Immunophenotyping of hereditary breast cancer

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Cover design: P. van der Groep
Lay-out: Gildeprint Drukkerijen - Enschede, The Netherlands
Printed by: Gildeprint Drukkerijen - Enschede, The Netherlands

ISBN: 978-90-393-5092-8

This thesis is the third to result from the 1st AEGON international Scholarship in Oncology, a joint research program between the Breast Cancer Research Group Utrecht (Department of Pathology, and the Division of Internal Medicine and Dermatology), the VU Oncology School (Departments of Pathology and Oncology, VU University Medical Center, Amsterdam), and the Sidney Kimmel Comprehensive Cancer Center at John Hopkins School of Medicine Baltimore, Maryland, USA. This joint research program is coordinated by Prof. dr. E. van der Wall and Prof. M.A. Carducci. It is funded by an unrestricted educational grant from Aegon Inc.

Financial support for printing this thesis was kindly provided by:
Division of Internal Medicine and Dermatology UMCUtrecht, Mieke and Leo, Jan and Paula, Department of Pathology UMCUtrecht, A.Menarini diagnostics.

Immunophenotyping of hereditary breast cancer

Immunofenotypering van erfelijke borstkanker (met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op dinsdag 23 juni 2009
des middags te 2.30 uur

door

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geboren op 21 mei 1970
te Amsterdam

Promotoren: Prof.dr. E. van der Wall
Prof.dr. G.J.A. Offerhaus

Contents

General Introduction

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In the Netherlands, one out of nine women will be diagnosed with breast cancer. Worldwide, over 1 million women each year are diagnosed with this disease and the numbers are still increasing. About 5% of all breast cancer cases are hereditary, mainly caused by germline mutations in the *BRCA1* and *BRCA2* genes. Since the discovery of the involvement of the *BRCA1* and *BRCA2* genes in breast cancer, their role in DNA repair, their structure, the mutations and the effect on the function of these genes has bit by bit been elucidated. In recent years, more information on the role of other susceptibility genes has become available, but still *BRCA1* and *BRCA2* are the main targets for mutation screening.

Positive family history for breast, ovarian and fallopian tube cancer, especially when occurring at younger age, is the usual criterion for starting genetic screening. However, even with refined genetic screening techniques available today, mutations in *BRCA1* and *BRCA2* may be missed. Furthermore, due to small families and sometimes insufficient medical records, family history may not be very informative. This may mean that germline mutations are not found. Lastly, for some mutations found in *BRCA1* and *BRCA2* it is yet unclear whether they are pathogenic, the so called "unclassified variants". All this is potentially serious as patients with a germline *BRCA1* or *BRCA2* mutation are at high risk of (bilateral) breast cancer, ovarian cancer, and fallopian tube cancer. This necessitates at least intensive screening for these malignancies to detect tumors in an early phase, but often women will opt for preventive bi- or contralateral mastectomy and salpingo-oophorectomy to maximally reduce their risk of getting cancer. Biomarkers pointing to a hereditary cancer syndrome are therefore warranted. Such biomarkers can e.g. be found in the breast cancer tissue of affected patients or their relatives. Biomarker profiling of tumour tissues, whether it is done in an expensive molecular or inexpensive immunohistochemical way, could therefore help to identify patients and families eligible for mutation screening or to establish whether an unclassified variant is pathogenic-or-not. In addition, new therapeutic targets may be identified in this way.

The aim of this thesis was therefore to investigate the immunoprofile of hereditary breast cancers caused by a germline mutation in the *BRCA1* or *BRCA2* gene to help to identify patients and families eligible for mutation screening, to provide a pathogenic mutation reference for research into unclassified variants. In addition, such studies on hereditary breast cancers and its precursors could help to better understand hereditary breast carcinogenesis and identify new therapeutic targets.

In *Chapter 2*, an overview of the literature concerning the pathology of hereditary breast cancer is given. In *Chapter 3*, we compared different clinicopathological features between breast cancers from carriers of proven *BRCA1* mutation and cancers from sporadic controls and used this to create decision trees. These decision trees where then used to classify five different groups of breast cancer cases at increasing risk of having the hereditary form of breast cancer. In *Chapter 4*, we report in two letters for the first time the expression of the epidermal growth factor receptor (EGFR) in *BRCA1* and *BRCA2* related breast cancers. In *Chapter 5*, we describe for the first time the immunoprofile of a premalignant lesion called ductal carcinoma in situ (DCIS) in *BRCA1* and *BRCA2* germline mutated patients and we compare this with the profile of the accompanying invasive breast cancers. In *Chapter 6,* we describe the novel finding of loss of expression of FANCD2, a Fanconi anaemia gene previously implicated in breast cancer in mice, in hereditary and sporadic invasive breast cancer. In *Chapter 7*, the expression of HIF-1α, the key regulator of the hypoxia response, in hereditary breast cancer is for the first time reported in comparison with sporadic breast cancer. All the findings in the studies mentioned above are discussed and the main conclusions of these studies are presented in *Chapter 8*.

Pathology of hereditary breast cancer

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Abstract

Hereditary breast cancer runs in families where several members in different generations are affected. Most of these breast cancers are caused by mutations in the high penetrance genes *BRCA1* and *BRCA2* accounting for about 5% of all breast cancers. Other genes like *CHEK2* , *PTEN*, *TP53* , *ATM*, *STK11/LKB1*, *CDH1*, *NBS1*, *RAD50*, *BRIP1* and *PALB2* have been described to be high or moderate penetrance breast cancer susceptibility genes, all contributing to the hereditary breast cancer spectrum. However, still a part of familial hereditary breast cancers is not related to any of these breast cancer susceptibility genes. Research on new susceptibility genes is therefore ongoing. In this review we will focus on the function of the today known high or moderate penetrance breast cancer susceptibility genes and the consequences of their mutated status. Furthermore, we will highlight differences in histological markers, the expression of certain proteins and the gene expression profile of the breast cancers caused by mutations in *BRCA1* and *BRCA2* genes and, where possible, the other high or moderate penetrance breast cancer susceptibility genes. Finally, an overview on diagnosis, treatment and survival of these hereditary breast cancer patients will be provided. This information should lead to a better understanding of the characteristics of different types of hereditary breast cancers.

Introduction

"In 1866 a French surgeon by the name of Broca was the first to describe a family with a high prevelance of carcinoma of the breast. His wife suffered from early onset of breast cancer and when he made a pedigree of her family, four generations with breast cancer could be identified[1]"

The "Broca" report is the first of many that pointed out that breast cancer can be inherited, passing through from one generation to the other. Family history of breast cancer is now an established risk factor for the development of the disease. In fact, among those variables that have been shown to bear a causal relationship with breast cancer, the highest increased risk, after age, is a positive family history of breast cancer[2]. With the knowledge of today, only in about 5% of all the breast cancer cases, the disease will occur as part of a hereditary cancer susceptibility syndrome, caused by mutations in high penetrance susceptibility genes. A substantial proportion of hereditary breast cancers, about 16%[3,4], can be attributed to germline mutations in either of the *BRCA* (breast cancer 1 and 2) early onset genes. Since the finding of the *BRCA1* and *BRCA2* genes in 1994, several studies have been undertaken to find other high penetrance breast cancer susceptibility genes like *BRCA1* and *BRCA2*, with no results so far[5].

Nevertheless, various other genes conferring an increased risk of breast cancer involved in hereditary cancer syndromes have been found, like *CHEK2*, *PTEN*, *TP53*, *ATM*, *STK11/LKB1*, *CDH1*, *NBS1*, *RAD50*, *BRIP1* and *PALB2*. Some of these genes are involved in multiple cancer syndromes like Li-Fraumeni (*TP53*), Peutz-Jeghers (*STK11/LKB1*) and Cowden syndrome (*PTEN*)[6-10]. In table 1, an overview of the hereditary cancer susceptibility syndrome genes is shown, including the chromosomal location of the causal genes and the clinical features of these syndromes.

In this paper, we will mainly focus on the hereditary breast cancer syndromes caused by germline mutations in the *BRCA1* and *BRCA2* genes since these have been well studied for the pathology of their cancers. Therefore, we will first discus briefly the rarer hereditary cancer susceptibility syndrome genes mentioned earlier (figure1) where yet is little known on tumor pathology [11-13].

Table 1. An overview of the hereditary cancer susceptibility syndrome genes is shown, including the chromosomal location of the causal genes and the clinical features of these syndromes.

Gene involved	Cytoband	Breast cancer risk	Syndrome	Clinical features
BRCA1	17q21	High	Hereditary breast cancer and ovarian syndrome	Breast cancer Ovarian cancer
BRCA2	13q12.3	High	Hereditary breast cancer and ovarian syndrome	Breast cancer Ovarian cancer Prostate cancer Pancreatic cancer Melanoma
TP53	17p13.1	High	Li-Fraumeni syndrome	Breast cancer Sarcomas Brain tumors
ATM	11q22.3	Intermediate	Louis-Bar syndrome	Lymphoma Cerebellar ataxia Immune deficiency Glioma Medulloblastoma Breast cancer
CDH1	16q22.1	Intermediate	Familial diffuse gastric cancer syndrome	Gastric cancer Lobular breast cancer
PTEN	10q23.31	Intermediate	Cowden syndrome	Increased risk of neoplasms: Breastcancer, thyroid carcinoma, endometrial carcinomas Hamartomatous polyps of the gastrointestinal tract
			Bannayan-Riley-Rivalcaba syndrome	Breast cancer Meningioma

STK1	19p13.3	Intermediate	Peutz-Jeghers syndrome	Melanocytic macules of the lips and others multiple gastrointestinal hamartomatous polyps increased risk of neoplasms; breast, testis, pancreas and cervix
NBS1	8q21	Intermediate	Nijmegen breakage syndrome	Microcephaly,growth retardation, immunodeficiency and a marked susceptibility to cancer Moderate risk of breast cancer
BRIP/FANCJ	17q22	Intermediate	Fanconi anemia	Developmental anomalies affecting the skeleton (absent or abnormal thumbs and radii), kidneys, heart or any other major organ system Aplastic anaemia Acute myeloid leukaemia and squamous cell carcinoma Low risk of breast cancer
PALB2/FANCN	16p12	Intermediate		
FANCA	16q24.3	Low		
FANCE	6p22-p21	Low		
MSH2	2p22-p21	Low	Lynch cancer family syndrome	increased risk of endometrial and colorectal carcinomas high risk of multiple primary malignant neoplasms including breast, ovarian, astrointestinal and genitourinary carcinomas, sarcomas, glioblastomas and leukaemia
MSH3	5q11-q12	Low		
MSH6	2p16	Low		
MLH1	3p21.3	Low		
PMS1	2q31-q33	Low		
PMS2	7p22	Low		

TP53 (tumor protein p53) is a tumor suppressor gene located on chromosome 17p13.1 encoding a nuclear phosphoprotein (p53). *TP53* acts as a transcription factor involved in the control of cell cycle progression, repair of DNA damage, genomic stability, and apoptosis[14]. *TP53* is constitutionally mutated in the Li-Fraumeni syndrome, an autosomal dominant predisposition to breast cancer and other forms of cancer (see table1). Most mutations concern point mutations leading to proteins defective for sequence-specific DNA binding and activation of p53 responsive genes[6-8]. The *TP53* gene is more commonly altered in *BRCA1* (56%) and *BRCA2* (29%) related breast cancer in comparison with non-*BRCA* related breast cancer, denoting *TP53* to be a high breast cancer susceptibility gene[15]. In *BRCA1* or *BRCA2* deficient cells, changes were seen at *TP53* codons that are not the mutation hotspots. Structural modelling showed that most of these p53 non-hot spot aminoacids are distributed in a region of the protein on the opposite side of the p53 DNA-binding surface in these BRCA1 or BRCA2 deficient cells[16]. Breast cancers with these *TP53* non-hot spot mutations were associated with a significantly better prognosis when compared with *TP53* mutations in conserved or structural domains[17]. Preliminary data suggest that *BRCA1* or *BRCA2* mutations influence the distribution of the *TP53* mutations and the way of carcinogenesis, but additional studies must be performed to support this[16].

The *CHEK2* (checkpoint kinase 2) gene is located on chromosome 22q12.1 and encodes a cell cycle checkpoint kinase which is a key mediator in DNA damage response[18-20]. Mutations in *CHEK2* were originally thought to result in the Li-Fraumeni syndrome or in a Li-Fraumeni-like syndrome (mentioned above and described in table 1), since the first *CHEK2* mutations were found in these Li-Fraumeni families[21]. More recent studies question this association, following the identification of the 1000delC and 1157T *CHEK2* germline variants among breast cancer patients that otherwise show no signs of Li-Fraumeni symptoms[22, 23]. The *CHEK2* gene has been proposed to be a low penetrance breast cancer susceptibility gene. The 1000delC variant results in an approximately two fold risk of breast cancer in women and a tenfold risk in men. In these cases, there is no mention of

co-existence of BRCA1 and BRCA2 mutations[18, 24]. So far, beside the 1000delC and 1157T mutations, no additional *CHEK2* mutations have yet been found[25].

The *ATM* (ataxia teleangiectasia mutated) gene is located on chromosome 11q22.3 and encodes a checkpoint kinase that plays a role in DNA repair. Biallelic mutations in this gene are linked to the rare human autosomal recessive disorder called ataxia teleangiectasia (AT)[26], causing a variety of somatic disorders as described in table 1. A heterozygous mutation of *ATM* does not lead to the AT phenotype but carriers do have a two to five fold risk of breast cancer[27, 28] (table1).

CDH1 (Cadherin 1, E-cadherin) is a gene located on chromosome 16q22.1 coding for E-cadherin, a calcium dependent cell adhesion glycoprotein, which is important for cell-to-cell adhesion[29] in epithelial cells. Familial diffuse gastric cancer, an autosomal dominant cancer syndrome, is caused by mutations in the *CDH1* gene, and affected women are also predisposed to lobular breast cancer with an about 50% life time risk of getting breast cancer[30, 31] (table1).

PTEN (phosphatase and tensin homolog), is a tumor suppressor gene located on chromosome 10q23.3. *PTEN* codes for the protein phosphatidylinositol phosphate phosphatase and has multiple yet incompletely understood roles in cellular regulation[32, 33]. Germline mutations in *PTEN* can lead to a rare autosomal dominant inherited cancer syndrome, Cowden disease, characterized by a high risk of breast, thyroid and endometrial carcinomas and hamartomas[10]. Mutations in *PTEN* also cause the related Bannayan-Riley-Rivalcaba syndrome[34], see for more details table 1.

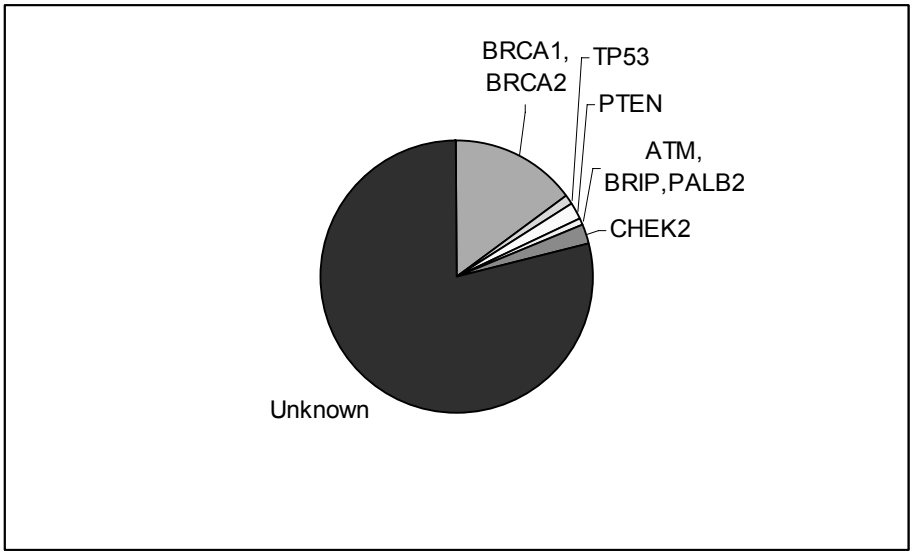
STK11 (LKB1) (Serine/theronine kinase 11) is a gene located on chromosome 19p13.3 that encodes a serine/threonine kinase and functions mainly through inhibition of the mTOR pathway. *STK11* is mutated in the autosomal dominant condition Peutz-Jeghers syndrome, characterized by perioral pigmentation and hamartomatous polyposis[9]. Patients with this syndrome have a 30 to 50% risk of developing breast cancer[35-37] (table1).

NBS1 is a gene located on chromosome 8q21 and involved in the Nijmegen breakage syndrome, a chromosome instability syndrome. Proteins of the gene *NSB* together with proteins of the genes *RAD50* and *MRE11*, form the so called MRN complex.

The MRN complex is involved in the recognition and repair of DNA double strand breaks[38]. The estimated prevalence of the most common mutation is very low and the breast cancer risk conferred by a NBS1 mutation is estimated to be low[39].

A rare recessive repair defect disorder called Fanconi anaemia (FA) is linked to a number of genes, in total 12 so far, that, together with *BRCA1*, are involved in homologous recombination DNA repair mechanisms[40-42]. Mutations in *FANCJ* (=*BRIP1*) and *FANCN* (= *PALB2*) are associated with a two fold increased risk of breast cancer [43, 44]. The remaining ten FA genes may likewise be involved in breast carcinogenesis but their role has not been elucidated yet. It has been suggested that the remaining FA genes are inactivated through epigenetic/transcriptional mechanisms. For example, the FANCD2 protein is down regulated in sporadic and in hereditary breast carcinomas[45].

Figure1. Affected genes in hereditary breast cancer.



The *Mismatch repair genes (MMR), MLH1, MSH2* and *MSH6,* play a role in hereditary non-polyposis colorectal cancer, the Lynch-syndrome. In a few of these families,

breast cancer is part of this syndrome, which seems to be related to the absence of the MLH1and MSH2 proteins[46]. Furthermore, a causative role of MSH6 in the occurrence of breast cancer has been suggested but only one case has been reported so far [47].

Together with *BRCA1* and *BRCA2*, the above described genes account for almost 25% of all hereditary breast cancers (figure 1). Obviously, the search for other genes involved in hereditary breast cancer is still continuing[48].

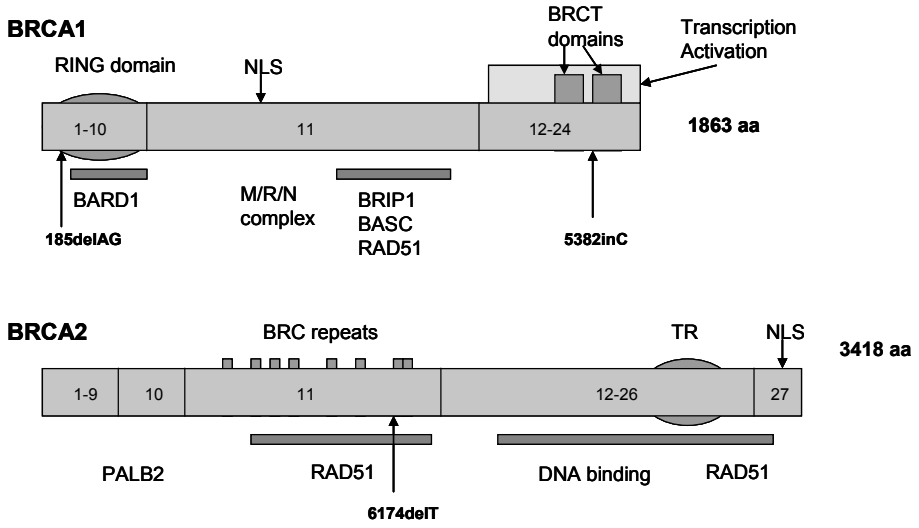
The *BRCA1* and *BRCA2* genes

Discovery

The *BRCA1* and *BRCA2* genes were discovered in the nineties, starting in 1990 where *BRCA1* was for the first time linked to breast cancer through linkage analysis in a large group of early onset breast cancer families. The *BRCA1* gene was mapped to chromosome 17q21[49]. Furthermore, this gene was also linked to families with ovarian cancer [50]. In 1994, the *BRCA1* gene was cloned and truncating mutations were identified in the coding sequence of the *BRCA1* gene in families with multiple cases of breast cancer [51]. Search for more genes that might be involved in these hereditary susceptibility breast cancer families led in 1995 to the discovery of the *BRCA2* gene. The *BRCA2* gene is located on chromosome 13q12.3, and was discovered also by linking analysis and positional cloning using familial breast cancer pedigrees in successive generations[52, 53]. At the same time, families with high frequencies of male breast cancer were found to carry the *BRCA2* mutation[54].

Carriers of the *BRCA1* and *BRCA2* mutations not only develop breast cancer and have a high risk for ovarian cancer but also bear an increased risk for developing fallopian tube, colon, melanoma, prostate and pancreatic cancer [55-60].

Figure 2. Schematic representation of *BRCA1* and *BRCA2* functional domains and selected binding partners.



NLS = Nuclear Localisation signal
Some of the proteins interacting with BRCA1 or BRCA2 are marked below the site of interaction

Structure

Both *BRCA* genes bear rather complex genomic structures. *BRCA1* is composed of 24 exons and *BRCA2* of 27 exons. They both encode very large proteins: *BRCA1* consists of 1863 amino acids and *BRCA2* of 3418 amino acids. In both genes, exon 1 is noncoding and exon 11 is unusually large[52, 53, 61] (figure 2). *BRCA1* has a highly conserved zinc-binding RING finger domain which is located close to the amino-terminus. RING finger domain proteins are recognized as E3 ligase enzymes that participate in ubiquitination[62]. Mutations in the RING finger domain inactivate BRCA E3 ligase and have an effect on the other tumor suppressor activities of *BRCA1*[63]. Towards the carboxyl terminus of *BRCA1* two tandem copies of the same motif are found, designated the BRCT domains. These BRCT domains are regions reported to activate transcription when fused to a DNA binding domain[64]. *BRCA2*

contains a number of recognizable motifs, the eight copies of a 20-30 amino acid repeat termed BRC repeats and the ssDNA binding region. Their function is to bind to RAD51 to regulate DNA repair (figure 2)[65, 66].

