CHEK2 1100delC and polygenic susceptibility to breast cancer and colorectal cancer

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CHEK2 1100delC and polygenic susceptibility to breast cancer and colorectal cancer

CHEK2 1100delC en polygene predispositie voor borstkanker en darmkanker

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CHAPTER 1

General introduction

1.1 Breast cancer

Breast cancer is the most commonly diagnosed cancer among women, with worldwide approximately 1.2 million new cases every year. In the Netherlands, 12,071 new breast cancers were diagnosed in women and 3,301 women died of the disease in 2005. Currently, about 1 in every 8 to 9 women in the Netherlands will be diagnosed with breast cancer during her lifetime and approximately 85% of breast cancer patients are still alive 5 years after diagnosis (www.ikcnet.nl).

Risk factors

The main risk factors for breast cancer are gender (male vs. female 1:150), older age, a family history of breast cancer and the presence of germline mutations in cancer susceptibility genes. Other breast cancer risk factors include younger age at menarche, nulliparity, older age at first childbirth, older age at menopause, no breast-feeding, use of hormone replacement therapy, use of oral contraceptives, exposure to ionising radiation, a history of benign breast disease and high mammographic tissue density. Lifestyle factors like alcohol use, diet, body mass index and low physical activity have also been associated with an increased breast cancer risk.[1-3]

Prognosis

Treatment options for women diagnosed with breast cancer are influenced by breast cancer prognosis, particularly the risk of disease recurrence. Breast cancer prognosis is strongly correlated to tumor characteristics, with the most important ones being tumor size, axillary lymph node involvement and tumor grade.[4-6] Tumor size and axillary lymph node status are closely related as larger tumors are more frequently lymph node positive. Generally, women with larger breast tumors and/or increasing numbers of positive lymph nodes have a worse breast cancer survival.[7] Tumor size and axillary lymph node involvement are combined in tumor staging according to the TNM system, where T refers to tumor size, N to the presence of local lymph node metastases and M to the presence of distant metastases. Together, these three tumor characteristics define four breast cancer stages that provide powerful tools for breast cancer prognosis as approximately 85% of women with stage I breast tumors will survive the disease compared to approximately 45% of women with stage III breast tumors.[8] Another commonly used prognostic breast cancer factor is tumor grade. Tumor grade is usually determined according to the Bloom and Richardson system which classifies breast tumors as well differentiated (grade I), moderately differentiated (grade II) or poorly differentiated (grade III), based on the degree of glandular differentiation, mitotic index and the degree of pleomorphism. Generally, women with high-grade breast tumors have a worse breast cancer survival.[9, 10]

Treatment

Breast cancer treatment involves surgery of the primary breast tumor, resulting in either partial resection (lumpectomy) or total resection (mastectomy) of the breast. The sentinel node and, when necessary, the axillary nodes are also surgically resected. Lumpectomy is always accompanied by radiation therapy. In contrast, mastectomy is accompanied by radiation therapy, depending on the size of the primary breast tumor and the risk of local recurrences. In addition, adjuvant systemic therapy involving hormonal therapy, chemotherapy, molecular targeted therapy (antibody or small molecule), or a combination of these therapies can be offered. Currently, three factors that predict response to breast cancer therapy are clinically implemented. The most widely accepted predictive factors for response to hormone treatment are estrogen receptor (ER) and progesterone receptor (PR) status. Approximately 50% to 60% of women with ER-positive breast tumors respond to hormone treatment. Combining ER status with PR status further improves the accuracy of predicting response to hormone treatment. Nearly 80% of women with ER- and PR-positive breast tumors will benefit from hormone therapy compared to only about 5% to 10% of women with ER- and PR-negative breast tumors.[11, 12] A third predictive factor is the human epidermal growth factor receptor 2 (also known as ERBB2, HER2 or NEU).[13] ERBB2 overexpression or amplification predicts response to treatment with the therapeutic antibody trastuzumab, both in the adjuvant setting and in metastatic disease.[14-17] Additionally, it had been suggested that ERBB2 overexpression might be associated with altered response to hormonal therapy and chemotherapy.[18-22]

1.2 Familial breast cancer

Familial breast cancer refers to a clustering of breast cancer cases in families that is higher than expected compared to the breast cancer incidence in the general population. Unfortunately, clinical and research studies have employed different, relatively arbitrary criteria to define familial breast cancer, thereby severely complicating comparison of studies on familial breast cancer. Familial breast cancer constitutes approximately 15-25% of all breast cancers. In the late twentieth century, the first high-risk breast cancer susceptibility genes were identified. Over the recent years technology has allowed the identification of moderate,- and low-risk breast cancer susceptibility alleles. In contrast to the very rare (e.g. <0.1%) population frequency of variants in high-risk breast cancer susceptibility genes, variants in moderate,- and low-risk breast cancer susceptibility alleles are present at rare (e.g. ~1%) to common (e.g. up to 40%) frequencies in the general population (Figure 1.1).[23] In the past, genetic variants with population frequencies of 1% or more were classified as polymorphisms. Currently it is considered more appropriate to classify variants that have been associated with disease as mutations, irrespective of their prevalence. Confusingly, however, single nucleotide

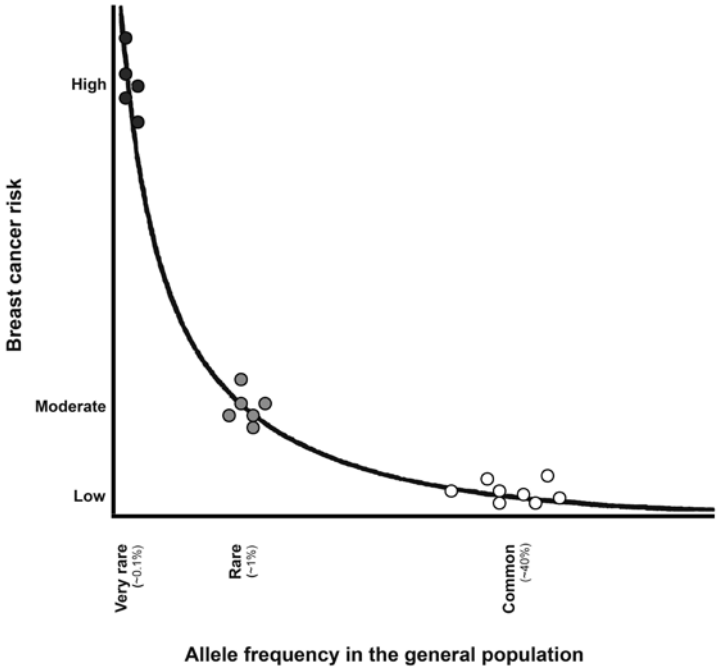

Figure 1.1 Correlation between breast cancer risks conferred by germline mutations in low-, moderate-, and high-risk breast cancer genes and population frequency.
Germline mutations in high-risk breast cancer genes, including *BRCA1*, *BRCA2*, *p53*, *PTEN* and *STK11,* generally are very rare in the population (e.g., <0.1%) but confer high breast cancer risks (e.g., 6- to 8-fold). Germline mutations in moderate-risk breast cancer genes, including *CHEK2*, *ATM*, *RAD50*, *NBS1*, *PALB2* and *BRIP1*, are rare in the population (e.g., ~1%) and confer moderate breast cancer risks (e.g., ~2-fold). Variants in common breast cancer risk alleles, including SNPs near or in the *RAD51*, *FGFR2*, *TNRC9*, *MAP3K1*, *LSP1, MRPS30* genes and the 8q24 and 2q35 geneless regions, are common in the population (e.g., up to 40%) and confer low breast cancer risks (on average 1.3-fold). Black, grey and white circles indicate high-risk, moderate-risk and low-risk genes, respectively.

polymorphisms (SNPs) comprise the majority of variants that have been shown to correlate with disease.

Germline mutations in high-risk, moderate-risk or low-risk breast cancer susceptibility alleles account for about one-third of familial breast cancer families. At present, it is unclear what underlies the breast cancer clustering in the remaining two-thirds of breast cancer families. Chance clustering, and shared environmental and lifestyle factors may account for part of the familial breast cancer cases.[24, 25] However, twin studies have shown that the breast cancer risk for unaffected monozygotic twins with a co-twin diagnosed with breast cancer is higher than the breast cancer risk for unaffected dizygotic twins with a co-twin diagnosed with breast cancer. As monozygotic twins are genetically identical and dizygotic twins share only approximately half of their genes, these studies have suggested that most of the remaining familial breast cancer risk is likely due to genetic factors.[26, 27] It has been postulated that most

of the remaining familial breast cancer clustering is best explained by polygenic breast cancer susceptibility. In a polygenic model, several common low-risk breast cancer susceptibility alleles together confer increased breast cancer risks.[24, 28]

1.2.1 High-risk breast cancer susceptibility genes

Currently, five high-risk breast cancer susceptibility genes, *BRCA1*, *BRCA2*, *p53*, *PTEN* and *STK11*, have been identified. The majority of disease-causing mutations in these high-risk breast cancer susceptibility genes are frameshift mutations or nonsense mutations, leading to the insertion of a premature termination codon in the encoded transcript. The lifetime breast cancer risk in women carrying an oncogenic mutation in the high-risk breast cancer susceptibility genes *BRCA1* or *BRCA2* is about 6- to 8-fold higher compared to the approximately 10% breast cancer risk estimated for the general population. Germline *BRCA1/BRCA2* mutations are very rare in the general population with population carrier frequencies of about 0.1% (Figure 1.1).[23, 29, 30] Yet, germline *BRCA1/BRCA2* mutations are identified in about 20% of breast cancer families.[25, 31] In contrast, germline mutations in the high-risk cancer susceptibility genes *p53*, *PTEN* and *STK11* together are identified in less than 1% of all familial breast cancer cases.[23, 31]

BRCA1 and *BRCA2*

The two major high-risk breast cancer susceptibility genes, *BRCA1* (located at chromosome 17q21) and *BRCA2* (located at chromosome 13q12), were identified through linkage analysis in 1994 and 1995.[32-35] In a linkage analysis, polymorphic markers that are located throughout the genome are genotyped within a family. Subsequent correlation of the generated genotype with the observed segregation pattern of disease in a family may reveal linkage of a chromosomal locus with the disease phenotype. The outcome of a linkage analysis is calculated as a logarithm of odds (LOD) score with a LOD score of 3.0 indicating evidence for linkage (i.e. the odds are a thousand to one in favor of genetic linkage).

Germline *BRCA1/BRCA2* mutations have been identified among familial breast and/or ovarian cancer cases. For example, Verhoog *et al* reported in 2001 that they identified oncogenic germline *BRCA1* and *BRCA2* mutations in 19% and 4% of families with a clustering of breast and/or ovarian cancer cases from the Southwestern Netherlands, respectively.[36] Germline *BRCA1/BRCA2* mutations had been identified among 52% of families with hereditary breast and ovarian cancer (HBOC) and 36% of families with hereditary ovarian cancer (HOC). In contrast, germline *BRCA1/BRCA2* mutations had been detected in only 13% hereditary breast cancer (HBC) families.[36] The strongest predictors for the presence of germline *BRCA1/BRCA2* mutations are a personal and/or family history of ovarian cancer and male breast cancer. Early age at breast cancer diagnosis, occurrence of bilateral breast cancer and presence of multiple breast

cancer cases in a family also increase the likelihood for detection of germline *BRCA1/BRCA2* mutations.[36-38]

Multiple studies have evaluated the cumulative risk of breast and/or ovarian cancer in *BRCA1/BRCA2* mutation carriers. Yet, the estimated cancer risks for *BRCA1/BRCA2* mutation carriers differs considerably among the various studies, ranging from 45% to 90% cumulative breast cancer risks at age 70 years.[39-45] Cancer risk estimates obtained from population-based studies [39, 44, 45] are usually lower than those obtained from studies using families with multiple affected individuals.[43] The higher cancer risk estimates obtained from family-based studies are most likely related to the presence of additional genetic risk factors in these families that may modify *BRCA1/BRCA2* associated cancer risks.[24, 30] Indeed, two recent studies have shown that SNPs near or in the *FGFR2*, *TNRC9* and *RAD51* genes further increase the breast cancer risk for *BRCA2* mutation carriers.[40, 41] In addition, differences in study design and analysis, or differences in study population and mutation detection methods may underlie the variations observed in *BRCA1/BRCA2*-related cancer risk estimates. Recently, a meta-analysis including ten studies that had evaluated breast and ovarian cancer risks for *BRCA1/BRCA2* mutation carriers has been conducted.[46] The aim of this meta-analysis was to integrate the cancer risk estimates of the ten studies into consensus breast and ovarian cancer risk estimates for *BRCA1/BRCA2* mutation carriers. The meta-analysis revealed a mean estimated cumulative breast cancer risk at age 70 years of 57% and 49% for *BRCA1* and *BRCA2* mutation carriers, respectively. The mean estimated cumulative risk for ovarian cancer at age 70 years was 40% for *BRCA1* and 18% for *BRCA2* mutation carriers.

It has also been shown that *BRCA1/BRCA2* mutation carriers have an estimated 50% to 60% cumulative risk to develop a second primary breast cancer at age 70 years. The cumulative risk of developing a primary ovarian cancer at age 70 years after breast cancer diagnosis was strongly dependent on *BRCA1* or *BRCA2* mutation status with a 40% risk for *BRCA1* and a 16% risk for *BRCA2* mutation carriers.[47, 48]

Germline *BRCA1* mutation carriers also have increased risks of pancreas cancer, cervix cancer and uterus cancer. Additionally, borderline evidence suggests that male *BRCA1* mutation carriers younger than 65 years are at increased risk of developing prostate cancer.[49] In contrast, a strong association between prostate cancer risk (irrespective of age) and germline *BRCA2* mutation status has been reported. Apart from prostate cancer, germline *BRCA2* mutation carriers also are at increased risk for pancreas cancer and possibly cancers of the biliary tract, stomach and melanoma.[47, 50]

p53, *PTEN* and *STK11*

Germline mutations in the *p53*, *PTEN* and *STK11* genes have also been associated with increased breast cancer risks. Germline *p53* mutations are identified in 50% to 70% of families with the Li-Fraumeni syndrome (LFS).[51, 52] The most common cancer types associated with this rare famil-

ial cancer syndrome include breast cancer, osteosarcoma, soft tissue sarcoma, leukaemia, brain cancer and adrenal cortical cancer.[53, 54] Germline mutations in *PTEN* are identified in more than half of the families with the rare hamartoma polyposis syndrome Cowden's disease (CD). This syndrome is characterized by multiple hamartomatous lesions, especially of the skin, mucous membranes, breast and thyroid. Additionally, patients with CD are at increased risk of breast cancer, thyroid cancer, endometrium cancer and brain cancer.[55, 56] Germline *STK11* mutations predispose to the rare hamartoma polyposis syndrome syndrome Peutz-Jeghers (PJS) that is characterized by the development of hamartomatous polyps in the gastrointestinal tract and by mucocutaneous-pigmented lesions. Additionally, PJS is associated with an increased risk for breast cancer, pancreas cancer, ovarian cancer and cancers of the gastrointestinal tract.[57, 58] Although *p53*, *PTEN* and *SKT11* germline mutations are associated with highly penetrant cancer syndromes, they are only accounting for less than 1% of familial breast cancer cases and are rarely observed in breast cancer cases outside the context of these rare cancer syndromes.[23, 31]

1.2.2 Moderate-risk breast cancer susceptibility genes

Moderate-risk breast cancer susceptibility genes often have been discovered through candidate gene association studies, involving direct evaluation of putative breast cancer susceptibility genes in large cohorts of familial breast cancer cases and controls. Statistically significant differences in variant frequencies between cases and controls subsequently may indicate if a genetic variant is associated with disease. Thus far, six moderate-risk breast cancer susceptibility genes have been identified, including the *CHEK2*, *ATM*, *RAD50*, *NBS1*, *BRIP1* and *PALB2* genes.[59-64] Disease-causing mutations in these six genes typically result in premature protein truncation and are associated with approximately 2-fold increased breast cancer risks. Mutations in moderate-risk breast cancer susceptibility genes are more common in the general population compared to high-risk breast cancer susceptibility genes. Yet, they are still considered rare with population carrier frequencies up to 1.5% (Figure 1.1). Currently, germline mutations in these six moderate-risk breast cancer susceptibility genes account for approximately 5% of familial breast cancer risk.[23]

CHEK2

In 2002, our research group had participated in an international consortium that has identified the truncating *CHEK2* 1100delC mutation as the first moderate-risk breast cancer allele, through a combined linkage and association study. The mutation had been identified in 5.1% of non-*BRCA1/BRCA2* familial breast cancer cases compared to 1.1% of population controls.[59] Independently of our study, Vatheristo *et al*. had reported similar frequencies of *CHEK2* 1100delC among non-*BRCA1/BRCA2* familial breast cancer cases and controls from the Finnish popula-

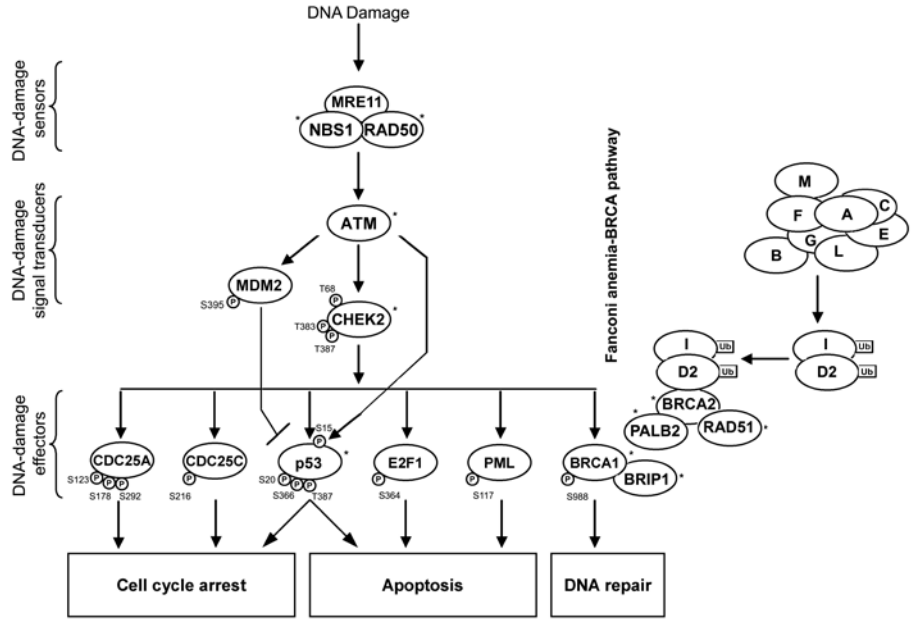

Figure 1.2 Schematic overview of CHEK2 and Fanconi anemia-BRCA signaling pathways.
CHEK2 signaling pathway; DNA damage is sensed by the MRN (MRE11-RAD50-NBS1) protein complex that is required for activation of ATM kinase. The ATM protein may phosphorylate CHEK2 kinase which subsequently phosphorylates the various DNA-damage signal effectors, including CDC25A, CDC25C, p53, E2F1, PML and BRCA1 that are involved in cell cycle arrest, apoptosis and DNA repair. Additionally, ATM kinase may also phosphorylate MDM2 proteins thereby preventing MDM2-p53 complex formation, leading to accumulation and increased p53 activity. Fanconi anemia-BRCA pathway; the Fanconi-anemia (FA) core complex consists of eight FA proteins (FA-A, FA-B, FA-C, FA-E, FA-F, FA-G, FA-L and FA-M) and is required for the monoubiquitination of the FA-D2 and FA-I protein complex. The activated FA-D2 and FA-I complex is translocated to DNA repair foci, where it co-localizes with other DNA damage proteins, including BRCA2, RAD51 and PALB2. The FA-BRCA pathway is depicted right-sided of the CHEK2 substrate BRCA1 because of the close functional relation in homologous DNA repair of the BRCA1 and BRCA2 proteins. Of note bi-allelic *BRCA2*, *BRIP1* and *PALB2* mutations cause Fanconi anemia subtypes FA-D1, FA-J, FA-N, respectively. The small round-shaped symbols enclosing the letter "P" next to the various proteins indicate known phosphorylation sites of each protein. The numbers below these phosphorylation sites refer to the residues involved with "S" referring to serine residues and "T" to threonine residues. The squares next to the FA-D2 and FA-I symbols indicate monoubiquitination sites , with Ub referring to monoubiquitination. *, Germline mutations in these genes are associated with increased breast cancer risks. In addition, somatic *p53* mutations and *MDM2* amplifications are frequently observed in breast cancer. Figure adapted from references 61, 181 and 197.

tion, therewith confirming our discovery of *CHEK2* 1100delC as a breast cancer susceptibility allele.[65] By now, *CHEK2* is firmly established as a breast cancer susceptibility gene, conferring an approximately 2-fold increased breast cancer risk to female *CHEK2* 1100delC carriers.[66] More in-depth details about *CHEK2* and the *CHEK2* 1100delC mutation are described in sections 1.6 through 1.11 of this thesis.

