfhydryl group. Arginine at position 309 locates to the N-terminal part of the protein, adjacent to the second BRCT domain (Figure 3 in chapter 2.6), and some lines of evidence suggest that the amino terminal region of TOPBP1 is responsible for transcriptional co-activation functions. The activity of human papilloma virus (HPV) transcription/replication factor E2 was shown to be enhanced by TOPBP1, and removal of the amino terminal portion of this protein abolishes this function (Boner *et al.* 2002). Previously the amino terminus of TOPBP1 was also shown to activate transcription in yeast (Mäkiniemi *et al.* 2001).

Segregation analysis of the Arg309Cys variant was performed with a limited number of samples from additional family members and revealed incomplete segregation of the alteration with cancer, as both affected non-carriers and healthy carriers were identified. The results of study I suggest that heterozygosity for the commonly occurring *TOPBP1* Arg309Cys allele may confer a moderately elevated risk of breast and/or ovarian cancer, perhaps by acting in concert with other predisposing factors. These findings fit the polygenic model of breast cancer susceptibility, according to which several low-penetrance alleles with multiplicative effects account for a significant portion of the familial clustering of breast cancer in the absence of mutations in the *BRCA1* and *BRCA2* genes (Pharoah *et al.* 2002, Antoniou & Easton 2003). The relatively high incidence of *TOPBP1* Arg309Cys carriers suggests that it is likely to be a low-penetrance alteration. No other pathogenic mutations in *TOPBP1* have been reported but as study I is, to date, the only one that has investigated mutations in the entire *TOPBP1* gene in relation to breast cancer susceptibility, the presence of high penetrance mutations cannot be ruled out.

Subsequently to performing study I, *TOPBP1* has been shown to be aberrantly expressed in breast cancer, and in some tumours (2/61) no *TOPBP1* expression was detected, suggesting that suppression of *TOPBP1* expression may contribute to cancer development (Going *et al.* 2007). Aberrations in *TOPBP1* yeast homologues have also been shown to lead to chromosome instability (Strome *et al.* 2008) and could, therefore, potentially also cause cancer in humans. One polymorphism in *TOPBP1* (S730L) has been studied in relation to chronic

lymphocytic leukaemia progression. Homozygosity for this variant was shown to result in poorer disease outlook (Sellick *et al.* 2008). The S730L variant was not observed in study I.

It was recently reported that TOPBP1 interacts with NBS1 (Morishima *et al.* 2007). *NBS1* is the gene mutated in Nijmegen breakage syndrome (Carney *et al.* 1998). The results obtained from this study suggested that NBS1 regulates TOPBP1 recruitment to sites of DNA damage. TOPBP1-depleted cells also exhibited hypersensitivity to MMC and ionizing radiation, an increased frequency of SCEs and a reduced homologous recombination repair (Morishima *et al.* 2007). Mutations in *NBS1* have been associated with breast cancer susceptibility and genomic instability (Heikkinen *et al.* 2006) and current results suggest that *TOPBP1* variants might have similar consequences. However, this observation requires further investigations to be verified.

6.2 *CLSPN* alterations and breast cancer predisposition (II)

CLSPN interacts with *BRCA1*, one of the two major breast cancer susceptibility genes, in DNA damage repair (Lin *et al.* 2004) Mutations in other BRCA1-interacting genes (e.g. *BARD1* and *BRIP1*) have previously been linked to breast cancer susceptibility (Ghimenti *et al.* 2002, Karppinen *et al.* 2006, Seal *et al.* 2006). In addition, the importance of claspin in various functions governing the maintenance of genomic integrity and activation of CHEK1 suggests that *CLSPN* could be a plausible candidate gene for hereditary cancer predisposition.

Altogether seven different sequence changes were observed. Out of these only one (1195delGlu) was novel. The deletion of glutamic acid 1195 in an amino acid repeat tract of several glutamic acids was found to be almost twice as common in cancer patients compared to controls ($p = 0.05$). Interestingly, a similar trinucleotide insertion (insGAA) in the same repeat region has been reported in an online sequence variation database (http://pga.gs.washington.edu/data/clspn/clspn.csnps.txt). A deletion in the same repeat was also observed in the study of Wang *et al.* (2008). The length of the glutamic acid repeat tract thus appears to be unstable, as both deletions and insertions have been detected. The human reference sequence lists five glutamic acids but the repeat length seems to vary between four and six repeats. Some short tandem repeat polymorphisms have been linked to breast cancer susceptibility (Zhang & Yu 2007) and can in certain instances influence gene transcription efficiency (Gerber *et al.* 1994). Although our results did not support a major role for the haplotype containing