Function

Both *BRCA* genes are involved in DNA repair. They form complexes that will activate the repair of double strand breaks (DSBs) and initiate homologous recombination (HR). *RAD51* is the key component of this mechanism. Co-localization of *BRCA1* and *BRCA2* with *RAD51* at the site of recombination and DNA damaged induced foci strongly suggest that they are involved in the detection and the repair of DSBs. The roles played by *BRCA1* and *BRCA2* in this process appear to differ. *BRCA1* will associate with *RAD51* upon DNA damage and subsequently will be phosphorylated in this process, but the nature of the interaction with *RAD51* is yet unknown[67]. *BRCA2* has a more direct role through its strict interaction with *RAD51* via the BRC repeats[68]. In addition, *RAD51* also interacts with the C-terminal region of *BRCA2*, TR2[69, 70]. This part of *BRCA2* is thought to serve a regulatory role in recombinational repair. Phosphorylation of this part of *BRCA2* can provide a dual function, resulting in inhibition or activation during HR[71, 72]. *BRCA2* also has a role in HR in meiosis via an interaction with *RAD51* and *DMC1*. Given the fact that they have distinct non-overlapping binding sites, it has been suggested that there might well be a BRCA2-RAD51-DMC1 complex. However, more data has to be obtained to confirm this. It does suggest that *BRCA2* not only plays a role in carcinogenesis but in addition contributes to fertility problems in affected carriers[73]. Cells that are defective for *BRCA1* and *BRCA2* are hypersensitive for crosslinking agents that will produce double strand breaks like mitomycin C and cisplatin[74-76]. Also, ionizing radiation will produce these same breaks and both will be resolved by error-prone repair, such as non homologous end joining[77, 78]. The level of expression of *BRCA1*, *BRCA2* and *RAD51* increase in cells when they enter the S- phase, indicating that they function during or after DNA replication. This means that *BRCA1* and *BRCA2* function in a common pathway that is responsible for the integrity of the genome and the maintenance of

chromosomal stability[79]. *BRCA1* is part of the *BRCA1* associated genome surveillance complex (BASC) This complex includes *MSH2*, *MSH6*, *MLH1*, *ATM*, *BLM* , the RAD50-MRE11-NBS1 complex and the DNA replication factor C. All the members of this complex have roles in recognition of abnormal or damaged DNA, suggesting that the BASC may serve as a sensor for DNA damage and as a regulator of the post-replicational repair process[80]. *BRCA1* functions also as a checkpoint control, playing an essential role in cell survival by preventing the propagation of DNA damage through cell cycle progression before DNA repair has taken place[80]. Taken together, *BRCA1* is an integral part of the DNA damage signalling cascade; downstream of *ATM* and ATR kinases and both downstream and upstream of the checkpoint protein kinases *CHK1* and *CHK2*, suggesting that there is a positive feedback loop to increase the magnitude of DNA damage response. In addition, *BRCA1* regulates the expression of additional G2M cell cycle checkpoint proteins, thereby preventing unscheduled transition into mitosis at multiple levels of regulation. Ubiquitination is the process by which proteins are tagged for degradation by the proteasome. *BRCA1* functions with BARD1 in this ubiquitination process[81]. It has been suggested that *BRCA1* plays a role in both transcription coupled repair[82] and global genome repair[83]. So, in conclusion, both *BRCA* genes are involved in DNA repair and both function in a common pathway that is responsible for the integrity of the genome and the maintenance of chromosomal stability.

Mutations

The Breast Cancer Information Core (BIC) database has recorded 1643 and 1856 distinct mutations, polymorphisms and variants in the *BRCA1* and *BRCA2* genes respectively (BIC data 2008). Mutations appear to be reasonably evenly distributed across the coding sequences, with no obvious "mutation hot spots". Most mutations found in the breast and ovarian cancer families are predicted to truncate the protein product, which will lead to shortened and non-functional *BRCA1* and *BRCA2* proteins. The most common types of mutations are small frameshift insertions or deletions, non-sense mutation or mutations affecting splice sites,

resulting in deletion of complete or partial exons or insertion of intronic sequence. These mutations will cover approximately 70% of the *BRCA1* mutations and 90% of the *BRCA2* mutations in linked families, as estimated by the Breast Cancer Linkage Consortium (BCLC)[12]. Large-scale rearrangements including insertions, deletions or duplications of more than 500kb of DNA have also been identified. There have been reports of at least 19 distinct large genomic rearrangements in *BRCA1* and two genomic rearrangements in *BRCA2*, identified using multiplex ligation dependent probe amplification (MLPA). The majority of the rearrangements are deletions of one or more exons[84, 85]. These mutations can be all classified with reasonable confidence but classification of rare missense changes is still a challenge. According to the BIC database, approximately half of the unique *BRCA1* and *BRCA2* variants detected (excluding common polymorphisms) are protein-truncating or known deleterious missense mutations, while the remaining are missense variants of unknown pathogenic potential, termed "unclassified variants". A matter of concern here is that the BIC database did not take into account the frequency in which these variants were found in the population undergoing testing. Furthermore, the clinical relevance of only a few unclassified variants has been established. For the others, the subtle alteration might not alter the function of the protein and there might also be insufficient information about the family history to classify these unclassified variants as cancer predisposed changes. However, these alterations can provide indications to do further functional and familial studies[86,87]. It has been stated that a new approach is needed to improve the association between these unclassified variants and breast cancer risk, and that using histopathology data of tumors from carries of an unclassified variant could be helpful[88].

Loss of heterozygosity (LOH) of the wildtype allele has been robustly demonstrated for *BRCA* linked breast cancer. Although some of the studies mentioning a role of LOH in approximately 80% of the cases included in the studies, for the rest of the cases LOH affecting the *BRCA* gene could not be detected[89-92]. This might be caused by the practical and conceptual problems associated with LOH studies[93]. Furthermore, LOH of the wild type allele is not required for *BRCA* linked breast tumorigenesis

and when it occurs it is probably a late event[94]. Another consideration is whether a second somatic mutation or methylation dependent silencing affecting the wild type allele accounts for these findings. However, no evidence of a second somatic mutation in *BRCA* linked breast cancer has been found until now. In addition, with respect to promotor methylation, so far this has only been reported in sporadic breast cancer [95, 96].

Population specific occurrence

The majority of all the mutation described above are found throughout the population. However, certain mutations in *BRCA1* and *BRCA2* have been observed to be common in specific populations. Such "founder mutations" in *BRCA1* and *BRCA2* have been described in French Canadian women[97], Swedes[98], Icelandic women[99], Norwegians[100], Finns[101], Dutch women[102, 103], Russians[104], Japanese women[105], African Americans[106], and Ashkenazi Jewish women[107, 108]. Three mutations are commonly found in the Ashkenazi Jewish population, the 185delAG[107, 109] and 5382insC in BRCA1 and 6147delT in *BRCA2*[110]. The 185delAG is prevalent in 1% of all Ashkenazi Jews but has also been reported in other Jewish groups[111]. The 5382insC mutation found in 0.1% of the Ashkenazi Jews is described to occur more widespread, being common in Poland, Russia, and other parts of Eastern Europe and occurring in most European populations. In Ashkenazi Jewish women with breast cancer, the 185delAG mutation in *BRCA1* is found in 20% [112]. The 6147delT mutation in *BRCA2* is found to be present in 8% of the Ashkenazi Jews with breast cancer[108, 113]. In Iceland, a single *BRCA2* mutation 999del5 has been identified and is present in the majority of familial breast cancer cases in this population[99, 114].

BRCA1 related breast cancer

Histology

The histology of *BRCA1* associated breast cancers differs from the histopathological features of sporadic breast cancers in various aspects. The majority of the *BRCA1* associated tumors are invasive ductal adenocarcinomas (74%). However, compared to sporadic breast cancer, a significantly higher frequency of the *BRCA1* associated tumors are classified as medullary or atypical medullary carcinomas, 2% versus 13% respectively[115, 116]. The remaining histological types of breast cancer occur about equally in *BRCA1* mutation associated tumors and in sporadic breast cancer [115]. With regard to other histopathological characteristics it is observed that *BRCA1* tumors are more frequently poorly differentiated (grade 3), have a high mitotic count and show an increased frequency of necrotic areas. Tubule formation is decreased, but a higher degree of pleiomorphism is observed, all aspects pointing at a more aggressive phenotype[116-119]. In addition, tumors are often well demarcated and show a remarkable degree of lymphoplasmocytic infiltration.

When considering the age of onset of these *BRCA1* mutation carriers, less than 50 years of age compared to age above 50 years, significantly higher grade and more medullary type breast cancer are seen in the younger population[120]. With regard to pre-invasive breast lesions, it has initially been reported that ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) are seen less frequently next to invasive breast cancers in *BRCA1* mutation carriers, being 41% and 2% respectively versus 56% and 6% in non-carriers[115, 121]. However, studies investigating the occurrence of premalignant lesions in prophylactic mastectomies of *BRCA1* mutation carriers showed different results, describing a usually more frequent occurrence of premalignant lesions in prophylactic mastectomies. These premalignant lesions concern DCIS[122-124], LCIS[123], atypical ductal (ADH)[122-125] and atypical lobular hyperplasia (ALH)[122-125], usual ductal hyperplasia[125], columnar cell lesions[122] and fibroadenoma[125, 126]. Interestingly, the remarkable lymphocytoplasmic infiltrate described in invasive *BRCA1* related cancers has also been described in DCIS lesions[115] and even the normal breast shows often T-cell lobulitis[127].

Immunophenotype

The immunophenotype of the *BRCA1* mutation related breast cancers is first of all characterized by a low expression of the estrogen receptor alpha (ERα). In 1997 the first reports about the low expression of ERα in *BRCA1* tumors compared to sporadic tumors were described. Subsequent reports confirmed this observation and in addition described a significant relationship between low ERα on the one hand and high grade[120, 128-134] and an earlier age of onset[120, 134, 135] on the other. In contrast, overexpression of estrogen receptor beta (ERβ) is seen in breast cancers of *BRCA1* mutation carriers[136] Expression of the progesterone receptor (PR) has been reported to be low[120, 128, 129, 132, 133].

Overall, the expression of the human epidermal growth receptor 2 (HER-2/*neu*) is low in BRCA1 related breast cancers when compared with controls[118, 129, 132]. Furthermore no HER-2/*neu* amplifications among *BRCA1* tumors have been reported. One explanation could be that in the background of a *BRCA1* germline mutation, HER-2/*neu* is lost during loss of heterozygosity (LOH) at the *BRCA1* locus since HER-2/*neu* is localized close to *BRCA1* on chromosome 17[128, 137, 138].

In contrast to HER-2/*neu*, overexpression of the epidermal growth factor receptor (EGFR) has been strongly associated with *BRCA1* associated breast cancers [139-143].

BRCA1 related breast cancers often lack cyclin D1 (CCND1) expression[132, 144], and no CCND1 amplification have been reported in *BRCA1* breast cancers[135]. Also the expression of $p27^{Kip1}$ is very low in *BRCA1* related breast cancers and this is seen together with high levels of cyclin E[134]. Several studies report that *TP53* mutations and p53 protein expression is frequently seen in *BRCA1* related breast cancers compared to sporadic breast cancers. Mutations in the *TP53* gene are seen in 30-77% of *BRCA1* tumors whereas they are only present in about 20% of sporadic controls. As a consequence, accumulation of p53 is often seen in *BRCA1* related breast cancer. Furthermore, the distribution of the TP53 mutations might be influenced by the *BRCA1* and *BRCA2* genes[16, 132, 145, 146].

Evaluating the expression of several basal markers in *BRCA1* related breast cancers, it was observed that most of these tumors are positive for cytokeratins CK5/6

and CK14[140, 143], caveolin-1[147], and vimentin and laminin[148]. In breast cancers with a *BRCA1* mutation the expression of p-cadherin is also frequently increased and shows a correlation with all the other basal markers[149].

Expression of the apoptosis related proteins BAX and BCL2 in *BRCA1* related breast cancers is lower compared to sporadic breast cancers is reported[128, 150, 15]. In contrast, high levels of active caspase-3 were observed in *BRCA1* tumors. Hypoxia inducible factor-1α (HIF-1α) is the key regulator of the hypoxia response. HIF-1α is overexpressed during sporadic breast carcinogenesis[152] and correlated with poor prognosis[153, 154]. It appears to be involved in *BRCA1* related breast cancers, where HIF-1α is overexpressed in most of these tumors[155].

Altogether, this immunophenotype indicates that *BRCA1* related invasive breast cancer largely shows a "basal" immunophenotype like the stem cells of the breast, indicating that they initially may (in contrast to *BRCA2* related cancers, see below) derive from these stem cells.

While the immunophenotype of invasive *BRCA1* related cancers has been well studied little is yet known on the immunophenotype of pre-invasive lesions from the *BRCA1* carcinogenetic spectrum.

Genetic profile

Gene expression profile analysis has provided a tool to distinguish distinct subtypes of breast cancers[156, 157]. The gene expression profile of *BRCA1* associated tumors involves genes that were found to have functions in proliferation, angiogenesis, cell motility, cell adhesion, transcription and DNA repair. Based on these data *BRCA1* associated breast cancers are also genetically classified as basal. As mentioned above, *BRCA1* related breast tumors express basal markers like CK 5/6, CK14, EGFR, P-cadherin and caveolin-1, vimentin and laminin, thereby confirming the basal subtype as established by gene expression[140, 143, 148]. These data further underline that carcinogenesis in *BRCA1* germline mutation carriers very often occurs within the "basal" progression route.

Promotor hypermethylation has been shown to be somewhat less abundant in

BRCA1 germline mutation related breast cancers[158], although it is still clearly higher than in normal tissue.

As to copy number changes, a different pattern of chromosomal copy-number gains and losses compared to sporadic controls has been found. Copy number changes frequently occurring in BRCA1 related breast cancers are gains of 3q, 7p, 8q 10p, 12p, 16p and 17q and loss of 2q, 3p, 4p, 4q, 5q, 12q, 16p and 18q. This only partly overlaps with copy number changes found in sporadic and *BRCA2* germline mutation related breast cancers (table 2)[159-161].

Table 2. Chromosomal loci showing significant differences in frequency of gain or loss by array comparative genomic hybridization between *BRCA1* and *BRCA2* related and sporadic breast cancers.

	Frequency			p-value		
Locus	***BRCA1***	***BRCA2***	***Sporadic***	***BRCA1* vs sporadic**	***BRCA2* vs sporadic**	***BRCA1* vs *BRCA2***
Gains						
1cen-p13	89	68	87			0.054
3pter-p22	33	16	0	0.006		
3q13-q27	67	56	13	0.000	0.073	
8p12-cent	11	16	47	0.012		
9p	33	16	3	0.078		
9q22-q34	0	32	3			0.013
10pter-p12	50	20	7	0.000		
10p12-q21	36	4	3	0.089		
13q3	25	8	0	0.059		
16p	17	24	57	0.019		
18p	28	16	3	0.025		
Losses						
5cent-q23	72	40	27	0.025		
14q1-q2	39	8	10			0.048

BRCA2 related breast cancer

Histology

Similar to *BRCA1* related breast cancers, the most common histological type in *BRCA2* tumors is invasive ductal carcinoma (76%)[115]. Reports of a higher incidence of tumors belonging to invasive (pleiomorphic) lobular, tubular and cribriform carcinomas in *BRCA2* related breast cancers compared to sporadic breast cancer have been published[116, 117, 119, 162-164]. *BRCA2* tumors are more frequently moderately or poorly differentiated carcinomas (grade 2 and 3)[116, 128, 133, 162] due to less tubule formation[115], more nuclear pleiomorphism and higher mitotic rates compared to controls[162, 163]. *BRCA2* related breast cancers have, as *BRCA1* related cancers, a higher proportion of continuous pushing margins in comparison to sporadic breast cancers[116, 163]. With regard to pre-invasive breast lesions it has been described that DCIS and LCIS in *BRCA2* mutation carriers occur in about the same frequency, 52% and 3% respectively compared to 56% and 6% in control individuals[115, 121, 165]. The occurrence of premalignant lesions in prophylactic mastectomies of *BRCA2* mutation carriers show different results, similar to what has been observed in *BRCA1* mutation carriers, ranging from no differences to the more frequent occurrence of DCIS[122, 123], LCIS[123, 124], ADH[122-125], ALH[122-125] and columnar cell lesions[122]. Interestingly, in line with the remarkable lymphocytoplasmic infiltrate described in invasive *BRCA2* related cancers, even the normal breast shows often T-cell lobulitis[127].

Immunophenotype

The immunophenotype of *BRCA2* related breast cancers is largely similar to the immunophenotype of sporadic breast cancers. As a consequence, most *BRCA2* tumors show a different immunophenotype compared to *BRCA1* related breast tumors discussed above with more frequently expression of ERα and PR[119, 120, 143, 162, 163]. ERα expression has been known to be inversely correlated with grade. Furthermore, these ERα positive *BRCA2* related breast cancers decrease in frequency with increasing age[134]. In *BRCA2* related breast cancer different studies report no

or low expression of HER-2/*neu* compared to sporadic breast cancer and absence of HER-2/*neu* amplification similar to *BRCA1* germline mutation related breast cancers[128, 129, 144, 163]. Furthermore, a more recent study described that *BRCA2* related breast cancers are characterized by a higher expression of fibroblast growth factor 1 (FGF1) and fibroblast growth factor receptor 2 (FGFR2) compared to *BRCA1* related breast cancers. This could help to distinguish *BRCA2* related breast cancers from *BRCA1* related and sporadic breast cancers[166]. The *BRCA2* related breast cancers usually express only "luminal" cytokeratins like CK8 and CK18 and not CK5/6 and CK14[150]. In *BRCA2* related breast cancers no expression of caveolin-1 has been described in contrast to the expression of caveolin-1 in *BRCA1* related tumors[147]. No differences or even lower levels of the incidence of p53 have been reported for *BRCA2* related breast cancers in comparison with *BRCA1* related breast cancers[150]. Higher expression of cyclin D1, BAX and BCL2 in *BRCA2* related breast cancers compared to *BRCA1* and non *BRCA* carriers have been described[128, 150]. Anecdotic data suggest that EGFR is high in *BRCA2* related cancers similar to *BRCA1* related cancers[142]. While BRCA1 related cancers have been described to be frequently positive for P-cadherin, vimentin and HIF-1α, no such data are yet available for *BRCA2* related cancers.

While the immunophenotype of invasive *BRCA2* related cancers has been well studied, little is yet known on the immunophenotype of pre-invasive lesions from the *BRCA2* carcinogenetic spectrum.

In conclusion, most of the *BRCA2* related breast cancers are of the so called "luminal" type with overexpression of ERα, CK8 and CK18. This is clearly different from the observations in *BRCA1* related breast cancers[128, 129], pointing to a different origin from the luminal cells of the breast rather than the stem cells as in *BRCA1* related breast cancer.

Genetic profile

In a recent study using gene expression analysis to distinguish *BRCA2* associated tumors, discriminating genes were those related to transcription, signal

transduction, cell proliferation, cell adhesion and extracellular matrix remodelling. In this study, a relative high expression of FGF1 and FGFR2 was observed and this was confirmed by immunohistochemistry[159, 166-168]. According to the gene expression profile mentioned before, most of the *BRCA2* related breast cancers were classified as luminal[156, 157]. Based on the data so far, the overall conclusion can be made that there are not many genetic factors yet available to distinguish *BRCA2* related breast cancers from *BRCA1*-related and sporadic breast cancer cases. Further studies on distinguishing *BRCA2* related breast cancers from *BRCA1* related or sporadic breast cancers should be undertaken to establish new strategies in prevention or the development of specific therapies. At the chromosomal level, *BRCA2* related breast cancers show patterns of chromosomal copy-number gains and losses that are not found in sporadic controls. Copy number changes more frequently occurring in *BRCA2* related breast cancers are gains of 8q, 17q22-q24 and 20q13 and loss of 8p, 6q, 11q and 13q[160, 161] (see table 2).

Non-*BRCA1* or *BRCA2* germline mutation related breast cancers

Phenotypic characteristics of cancers developing in patients with a strong family history without a *BRCA1* or *BRCA2* germline mutation are various. Although *BRCA1* and *BRCA2* germline mutations may remain undetected in some families, most of these non-*BRCA1* and *BRCA2* breast cancers likely develop as a consequence of mutations in different moderate to low penetrance genes, like the genes mentioned earlier (see table1), or in genes yet to be discovered. It has been established that non-*BRCA1* and *BRCA2* tumors have even a lower grade compared to sporadic breast cancers. Furthermore, the immunophenotype is more or less the same as shown in sporadic breast disease[169, 170].

Morphologic and immunophenotypic studies of breast cancer in patients with a *CHEK2* mutation have yielded conflicting results, probably largely due to the

limited cases of breast cancers that have been found being related to this mutation. Studies on ER and PR expression have reported contradictory results, ranging from similar to increased expression of ER and PR. Patients with a U157T mutation have been associated with an increased incidence of lobular carcinomas[171, 172]. One study describing a gene expression profile of non-*BRCA* related breast cancer was able to classify these tumors into two homogenous subsets, one group characterized by higher presentation of ribosomal genes. Additional experiments should be done on these non-*BRCA* related tumors to further describe their molecular characteristics[173]. In conclusion, in patients with a strong family history but without a detectable *BRCA1* or *BRCA2* or *CHEK2* germline mutation specific information about the pathological characteristics of their tumors is yet lacking.

Clinical relevance

Incidence

In The Netherlands, one out of nine women will be diagnosed with breast cancer. Worldwide, over 1 million women each year are diagnosed with this disease and the numbers are still increasing. While the incidence is rising, the number of women that die as a consequence of breast cancer has been declining over the past decade [World cancer report, WHO, P. Boyle and B. Levin]. Worldwide, using data from the 2004 Global Burden of Disease, the lifetime risk of dying from breast cancer is estimated at about 33% per thousand in high income, 25% in upper/middle and 15% in low income countries. In 2007, in The Netherlands less than 30 per 100,000 women of all ages have died of breast cancer [WHO European health for all database]. About 5% of all breast cancer cases are hereditary, with germline mutations in the *BRCA1* and *BRCA2* genes accounting for 15-25% of these cases. The lifetime risk of breast cancer for *BRCA1* or *BRCA2* mutation carriers is estimated to be 82% and the lifetime risk of ovarian cancer is 54% for *BRCA1* and 23% for *BRCA2* carriers[174]. Investigating overall survival in *BRCA1* and *BRCA2* associated

breast cancer versus age matched sporadic breast cancer patients have yielded contradicting results with some studies describing a worse survival and others a similar survival[175-180].

Diagnosis

In families where multiple (young) members are affected with breast or ovarian cancer, screening for *BRCA1* and *BRCA2* mutations will often be advised. The scope of the initial testing is a full mutation screening of the *BRCA* genes. In a significant proportion of breast or ovarian/tubal cancer patients with a family history strongly pointing to hereditary disease, a *BRCA1* or a *BRCA2* mutation can however not be demonstrated[181, 182]. If no mutation in the *BRCA1* or *BRCA2* is found, mutation analysis of the other breast cancer susceptibility genes is usually performed.

In women with a strong family history but with no established mutation in the *BRCA*-genes, a four fold risk of getting breast cancer has been described[183, 184].

Several computational models have been developed to predict the probability of finding a *BRCA1* and *BRCA2* gene mutation[185-187]. These models are however not practical for daily use. Furthermore, the lack of a family history or the small size of the family involved is of influence on the result of being a candidate for predictive testing. Other phenotypic, non-genetic tests are clearly warranted[181]. The pathology results described above could be useful in this respect.

The presently recommended surveillance plan consists of a monthly self-breast examination (breast awareness), a biannual clinical breast examination by a professional and an annual screening mammography/ultrasound and MRI of the breasts from the age of 25 years. Breast MRI is the key examination in young women, as the sensitivity of mammography in mutation carriers is lower in young women than in the general population due to dense breast tissue[188, 189]. In addition, recently reports have been published on the potential risk of radiation induced breast cancer in young *BRCA* mutation carriers as a consequence of these annual mammograms[190, 191]. This has even more supported the use of MRI in young women with a *BRCA* mutation. The results of clinical studies assessing the value of screening

through genetic analysis of nipple fluid are eagerly awaited[192].

Surgical options for surveillance include prophylactic bilateral mastectomy and prophylactic bilateral salpingo-oophorectomy (BSO)[193]. Prophylactic bilateral mastectomy reduces the risk of breast cancer by almost 100% in mutation carriers[194, 195]. In view of the additional high lifetime risk of ovarian cancer, especially in *BRCA1* mutation carriers, these women are strongly advised to undergo BSO including the removal of the fallopian tubes after the completion of childbearing[196-198]. The current use of oral contraceptives is not associated with an increased risk of breast cancer among *BRCA1* and *BRCA2* mutation carriers compared to the general population. However, the duration and period of use of the oral contraceptives, i.e. more than 5 years and before the age of 30 and especially before first full pregnancy, may be associated with an increased risk of breast cancer. The use of oral contraceptives has however a protective effect on the risk of ovarian cancer. So, in conclusion, it is difficult to give an advice whether or not to use or not use oral contraceptives in *BRCA1* and *BRCA2* carriers, although switching after 5 years to an alternative might be preferred[199-202]. Preliminary results from one study suggests that the use of hormone therapy in postmenopausal women with a *BRCA1* mutation was associated with a decreased risk of breast cancer. It is important to confirm this in a larger study including different populations and a longer study period[203].