ATM, *RAD50* and *NBS1*

Genes encoding for proteins involved in maintenance of genomic integrity have since long been recognised as putative breast cancer susceptibility genes. A particularly interesting candidate was the ATM protein kinase that is involved in repair of DNA damage (Figure 1.2). Compound heterozygous or homozygous germline *ATM* mutations were identified as the cause for the rare autosomal recessive neurological disorder ataxia-telangiectasia (A-T). Moreover, it had been suggested that female heterozygous *ATM* mutation carriers in A-T families had an increased risk of breast cancer.[67-69] However, studies evaluating *ATM*-associated breast cancer risks for a long time failed to conclusively identify *ATM* as a breast cancer susceptibility gene.[70, 71] Conclusive evidence that heterozygous germline *ATM* mutations are indeed associated with an increased breast cancer risk was provided by an extensive association study of Renwick *et al*.[60] Mutational screening of the entire *ATM* coding sequence in familial breast cancer cases and controls had shown that heterozygous oncogenic *ATM* mutations were significantly more often identified in familial breast cancer cases compared to controls. It was estimated that heterozygous disease-causing A-T mutations would confer an approximately 2-fold increased breast cancer risk.[60]

The MRE11-RAD50-NBS1 (MRN) protein complex plays a central role in the cellular responses activated upon DNA double-stranded breaks (Figure 1.2). Bi-allelic *MRE11* and *NBS1* mutations have been identified in the genetic instability disorders ataxia-telangiectasia like disorder (ATLD) and Nijmegen breakage syndrome (NBS), respectively.[72, 73] Recently, association studies have shown that the protein truncating mutations, *NBS1* 657del5 and *RAD50* 687delT are associated with increased breast cancer risks among the Eastern European and Finnish populations.[63, 64] It was estimated that *NBS1* 657del5 and *RAD50* 687delT confer an approximately 3-fold and a nearly 4-fold increased familial breast cancer risk to female mutation carriers, respectively.

BRIP1 and *PALB2*

Fanconi anemia (FA) is a genetic disease that is characterized by severe developmental abnormalities, bone marrow failure and a high predisposition to cancer.[74, 75] Currently, thirteen FA subtypes have been identified.[76] In 2002, it was discovered that germline bi-allelic truncating *BRCA2* mutations were associated with the FA-D1 subtype.[77] As heterozygous *BRCA2* mutations had been associated with high breast cancer risks, it was hypothesized that heterozygous germline mutations in other FA genes might also confer breast cancer risks. Thus far, association studies have identified two other FA-associated genes as breast cancer susceptibility genes. Rahman and colleagues had screened the FA-J associated *BRIP1* gene, which encodes for a helicase that interacts with the C-terminal BRCT domain of BRCA1 proteins, for germline mutations in familial breast cancer cases and controls (Figure 1.2). The

frequency of heterozygous truncating *BRIP1* mutations was significantly higher among the familial breast cancer cases compared to the controls, showing that heterozygous truncating *BRIP1* mutations are breast cancer susceptibility alleles. It was estimated that heterozygous truncating *BRIP1* mutations were associated with an approximately 2-fold increased breast cancer risk.[62]

Soon after the discovery of *BRIP1* as a breast cancer susceptibility gene, it was shown that the FA-N associated *PALB2* gene also is a breast cancer susceptibility gene. *PALB2* encodes for a BRCA2 binding protein that is involved in nuclear localization and stabilization of BRCA2 proteins, a process required for BRCA2-associated DNA repair (Figure 1.2). Rahman *et al*. conducted a *PALB2* association study among familial breast cancer cases and controls, revealing that heterozygous truncating *PALB2* mutations are associated with an approximately 2-fold increased breast cancer risk.[61] Around the same time, an independent study reported that the Finnish *PALB2* 1592delT mutation conferred an approximately 4-fold increased breast cancer risk.[78] Together, these two studies had thus provided conclusive evidence that *PALB2* is breast cancer susceptibility gene.

1.2.3 Low-risk breast cancer susceptibility alleles

Low-risk breast cancer susceptibility alleles are common in the general population, with population frequencies ranging from 5% to 40%. Typically, these low-risk breast cancer susceptibility alleles confer small, about 1.3-fold, increased breast cancer risks (Figure 1.1). Low-risk breast cancer susceptibility alleles are usually identified though genome-wide association (GWA) studies. GWA studies analyse hundreds of thousands SNPs located throughout the humane genome in samples from diseased (cases) and disease-free (controls) individuals.[79, 80] Generally, GWA studies are performed in three stages, with a relatively small initial cohort of hundreds of cases and controls to identify statistically significant differences in SNP frequency between cases and controls, which are then replicated in an independent second and third cohort with up to tens of thousands cases and controls. The identification of SNPs associated with disease is based on a large number of statistical analyses and therefore stringent acceptance levels for statistical significance are used.[81] For example, a threshold of $P < 5 \times 10^{-8}$ is considered appropriate for a one million SNP analysis.[79] Another important aspect of GWA studies is the availability of large cohorts of cases and controls. The requirement of extensive cohorts of cases and controls has led to the establishment of large international consortia.[82] Currently, the largest breast cancer consortium is the Breast Cancer Association Consortium (BCAC) that includes more than 20 international collaborative research groups, with a combined sample size of about 30,000 breast cancer cases and 30,000 controls.[83] To date, eight SNPs have convincingly been identified as low-risk breast cancer susceptibility alleles, including SNPs near or in the 8q24 and 2q35 geneless regions and the *FGFR2*, *LSP1*,

MAP3K1, *MRPS30*, *TNRC9* and *RAD51* genes. Of note, the SNP identified in the *RAD51* gene is specifically associated with an increased breast cancer risk for *BRCA2* mutation carriers.[40, 84-88] Together, these eight SNPs have been estimated to account for approximately 5% of familial breast cancer risk.[23]

RAD51 (rs1801320, 135G>C)

The *RAD51* gene is required for repair of double-strand DNA breaks and interacts with both BRCA1 and BRCA2.[89, 90] It had been suggested that the *RAD51* 135G>C SNP in the 5'-untranslated region of *RAD51* was associated with an increased breast cancer risk among *BRCA2* mutation carriers.[91-93] In contrast, it had also been reported that the *RAD51* 135G>C SNP conferred a decreased breast cancer risk for *BRCA1* mutation carriers.[94, 95] To clarify the effect of *RAD51* 135G>C for *BRCA1* and *BRCA2* mutation carriers, the Consortium of Investigators of Modifiers of *BRCA1/2* (CIMBA) had genotyped *RAD51* 135G>C in 8,512 female *BRCA1/BRCA2* mutation carriers including 4,069 unaffected cases and 4,443 cases with breast cancer. No association between *RAD51* 135G>C and *BRCA1* mutation carriers had been identified. Yet, *BRCA2* mutation carriers homozygous for the *RAD51* 135 C-allele had an approximately 3-fold increased risk of breast cancer.[40]

SNPs near or in *FGFR2* (rs2981582), *LSP1* (rs3817198), *TNRC9* (rs3803662), *MAP3K1* (rs889312) and 8q24 (rs13281615)

In 2007, BCAC conducted a GWA study to identify common low-risk breast cancer susceptibility alleles.[85] Using a three-stage study design, including 26,258 breast cancer cases and 23,294 controls, five SNPs were discovered as low-risk breast cancer susceptibility alleles. Four SNPs were located near or in regions that contain protein-encoding genes, including SNPs in or nearby *FGFR2* (rs2981582), *LSP1* (rs3817198), *TNRC9* (rs3803662) and *MAP3K1* (rs889312). The fifth SNP (rs13281615) was located at chromosome 8q24 in a 110kb region that did not contain known genes. It was estimated that these low-risk breast cancer SNPs conferred approximately 1.3-fold increased breast cancer risks, with the highest breast cancer risks for the *FGFR2* SNP.[85] The function of low-risk SNPs in breast cancer susceptibility is currently unknown as no clear disease-causing mutations have been identified in genes within the vicinity of the low-risk SNPs.[85] Therefore, it has been speculated that low-risk SNPs may function by modulation of transcript expression of nearby-located genes or by mediation of non-coding RNAs located in the genomic loci associated with these SNPs.

Independently of the BCAC study, the *FGFR2* and *TNRC9* SNPs had been identified as low-risk breast cancer susceptibility loci in two other GWA studies.[86, 87] With regard to the 8q24 breast cancer susceptibility locus, it is interesting that GWA studies involving prostate and colorectal cancer cases have also identified susceptibility loci on 8q24. Yet, the SNPs associated with

these loci do not confer increased breast cancer risks suggesting that the 8q24 SNPs are each associated with specific tumor types.[84, 96]

SNPs near or in 2q35 (rs13387042) and *MRPS30* (rs4415084/rs10941679)

In addition to the five low-risk breast cancer susceptibility loci identified by BCAC, an Icelandic GWA study had identified a low-risk breast cancer SNP (rs13387042) on 2q35. Similar to the 8q24 SNP, this region also did not contain known genes.[87] More recently, the same research group reported two additional low-risk breast cancer susceptibility SNPs on 5p12 that were near the genomic region containing the *MRPS30* gene (rs4415084 and rs10941679*)*.[88] It was estimated that these SNPs confer about 1.3-fold increased breast cancer risks.

1.2.4 Protective breast cancer allele

In contrast to alleles that confer increased breast cancer risks, one could envision the existence of genetic variants that confer decreased breast cancer risks. However, as most studies are designed for the identification of novel breast cancer risk alleles, the identification of protective risk alleles is lagging behind. Thus far, only one SNP in the *CASP8* gene (SNP D320H, rs1045485) has been suggested as a protective breast cancer allele.[97, 98] Conclusive evidence that the *CASP8* D320H SNP is indeed a protective breast cancer allele was generated by BCAC. BCAC had evaluated *CASP8* D320H SNP in 16,423 breast cancer cases and 17,109 controls, showing that the *CASP8* D320H SNP is associated with an approximately 0.7-fold breast cancer risk.[99]

1.2.5 Summary familial breast cancer

Approximately 15-25% of all breast cancer cases are defined as familial breast cancer cases. To date, three classes of breast cancer susceptibility genes have been identified, including high-, moderate-, and low-risk breast cancer susceptibility genes (Figure 1.1). The two major high-risk breast cancer susceptibility genes are *BRCA1* and *BRCA2*. Women carrying oncogenic germline mutations in one of these two genes have about 6- to 8-fold higher lifetime breast cancer risks compared to the general population. Germline mutations in *BRCA1* and *BRCA2* are identified in about 20% of breast cancer families. Other high-risk breast cancer susceptibility genes include *p53*, *PTEN* and *STK11* but germline mutations in these three genes are only identified in less than 1% of familial breast cancer cases. The second class of breast cancer susceptibility genes is comprised of moderate-risk breast cancer susceptibility genes. Generally, mutations in moderate-risk breast cancer susceptibility genes are associated with

approximately 2-fold increased breast cancer risks. Currently, six moderate-risk breast cancer susceptibility genes have been discovered, including the *CHEK2*, *ATM*, *RAD50*, *NBS1*, *BRIP1* and *PALB2* genes. Germline mutations in these six genes together account for 5% of familial breast cancer risk. The last class of breast cancer susceptibility genes comprises low-risk breast cancer susceptibility alleles. Typically, these low-risk breast cancer susceptibility alleles confer approximately 1.3-fold increased breast cancer risks. Thus far, eight common low-risk breast cancer susceptibility alleles have been discovered, including SNPs that are near or in the *RAD51*, *FGFR2*, *TNRC9*, *MAP3K1*, *LSP1, MRPS30* genes and the 8q24 and 2q35 geneless regions. Together, these eight SNPs have been estimated to account for approximately 5% of familial breast cancer risk. In addition, a SNP in the *CASP8* gene has been associated with an approximately 0.7-fold breast cancer risk. As a whole, germline variants in high-, moderate-, and low-risk breast cancer susceptibility genes or alleles account for about one-third of familial breast cancer cases.

1.3 Colorectal cancer

Colorectal cancer is the second and third most commonly diagnosed cancer among women and men in Western countries, with about one million new colorectal cancer patients every year. In the Netherlands, 10,855 new colorectal cancer patients (both female and male) were identified and 4,562 colorectal cancer patients died of the disease in 2005. Current figures estimate that approximately 1 in every 16 men and 1 in every 18 women will be diagnosed with colorectal cancer and around 50% of patients will die of the disease within 5 years after diagnosis (www.ikcnet.nl).

Risk factors

The strongest risk factors for colorectal cancer are a family history of colorectal cancer and the presence of germline mutations in known colorectal cancer susceptibility alleles. In addition, it has been suggested that certain environmental factors, mainly related to dietary and lifestyle habits are associated with colorectal cancer (risk factors include alcohol consumption and smoking, protective factors include intake of fibres and physical exercise).[100]

Prognosis

Colorectal cancer prognosis is predominantly determined by stage of the disease at the time of diagnosis. Stage depends on the degree of invasion of tumor cells into the surrounding tissue of the colorectal tumor, the presence of positive regional lymph nodes and the presence of distant metastasis.[101] Similar to staging of breast cancer, the most commonly used colorectal cancer

staging system is the TNM system, where T refers to tumor size, N to lymph node involvement and M to the presence of distant metastasis. Staging according to the TNM system is closely related to colorectal cancer survival as the 5-year survival decreases from 90% for individuals with stage I colorectal cancer to only 10% for those with stage IV colorectal cancer.[102] Tumor grade, based on the percentage of glandular formation, is also commonly used as prognostic factor for colorectal cancer. Additionally, several molecular features, including microsatellite instability, chromosomal loss at 18q and *p53* mutation status have been suggested as possible prognostic colorectal cancer factors. However, the true prognostic value of these molecular factors is largely unknown and they are therefore not recommended as prognostic tools in routine clinical practice.[101]

Treatment

Generally, treatment for colorectal cancer patients without distant metastasis involves surgical resection of the primary tumor and lymph nodes. Surgical resection is preceded by radiation therapy in patients with rectal cancer, with additional chemotherapy in case of large tumors and/or positive lymph nodes (www.oncoline.nl; landelijke CBO richtlijn coloncarcinoom en rectumcarcinoom). There are several chemotherapeutic agents with proven activity against colorectal cancer. Adjuvant chemotherapy was shown to be beneficial for colon cancer patients with positive lymph nodes and/or perforation of the colon. In contrast, the effectiveness of adjuvant chemotherapy for rectal cancer patients with positive lymph nodes has not been firmly established (www.oncoline.nl; landelijke CBO richtlijn coloncarcinoom en rectumcarcinoom). Recently, it has been shown that monoclonal antibodies against the vascular endothelial growth factor (VEGF) and the epidermal growth factor-receptor (EGFR) exhibit anti-tumor activity.[103] However, the anti-tumor activity of monoclonal antibodies targeting EGFR is absent in colorectal cancers with mutated *KRAS* and are therefore only administered to patients with wild-type *KRAS* colorectal cancers.[104, 105]

1.4 Familial colorectal cancer

The majority of colorectal cancer cases can be classified as sporadic colorectal cancer cases with no evidence for an inherited colorectal cancer predisposition.[106] About 25% of colorectal cancer cases have a familial basis.[107-109] Unfortunately, due to the absence of a general consensus definition for familial colorectal cancer, it has become problematic to compare the various clinical and research studies regarding familial colorectal cancer. Yet, similar to familial breast cancer, familial colorectal cancer generally refers to a clustering of colorectal cancer cases in families that is higher than the colorectal cancer incidence in the general population. Major familial colorectal cancer syndromes include (attenuated) familial adenomatous polyposis ((A)

FAP), MUTYH-associated polyposis (MAP) and Lynch syndrome, previously annotated as hereditary non-polyposis colorectal cancer (HNPCC)†.[110-112] Germline mutations in the *APC*, *MUTYH* and DNA mismatch repair (MMR) genes that predispose to (A)FAP, MAP and HNPCC, respectively, together have been identified in approximately 15% of familial colorectal cancer cases.[106, 109, 110] Additionally, several hamartoma polyposis syndromes such as Peutz-Jeghers have been identified. These syndromes are however rare and therefore only a small fraction of familial colorectal cancer cases is due to germline mutations that predispose to these syndromes. Hence, it is still unclear what underlies the colorectal cancer clustering in the majority of familial colorectal cancer cases. Similar to breast cancer, part of this remaining familial colorectal cancer is likely due to mutations in moderate- and low-risk colorectal cancer genes. The discussion of these moderate- and low-risk colorectal cancer genes is, however, outside the scope of this thesis.