1195delGlu and Ser1280Leu alterations in cancer predisposition, we did observe an unexpectedly high degree of cosegregation (56%) with previously detected susceptibility gene mutations in the studied set of cancer families, which could indicate a slight modifier effect. The other exonic changes, Gly6Asp and Ser1280Leu, were shown to have some effect on ESE motifs as determined by ESEfinder software, but similar frequencies in cases and controls suggested no relationship between these alterations and predisposition to cancer. Neither did any of the observed intronic variants prove to be associated with increased cancer risk.

The results of study II are supported by a recent report investigating *CLSPN* alterations in breast and pancreatic tumours. The Ser1280Leu alteration was initially predicted to affect protein function when analysed with Pmut prediction scores (Wang *et al.* 2008). Similar results were obtained by the PolyPhen analysis software in study II. Wang *et al.* (2008) initially observed an elevated prevalence of Ser1280Leu in breast cancer cases compared to controls (25% vs. 2%) based on a relatively small sample size. However, further genotyping showed this change to be most likely non-pathogenic. The non-pathogenicity of this variant corresponds to results obtained in study II. Ser1280Leu was invariably observed in a haplotype also containing the deletion in exon 22 (1199delGlu) (Wang *et al.* 2008). A similar haplotype pattern was observed in study II for 1195delGlu and Ser1280Leu alterations.

None of the observed *CLSPN* variants resulted in aberrant splicing or translational frameshift and all missense variants located outside of currently known functional domains (Figure 4 in chapter 2.7). However, since these domains only cover a small portion of the whole protein sequence and the entire range of *CLSPN* function remains to be determined, possible new functional motifs are likely to be discovered. Only three changes have been observed in the 25 exons of *CLSPN,* reflecting extreme conservation of the coding regions in this gene. Similarly, no germline mutation has been reported in the *CHEK1* gene, an important claspin binding partner. The preservation of this pathway could result from the numerous essential and unique cellular functions it performs such as monitoring DNA replication and repair. This could cause poor tolerance for germline *CLSPN* alterations and complete intolerance for *CHEK1* mutations.

Overall, our results showed no evidence of significant involvement of *CLSPN* alterations in familial breast and breast-ovarian cancer susceptibility. However, further research is needed to confirm these findings in other populations and cancer types.

6.3 *PALB2* as a breast cancer susceptibility gene (III)

Several recent studies have demonstrated that monoallelic truncating *PALB2* mutations are associated with breast cancer susceptibility (Rahman *et al.* 2007, Foulkes *et al.* 2007, Tischkowitz *et al.* 2007, Cao *et al.* 2008, García *et al.* 2008). These mutations are shown in Figure 7. *PALB2* mutations are generally considered to result in two- to four-fold increased risk of breast cancer (Walsh & King 2007). However, there may be significant genetic heterogeneity between the risks conferred by different mutations.

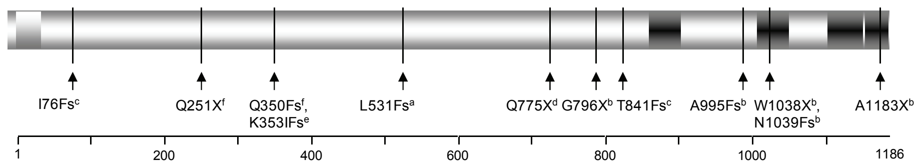

Fig. 7. The approximate locations of the currently reported *PALB2* mutations. Data from study III[a], Rahman *et al.* 2007[b], Tischkowitz *et al.* 2007[c], Foulkes *et al.* 2007[d], García *et al.* 2008[e] and Cao *et al.* 2008[f]. The black boxes represent the WD40 domains and the grey box corresponds to the putative coiled coil-domain at the N-terminal part. Figure modified from Figure 1a of study III.