Due to the important role of the *BRCA* genes in DNA repair it could be expected that DNA cross linking agents, like cisplatin and mitomycine C, would have an effect especially in those diseases that occur as a consequence of mutated and therefore dysfunctional *BRCA1* and *BRCA2* genes[204]. Higher tumor responses to platinum based chemotherapy have indeed been observed in patients with *BRCA1* mutated ovarian cancers when compared with the effects observed in non-hereditary ovarian cancer[205, 206].

The association of negativity ER/PR/HER2 status classifies many of *BRCA1* related cancers in the "triple negative" category, that is clinically under scrutiny as these cancers may require an alternate chemotherapeutic approach. A potentially new strategy that has emerged for treatment of *BRCA1* and *BRCA2* related tumors is

the use of poly(ADP-ribose) polymerase 1 (PARP1) inhibitors. *BRCA1* and *BRCA2* are both involved in DNA double strand break repair, as mentioned before. PARP1 is involved in base excision repair, a key pathway in the repair of DNA single strand break. The absence of PARP leads to spontaneous single strand breaks which collapse replication forks into double strand breaks, triggering homologous recombination for repair. However, with the loss of functional *BRCA1* or *BRCA2*, cells will be sensitized to inhibit the PARP activity, apparently leading to the persistence of the DNA lesions which are usually repaired by homologous recombination. When both pathways are defect this will result in chromosomal instability, cell cycle arrest and finally apoptosis. Cell survival assays showed that cell lines lacking wildtype *BRCA1* or *BRCA2* were extremely sensitive to PARP inhibitors compared to heterozygous mutant or the wildtype cells[207]. Similar results were obtained using nonembryonic cells deficient for *BRCA2*. These results suggest the potential use of PARP inhibitors in the treatment of *BRCA1* and *BRCA2* related breast cancer. This is presently evaluated in various clinical trials in BRCA carriers suffering from breast and/or ovarian cancer[207-210].

Differences in patterns of metastatic spread and survival following recurrent disease between *BRCA1* and *BRCA2* associated breast cancers and sporadic tumors have been observed. In *BRCA1* associated tumors, a lower rate of bone metastases and a higher frequency of lung and brain metastases have been described. In contrast, in women with *BRCA2* associated breast cancer, bone and soft tissue metastases are observed more frequently likely associated with their more frequent ER positivity[211].

As has been described earlier, *BRCA1* related breast cancers are well characterized by morphological, immunohistochemical and molecular features that clearly help to differentiate them from sporadic tumors and identify high risk patients for mutation testing. *BRCA2* related breast cancers on the other hand, offer yet only a few morphological, immunohistochemical or molecular features to separate them from sporadic controls. Finally, although numbers studied so far are small, breast cancers caused by other breast cancer susceptibility genes do not, as in *BRCA2*

related disease, seem to differ significantly from sporadic breast cancers.

More studies should be performed on morphological, immunohistochemical and molecular characterization of *BRCA2* related breast cancers, breast cancers caused by unclassified variants of *BRCA1* and *BRCA2*, and breast cancers caused by other breast cancer susceptibility genes. This would lead to more insight into the development of these breast cancers, and therby to clues for diagnosis and new therapeutic approaches.

Table 3. Expression of different immunohistochemical markers in *BRCA1* and *BRCA2* germline mutation related breast cancers relative to sporadic controls (? indicates not known)

	BRCA1 related cancers	BRCA2 related cancers
CK5/6	↑	↓
CK8	↓	↑
CK18	↓	↑
CK14	↑	↓
ERα	↓	↑
ERβ	↑	?
PR	↓	↑
HER2	↓	↓
EGFR	↑	↑
HIF-1α	↑	?
p53	↑	↓
Vimentin	↑	?
Laminin	↑	?
P-cadherin	↑	?
Caveolin1	↑	↓
Bax	↑	↑
BCL2	↑	↑
Active caspase 3	↑	↑
FGF1	↓	↑
FGFR2	↓	↑

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Distinction between hereditary and sporadic breast cancer based on clinicopathological data

3

J Clin Pathol 2006;59;611 617

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Abstract

Background: About 5% of all breast cancer cases are attributable to germline mutations in BRCA1 or BRCA2 genes. BRCA mutations in suspected carriers, however, may be missed, which hampers genetic counselling.

Materials and methods: Different clinicopathological features were compared between 22 breast cancers from carriers of proved BRCA1 mutations and 604 cancers from sporadic controls. In addition, 5 BRCA2-related breast cancers and 66 breast cancers of untested patients at intermediate risk and 19 breast cancers of untested patients at high risk of hereditary disease on the basis of family history were evaluated.

Results: A "probably sporadic" class (age >54 years and epidermal growth factor receptor (EGFR) negative; 68% of cases) with a 0% chance of BRCA1-related breast cancer containing 79% of the sporadic cases was yielded by using a decision tree with age, Ki67 and EGFR. A 75% chance of BRCA1-related breast cancer was shown by the "probably BRCA1-related" class (age < 54 years and Ki67 ≥ 25%; 8% of cases) with 82% of the BRCA1-related cases but only 1.4% of the sporadic cases. Most cases at intermediate or high risk of hereditary disease on the basis of family history could be classified with high probability as either probably BRCA1 related or probably sporadic.

Conclusion: Breast carcinomas can be classified with a high level of certainty as sporadic or related to BRCA1 germline mutations by using a decision tree with age, Ki67 and EGFR. This can be clinically useful in mutation analysis in families with a borderline risk of hereditary disease.

Introduction

Family history of breast cancer is an established risk factor for the development of the disease. Among those variables that have been shown to bear a causal relationship with breast cancer, the highest increased risk, after age, is a positive family history of breast cancer[1]. In 5% of the breast cancer cases, the disease occurs as part of a hereditary cancer susceptibility syndrome. In contrast with acquired (somatic) BRCA1 and BRCA2 mutations that do not seem to be an important factor in the development of most sporadic breast cancers, a substantial proportion of hereditary breast cancers can be attributed to germline mutations in either of these genes. Establishment of a BRCA1 or BRCA2 germline mutation has important consequences. Mutation carriers are at high risk not only of breast cancer but also of cancers of the contralateral breast, ovary and fallopian tube[2, 3], which necessitates preventive strategies in these patients. Furthermore, hereditary breast cancer is associated with a poorer survival and hereditary ovarian cancer with a better survival[4] than their sporadic counterparts, which may have consequences for treatment. Screening for BRCA1 or BRCA2 mutations is difficult. In a large proportion of patients with breast cancer, or ovarian or tubal cancer, who have a family history strongly pointing to hereditary disease, a BRCA1 or BRCA2 mutation cannot be shown. This may partly be explained by germline mutations in genes other than BRCA1 or BRCA2, such as CHEK2[5], but mutations in BRCA1 or BRCA2 may be missed in current screening procedures, even with complete sequencing[6]. It is currently possible to detect about 90% of all BRCA1 or BRCA2 mutations by using standard diagnostic procedures, and a mutation is detected in almost 25% of cases presenting with familial cancer. Clinically, a hereditary basis of breast cancer is recognised by early age at onset, family history, bilateral breast cancer, breast cancer in men and cancer of the ovary or of the fallopian tube. Also, family history may be incomplete, even in developed countries. In The Netherlands, incompleteness of medical history is an increasing problem as a law has been introduced that does not permit keeping patient data and material for more than 10 years after the initial

diagnosis[7]. Furthermore, families may be small, inheritance may occur through non-affected men and penetrance may be incomplete. Therefore, in patients at high risk on the basis of family history, additional features pointing to hereditary disease in case of negative mutation screening are useful, for example, to decide on preventive strategies and diagnostic procedures. In patients at an intermediate risk of hereditary disease on the basis of family history, additional features may help to decide on mutation screening. Inversely, such features may rule out the need for mutation screening in case of a suspected family history. Besides young age, poor tumour differentiation, high proliferation, negative steroid receptor status, p53 and HER-2/*neu* positivity[8-13], overexpression of epidermal growth factor receptor (EGFR)[14] and cytokeratin 5/6 positivity[15] may point to hereditary breast cancer associated with BRCA1. BRCA2-related tumours show a less conspicuous phenotype[16] Few studies, however, have aimed at integrating all these features into a multivariate model to classify breast cancer in patients as hereditary or sporadic, as an aid for genetic counseling[17-19]. We therefore aimed at evaluating a panel of clinicopathological variables to classify breast cancers as hereditary or sporadic using a multivariate approach.

Subjects and methods

Patients

The study group comprised 22 stage I or stage II invasive breast cancers from 22 patients with a proved BRCA1 germline mutation and five cancers from five patients with a proved BRCA2 germline mutation. In addition, we studied 19 patients with invasive breast cancer, who were not screened for mutations, but were known to have a proved BRCA1 (n=17) or BRCA2 (n=2) mutation in their families, further denoted as being at high risk of hereditary disease. The final group comprised 66 patients with invasive breast cancer "at intermediate risk" of hereditary disease on the basis of family history according to Claus's criteria[20]. These patients were not

tested for BRCA1 or BRCA2 mutations. All these patients were from the Familial Cancer Clinic of the VU University Medical Center, Amsterdam, The Netherlands. The control group comprised 604 patients with stage I or stage II invasive breast cancer unselected for family history, further denoted as sporadic cases, from the archives of the Department of Pathology of the VU University Medical Center.

Histopathology

Tumour size was measured in the freshly resected specimens, and tumour samples were subsequently fixed in neutral buffered formaldehyde and processed to blocks of paraffin wax according to standard procedures. Four-micrometre thick sections were cut and stained with haematoxylin and eosin for histopathology. Tumour type was assessed according to the World Health Organization criteria, and tumours were graded according to the criteria by Elston and Ellis. To assess the mitotic activity index (MAI), mitoses were counted as described previously[21].

Immunohistochemistry

Immunohistochemical analysis was carried out on 4-μm thick sections. After deparaffination and rehydration, endogenous peroxidase activity was blocked for 30 min in a methanol solution containing 0.3% hydrogen peroxide. After antigen retrieval in citrate buffer (autoclaved, except for oestrogen receptor when the microwave was used, and HER-2/neu for which no retrieval was done), a cooling-off period of 30 min preceded the incubation (overnight at 4°C) with the primary antibodies (p53: DO7, Dako, (Glostrup, Denmark) 1:500; p21: Pharmingen, (San Diego, California, USA) 1:500; p27: Transduction Laboratories, 1:1000; cyclin A: Novocastra, (Newcastle-Upon-Tyne, UK) 1:100; cyclin D1: Neomarkers, 1:400; EGFR: Novocastra, 1:10; oestrogen receptor: Dako, 1:50; progesterone receptor: Novocastra, 1:50; HER-2/*neu*: courtesy Dr Marc van den Vijver, Netherlands Cancer Institute, Amsterdam, 1:25; Ki67: Dako, 1:40). The primary antibodies were detected with a biotinylated rabbit antimouse antibody (Dako). The signal was amplified by avidin–biotin complex formation and developed with diaminobenzidine,

followed by counterstaining with haematoxylin, dehydrated in alcohol and xylene and mounted. For Ki67, p53, p21, cyclin D1, cyclin A, p27, oestrogen receptor and progesterone receptor, only nuclear staining was considered and diffuse cytoplasmic staining was ignored, leading to an estimated percentage of positively stained nuclei. Stainings of HER-2*/neu* and EGFR were scored positive when a clear membrane staining pattern was seen. Scoring was carried out by a single experienced pathologist (PJvD) who was blinded to BRCA1 or BRCA2 mutation status.

Statistics

Continuous variables were tested for differences between the hereditary and sporadic cases with the Mann–Whitney test, and discrete variables were tested with the chi-square test using logical classes. Significance level was set at $p<0.05$. Finally, decision tree analysis based on recursive partitioning was carried out with the OMEGA Analytical Engine (KiQ, Amsterdam, The Netherlands) to discern BRCA1 and sporadic cases, by using a maximum of three variables with the highest univariate differences between BRCA1 and sporadic cases. Besides the optimal model that was composed of all features in the analysis, two alternate decision trees were designed: one excluding age, to gain insight into the most important primary tumour features pointing to BRCA1-related breast cancer, and the other excluding immunohistochemical variables, which may be useful when tissue blocks are not available for immunohistochemistry. These decision trees had four end points, and for each of these end points the chance for BRCA1-related disease was calculated. This classification model resulted in four classes at an increasing risk of BRCA1-related disease. The class at the lowest risk of BRCA1-related disease was denoted as probably sporadic, and the class at the highest risk as probably BRCA1 related. Next, the cases at intermediate and high risk and the BRCA2-related cancers were classified with these decision trees. The decision tree approach was chosen as such trees are easy to use in clinical practice. For further statistical clarity, however, we also carried out logistic regression.

Results

Morphologically, tubule formation was virtually absent in cases with BRCA1 and BRCA2 mutations and lymphocytic infiltration was often seen. Mitotic activity was higher and nuclear atypia more outspoken in cases with hereditary cancer. The medullary histological type was relatively frequent in cases with BRCA1 mutations (4/22 v 10/604 of sporadic cases, Fisher's exact p=0.001). All remaining cases with BRCA1 mutations were of the ductal type (18/22). All cases with BRCA2 mutations had cancers of the invasive ductal type. Tables 1 and 2 show the median values (and ranges) of the different continuous variables and the values for the discrete variables for the different risk groups, respectively. With increasing risk of hereditary disease, age, oestrogen and progesterone receptor expression decreased, whereas MAI, grade and Ki67, p53, and cyclin A expression increased. We found a significant ($p<0.0001$) increase in the frequency of EGFR expression in cancers associated with BRCA1 (14/21, 67%) and BRCA2 mutations (5/5, 100%) than in sporadic cancers (70/430, 16%). EGFR expression in the intermediaterisk group (7/31, 23%) was comparable with that in the group with sporadic cancers, but in the high-risk group EGFR expression (12/15, 80%) was similar to that in the hereditary cases. We detected no relationship between tumour size and HER-2/neu, p27, p21 and cyclin D1 expression and no risk of hereditary disease. For many variables, the five cases associated with BRCA2 mutations showed values between those of cases associated with the BRCA1 mutation and the sporadic cancers. Age was on average 55 years for cases with BRCA2 mutations, compared with 64 years for sporadic cancers and 42 years for cases with BRCA1 mutations. The same intermediate values were seen for MAI, Ki67, oestrogen receptor and progesterone receptor expression. All cases with BRCA2 mutations showed very low p53 expression, with an average of 0.4% positive nuclei, whereas cases with sporadic cancer and BRCA1 mutations showed an average of 11% and 46% of positive nuclei, respectively. Average cyclin A values of cases with BRCA2 mutations were comparable to those of sporadic cases, but were much lower than those of cases associated with BRCA1 mutations.

Table 1. Median values (ranges) of different continuous clinicopathologic features for sporadic breast cancers, cancers of BRCA1/2 mutated patients, and breast cancer patients at different risk of hereditary disease based on family history only.

Feature	Sporadic		Intermediate risk		High risk		BRCA1 mutation		BRCA2 mutation	
	N	median (range)	N	median (range)	N	median (range)	N	median (range)	N	median (range)
Age (years)	579	**64** (30-87)	66	**51** (26-76)	19	**47** (31-77)	22	**38** (31-57)	5	**56** (38-66)
MAI	567	**10** (0-151)	63	**7** (0-130)	19	**28** (0-104)	22	**30** (0-105	5	**11** (4-56)
T-size (cm)	554	**2.0** (0.1-10)	63	**2.0** (0.2-8.0)	17	**3.2** (1-22)	17	**2.0** (0.5-4)	5	**1.5** (1-3)
Ki67 (%)	421	**10** (0-80)	62	**10** (1-90)	18	**58** (0-100)	22	**75** (2-100)	5	**20** (10-70)
p53 (%)	419	**0** (0-100)	57	**1** (0-100)	19	**5** (0-100)	22	**39** (0-100)	5	**0** (0-2)
ER (%)	423	**80** (0-100)	62	**78** (0-100)	19	**0** (0-90)	22	**0** (0-100)	5	**0** (0-90)
PR (%)	420	**0** (0-100)	65	**25** (0-100)	19	**0** (0-80)	22	**0** (0-90)	5	**35** (0-80)
p27 (%)	412	**30** (0-100)	0	**-**	14	**10** (0-90)	17	**50** (0-100)	2	**28** (5-50)
p21 (%)	459	**1** (0-65)	56	**2** (0-90)	18	**2** (0-20)	20	**1** (0-80)	4	**3** (0-25)
Cyclin E (%)	424	**0** (0-20)	59	**0** (0-50)	4	**0** (0-0)	5	**0** (0-1)	3	**1** (0-1)
Cyclin A (%)	457	**5** (0-50)	48	**5** (1-60)	19	**25** (2-50)	21	**35** (5-90)	5	**10** (2-20)
Cyclin D1 (%)	427	**1** (0-90)	59	**0** (0-60)	18	**1** (0-35)	22	**0** (0-50)	5	**0** (0-80)

Table 2. Breakdown of sporadic breast cancers, cancers of BRCA1/2 mutated patients, and breast cancer patients at different risk of hereditary disease based on family history only (Claus criteria) over different discrete clinicopathologic features.

	Type	Sporadic (N, %)	Intermediate risk of hereditary disease (N, %)	High risk of hereditary disease (N, %)	BRCA1 mutated (N, %)	BRCA2 mutated (N, %)
Grade	1	119 (22%)	9 (16%)	0 (0%)	0 (0%)	0 (0%)
	2	181 (34%)	25 (46%)	5 (26%)	4 (18%)	1 (20%)
	3	232 (44%)	21 (38%)	14 (74%)	18 (82%)	4 (80%)
Histologic	Ductal	474 (79%)	55 (84%)	14 (74%)	18 (82%)	5 (100%)
type	Lobular	56 (9%)	8 (12%)	1 (5%)	0 (0%)	0 (0%)
	Medullary	10 (2%)	0 (0%)	1 (5%)	4 (18%)	0 (0%)
	Tubular	22 (4%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)
	Other	42 (7%)	1 (2%)	3 (16%)	0 (0%)	0 (0%)
EGFR	Neg	360 (84%)	24 (77%)	3 (20%)	7 (33%)	0 (0%)
	Pos	70 (16%)	7 (23%)	12 (80%)	14 (67%)	5 (100%)
HER-2/*neu*	Neg	374 (87%)	52 (88%)	15 (83%)	17 (81%)	3 (75%)
	Pos	55 (13%)	7 (12%)	3 (17%)	4 (19%)	1 (25%)

Cyclin D1 values were remarkably high in cases with BRCA2 mutations. The grade of cases with BRCA2 mutations was similar to those with BRCA1 mutations. Table 3 shows the markers for major differences when comparing the different groups: cases with sporadic cancer, groups at intermediate risk and high risk, and cases with BRCA1 and BRCA2 mutations. In decision tree analysis, age was the best univariate predictor, followed by Ki67, oestrogen receptor and EGFR. By using bivariate analysis, the best combinations of variables were found to be age/Ki67, followed by age/EGFR and age/MAI. Logistic regression also yielded age and Ki67 as important predictors. The best combination without including age was Ki67/EGFR. Logistic regression also yielded Ki67 and EGFR as relevant predictors. The optimal decision tree model was composed of age, Ki67 and EGFR.

Table 3. Clinicopathologic features that are significantly different ($p<0.05$) between the various groups of sporadic breast cancers, cancers of BRCA1/2 germline mutated patients, and breast cancer patients at different risk of hereditary disease based on family history only (Claus criteria). Continuous variables were compared with the Mann-Whitney test, discrete variables with the Chi-square test.

	Breast cancer in patients at intermediate risk of hereditary disease	Breast cancer in patients at high risk of hereditary disease	BRCA1 germline mutated breast cancer	BRCA2 germline mutated breast cancer
Sporadic breast cancer	Age, T-size, p53, PR, p21, cyclin E, cyclin D1	Age, MAI, T-size, Ki67, p53, ER, cyclin A, EGFR, Grade	age, MAI, Ki67, p53, ER, PR, cyclin A, EGFR, Grade	Age, cyclin E, EGFR, Grade
Breast cancer in patients at intermediate risk of hereditary disease		MAI, T-size, Ki67, ER, PR, cyclin A, EGFR, Grade	age, MAI, Ki67, ER, PR, p21, cyclin A, EGFR, Grade	Ki67, cyclin E, EGFR, Grade
Breast cancer in patients at high risk of hereditary disease			T-size, p27	T-size, p53, ER, cyclin A,
Breast cancer in BRCA1 germline mutated patients				age, Ki67, PR, cyclin A

Table 4 shows the probability of hereditary disease for the different groups used in this decision tree. The probably sporadic and probably BRCA1-related classes

contained 68% and 8% of cases, respectively. In the high-age group (≥54 years), the chance of hereditary disease was 0% (probably sporadic class) when EGFR was negative and 3% when EGFR was positive. In the low-age group, the chance of hereditary disease was 9% when Ki67 was low and 75% (probably BRCA1-related class) when Ki67 was high. This decision tree classified 79% of the sporadic cases as probably sporadic, and only 1.4% as probably BRCA1 related. Of the BRCA1 cases, none were classified as probably sporadic and 82% as probably BRCA1 related. When classifying the intermediate-risk cases with the decision tree, 41% were classified as probably sporadic and 12% as probably BRCA1 related. Of the high-risk cases, 16% were classified as probably sporadic and 63% as probably BRCA1 related. All cases with BRCA2 mutations were classified into the intermediate categories and none into the probably sporadic or probably BRCA1-related classes. The patients at high risk with proved BRCA2 mutations in their families were also classified into the intermediate-risk category.

Table 4. Probability of BRCA1 germline mutation related breast cancer for different groups of breast cancer patients based on a combination of age at diagnosis, %Ki67 positive cells in and EGFR status of the primary tumour

Classification	Decision tree	# sporadic cases	# BRCA1 mutated cases	Probability of BRCA1 related disease	# inter-mediate risk cases	# high risk cases	# BRCA2 mutated cases
Sporadic	Age ≥ 54 and EGFR = neg	332	0	0%	24	3	0
Intermediate	Age ≥ 54 and EGFR = pos	62	2	3%	2	2	3
Intermediate	Age < 54 and Ki67 < 25 %	21	2	9%	26	2	2
BRCA1 related	Age < 54 and Ki67 ≥ 25 %	6	18	75%	7	12	0

By allowing only primary tumour-related features, a decision tree was composed of Ki67, EGFR and the percentage of progesterone receptor-positive cells (table 5). In the group with low Ki67, the chance of hereditary disease was 0% (probably sporadic class) when progesterone receptor was positive, and 2% when progesterone

receptor was completely negative. In the high-Ki67 group, the chance of hereditary disease was 8% when EGFR was negative, but 33% (probably BRCA1-related class) when EGFR was positive. With this decision tree, 44% of the sporadic cases were classified as probably sporadic and only 6% as probably BRCA1 related. Of the cases with BRCA1 mutations, none were classified as probably sporadic class and 59% as probably BRCA1-related class. When classifying the intermediate-risk cases with the decision tree, 56% were classified as probably sporadic and 3% as probably BRCA1 related. Of the high-risk cases, 11% were classified as probably sporadic and 53% as probably BRCA1 related.