1.4.1 Familial adenomatous polyposis (FAP)

Familial polyposis syndromes are identified in approximately 5% of all familial colorectal cancer cases.[113, 114] The majority of polyposis cases is diagnosed with the autosomal dominant inherited colorectal cancer syndrome familial adenomatous polyposis (FAP). The main phenotype of FAP is the presence of hundreds to thousands of colorectal adenomas that typically manifest at a very young age, usually during the second and third decades of life. Unless surgical intervention has interrupted the progression of one or several of these adenomatous polyps to colorectal cancer, the lifetime colorectal cancer risk of FAP cases is almost 100%. FAP cases also have a high lifetime risk for development of duodenal adenomas.[115-117] Yet, despite of the high lifetime risk for duodenal adenomas, FAP cases only have a lifetime risk of about 5% for duodenal carcinoma.[115-117] In addition to colorectal and duodenal adenomas, FAP cases are frequently diagnosed with desmoid tumors.[114] Desmoid tumors and duodenal carcinomas are currently the major causes of death in FAP patients who have undergone prophylactic colectomy.[118] Other clinical features associated with FAP include osteomas, dental abnormalities and congenital hypertrophy of the retinal pigment epithelium (CHRPE).[111, 114]

Apart from classical FAP, an atypical form termed attenuated familial adenomatous polyposis (AFAP) has been identified. Compared to FAP, the most important features of AFAP are a de-

† During the course of this thesis, the term HNPCC has been replaced by Lynch syndrome, referring to cancer families with a germline oncogenic mutation in one of the mismatch repair (MMR) genes and/or evidence for microsatellite instability (MSI) in one of the cancers. All published chapters in this thesis refer to HNPCC and/or HNPCC-related disease with the first referring to Lynch syndrome families and families that fulfill the revised Amsterdam criteria and the latter referring to non-Lynch syndrome families (and families that do not fulfill the revised Amsterdam criteria). For clarity and consistency, the terms HNPCC and/or HNPCC-related disease have also been used in all unpublished parts of this thesis, including this introduction.

layed disease onset, on average 15 years later as reported for FAP cases, reduced polyp count (5 to 100 polyps) and the occurrence of less extracolonic manifestations.[119, 120]

In 1991, germline mutations in the *APC* gene were discovered as the major genetic cause for the (A)FAP syndrome. The *APC* gene was identified through linkage analysis and its encoded protein is involved in Wnt signaling and maintenance of chromosomal stability.[121-126] The majority of germline *APC* mutations associated with (A)FAP are insertions, deletions and nonsense mutations that result in truncated APC proteins. Germline *APC* mutations have been identified in approximately 60% to 80% and 10% to 30% of FAP and AFAP cases, respectively.[114]

1.4.2 MUTYH-associated polyposis (MAP)

MUTYH-associated polyposis (MAP) has been identified as the first recessively inherited colorectal cancer syndrome, with inheritance patterns that commonly manifest as multiple affected family members within a single generation.[127] MAP is associated with compound heterozygous or homozygous germline mutations in the base excision repair gene *MUTYH*. These mutations may induce increased frequencies of somatic G:C>T:A transversions in genes involved in colorectal cancer carcinogenesis, such as *APC* and *KRAS*.[127-130]

Clinically, it is difficult to distinguish MAP from FAP, and particularly from AFAP.[114, 131] Generally, MAP tends to present at later age then (A)FAP, usually in the mid-50s. The number of polyps observed in MAP cases ranges from five to hundreds but rarely equals the thousands of polyps seen in classical FAP cases.[114] Colorectal cancer has been identified in approximately 50% of MAP cases.[132, 133] Extracolonic features appear to be rare but two studies have suggested that MAP cases may be at increased risk of adenomas in the duodenum.[131, 134]

Bi-allelic germline *MUTYH* mutations have been detected in about 10% of FAP cases and 10% to 25% of AFAP cases without inherited *APC* mutations.[134] The most common disease-causing *MUTYH* mutations in Western populations are the missense mutations *MUTYH* Y179C and *MUTYH* G396D, together accounting for 75% of disease-causing *MUTYH* mutations reported thus far. In the Netherlands, these two missense mutations and the Dutch *MUTYH* P405L founder mutation represent nearly 90% of *MUTYH* mutations identified in MAP cases.[134-136]

1.4.3 Hereditary non-polyposis colorectal cancer (Lynch syndrome)

Hereditary non-polyposis colorectal cancer (HNPCC, currently known as Lynch syndrome) is the most common familial colorectal cancer syndrome. The syndrome is inherited in an autosomal dominant manner and represents approximately 20% of all familial colorectal cancer cases.[137, 138] HNPCC is characterized by a familial clustering of colorectal cancer and a variety of extracolonic cancers including cancers of the endometrium, ovary, small intestine, stomach, and transitional

Table 1.1 Revised Amsterdam and revised Bethesda criteria.[144, 148]

Revised Amsterdam criteria for HNPCC families:

A family should include at least three relatives with a HNPCC-associated tumor* and

1. One relative should be a first-degree of the other two and
2. At least two successive generations should be affected and
3. At least one individual should be diagnosed at age <50 years and
4. Tumors should be verified by pathological examination and
5. Familial adenomatous polyposis should be excluded in the individual(s) with colorectal cancer, if any

* HNPCC-associated tumors include colorectal, small bowel, endometrial, ureter and renal pelvis tumors.

Revised Bethesda criteria for eligibility for MSI testing:

1. Individuals with colorectal cancer diagnosed at age <50 years or
2. Individuals with synchronous or metachronous colorectal or other HNPCC-associated tumors**, regardless of age at diagnosis or
3. Individuals with the MSI-high histology† diagnosed at age <60 years or
4. Individuals with colorectal cancer and at least one first-degree relative with a HNPCC-associated tumor**, with one of the cancers diagnosed at age <50 years or
5. Individuals with colorectal cancer and at least two first- or second-degree relatives with HNPCC-associated tumors**, regardless of age at diagnosis

** HNPCC-associated tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome, and carcinoma of the small bowel.

† presence of tumor infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern.

The revised Amsterdam criteria were formulated to guide international collaborative studies regarding HNPCC families. The revised Bethesda criteria describe all clinical conditions that lead to eligibility for MSI testing. HNPCC, hereditary non-polyposis colorectal cancer; MSI, microsatellite instability.

cell carcinoma of the ureter and renal pelvis. Additionally, brain and skin tumors may occur.[139-142] To provide standardized clinical criteria that would guide international collaborative studies regarding HNPCC families, the Amsterdam criteria were established in 1990 and revised in 1999 (Table 1.1).[143, 144]

Germline mutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2,* predispose to HNPCC.[145] One of the key features of a defective DNA MMR system is the occurrence of instable microsatellite marker lengths throughout the tumor genome. This microsatellite instability (MSI) phenotype can therefore be used as a diagnostic tool to identify cases that are likely carriers of germline mutations in one of the *MMR* genes.[146] The clinical conditions that lead to eligibility for MSI testing were formulated in the Bethesda criteria in 1997, that

subsequently were revised in 2004 (Table 1.1).[147, 148] Nowadays, the revised Bethesda criteria are the most widely used criteria for identification of HNPCC families.[149] Yet, approximately 25% of HNPCC cases fails to fulfill the revised Bethesda criteria [150] and it has therefore been proposed that MSI together with immunohistochemistry of MMR proteins may provide more accurate means for identification of HNPCC families.[106, 150]

Germline *MMR* mutations have been identified in about 50% of HNPCC families that meet the revised Amsterdam criteria with approximately 50%, 40% and 10% of the detected mutations in the *MLH1*, *MSH2*, and *MSH6* genes, respectively.[108, 145, 151] Germline *PMS2* mutations have so far been identified in less than 1% of HNPCC families.[108, 145, 152] Yet, this figure may represent an underestimation of the actual contribution of *PMS2* mutations to HNPCC as *PMS2* mutation analysis is complicated by the presence of pseudogenes in the genome.[153-155] The mutation spectrum of the *MLH1*, *MSH2*, *MSH6* and *PMS2* genes appears to be very broad, including missense, nonsense, frameshift and splice site mutations. In addition, large genomic deletions, duplications and genomic rearrangements have been identified in the *MLH1*, *MSH2* and *PMS2* genes (www.insight-group.org).

Several studies have estimated the cumulative colorectal cancer risks in HNPCC cases with germline *MLH1*, *MSH2* and *MSH6* mutations. Depending on gender and gene involved, the estimated cumulative colorectal cancer risks vary from 30% to 75% at age 70 years.[156-159] Generally, lower cumulative colorectal cancer risks at age 70 years are reported for women.[160] Age at colorectal cancer diagnosis is approximately five years later in *MSH6* mutation carriers compared to *MLH1* and *MSH2* mutation carriers (50 years vs. 45 years, respectively).[158, 159, 161] The cumulative risk of endometrial cancer at age 70 years ranges from 30% to 70%.[156, 157, 159, 160, 162] Typically, higher risks for endometrial cancer are reported for *MSH6* mutation carriers. In addition, an approximately 5-year earlier diagnosis of endometrial cancer has been shown for *MSH6* mutation carriers compared to *MLH1* and *MSH2* mutation carriers (54 years vs. 59 years, respectively).[55, 158, 159, 161] The lifetime risks of other extracolonic cancers associated with HNPCC, such as ureter and renal pelvis cancer, are usually below 10%.[156, 157, 161, 162] Of note, several studies have evaluated whether breast cancer is an integral tumor type of HNPCC. Yet, no consensus has been obtained, resulting in an ongoing debate regarding the inclusion of breast cancer as part of the HNPCC syndrome.[163-168]

1.4.4 Summary familial colorectal cancer

Approximately 25% of all colorectal cancer cases have a familial basis. Similar to familial breast cancer, familial colorectal cancer refers to families with an increased incidence of colorectal cancer cases compared to the general population. Familial polyposis syndromes are identified in approximately 5% of familial colorectal cancer cases and the majority of these polyposis cases are characterized as FAP and AFAP patients. Germline mutations in the *APC* gene are identified

in 60% to 80% of FAP and 10% to 30% of AFAP cases. A third polyposis syndrome is MAP, which is associated with compound heterozygous or homozygous germline *MUTYH* mutations. Currently, approximately 10% of FAP cases and 20% to 25% of AFAP cases without germline *APC* mutations have bi-allelic *MUTYH* mutations. HNPCC is a third major syndrome which is diagnosed in nearly 20% of familial colorectal cancer cases. Germline mutations in the mismatch repair genes *MLH1*, *MSH2*, *MSH6* and *PMS2* predispose to HNPCC. Among these four genes, mutations in *MLH1*, *MSH2* and *MSH6* account for about 50%, 40% and 10% of all identified HNPCC-associated mutations. Mutations in *PMS2* have, thus far, been identified in less than 1% of HNPCC families. Apart from (A)FAP, MAP and HNPCC, several rare hamartoma polyposis syndromes with an increased predisposition to colorectal cancer have been identified, accounting for only a small fraction of familial colorectal cancer cases. Together, approximately 15% of familial colorectal cancer cases are associated with germline mutations in known colorectal cancer genes that predispose for high-risk familial colorectal cancer syndromes. Thus, the genetic cause for the majority of familial colorectal cancer cases is currently unknown. Similar to familial breast cancer, part of the remaining familial colorectal cancer risk is likely due to mutations in moderate and low-risk colorectal cancer genes.

1.5 Cancer susceptibility and DNA damage repair

Cancer susceptibility genes have been subdivided in three classes. The first class includes genes that function in control of cell proliferation and tumor growth, the so-called gatekeeper genes. The second class consists of genes that control the integrity of the genome and have been termed caretaker genes. The third class of genes, called landscaper genes, are mutated in stromal cells surrounding the tumor, thus providing an optimal environment for tumor growth.[169, 170] Most of the breast cancer and colorectal cancer susceptibility genes can be classified as caretaker genes, encoding for proteins that function in DNA damage responses, particularly cell-cycle arrest and DNA repair pathways.

Interestingly, the majority of breast cancer susceptibility genes have been implicated in repair of double-strand DNA breaks (Figure 1.2). Double-strand DNA breaks are generally sensed by the MRE11-RAD50-NBS1 (MRN) protein complex. This MRN complex activates ATM kinase that serves as signal transducer and activator of CHEK2 kinase.[171] Activated CHEK2 signals to a number of downstream proteins, of which p53 is a main target. Another prominent signaling target of CHEK2 is the BRCA1 protein.[172] The BRCA1 protein functions in repair of DNA damage by homologous recombination, a process also involving the BRIP1, BRCA2, RAD51 and PALB2 proteins.[173, 174] Additionally, the BRCA1 protein has been identified as a scaffold protein for over 40 proteins.[175] This BRCA1-associated genome surveillance complex (BASC) included the DNA damage response proteins ATM, MRE11, RAD50, NBS1, MLH1, MSH2 and MSH6.[175] Together, it

had therefore been suggested that the BRCA1 protein plays a pivotal role in the organization and execution of DNA damage repair processes upon various types of DNA damage.

Apart from the association of breast cancer susceptibility genes with DNA repair, the known colorectal cancer susceptibility genes *APC*, *MLH1*, *MSH2*, *MSH6*, *PMS2* and *MUTYH* have also been implicated in preserving genomic integrity. In addition to its function in the Wnt pathway, the APC protein is involved in spindle formation and chromosomal segregation during cell division. Loss of APC protein function has been shown to elicit chromosomal instability, resulting in several chromosomal abnormalities.[122, 123] The MLH1, MSH2, MSH6 and PMS2 proteins are the main components of the mismatch repair (MMR) protein complex. The MMR complex recognizes and repairs base-pair mismatches and small nucleotide insertions/deletions (1 to several basepairs) that occur during replication.[176] Another important DNA repair process is the base excision repair (BER) pathway that is involved in repair of small chemical alterations of bases.[177] The *MUTYH* colorectal cancer susceptibility gene is one of the key players in the BER pathway.[127] Interestingly, it was suggested that the MUTYH protein interacts with the MSH6 protein, enhancing DNA binding and glycosylase activities of the MUTYH protein.[178] Apart from the functional link between the BER and MMR pathways, it has also been shown that the MMR proteins MLH1, MSH2 and MSH6 are components of the BASC-complex.[175] Together this suggests an extensive crosstalk between DNA repair mechanisms and provides a functional link between biological pathways involved in the maintenance of genomic stability and susceptibility to breast cancer and colorectal cancer.

1.6 Cell cycle checkpoint kinase 2

Cells are continuously exposed to endogenous and exogenous stress factors that may cause DNA damage. To maintain genomic integrity, a network of proteins is functioning throughout the cell cycle to regulate cellular pathways involved in cell cycle arrest, DNA repair and apoptosis.[179] Defects in these pathways will lead to the accumulation of DNA damage which, in turn, may enhance cancer development. One of the central players in DNA damage response pathways is cell cycle checkpoint kinase 2 (CHEK2, also known as Chk2 and as Cds1 in *S. pombe* and Rad53 in *S. cerevisiae*). In this chapter, CHEK2 structure and function as well as various aspects of *CHEK2*-associated carcinogenesis will be discussed.

CHEK2 structure and function

The cell cycle checkpoint kinase 2 gene is located on chromosome 22q12.1. The gene encompasses approximately 50 kilobases and consists of 14 exons that encode for a serine/threonine kinase of 543 amino acids. The CHEK2 protein is characterized by four functional domains; an amino-terminal serine-glutamine/threonine-glutamine (SQ/TQ) domain (residues 19-69), a fork

head-associated (FHA) domain (residues 115 to 175), a serine/threonine kinase domain (residues 226 to 486) and a carboxy-terminal nuclear localisation signal (NLS, residues 515 to 522, Figure 1.3).[172, 180-182] The SQ/TQ domain contains phosphorylation sites that are necessary for activation of the CHEK2 protein. The FHA domain participates in phosphoprotein interactions during CHEK2 protein activation and in binding to other phosphorylated proteins, particularly through recognition of phosphothreonine residues. The catalytic serine/threonine kinase domain contains an activation T loop that comprises two threonine residues (T383 and T387). Together with the serine residue at codon 516 (S516) located in the NLS domain, phosphorylation of T383 and T387 is essential for full CHEK2 protein activation (Figure 1.3)[172, 180-182]

The CHEK2 protein is activated by ionising radiation, chemotherapeutic agents, or other compounds that elicit DNA damage. Upon DNA damage, specific DNA damage sensor protein complexes are recruited that subsequently activate DNA damage signal transducers (Figure 1.2). In response to double-strand breaks, the MRE11-RAD50-NBS1 (MRN) protein complex (sensor) may activate ATM kinase (signal transducer).[181] One of the main signaling routes of ATM involves phosphorylation of specific residues in the SQ/TQ-domain of CHEK2, particularly threonine 68. Phosphorylation of threonine 68 promotes binding of the SQ/TQ domain of one CHEK2 molecule to the FHA domain of another CHEK2 molecule. This dimerisation is essential for full CHEK2 activation, involving autophosphorylation of threonine 383 and threonine 387 in the activation T loop and serine 516 in the NLS domain.[172, 180]

Activated CHEK2 proteins function as signal transducers by phosphorylating a number of substrates involved in cell-cycle arrest, DNA repair or apoptosis (Figure 1.2).[171, 181, 182] G1-phase cell-cycle arrest is mainly regulated through the ATM-CHEK2-p53-MDM2-p21 pathway. Under normal conditions, MDM2 binds to p53 which promotes p53 ubiquitination and p53 export from the nucleus to the cytoplasm. In the cytoplasm, p53 can then be degraded by cytoplasmic proteosomes.[183] Upon DNA damage, ATM and CHEK2 kinases phosphorylate p53 at serine 15 and 20, respectively. Additionally, ATM phosphorylates MDM2 at serine 395. These ATM and CHEK2-mediated phosphorylations prevent binding of MDM2 to p53, resulting in abrogation of p53 export to the cytoplasm and degradation.[184-188]The subsequent nuclear accumulation and increased transcriptional activity of p53 will, amongst others, enhance p21 function as inhibitor of cyclin-dependent Cdk2/Cyclin E kinases, causing G1-phase cell cycle arrest.[189] Late G1-phase cell cycle arrest is partly induced by CHEK2-mediated phosphorylations of CDC25A at serine 123, serine 178 and serine 292. Phosphorylations at these CDC25A residues promote CDC25A proteasomal degradation, leading to inhibition of Cdk2/Cyclin E protein function and consequently late G1-phase cell cycle arrest.[190] Finally, CHEK2 kinase has been implicated in cell cycle arrest during G2-phase. CHEK2 then phosphorylates CDC25C phosphatase at serine 216, promoting binding of 14-3-3δ proteins that facilitate nuclear export of CDC25C to the cytoplasm. Reduced CDC25C activity due to cytoplasmic localization results in G2-phase cell cycle arrest as the downstream Cdk1/Cyclin B complex is no longer activated.[191-193] Apart from cell cycle arrest, CHEK2 kinase has been implicated in DNA repair through phosphorylation of

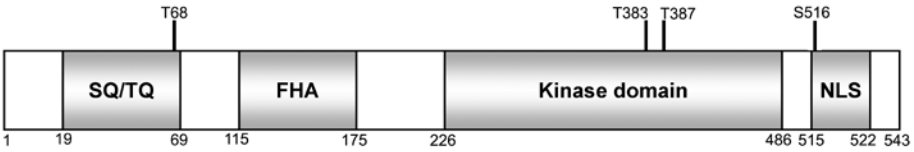

Figure 1.3 Schematic representation of the CHEK2 protein structure.
The four functional domains of the CHEK2 protein are indicated by shaded boxes including an amino-terminal serine-glutamine/threonine-glutamine (SQ/TQ) domain, a fork head-associated (FHA) domain, a serine/threonine kinase domain and a carboxy-terminal nuclear localisation signal (NLS). The numbers below the figure indicate locations of the amino acid residues corresponding to the four CHEK2 protein domains. The symbols above the figure indicate the most important CHEK2 phosphorylation residues required for full activation of the CHEK2 protein. These residues include threonine 68 (T68), threonine 383 (T383), threonine 387 (T387) and serine 516 (S516). Figure adapted from Antoni *et al*. 2007.[181]

BRCA1 at serine 988 and in apoptosis through phosphorylations of the E2F1 transcription factor at serine 364 and the PML tumor suppressor at serine 117.[194-196]

1.7 *CHEK2* 1100delC and breast cancer

Identification of *CHEK2* 1100delC as a breast cancer susceptibility allele

In 2002, our research group had participated in an international study that led to the discovery of the *CHEK2* gene as a novel breast cancer susceptibility gene.[59] The start for identification of *CHEK2* as a breast cancer susceptibility gene had been a genome-wide linkage analysis in the EUR60 family, which is the largest non-*BRCA1/BRCA2* breast cancer family registered at the Rotterdam Family Cancer Clinic (Figure 1.4). Although the maximum possible logarithm of odds (LOD) score of the EUR60 family was 4.7, the linkage analysis revealed a 1.2 LOD score on chromosome 22q as the highest score. Yet, no obvious candidate genes were located in the identified 22q region. Soon after this linkage analysis had been conducted, however, it was reported that Li-Fraumeni syndrome (LFS) families without *p53* mutations carried germline *CHEK2* mutations.[198] As *CHEK*2 is located on chromosome 22q in vicinity of the identified linkage region, the *CHEK2* gene was screened for germline mutations in individuals of the EUR60 family. The protein truncating *CHEK2* 1100delC mutation was identified in six of eight breast cancer cases in the EUR60 family for which *CHEK2* 1100delC status could be determined or inferred (Figure 1.4). Interestingly, the *CHEK2* 1100delC mutation was also identified in one of the LFS families previously screened for germline *CHEK2* mutations, suggesting that the *CHEK2* 1100delC mutation represents a founder mutation derived from a common ancestor. To further evaluate the association of *CHEK2* 1100delC with breast cancer, the mutation was genotyped in 718 familial non-*BRCA1/BRCA2*, 318 familial *BRCA1/BRCA2* and 636 unselected breast cancer