Based on the results of study III, it seems clear that the *PALB2* c.1592delT mutation contributes significantly to familial and unselected breast cancer risk in Finland. Similar results for other *PALB2* mutations have been obtained for a few other populations. The French Canadian population was investigated for the presence of *PALB2* mutations employing a similar study approach as in study III. They discovered a *PALB2* founder mutation Q775X which accounted for 0.5% of breast cancers diagnosed under the age of 50 years (Foulkes *et al.* 2007). However, a somewhat smaller contribution for familial breast cancer was estimated for the British population. *PALB2* mutations were assessed to result in a two-fold increased breast cancer risk and the population attributable fraction (PAF) of *PALB2* mutations was estimated to be 0.23% (Rahman *et al.* 2007). In contrast, one mutation identified in the Canadian population, c.229delT, was found to have high penetrance (Tischkowitz *et al.* 2007). Two carriers of this mutation developed three separate breast cancers each. One carrier developed one breast cancer (Tischkowitz *et al.* 2007) which could suggest the presence of modifiers affecting the penetrance of *PALB2* mutations.

In the Spanish population, a truncating mutation in *PALB2* has also been observed, but only in a single family, and it is thus not a very reliable estimate of

the frequency of *PALB2* mutations in Spain (García *et al.* 2008). It seems that certain populations, such as the Finns and French Canadians in this case, harbour *PALB2* founder mutations, while mutations in this gene appear to be less common in other populations or may even be completely absent (Gunnarsson *et al.* 2008). Additional research on this is needed to determine the effect of *PALB2* mutations in other populations. These populations are likely to display different mutations as no clear mutational hotspots of *PALB2* or recurrent mutations occurring in several populations have been discovered so far (Figure 7). Interestingly, however, all the mutations discovered in the Chinese population occurred in exon four of *PALB2,* which could suggest a potential hotspot (Cao *et al.* 2008). The *PALB2* c.1592delT mutation is also located in exon four. It must be noted, though, that exon four is the largest exon of *PALB2* and co-occurrence of a few truncating variants and some missense changes in this exon could also be merely coincidental.

Interestingly, all the pathogenic *PALB2* alterations currently reported have been truncating ones. Rare missense variants or in frame-deletions or -insertions of *PALB2* could also cause cancer, especially if they occur in conserved sequence regions or in important functional domains of this protein. Not all functions of PALB2 are currently known and novel binding partners are likely to be discovered. Therefore, testing the functional effect of a rare missense alteration can be difficult if the variant does not directly affect BRCA2 binding. For example, the G1145R alteration observed in study III occurs at the conserved WD40 domain of PALB2. WD40 domains are often involved in protein-protein interactions. The G1145R variant was only observed in a single breast cancer family but in none of the 971 controls, making statistical comparisons unfruitful. The family, however, exhibits a markedly increased cancer predisposition, particularly for breast cancer. The only additional available DNA sample was from a maternal cousin with both breast and uterine cancer, and it tested negative for this alteration. In functional analyses G1145R behaved like wild-type PALB2 as BRCA2 binding was not affected and HR function was normal. Bioinformatic and functional studies performed in study III suggest that G1145R is a rare variant not associated with cancer, but its potential pathogenicity cannot be completely excluded by these investigations.

The only large rearrangement in *PALB2* currently reported was observed in the family of a FA-N individual with several family members who had cancer (Xia *et al.* 2007). Subsequently two studies have assessed the effect of large *PALB2* rearrangements on breast cancer susceptibility. Further rearrangements in *PALB2* have not been detected and, therefore, these are unlikely to contribute

significantly to breast cancer predisposition (Tischkowitz *et al.* 2007, Pylkäs *et al.* 2008). Further investigations on the potential relationship between *PALB2* rearrangements and cancer risk are still needed, although large rearrangements of this gene seem to be very rare.

The presence of *PALB2* c.1592delT was discovered in approximately 1% of unselected breast cancer cases in Finland. The observed prevalence of this allele especially in breast cancer cases from the northern part of the country is significant considering that the 19 most common founder mutations in *BRCA1* and *BRCA2* combined only account for 1.8% of unselected cases in Finland (Syrjäkoski *et al.* 2000). Cosegregation of *PALB2* c.1592delT and *BRCA1* or *BRCA2* mutations was not observed in any of the 16 individuals tested for this mutation and *BRCA1* or *BRCA2* mutations, but since most individuals were only screened for the common *BRCA1* and *BRCA2* founder mutations, cosegregation with less common mutations cannot be ruled out.