Table 5. Probability of BRCA1 germline mutation related breast cancer for different groups of breast cancer patients based on a combination of %Ki67 positive cells, percentage PR positive cells in and EGFR status of the primary tumour

Classification	Decision tree	# sporadic cases	# BRCA1 mutated cases	Probability of BRCA1 related disease	# inter-mediate risk cases	# high risk cases	# BRCA2 mutated cases
Sporadic	Ki67 < 25% and %PR > 0	184	0	0%	33	2	3
Intermediate	Ki67 < 25% and %PR = 0	128	2	2%	15	2	0
Intermediate	Ki67 ≥ 25 % and EGFR=neg	82	7	8%	9	5	0
BRCA1 related	Ki67 ≥ 25 % and EGFR=pos	27	13	33%	2	10	2

Three cases with BRCA2 mutations were classified as probably sporadic and two as probably BRCA1 related. When excluding immunohistochemical features from recursive partitioning, a bivariate model composed of age and MAI was obtained (table 6). In the high-age group, the chance of hereditary disease was 0% (probably sporadic class) with low MAI and 2% with high MAI. In the low-age group, the chance of hereditary disease was 22% with low MAI and 63% (probably BRCA1-related class) with high MAI. With this decision tree, 64% of the sporadic cases were classified as probably sporadic and only 2% as probably BRCA1 related. Of the cases with BRCA1 mutations, none were categorised as probably sporadic and

68% as probably BRCA1 related. When classifying the intermediate-risk cases with the decision tree, 36% were classified as probably sporadic and 19% as probably BRCA1 related. Of the high-risk cases, 21% were classified as probably sporadic and 63% as probably BRCA1 related. One case with BRCA2 mutation was categorised as probably sporadic and none as probably BRCA1 related.

Table 6. Probability of BRCA1 germline mutation related breast cancer for different groups of breast cancer patients based on a combination of classical features: age at diagnosis and MAI.

Classification	Decision tree	# sporadic cases	# BRCA1 mutated cases	Probability of BRCA1 related disease	# inter-mediate risk cases	# high risk cases	# BRCA2 mutated cases
Sporadic	Age ≥ 54 and MAI < 17	269	0	0%	21	4	1
Intermediate	Age ≥ 54 and MAI ≥ 17	125	2	2%	5	1	2
Intermediate	Age < 54 and MAI < 17	18	5	22%	22	2	2
BRCA1 related	Age < 54 and MAI ≥ 17	9	15	63%	11	12	0

Discussion

In line with previous studies[8-13,16], the BRCA1-related cancers showed, morphologically, virtually no tubule formation, lymphocytic infiltration remarkably often, more nuclear atypicality and higher mitotic activity. The medullary histological type was relatively frequent in cases with BRCA1 mutations with the remaining BRCA1 cases being of the ductal type. All cases with BRCA2 mutations had cancers of the invasive ductal type. We found a clear inverse relationship between age and risk for hereditary disease, as expected[11, 22-24] The mean age of patients with sporadic breast cancer was 64 years, whereas the mean age of patients with a proved BRCA1 mutation was 42 years. As age is a powerful discriminator between patients with BRCA1-related and sporadic breast cancer, we have deliberately not matched

for age in this study. Primary tumour features that were associated with BRCA1-related cancers were low expression of oestrogen and progesterone receptors, overexpression of EGFR, high MAI and grade, and high expression of Ki67, p53 and cyclin A. Although some studies showed no differences between BRCA-related breast cancers and sporadic cancers[25], low expression of oestrogen and progesterone receptors has been described previously in BRCA1-related cancers [8, 9, 12, 16, 24, 25]. We recently described for the first time the high expression of EGFR in breast cancers related to BRCA1 or BRCA2 mutations[14]. With regard to the proliferation markers, we found high expression levels of Ki67 and high MAI in BRCA-associated tumours as in previous studies[12, 23-25], but we describe here, for the first time, high cyclin A expression as another useful marker of proliferation[26]. Accumulation of p53 in BRCA related tumours is also in agreement with other studies[8, 24, 27], indicating that p53 inactivation is, next to BRCA1 inactivation, an important event in BRCA1-associated carcinogenesis or progression. We found no relevant differences for p21, cyclin D1, p27 and HER-2*/neu* between BRCA1-related cases and sporadic controls. For p21[27] and cyclin D1[11] these results are in agreement with previous studies. Chappuis et al.[9] found in general a lower percentage of HER-2*/neu* positivity in BRCA1-related cancers. Therefore, p21, cyclin D1 and HER-2*/neu* do not seem to have a differential role in sporadic and BRCA1-related breast cancers. Although we studied only five cases of BRCA2-related breast cancers, it appeared nevertheless that these cancers show a phenotype between sporadic and BRCA1-associated breast cancers. Age at presentation, MAI, Ki67[9, 24], oestrogen and progesterone receptor values[8, 9, 12, 16, 24] of the cases with BRCA2 mutations were between those of sporadic and BRCA1-mutated cases. All cases with BRCA2 mutations showed very low p53 expression, which has also been shown previously,[24] although some disagree[9]. As low p53 expression usually indicates the presence of the wild-type gene, p53 inactivation seems to be much less relevant as a next hit for cases with BRCA2 mutations compared with those with BRCA1 mutations. Further, cyclin A values were comparable to values in sporadic cases, and cyclin D1 expression was remarkably high. The grade and frequency of EGFR expression were similar to those

of BRCA1-related cases, and for cyclin D1, BRCA2-related cases showed remarkably high values unlike those in BRCA1-related cancers and in contrast with a previous study[28]. HER-2/*neu* overexpression seems to be rare in BRCA2-related cancers[9, 28].
In decision tree analysis, the best classifier was composed of age, Ki67 and EGFR. In the age group ≥ 54 years, the chance of BRCA1-related cancer was as low as 0% when EGFR was negative and 3% when EGFR was positive. In the age group< 54 years, the chance of BRCA1-related disease was only 9% when Ki67 was low and as high as 75% when Ki67 was high. Most sporadic cases were classified as probably sporadic, and only a few as probably BRCA1 related, which can be expected as our group of sporadic cases will also inherently contain some BRCA1-related cancers because the controls were "unselected for family history" owing to nonavailability of data on family history. None of the BRCA1-related cases were categorised as probably sporadic and as many as 82% were classified as probably BRCA1 related, which is not unexpected, as patients with BRCA1 mutations also have the baseline breast cancer risk of the population without mutations, which is about 10% in The Netherlands. Therefore, some breast cancers in the population with BRCA1 germline mutations, especially in elderly patients, will not be attributable to BRCA1, but will arise according to sporadic carcinogenetic pathways. Obviously, the 75% chance of BRCA1-related disease in the probably BRCA1-related group is relative, because this chance is influenced by the ratio between BRCA1-related cancers and controls in this study. A ratio of 22 cases of BRCA1-related cancers to 604 controls, however, is in the order of 3–4% of BRCA1-related cases we would expect in a random Western population of patients with breast cancer. Therefore, we believe that the 75% chance may reflect the actual chance of BRCA1-related disease for patients with the described profile. When the decision tree was used to classify cases at intermediate risk of hereditary disease, 41% were classified as probably sporadic and only 12% as probably BRCA1 related. Although we have no gold standard for this group, this distribution makes sense and shows that at least many of these patients can be classified by using the decision tree with high probability. Of the cases at high risk of hereditary disease (with family members having a proved BRCA1

mutation), 16% were classified as probably sporadic and 63% as probably BRCA1 related. The patients with a BRCA2 mutation in the family could not be classified as either probably BRCA1 related or probably sporadic. This distribution makes sense also because most breast cancers in this group (especially those arising at a young age) will be due to BRCA1, as many of these women will have the BRCA1 mutation that has been established in their family. All cases with BRCA2 mutations fell into the intermediate categories, underlining their intermediate nature with regard to age at presentation and biological characteristics of the tumour. Therefore, this decision tree seems to be quite useful. It may help to decide on the need for DNA testing in families at intermediate risk. Further, it may be of great value to decide on preventive strategies for women in families that are most likely carrying a germline mutation in one of the genes associated with hereditary breast cancer syndromes, but have not shown a mutation on DNA testing. As the differences between BRCA1-related and sporadic cancers with regard to some of the clinicopathological variables may be related to age, we also designed a decision tree excluding age. Allowing only primary tumour-related features, a decision tree emerged with Ki67, EGFR and the percentage of progesterone receptor-positive cells. Also, here, grade did not emerge. This indicates that these are the features most strikingly different between hereditary and sporadic cancers. By using this decision tree, a group at 0% chance of hereditary disease can be identified, but the chance of BRCA1-related disease in the probably BRCA1-related class was much lower than when age was included. This underlines the significance of young age as a feature of BRCA1-related breast cancer. The significance of young age is further strengthened as only 2 of the 396 patients aged >54 years were BRCA1 carriers, in contrast with 20 of 47 carriers in the younger group. As Eerola et al.[29] recently pointed out, breast cancers occurring at a higher age in BRCA1 carriers do not have the typical BRCA1 phenotype and are therefore probably due to the baseline sporadic risk and are not related to the BRCA1 germline mutations. A further improvement in classification functions may be expected from molecular techniques. Wessels et al.[30] reliably (84% accuracy) classified sporadic and hereditary breast cancers by using chromosomal

gains and losses assessed with comparative genomic hybridisation. Differences in gene expression between sporadic and hereditary breast cancers are also shown in two studies[31, 32]. These techniques, however, are much more complicated, quite expensive or require fresh tissue, owing to which simple immunohistochemistry on blocks of paraffin wax will remain a practical approach for many laboratories. In conclusion, most invasive breast carcinomas can be classified as sporadic or BRCA1-related with a high degree of certainty by using a decision tree based on age, Ki67 and EGFR. This could be clinically useful to decide on mutation testing in families at a borderline risk of hereditary disease.

Acknowledgements

Dr. RJAM Michalides, Department of Cell Biology, Netherlands Cancer Institute, Amsterdam, contributed to the immunohistochemistry of the control patients. We thank Dr. Arno Kuijper for critically reading the manuscript.

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Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer

JNCI 2004;96:712-713

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4

EGFR expression predicts BRCA1 status in patients with breast cancer

Clin Cancer Res 2006;12:670

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Re: Germline BRCA1 Mutations and a Basal Epithelial Phenotype in Breast Cancer

Foulkes et al.[1] reported that the expression of cytokeratin 5/6, indicating a basal epithelial phenotype, was statistically significantly associated with germline BRCA1 mutations in estrogen receptor (ER)- and erbB2-negative invasive breast cancers. It has recently been shown that the epidermal growth factor receptor (EGFR) is often expressed in basal-type ("stem") cells of the breast[2], extending the phenotype of basal cells to $ER^-/erbB2^-/EGFR^+$ breast cells. We examined the expression of EGFR by immunohistochemistry in the invasive breast cancers from 21 proven carriers of BRCA1 germline mutations and five proven carriers of BRCA2 germline mutations, as well as from a control group of 430 invasive breast cancers from patients unselected for a family history of breast cancer. Only clear membrane staining for EGFR was considered as overexpression. Of the 21 BRCA1-related breast cancers, 14 (67%) showed EGFR overexpression, 19 (90%) were ER negative, and 17 (81%) were erbB2 negative. Eleven (52%) of 21 tumors were $ER^-/erbB2^-/EGFR^+$. All five (100%) breast cancers in BRCA2 mutation carriers showed EGFR overexpression, four (80%) were ER negative, and three (75%) of four were erbB2 negative (one could not be characterized). Two (50%) of four tumors were $ER^-/erbB2^-/EGFR^+$. In the control group of 430 tumors, EGFR overexpression was found in only 70 (16%), and only 28 (7%) of 422 tumors were $ER^-/erbB2^-/EGFR^+$. (Eight tumors could not be characterized for both proteins.) EGFR overexpression was statistically significantly higher in breast cancers in BRCA1 ($P<.001$, two-sided Fisher's exact test) and BRCA2 ($P<.001$) mutation carriers than in the control tumors. Also, the full $ER^-/erbB2^-/EGFR^+$ phenotype was statistically significantly more frequent in BRCA1/2 mutation carriers ($P<.001$). The high frequency of EGFR overexpression fits with the poor prognosis for patients with hereditary breast cancer, but the underlying mechanism is yet unclear. Amplification of EGFR seems to be very rare in invasive breast cancer (Buerger H, unpublished data). Further, by comparative genomic hybridization, amplification at the EGFR locus was not

observed[3]. Genetic alterations in an expression-regulating CA repeat in the first intron of the EGFR gene[4] have not yet been studied in hereditary breast cancer. In two gene expression studies in hereditary breast cancer[5, 6], increased EGFR mRNA expression was not observed, indicating that EGFR expression is probably largely posttranscriptionally regulated. We conclude that invasive breast carcinomas in patients with a BRCA1 or BRCA2 germline mutation show a high frequency of EGFR overexpression, compatible with the previously[7] established predominantly basal phenotype (ER^-/$erbB2^-$) of these cancers and their aggressive clinical behavior. Thus, we urge further investigation into the mechanisms of EGFR overexpression and into new preventive strategies through EGFR targeting.

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EGFR Expression Predicts BRCA1 Status in Patients with Breast Cancer

To the Editor: In their article, Lakhani et al.[1] report on the value of basal phenotype markers for the prediction of BRCA1 status. One of the useful features pointing to "BRCA1-ness" appeared to be high expression of the epidermal growth factor receptor (EGFR). No rationale is given by the authors for including EGFR in the analysis. There is a clear one, however: EGF (likely acting through EGFR) was related to the basal phenotype in breast cancer by DiRenzo et al.[2]. Furthermore, the finding of high EGFR expression in BRCA1-related breast cancer confirms previous data from our published study[3]. We examined EGFR expression by immunohistochemistry in the invasive breast cancers from 21 proven carriers of BRCA1 germ line mutations, as well as from a control group of 430 invasive breast cancers from patients unselected for a family history of breast cancer. Of the 21 BRCA1-related breast cancers, 14 (67%) showed EGFR overexpression. In the control group of 430 tumors, EGFR overexpression was found in only 70 (16%) ($P < 0.001$, two-sided Fisher's exact test). Also, the full ER-erbB2-/EGFR+ phenotype was statistically significantly more frequent in BRCA1 mutation carriers ($P < 0.001$). We hypothesize that this EGFR overexpression is probably largely posttranscriptionally regulated, as amplification of the EGFR gene seems to be very rare in invasive breast cancer[4], gains at the EGFR locus were not observed by comparative genomic hybridization[5], and increased EGFR mRNA expression was not observed in two gene expression studies in hereditary breast cancer [6, 7]. We therefore urge further investigation into the mechanisms of EGFR overexpression in hereditary breast cancer and into new preventive strategies through EGFR targeting. As the first article relating EGFR to the basal phenotype and the previously published EGFR expression results in BRCA1-related breast cancer were not referred to, and mechanisms behind the interesting finding of high EGFR expression in BRCA1-related breast cancer were not discussed, we would like to bring this to the attention of the Clinical Cancer Research readership.

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Molecular profile of ductal carcinoma in situ of the breast in *BRCA1* and *BRCA2* germline mutation carriers

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Submitted

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Abstract

Ductal carcinoma *in situ* (DCIS) is an established late precursor of sporadic invasive breast cancer and to a large extent parallels its invasive counterpart with respect to molecular changes and immunophenotype. Invasive breast cancers in germline *BRCA1* mutation carriers have a distinct ("basal") immunophenotype characterized by high expression of stem cell cytokeratins (CK) 5/6 and 14 and epidermal growth factor receptor (EGFR) and low expression of the estrogen (ER), progesterone (PR) and HER-2/*neu* receptors. The immunophenotype of *BRCA2* related breast cancers resembles more the immunophenotype of sporadic cancers ("luminal") with a frequent expression of ER, PR and only "luminal" cytokeratins such as CK8/18. Precursor lesions for these hereditary breast cancers like DCIS have hardly been studied and the immunophenotype of these lesions has not yet been established. The aim of this study was therefore to evaluate the immunophenotype of DCIS of *BRCA1* and *BRCA2* mutation carriers and to compare the immunophenotype and molecular classification of these DCIS lesions with their available invasive counterparts.

DCIS lesions of 25 proven *BRCA1* and 9 *BRCA2* germline mutation carriers and their respectively 22 and 6 accompanying invasive lesions were stained by immunohistochemistry for ER, PR, HER-2/*neu*, CK5/6, CK14, EGFR and Ki67.

DCIS lesions in *BRCA1* mutation carriers were mostly of the "basal" molecular type with low ER, PR and HER2 expression, while they frequently expressed CK5/6, CK14 and EGFR. They were mostly grade 3 and showed high proliferation. DCIS lesions in *BRCA2* mutation carriers were mostly of Luminal A molecular type with frequent expression of ER and PR, and infrequent expression of CK5/6, CK14 and EGFR. These lesions were mostly grade 3 and showed low proliferation. There was in both *BRCA1* and *BRCA2* mutation carriers a high concordance between DCIS lesions and their concomitant invasive counterpart with regard to expression of individual markers as well as molecular subtype.

In conclusion, DCIS lesions in *BRCA1* and *BRCA2* mutations carriers are usually of

the Basal and Luminal A molecular type, respectively. Their molecular phenotype is similar to their accompanying invasive cancers, thereby providing evidence that DCIS is a direct precursor lesion in these hereditary predisposed patients. This also suggests that crucial carcinogenetic events leading to these phenotypes occur at an early stage in hereditary predisposed patients, possibly even before the stage of DCIS.

Introduction

Carriers of germline mutations in *BRCA1* or *BRCA2* have a hereditary predisposition for developing breast[1, 2] and/or ovarian/fallopian tube cancer [3, 4]. Several studies have indicated that the genetic makeup of *BRCA1/2* related breast cancers is different from that of sporadic breast cancer. These differences comprise gains and losses of specific parts of chromosomes as well as differences in gene expression[5-11]. In line with this, the morphological and immunohistochemical phenotype of *BRCA1* and *BRCA2* related breast cancers is also different[12, 13, 14]. Common features of both *BRCA1* and *BRCA2* related cancers are that most of these cancers are high grade ductal cancers with conspicuous lymphocytoplasmic infiltrates[15, 16]. In addition, *BRCA1* related cancers show a high proliferation rate[16] and more often a medullary differentiation. The immunophenotype of these cancers comprises low expression of estrogen (ER), progesterone (PR) and HER-2/*neu* receptors[17] They often lack $p27^{Kip1}$ and cyclin D1 expression[13, 18, 19], but frequently accumulate p53 [20] and overexpress cyclin E [18], "stem cell" cytokeratins (CK) 5/6[20, 21] and CK14[22, 23], EGFR[24-26], HIF-1α[27] and p-cadherin[28], in general referred to as "of basal type". The immunophenotype of *BRCA2* related breast cancers resembles more the immunophenotype of sporadic cancers with a frequent expression of ER, PR and only "luminal" cytokeratins such as CK8/18[29].

Sporadic invasive breast cancer is generally thought to derive through different morphologically recognizable pre-invasive stages like atypical hyperplasia and ductal carcinoma *in situ* (DCIS), and the molecular profile and immunophenotype of DCIS parallels that of its invasive counterpart[30-32]. DCIS is relatively rarely found next to *BRCA1* and *BRCA2* related invasive breast cancer, but DCIS, fibroadenoma, and ductal hyperplasia seem to be more common in prophylactic mastectomy (PM) specimens of *BRCA1* and *BRCA2* mutation carriers than observed in control mammoplasty specimens[16, 33-42]. Lymphocytoplasmic infiltrate, often seen in hereditary invasive cancers, has also been observed in DCIS of hereditary patients[43], and T-cell lobulitis is seen frequently in PM specimens of these women[44]. No studies

have so far been performed on the immunophenotype or the molecular profile of premalignant breast lesions in *BRCA1* and *BRCA2* germline mutation carriers. The aim of this study was therefore to examine the immunophenotype of DCIS in *BRCA1* and *BRCA2* mutation carriers and to compare this profile with its invasive counterpart (if available) to establish DCIS as a precursor of hereditary breast cancer**.**

Materials and methods

Patients

The original study group comprised 134 invasive breast cancer patients with a known *BRCA1* and *BRCA2* mutation. Of these, DCIS material was available from 25 and 9 patients with a *BRCA1* and *BRCA2* germline mutation, respectively. For some women no invasive cancer tissue was left in the available blocks, leaving 22 *BRCA1* and 6 *BRCA2* patients with concomitant invasive cancer. Paraffin embedded blocks of these patients were derived from the archives at the UMC Utrecht, the VU University Medical Center, Amsterdam, and the University Medical Center Groningen, The Netherlands. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in the Netherlands[45].

Histopathology

Tumor samples had been fixed in neutral buffered formaldehyde, and processed to paraffin blocks according to standard procedures. Four µm thick sections were cut and stained with H&E for histopathology. DCIS was graded according to Holland et al[46].

Immunohistochemistry

After deparaffinization and rehydration, antigen retrieval was performed at boiling temperature in citrate buffer pH 6 (ER, PR, HER-2/*neu*, Ki67) or EDTA buffer pH 9 (CK5/6, CK14). For EGFR, antigen retrieval was performed by incubating the slides with Prot K solution (ready to use, DAKO, Glostrup, Denmark) for 5 minutes at room temperature. A cooling off period of 30 minutes preceded the incubation (60 minutes at room temperature) with the following mouse monoclonal antibodies: ER (1:50, DAKO), PR (1:50, Novocastra, Newcastle upon Tyne, United Kingdom), HER-2/*neu* (1:100 Neomarkers, Lab Vision Corp, Fremont, CA, USA), CK5/6 (1:3000, Chemicon, Temecula, USA), CK14 (1:400, Neomarkers), proliferation marker Ki67 (1:40, MIB-1, Immunotech, Marseille Cedex, France). For EGFR (1:30, Zymed, South San Francisco, CA, USA) the incubation was done overnight at 4°C. For detection of the primary antibodies a Goat anti Ms/Rb/Rt-poly-HRP (ready to use, Powervision, Immunologic, Immunovision Technologies, Brisbane, USA) was used except for EGFR for which a Novolink Max Polymer detection system (ready to use, Novocastra) was applied. All slides were developed with diaminobenzidine followed by haematoxylin counterstaining. Before the slides were mounted all sections were dehydrated in alcohol and xylene.

Scoring was performed by one observer (PJvD) blinded to the origin of the breast lesion. For ER, PR and Ki67 the percentage of positively stained nuclei was estimated. Cases with 10% or more nuclei stained were denoted ER/PR positive, and DCIS cases with ≥25% and invasive cancers ≥35% Ki67 staining as high proliferation (based on median values). HER-2/*neu* was scored according to the DAKO system, regarding 3+ cases as positive. EGFR was scored positive when a clear membrane staining pattern was seen, and CK5/6 and CK14 when clear cytoplasmic staining was observed.

Molecular classification

DCIS and invasive lesions were also classified as Luminal A (ER and/or PR-positive, HER2/*neu* negative), Luminal B (ER and/or PR-positive, HER2/*neu* positive), HER2

(HER2/*neu* positive and negative for ER and PR) or basal (CK5/6 or CK14 or EGFR positive and negative for ER, PR and HER2/*neu*) according to the new molecular classification of breast cancer [21, 47-50].

Results

As shown in Table 1, DCIS in *BRCA1* cases (N=25) showed very frequent expression of CK5/6 (72%), frequent expression of CK14 (44%) and EGFR (44%), while expression of ER (32%), PR (16%) and HER2/*neu* (0%) was infrequent. 21/25 cases (84%) were either CK5/6 or CK14 or EGFR positive. The mean % of Ki67 staining in these cases was 27% (range 0-100). Ten cases were grade 2, 15 were grade 3.

Table 1. Immunophenotype of DCIS and concomitant invasive breast cancers in women with a BRCA1 or BRCA2 germline mutation.