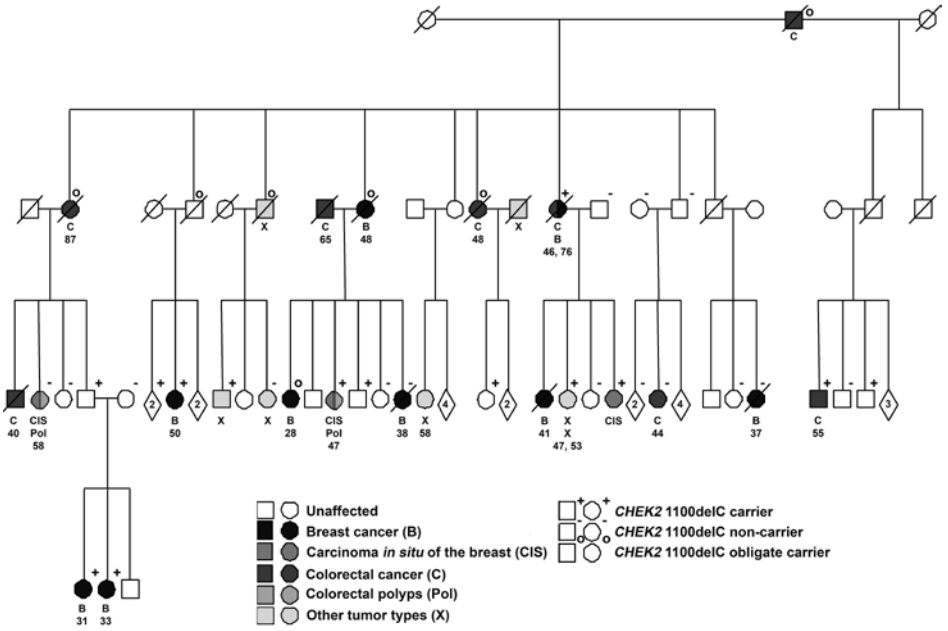

Figure 1.4 Pedigree of the *CHEK2* 1100delC breast cancer family EUR60.
Cancer types and ages at diagnosis are indicated below each affected family member.To ensure anonymity, unaffected cases in the youngest generation are omitted from the pedigree and unaffected cases who are not obligate *CHEK2* 1100delC carriers are not indicated. The EUR60 family is designated as a HBCC family, fulfilling all three HBCC criteria. In short these criteria include presence of a double breast and colorectal cancer case and/or an early-onset colorectal cancer case and/or multiple colorectal cancer cases in the family. Note that the EUR60 family has a severe cancer phenotype including 8 breast cancer cases, 6 colorectal cancer cases, 2 cases with double tumors, 4 non-breast/colorectal cancer cases and 3 cases with premalignant lesions. Also note the incomplete *CHEK2* 1100delC genotype/phenotype segregation as illustrated by the negative *CHEK2* 1100delC status of two breast cancer cases (B:38, B:37) and a single colorectal cancer case (C:44) in the third generation. Together, these two observations suggest that other cancer susceptibility alleles, in addition to *CHEK2* 1100delC, are present in the EUR60 family.

cases as well as 1,620 controls. Breast cancer cases and controls had originated from three geographical regions including the Netherlands, United Kingdom and North America. The *CHEK2* 1100delC mutation was identified in 1.1% of controls with no apparent difference in *CHEK2* 1100delC frequencies among the three control populations. The 1.1% *CHEK2* 1100delC population frequency was not significantly different compared to the 0.3% and 1.4% *CHEK2* 1100delC frequency among familial *BRCA1/BRCA2* and unselected breast cancer cases, respectively. In sharp contrast, the 5.1% prevalence of *CHEK2* 1100delC among familial non-*BRCA1/BRCA2* breast cancer cases was significantly increased, suggesting that *CHEK2* 1100delC conferred an increased breast cancer risk. Based on the difference in breast cancer incidence among *CHEK2* 1100delC carriers and non-carriers, it was estimated that female *CHEK2* 1100delC carriers had an approximately 2-fold increased breast cancer risk. Accordingly, the *CHEK2* 1100delC

mutation had been identified as the first moderate-risk breast cancer susceptibility allele.[59] Moreover, as the prevalence of *CHEK2* 1100delC in the general population is relatively high, but LFS is very rare, these results had shown that the *CHEK2* 1100delC mutation was not a high-risk LFS susceptibility allele as had been suggested previously.[198]

Interestingly, haplotype analysis had shown that all *CHEK2* 1100delC carriers, irrespective of their geographical origin, shared the same allele of the D22S275 microsatellite marker located in intron 4 of the *CHEK2* gene. The occurrence of a single allele among all *CHEK2* 1100delC carriers confirmed that the *CHEK2* 1100delC mutation indeed represents a founder mutation.

Independently of our study, Vahteristo *et al*. reported a similar association between *CHEK2* 1100delC and Finnish non-*BRCA1/BRCA2* familial breast cancer cases.[65] They had evaluated *CHEK2* 1100delC in 507 familial non-*BRCA1/BRCA2* cases, 1,035 unselected breast cancer cases and 1,885 controls. Comparable to our results, they did not detect a significant difference between the frequency of *CHEK2* 1100delC in unselected breast cancer cases and controls. Yet, the *CHEK2* 1100delC frequency was 5.5% in familial non-*BRCA1/BRCA2* breast cancer cases compared to 1.4% in controls, thus confirming our results that *CHEK2* 1100delC is a moderate-risk breast cancer susceptibility allele.[65]

The deleterious nature of the *CHEK2* 1100delC mutation was subsequently confirmed by functional studies that used cell lines transfected with mutant *CHEK2* 1100delC constructs. Cells expressing exogenous *CHEK2* 1100delC had low to no CHEK2 protein expression, were unable to undergo ATM-mediated phosphorylation at threonine 68 and had abolished CHEK2 kinase function.[199-201] These experiments further emphasized that the *CHEK2* 1100delC mutation represents a deleterious oncogenic mutation.

CHEK2 1100delC breast cancer risks

After the discovery of *CHEK2* 1100delC as a breast cancer susceptibility allele, a wealth of studies have evaluated this founder mutation in breast cancer cases and controls (Table 1.2). It then became evident that the *CHEK2* 1100delC frequency varied substantially among the various populations. For example, the *CHEK2* 1100delC frequency varied from 0.4% to 1.3% in Northern European populations whereas Eastern European populations reported *CHEK2* 1100delC frequencies of 0.2% to 0.3%. Additionally, *CHEK2* 1100delC was virtually absent in Southern European populations (Figure 1.5, Figure 1.6). *CHEK2* 1100delC also appears to be less common among non-European populations compared to European populations and populations of European descent, suggesting that the *CHEK2* 1100delC founder mutation originated in Europe (Figure 1.6). Interestingly, although the *CHEK2* 1100delC population frequency varied among the various European populations, the *CHEK2* 1100delC mutation frequency among breast cancer cases was always nearly twice as high compared to geographically-matched controls, thereby reflecting the estimated 2-fold increased breast cancer risk for female 1100delC carriers (Figure 1.5). The estimated *CHEK2* 1100delC breast cancer risk had been based on the difference in breast cancer

Table 1.2 Summary of reported *CHEK2* 1100delC genetic screens in breast cancer.

	Unselected BRC cases		**Familial BRC cases**		**Familial BRC cases**	
			(unknown or non- *BRCA1/BRCA2)*		**(*BRCA1/BRCA2* mutant)**	
	+ve/tested	**(%)**	**+ve/tested**	**(%)**	**+ve/tested**	**(%)**
The Netherlands	168/5023	(3.3)	25/435	(5.8)	2/211	(1.0)
United Kingdom	42/3450	(1.2)	15/279	(5.4)	0/52	-
Denmark	13/1088	(1.2)	n.a.		n.a.	
Sweden	23/1898	(1.2)	10/450	(2.2)	n.a.	
Finland	45/1761	(2.6)	28/507	(5.5)	0/19	-
Germany	18/2193	(0.8)	12/690	(1.7)	0/30	-
Poland	20/4454	(0.4)	n.a.		n.a.	
Czech Republic	3/688	(0.4)	1/358	(0.3)	n.a.	
Russia	14/660	(2.1)	n.a.		n.a.	
Belgium	1/100	(1.0)	3/78	(3.8)	n.a.	
Spain	0/56	-	2/689	(0.2)	0/6	-
Italy	n.a.		0/756	-	1/183	(0.5)
United States	31/2700	(1.1)	11/649	(1.7)	0/157	-
Canada	41/3648	(1.1)	11/453	(2.4)	0/307	-
Australia	10/1474	(0.7)	2/300	(0.7)	n.a.	
South America	2/517	(0.4)	n.a.		n.a.	
Asia	0/1300	-	0/601	-	0/42	-
Israel	n.a.		n.a.		1/219	(0.5)

The summary includes data from 44 reported genetic screens that have evaluated the breast cancer risk conferred by *CHEK2* 1100delC. Breast cancer cases were classified as familial when indicated as such in the reported studies. Generally, the majority of familial breast cancer cases had reported at least one first or second degree relative diagnosed with breast cancer or at least one relative with ovarian cancer. BRC, breast cancer; MBC, male breast cancer; +ve, *CHEK2* 1100delC positive; n.a., not available.

Bilateral BRC cases		MBC cases and/or families with MBC		Population controls		References
+ve/tested	(%)	+ve/tested	(%)	+ve/tested	(%)	
15/233	(6.4)	3/94	(3.2)	23/2059	(1.1)	59, 66, 203-208, chapter 2
7/469	(1.5)	1/169	(0.6)	26/4559	(0.6)	59, 66, 209-213
n.a.		n.a.		22/4643	(0.5)	214
n.a.		n.a.		16/2394	(0.7)	215-217
6/68	(8.8)	2/150	(1.3)	31/2332	(1.3)	65, 66, 218-220
0/43	-	0/15	-	14/3617	(0.4)	59, 66, 221, 222
n.a.		n.a.		12/5496	(0.2)	223
n.a.		n.a.		2/730	(0.3)	224
8/155	(5.2)	n.a.		1/821	(0.1)	225
n.a.		n.a.		0/100	-	226
n.a.		n.a.		0/520	-	227-229
0/115	-	0/102	-	0/597	-	230, 231
1/92	(1.1)	0/128	-	14/3440	(0.4)	59, 211, 232-237
1/137	(0.7)	n.a.		23/8359	(0.3)	232, 238
n.a.		0/7	-	1/736	(0.1)	66, 239
n.a.		n.a.		0/1261	-	238, 240
n.a.		0/5	-	0/435	-	238, 240-243
n.a.		0/54	-	0/146	-	244

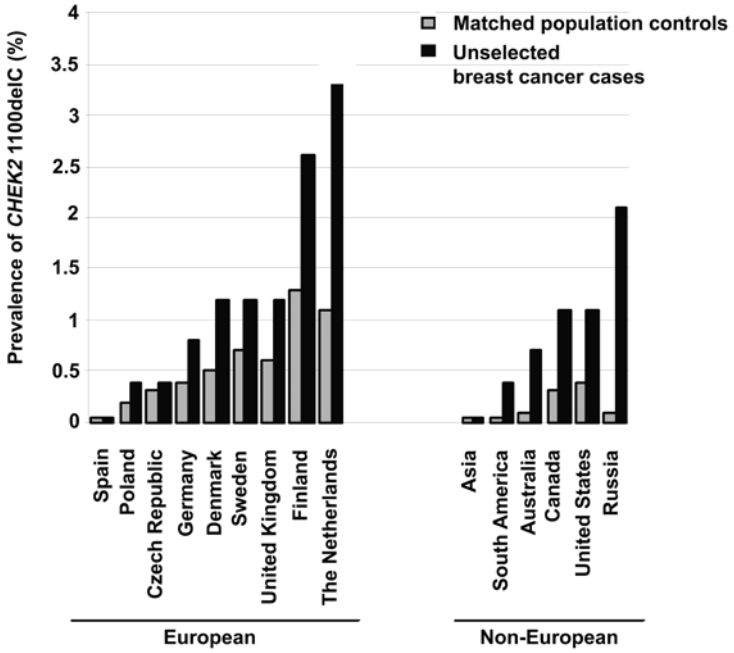

Figure 1.5 Prevalence of *CHEK2* 1100delC in unselected breast cancer cases and matched population controls. The figure has been based on the prevalence of *CHEK2* 1100delC in unselected breast cancer cases and controls as reported in Table 1.2. Depicted are the geographical areas for which more than 200 unselected breast cancer cases and controls have been analysed. The data have been grouped in European and non-European populations with the different populations being arranged at increasing *CHEK2* 1100delC frequencies among unselected breast cancer cases within these groups. Note that the *CHEK2* 1100delC mutation appears most common among Northern and Western European populations, suggesting that the *CHEK2* 1100delC founder mutation originated in these populations. Also note that the *CHEK2* 1100delC mutation frequency among the majority of unselected breast cancer cases is nearly twice as high as in geographically-matched controls, reflecting the estimated 2-fold increased breast cancer risk for female *CHEK2* 1100delC carriers.

incidence between familial breast cancer cases carrying the *CHEK2* 1100delC mutation and familial breast cancer cases negative for the mutation. Consequently, it had been uncertain if the estimated 2-fold *CHEK2* 1100delC breast cancer risk also applies to women without a family history of breast cancer. To evaluate the breast cancer risk of *CHEK2* 1100delC at population level, the CHEK2 Breast Cancer Case-Control Consortium had genotyped *CHEK2* 1100delC in 10,860 unselected breast cancer cases and 9,065 matched controls from five different geographical areas. The *CHEK2* 1100delC mutation was detected in 1.9% of unselected breast cancer cases compared to 0.7% of controls (OR=2.3). Together these analyses had provided

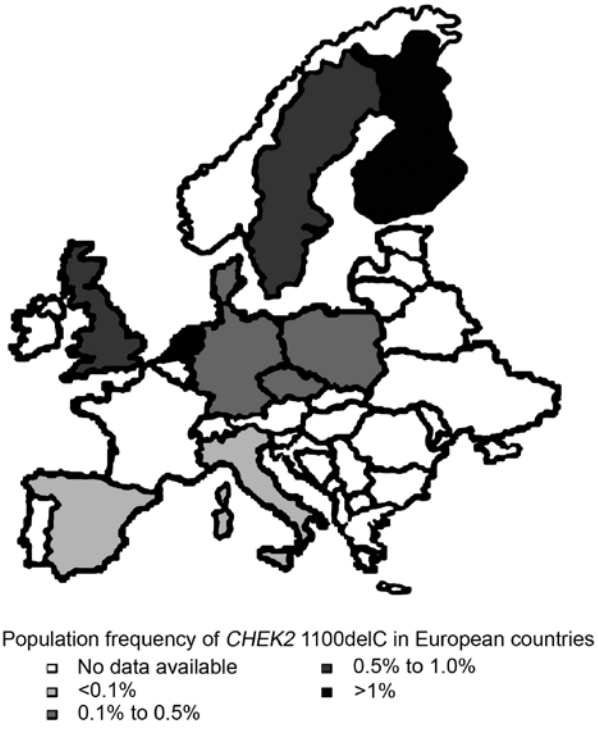

Figure 1.6 Population frequency of *CHEK2* 1100delC in European countries.

conclusive evidence that *CHEK2* 1100delC is associated with an approximately 2-fold increased breast cancer risk for female *CHEK2* 1100delC carriers.[66]

Recently, a meta-analysis of 16 *CHEK2* 1100delC studies has been conducted, including 26,488 breast cancer cases and 27,402 controls of Northern/Eastern European decent.[202] This analysis evaluated the breast cancer risk of *CHEK2* 1100delC among unselected breast cancer cases diagnosed before and after age 51 years and among familial breast cancer cases. In line with the study of the CHEK2 Breast Cancer Case-Control Consortium, an odds ratio of 2.7 was identified for unselected breast cancer cases diagnosed before and after age 51 years.[66] In line with the initial *CHEK2* 1100delC identification studies [59, 66], the *CHEK2* 1100delC mutation conferred an approximately 5-fold increased breast cancer risk for familial breast cancer cases (i.e., OR=4.8). Assuming a cumulative breast cancer risk of 7.8% at age 70 years among the general population, the OR of 4.8 for breast cancer in familial *CHEK2* 1100delC carriers corresponded to a cumulative breast cancer risk of 37% at age 70 years.[202]

CHEK2 1100delC and bilateral breast cancer

In addition to an increased breast cancer risk, it was reported that *CHEK2* 1100delC carriers are at an increased risk for bilateral breast cancer. This was first noted by Vahteristo *et al.*, who reported a 6-fold increased *CHEK2* 1100delC frequency among Finnish bilateral breast cancer cases compared to unilateral breast cancer cases.[65] This observation was confirmed by three Dutch studies, that also found increased risks for bilateral breast cancer among Dutch bilateral breast cancer cases carrying *CHEK2* 1100delC.[204, 208, 245] It was also suggested that the risk of contralateral breast cancer in *CHEK2* 1100delC breast cancer cases may be modified by radiation therapy.[203, 204] This hypothesis had been conceivable as CHEK2 plays an important role in maintaining genomic integrity by facilitating, amongst others, repair of radiation-induced DNA damage. Our research group has participated in an international collaborative study that has evaluated the frequency of *CHEK2* 1100delC among 1,828 bilateral breast cancer cases and 7,030 population controls. This study also found a 6-fold increased risk of *CHEK2* 1100delC among bilateral breast cancer cases as compared to population controls, thus confirming that *CHEK2* 1100delC confers a significant risk for bilateral breast cancer.[246]

CHEK2 1100delC and male breast cancer

In the initial *CHEK2* 1100delC identification report, it had been suggested that *CHEK2* 1100delC might also confer a 10-fold increased risk for male breast cancer (MBC).[59] To date, the *CHEK2* 1100delC mutation has been identified in only 3 of 660 additionally screened MBC cases worldwide, questioning the role of *CHEK2* 1100delC in MBC.[59, 209, 211, 213, 218, 220, 221, 231, 233, 236, 239, 242, 244] To evaluate if *CHEK2* 1100delC is a risk allele for MBC in the Dutch population, we have genotyped *CHEK2* 1100delC in a cohort of 94 MBC cases (chapter 2).

1.8 Tumor and clinical characteristics of *CHEK2* 1100delC breast tumors

Loss of CHEK2 function in *CHEK2* 1100delC breast tumors

Genetic inactivation of tumor suppressor genes generally results in loss of function of the encoded proteins. Immunohistochemical analyses of *CHEK2* 1100delC breast tumors revealed absent and/or reduced CHEK2 protein expression in 80% to 100% of *CHEK2* 1100delC breast tumors. In contrast, only 5% to 15% of non-*CHEK2* 1100delC breast tumors had decreased CHEK2 protein expression, suggesting that the protein-truncating *CHEK2* 1100delC mutation abrogates CHEK2 function.[65, 207] Accordingly, it has been postulated that the *CHEK2* gene is a tumor suppressor gene.