It would be interesting to investigate whether *BRCA2* and *PALB2* mutations can co-exist without resulting in phenotypic consequences such as embryonic lethality or Fanconi anaemia, as observed in biallelic mutation carriers of both *PALB2* and *BRCA2* (Reid *et al.* 2007, Xia *et al.* 2007, Howlett *et al*. 2002). Curiously, there are some monoallelic *BRCA2* mutation carrying FA cases that do not belong to any of the currently known subtypes. Naturally, this could be the result of an undetected mutation in *BRCA2* or alternatively, the patient actually belongs to a novel subtype yet to be defined and the individual, simply by chance, also happens to be a *BRCA2* mutation carrier. Reverse mosaicism, a common event in FA, could also explain this phenomenon (Waisfisz *et al.* 1999, Ameziane *et al.* 2008). However, it remains possible that mutations in two functionally linked genes such as *BRCA2* and *PALB2* might also produce a FA-like phenotype. Families co-segregating known susceptibility mutations in *CHEK2* (1100delC) and *RAD50* (c.687delT) and *PALB2* c.1592delT have been observed, but these families are extremely rare and do not appear to exhibit a significantly more pronounced cancer phenotype than the other *PALB2* c.1592delT families (unpublished results).

Several studies have investigated whether *PALB2* mutations are more likely to produce certain subtypes of breast tumours (Tischkowitz *et al.* 2007, García *et al.* 2008, Foulkes *et al.* 2007), a phenomenon most often observed in *BRCA1*-related breast cancer (Sorlie *et al.* 2003). The results of these studies are summarized in Table 6. In study III, it was observed that all except one of the seven tumours analysed (86%) revealed strong expression of the oestrogen

receptor, and five of seven (71%) showed expression of the progesterone receptor. These results imply that *PALB2* tumours mainly share the above phenotypic properties with those generated by *BRCA2* mutations (Borg 2001, Hedenfalk *et al.* 2003). However, the phenotype of *BRCA2* mutated tumours is not as clearly defined as that of *BRCA1* mutation related breast tumours. In addition, because of the limited number of specimens analysed in study III, more extensive analysis is needed to determine whether these expression patterns can be used to distinguish *PALB2* mutated tumours from sporadic ones. In the study of Tischkowitz *et al.* (2007) similar results were obtained, the majority of tumours having strong expression of oestrogen receptor and progesterone receptor. In contrast, in a Spanish study the oestrogen receptor and progesterone receptor statuses were negative in the single tumour analysed (García *et al.* 2008). The vast majority of *PALB2* tumours seem to be infiltrating ductal carcinomas (Table 6), but some cases of infiltrating lobular carcinoma and tumours with medullary features have also been described, in addition to premalignant ductal carcinoma *in situ* and lobular carcinoma *in situ* (Foulkes *et al.* 2007, Tischkowitz *et al.* 2007, García *et al.* 2008, Cao *et al.* 2008). Most breast tumours of carriers of *PALB2* c.1592delT in study III were also infiltrating ductal carcinomas (Table 5 in chapter 5.3), which are the most common histological subtype of breast cancer. *PALB2* tumours also appear to be of relatively high grade (mostly grade III). More research on this is needed to make more accurate predictions on the tumour subtype(s) associated with *PALB2* mutations, as there could also be a genotype-phenotype correlation between tumours with different *PALB2* mutations.

Table 6. Clinical characteristics of tumours of *PALB2* mutation carriers. Data from (1) Tischkowitz *et al.* 2007, (2) Foulkes *et al.* 2007, (3) García *et al.* 2008 and (4) Cao *et al.* 2008.

ID	Mutation	Effect on protein	Tumour	Age (years)	Cancer type	Grade	IHC	Ref.
AIII.1	229delT	C77Fs	T1	39	IDC	N/A	ER+ ,PR+	1
	229delT	C77Fs	T2	42	IDC	3*	ER+,PR+	1
	229delT	C77Fs	T3	60	IDC	2‡	ER+, PR-,Her2-	1
AII.2	229delT	C77Fs	T1	64	IDC	N/A	N/A	1
AII.4	229delT	C77Fs	T1	56	N/A	N/A	N/A	1
	229delT	C77Fs	T2	81	IDC	2*	ER+ PR+	1
	229delT	C77Fs	T3	85	HS	N/A	N/A	1
	229delT	C77Fs	T4	98	IDC	3*	ER+, PR-, Her2+	1
BIII.2	2521delA	T841Fs	T1	29	IDC	2‡	ER+ PR+	1
	2521delA	T841Fs	T2	46	ILC/ LCIS	2‡	ER+ PR+	1
BIII.5	2521delA	T841Fs	T1	46	IDC	2‡	ER+ ,PR+	1
P28031	2323C>T	Q775X	T1	54	IDC	2‡	ER+,PR+, Her2-	2
P31010	2323C>T	Q775X	T1	49	DCIS	N/A	N/A	2
	2323C>T	Q775X	T2	49	IDC	3‡	ER- PR-, Her2-	2
P26007	2323C>T	Q775X	T1	36	IDC[a]	3‡	ER-,PR-, Her2+	2
1	1056_1057delGA	K353Fs	T1	54	IDC	3	ER-,PR-, Her2-	3
1	751C>T	Q251X	T1	34	IDC	2‡	ER+,PR+, Her2+	4
2	751C>T	Q251X	T1	32	IDC	2‡	ER+,PR+, Her2+	4
3b	1050_1051delAAinsCT	Q350Fs	T1	44	IDC	3‡	ER-,PR+, Her2-	4
4b	1050_1051delAAinsCT	Q350Fs	T1	44	ILC	N/A	N/A	4