		DCIS		Invasive	
		BRCA1	BRCA2	BRCA1	BRCA2
ER	-	17	1	16	2
	+	8	8	6	4
PR	-	21	5	19	2
	+	4	4	3	4
HER2	-	25	6	20	5
	+	0	3	2	1
CK5/6	-	7	8	7	6
	+	18	1	15	0
CK14	-	14	8	11	6
	+	11	1	11	0
EGFR	-	14	6	7	5
	+	11	3	15	1
Grade	2	10	1	3	2
	3	15	8	19	3
Ki67	Low	12	8	3	5
	High	13	1	19	1

As to the molecular classification, eight *BRCA1* mutated DCIS cases were of the Luminal A type, 17 cases were categorized as Basal. When comparing *BRCA1* related DCIS with its invasive counterpart (N=22), expression of ER and PR was similar in all cases (table 3), while HER2*/neu*, CK5/6, CK14, EGFR, and Ki67 showed concordance in 91%, 91%, 77%, 77%, and 59% of cases, respectively. As to the molecular classification, 6 and 14 cases were Luminal A respectively Basal type concordant in both DCIS and invasive, while one case was classified Luminal B in the invasive lesion and Basal in DCIS, one case was classified HER2 in the invasive lesion and Basal in DCIS (concordance 91%) (see Table 2).

Table 2. Molecular classification of DCIS versus concomitant invasive cancers in BRCA1/2 germline mutated patients.

		Invasive			
DCIS		Luminal A	Luminal B	HER2	basal
BRCA1	Luminal A	6			
	Luminal B				
	HER2				
	Basal		1	1	14
BRCA2	Luminal A	4			
	Luminal B	1		1	
	HER2				
	Basal				

DCIS in *BRCA2* cases (N=9) showed very frequent expression of ER (89%), frequent expression of PR (44%), while expression of HER2*/neu* (33%), EGFR (33%), CK5/6 (11%) and CK14 (11%) was infrequent (Table 1). Three out of nine cases (33%) were either CK5/6 or CK14 or EGFR positive. The mean % of Ki67 staining in these cases was 14% (range 0-100). One case was grade 2, eight were grade 3. As to the molecular classification, five cases were Luminal A, three Luminal B, and one case was classified as basal. When comparing *BRCA2* related DCIS with its invasive

counterpart (N=22), expression of Ki67 was similar in all cases (Table 4), while the concordance of expression of HER2/*neu*, CK5/6, CK14, ER, PR, and EGFR was 83%, 83%, 83%, 66%, 66%, and 50%, respectively (Table 4). As to the molecular classification, four cases were Luminal A concordant in the DCIS and invasive lesion, one case was Luminal A in the invasive lesion while the DCIS was Luminal B, and one case was HER2/*neu* in the invasive lesion and Luminal B in the DCIS (concordance 67%) (table 3).

Table 3. Immunophenotype of DCIS versus concomitant invasive breast cancer in BRCA1 germline mutated patients.

			Invasive													
			ER		PR		HER2		CK5/6		CK14		EGFR		Ki67	
			-	+	-	+	-	+	-	+	-	+	-	+	low	high
	ER	-	16	0												
		+	0	6												
	PR	-			19	0										
		+			0	3										
	HER2	-					20	2								
DCIS		+					0	0								
	CK5/6	-							5	0						
		+							2	15						
	CK14	-									9	3				
		+									2	8				
	EGFR	-											7	5		
		+											0	10		
	Ki67	Low													6	5
		high													4	7

Table 4. Immunophenotype of DCIS versus concomitant invasive breast cancer in BRCA2 germline mutated patients.

			Invasive													
			ER		PR		HER2		CK5/6		CK14		EGFR		Ki67	
			-	+	-	+	-	+	-	+	-	+	-	+	low	High
	ER	-	0	0												
		+	2	4												
	PR	-			2	2										
		+			0	2										
	HER2	-					4	0								
DCIS		+					1	1								
	CK5/6	-							5	0						
		+							1	0						
	CK14	-									5	0				
		+									1	0				
	EGFR	-											3	1		
		+											2	0		
	Ki67	Low													5	0
		high													0	1

Discussion

The molecular- and immunophenotype of invasive sporadic and *BRCA1* and *BRCA2* related breast cancers has fairly well been unraveled. However, direct breast cancer precursor lesions and genetic progression routes have only been identified for sporadic breast cancer, with DCIS as the final stage before invasion takes place[30-33]. The aim of the present study was to evaluate the immunophenotype of DCIS lesions in *BRCA1* and *BRCA2* mutation carriers and to compare this profile with their invasive counterparts (if available) to establish whether DCIS is a precursor of hereditary breast cancer.

We were able to compile a group of 25 respectively 9 DCIS lesions in *BRCA1* and *BRCA2* germline mutation carriers from an original group of 134 patients with

BRCA1/2 related invasive breast cancer. The percentage of cases DCIS in this group is thereby relatively low (20%) compared to studies in non *BRCA* mutation carriers (40% or even 59%)[16, 42], indicating that DCIS is rarely present next to invasive *BRCA1* and *BRCA2* related cancers.

In the present study, DCIS lesions in *BRCA1* mutation carriers were mostly of the basal type with low expression of ER, PR and HER2/*neu*, while frequently expressing CK5/6, CK14 and EGFR. Further, they were mostly grade 3 and showed high proliferation. This is a similar immunophenotype as has been described for invasive *BRCA1* related invasive breast cancers[13, 25, 26]. Indeed, DCIS lesions showed a high similarity in immunophenotype and molecular subtype with their concomitant invasive counterparts. DCIS lesions in *BRCA2* mutation carriers were mostly of Luminal A type with frequent expression of ER and PR, and infrequent expression of CK5/6, CK14 and EGFR. Further, they were mostly grade 3 and showed low proliferation. This is also similar to the immunophenotype of invasive *BRCA2* related breast cancers[29]. The present study showed indeed a high concordance between *BRCA2* related DCIS lesions and their concomitant invasive counterparts with regard to expression of individual markers as well as molecular subtype. This illustrates that DCIS is very likely a direct precursor lesion of invasive *BRCA1* and *BRCA2* related breast cancers, for *BRCA1* mutation carriers mostly following the Basal progression route with retained stem cell properties, while for *BRCA2* mutation carriers predominantly following the Luminal A progression route. However, in view of the relatively low number of *BRCA2* related DCIS lesions that in the present study could be studied, these results have to be interpreted with more care.

These observations suggest that crucial carcinogenetic events leading to these apparently dedicated phenotypes occur at an early stage in *BRCA1* and *BRCA2* mutation carriers, possibly even before the stage of DCIS. However little is known about the earliest precursor lesions for *BRCA1* and *BRCA2* related breast cancers, although a variety of lesions proposed to be precursor lesions for sporadic breast cancer has been described in increased frequency in PM specimens in hereditary predisposed patients. These lesions encompass fibroadenoma, and atypical ductal

and lobular hyperplasia[33-40]. In view of the Luminal A nature of *BRCA2* related more advanced lesions, it may well be that these are preceded by luminal A type precursors like cylindric cell lesions, atypical ductal hyperplasia and lobular neoplasia. These lesions are on the other hand unlikely precursors in the Basal *BRCA1* related progression route with its retained stem cell properties. The search for earlier basal precursors is therefore on. Whether a subgroup of ductal hyperplastic lesions may have clonal basal properties and thereby be a precursor of *BRCA1* related DCIS, or that *BRCA* related DCIS almost directly derives from breast stem cells is yet a matter of speculation.

Since the *BRCA1* and *BRCA2* phenotype is apparently already present in the pre-invasive stage, preventive systemic treatment should take this into account. While tamoxifen may help to prevent or delay development of DCIS/invasive cancer in *BRCA2* mutated patients, it may be ineffective in *BRCA1* carriers that may be more sensitive to EGFR inhibitors. Further, targeted imaging strategies to detect DCIS lesions in these hereditary patients in an early phase by e.g. PET or optical molecular imaging techniques using targeted tracers may have to be fine tuned based on molecular phenotype.

In conclusion, the immunophenotype and (consequently) the molecular phenotype of DCIS in *BRCA1* and *BRCA2* mutation carriers is similar to their accompanying invasive cancers, thereby providing evidence that DCIS is a direct precursor lesion in these hereditary predisposed patients. DCIS lesions in *BRCA1* mutation carriers are usually of the Basal type, while DCIS in *BRCA2* mutation carriers is predominantly of the Luminal A phenotype. This suggests that crucial carcinogenetic events leading to these phenotypes occur at an early stage in these mutation carriers, possibly even before the stage of DCIS. Further studies are required to identify the earliest precursors of *BRCA1* and *BRCA2* related breast cancer.

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Loss of expression of FANCD2 protein in sporadic and hereditary breast cancer

6

Breast Cancer Research and Treatment, 2008 Jan;107(1):41-7

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Abstract

Fanconi anemia (FA) is a recessive disorder associated with progressive pancytopenia, multiple developmental defects, and marked predisposition to malignancies. FA is genetically heterogeneous, comprising at least 12 complementation groups (A–M). Activation of one of the FA proteins (FANCD2) by mono-ubiquitination is an essential step in DNA damage response. As FANCD2 interacts with BRCA1, is expressed in proliferating normal breast cells, and FANCD2 knockout mice develop breast tumors, we investigated the expression of FANCD2 in sporadic and hereditary invasive breast cancer patients to evaluate its possible role in breast carcinogenesis. Two tissue microarrays of 129 and 220 sporadic breast cancers and a tissue microarray containing 25 BRCA1 germline mutation-related invasive breast cancers were stained for FANCD2. Expression results were compared with several clinicopathological variables and tested for prognostic value. Eighteen of 96 (19%) sporadic breast cancers and two of 21 (10%) BRCA1-related breast cancers were completely FANCD2-negative, which, however, still showed proliferation. In the remaining cases, the percentage of FANCD2-expressing cells correlated strongly with mitotic index and percentage of cells positive for the proliferation markers Ki-67 and Cyclin A. In immunofluorescence double staining, coexpression of FANCD2 and Ki-67 was apparent. In survival analysis, high FANCD2 expression appeared to be prognostically unfavorable for overall survival ($p = 0.03$), independent from other major prognosticators ($p = 0.026$). In conclusion, FANCD2 expression is absent in 10–20% of sporadic and BRCA1-related breast cancers, indicating that somatic inactivating (epi)genetic events in FANCD2 may be important in both sporadic and hereditary breast carcinogenesis. FANCD2 is of independent prognostic value in sporadic breast cancer.

Introduction

Fanconi anemia (FA) is a recessive disease with both autosomal and X-linked inheritance. FA is associated with progressive pancytopenia, developmental defects, and marked predisposition to malignancies, especially acute myeloid leukemia and squamous cell carcinoma of the head and neck[1, 2]. FA cells are characterized by spontaneous chromosomal instability and hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC). FA is genetically heterogeneous and comprises at least 12 complementation groups (A–M). Eleven of the FA genes have been identified sofar: FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCJ, FANCL, and FANCM[3-18]. Eight FA proteins (A, B, C, E, F, G, L, and M) form a nuclear protein complex which is required for mono-ubiquitination of the downstream FA protein, FANCD2. Activation of FANCD2 by mono-ubiquitination is an essential step in the DNA damage response induced by MMC or ionizing irradiation[2, 10]. This DNA damage response pathway also includes the breast cancer susceptibility genes BRCA1 and BRCA2, also referred to as the FA-BRCA pathway. Following ionizing radiation, FANCD2 and BRCA1 accumulate and colocalize in nuclear foci, which reflect sites of DNA damage and repair[10, 19]. Like FA cells, cells lacking BRCA1/2 proteins are hypersensitive to DNA crosslinking agents. D'Andrea et al. showed that FANCD1 and BRCA2 are the same proteins. BRCA2 is a direct regulator of RAD51, a protein essential for homologous recombination repair[20]. Although BRCA1 is mainly involved in hereditary breast cancer[21], it has also been implicated in sporadic breast cancer[22]. In an immunohistochemical analysis, we have previously shown that FANCD2 is expressed in proliferating cells of different organs, including the premenopausal breast duct epithelium[23]. This is in line with the role of FANCD2 in DNA repair which is important to guarantee the integrity of the genome during cell replication[10] As deregulation of proliferation is one of the crucial processes of carcinogenesis, these observations imply a potential role for FANCD2 in the pathogenesis of breast cancer. Indeed, FANCD2 knockout mice develop breast tumors[24]. These considerations prompted us to investigate

the expression of FANCD2 in sporadic and hereditary invasive breast cancers by immunohistochemistry in relation to several other proliferation-related biomarkers and survival.

Materials and methods

Tissue microarray

Paraffin blocks containing formaldehyde-fixed breast cancer tissues of 129 cases of invasive breast cancer not selected for family history (further denoted "sporadic") were obtained from the archives of the Department of Pathology of the VU University Medical Center, Amsterdam. For all breast cancer cases, age, lymph node status, and tumor size were documented. A second array block was constructed containing 24 cases with a proven BRCA1 germline mutation identified through the Family Cancer Clinic of the VU University Medical Center as previously described[25]. Patient characteristics are shown in Table 1. The hematoxylin–eosin stainings were used to identify representative areas of tumor tissue in the blocks. A tissue microarray was then constructed by transferring tissue cylinders of 4–5 mm from the representative tumor area of each donor block to the recipient block using a tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA) as described before[26]. A third tissue array block of 220 sporadic breast cancer patients with long-term follow-up was obtained from the archives of the Gerhard-Domagk Institute of Pathology, University of Muenster, as previously described[27]. Sections of 4 μm were cut and transferred on SuperFrost+ (Menzel&Glaeser, Germany) slides for immunohistochemistry. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in our hospital[28].

Immunohistochemistry

Immunohistochemical analysis had been previously performed on conventional sections for the following markers: Ki-67, Cyclin A, p21, p27, p53, estrogen receptor

(ER), progesterone receptor (PR), HER-2/*neu*, and EGF-receptor[29]. Rabbit polyclonal antiserum against FANCD2 was generated as previously described[23]. Tissue sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with methanol/0.3% H_2O_2 (20 min). Sections were heated (30 min, 120ºC) in 0.1 M citrate buffer pH 6. Unspecific binding was blocked with a 1:50 normal goat serum in PBS pH 7.4/ 1% BSA. Polyclonal rabbit anti-FANCD2 (200 lg/ml) was diluted 1:500 in PBS/1% BSA, and sections were incubated overnight (4ºC) in a humidified chamber. Subsequently, sections were incubated with HRPconjugated secondary antibodies (EnVision, DAKO) and diaminobenzidin (10 min), counterstained with hematoxylin (20 s), dehydrated, and cover-slipped.

Table 1. Patient characteristics and histology of 120 sporadic and hereditary breast cancers.

	Sporadic	BRCA1mutation	Total
No. of patients	96	24	120
Age			
Mean	65	42	62
Lymph node status			
Negative	58	9	67
Positive	38	11	49
Tumor size			
Mean	2.34	2.78	2.40
Histological type			
ductal	85	17	102
lobular	7	1	8
medullary	1	2	3
tubular	1	-	1
cribriform	1	-	1
apocrine	1	-	1
metaplastic	-	4	4

Appropriate positive controls were used throughout, and negative controls were obtained by omission of the primary antibodies. Percentages of positively stained nuclei were estimated by an experienced observer (PJvD), except for HER-2/*neu* and EGF-receptor where membrane staining was scored as positive. In addition, FANCD2 intensity was scored semiquantitatively as 0–3, and an FANCD2 score was

calculated for each case by multiplying the % FANCD2-positive cells by the staining intensity. For FANCD2/Ki-67 double staining, anti-FANCD2 was diluted 1:150, incubated overnight, followed by incubation with swine anti-Rabbit HRP 1:200 (Dako, Glostrup, Denmark), and detected with the TSATM Tetramethylrhodamine system (PerkinElmer Life Sciences, Boston, USA). This was immediately followed by incubation with mouse anti-Ki-67 1:50 (MIB1, Immunotech, Marseille, France) followed by rabbit anti-mouse FITC 1:40 (Dako, Glostrup, Denmark). Nuclei were counterstained by incubation with TO-PRO-3 (Molecular Probes, Eugene, OR, USA) 1:5,000 as previously described[30].

Statistics

Bivariate scatter plots were generated between the percentage of FANCD2-expressing cells and the other continuous features. For the proliferation-associated features Ki-67, Cyclin A and MAI, the cases with no FANCD2 expression were excluded from the analysis, assuming that FANCD2 is, by some mechanisms, no longer expressed in these cases. By linear regression analysis, the correlation coefficient *R* and related *p* values were calculated. Student's t test was used to compare FANCD2 expression levels between the low-level vs. high-level groups for HER-2/neu, the EGFreceptor, ER (cut off 10%) and PR (cut off 10%), p53 (cut off 10%), and Cyclin D1 (cut off 5%). Prognostic value of FANCD2 (Muenster cases) was assessed by computing Kaplan–Meier curves, and differences between the curves were evaluated with the log-rank test. Multivariate survival analysis was performed by Cox regression.

Results

Successful FANCD2 staining was performed in 96 of the 129 cases of the VUmc sporadic array block. The drop outs were caused by damaged or detached cores during cutting, mounting, or staining, or did not contain tumor. Eighteen of these

cases (19%) were completely FANCD2-negative. The other cases showed variable staining from 1 to 85% of the nuclei. In 21 of the 24 BRCA1 cases, FANCD2 staining was performed successfully. In two of these (9.5%), FANCD2 expression was completely negative. Figure 1 shows some representative examples of FANCD2 staining. The mean percentage of FANCD2-expressing cells was significantly higher in ER-negative patients ($p = 0.019$), PR-negative patients ($p = 0.016$), EGFR positive patients ($p = 0.002$), Cyclin-D1-negative patients ($p = 0.002$), and p53-positive patients ($p < 0.001$) (Table 2). No statistically significant difference was seen for HER-2/neu. When analyzing the sporadic and hereditary subgroups, similar associations were seen. FANCD2 staining intensity yielded no useful correlations and no prognostic value, and the FANCD2 score yielded essentially the same correlations and prognostic value as the % FANCD2-positive cells (data not shown).

Table 2. Mean percentage of FANCD2 positive cells in high and low-level expression groups of hormone receptors, growth factor receptors, cyclin D1 and p53 in sporadic and hereditary breast cancers.

	# (%)	FANCD2% Mean (SE)	T-test p-value
Total	117 (100)		
Estrogen receptor			
Low	56 (48)	14 (2.5)	0.019
High	61 (52)	8 (1.6)	
Progesterone receptor			
Low	81 (69)	17 (1.9)	0.016
High	36 (31)	12 (1.9)	
HER-2/*neu*			
Negative	93 (79)	16 (1.6)	0.797
Positive	24 (21)	17 (3.5)	
EGF receptor			
Negative	78 (66)	13 (1.5)	0.002
Positive	39 (33)	19 (3.1)	
Cyclin D1			
Low	85 (73)	12 (1.9)	0.002
High	32 (27)	7 (2.6)	
p53			
low	88 (75)	8 (1.3)	< 0.001
high	29 (25)	19 (4.3)	

Figure 1. Examples of FANCD2 staining in sporadic (A/B, A=negative control) and BRCA1 related breast cancer (C/D, C=negative control).

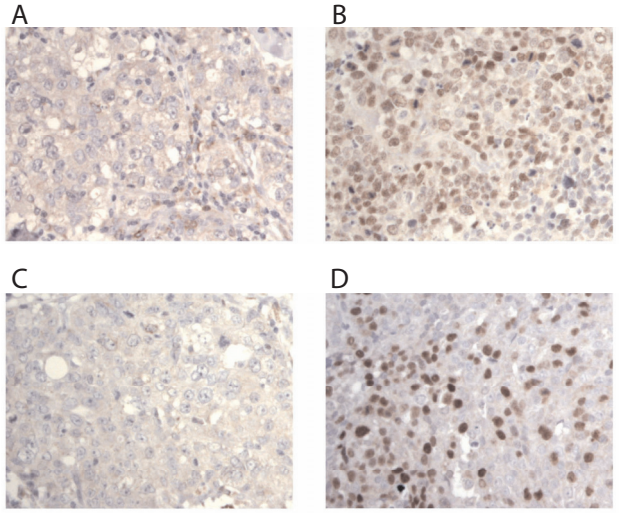


Figure 2. FANCD2/Ki67 immunofluorescence double staining in a representative case of invasive breast cancer. Top left: Ki67 staining. Top right: FANCD2 staining. Bottom left: TO-PRO staining. D. Triple exposure showing co-expression of FANCD2 and Ki67.

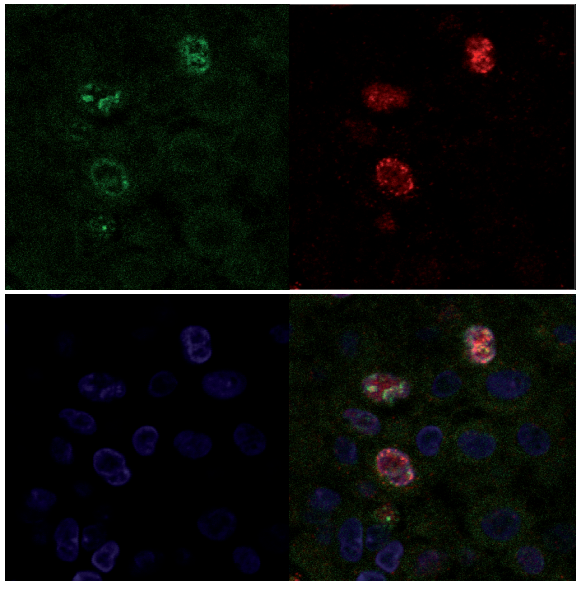


Table 3. Correlation between mean percentage of FANCD2 expressing cells and other continuous clinicopathological variables in sporadic and hereditary breast cancers.

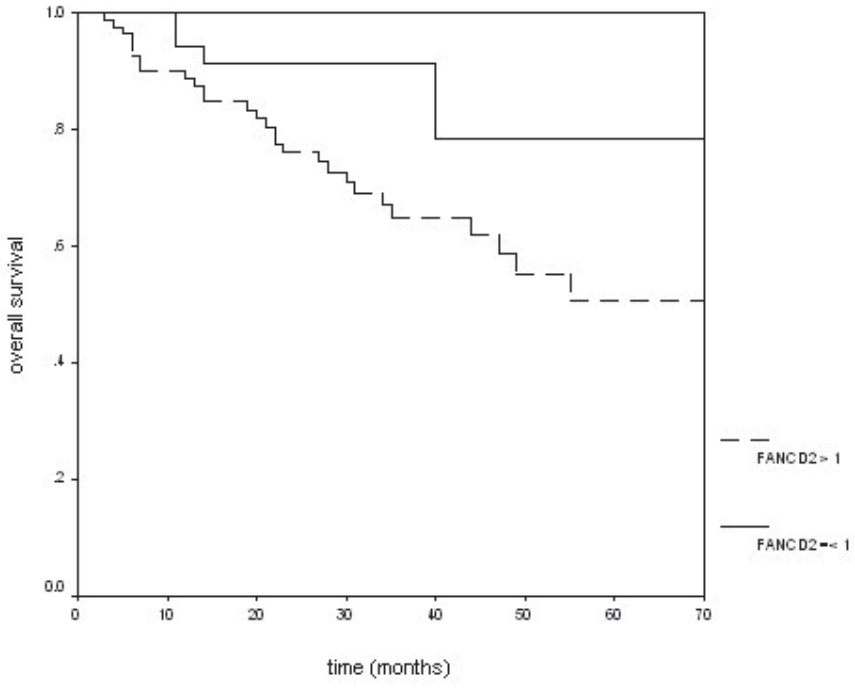
Feature	R	p-value
MAI*	0.506	<0.001
Ki67*	0.502	<0.001
Cyclin A*	0.482	<0.001
Age	-0.197	0.033
ER	-0.221	0.017
PR	-0.204	0.028
Tumour size	0.018	0.852
p27	-0.074	0.430
p21	-0.171	0.065
p53	0.379	<0.001
Cyclin D1	-0.126	0.176

*FANCD2 negative cases excluded.