Inactivation of both tumor suppressor alleles is required for complete loss of function of the encoded protein. Knudson's original two-hit hypothesis for inactivation of the *RB1* tumor suppressor gene involved a germline mutation in one allele and (partial) deletion of the chromosomal region containing the other allele (termed loss of heterozygosity (LOH)).[247] Thus far, however, LOH studies in *CHEK2* 1100delC breast tumors had shown inconsistent results with some breast tumors showing loss of the wild-type *CHEK2* allele, whereas others had lost the mutant *CHEK2* 1100delC allele or had no loss at all.[65, 207, 212, 239] Together, these observations had suggested that 22q LOH may not be the main mechanism of inactivation for the *CHEK2* wild-type allele in *CHEK2* 1100delC breast tumors. Possible other inactivation mechanisms for the other *CHEK2* allele include epigenetic inactivation through promoter hypermethylation and the presence of somatic or germline *CHEK2* mutations. Alternatively, the *CHEK2* 1100delC mutation may act in a dominant-negative fashion whereby the mutant protein abrogates the function of the wild-type allele. Several studies have shown that none of these inactivation mechanisms appear to be relevant in *CHEK2* 1100delC-related cancers [200, 248, 249], suggesting that *CHEK2* may not be a classical tumor suppressor gene after all. It had therefore been suggested that *CHEK2* may act by haploinsufficiency, a process in which inactivation of a single allele contributes to tumor progression even though a wild-type allele is present.

ER status and intrinsic subtype of *CHEK2* 1100delC breast tumors

Thus far, three independent studies have evaluated the estrogen receptor (ER) status of *CHEK2* 1100delC tumors.[208, 219, 245] Interestingly, all three studies reported higher frequencies of ER-positive tumors among *CHEK2* 1100delC carriers compared to non-*CHEK2* 1100delC carriers (~90% versus ~70%, respectively). Our research group has recently determined a gene expression profile on 26 *CHEK2* 1100delC breast tumors. One of the outcomes of this analysis had been that all 26 *CHEK2* 1100delC breast tumors could be classified as ER-positive, confirming the association of *CHEK2* 1100delC and positive ER status.[250]

Our profiling study also had shown that, according to the intrinsic gene signatures generated by Perou and colleagues [251-253], all *CHEK2* 1100delC tumors could be classified as luminal subtype breast cancers.[250] The identification of a 40-gene *CHEK2* profile for the *CHEK2* 1100delC breast tumors further suggested biological homogeneity among the *CHEK2* 1100delC breast tumors. This biological homogeneity may suggest that only a few additional cancer susceptibility alleles or only a restricted number of biological pathways are involved in *CHEK2* 1100delC associated breast cancer susceptibility.[250]

Clinical outcome of *CHEK2* 1100delC-associated breast cancer

At present, two studies have evaluated clinical outcome associated with *CHEK2* 1100delC breast cancers.[208, 245] Our research group conducted a prospective analysis including 1,084 unselected

breast cancer cases with a median follow-up of 3.8 years, revealing that distant metastasis had occurred in 13% of non-carriers and 29% of *CHEK2* 1100delC carriers. In addition, at this median follow-up, 92% of non-carriers were still disease-free as opposed to only 44% of *CHEK2* 1100delC carriers. In conclusion, *CHEK2* 1100delC carriers had a worse prognosis regarding disease-free survival and distant metastasis-free survival.[245] These data were confirmed by an independent retrospective study of 1,479 breast cancer cases with a median follow-up of 10.1 years. *CHEK2* 1100delC carriers had a worse metastasis-free survival (local, regional or distant) compared to non-carriers. Additionally, breast cancer-specific survival was worse in *CHEK2* 1100delC carriers compared to non-carriers.[208] Together, both studies show that a positive *CHEK2* 1100delC status is associated with a worse breast cancer prognosis.

1.9 Non-1100delC *CHEK2* mutations and breast cancer

CHEK2 I157T and breast cancer

The isoleucine to threonine missense mutation at codon 157 (I157T) in the FHA domain of the *CHEK2* gene was first identified in a Li-Fraumeni-like case that had been diagnosed with early-onset breast cancer, melanoma and lung cancer.[198] Functional studies showed that the CHEK2 I157T mutant protein was stably expressed and normally phosphorylated upon exposure to ionizing radiation. Yet, CHEK2 I157T mutant proteins appeared to be deficient in binding and/or phosphorylating several downstream targets including p53, CDC25A and BRCA1, suggesting that the I157T mutation abolishes CHEK2 function.[190, 199, 201, 254, 255] The association of *CHEK2* I157T with an increased risk for breast cancer was first reported by Kilpivaara *et al.*, who identified *CHEK2* I157T in 5.3% of population controls compared to 7.4% of unselected Finnish breast cancer cases (Figure 1.7).[254] Soon thereafter, it had been reported that *CHEK2* I157T also confers increased breast cancer risks for Polish, German and Byelorussian unselected breast cancer cases (Figure 1.7).[223, 256] Together, these studies had shown that *CHEK2* I157T is associated with an approximately 1.5-fold increased breast cancer risk. In contrast, however, the *CHEK2* I157T mutation was virtually absent among familial breast cancer cases and population controls from the Netherlands, the United Kingdom and North America [257], suggesting that *CHEK2* I157T, similar to *CHEK2* 1100delC, is confined to specific populations.

CHEK2 IVS2+1G>A and breast cancer

The splice site mutation *CHEK2* IVS2+1G>A is predicted to generate a premature termination codon in exon 3 of the *CHEK2* gene, leading to elimination of part of the FHA domain as well as the entire CHEK2 kinase domain.[258] The mutation was first reported among unselected German breast cancer cases.[256] Yet, the frequency of *CHEK2* IVS2+1G>A in unselected German breast

cancer cases was similar to the 0.3% German population frequency (Figure 1.7). An increased *CHEK2* IVS2+1G>A frequency was however observed in unselected Byelorussian breast cancer cases where the mutation was present in 0.9% of unselected breast cancer cases compared to none of the population controls (Figure 1.7).[256] Further support for the association of *CHEK2* IVS2+1G>A with breast cancer was generated by Cybulski *et al.*, who identified the *CHEK2* IVS2+1G>A mutation in 1.0% of unselected Polish breast cancer cases compared to only 0.4% among the general population. This study suggested that *CHEK2* IVS2+1G>A confers a nearly 2.5-fold breast cancer risk (Figure 1.7).[223]

CHEK2 del5395 and breast cancer

The 5.4-kb genomic *CHEK2* del5395 deletion results in loss of exons 9 and 10 and is predicted to abolish the CHEK2 kinase domain by generating a premature stop codon at residue 381. This deletion was first identified in two high-risk breast cancer families of Czechoslovakian ancestry.[237] Genotyping of *CHEK2* del5395 in unselected Czechoslovakian breast cancer cases and controls subsequently showed that the deletion was present in 1.3% of breast cancer cases and none of the controls (Figure 1.7).[237] The *CHEK2* del5395 deletion had also been identified among 0.9% of unselected Polish breast cancer cases compared to 0.4% of matched-population controls, suggesting that *CHEK2* del5395 carriers have an approximately 2-fold increased breast cancer risk (Figure 1.7).[223]

CHEK2 S428F and breast cancer

The serine to phenylalanine missense mutation at codon 428 (S428F) is localized in the kinase domain of the *CHEK2* gene. The human CHEK2 S428F mutant protein was unable to complement lethality of a deletion in the CHEK2 homologue *Rad53* in the yeast *S. cerevisiae*, suggesting that the *CHEK2* S428F mutation abrogates normal CHEK2 function.[259] The *CHEK2* S428F mutation was identified in 2.9% of unselected Ashkenazi Jewish breast cancer cases that were wild-type for the Ashkenazi Jewish *BRCA1* (185delAG and 5382insC) [260, 261] and *BRCA2* (6174delT) [262] founder mutations. In contrast, the *CHEK2* S428F mutation was only identified in 1.4% of Ashkenazi Jewish controls, suggesting a 2.1-fold increased breast cancer risk for the mutation (Figure 1.7).[259] Interestingly, this mutation was not identified among 768 unselected non-Jewish breast cancer cases suggesting that the *CHEK2* S428F mutation represents a Jewish founder mutation.[237, 259]

CHEK2 mutations and breast cancer in the Netherlands

In sharp contrast to the hundreds of oncogenic mutations reported for the high-risk breast cancer susceptibility genes *BRCA1* and *BRCA2*, thus far only five oncogenic germline *CHEK2*

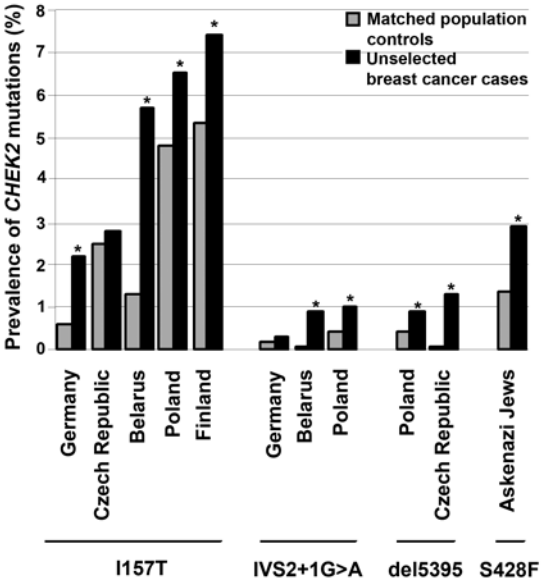

Figure 1.7 Frequency of non-1100delC *CHEK2* mutations among unselected breast cancer cases and matched population controls.
The figure includes data from the 6 reports that have evaluated the prevalence of *CHEK2* I157T, IVS2+1G>A, del5395 and S428F in at least 500 unselected breast cancer cases and controls.[223, 224, 237, 254, 256, 259] The data have been grouped according to four oncogenic *CHEK2* mutations, I157T, IVS2+1G>A, del5395 and S428F. Within each group, the different populations have been arranged at increasing mutation frequencies among breast cancer cases. *, significant difference between unselected breast cancer cases and controls.

mutations have been identified in breast cancer cases: 1100delC, I157T, IVS2+1G>A, del5395 and S428F. Of these oncogenic *CHEK2* mutations, only *CHEK2* 1100delC, I157T and IVS2+1G>A have been evaluated in Dutch breast cancer cases and controls. Genotyping of *CHEK2* I157T has shown that the mutation is virtually absent in the Dutch population (i.e., 0.12% of 803 cancer cases and controls; M. Wasielewski unpublished data).[257] Similarly, the *CHEK2* IVS2+1G>A mutation was not identified among 278 bilateral breast cancer cases and controls (M. Wasielewski unpublished data). In conclusion, thus far, only the *CHEK2* 1100delC mutation has been identified in the Dutch population.

1.10 *CHEK2* mutations and susceptibility to other cancer types

CHEK2 1100delC and colorectal cancer

We had noted that Dutch *CHEK2* 1100delC breast cancer families frequently also included colorectal cancer cases, suggesting that *CHEK2* 1100delC might be associated with a cancer phenotype involving breast cancer and colorectal cancer.[59, 206] For example, the EUR60 family had included six colorectal cancer cases of which four had been diagnosed before the age of 50 years (Figure 1.4). We therefore formulated clinical criteria to identify breast cancer families with hereditary breast and colorectal cancer (HBCC).[206] HBCC families were defined by at least two breast cancer cases in first- or second-degree relatives of whom at least one was diagnosed before age 60 years *and* (1) at least one case with breast and colorectal cancer diagnosed at any age *or* (2) at least one early-onset colorectal cancer case (diagnosed before age 50 years) who was a first- or second-degree relative of a breast cancer case *or* (3) at least two colorectal cancer cases diagnosed at any age of whom at least one was a first- or second-degree relative of a breast cancer case.[206] The EUR60 family represents a prime example of an HBCC family, fulfilling all three criteria for identification of HBCC families (Figure 1.4). Application of these criteria to a cohort of 435 Dutch non-*BRCA1/BRCA2* breast cancer families led to the identification of 55 HBCC families of which 18% was *CHEK2* 1100delC positive compared to only 4% among non-HBCC breast cancer families. These results thus showed that *CHEK2* 1100delC serves as a genetic marker to identify HBCC families. Of course, this raised the question whether *CHEK2* 1100delC also was a colorectal cancer risk allele, independent of its risk for breast cancer. To date, ten independent studies have evaluated the colorectal cancer risk associated with the *CHEK2* 1100delC mutation and some studies indeed suggested that *CHEK2* 1100delC might confer a colorectal cancer risk.[216, 234, 263-270] Yet, due to the moderate cancer risk associated with *CHEK2* 1100delC and the variable prevalence of the mutation among different populations, none of the reported studies had sufficient statistical power to provide evidence that *CHEK2* 1100delC is a colorectal cancer susceptibility allele. By taking advantage of the relatively high Dutch population frequency of *CHEK2* 1100delC and by using a well-characterized cohort of familial colorectal cancer cases, we were the first to conclusively show that *CHEK2* 1100delC indeed confers a colorectal cancer risk (chapter 3).

Non-1100delC *CHEK2* mutations and colorectal cancer

Evaluation of the oncogenic *CHEK2* IVS2+1G>A, del5395 and S428F mutations had shown that none were associated with an increased colorectal cancer risk.[259, 264] In contrast, *CHEK2* I157T has also been identified as a colorectal cancer susceptibility allele. Thus far, the association of *CHEK2* I157T and colorectal cancer has been reported for the Polish and Finnish populations. The *CHEK2* I157T had been identified in 4.8% and 7.1% of Polish population controls and

unselected colorectal cancer cases, respectively. In the Finnish population, the mutation was present in 5.3% of population controls and 7.8% of unselected colorectal cancer cases. Alike the nearly 1.5-fold increased *CHEK2* I157T breast cancer risk [223, 254], both studies estimated that *CHEK2* I157T also confers an approximately 1.5-fold increased colorectal cancer risk.[264, 271, 272]

CHEK2 mutations and prostate cancer

CHEK2 mutations have also been shown to confer increased prostate cancer risks. The first indication that *CHEK2* mutations might be associated with increased prostate cancer risks had been provided by Dong *et al*, who had screened the entire *CHEK2* coding sequence for mutations among 698 prostate cancer cases originating from Northern America. Thirteen unique *CHEK2* mutations were identified, including the well-known *CHEK2* 1100delC, I157T and IVS2+1G>A mutations. Together, these thirteen unique *CHEK2* mutations had been significantly more prevalent among prostate cancer cases compared to controls. The study had, however, been underpowered to detect the prostate cancer risk associated with each individual *CHEK2* mutation.[258] Shortly after the study by Dong *et al*., the *CHEK2* 1100delC and I157T mutations were evaluated in Finnish familial prostate cancer cases, providing borderline evidence that *CHEK2* 1100delC and *CHEK2* I157T confer increased prostate cancer risks.[273] Conclusive evidence that *CHEK2* 1100delC and *CHEK2* I157T are indeed associated with increased prostate cancer risks was generated by Cybulski *et al*. The *CHEK2* 1100delC mutation was identified in 0.8% prostate cancer cases compared to 0.2% of population controls, suggesting an approximately 3.5-fold increased prostate cancer risk. A more moderate ~1.5-fold increased prostate cancer risk was detected for carriers of the *CHEK2* I157T mutation. This mutation had been identified among 7.6% of prostate cancer cases and 4.8% of population controls. The same cohort of Polish unselected prostate cancer cases and population controls had also been genotyped for *CHEK2* del5395 and *CHEK2* IVS2+1G>A. Both mutations were, however, not associated with an increased prostate cancer risk.[271, 274]

CHEK2 mutations and other cancers

Cancer risks associated with *CHEK2* mutations have been studied mainly in breast cancer, colorectal cancer and prostate cancer cases. It had however been suggested that *CHEK2* mutations may predispose to cancers at other anatomical sites. A landmark study in this area was the study by Cybulski *et al*., that evaluated *CHEK2* 1100delC, I157T and IVS2+1G>A in a cohort of 4,008 unselected cancer cases from 13 different cancer types and 4,000 controls. Cancers of the kidney and thyroid were associated with the *CHEK2* I157T and IVS2+1G>A mutations, respectively. In addition, it was also suggested that *CHEK2* I157T predisposes to thyroid cancer and that *CHEK2* IVS2+1G>A confers increased risks for kidney cancer, but these associations

had been of borderline significance.[271] Together, these results had suggested that *CHEK2* acts as multiorgan susceptibility gene.[182, 271, 272]

In summary, *CHEK2* mutations have been associated with increased risks for cancers of the breast, colon, prostate, kidney and thyroid. Yet, it may well be that the spectrum of *CHEK2*-associated cancer risks is much broader, as different populations have specific *CHEK2* founder mutations and distinct *CHEK2* mutations may be associated with different cancer types. Establishment of the full range of *CHEK2*-associated cancer risks therefore requires whole *CHEK2* gene screens among large cohorts of cancer cases and controls from different populations.

1.11 *CHEK2* 1100delC and polygenic breast cancer susceptibility

Two peculiar observations were made during analysis of *CHEK2* 1100delC breast cancer families. First, although classified as a moderate-risk breast cancer susceptibility allele, the *CHEK2* 1100delC mutation typically associated with breast cancer families that had a high-risk breast cancer inheritance pattern (i.e., similar to those observed for *BRCA1* and *BRCA2* families).[59, 65, 66, 206] For example, in our Dutch breast cancer family cohort, the prevalence of *CHEK2* 1100delC increased from 3.5% among unselected breast cancer cases to >10% among breast cancer families with at least three breast cancer cases diagnosed before age 60 years.[66, 206, 207] Second, the *CHEK2* 1100delC mutation only partially segregated with the disease phenotype in *CHEK2* 1100delC breast cancer families.[59, 65, 206, 207] For instance, two of eight evaluated breast cancer cases in the EUR60 family had been negative for *CHEK2* 1100delC (Figure 1.4, third generation). These two cases were diagnosed with breast cancer at ages 37 years and 38 years, respectively. The absence of *CHEK2* 1100delC in these two early-onset cases had been suggestive for the presence of germline mutations in other, putative breast cancer susceptibility genes. The observation of incomplete genotype/phenotype segregation had also been consistent with the weak LOD score that we identified in the linkage analysis of the EUR60 family (section 1.7). Together, the association of *CHEK2* 1100delC with high-risk breast cancer families and the observed incomplete *CHEK2* 1100delC genotype/phenotype association had suggested that other as-yet unknown putative breast cancer susceptibility allele(s) might also be inherited in *CHEK2* 1100delC breast cancer families. Such polygenic model could account for the observed incomplete genotype/phenotype segregation as well as the differences in prevalence of *CHEK2* 1100delC among low-risk and high-risk breast cancer families. In addition, it has been hypothesized that *CHEK2* 1100delC may act in concert with the additional putative cancer risk allele(s) and that *CHEK2* 1100delC and these putative cancer risk alleles operate in biologically-related pathways. Currently, none of the putative additional cancer risk alleles involved in the polygenic *CHEK2* 1100delC model have been identified. In part III of this thesis, we have evaluated

the *MDM2* SNP309 allele and the *MUTYH* gene for involvement in the polygenic CHEK2 cancer model (chapters 6 and 7).

1.12 Aims and outline of this thesis

Familial breast cancer comprises about 15-25% of all breast cancer cases and in the majority of these cases the genetic cause underlying the disease is unknown. The *CHEK2* 1100delC mutation had been identified as the first moderate-risk breast cancer susceptibility allele, conferring an approximately 2-fold increased breast cancer risk for female carriers. Although classified as a moderate-risk breast cancer susceptibility allele, the mutation typically was identified in breast cancer families with a high-risk breast cancer inheritance pattern. Moreover, the mutation only partially segregated with the breast cancer phenotype in *CHEK2* 1100delC positive families. Together, these two peculiarities have suggested that *CHEK2* 1100delC acts in concert with other as-yet-unknown cancer susceptibility genes to confer high cancer risks.