Ref. reference, HS hemangiosarcoma, a, with medullary features, b, patients belong to the same family * nuclear grade, ‡ histological grade, N/A, not available.

Loss of heterozygosity was not observed in the breast tumours analysed in study III. A similar result was obtained in the Canadian population (Tischkowitz *et al.* 2007), whereas the single tumour analysed in the Spanish study did exhibit signs of loss of the wild-type allele (García *et al.* 2008). A relatively small number of samples was analysed in each of the studies and further research is needed to determine whether LOH is an important event in tumours of *PALB2* mutation carriers. Some evidence of LOH in the *PALB2* c.1592delT carriers has been observed, but it is restricted to ovarian cancer cases (unpublished results). The observed difference in LOH prevalence between breast and ovarian cancers may

be the result of differential pathways of tumourigenesis or simply reflect the fact that ovarian tumours are structurally more homogenous and LOH is therefore more easily detected in ovarian tumours.

In addition to LOH, other mechanisms of tumourigenesis have also been suggested to account for *PALB2* tumours, including haploinsufficiency and dominant-negative effect of the truncated protein product (Rahman *et al.* 2007, study III). Recently, hypermethylation of the *PALB2* core promoter region has been reported in familial and sporadic breast and ovarian cancers. Two primary breast tumours of *BRCA2* mutation carriers, four sporadic breast and four ovarian tumours displayed promoter hypermethylation. All samples from normal tissue were unmethylated (Potapova *et al.* 2008). This suggests that *PALB2* inactivation may also occur through hypermethylation. A similar frequency of hypermethylation has been observed for *BRCA1* (Esteller *et al.* 2000, Esteller *et al.* 2001). Therefore, it will be important to investigate whether the wild-type allele may be inactivated by promoter hypermethylation in tumours of *PALB2* mutation carriers (Potapova *et al.* 2008). Immunohistochemistry could also be used to detect expression levels of PALB2 protein.

The age of cancer onset for *PALB2* c.1592delT carriers appears to be somewhat lower than for breast cancer cases without this mutation. However, this finding does not reach statistical significance. Naturally, this may be due to the low number of carriers or because there really is no significant difference between the two groups. Rahman *et al.* (2007) also investigated the effect on the age of onset for *PALB2* mutation carriers and observed that while the average age of onset was slightly lower for carriers this difference was not statistically significant. Age of cancer onset for *PALB2* c.1592delT carriers appears to be somewhat higher when compared to *BRCA1/2* mutation carriers in Finland, but this variation has not been shown to be statistically significant.

6.4 Involvement of *PALB2* mutations in other cancer types (III, IV)

Most *PALB2* mutation screenings have focused on breast cancer, but putative associations with other cancer types belonging to the BRCA-related cancer spectrum, such as male breast and colorectal cancer, have also been proposed (Rahman *et al.* 2007, Tischkowitz *et al.* 2007, Foulkes *et al.* 2007, García *et al.* 2008, Cao *et al.* 2008)