In linear regression analysis (Table 3), the percentage of FANCD2-expressing cells was significantly positively correlated to Ki-67 ($R = 0.502$, $p < 0.0001$), Cyclin A ($R = 0.482$, $p < 0.0001$), MAI ($R = 0.506$, $p < 0.001$), and p53 ($R = 0.379$, $p < 0.0001$), and significantly negatively correlated to age ($R = 0.197$, $p = 0.033$), ER ($R = 0.221$, $p = 0.017$), and PR ($R = 0.204$, $p = 0.028$). There was no correlation between FANCD2 and the other continuous features. Figure 2 shows examples of FANCD2/Ki-67 immunofluorescence double staining, underlining the coexpression of FANCD2 and Ki-67 in invasive breast cancers cells. In the Muenster cases, most of these correlations could be reproduced (age: $p = 0.063$, Ki-67: $p = 0.001$, p53: $p = 0.003$, ER: $p = 0.034$). Only PR was not significant here, and Cyclin A was not performed. In the regression analysis between the percentages of FANCD2-expressing cells and Ki-67 and Cyclin A, the completely negative FANCD2 cases were excluded. These FANCD2-negative cases had Ki-67 values between 1 and 65 (mean 16%), Cyclin A values between 0 and 50 (mean 10%), and MAI values between 0 and 37 (mean 11), indicating that these cases had (sometimes even high) FANCD2-independent proliferation. In survival analysis (sporadic Muenster cases), high FANCD2 expression appeared to be prognostically unfavorable ($p = 0.03$). Figure 3 shows

the survival curves. In Cox regression including tumor size, lymph node status, ER, and grade, FANCD2 staining appeared to have independent prognostic value for overall survival (p = 0.026).

Figure 3. Prognostic value of FANCD2 expression in sporadic invasive breast cancer. Low expressors have a better survival than high expressors (p=0.03, N=122).



Discussion

The aim of this study was to investigate the expression of FANCD2 in sporadic and hereditary breast cancers. This was inspired by several observations. First, FANCD2 and BRCA1/2 are functionally closely linked in the DNA repair response, and BRCA1 and BRCA2 are implicated in hereditary and sporadic breast cancers[21, 22]. Second, targeted deletion of FANCD2 in mice resulted in an increased rate of breast tumors[24]. Third, we have shown that FANCD2 is expressed in proliferating cells in

the duct epithelium of the normal breast[23]. It appeared that 19% of sporadic breast cancers completely lacked FANCD2 expression. Yet, these FANCD2-negative cases had high mean Ki-67, Cyclin A and MAI values, indicating that the low FANCD2 levels in these cases cannot be explained by low proliferation. The fact that these FANCD2-negative cases stained for other proteins makes it quite unlikely that the FANCD2 negativity is due to fixation problems. In the FANCD2-negative cases, FANCD2 inactivation may, in view of its important function, have been a hit in carcinogenesis. BRCA1 germline mutation-related breast cancers showed lack of FANCD2 expression in only 9.5% of cases, which fits with the concept that a major hit in an important pathway (in these cases the BRCA1 germline mutation) is usually not associated with further hits in this pathway. It is yet unclear what the mechanism behind the lack of FANCD2 expression in these cases is. It needs to be further studied whether there are inactivating somatic mutations in these cases or whether promoter methylation plays a role. FANCD2 expression was strongly correlated with expression of the proliferation-associated features Ki-67, Cyclin A, and MAI, and FANCD2 and Ki-67 were coexpressed in invasive cancer cells. This is likely a reflection of the physiological function of FANCD2 in DNA repair of proliferating cells, rather than an independent overexpression of an altered gene, in line with our previous study where we found a coexpression of FANCD2 and Ki-67 in proliferating cells of various normal human tissues[23]. The observation that high FANCD2 expression indicated poor prognosis fits within the same concept, as rate of proliferation (and thereby FANCD2 expression) is a major cell biological phenomenon determining prognosis[31-33]. FANCD2 had prognostic value independent of stage and grade, which can be explained by the fact that proliferation and stage are not strongly correlated, and that grade includes nuclear atypia and tubule formation besides rate of proliferation as measured by mitotic index. Although heterozygosity for p53 was shown to accelerate epithelial tumor formation in FANCD2 knockout mice[34], a functional link between p53 and FANCD2 has not been described to explain the association found in the present study, which is likely caused by the fact that p53 mutated and thereby p53 protein accumulated

tumors show, in general, higher proliferation and therefore more proliferating FANCD2-expressing cells. The same may also hold for the relation between FANCD2 and EGFR expressions, for which also no functional relationship has been described. The negative relation between FANCD2 and age can likely be explained by the fact that BRCA1-related patients that have higher FANCD2 expression are younger. Within the light of the above observations, the question remains why FANCD2 patients do not seem to be predisposed to breast cancer in clinical practice. FA itself is a rare genetic disease where the complementation group D2 constitutes only 1–2% of all FA cases and these patients generally have a more severe clinical course. They may therefore simply not live to get breast cancer in an apparent increased frequency. Our results do not indicate that somatic (epi)genetic changes in FANCD2 are a frequent secondary carcinogenetic event in BRCA1 germline-mutated patients, although this needs to be confirmed in a larger study group. In conclusion, FANCD2 expression is absent in 10–20% of sporadic and BRCA1-related breast cancers, indicating that somatic inactivating (epi)genetic events in FANCD2 may be important in both sporadic and hereditary breast carcinogenesis. FANCD2 is of independent prognostic value in sporadic breast cancer.

Acknowledgments

We thank Dick van Wichen, Jurryt van der Gugten, and Lennert Ploeger for their expert help with the immunofluorescence double staining. This work was supported by grants from the Maurits Anna de Kock foundation, the Dutch Organization for Scientific Research and the Dutch Cancer Society (VU2001–2505), and the First Aegon International Scholarship in Oncology.

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High frequency of HIF-1α overexpression in BRCA1 related breast cancer

7

Breast Cancer Research and Treatment, 2008 Oct;111(3):475-80

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Abstract

Hypoxia is a hallmark of cancer. Hypoxia inducible factor-1a (HIF-1α) is the key regulator of the hypoxia response. HIF-1α is overexpressed during sporadic breast carcinogenesis and correlated with poor prognosis. Little is known on the role of HIF-1α in hereditary breast carcinogenesis. A recent study suggests a role for BRCA1 in HIF-1α regulation. We therefore examined the expression of HIF-1α in BRCA1 related breast cancers. By immunohistochemistry we studied expression of HIF-1α and some of its downstream targets in 30 BRCA1 related breast cancers in comparison with 200 sporadic controls. HIF-1α overexpression was significantly more frequent in BRCA1 related breast cancers (27/30, 90%) than in sporadic controls (88/200, 44%) ($P<0.0001$). 19/30 (63%) of BRCA1 tumors showed perinecrotic (hypoxia induced) and 8/30 (27%) a diffuse HIF-1α overexpression pattern, the latter more likely related to genetic alterations in oncogenes and tumor suppressor genes. In contrast, sporadic breast cancer HIF-1 expressing tumors showed an inverse ratio of perinecrotic/diffuse expression (31 vs. 69%, $P = 0.0002$). Glut-1 and CAIX, downstream HIF1 targets, were expressed in 27/30 (90%) and 15/21 (71%) of hereditary cases versus 54/183 (29%) and 24/183 (13%) in sporadic cases. p300 levels, necessary for HIF-1 downstream activation, were significantly higher in hereditary cases (20/21, 95%) compared to sporadic cases (91/183, 50%, $P = 0.0001$). In conclusion, in BRCA1 germline mutation related breast cancer, functional HIF-1α overexpression is seen at a much higher frequency than in sporadic breast cancer, mostly hypoxia induced. This points to an important role of hypoxia and its key regulator HIF-1α in hereditary breast carcinogenesis.

Introduction

Carriers of germline mutations in BRCA1 or BRCA2 have a hereditary predisposition for developing breast and/or ovarian cancer. Several studies have indicated that the genetic makeup of BRCA1/2 related breast cancer is different from that of sporadic breast cancer. These differences comprise gains and losses of specific parts of chromosomes as well as differences in gene expression[1-6]. In line with this, the morphological and immunohistochemical phenotype of BRCA1 related breast cancer is also different[7, 8]. They often concern well demarcated medullary and poorly differentiated ductal cancers with conspicuous lymphocytoplasmic infiltrates[9, 10] that are of high-grade[11] and show high proliferation[12]. In addition, they do not express estrogen (ER), progesterone (PR) or HER-2/*neu* receptors[13], often lack p27Kip1[14], but do accumulate p53[15], and overexpress cyclin E[16], cytokeratins (CK) 5/6 and 14[17, 18], and EGFR[19-21]. These observations point to a carcinogenetic pathway of BRCA1 related breast cancers different from that in sporadic cancers. Hypoxia is a hallmark of many sporadic cancers[22]. Hypoxia inducible factor-1 (HIF-1) is the key regulator of the hypoxia response. HIF-1 consists of 2 subunits, HIF-1α and HIF-1β. While HIF-1β is constitutively expressed, the HIF-1α protein is continuously degraded under normoxia by the ubiquitin-proteasome pathway[23, 24]. Under hypoxia, HIF-1α protein degradation is inhibited resulting in its overexpression and subsequent binding to HIF-1β[24]. This HIF-1 complex then regulates the expression of its target genes through binding with hypoxia responsive elements in the promoter regions of these genes[25]. The overexpression of HIF-1α has been demonstrated in several types of cancer, with a negative impact on therapy response and prognosis[26-28]. In sporadic breast cancer, previous studies have demonstrated that HIF-1α overexpression plays a role in breast carcinogenesis [29-32] and is correlated with a poor prognosis in invasive breast cancer[31, 33, 34]. Little is known of the putative role of HIF-1α in hereditary breast carcinogenesis. A recent study suggested that BRCA1 plays a role in the hypoxic response by regulating HIF-1α stability and by modulating expression of vascular endothelial growth factor, a major downstream

target of HIF-1α[35]. The aim of this study was therefore to examine the expression of HIF-1α in BRCA1 related breast cancer to find clues for its putative role in the BRCA1 carcinogenesis.

Materials and methods

Patients

The study group comprised 30 invasive breast cancer cases from 17 patients with a proven BRCA1 germline mutation and 13 patients with invasive breast cancer who were not screened for mutations themselves, but were known to have a BRCA1 mutation in their family. All these patients were derived from the Familial Cancer Clinic of the VU University Medical Centre, Amsterdam. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients[36]. As sporadic controls, data from our previous study[33] on invasive breast cancers from patients unselected for family history were used.

Histopathology

Tumor size was measured in the fresh resection specimens, and tumor samples were subsequently fixed in neutral buffered formaldehyde, and processed to paraffin blocks according to standard procedures. A tissue array block was made as previously described[37]. About 4 μm thick sections were cut and stained with H&E for histopathology. Tumor type was assessed according to the WHO, and tumors were graded according to the Nottingham grading system. Mitoses counting was performed as previously described[38]. Presence of necrosis was noted. Scoring was performed by one observer (PJvD) who was blinded to the origin of the tumors.

Immunohistochemistry

After deparaffination and rehydration, target retrieval solution (DAKO) was used for antigen retrieval with all slides placed in a water bath for 45 min at 97°C. A cooling

off period of 20 min preceded the incubation of the HIF-1α mouse monoclonal (BD Biosciences, Pharmingen, Lexington, USA), at a dilution of 1:500. The catalysed signal amplification system (DAKO) was used to detect HIF-1α as before[25]. For ER, PR, HER-2/*neu*, EGFR, Ki67, p53, p27 and p21 antigen retrieval was performed in an autoclave with the slides placed in a citrate buffer (pH 6). For Glut-1, CAIX and P300 antigen retrieval was performed in citrate buffer, pH = 6.0, for 20 min at 100ºC and for CK5/6 and CK14 an EDTA buffer (pH 9) was used. A cooling off period of 30 min preceded the incubation (60 min at room temperature) with the primary antibodies. Mouse monoclonal antibodies used were: ER (1:50, DAKO), PR (1:50, Novocastra, Newcastle upon Tyne, United Kingdom), HER-2/*neu* (1:10,000, Prof. M. van der Vijver, Dutch Cancer Institute, Amsterdam, The Netherlands), EGFR (1:10, Novocastra), CK5/6 (1:3000, Chemicon, Temecula, USA), CK14 (1:400, Neomarkers, Lab Vision Corp, Fremont, CA, USA), Ki67 (1:40, MIB-1, Immunotech, Marseille Cedex, France), p53 (1:500, DAKO), p27 (1:1000, BD Biosciences Transduction laboratories, Lexington, USA), p21 (1:50, BD Biosciences, Pharmingen). Polyclonal primary antibodies used were: Glut-1 (1:200, DAKO), CAIX (1:1000, Abcam, Cambridge Science Park, Cambridge, UK), P300 (1:200, clone N15, Santa Cruz, CA, USA) For detection of the primary antibodies against CK5/6, CK14, CAIX and p300, a poly HRP anti Mouse/Rabbit/Rat IgG (ready to use, ImmunoLogic, ImmunoVision Technologies, Brisbane, USA) was used for the other primary antibodies a biotinylated rabbit anti-mouse antibody (DAKO) or a biotinylated swine anti-rabbit antibody was used. The signal was amplified by avidin-biotin complex formation. All slides were developed with diaminobenzidine followed by haematoxylin counterstaining. Before the slides were mounted all sections were dehydrated in alcohol and xylene. Scoring was performed by one observer (PJvD). HIF-1α, ER, PR, Ki67, p53, p27 and p21 staining was usually confined to the nucleus. Diffuse cytoplasmic staining was sometimes seen but ignored, estimating the percentage of positively stained nuclei. p300 nuclear staining intensity was in accordance with our previous study[39] scored as negative, 1+, 2+ or 3+ and for further statistical analysis grouped as negative (neg, 1+) or positive (2+, 3+). HIF-1α was regarded overexpressed when >1% of nuclei

were positive as described before[27], and the expression pattern (perinecrotic or diffuse) was noted[32]. HER-2/*neu*, EGFR, and CAIX stainings were scored positive when a clear membrane staining pattern was seen. Glut-1 expression was scored positive if a clear membrane or a distinct cytoplasmic staining was seen, and Ck5/6, Ck14 were scored positive in case of cytoplasmic staining.

Table 1. Expression of HIF-1α in hereditary and sporadic breast cancers in relation to various clinicopathologic features and HIF-1α downstream genes.

		hereditary				sporadic			
			HIF-1α				HIF-1α		
		N	< 1%	> 1%	p-value	N	< 1%	> 1%	p-value
Total		30	3	27		200	112	88	
Tumor type	ductal	21	2	19	0.019	144	76	68	0.0087
	lobular	1	1	0		30	22	8	
	medullary	5	0	5		4	0	4	
	metaplastic	3	0	3		0	0	0	
	tubular					11	9	2	
	papillary					2	1	1	
	mucinous					4	1	3	
	apocrine					2	0	2	
	cribriform					3	3	0	
Grade	I	0	0	0	0.061	61	46	15	<0.001
	II	7	2	5		78	48	30	
	III	23	1	22		61	18	43	
Tumor size	0-2 cm	7	1	6	0.923	97	61	36	0.08
	2-5 cm	15	2	13		89	42	47	
	> 5 cm	1	0	1		14	9	5	
Glut-1	negative	2	2	0	<0.001	143	97	46	<0.001
	positive	28	1	27		57	15	42	
CAIX	negative	6	2	4	0.019	175	108	67	<0.001
	positive	15	0	15		25	4	21	

Results

High levels of HIF-1α expression were detectable in 27/30 (90%) of the hereditary breast cancer cases, compared to 88/200 (44%) of the sporadic controls (P < 0.0001) (Table 1). Necrosis was present in 19/30 (63%) of the hereditary cases compared to 38/200 (19%) of controls (P < 0.0001). In 19/27 of the hereditary cases that showed HIF-1α expression, a perinecrotic staining pattern was observed and in 8/27 a diffuse pattern was seen, compared to 27/88 (31%) and 61/88 (69%) of sporadic cases, respectively (P = 0.0002).

Table 2. Expression of HIF-1α in hereditary breast cancers in relation to various other immunophenotypic markers.

			HIF-1α		
		N	<1%	>1%	P
ER	negative	24	1	23	0.033
	positive	6	2	4	
PR	negative	23	0	23	0.001
	positive	7	3	4	
HER-2/*neu*	negative	27	1	26	0.001
	positive	3	2	1	
EGFR	negative	8	2	6	0.099
	positive	22	1	21	
Ck5/6	negative	8	2	6	0.058
	positive	13	0	13	
Ck14	negative	7	2	5	0.035
	positive	14	0	14	
Ki67	negative	16	3	13	0.088
	positive	14	0	14	
p53	negative	16	2	14	0.626
	positive	14	1	13	
p27	negative	23	3	20	0.314
	positive	7	0	7	
p21	negative	18	1	17	0.320
	positive	12	2	10	

Glut-1 expression was detected in 27/30 hereditary cases and CAIX in 15/21 cases, and both were correlated with HIF-1α overexpression (P-value < 0.001 for both). In the sporadic cases the expression of Glut-1 and CAIX was 29% (54/183) and 13% (24/183) respectively. p300 levels were significantly higher in hereditary cases 95% compared to sporadic cases 50% (91/183), (P = 0.0001). Furthermore, high levels of HIF-1α expression in these hereditary breast cancers were associated with a poor histological grade (P = 0.061) and EGFR expression (P = 0.099) (Table 2). HIF-1α correlated significantly negatively with the presence of ER (P = 0.033), PR (P = 0.001), and HER-2/*neu* (P = 0.001). For the remaining markers no significant correlations with HIF-1α expression were found.

About 21/30 (70%) cases were both HIF-1α and EGFR positive. All of these HIF-1α and EGFR positive cases were Glut-1 positive, 14/21 of these cases showed a perinecrotic HIF-1α expression pattern and the remaining cases showed a diffuse HIF-1α expression pattern. In nine of the perinecrotic HIF-1α cases CAIX staining was also present. In the seven diffuse HIF-1α cases four cases were CAIX positive.

Discussion

The aim of this study was to examine the expression of HIF-1α in BRCA1 related breast cancers to establish whether the HIF-1α pathway plays a role in the BRCA-1 carcinogenesis and progression. About 90% of BRCA1 related breast cancers showed expression of HIF-1α, a percentage significantly higher than in sporadic controls. Mostly, this concerned the perinecrotic type of HIF-1α expression. Necrosis, likely caused by the well known rapid tumor cell proliferation of these hereditary cancers while the vasculature is lagging behind, was clearly more present than in the sporadic cancers. Likewise, a perinecrotic pattern of overexpression of HIF-1α was more frequent in BRCA1-related than in sporadic breast cancers. The perinecrotic type of HIF-1α expression was accompanied by overexpression of the HIF-1α downstream genes Glut-1 and CAIX, pointing towards

functional HIF-1α. This type of HIF-1α overexpression is thought to be caused by (severe) hypoxia, whereas diffuse HIF-1α overexpression at (relative) normoxia is thought to be induced by growth factors like HER2/*neu*[39], HIF-1α gene amplifications[40] or mutations[41], or by other oncogenes or loss of tumour suppressor genes. We have previously shown that, compared to a diffuse staining pattern, perinecrotic HIF-1α overexpression is associated with the worst survival of sporadic breast cancer patients[33]. The present results are in contrast with a recent in vitro study where increased levels of BRCA1 were seen to increase the response of the VEGF promoter to hypoxia in a HIF-1α dependent fashion[35]. In that study, reduced levels of BRCA1 protein reduced the ability of hypoxia to induce VEGF. We, however, observed marked upregulation of HIF-1α in human BRCA1 related breast cancers. In view of the frequent presence of necrosis and perinecrotic HIF-1α expression, we hypothesize that in breast cancers in BRCA1 germline mutation carriers, hypoxia overrides the potential negative effect of BRCA1 expression loss on HIF-1α expression, yet leading to frequent perinecrotic HIF-1α expression and subsequently to activation of HIF-1 downstream genes. In line with this, p300 expression levels, a prerequisite for HIF-1 downstream activation, were high in hereditary cancers. Whilst we previously reported frequent overexpression of EGFR in hereditary breast cancers[19, 42], we now find concomitant expression of HIF-1α and EGFR in 70% of BRCA1 related breast cancers. Furthermore, all of these cases had evidence of HIF1 downstream activation, suggesting that EGFR enhances the hypoxic response. Several previous studies have elucidated in vitro the role of both the PI3K and the MAPK pathway in the induction of HIF-1α, including its upregulation by HER-2/*neu*. In addition, the upregulation of EGFR has been related to elevated levels of downstream targets of HIF-1α, like VEGF and survivin[43]. This suggests a role for specific oncogenes in the (normoxic) induction of HIF-1α. The association between HIF-1α and EGFR might be explained by the EGFR induced activation of the PI3K/PTEN/AKT/FRAP pathway, through which HER-2/*neu* also acts on HIF-1α[44, 45]. Further studies will have to elucidate the role of EGFR in the carcinogenesis of BRCA1 related breast cancer. Recent in vitro studies on breast

basal-like cell lines showed that these cell lines are more sensitive for EGFR inhibitors and for carboplatin with a synergistic effect when these are combined[46]. This might lead to new therapy strategies for BRCA1 related breast cancer patients. In contrast to EGFR, an association between HIF-1α and HER-2*/neu* as observed in previous studies[27, 39] could not be confirmed in this study. 26/27 (96%) of HIF-1α positive cases were HER-2*/neu* negative, in which the usual HER-2*/neu* negativity of hereditary breast cancers likely plays a role. We conclude that the BRCA1 germline mutation related breast cancers show a high frequency of HIF-1α overexpression. In view of the predominantly perinecrotic staining pattern, overexpression of HIF-1α in hereditary breast cancer seems to be caused by hypoxia rather than by activation of oncogenes or inactivation of tumor suppressor genes. However, the frequent overexpression of EGFR and concomitant expression of EGFR and HIF-1α may open up new ways of treatment of BRCA1 related breast cancer by targeting EGFR.

Acknowledgement

Supported by an unrestricted educational grant from Aegon Inc.

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General Discussion and Summary

8

About 5% of all breast cancers are caused by the germline mutations in the Breast Cancer gene1 (BRCA1) and Breast Cancer gene2 (BRCA2) genes. In this thesis the immunoprofile of these hereditary breast cancers and their precursors was studied by immunohistochemistry and related to clinicopathological features and compared with data found in sporadic breast cancer. Further establishing the immunoprofile of hereditary breast cancer and its precursors could lead to new practical tools to more accurately distinguish hereditary from sporadic breast cancers. This could help to identify patients and families eligible for mutation screening, and to provide a pathogenic mutation reference for research into unclassified variant mutations in BRCA1 and BRCA2. In addition, the data could provide clues on hereditary breast carcinogenesis and thereby provide new targets for the treatment and/or prevention of hereditary breast cancer.