The main aims of this thesis were: first, to evaluate the association of *CHEK2* 1100delC with male breast cancer and familial colorectal cancer; second, to gain insight in the CHEK2-p53 tumor suppressor pathway through mutation analyses of the *CHEK2* gene and its downstream target *p53* in human breast cancer cell lines; and third, to identify genes involved in polygenic *CHEK2* 1100delC cancer susceptibility by using a candidate gene approach.

Chapter 1 of this thesis comprises a general introduction in familial breast cancer, familial colorectal cancer and the *CHEK2* gene. Furthermore, the relation between defects in DNA damage repair pathways and cancer susceptibility has been addressed.

Part I of the results concerns the evaluation of cancer phenotypes associated with the *CHEK2* 1100delC mutation. The association of *CHEK2* 1100delC with male breast cancer was studied in chapter 2 whereas chapter 3 describes the risk of *CHEK2* 1100delC for familial colorectal cancer.

Part II of the results focuses on the CHEK2-p53 tumor suppressor pathway. A mutation analysis of the *p53* and *CHEK2* genes in human breast cancer cell lines is described in chapter 4 and chapter 5, respectively.

Part III of the results focuses on the identification of putative risk alleles involved in the polygenic CHEK2 cancer model by using a candidate gene approach, involving evaluation of the *MDM2* SNP309 allele (chapter 6) and the *MUTYH* Y179C, G396D and P405L mutations (chapter 7).

The thesis is concluded with a general discussion regarding the polygenic CHEK2 cancer model, the use of genetic *CHEK2* 1100delC screening and perspectives for future *CHEK2* research (chapter 8). The studies described in this thesis are summarized in chapter 9.

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CHAPTER 2

CHEK2 1100delC and male breast cancer in the Netherlands

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Abstract

Mutations in the breast cancer susceptibility genes *BRCA1*, *BRCA2* and *CHEK2* are known risk factors for female breast cancer. Mutations in *BRCA1* and *BRCA2* also are associated with male breast cancer (MBC). Similarly, it had been suggested in the original *CHEK2* identification report that the *CHEK2* 1100delC mutation confers an increased risk for MBC. Here, we have evaluated the risk of *CHEK2* 1100delC for MBC by genotyping *CHEK2* 1100delC in 23 familial and 71 unselected Dutch MBC cases. None of the 23 familial MBC cases carried the *CHEK2* 1100delC mutation. In contrast, *CHEK2* 1100delC was present in 3 of the 71 (4.2%) unselected MBC cases, which was significantly more prevalent than the 1.1% Dutch population frequency assessed in 1692 individuals (P=0.05, OR=4.1, 95%CI 1.2-14.3). Our data suggest that, in the Netherlands, *CHEK2* 1100delC is associated with an increased risk for MBC.

Introduction

Male breast cancer (MBC) is a rare disease that accounts for less than 1% of all breast cancers. Major risk factors for MBC are Klinefelter syndrome, disorders associated with high estrogen levels, testicular disorders and a family history of breast cancer. Germline mutations in the breast cancer susceptibility genes *BRCA2* and *BRCA1* account for approximately 4-14% of familial MBC cases, with higher frequencies found in *BRCA2* founder populations.[1] Apart from *BRCA2* and *BRCA1* mutations, mutations in the androgen receptor, the *CYP17* and the *PTEN* genes have also been implicated in MBC.[1-3] Yet, it is still unclear what the underlying predisposition in the majority of MBC cases is.

In 2002, we and others identified the *CHEK2* 1100delC mutation as a breast cancer susceptibility allele.[4, 5] We estimated that female *CHEK2* 1100delC carriers had a 2-fold increased breast cancer risk compared with non-carriers, classifying the mutation as a moderate-risk breast cancer susceptibility allele.[6] In addition to a female breast cancer risk, four of 33 (12%) index cases of families with at least one MBC case were *CHEK2* 1100delC positive suggesting that the mutation also might confer a risk for MBC.[4] Thus far, the prevalence of *CHEK2* 1100delC in MBC has been evaluated in twelve additional studies.[7-18] Yet, only 3 of 627 genotyped samples carried the *CHEK2* 1100delC mutation, raising doubts on the association of *CHEK2* 1100delC with MBC. Evaluation of *CHEK2* 1100delC in MBC is however severely hampered by the moderate cancer risks associated with *CHEK2* 1100delC, the large differences in *CHEK2* 1100delC population frequencies and the rarity of the MBC phenotype.

In the Netherlands, the relatively high *CHEK2* 1100delC population frequency of 1.1% previously had allowed the identification of *CHEK2* 1100delC as a breast cancer allele and a colorectal cancer allele.[4, 19, 20] Here, we have exploited our unique opportunity to assess *CHEK2* 1100delC-associated cancer risks by screening *CHEK2* 1100delC in 94 Dutch MBC cases.

Materials and methods

Male breast cancer cases and controls

The evaluated MBC cohort consisted of 23 familial and 71 unselected MBC cases, together including all available MBC cases registered at Erasmus MC. All familial breast cancer cases were ascertained through the Rotterdam Family Cancer Clinic at Erasmus MC (Table 2.1). A family history of MBC was defined by the presence of at least two breast cancer cases in first or second-degree relatives of whom at least one was a man (21 families) or by the presence of at least one MBC case who had two or more first-degree relatives diagnosed with cancer types other than breast carcinoma (2 families). Screening of all 23 MBC families had identified *BRCA2* and *BRCA1* germline mutations in five and three families, respectively. The *CHEK2* 1100delC

Table 2.1 Inclusion criteria of familial MBC cases.

Inclusion criteria MBC families	Mutation status		
	Non-*BRCA1/2*	*BRCA1*	*BRCA2*
> 1 MBC with > 1 FBC 1st/2nd DGR (MBC tested)	9	1	2
> 1 MBC with > 1 FBC 1st/2nd DGR (BRC in 1st DGR tested)	4	2	3
> 1 MBC with > 2 non-BRC 1st DRG (MBC tested)	2	-	-
Total (n=23)	15	3	5

FBC, female breast cancer; MBC, male breast cancer; non-BRC, cancer types other than breast carcinoma; 1st/2nd DGR's, first and/or second-degree relative

mutation was tested in blood-derived DNA of 14 MBC cases and in 17 first-degree relatives of nine MBC cases of whom no DNA was available (Table 2.1). Unselected MBC cases included all available MBC cases from the Erasmus MC Pathology archive (16 paraffin-embedded tumors), the Rotterdam Family Cancer Clinic (3 blood-derived DNA samples) and the Rotterdam Medical Oncology Tumor Bank (52 fresh-frozen tumors). None of the unselected MBC cases had been screened for *BRCA1* or *BRCA2* mutations. Of note, 25 fresh-frozen MBC tumors had also been part of a previous study that had evaluated the clinical relevance of steroid hormones and factors involved in the urokinase system among male and female breast cancers.[21] The Medical Ethical Review Board at Erasmus MC has approved the study, which was carried out according the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl/).

Population controls were previously reported and included a hospital-based cohort of 967 spouses of heterozygous cystic fibrosis mutation carriers registered by the department of Clinical Genetics at Erasmus MC, a population-based cohort of 460 persons without cancer after age 55 years from the Erasmus Rotterdam Health and the Elderly Study (ERGO) and a cohort of 265 randomly-selected blood donors.[6, 22]

CHEK2 1100delC genotyping

CHEK2 1100delC genotyping was performed on DNA by allele-specific oligonucleotide hybridization, with confirmation of mutant cases by long-range PCR and sequencing of an independent template, as described.[4]

Statistics

The difference between *CHEK2* 1100delC frequency in cancer cases and controls among *CHEK2* 1100delC carriers versus non-carriers was tested by using Fisher's Exact Test. Odds ratios (OR) and corresponding 95% confidence intervals (95%CI) were calculated using Woolf's method. All statistical analyses were performed with STATA statistical package, release 10 (STATA Corp, College Station, TX).

Results and discussion

Genotyping of *CHEK2* 1100delC identified three of 94 MBC cases (3.2%) as heterozygous carriers of the *CHEK2* 1100delC mutation (Table 2.2). All three *CHEK2* 1100delC cases were identified among the 71 unselected MBC cases (4.2%, Table 2.2), which was significantly more prevalent compared with the 1.1% Dutch population frequency (P=0.05, OR=4.1, 95%CI 1.2-14.3). Our results thus suggest that *CHEK2* 1100delC is associated with an increased risk for MBC in the Dutch population.

Age at diagnosis and cytosolic estrogen receptor (ER) and progesterone receptor (PR) levels were available for 50 unselected MBC cases including all three *CHEK2* 1100delC MBC cases. The

Table 2.2 Summary of reported *CHEK2* 1100delC genetic screens in male breast cancer cases.

Ref.	Population	*CHEK2* 1100delC+ / Total tested (%)							
		Population controls		Unselected MBC cases		Familial MBC cases		All MBC cases	
Present study	NL	18/1692	(1.1)	3/71	(4.2)	0/23		3/94	(3.2)
4	UK/NL/US/Germany	18/1620	(1.1)			4/33	(12.1)	4/33	(12.1)
13,18	Finland	26/1885	(1.4)	2/132	(1.5)	0/18		2/150	(1.3)
9,14,17	UK	20/3749	(0.5)	0/79		1/90	(1.1)	1/169	(0.6)
11,14,15	USA	8/2261	(0.4)	0/109		0/19		0/128	
16	Israel	0/146		0/31		0/23		0/54	
10	Italy	0/263		0/77		0/25		0/102	
8	Germany	6/1315	(0.5)			0/12		0/12	
12	Australia	1/736	(0.1)[a]			0/7		0/7	
7	South Korea	n.a.				0/5		0/5	
	Total			5/499		5/255		10/754	

The summary includes data from the present study and 12 studies that have thus far screened *CHEK2* 1100delC in MBC cases. NL, the Netherlands; UK, United Kingdom; USA, United States of America; MBC, male breast cancer; [a], controls were obtained from reference 6; n.a., not available.

CHEK2 1100delC MBC cases were diagnosed with breast cancer at age 64, 66 and 73 years, which was not different from the average age at diagnosis of 67 years among 47 *CHEK2* 1100delC negative MBC cases. All three *CHEK2* 1100delC cases were ER-positive as were 41 of 47 (87%) *CHEK2* 1100delC negative cases. Similar, two of the three *CHEK2* 1100delC MBC cases (67%) were PR-positive compared to 37 of 47 (78%) *CHEK2* 1100delC negative cases. Clinical follow-up including tumor stage, lymph node involvement, local and/or distant metastasis data were available for two *CHEK2* 1100delC and 33 non-*CHEK2* 1100delC unselected MBC cases. Overall, the median follow-up time of MBC cases still alive was 47 months (range 2-165 months). The first *CHEK2* 1100delC case was diagnosed with a lymph node-negative T1 breast tumor and the second case had a lymph node-positive T4 breast tumor. Both cases died of bone metastasis, 24 and 36 months after their diagnosis, respectively. In contrast, distant metastasis at diagnosis and during follow-up was reported in 12 of 33 (36%) *CHEK2* 1100delC negative MBC cases and only two of these had a sole relapse to the bone. In agreement with previous reports on bone metastatic disease and ER status, all four MBC cases diagnosed with bone metastasis were ER-positive, affirming the strong association between relapse to bone and positive ER status.[23, 24]

In the initial *CHEK2* 1100delC identification report, four unrelated *CHEK2* 1100delC cases had been identified among 33 (12%) high-risk non-*BRCA1/BRCA2* MBC families, suggesting an estimated 10-fold increased MBC risk for *CHEK2* 1100delC MBC carriers.[4] Yet, here we did not identify *CHEK2* 1100delC among the 23 familial MBC cases analyzed. The analyzed familial MBC cohort included eight confirmed *BRCA1/BRCA2* positive MBC cases (Table 2.1). These cases were *a priori* unlikely to carry *CHEK2* 1100delC as the *CHEK2* 1100delC mutation is quite uncommon among *BRCA1/BRCA2* mutation carriers.[4, 6] Additionally, four non-*BRCA1/BRCA2* families had been included for which the MBC case was not available for *CHEK2* 1100delC genotyping (Table 2.1). As a whole, we thus had 11 non-*BRCA1/BRCA2* familial MBC cases available for *CHEK2* 1100delC analysis, a too low number to establish a possible correlation between *CHEK2* 1100delC and familial non-*BRCA1/BRCA2* breast cancer.

In addition to our previous 4 out 33 MBC cases [4], thus far, only three *CHEK2* 1100delC cases have been identified worldwide among the additional 627 MBC cases genotyped (Table 2.2).[7-18] In contrast to the initial *CHEK2* 1100delC identification report [4] and our current report, this low frequency of 0.5% (3 of 627 MBC cases) had suggested that *CHEK2* 1100delC is not a risk allele for MBC. However, most of the reported studies had low *CHEK2* 1100delC population frequencies and also had used small MBC cohorts [4, 7-18], resulting in a lack of statistical power to detect *CHEK2* 1100delC-associated cancer risks. Interestingly, the Finnish *CHEK2* 1100delC MBC study had comparable power to our study but failed to identify a *CHEK2* 1100delC related MBC risk.[13, 18] Similarly, we previously had identified *CHEK2* 1100delC as a risk allele for Dutch colorectal cancer cases whereas no such risk could be detected for Finnish colorectal cancer cases.[20, 25] These observations might suggest the presence of additional susceptibility alleles in the Dutch population that are absent in the Finnish population. One might speculate that the same putative Dutch cancer susceptibility allele acts in synergy with *CHEK2* 1100delC to

confer a higher risk for colorectal cancer as well as male breast cancer. Or, are there two or more cancer susceptibility alleles present in the Dutch population that, together with *CHEK2* 1100delC, are responsible for the Dutch *CHEK2* 1100delC phenotype of female breast cancer, male breast cancer and colorectal cancer?

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CHAPTER 3

CHEK2 1100delC is a susceptibility allele for HNPCC-related colorectal cancer

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Abstract

The pathogenic *CHEK2* 1100delC variant is firmly established as a breast cancer susceptibility allele. Dutch *CHEK2* 1100delC breast cancer families frequently also include colorectal cancer cases and the variant is particularly prevalent among breast cancer families with hereditary breast and colorectal cancer. Yet, it is still unclear whether *CHEK2* 1100delC also confers a colorectal cancer risk independent of its breast cancer risk. *CHEK2* 1100delC was genotyped in the index cases of 369 Dutch colorectal cancer families that had been excluded for familial breast cancer. The cohort included 132 cases with familial adenomatous polyposis (FAP) and FAP-related disease and 237 cases with hereditary non-polyposis colorectal cancer (HNPCC) and HNPCC-related disease[†]. None of the FAP/FAP-related cases carried the *CHEK2* 1100delC variant. In contrast, *CHEK2* 1100delC was present in 10 of 237 (4.2%) HNPCC/HNPCC-related cases which was significantly more prevalent than the 1.0% Dutch population frequency (OR=4.3; 95%CI, 1.7-10.7; *P*=0.002). Nine of the ten *CHEK2* 1100delC colorectal cancer cases met the revised Amsterdam and/or Bethesda criteria. The ten *CHEK2* 1100delC colorectal cancer families had a high-risk cancer inheritance pattern, together including 35 colorectal cancer cases, 9 cases with polyps, and 21 cases with other tumor types. Our analysis provides strong evidence that the 1100delC variant of *CHEK2* confers a colorectal cancer risk in HNPCC/HNPCC-related families, supporting the hypothesis that *CHEK2* is a multiorgan cancer susceptibility gene.

[†] During the course of this thesis, the term HNPCC has been replaced by Lynch syndrome, referring to cancer families with a germline oncogenic mutation in one of the mismatch repair (MMR) genes and/or evidence for microsatellite instability (MSI) in one of the cancers. All published chapters in this thesis refer to HNPCC and/or HNPCC-related disease with the first referring to Lynch syndrome families and families that fulfill the revised Amsterdam criteria and the latter referring to non-Lynch syndrome families (and families that do not fulfill the revised Amsterdam criteria). For clarity and consistency, the terms HNPCC and/or HNPCC-related disease have also been used in all unpublished parts of this thesis, including this chapter.

Introduction

In 2002, we and others identified *CHEK2* 1100delC (MIM 604373) as a breast cancer susceptibility allele.[1-4] The *CHEK2* 1100delC variant was present in 5% of non-*BRCA1/BRCA2* breast cancer families as compared to only 1% in the general population. Although variants with a population frequency of 1% or more are often considered polymorphisms, the estimated 2-fold increased breast cancer risk for female *CHEK2* 1100delC carriers had classified *CHEK2* 1100delC as a pathogenic moderate-risk mutation. Strikingly, the prevalence of *CHEK2* 1100delC increased with increasing numbers of breast cancer cases in the families, suggesting a rather high-risk breast cancer inheritance pattern for the variant. Also, the *CHEK2* 1100delC genotype only partially segregated with the breast cancer phenotype, particularly so in families that were more severely affected with breast cancer. Together, these apparent discrepancies have suggested a polygenic breast cancer susceptibility model for the *CHEK2* 1100delC variant, in which *CHEK2* 1100delC acts in concert with another as-yet-unknown breast cancer susceptibility allele or alleles.[1-5]

We had noted that near half of Dutch *CHEK2* 1100delC breast cancer families also included colorectal cancer cases (11 of 25 families, with together 21 cases).[1, 6] Indeed, when we applied stringent clinical criteria to define a new subtype of breast cancer families with a "hereditary breast and colorectal cancer" (HBCC) phenotype, the *CHEK2* 1100delC frequency was almost five times higher among the HBCC families as compared to breast cancer families not fulfilling the HBCC criteria (18% versus 4%).[6] These results thus suggested that *CHEK2* 1100delC carriers may also be predisposed to develop colorectal cancer. To evaluate the colorectal cancer risk conferred by *CHEK2* 1100delC independent of its breast cancer risk, we here have genotyped *CHEK2* 1100delC in 369 Dutch familial colorectal cancer cases.

Materials and methods

Colorectal cancer families

The selected familial colorectal cancer cohort (n=385) included all colorectal cancer families registered by the Dutch Foundation for Detection of Hereditary Tumors (STOET) and those that were registered by the Rotterdam Family Cancer Clinic at Erasmus MC. Sixteen HNPCC-related families had been excluded because they met our criteria for familial breast cancer (i.e., at least two first or second degree relatives (DGRs) with breast cancer of whom at least one was diagnosed before age 60 years). The evaluated STOET cohort (cohort I) included 114 HNPCC/HNPCC-related and 91 FAP/FAP-related families that had been reported as part of another *CHEK2* 1100delC study.[6] The evaluated Erasmus MC cohort (cohort II) included 123 HNPCC/HNPCC-related and 41 FAP/FAP-related families. All cases have consented to search for cancer susceptibility genes and the Medical Ethical Review Board at Erasmus MC has approved the study.