In study III, *PALB2* c.1592delT was screened in 141 Finnish male breast cancers, 639 prostate and 476 colon cancers. No carriers were identified among

male breast cancer patients or colon cancer patients. Additionally, approximately 30 male breast cancer cases were screened for mutations in the entire *PALB2* gene but no pathogenic changes were identified (unpublished results). Therefore, *PALB2* c.1592delT is unlikely to contribute significantly to male breast cancer or colorectal cancer in Finland. This is further supported by the fact that in the families of *PALB2* c.1592delT carriers, there were no cases of male breast cancer. In the British and Spanish populations, it was observed that *PALB2* mutations appeared slightly more often in families with both male and female breast cancer than merely female breast cancer. However, this difference was not statistically significant. The estimates were derived from analysis of just one family in each study and no male breast cancer patients were actually genotyped (Rahman *et al.* 2007, García *et al.* 2008). Therefore, this putative association remains to be explored. Naturally, possible involvement of *PALB2* mutations other than c.1592delT in Finnish male breast or colorectal cancers cannot be ruled out and they may affect the genetic makeup of these cancers. In fact, a recent study of a *PALB2* mutation carrier family exhibited several cases of colorectal cancer in addition to other cancer types (Foulkes *et al.* 2007). However, no colorectal cancer cases were genotyped in this study and therefore the potential association of these cancers with *PALB2* mutation remains unresolved. Colorectal cancer patients were also observed in some of the *PALB2* c.1592delT carrier families in study III, but the lack of DNA specimens from these family members prevented further analysis.

Among the familial prostate cancer cases, one family carrying the *PALB2* c.1592delT mutation was observed. A segregation study was performed on the family of the mutation-positive patient with prostate cancer. High penetrance of this mutation was observed in the two generations of this family that were studied. Subsequently, *PALB2* alterations have been investigated in 95 hereditary prostate cancer families. No truncating mutations were discovered and the study only detected two novel changes that were considered to be non-pathogenic (Tischkowitz *et al.* 2008). This suggests that *PALB2* mutations are unlikely to be common in hereditary prostate cancer, but they may account for some families with several affected family members.

Pedigrees of *PALB2* c.1592delT carrier families also displayed increased prevalence for e.g. stomach and ovarian cancers. In fact, some carriers of *PALB2* c.1592delT have been observed among ovarian cancer and stomach cancer patients, but the incidence is much lower than among breast cancer patients

(unpublished results). Therefore, investigating the role of *PALB2* mutations in other cancer types is of great importance.

6.5 Hazard ratio and penetrance of *PALB2* c.1592delT (IV)

Approximately a 6-fold increased incidence of breast cancer for *PALB2* c.1592delT carriers was observed using the families of all mutation carriers identified in a series of unselected breast cancer cases. Mutations in *BRCA1* and *BRCA2* have been estimated to have approximately 10-fold increased relative risk (Hopper *et al.* 1999). The evidence for increased breast cancer risks obtained in study IV is robust because it is fairly insensitive to the exact allele frequency and degree of family histories used. The estimate is also conservative because of the assumption that the seven carriers without a recorded family history had no relatives with cancer. Our analyses suggest that the risk of breast cancer for mutation carrying relatives of unselected carriers is about 40% by age 70 years (Figure 6 in chapter 5.4). This result has a relatively wide confidence interval ranging from 17–77% and it should therefore be verified using a larger sample size. There was no statistically significant evidence for an increased risk of NBC for carriers, but the study had little power to address this issue as it was focused on breast cancer patients. Further analyses are needed to determine the magnitude of risk for other cancer types in *PALB2* c.1592delT carriers. For comparison, a recent large meta-analysis of penetrance of the *CHEK2* 1100delC mutation revealed a cumulative risk of breast cancer of 37% by age 70 years (Weischer *et al.* 2008). This estimate was based on familial cases, however, and the risk associated with *CHEK2* 1100delC mutation might be lower in unselected cases.

The penetrance estimate and hazard ratio obtained in study IV should be interpreted with caution, considering that the estimates are based on a relatively small number of carriers and their families compared to published population-based estimates for the penetrance of mutations in *BRCA1* and *BRCA2*. Some assumptions also had to be made about missing data on family members, most particularly those for which family structure and cancer history had not been recorded. It would have been advantageous to know the mutation status for more relatives of the carriers. However, this information is not essential; the statistical approach used in study IV provides valid estimates no matter how many, if any, relatives are genotyped. Furthermore, having missing ages for some unaffected relatives is not optimal, but most of the information on penetrance comes from the affected women whose age at diagnosis was known.