Immunoprofile of hereditary breast cancer

In **Chapter 2** we review the literature on the pathology of hereditary breast cancer. Hereditary breast cancer runs in families where several family members in different generations are affected. Most of these breast cancers are caused by mutations in the high penetrance genes BRCA1 and BRCA2 which account for about 5% of all breast cancers. In families with a clear history of breast cancer, mutations in the BRCA genes account for 20-25% of breast cancers. Other genes like CHEK2, PTEN, TP53, ATM, STK11/LKB1, CDH1, NBS1, RAD50, BRIP1 and PALB2 have all been described to be high or moderate penetrance breast cancer susceptibility genes and are also a part of the hereditary breast cancer spectrum. However, still a part of these familial hereditary breast cancers can not be attributed to mutations in any of the presently known high or moderate penetrance breast cancer susceptibility genes. In this review we focus, after describing the function of these different genes, on differences in histology, the expression of certain proteins, gene expression profiles of the breast cancers caused by mutations in BRCA1 and BRCA2

genes or by the other breast cancer susceptibility genes. Finally, a short overview of diagnosis, treatment and prognosis of these hereditary breast cancer patients is provided. To conclude, quite much is known on the pathology of BRCA1 related breast cancers, but clearly more information is needed on the pathogenicity of unclassified variant mutations in BRCA1 and BRCA2, on the phenotype of BRCA2 and other high or moderate penetrance breast cancer susceptibility genes, and on the immunophenotype of precursor lesions. It should be mentioned that low numbers of available cases may be the limiting factor to provide satisfactory data. This calls for international consortia to arrive at large enough collections.

Several previous studies have indicated that the genetic makeup of BRCA1and BRCA2 related breast cancer is different from that of sporadic breast cancer. These differences comprise gains and losses of specific parts of chromosomes as well as differences in gene expression In line with this, the morphological and immunohistochemical phenotype of BRCA1 related breast cancer is different. They often concern well demarcated medullary and poorly differentiated invasive ductal cancers with conspicuous lymphocytoplasmic infiltrates that are of high-grade and show high proliferation. In addition, they show low expression of the estrogen (ER), progesterone (PR) and HER-2/*neu* receptors, often lack $p27^{Kip1}$, but do accumulate p53, and overexpress cyclin E, cytokeratins (CK) 5/6 and 14. The phenotype of BRCA2 related cancers is however less clearly defined. Before, few studies had aimed at integrating the various clinicopathological features into a multivariate model to classify breast cancer in patients as hereditary or sporadic, as an aid to genetic counselling. We therefore aimed at evaluating a panel of clinicopathological variables to classify breast cancers as hereditary or sporadic using a multivariate approach in **Chapter 3**. Different clinicopathological features were compared between breast cancers from carriers of a proven BRCA1 or BRCA2 mutation, breast cancer from patients at an intermediate- or high risk of hereditary disease on the basis of family history but untested for BRCA mutations, and cancers from sporadic controls. Most of the investigated markers were expressed in line with earlier data. However, we were the first to describe the high expression of

EGFR in breast cancers related to BRCA1 and BRCA2 mutations, as is further detailed in **Chapter 4**. In addition, the observed high cyclin A expression is another useful marker of proliferation in BRCA1 related breast cancers and has been herewith established for the first time. Although our data were based on only five cases of BRCA2-related breast cancers, it appeared nevertheless that these cancers stand midway between sporadic and BRCA1-associated breast cancers. In the BRCA2 related breast cancers cyclin D1 expression was remarkably high. In the decision tree analysis, the best classifier appeared to be composed of age, Ki67 and EGFR. In the age group ≥54 years, the chance of a BRCA1-related breast cancer was as low as 0% when EGFR was negative and 3% when EGFR was positive. In the age group <54 years, the chance of BRCA1-related disease was only 9% when Ki67 was low and as high as 75% when Ki67 was high. The majority of sporadic cases were classified as probably sporadic, and only a few as probably BRCA1-related. This is in line with the fact that the controls were "unselected for family history" owing to non-availability of data on family history and could therefore potentially include some patients with hereditary breast cancer. Of note, none of the BRCA1-related cases were categorized as probably sporadic and as many as 82% were classified as probably BRCA1-related. A remaining percentage of 18 percent that could not be categorized is not unexpected since some breast cancers in women with BRCA1 germline mutations, especially in elderly patients, will not be attributable to BRCA1, but will arise according to a sporadic carcinogenetic pathway. Obviously, the 75% chance of BRCA1-related disease in the probably BRCA1-related group is relative, because this chance is influenced by the ratio between BRCA1-related cancers and controls in this study. When the decision tree was used to classify cases at intermediate risk of hereditary disease, 41% were classified as probably sporadic and only 12% as probably BRCA1-related. Although we have no golden standard in this group, this distribution makes sense and shows that at least many of these patients can be classified with high probability using the decision tree composed, as mentioned earlier, of age, Ki67 and EGFR. Tumors of patients at high risk of hereditary disease (with family members having a proven BRCA1 mutation, but

without having undergone mutation analysis themselves) were classified in 16% as probably sporadic and 63% as probably BRCA1-related. This distribution seems realistic, assuming that the majority of breast cancers in this group (especially those arising at a young age) will be due to BRCA1, since these women are part of a family where a BRCA1 mutation has been established.

In patients with a BRCA2 mutation in the family the classification as either probably BRCA1-related or probably sporadic could not be performed. All cases with BRCA2 mutations fell into the intermediate categories, underlining their intermediate nature with regard to age at presentation and biological characteristics of the tumor.

As the differences between BRCA1-related- and sporadic cancers with regard to some of the clinicopathologic variables could be solely related to age, we also designed a decision tree excluding age. Allowing only primary tumor-related features, a decision tree emerged with Ki67, EGFR and the percentage of progesterone receptor-positive cells. With this decision tree, a group at 0% chance of hereditary disease could be identified. However, the chance of BRCA1-related disease in the probably BRCA1-related class was much lower , 33%, following the exclusion of age as a decision factor. We concluded that most invasive breast carcinomas can be classified as sporadic or BRCA1-related with a high degree of certainty by using a decision tree based on age, Ki67 and EGFR. This could be clinically useful to decide on additional mutation analysis in families at a borderline risk of hereditary disease. The mechanism of the high EGFR expression is yet unclear and deserves to be further studied.

Further improvement in classification is expected from new immunohistochemical markers like CK5/6, CK14, caveolin-1, laminin, vimentin, p-cadherin, FGF1, FGFR2, cyclin D1, BAX and BCL2. In addition, molecular techniques may help. Gene expression analysis is promising but expensive and requires fresh or frozen material, so is rather impractical. Also comparative genomic hybridization (CGH) is complicated, but it is to be expected that MLPA kits, that are easier to use and cheaper, will be developed containing probes directed to the chromosomal loci

with gains and losses found with CGH. Until then, especially immunohistochemistry using paraffin blocks will remain a practical approach for many laboratories.
The immunoprofile of ductal carcinoma *in situ* (DCIS) in BRCA1 and BRCA2 mutation carriers is described in **Chapter 5**. DCIS is an established late precursor of sporadic invasive breast cancer and to a large extent parallels its invasive counterpart with respect to molecular changes and immunophenotype. Invasive breast cancers in germline BRCA1 mutation carriers have a distinct ("basal") immunophenotype characterized by high expression of stem cell cytokeratins (CK) 5/6 and 14 and epidermal growth factor receptor (EGFR) and low expression of the estrogen (ER), progesterone (PR) and HER-2/*neu* receptors. The immunophenotype of BRCA2 related breast cancers resembles more the immunophenotype of sporadic cancers ("luminal") with a frequent expression of ER, PR and only "luminal" cytokeratins such as CK8/18. Here we report for the first time that the immunoprofile of this premalignant lesion is similar to the accompanying invasive cancer lesion. DCIS lesions in BRCA1 and BRCA2 mutations carriers are usually of the Basal and Luminal A molecular type, respectively, suggesting that DCIS is a direct precursor lesion in these hereditary predisposed patients. In addition, chances are high that crucial carcinogenetic events leading to these phenotypes occur at an early stage in hereditary predisposed patients, most likely even before the stage of DCIS. Further studies will focus on the immunoprofile of putative earlier lesions in the hereditary carcinogenetic route.

Novel biomarkers in hereditary breast cancer

Fanconi anemia (FA) is a recessive disorder associated with progressive pancytopenia, multiple developmental defects and marked predisposition to malignancies. FANCD2 is one of twelve (so far described) genes that is responsible for this disease when mutated. Together with BRCA1 and BRCA2 (also known as FANCD1) these proteins are functionally closely linked in the DNA repair response.

Targeted deletion of FANCD2 in mice results in an increased rate of breast tumors in these mice. FANCD2 is expressed in proliferating cells in the duct epithelium of the normal breast, as has previously been shown by our group. These features of FANCD2 led us to investigate the expression of FANCD2 in sporadic and hereditary breast cancer to evaluate its possible role in various forms of breast carcinogenesis.

In **Chapter 6** we describe the expression of FANCD2 in sporadic and hereditary breast cancer. In most of the sporadic and hereditary breast cancer cases a high FANCD2 expression was observed. In the sporadic breast cancers, high FANCD2 expression was correlated with poor prognosis. This was expected, as FANCD2 is normally expressed in proliferating cells, and high proliferation was many times before shown to be prognostically unfavorable. Interestingly, FANCD2 expression was completely absent in 10-20% of sporadic and BRCA1 related breast cancers, even in proliferating cells, indicating that somatic inactivating (epi)genetic events in FANCD2 may occur in both sporadic and hereditary breast carcinogenesis. This is potentially important in view of the crucial role of FANCD2 in DNA repair. Our results do however not indicate that somatic (epi)genetic changes in FANCD2 are a frequent secondary carcinogenetic event in BRCA1 germline mutated patients. These data warrant further investigation in a larger study group.

In **Chapter 7,** we evaluated the expression of Hypoxia inducible factor-1α (HIF-1α) in hereditary breast cancer. Hypoxia is a hallmark of cancer and HIF-1 is the key regulator of the hypoxia response. HIF-1α was previously by us shown to be overexpressed during sporadic breast carcinogenesis and to be correlated with poor prognosis. Little was however known on the role of HIF-1α in hereditary breast carcinogenesis. A recent study suggested that BRCA1 plays a role in the hypoxic response by regulating HIF-1α stability and by modulating expression of vascular endothelial growth factor, a major downstream target of HIF-1α. In the present study, 90% of BRCA1 germline mutation related breast cancers showed overexpression of HIF-1α, a percentage significantly higher than in sporadic controls. Necrosis, likely caused by the well known rapid tumor cell proliferation of these hereditary cancers while the vasculature is lagging behind, was clearly more

present than in sporadic cancers. Likewise, a perinecrotic pattern of overexpression of HIF-1α was more frequent in BRCA1-related than in sporadic breast cancers. The perinecrotic type of HIF-1α expression was accompanied by overexpression of the HIF-1α downstream genes Glut-1 and CAIX, pointing towards functional HIF-1α. This type of HIF-1α overexpression is thought to be caused by (severe) hypoxia. The present results are in contrast with a recent *in vitro* study where increased levels of BRCA1 were seen to increase the response of the VEGF promoter to hypoxia in a HIF-1α dependent fashion. In that study, reduced levels of BRCA1 protein reduced the ability of hypoxia to induce VEGF. We, however, observed marked upregulation of HIF-1α in human BRCA1 related breast cancers where functional BRCA1 levels are down due to the mutation. In view of the frequent presence of necrosis and perinecrotic HIF-1α expression, we hypothesize that in breast cancers in BRCA1 germline mutation carriers, hypoxia overrides the potential negative effect of BRCA1 expression loss on HIF-1α expression, thereby leading to frequent perinecrotic HIF-1α expression and subsequent activation of HIF-1 downstream genes. In line with this, p300 expression levels, a prerequisite for HIF-1 downstream activation, were high in hereditary cancers. Whilst we previously reported frequent overexpression of EGFR in hereditary breast cancers, (Chapters 3 and 4) we now also find concomitant expression of HIF-1α and EGFR in seventy percent of BRCA1 related breast cancers. Furthermore, all of these cases had evidence of HIF1 downstream activation, suggesting that EGFR enhances the hypoxic response. The association between HIF-1α and EGFR might be explained by the EGFR induced activation of the PI3K/PTEN/AKT/FRAP pathway, through which HER-2/*neu* also acts on HIF-1α. Further studies will have to elucidate the role of EGFR in the carcinogenesis of BRCA1 related breast cancer. Recent *in vitro* studies on breast basal-like cell lines showed that these cell lines are more sensitive for EGFR inhibitors and for carboplatin with a synergistic effect when these are combined. This might lead to new therapy strategies for BRCA1 related breast cancer patients. In contrast to EGFR, an association between HIF-1α and HER-2/*neu* as observed in previous studies could not be confirmed in this study; 96% of HIF-1α positive cases

were HER-2/*neu* negative.

We conclude that overexpression of HIF-1α in BRCA1 related breast cancers is a frequent event, suggesting that the HIF-1α pathway plays a role in the BRCA-1 carcinogenesis and progression. The overexpression of HIF1-α is likely hypoxia related, and is correlated with EGFR expression. This opens up news ways for imaging and development of novel strategies for prevention and therapy of hereditary breast cancer.

Major conclusions of this thesis

1 While much progress has been made in unravelling the immunophenotype of breast cancers in BRCA1 germline mutation carriers, much less is yet known on BRCA2 related cancers, few data are available on CHEK2 related cancers, and hardly anything is known on cancers due to the other high or moderate penetrance breast cancer susceptibility genes. This urges for international consortia to arrive at large enough tumour collections in these rarer syndromes.

2 Most invasive breast carcinomas can be classified as sporadic or BRCA1 related with a high degree of certainty using a decision tree based on age, Ki67 and EGFR. This could be clinically useful to decide on additional mutation testing in families at borderline risk of hereditary disease.

3 High expression of EGFR is observed in breast cancers related to BRCA1 and BRCA2 mutations, opening up a possible new strategy for therapeutic interventions.

4 DCIS is a direct precursor lesion in hereditary predisposed breast cancer patients, with a similar phenotype (basal in BRCA1 carriers and luminal in BRCA2 carriers) as accompanying invasive cancers. This suggests that crucial carcinogenetic events leading to these phenotypes occur at an early stage in hereditary predisposed patients, possibly even before the stage of DCIS.

5 FANCD2 expression is absent in 10-20% of sporadic and BRCA1 related breast cancers, indicating that somatic inactivating (epi)genetic events in FANCD2 may be important in both sporadic and hereditary breast carcinogenesis.

6 Most BRCA1 related breast cancers show functional overexpression of HIF-1α, significantly higher than in sporadic controls. The overexpression of HIF-1α in hereditary breast cancer seems to be caused by hypoxia rather than by activation of oncogenes or inactivation of tumor suppressor genes. The concomitant expression of EGFR and HIF-1α may open up news ways of imaging, prevention and treatment of BRCA1 related breast cancer by targeting EGFR and HIF-1α.

Addendum

9

Nederlandse samenvatting
Curriculum vitae
List of publications
Dankwoord

Jaarlijks krijgen bijna 12.000 vrouwen de diagnose borstkanker te horen. Daarmee is borstkanker in Nederland de meest voorkomende soort kanker bij vrouwen. Ongeveer 60 mannen krijgen jaarlijks de diagnose borstkanker. Van alle borstkankers in Nederland is ongeveer 5 procent erfelijk bepaald. De erfelijke informatie van de mens is te vinden in de genen (dragers van een erfelijke eigenschap). Genen zijn opgebouwd uit DNA en liggen verspreid over de 22 paar chromosomen die in iedere menselijke cel aanwezig zijn. Men schat dat ieder mens ongeveer 25.000 genen heeft. Een gen is een stuk DNA dat de code voor een bepaald eiwit bevat. De verschillende eiwitten bepalen uiteindelijk de functie van de cel. Een gen kan door een verandering (mutatie) in de volgorde van het DNA uiteindelijk minder goed of anders gaan werken. In sommige families komen mutaties in genen voor die tot een verhoogde kans op kanker leiden, zoals mutaties in de BRCA1 en BRCA2 genen (BRCA is afkomstig van het Engelse woord BReast CAncer (borstkanker)), die niet alleen een verhoogd risico op borstkanker veroorzaken maar ook tot een toename in het optreden van eierstok- en eileiderkanker leiden. Als vrouwen een mutatie in het BRCA1 of BRCA2 gen hebben, dan hebben zij 55-85% kans om borstkanker te ontwikkelen en 15-65% kans om eierstokkanker te ontwikkelen. Mannen die drager zijn van een mutatie in het BRCA1 of BRCA2 gen hebben een verhoogde kans op o.a. borstkanker en prostaatkanker, maar de kans dat een man borstkanker ontwikkelt is kleiner dan 5%. Inmiddels kennen we veel borstkanker families, waarbij in één van de borstkankergenen een mutatie is aangetoond. Op grond hiervan weet men dat de aandoening overerft waarbij zowel mannen als vrouwen het gen kunnen erven en doorgeven. Ieder kind van een mutatie-dragende ouder heeft een kans heeft van 50% om zelf deze mutatie te erven, met dus als gevolg een verhoogde kans op de beschreven vormen van kanker.

In dit proefschrift werd het immunohistochemisch profiel van deze erfelijke borstkankers en de voorstadia daarvan bepaald en gerelateerd aan klinisch-pathologische kenmerken. De resultaten van dit onderzoek werden vergeleken met bevindingen gedaan in “sporadische” (niet erfelijke) borstkanker. Het vastleggen van een kenmerkend immunohistochemisch profiel van erfelijke borstkanker

en haar voorstadia zou een aanvullende, eenvoudige methode kunnen zijn om erfelijke en sporadische borstkankers nauwkeuriger van elkaar te onderscheiden. Dit biedt de mogelijkheid om daar waar bijvoorbeeld familieonderzoek niet bijdragend kan zijn, patiënten en families te identificeren die in aanmerking zouden moeten komen voor mutatieonderzoek. Tevens kan dit toepasbaar zijn op borstkankermateriaal van patiënten met een mutatie in de BRCA1 of BRCA2 genen waarvan niet vaststaat of ze tot kanker leiden. Bovendien kunnen deze gegevens aanwijzingen opleveren over de ontstaanswijze van erfelijke borstkanker en voor nieuwe aangrijpingspunten zorgen voor de behandeling en/of preventie van erfelijke borstkanker.

Immunoprofiel van erfelijke borstkanker

Hoofdstuk 2 geeft een overzicht van de literatuur betreffende de pathologie van erfelijke borstkanker. Erfelijke borstkanker komt in families voor waarbij meerdere familieleden in verschillende generaties, vaak op jonge leeftijd, getroffen zijn. De meeste van deze borstkankers zijn een gevolg van mutaties in de BRCA1 en BRCA2 genen; deze mutaties komen bij ongeveer 5 procent van alle borstkanker patiënten voor. In families waar een duidelijke geschiedenis van borstkanker aanwezig is, zijn mutaties in de BRCA genen verantwoordelijk voor 20-25% van de borstkankers. Andere genen zoals CHEK2, PTEN, TP53, ATM, STK1/LKB1, CDH1, NSB1, RAD50, BRIP1 en PALB2 zijn genen die, indien gemuteerd, allen deel uitmaken van het erfelijke borstkanker spectrum. Echter, een flink deel van de familiaire erfelijke borstkanker kan niet herleid worden tot mutaties in bovengenoemde genen. De genen die ten grondslag liggen aan dit familiair voorkomen zijn nog niet bekend. In dit overzichtsartikel wordt, na het beschrijven van de functies van de verschillende genen, uitgebreid ingegaan op de histologie en de expressie van verschillende eiwitten, en de gen-expressie patronen van borstkankers die ontstaan als een gevolg van een mutatie in de BRCA1 en BRCA2 genen of door de andere genen.

Het artikel wordt afgesloten met een kort overzicht van het tot stand komen van de diagnose, de behandeling en de prognose van erfelijk borstkanker. Concluderend, alhoewel veel bekend is over de pathologie van BRCA1 gerelateerde borstkanker, meer informatie over de pathogeniciteit van de ongeclassificeerde varianten van BRCA1 en BRCA2 is nodig. Daarnaast is meer inzicht nodig in het fenotype van BRCA2 gerelateerd borstkanker en de andere erfelijke vormen van borstkanker als ook een nadere bepaling van het immunofenotype van de voorstadia van erfelijke borstkanker. Internationale samenwerking is nodig om grotere groepen van deze laatstgenoemde patiënten met erfelijk borstkanker beschikbaar te hebben om de gegevens betreffende het immunofenotype van deze borstkankers zo volledig mogelijk in kaart te brengen.

Verschillende eerdere studies hebben aangetoond dat de genetische opmaak van de BRCA1 en BRCA2 gerelateerde borstkanker verschillend is in vergelijking met die van sporadische borstkanker. Deze verschillen omvatten het Verlies ("losses") en toename ("gains") specifieke stukken van chromosomen met een grote genetische diversiteit tot gevolg. Het morfologische en immunohistochemische fenotype van BRCA1 gerelateerde borstkanker is verschillend van dat van BRCA2 gerelateerde borstkanker. Het BRCA1 gerelateerde borstkanker kenmerkt zich histologisch als een goed begrensde tumor, meestal van het medullaire of slecht gedifferentieerde ductale type, met uitgebreide lymfocytaire infiltraten. Daarnaast suggereert de hoge delingsgraad en de hoge expressie van proliferatie markers een meer agressief verloop. Tevens is er een lage expressie van de oestrogeen-, progesteron- en HER-2/*neu* receptoren, weinig expressie van p27, maar juist wel accumulatie van p53 en overexpressie van Cycline E, Cytokeratine 5/6 en 14. Het fenotype van BRCA2 gerelateerde borstkanker is minder duidelijk omschreven. Een gering aantal studies hebben geprobeerd om de verschillende klinisch-pathologische kenmerken van borstkanker te gebruiken om de sporadische van de erfelijke borstkankers te onderscheiden. Deze toegevoegde informatie zou nuttig kunnen zijn voor klinisch genetici die daarmee hoog risico families beter kunnen helpen. Het doel van onze volgende studie was daarom om met behulp

van een multivariate methode en gebruik makend van een groot aantal klinisch-pathologische markers borstkanker te kunnen classificeren als erfelijk dan wel sporadisch. Dit is beschreven in **hoofdstuk 3**. Hiervoor hebben we borstkanker weefsel van verschillende groepen vrouwen vergeleken. Dit betrof vrouwen met een bewezen BRCA1 of BRCA2 mutatie, vrouwen met een gemiddeld risico op het krijgen van erfelijke borstkanker (op basis van familie geschiedenis), vrouwen met een hoog risico op het krijgen van erfelijke borstkanker (op basis van familie geschiedenis waar in meerdere familieleden een mutatie in het BRCA1 of BRCA2 gen is aangetoond maar waarbij de patiënt zelf geen mutatie analyse heeft ondergaan) en, als laatste groep, vrouwen met sporadische borstkanker. Bij de analyse van alle klinisch-pathologische markers werden vergelijkbare expressie niveaus gezien als eerder beschreven. Echter, de overexpressie van de epidermale groei factor receptor (EGFR) in BRCA1 en BRCA2 gerelateerde borstkanker wordt hier door ons als eerste beschreven. Dit wordt verder uitgewerkt in **hoofdstuk 4**. Verder wordt in deze studie voor het eerst aangetoond dat Cycline A, een andere bruikbare proliferatie marker, verhoogd tot expressie komt in BRCA1 gerelateerde borstkanker. Ondanks dat het onderzoek in het weefsel van slechts vijf patiënten met BRCA2 gerelateerde borstkanker kon worden uitgevoerd, lijkt het erop dat het profiel van deze erfelijke vorm van borstkanker zowel aspecten van sporadische als van BRCA1 gerelateerde borstkanker bij zich draagt. In BRCA2 gerelateerde borstkanker was verder expressie van Cycline D1 opmerkelijk hoog.