Population controls

The 909 population-matched controls all originated from the Southwestern Netherlands and included a hospital-based cohort of 184 spouses of heterozygous cystic fibrosis mutation carriers ascertained through the department of Clinical Genetics at Erasmus MC, a population-based cohort of 460 persons without cancer after age 55 years from the Erasmus Rotterdam Health and the Elderly Study (ERGO) and a cohort of 265 randomly-selected blood donors.[5]

Mutation screening of colorectal cancer families

The complete coding sequences of the *APC*, *MUTYH*, *MLH1*, *MSH2* and *MSH6* genes were analyzed for mutations, including all known Dutch founder mutations, deletions and rearrangements, in a routine clinical genetics diagnostic setting, as described.[6, 7] For each family, mutation screening was performed in blood-derived DNA of at least the colorectal cancer case with the youngest age at diagnosis for whom DNA was available (index case). *CHEK2* 1100delC screening was performed on blood-derived DNA of the index case by allele-specific oligonucleotide hybridization. This method includes PCR amplification of *CHEK2* (ENSG00000183765) exon 10, application of the amplified products to nylon filters and hybridization under high stringency to [^{32}P] oligonucleotides that are complementary to the *CHEK2* 1100delC or *CHEK2* wild-type sequence. Amplification of exons 9 through 14 by long-range PCR and subsequent sequencing of a nested PCR product using primers flanking exon 10 confirmed all positive *CHEK2* 1100delC cases. *CHEK2* 1100delC screening methods are described in detail in reference 1.

Statistical analysis

The difference between *CHEK2* 1100delC carriers in colorectal cancer cases and controls was analyzed using Fisher's Exact Test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated from two-by-two tables. All analyses were performed with SPSS version 11.

Results and discussion

Characterisation of colorectal cancer families

The 369 colorectal cancer families screened for *CHEK2* 1100delC were classified as familial adenomatous polyposis (FAP, MIM 175100) and FAP-related families or as hereditary non-polyposis colorectal cancer (HNPCC, MIM 120435) and HPNCC-related families. Of the 132 FAP/FAP-related families, 86 (65%) had a pathogenic germline mutation in *APC* (MIM 175100), ten (8%) had a pathogenic germline mutation in *MUTYH* (MIM 604933) and another 36 (27%) families included

cases that had been diagnosed with more than 20 adenomatous polyps. The 237 HNPCC/HNPCC-related families were classified according to the presence of a pathogenic germline mismatch repair (MMR) gene mutation or otherwise by distribution and mode of inheritance of colorectal cancer cases in the family. The 83 MMR mutation positive families included 38 (46%) *MLH1* (MIM 120436), 34 (41%) *MSH2* (MIM 120435) and 11 (13%) *MSH6* (MIM 600678) mutation positive HNPCC families. Analysis of microsatellite instability (MSI) was performed in approximately one-third of these families showing that 22 (81%) index cases had an MSI-high, 4 (15%) had an MSI-low and one (4%) had an MSI-stable phenotype. Extensive mutational screening of *MLH1*, *MSH2* and *MSH6* had failed to identify pathogenic mutations, deletions or rearrangements in 154 HNPCC/HNPCC-related families. These 154 MMR mutation negative families were classified by either the presence of at least two first-degree relatives (DGRs) with colorectal cancer of whom at least one was diagnosed before age 50 years (n=95, 62%) *or* by the presence of at least two first DGRs with colorectal cancer but none of them diagnosed before age 50 years (n=36, 23%) *or* by the presence of a single colorectal cancer case diagnosed before age 50 years (n=23, 15%). MSI data was available for 100 (65%) MMR mutation negative HNPCC/HNPCC-related families. The majority of index cases (74%) among these families had an MSI-stable phenotype. MSI-high and MSI-low phenotypes were reported for 18% and 8% HNPCC/HNPCC-related families, respectively. Fifty-eight (38%) of the MMR mutation negative families were classical HNPCC families as they fulfilled the revised Amsterdam criteria, and another 73 (47%) families had index cases that met the revised Bethesda criteria for HNPCC screening.[8, 9] The cancer incidences among the MMR mutation negative HNPCC/HNPCC-related families are detailed in Table 3.1, showing that the majority of families had at least three colorectal cancer cases and thus can be assigned as high-risk colorectal cancer families.

Table 3.1 Cancer incidences among MMR mutation negative HNPCC/HNPCC-related families.

	Number of families	Number of cases with cancer				
		CRC > 1960*	CRC < 1960*	Polyps	BRC	Other cancers
Families with colorectal cancer:	154	488	53	200	52	279
One case with CRC	11	11	4	3	4	21
Two cases with CRC	54	108	12	50	19	85
Three cases with CRC	39	117	12	48	9	52
Four cases with CRC	28	112	15	53	13	62
Five or more cases with CRC	22	140	10	46	7	59

Data include all cancer cases of families negative for mutations in the mismatch repair genes *MLH1*, *MSH2* and *MSH6*. We considered an amnestic report of colorectal cancer more reliable when the diagnosis had been made after 1960 (*). Families were therefore classified according to the number of colorectal cancer cases diagnosed after 1960, showing that the majority of families have at least three colorectal cancer cases. The eleven single colorectal cancer cases were all diagnosed before age 50 years and after 1960. CRC, colorectal cancer; Polyps, adenomatous polyps in the colorectum; BRC, breast cancer.

CHEK2 1100delC confers HNPCC/HNPCC-related colorectal cancer

Genetic screening for *CHEK2* 1100delC revealed that none of 132 FAP/FAP-related cases carried the variant (Table 3.2). In contrast, 10 of 237 (4.2%) HNPCC/HNPCC-related cases carried *CHEK2* 1100delC as compared to only 9 of 909 (1.0%) of population controls, implying a strong association of the variant with familial colorectal cancer (*P*=0.002, Table 3.2). Our results thus indicate that *CHEK2* 1100delC confers a colorectal cancer risk. Moreover, we show that this risk is associated with HNPCC/HNPCC-related colorectal cancer rather than FAP/FAP-related colorectal cancer.

Progressive insight in *CHEK2* cancer susceptibility has revealed that various *CHEK2* gene variants confer only low to moderate cancer risks and that the prevalences of these variants are highly variable among populations. These characteristics severely hamper evaluation of

Table 3.2 Prevalence of *CHEK2* 1100delC among colorectal cancer families from the Southwestern Netherlands.

Case cohorts	***CHEK2* 1100delC+ / Total tested (%)**					
	Cohort I		**Cohort II**		**Cohort I+II**	
Controls					9/909	(1.0)
Families FAP/FAP-related families	0/91	(0.0)	0/41	(0.0)	0/132	(0.0)
APC mutation positive	0/64		0/22		0/86	
MUTYH mutation positive	0/6		0/4		0/10	
>20 polyps	0/21		0/15		0/36	
HNPCC/HNPCC-related families	4/114	(3.5)	6/123	(4.9)	10/237	(4.1)
MLH1 mutation positive	1/29		0/9		1/38	
MSH2 mutation positive	0/26		0/8		0/34	
MSH6 mutation positive	1/4		0/7		1/11	
>1 CRC <50y	not included		2/23		2/23	
≥2 CRC 1st DGRs	not included		3/36		3/36	
≥2 CRC 1st DGRs with ≥1 CRC <50y	2/55		1/40		3/95	
P-value [HNPCC (-related) vs. Controls]	0.05		0.005		0.002	

Cohort I, cohort of colorectal cancer cases registered at the STOET; data on cohort I have also been published in reference 6. Cohort II, cohort of colorectal cancer cases registered at Erasmus MC. FAP, familial adenomatous polyposis; HNPCC, hereditary non-polyposis colorectal cancer; CRC, colorectal cancer; <50y, diagnosed before age 50 years; 1st DGRs, first degree relatives.

CHEK2 cancer risks. In an initial screen for *CHEK2* 1100delC in familial colorectal cancer cases, for example, we had found some suggestion of an HNPCC/HNPCC-related colorectal cancer risk for the variant.[6] However, the familial colorectal cancer cases in this screen had been collected through the International Concerted Action Polyp Prevention (CAPP) and the Dutch Foundation for Detection of Hereditary Tumors (STOET), including 114 Dutch cases, 50 German cases, 37 American cases and several smaller cohorts from other countries. In retrospect, this case cohort was not only too divers regarding its geographical origins but also had not appropriately been stratified for population controls. The case cohort had included six *CHEK2* 1100delC mutant colorectal cancer cases, of which four originated from the Netherlands and the other two from Germany. When now properly compared to the Dutch population frequency of 1.0%, the 3.5% frequency of *CHEK2* 1100delC among the 114 Dutch HNPCC/HNPCC-related cases was significantly increased (P=0.05; Cohort I in Table 3.2). In the current study, we sought to further substantiate these initial findings and collected all available familial colorectal cancer cases from the Rotterdam Family Cancer Clinic at Erasmus MC. Indeed, *CHEK2* 1100delC was again identified in HNPCC/HNPCC-related cases but not in FAP/FAP-related cases, with six *CHEK2* 1100delC cases among 123 HNPCC/HNPCC-related cases (4.9% and P=0.005; Cohort II in Table 3.2). Clearly, reliable evaluation of *CHEK2* cancer risks requires sufficiently large and homogeneous case cohorts that are appropriately matched for population controls. Upon combining both Dutch colorectal cancer case cohorts, we were now able to provide strong evidence that *CHEK2* 1100delC confers a colorectal cancer risk (4.1% and P=0.002; Table 3.2).

To date, the colorectal cancer risk of *CHEK2* 1100delC has been evaluated in 908 familial colorectal cancer cases and in 2740 unselected colorectal cancer cases, derived from ten independent studies (reviewed in Table 3.3).[10-19] As a whole, there was some suggestion that *CHEK2* 1100delC may confer a colorectal cancer risk. Yet, none of the reported studies had sufficient statistical power to show, beyond doubt, that *CHEK2* 1100delC is associated with colorectal cancer. Unfortunately, the inclusion criteria for the colorectal cancer cases differed substantially among the reported studies and *CHEK2* 1100delC population frequencies were also highly variable, inducing us to refrain from meta-analysis of the combined results. Our current study therefore is the very first to establish an HNPCC/HNPCC-related colorectal cancer risk for the *CHEK2* 1100delC variant. One other *CHEK2* variant (I157T) has also been associated with colorectal cancer, in both the Finnish and the Polish populations.[10, 11, 20] The colorectal cancer risk of *CHEK2* is thus not limited to the 1100delC variant. Similar to what has recently been done for other breast cancer genes [21-25], the full spectrum of *CHEK2* associated cancer risks should therefore be established by whole *CHEK2* gene screens, in large sample cohorts that preferably also include cancer cases of other tumor types.

Table 3.3 Summary of reported *CHEK2* 1100delC genetic screens in colorectal cancer cases.

Area (Ref)	*CHEK2* 1100delC+ / Total Tested (%)							
	Population controls		Familial cases with CRC		OR (95% CI)	Non-familial cases with CRC		OR (95% CI)
NL-SW (present study)	9/909	(1.0)	10/237	(4.2)	4.3 (1.7-10.7)			
UK-SE (17)	21/4037	(0.5)†	3/149	(2.0)	3.9 (1.2-13.3)*			
Finland (16)	~36/1885	(1.9)	2/149	(1.3)	0.7 (0.2-2.9)*	15/513	(2.9)	1.5 (0.8-2.8)
NL-N (12)	1/230	(0.4)	7/236	(3.0)	7.0 (0.9-57.4)*	5/498	(1.0)	2.3 (0.3-20.0)*
Spain (19)	0/400	(0.0)	0/182	(0.0)	-			
Sweden (13)	5/760	(0.7)	2/192	(1.0)	1.6 (0.3-8.3)*	6/644	(0.9)	1.4 (0.4-4.7)*
Poland (10, 11)	12/5496	(0.2)				5/1085	(0.5)	2.1 (0.7-6.0)*
Sweden (15)	3/300	(1.0)						
USA-PA (14)	1/153	(0.7)						
UK-SE (18)	21/4037	(0.5)†						

The summary includes data from the present study and 10 studies that have thus far been published on *CHEK2* 1100delC in colorectal cancer cases.[10-19] Colorectal cancer families from NL-SW were excluded for families that fulfilled our familial breast cancer criteria. The Finnish data set included nine cases with a personal or family history of BRC (with one *CHEK2* 1100delC case). The breast cancer history of the familial case cohorts from the UK-SE and NL-N are not known. NL-SW, Southwestern Netherlands; UK-SE, Southeastern United Kingdom; NL-N, Northern Netherlands; US-PA, Pennsylvania area in the United States of America; CRC, colorectal cancer; BRC, breast cancer; AMS/Bethesda, Amsterdam and Bethesda criteria for hereditary non-polyposis colorectal cancer; DGRs, degree relatives; y, years of age at diagnosis. OR, odds ratio from two-by-two tables; 95%CI, (approximate) 95% confidence interval; *, 95%CI is unreliable due to small numbers; †, Controls were obtained from reference 5; ‡, *P*-values for familial colorectal cancer cases versus matched population controls, using Fisher's Exact Test.

***CHEK2* 1100delC+ / Total Tested (%)**			***P*-value‡**	**Criteria familial cases**
Cases with CRC & BRC		**OR (95% CI)**		
			0.002	AMS/Bethesda HNPCC, diagnosed <50y and/or ≥2 CRC first DGRs
			0.05	5-100 polyps and *APC*/*MUTYH*-negative
			1.0	≥2 CRC first DGRs
			0.07	AMS/Bethesda HNPCC, diagnosed <50y and/or ≥2 CRC second DGRs
			0.6	AMS/Bethesda HNPCC, or HNPCC-like ≥2 CRC and *MLH1*/*MSH2*-negative
2/75	(2.7)	2.7 (0.5-16.5)*		
0/24	(0.0)	-		
0/20	(0.0)	-		

Table 3.4 Cancer histories of *CHEK2* 1100delC colorectal cancer families.

Family Id	Inclusion Criterion	Index = delC+ (MSI status)	1st DGRs	2nd DGRs	3rd DGRs	Other family branch
EMC16517	CRC <50y	CRC:35 (MSI-stable)	Father double other Ca 1 polyps: <50	1 CRC (<1960) 2 other Ca	1 BRC:43	Mother other Ca (delC+) 2nd DGR: 1 other Ca
EMC19398	≥2 CRC 1st DGRs	CRC:55 (MSI-stable)	Father double CRC:79/91 1 CRC:50	1 BRC:74		Mother BRC:75 2nd DGR: 1 CRC:83
EMC21829	≥2 CRC 1st DGRs	CRC:53 (MSI-stable)	1 CRC:59 1 other Ca			
EMC22131	CRC <50y and ≥2 CRC 1st DGRs	CRC:41 (MSI-stable)	Mother CRC:59 2 polyps:41/47	1 CRC 2 other Ca		Father unaffected (delC+) 2nd DGR: 2 other Ca
EMC23748	CRC <50y	CRC:46 (MSI-stable)		1 CRC:60		
EMC50100	*MLH1* positive	CRC:34 Other Ca:55 (MSI-high)	Mother CRC:43 unaffected sibs (2 delC+)	1 CRC:43 1 other Ca	1 CRC:43 3 CRC (1 delC-) 1 other Ca (delC-)	
EMC52210	≥2 CRC 1st DGRs	CRC:53 CRC:56 (MSI-low)	Father other Ca 2 CRC:54/62 1 polyps:28 (delC+) 1 other Ca			2nd DGR: 1 CRC:65
NA15	CRC <50y and ≥2 CRC 1st DGRs	CRC:45 (n.a.)	Mother other Ca (delC+) unaffected sibs (1 delC+)	1 CRC (delC+) 1 CRC:42 1 other Ca	2 CRC:47/58 (2 delC-, with 2 delC+ unaffected sibs) 1 CRC:58 1 polyps (delC-)	
NA22	*MSH6* positive	CRC:65 (MSI-low)	Father CRC:69 1 CRC/other Ca:45/54 2 polyps:56/66 (1 delC+) 3 other Ca:45/47/57 (2 delC-)	1 polyps:40 (delC-)		Mother unaffected (delC+)
NA211	CRC <50y and ≥2 CRC 1st DGRs	CRC:48 (MSI-stable)	Mother polyps:70 (delC+) 1 CRC:45	1 BRC<60		Father other Ca

Detailed are the affected relatives from the main cancer-prone branch of the *CHEK2* 1100delC family, with the other family branch summarized. Families EMC50100, NA15, NA22 and NA211 have been reported previously.[6] The number of relatives with a particular tumor type are indicated before the tumor type, with the age at diagnosis for the CRC, polyps or BRC cases, after the semicolon, when known. Genetic screening results for *CHEK2* 1100delC are indicated between brackets, including delC+ unaffected sibs. CRC, colorectal cancer; Polyp(s), adenomatous polyp(s) in the colorectum; MSI, microsatellite instability; BRC, breast cancer; other Ca, cancer other than CRC or BRC; <50y, diagnosed before age 50 years; DGRs, degree relatives; n.a., not available.

CHEK2 1100delC and polygenic cancer susceptibility

The ten *CHEK2* 1100delC colorectal cancer families that we here have identified included eight MMR mutation negative HNPCC/HNPCC-related families and two families with a MMR mutation (*MLH1* and *MSH6;* both reported earlier in reference 6). Four *CHEK2* 1100delC families fulfilled the revised Amsterdam criteria for HNPCC disease and five other families had index cases that met the revised Bethesda criteria. Together, the ten *CHEK2* 1100delC colorectal cancer families included 35 colorectal cancer cases, 9 cases with polyps, and 21 cases with other tumor types (detailed in Table 3.4), implying that the *CHEK2* 1100delC families can be classified as high-risk colorectal cancer families.

We also genotyped *CHEK2* 1100delC in all non-index cases from the *CHEK2* 1100delC families of whom DNA was available, including four cases with colorectal cancer, five cases with polyps and four cases with other cancers in first, second or third DGRs from the main cancer-prone branch of the families (Table 3.4). One of four colorectal cancer cases carried *CHEK2* 1100delC, three of five cases with polyps were *CHEK2* 1100delC positive and also one of four other cancers carried *CHEK2* 1100delC. As expected, cosegregation was better among first DGRs, with three *CHEK2* 1100delC positive cases who had polyps and one case who was diagnosed with ovarian cancer among six affected first DGRs (no DNA was available of first DGRs with colorectal cancer). Altogether, our analysis thus shows incomplete cosegregation of the *CHEK2* 1100delC genotype with the cancer phenotype in these families, suggesting the presence of an additional cancer susceptibility allele or alleles.

Although our current study design does not allow for an accurate estimate of the colorectal cancer risk conferred by *CHEK2* 1100delC, the observed 4.3 odds ratio does not account for the high-risk colorectal cancer inheritance pattern observed in the *CHEK2* 1100delC families. The high-risk inheritance pattern together with the incomplete cosegregation of the *CHEK2* 1100delC variant suggests a polygenic colorectal cancer susceptibility model, similar to the current ideas on *CHEK2* related breast cancer susceptibility.[1-5] Consistent with a polygenic model, three of the *CHEK2* 1100delC colorectal cancer families had not inherited *CHEK2* 1100delC through the main cancer-prone family branch (Table 3.4). Further support for a polygenic *CHEK2* model involves the dramatic and unprecedented high *CHEK2* 1100delC prevalence among Dutch families with hereditary breast and colorectal cancer (HBCC) as compared to families with only breast cancer or colorectal cancer (18% versus each 4%).[1, 6] It appears that at least two cancer susceptibility alleles in addition to *CHEK2* 1100delC are inherited in these HBCC families. One thus might wonder whether *CHEK2* is a breast cancer gene, a colorectal cancer gene, or a multiorgan gene - as was rightly suggested before.[10, 20] Or, alternatively, is it the additional cancer susceptibility alleles that determine organ specificity? In the HBCC families, one of these would then determine the breast cancer phenotype whereas the other determines the colorectal cancer phenotype. In this scenario, *CHEK2* 1100delC would be a modifier that only increases cancer risks for carriers of the other susceptibility alleles. Comprehension of

the exact nature of polygenic *CHEK2* cancer susceptibility clearly awaits identification of the additional cancer susceptibility alleles. Our finding that *CHEK2* 1100delC specifically associates with HNPCC/HNPCC-related colorectal cancer suggests that the mismatch repair genes might also be involved in breast cancer families with the HBCC phenotype.