If there are familial factors, such as other genes, which modify risks, then the estimates of penetrance using families selected for increased cancer occurrence will on average be higher than found by an approach as the one used in study IV. Similarly, estimates based on the family histories of mutation carrying controls (unselected for family history) will on average be lower. At this stage there is no evidence for there being familial modifiers of penetrance for *PALB2* mutation carriers, as was assumed by Rahman *et al.* (2007). This scenario, however, is consistent with the marginally significant observations that the HR decreases with age and that the age at onset of the seven case carriers with no known family history was 8.5 yrs later compared to the probands of the ten families with family histories of cancer. Recently, low-penetrance alleles in *FGFR2* and *MAP3KI* were estimated to modify the breast cancer risk of *BRCA2* mutation carriers. *TNRC9* variants appeared to modify the cancer risk in both *BRCA1* and *BRCA2* mutation carriers (Antoniou *et al.* 2008). The same variants could also be modifiers for *PALB2* mutation carriers, but this has not been studied so far. Due to the relatively low prevalence of *PALB2* mutation carriers, collecting enough samples to identify and statistically verify modifier genes might be difficult.

In study IV about half of the 17 *PALB2* mutation carriers were observed to have a family history of breast cancer, which is consistent with the estimated 40% lifetime risk for carriers. For example, about half of the *BRCA1* and *BRCA2* mutation-carrying breast cancer families identified through population-based sampling of cases, such as in study IV, also do not have a family history of cancer. Although this observation has been found repeatedly in several investigations it is perhaps not well-recognized (Hopper 2001).

One of the strengths of study IV was its focus on one particular mutation. Larger studies in which the relative risks of different *PALB2* mutations can be compared are needed to determine whether the risks caused by other *PALB2* mutations are similar to those attributed here to c.1592delT. For example, high penetrance has been suggested for c.229delT, the most 5' mutation in *PALB2* currently reported (Tischkowitz *et al.* 2007). In contrast, a much lower penetrance estimate (about a two-fold increased risk) was calculated for mutations located in the 3' terminus of the gene (Rahman *et al.* 2007). Therefore, it is possible that mutations closer to the 5' terminus could confer higher relative risks than those in the 3' terminus. The observed penetrance could also reflect differences in protein stability. The *PALB2* c.1592delT mutation produces a somewhat stable but functionally defective product and could therefore confer a higher cancer risk compared to a mutation acting merely in a haploinsufficient manner.

The estimated 40% breast cancer risk for *PALB2* c.1592delT carriers is high considering that it has been estimated that for *BRCA2* mutation carriers the breast cancer risk by age 70 years is 45% (95% CI 31–56) (Antoniou *et al.* 2003). Given this substantially enhanced cancer risk observed for heterozygous carriers and the approximately 1% occurrence rate of *PALB2* c.1592delT in unselected breast cancer cases observed in study III, genetic testing for this germline mutation could be considered in Finland for women with breast cancer. Particularly, testing women with early-onset disease and a family history of breast cancer and other related cancers not explained by high-risk mutations in *BRCA1* and *BRCA2* might be considered. In addition, both affected and disease-free *PALB2* c.1592delT carriers could benefit from inclusion in extended cancer surveillance programs similar to those of identified BRCA1/2 mutation carriers.

At present, corresponding penetrance analyses for other *PALB2* mutations have not been reported. It will be necessary to investigate the risks associated with different *PALB2* mutations and what clinical implications, such as different treatment schemes based on the defective DNA damage response of *PALB2* tumours, these investigations may have. It should be noted, however, that c.1592delT is currently the most common recurrent *PALB2* founder mutation reported and *PALB2* mutations in other populations may be too rare to allow sufficient sample size for a precise penetrance estimate. Nevertheless, it appears that in the absence of *BRCA1* and *BRCA2* mutations testing for mutations in other genes might be beneficial for families with increased breast cancer risk.

7 Concluding remarks

The purpose of this thesis was to elucidate the background of familial breast cancer susceptibility and specifically, to study the involvement of the putative candidate genes chosen for this study: *TOPBP1*, *CLSPN* and *PALB2*. These candidate genes were chosen based on their biological function in the DNA damage response pathway and/or their interactions with known susceptibility genes. Based on the results of each study the following observations and conclusions were made:

1. A potentially pathogenic alteration Arg309Cys was discovered in the *TOPBP1* gene. The observed frequencies for C/T heterozygotes were 15.2% (19/125) in the familial breast cancer cases compared to 7.0% (49/697) in the controls ($p = 0.002$; OR 2.4; 95% CI 1.3–4.2). Only one homozygous individual for this change was observed among familial breast cancer cases and none in the 697 control samples analysed. Protein analyses did not display differences in protein levels and no aberrant sized protein products were observed in cell lines of heterozygous Arg309Cys carriers. This result should, however, also be verified on the RNA level. The Arg309Cys change is relatively common at the population level so it is likely to be associated with a slightly increased breast cancer risk.
2. No clearly pathogenic mutations were found in the *CLSPN* gene. Only three changes were detected in the entire coding region. Therefore, the *CLSPN* gene appears to be extremely conserved, possibly reflecting crucial biological involvement. A potential modifier effect was discovered for the haplotype containing the 1195delGlu and Ser1280Leu alterations. However, this observation needs to be verified by further investigations. Based on these results *CLSPN* does not appear to be a significant breast cancer susceptibility gene.
3. The *PALB2* c.1592delT founder mutation was identified and found to be associated with an approximately four-fold increased breast cancer risk. Interestingly, *PALB2* c.1592delT mutation was observed in about 1% of unselected breast cancer patients, therefore making a significant contribution to breast cancer predisposition even outside the families considered to have a high heritable risk of breast cancer. G1145R missense change was also discovered, but an association with breast cancer was not confirmed. This alteration, however, locates to the WD40 repeats of PALB2 and could

therefore potentially be involved in functions of this protein that are yet to be determined.

4. A potential association of *PALB2* c.1592delT with familial prostate cancer was detected. However, this observation needs additional studies to be confirmed. No carriers of *PALB2* c.1592delT were observed among male breast cancer patients or colorectal cancer patients. Thus, this mutation is unlikely to be a major susceptibility factor for male breast or colorectal cancer in Finland.
5. *PALB2* c.1592delT exhibited significantly decreased BRCA2-binding affinity and consequently was also defective in homologous recombination in PALB2 knockdown cells. It was also not able to re-establish crosslink repair in PALB2 deficient cells. Thus, c.1592delT appears to be a genuine loss-of-function mutation.
6. No reproducible evidence of loss of heterozygosity was observed in the breast tumours of the six patients analysed. This suggests that c.1592delT might not act as a classical tumour suppressor gene. The effect of c.1592delT might be mediated through *PALB2* haploinsufficiency, possibly combined with a dominant-negative effect of the truncated protein product. Promoter hypermethylation could also be involved in inactivation of *PALB2* and this should be verified by additional studies.
7. The penetrance of *PALB2* c.1592delT was determined to be 40% by age 70 years in unselected carrier families. The hazard ratio for breast cancer was 6.1 [95% CI 2.2–17.2; $p = 0.01$] signifying a markedly increased risk of breast cancer. Based on these results, it might be beneficial to offer mutation carriers genetic counselling regarding their cancer risk and potentially include them in extended surveillance programs.

8 Future directions

The main purpose of this thesis was accomplished as novel cancer causing mutations were discovered. However, much more research is needed before the genetic makeup of breast cancer is defined. Overall, the results of this study have to some extent elucidated the background of familial breast cancer and created novel research paths to be pursued.

The *PALB2* c.1592delT mutation was proven to be clearly pathogenic and impose a markedly increased cancer risk also outside the high risk cancer families. Similar research findings have also been obtained in other populations. Much work still remains to be done to define the cancer spectrum of *PALB2* mutation carriers and to determine whether *PALB2* mutation carriers could benefit from the newly developed potential cancer treatments which selectively kill tumour cells with a defective DNA damage response. This, however, would require large collaboration and long follow-up times and it was consequently outside the scope of this investigation.

The tumour properties of *PALB2* c.1592delT mutation carriers should be further investigated by immunohistochemical analyses. Also, as the tumour properties could not be statistically evaluated, no clear definition of the phenotype of *PALB2* c.1592delT breast tumours could be made although certain similarities were observed between carriers. Statistical analysis would also be complicated by the relatively low number of carriers identified so far.

While no clearly pathogenic alterations were found in the *CLSPN* gene, the result nevertheless is important to rule out the involvement of this gene in cancer predisposition. The Arg309Cys change in the *TOPBP1* gene was demonstrated to be associated with breast cancer susceptibility but this result needs to be confirmed using an independent sample. The potential effect of this change on the RNA level is also currently unknown. No high penetrance mutation were found in the *TOPBP1* gene but they cannot be completely excluded by this analysis and may potentially affect other cancer types or merely be absent from the Finnish population.

In this thesis mostly genetic methods were employed. These may not always accurately describe these genes at the cellular level. Many of these analyses would benefit greatly from additional studies investigating the expression of these genes at the RNA and protein levels. Also comparison of protein expression in normal tissue vs. in tumours requires additional studies.

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ISBN 978-951-42-8967-5 (Paperback)
ISBN 978-951-42-8968-2 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)

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