De klinisch-pathologische markers leeftijd (waarop de borstkanker ontstond), Ki67 en EGFR expressie zijn de markers die gebruikt worden in de beslisboom. Deze markers zorgen het beste ervoor dat de borstkankers in twee groepen te classificeren zijn, namelijk mogelijk BRCA1 gerelateerde en waarschijnlijk sporadische borstkanker. In de leeftijdsgroep ≥ 54 jaar was de kans dat er sprake is van een mogelijk BRCA1 gerelateerde borstkanker 0% wanneer er geen EGFR overexpressie kan worden aangetoond en 3% als EGFR overexpressie aanwezig was. In de leeftijdsgroep ≤ 54 jaar was de kans op BRCA1 gerelateerde borstkanker 9% als de Ki67 expressie laag is en 75% als de Ki67 expressie hoog is. Duidelijk is

dat de 75% kans op het krijgen van een BRCA1 gerelateerde ziekte in deze mogelijk BRCA1 gerelateerde groep relatief is omdat de kans beïnvloed wordt door de ratio tussen BRCA1 gerelateerde en de sporadische controle groep in deze studie. De meeste sporadische borstkankers werden correct geclassificeerd als mogelijk sporadisch en slechts een klein aantal als mogelijk BRCA1 gerelateerd (64% versus 2%). Dat deze "sporadische" borstkankers mogelijk toch BRCA1 gerelateerd zijn kan niet worden uitgesloten aangezien deze groep niet geselecteerd is op familie geschiedenis (door het niet aanwezig zijn van deze informatie) en dus bijna per definitie een paar procent patiënten bevat die erfelijk belast zijn. Belangrijk is dat geen van de BRCA1 gerelateerde borstkankers werd geclassificeerd als mogelijk sporadische van origine en 82% als mogelijk BRCA1 gerelateerd. De resterende 18% kon niet geclassificeerd worden. Dat kan mogelijk verklaard worden door het feit dat bij vrouwen met een BRCA1 mutatie, vooral op oudere leeftijd, de borstkanker toch niet het gevolg is van de BRCA1 mutatie maar het gevolg is van het "spontaan" ontstaan van borstkanker volgens de niet-erfelijke carcinogenetische routes. Wanneer we de beslisboom analyse gebruiken voor het classificeren van de groep met een gemiddeld risico op erfelijke borstkanker dan wordt 41% geclassificeerd als waarschijnlijk sporadisch en 12% als mogelijk BRCA1 gerelateerd. Ondanks dat er voor deze studiegroep geen gouden standaard is te maken, lijkt deze verdeling aannemelijk, en konden de patiënten in deze intermediaire risoco groep toch met een grote mate van waarschijnlijkheid geclassificeerd worden. Op basis van weefsel onderzoek van borsttumoren van patiënten met een hoog risico op het krijgen van erfelijke borstkanker werd 16% van de tumoren geclassificeerd als mogelijk sporadisch en 63% als mogelijk BRCA1 gerelateerd. Deze verdeling lijkt realistisch aannemende dat de meeste borstkankers in deze groep (gezien de jonge leeftijd) een gevolg zullen zijn van de BRCA1 mutatie.

In patiënten met een BRCA2 mutatie in de familie is de classificatie voor een mogelijke BRCA1 dan wel sporadische borstkanker niet uitgevoerd omdat het aantal patiënten hiervoor te laag was. Aangezien de verschillen tussen BRCA1 en sporadische borstkanker, als we kijken naar alle klinisch-pathologische kenmerken,

terug te voeren zijn op de leeftijd waarop de kanker ontstaan is, is er ook een beslisboom zonder leeftijd als factor gemaakt. Dit leverde een beslisboom op basis van de expressie van Ki67, EGFR en de progesteron receptor op. Met deze beslisboom werd er een groep borstkanker geclassificeerd die een kans heeft van 0% op BRCA1 gerelateerde kanker. Echter, het classificeren van BRCA1 gerelateerde borstkankers als mogelijk BRCA1 gerelateerde kanker was met behulp van deze beslisboom minder sterk, namelijk 33%. Het niet verdisconteren van de sterke factor leeftijd zal hier de oorzaak van zijn.

Concluderend, het grootste deel van de invasieve borsttumoren kan met grote mate van zekerheid geclassificeerd worden als mogelijk BRCA1 kiembaan mutatie gerelateerd dan wel sporadisch op basis van de factoren leeftijd, Ki67 en EGFR. Dit biedt de mogelijkheid van een eenvoudige methode om tot een beslissing over te gaan op mutatie analyse in families met een borderline risico op het hebben van erfelijke ziekte. Het mechanisme achter de hoge expressie van EGFR is niet duidelijk en dit zou verder uitgezocht moeten worden.

De classificatie kan mogelijk verbeterd worden als er gebruikt wordt gemaakt van nieuwe immunohistochemische markers zoals CK5/6, CK14, caveolin-1, laminine, vimentine, p-cadherin, FGF1, FGFR2, cycline D1, BAX, p63 en BCL2. Tevens kunnen moleculaire technieken hierbij aanvullend zijn zoals gen-expressie analyses. Dit laatste is vooralsnog een erg kostbare techniek en vereist de aanwezigheid van vers materiaal hetgeen meestal niet (in voldoende mate) beschikbaar is. Ook het uitvoeren van comparative genomic hybridization (CGH) is gecompliceerd, maar het is in de lijn der verwachting dat MLPA kits makkelijker en goedkoper worden ontwikkeld, zodat met behulp van probes direct gericht tegen chromosoom delen met verlies ("losses") of toename ("gains") van DNA hetzelfde resultaat kan worden behaald als met CGH. Tot die tijd zal vooral immunohistochemische analyse van coupes van paraffine blokken de meest praktische aanpak zijn, toepasbaar in vrijwel alle pathologische laboratoria.

Het immunohistochemisch profiel van een voorstadium van invasief carcinoom, ductaal carcinoma *in situ* (DCIS), in BRCA1 en BRCA2 mutatie dragers is beschreven

in **hoofdstuk 5**. DCIS is een laat voorstadium van invasieve borstkanker en heeft, in niet-erfelijk belaste vrouwen, veelal hetzelfde moleculaire patroon en immunofenotype als in het invasieve stadium. Invasieve borstkankers in BRCA1 mutatie dragers hebben een specifiek (basaal) immunofenotype, gekarakteriseerd door een hoge expressie van stamcel cytokeratinen (CK) 5/6 en 14, en epidermale groei factor receptor (EGFR), en een lage expressie van oestrogeen-, progesteron- en HER-2*/neu* receptoren. Het immunofenotype van BRCA2 gerelateerde borstkanker lijkt meer op die van sporadische borstkanker (luminaal) met een freqente expressie van de oestrogeen- en progesteron- receptoren en de luminale cytokeratinen CK 8/18. We beschrijven voor het eerst dat het immuno-histochemisch profiel van deze premaligne laesies gelijk is aan dat van de aangrenzende invasieve tumoren. DCIS laesies in BRCA1 en BRCA2 muatie dragers zijn meestal van respectievelijk het basale en luminale A moleculaire type, hetgeen suggereert dat in erfelijke belaste patiënten DCIS een direct voorstadium van de invasieve kanker is. Kennelijk vinden in deze erfelijk belaste patiënten cruciale carcinogenetische veranderingen al plaats het stadium voor invasie, en misschien zelfs wel eerder. Meer studies zouden zich moeten richten op het immunohistochemisch profiel van zeer vroege afwijkingen in de erfelijke carcinogenese route.

Nieuwe markers in erfelijke borstkanker

Fanconie anemie (FA) is een reccesieve afwijking geassocieerd met progressieve pancytopenie, multiple ontwikkelings-defecten en een verhoogd risico op het krijgen van allerlei ziekten. FANCD2 is een van de twaalf tot nu beschreven genen die, indien gemuteerd, verantwoordelijk is voor de ziekte. Samen met BRCA1 en BRCA2 (ook bekend als FANCD1) zijn Fanconi eiwitten functioneel met elkaar verbonden als een soort van DNA herstel apparaat. Gerichte deletie van FANCD2 in muizen resulteert in een verhoogde frequentie van borstkanker. FANCD2 komt tot expressie in prolifererende cellen in het epitheel van de normale borst, wat

al eerder door ons is beschreven. Deze kenmerken van FANCD2 deed ons er toe besluiten om de expressie van FANCD2 in sporadische en erfelijke borstkanker te bepalen om na te gaan wat de mogelijke rol van FANCD2 is in de carcinogenese. In **hoofdstuk 6** is de expressie van FANCD2 in sporadische en erfelijke borstkanker beschreven. De meeste sporadische en erfelijke borstkankers tonen een (variabele) expressie van FANCD2. In de sporadische tumoren was hoge FANCD2 expressie gecorreleerd met een slechte prognose. Dit is te verwachten, aangezien FANCD2 normaal aanwezig is in prolifererende cellen en hoge proliferatie al meerdere keren aangetoond is als een marker voor een slechte prognose. Een interessant punt is dat FANCD2 expressie totaal afwezig was in 10-20% van de sporadische en de BRCA1 gerelateerde borstkankers, zelfs in prolifererende cellen. Dit geeft aan dat somatische inactiverende (epi) genetisch veranderingen in FANCD2 kunnen voorkomen in zowel de sporadische als de erfelijke borst carcinogenese. Dit is potentieel belangrijk gezien de rol van FANCD2 in het herstel van DNA fouten. Onze resultaten geven echter niet aan dat somatische (epi)genetische veranderingen in FANCD2 een frequente tweede carcinogenetische verandering zijn bij patiënten met een BRCA1 kiembaan mutatie. Deze data verdienen nader onderzoek in een grotere groep patiënten.

In **hoofdstuk 7** hebben we naar de expressie van Hypoxie Induceerbare Factor-1α (HIF-1α) in erfelijke borst kanker gekeken. Hypoxie, oftewel zuurstoftekort, is een van de bekendste kenmerken van kanker en HIF-1 is de belangrijkste regulator van de hypoxische reactie. HIF-1 wordt samengesteld uit twee eiwitten namelijk HIF-1α en HIF-1β. HIF-1β is altijd aanwezig, het HIF-1α eiwit echter wordt onder normoxie (= normale zuurstof omstandigheden) continu afgebroken en onder hypoxie (= zuurstof te kort) gestabiliseerd waardoor het tot overexpressie komt en aan HIF-1β kan binden. HIF-1α overexpressie in sporadische invasieve borstkanker carcinogenese was door ons al eerder beschreven en correleert met een slechte prognose. Er is echter weinig bekend over de expressie van HIF-1α in de erfelijke borstkanker carcinogenese. In deze studie werd bij 90% van de BRCA1 kiembaan mutatie gerelateerde borsttumoren een overexpressie van HIF-1α gezien, een

percentage dat duidelijk hoger ligt dan voor de sporadische borstkanker controle groep. Weefselnecrose, ontstaan als gevolg van de verhoogde proliferatie en het daarbij achterblijven van de vaatstructuren, is veelvuldig aanwezig in erfelijke borstkankerweefsel en duidelijk meer dan in sporadische borstkanker. Een perinecrotische aankleuring van HIF-1α was eveneens meer aanwezig in BRCA1 gerelateerde tumoren dan in de sporadische controles. De perinecrotische aankleuring van HIF-1α valt samen met de overexpressie van de eiwitten van de genen die door HIF-1 worden geactiveerd, Glut-1 en CAIX, hetgeen wijst op functioneel HIF-1α. Men denkt dat dit type van overexpressie van HIF-1α te danken is aan ernstige hypoxie.

Deze resultaten komen niet overeen met de resultaten van een recente *in vitro* studie waar gesuggereerd wordt dat BRCA1 een rol speelt in de hypoxie reactie door het reguleren van de stabiliteit van HIF-1α en het beïnvloeden van de expressie van vasculair endotheliale groei factor (VEGF), een gen dat door HIF-1 aangezet wordt. In deze studie tonen zij aan dat lage hoeveelheden van BRCA1 ervoor zorgen dat de mogelijkheid om VEGF te activeren door hypoxie verlaagd wordt. Wij laten zien dat de HIF-1α expressie juist hoog is waar de functionele BRCA1 expressie laag is als gevolg van de mutatie. Tevens is de HIF-1α expressie perinecrotisch (in de buurt van necrose) waar de mate van hypoxie het hoogst is. Wij denken dat de mate van hypoxie een grote rol speelt in dit proces en dat hier het mogelijk negatieve effect van BRCA1, wat door de andere groep beschreven wordt, teniet gedaan wordt door de hypoxie. Tevens is de p300 expressie, een eiwit dat betrokken is bij de HIF-1 activerende functie, ook hoog in erfelijke borstkanker. Eerder werd genoemd dat wij vaak overexpressie zien van EGFR in erfelijk borstkanker (hoofdstukken 3 en 4), nu laten we in deze studie zien dat er zowel EGFR als HIF-1α expressie te zien is in 70 procent van de BRCA1 gerelateerde borstkankers. Tevens is in deze gevallen aangetoond dat er een HIF-1 activatie heeft plaatsgevonden wat suggereert dat EGFR de hypoxie reactie verhoogt. De samenhang tussen EGFR en HIF-1α kan uitgelegd worden doordat EGFR de PI3K/PTEN/AKT/FRAP route activeert; HER-2/*neu* activeert ook via deze route HIF-1α. Toekomstige studies moeten uitwijzen wat

de rol van EGFR is in de erfelijke borstkanker carcinogenese. Recente *in vitro* studies met basale borstkanker cellijnen laten zien dat deze cellijnen gevoelig zijn voor EGFR remmers en voor carboplatin; samen hebben ze extra effect. Dit zou kunnen leiden tot nieuwe therapieën voor BRCA1 gerelateerde borstkanker patiënten. In tegenstelling tot EGFR is er geen associatie gevonden tussen HIF-1α en HER-2/*neu*, terwijl dit eerder beschreven was, mogelijk doordat 96% van de HIF-1α positieve gevallen HER-2/*neu* negatief waren zoals gebruikelijk in BRCA1 gerelateerde mamma- carcinomen. Concluderend, de overexpressie van HIF-1α is een veel voorkomend fenomeen in BRCA1 gerelateerde borstkanker, wat suggereert dat HIF-1α een rol speelt in de BRCA1 carcinogenese en progressie. De overexpressie van HIF-1α is waarschijnlijk hypoxie gereguleerd en is gecorreleerd met EGFR expressie. Dit levert nieuwe mogelijkheden op voor het opsporen van deze kankers met beeldvormende technieken, het ontwikkelen van nieuwe strategieën om de kanker te voorkomen en nieuwe specifieke therapieën te ontwikkelen voor erfelijke borstkanker.

De belangrijkste conclusies van dit proefschrift

1 Ondanks het feit dat er veel vooruitgang geboekt is met het ontrafelen van het immunofenotype van borstkanker in BRCA1 kiembaan mutatie dragers, is er niet veel bekend over BRCA2 gerelateerde kanker, nog minder over CHEK2 gerelateerde kanker en eigenlijk weten we nauwelijks iets over het immunofenotype van kankers die ontstaan door andere genen die betrokken zijn bij erfelijke borstkanker. Dit zou internationale consortia er toe moeten brengen om grotere groepen van deze niet zo goed beschreven borstkankervormen beter te bestuderen.

2 De meeste invasieve borsttumoren kunnen met een hoge mate van zekerheid geclassificeerd worden als sporadisch dan wel BRCA1 gerelateerd door gebruik te maken van een beslisboom gebaseerd op leeftijd, Ki67 en EGFR. Dit is in de

kliniek eenvoudig te gebruiken om te bepalen of additionele mutatie analyses moeten worden uitgevoerd in families met een kans op erfelijke aanleg voor borstkanker.

3 Hoge expressie van EGFR wordt vaak gezien in BRCA1 en in BRCA2 gerelateerde borstkankers hetgeen een opening creëert voor de toepassing van middelen gericht tegen EGFR in deze groep van patiënten.

4 DCIS is een directe voorloper laesie in erfelijke belaste borstkanker patiënten, en deze laesies hebben veelal hetzelfde fenotype (basaal in BRCA1 en luminaal in BRCA2 dragers) als de aangrenzende invasieve tumor. Dit suggereert dat in deze erfelijk belaste patiënten cruciale carcinogenetische gebeurtenissen die leiden tot deze fenotypes al in een vroeg stadium, voor de ontwikkeling van invasie, tot stand komen.

5 FANCD2 expressie is afwezig in ongeveer 10-20% van de sporadische en BRCA1 gerelateerde borstkanker, hetgeen aangeeft dat somatische inactiverende (epi)genetische gebeurtenissen in FANCD2 belangrijk zouden kunnen zijn in zowel sporadische als erfelijke borst carcinogenese.

6 De meeste BRCA1 gerelateerde borstkankers laten een functionele overexpressie van HIF-1α zien die hoger is in vergelijking met sporadische tumoren. De overexpressie van HIF-1α in erfelijke borstkanker lijkt eerder het gevolg te zijn van hypoxie dan de activering van andere oncogenen of inactiveren van tumor suppressor genen.

Curriculum vitae

Petra van der Groep was born on May 21, 1970 in Amsterdam. Following her high-school graduation from the Waterlant College in Amsterdam in 1987, she started to study at the Hogeschool van Amsterdam and graduated in 1992 as a clinical chemistry technician. That same year she started to study Medical Biology at the Free University in Amsterdam. She graduated in 1995 with a specific specialization in pathology and oncology. From 1996 till 1999 she worked as a researcher on the topic of bone marrow tumor genetics at the department of Anthropogenetics of the Free University Medical Center in Amsterdam. In 2000 she took a position as a research technician at the Department of Oncology and Pathology of the same Medical Center being part of the researchgroup working on tumor hypoxia. In 2003, research on hereditary breast cancer became the second project. In 2004 she moved to the University Medical Center Utrecht, where she was appointed as the chief technician of pathology research laboratory. While being responsible for the logistics of the laboratorium and participating in the research of various PhD students, she continued her own research on hereditary breast carcinogenesis. In 2008 she therefore stept down as chief technician to become full-time research assistant in order to finish her own PhD. Following June 23 , 2009, she will continue her research on hereditary breast cancer and other cancers while also giving her support to the research projects of other scientists.

Petra van der Groep werd geboren op 21 mei, 1970 te Amsterdam. Nadat ze haar HAVO diploma behaalde op het Waterlant College in Amstedam in 1987, begon ze aan de opleiding tot klinisch chemisch analist aan de Hogeschool van Amsterdam en ze studeerde af in 1992. In hetzelfde jaar begon ze aan de doctoraal studie medische biologie aan de Vrije Universiteit te Amsterdam. Ze studeerde af in 1995 met als specialisatie pathologie/oncologie. Hierna werkte ze van 1996 tot 1999 als adjunct onderzoeker, beenmerg tumor genetica, bij de sectie chromosomenonderzoek van de afdeling antropogenetica, aan het VU medisch centrum in Amsterdam. In 2000 begon ze als research analist bij de afdelingen oncologie en pathologie van het VU medisch centrum, waar ze werkte aan een project over tumor hypoxie. In 2003 ging ze meer zelfstandig te werken aan een onderzoek betreffende erfelijke borstkanker. Toen in 2004 de onderzoeksgroep naar het Universitair Medisch Centrum in Utrecht verhuisde is ze meegegaan om daar hoofdanalist te worden van het research laboratorium van de afdeling pathologie. In de de laatste jaren werd het "bij" project aangaande erfelijke borstkanker steeds meer een project op zichzelf. Uiteindelijk besloot ze in 2007 op dit onderwerp te gaan promoveren. In 2008 legde ze daarvoor haar functie als hoofdanalist neer om voltijds te gaan werken als onderzoeks assistent. Na het verdedigen van haar proefschrift hoopt ze in deze ondersteunende functie verder te werken aan de verschillende projecten die onder andere betrekking zullen hebben op (erfelijke) borstkanker maar ook andere kankers.

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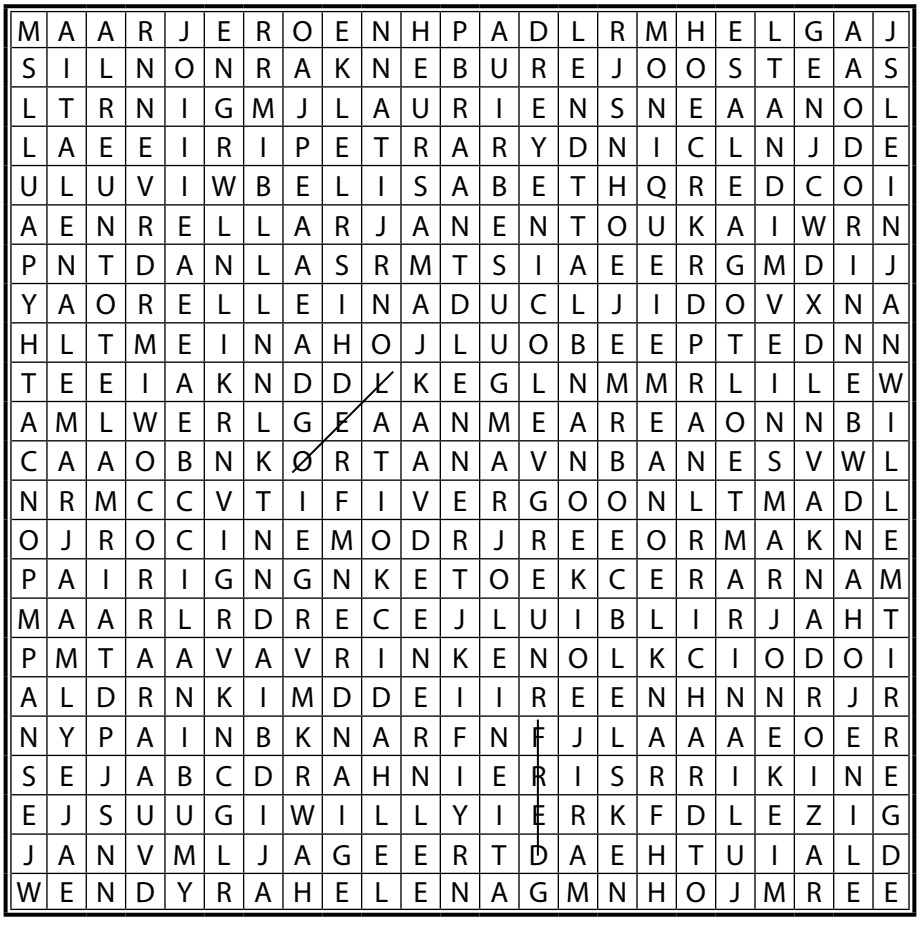
Dankwoord



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Los deze puzzel op en lees de verborgen boodschap….

ELSKEN
PAUL
JOHAN
PATIENTEN
JANVDG
PAULA
MIEKE
~~LEO~~
ROGIER
GUUSJE
ELINE
RUBEN
MARINA
MARJOLEIN
~~FRED~~
ALWIN
RICHARD
GERRIT
CINDY
INGRID
THEA
WIM
JANNEKE
NICOLEVG
MIREILLE
MARIO
REINHARD
ARNO
MARIJE
MARCB
LENNERT
JOHANDW
STEVEN

ANDRE
AAD
FRANKB
WILLY
IRMA
MARJON
PATRICIA
MONIQUE
MARJA
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SABRINA
KEVIN
HELGA
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ROELDW
DORINNE
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DOMENICO
JANB
JOHN
GLADYS
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ROELB
JOS
MARCV
ARJAN
EELKE
KARIJN
FOLKERT
WENDY
ROBERTS

GEERT
IORDANKA
FRANKL
JULIA
CIGDEM
ROBERTVD
JEROEN
ALEX
JANVM
LAURIEN
MARISE
RAZI
LODEWIJK
RENEEDV
PIETER
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ARNOUT
GUUS
NIELS
PAN
NICOLEH
LAURA
TRI
HELEN
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ELISABETH
JOOST
NICOLEN