Acknowledgements

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CHAPTER 4

Thirteen new *p53* gene mutants identified among 41 human breast cancer cell lines

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Abstract

The *p53* tumor suppressor gene is frequently mutated in breast cancer. Here, we used direct sequencing to screen the complete coding sequence of the *p53* gene from 41 human breast cancer cell lines. We identified 32 cell lines (78%) with a *p53* gene alteration that predicted a change in the encoded protein. Thirty-one of these mutations were accompanied by loss of the other *p53* allele. All mutations but one were unique and 27 mutations had previously been identified in uncultured human cancers. Ten mutations were predicted to encode a truncated p53 protein and 22 missense mutations were identified. *p53* transcript expression was analyzed by semi-quantitative RT-PCR and p53 protein expression was determined by Western blotting. Our analyses revealed three p53 expression patterns: wild-type *p53* cell lines had normal transcript levels and low or no detectable protein expression; cell lines with a *p53* truncating mutation had low transcript levels and low or no detectable protein expression; and cell lines with a *p53* missense mutation had highly variable transcript and protein expression levels. As a whole, our data represent a *p53* mutation profile in breast cancer cell lines, providing a model for structural, functional and pharmacological studies on p53 in human cancer.

Introduction

In normal cells, p53 proteins are phosphorylated as a result of stress signals such as DNA damage. Phosphorylated p53 then transactivates genes that function in cell cycle arrest or apoptosis, thereby allowing repair of DNA damage or elimination of the damaged cell. Inactivating p53 function therefore results in the accumulation of mutations, which ultimately can lead to the development of cancer.[1, 2] Many studies have shown that *p53* mutations are involved in breast carcinogenesis.[1, 2] However, the precise functional and clinical relevance of *p53* mutations is only partially understood, mainly because clinical breast cancer specimens hamper such studies. We therefore screened 41 human breast cancer cell lines for mutations in the complete coding sequence of *p53*, and analyzed the effects of these mutations on the expression of p53 transcripts and proteins. This characterization of breast cancer cell lines should be useful for ongoing p53 research.

Materials and methods

Breast cancer cell lines

The 41 human breast cancer cell lines used in this study are listed in Table 4.1. Cell lines EVSA-T, MPE600, SK-BR-5/7 and the SUM series were kind gifts of Dr. N. DeVleeschouwer (Institut Jules Bordet, Brussels, Belgium), Dr. H.S. Smith (California Pacific Medical Center, San Francisco, CA), Dr. E. Stockert (Sloan-Kettering Institute for Cancer Research, New York, NY), and Dr. S. Ethier (Barbara Ann Karmanos Cancer Institute, Detroit, MI), respectively. Cell line OCUB-F was obtained from Riken Gene Bank. All other cell lines were obtained from American Type Culture Collection.

PCR

All ten coding exons of *p53* (Genbank accession number X54156) and intron/exon boundaries were amplified from genomic DNA in three gene fragments as described.[3] Fragment one contained exons 2-5 and was amplified using primers 5′-TTGTGGTGAAACATTGGAAG-3′ and 5′-CACTCGGATAAGATGCTGAG-3′. Fragment two contained exons 5-9 and was amplified using primers 5′-GGTTGCAGAGGTGCTTAC-3′ and 5′-TGCCCCCTGATGGCAAATG-3′. The last fragment contained exons 10-11 and was amplified using primers 5′-AGGTACTTGAAGTGCAGTTTC-3′ and 5′-TGCAGGCCAACTTGTTCAG-3′. All forward primers also contained the M13(-29) 5′-caggaaacagctatgacc-3′ sequence at their 5′-end and all reverse primers contained the M13(-21) 5′-TGTAAAACGACGGCCAGT-3′ sequence. The annealing temperatures were 56°C (fragment 1), 66°C (fragment 2) and 64°C (fragment

3). Allelic loss of chromosome 17p13 was analyzed by radioactive PCR as described [4], using microsatellite markers *D17S1881*, *D17S1854*, *D17S1796*, *D17S786* and *D17S1805*.

Cycle sequencing

p53 amplification products were incubated with 10 units of exonuclease I and 2 units of shrimp alkaline phosphatase (Amersham) for 15 min at 37°C, and the enzymes were then inactivated for 15 min at 80°C. Bi-directional sequence reactions were performed using the Thermo Sequenase DYEnamic Direct Cycle Sequencing Kit (Amersham Pharmacia Biotech), using IRD700 and IRD800 labeled M13(-29) and M13(-21) primers. Approximately one-tenth of the reaction was sequenced for 5 min at 94°C and then 20 cycles of 1 min at 94°C, 1.5 min at 58°C and 1 min at 72°C, with a final extension of 5 min at 72°C. The reactions were analyzed using a LI-COR IR^2 automated sequencer.

Reverse transcription-PCR

A duplex *p53/HPRT* RT-PCR was performed using the Qiagen Onestep RT-PCR kit. *p53*-specific primers annealed to sequences in exons 9 and 11 (5'-GGAGAATATTTCACCCTTCAG-3' and 5'-GTCAGGCCCTTCTGTCTTG-3'). *HPRT*-specific primers annealed to sequences in exons 2-3 and 7 (5'-TATGGACAGGACTGAACGTC-3' and 5'-GTGGGGTCCTTTTCACCAG-3'). *p53* and *HPRT* transcript expression was analyzed by semi-quantitative intensity measurements using an AlphaImager (Alpha Innotech Corporation), where *HPRT* levels were used as an internal reference.

Western blotting

Cell lysates were prepared by resuspending cells in 10mM Tris-HCl pH 7.0, 0.1% SDS buffer and subsequent sonication for 10 s and denaturation for 10 min at 95°C. Proteins were separated by electrophoresis in a 10% acryl/bisacrylamide (29:1) gel and transferred to Hybond-P membranes (Amersham) by electroblotting. Blots were first incubated with anti-p53 monoclonal antibody DO-7, which is directed against an N-terminal epitope of p53 (residues 19-26), and then with anti-GAPDH monoclonal antibody MAB374 (both from Dako). Horseradish peroxidase-conjugated rabbit antimouse antibodies were used as secondary antibodies (Dako). Reactions were visualized through enhanced chemiluminescence (Amersham).

Results and discussion

Sequencing of the complete coding sequence of the *p53* gene identified 33 unique alterations among 41 breast cancer cell lines (Table 4.1 and Figure 4.1). Two alterations were reported

Table 4.1 p53 Mutation and expression analysis of 41 human breast cancer cell lines.

Breast cancer cell lines	17p	*p53* Gene sequence				p53 Expression	
	Allelic loss	Exon	Mutation	Predicted effect	No. cited db	Transcripts	Proteins
Wild-type (n=9)							
DU4475 *	retention		wild-type			0.8	n.d.
MCF-7	retention		wild-type			0.8	n.d.
MDA-MB-175VII	loss		wild-type			0.9	-
MPE600 *	loss		wild-type			1.0	n.d.
SK-BR-7	retention		wild-type			0.7	-
SUM102PT	retention		wild-type			1.0	+ / -†
UACC-812	loss		wild-type			0.6	-
ZR75-1	retention		wild-type			0.7	-
ZR75-30	loss		wild-type			0.7	n.d.
Missense mutations (n=22)							
BT20	loss	5	AAG › CAG #	K132Q	6	0.8	n.d.
SUM1315MO2	loss	5	TGC › TTC	C135F	22	1.0	n.d.
Hs578T	loss	5	GTC › TTC #	V157F	200	1.1	n.d.
SUM159PT	loss	5	TCC › TCCTCC	S158insS	0	0.7	+ / -†
SK-BR-5	loss	5	GCC › GAC	A161D	8	0.8	n.d.
SK-BR-3	loss	5	CGC › CAC #	R175H	650	1.1	+
T47D	loss	6	CTT › TTT #	L194F	14	1.0	++
MDA-MB-330	loss	7	TAT › TGT	Y220C	172	0.8	n.d.
MDA-MB-415 *	loss	7	TAC › TGC	Y236C	32	1.7	+ / -
SUM149PT	loss	7	ATG › ATA	M237I	103	1.0	++
EVSA-T	loss	7	TCC › TGC ##	S241C	20	1.4	++
OCUB-F	retention	7	GGC › AGC	G244S	35	1.0	-
BT483	loss	7	ATG › ATA##	M246I	25	0.7	n.d.
BT549	loss	7	AGG › AGC #	R249S	285	1.2	++†
SUM225CWN	loss	8	CTG › CCG	L265P	14	0.9	++
MDA-MB-435S	loss	8	GGA › GAA #	G266E	36	0.8	+†
MDA-MB-468	loss	8	CGT › CAT #	R273H	408	0.9	++
SUM229PE	loss	8	CGT › TGT	R273C	351	0.7	n.d.
CAMA-1	loss	8	AGA › ACA ##	R280T	55	0.9	+
MDA-MB-231	loss	8	AGA › AAA #	R280K	41	1.1	++†
BT474	loss	8	GAG › AAG #	E285K	88	1.1	-
MDA-MD-134VI *	loss	8	GAG › AAG ##	E285K	88	0.7	-
Truncating mutations (n=10)							
SUM44PE	loss	3	GAA › CA	E28fsX16	0	0.4	n.d.
MDA-MB-361	loss	4	GAA › TAA##	E56X	1	0.6	-
MDA-MB-157	loss	4	CCA › CCdel26 bp ##	P87fsX53	0	0.4	n.d.
SUM185PE	loss	5	CAG › TAG	Q144X	24	0.0	-
MDA-MB-436	loss	6	GAG › GCGTGTGG #	E204fsX45	0	0.3	+ / -†
SUM52PE	loss	6	CGA › TGA	R213X	155	0.3	-
HCC1937	loss	8	CGA › TGA #	R306X	63	0.3	-†
SUM190PT	loss	9	CAG › TAG	Q317X	13	0.4	n.d.
UACC-893	loss	10	CGA › TGA##	R342X	6	0.4	-
MDA-MB-453	loss	10/11	Homozygous deletion #	Exons 10/11	0	n.d.	-

*, Cell line with *MKK4* mutation [8-10]; #, *p53* mutation also reported in database http://p53.curie.fr; ##, *p53* mutation also reported in reference 7, 18, 19; -, no p53 protein expression detectable; + / -, low p53 protein expression; +, intermediate p53 protein expression; ++, high p53 protein expression; †, additional protein detected at 55-60 kD; No. cited db, number of times the mutation has been found in uncultured human cancers in database http://p53.curie.fr; bp, basepairs; del, deletion; fs, frameshift; ins, insertion; loss, allelic loss; n.d., not determined; retention, retention of both alleles; X, termination codon.

non-pathogenic polymorphisms, including one silent polymorphism in breast cancer cell line EVSA-T at Arg^{213}, exon 6 (CGA›CGG) [5], and an arginine to proline polymorphism at codon 72, exon 4 (CGC›CCC) [6] in cell lines DU4475, MDA-MB-157 and UACC-812. The remaining 31 alterations predicted changes in the encoded p53 proteins and were therefore considered mutations. Of these 31 *p53* mutations, 30 were unique and one was identified twice (E285K, Table 4.1)

All mutations except the one in OCUB-F were accompanied by loss of the other *p53* allele (Table 4.1). Because cell line OCUB-F has the microsatellite instability phenotype (F. E. and M. S., unpublished observations), the G244S mutation in OCUB-F may have been acquired as a result of impaired DNA repair in this cell line. Yet the G244S mutation has also been reported 35 times in uncultured tumor specimens (http://p53.curie.fr/), suggesting that it is relevant for human carcinogenesis.

Cell line MDA-MB-453 had a homozygous deletion of exons 10 and 11 of the *p53* gene sequence. The extent of the deletion in MDA-MB-453 was previously reported to be only about 30 base pairs at the 3'-end of exon 11.[7] We performed eleven different PCR reactions with primers designed to encompass exon 10 or 11 or both. Together, they unambiguously confirmed that both exons had been deleted (data not shown). The homozygous deletion was also confirmed by duplex PCR using primer pairs that amplified exons 5-9 and exons 10-11 of the *p53* gene (Figure 4.1).

All other *p53* sequence alterations were confirmed by sequencing of an independently-amplified template, and the nine *p53* wild-type cell lines and OCUB-F were analyzed twice for sequence alterations. Of the nine *p53* wild-type cell lines, four had 17p allelic loss. Another tumor suppressor gene located at 17p13 is *MKK4* (a.k.a. *MAP2K4*). Mutations in *MKK4* had previously been reported in two of the *p53* wild-type cell lines, as well as in two other cell lines from our collection (DU4475, MDA-MD-134VI, MDA-MD-415, and MPE600; Table 4.1).[8-10] Together, 91% of the 17p allelic losses in the 41 breast cancer cell lines were accompanied by mutations in the other allele of *p53* and/or *MKK4*. It is therefore unlikely that 17p contains another tumor suppressor gene that is of major importance for breast carcinogenesis.

RNA samples were available from 41 breast cancer cell lines and protein samples from 27 breast cancer cell lines. In duplo experiments, we analyzed transcript expression of the *p53* and *HPRT* genes by semi-quantitative duplex RT-PCR (Table 4.1). Expression of p53 and GAPDH proteins was analyzed by Western blotting (Table 4.1, Figure 4.2). All protein samples were analyzed at two dilutions and were scored by two investigators who were unaware of sample identifiers (M. W. and M. S.). Wild-type *p53* breast cancer cell lines (n=9) had intermediate transcript levels (median 0.8, range 0.6-1.0) and low or no detectable protein expression, in concordance with the high turnover of wild-type p53 proteins (Table 4.1, Figure 4.3).[11] Breast cancer cell lines with a *p53* missense mutation (n=22) had intermediate to high transcript levels (median 1.2, range 0.7-1.7) and variable protein expression levels from not detectable to high (Table 4.1 and Figure 4.3). Although not investigated in the present study, these observations could be due to failure of the MDM2 protein to interact with and down-regulate some of

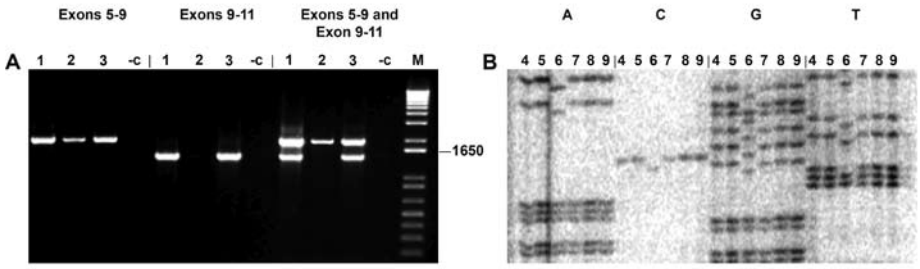

Figure 4.1 *p53* mutation analysis in breast cancer cell lines.
(A) Homozygous deletion of exons 10-11 in cell line MDA-MB-453, identified by PCR amplification of genomic DNA. Lane 1, MDA-MB-157; 2, MDA-MB-453; 3, BX5-1; -c, template-negative control; M, 1-kb-plus ladder (LifeTechnologies). (B), Deletion of a thymine residue in xenograft BX1-7, identified by PCR and direct cycle sequencing. Lane 4, UACC-812; 5, ZR75-1; 6, BX1-7; 7, BX2-3; 8, BT20; 9, HCC1937.

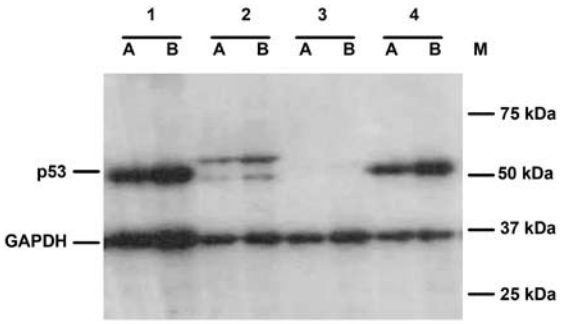

Figure 4.2 Western immunoblot analysis of p53 and GAPDH protein expression in breast cancer cell lines using antibodies DO-7 and MAB374, respectively.
Lane 1, SUM149PT (high expression); 2, SUM159PT (low expression); 3, SUM185PE (not detectable); 4, T47D (high expression); lanes A, 3 µg protein loaded; lanes B, 6 µg protein loaded; M, BIO-RAD precision plus marker.

the mutant p53 proteins.[12] Breast cancer cell lines with a *p53* truncating mutation (n=10) had relatively low transcript levels (median 0.3, range 0-0.6) and low or not detectable protein expression (Table 4.1 and Figure 4.3). This observation may be explained by down-regulation of transcripts with premature stop codons through nonsense mediated decay of mRNA.[13] Thus, if only *p53* transcript expression had been used to identify mutants of the *p53* gene, half of *p53* missense mutants would not have been identified (i.e., at least 15 of 32 mutations). Similarly, none of the *p53* truncating mutants and one-fourth of the *p53* missense mutants would have gone undetected if only p53 protein expression had been used to identify *p53* gene mutants (i.e., 5 of 22 missense mutations). In addition, almost one-fifth of the *p53* mutations were located outside the generally accepted "exons 5-9 hotspot region of mutation". As previously

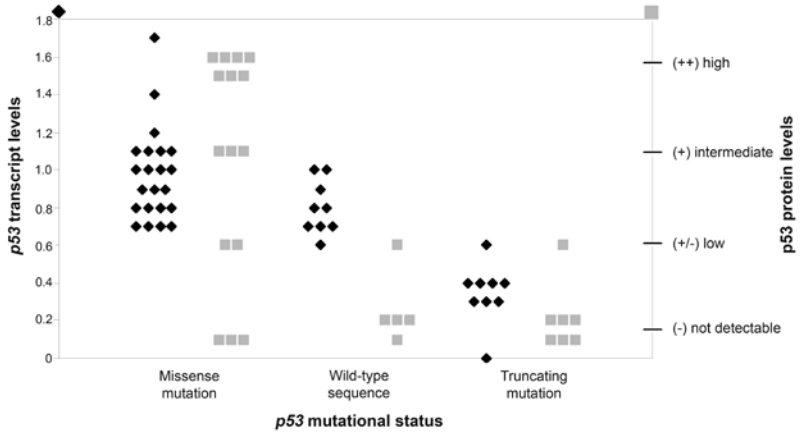

Figure 4.3 Correlation between *p53* mutational status and expression of p53 transcripts and proteins. *X*-axis, status of the *p53* gene represented by three groups: *p53* missense or truncating mutation and wild-type sequence; *Y*-axis (left ♦), semi-quantitative relationship between *p53* and *HPRT* transcript expression; *Y*-axis (right ■), p53 protein expression levels determined by Western blotting using antibody DO-7.

noted, we conclude that *p53* gene alterations are best identified by sequencing the complete coding sequence of the p53 gene.[14, 15]

The *p53* truncating mutations were located throughout the p53 protein, whereas all *p53* missense mutations clustered in the sequence-specific DNA binding region of p53 (residues 102 to 292; Table 4.1). This p53 core domain consists of a β sandwich that serves as a scaffold for the two large L2 and L3 loops and a loop-sheet-helix motif that together form the DNA binding surface of p53.[16] Of the 22 identified *p53* missense mutations, eight were located in the loop-sheet-helix motif (targeting Lys^{132}, Cys^{135}, Arg^{273}, Arg^{280}, Glu^{285}), two in the L2 loop (Arg^{175}, Leu^{194}) and six in the L3 loop (Tyr^{236}, Met^{237}, Ser^{241}, Gly^{244}, Met^{246}, Arg^{249}). Five of these missense mutations target residues Ser^{241}, Arg^{273} and Arg^{280}, which are thought to be crucial in the interaction of the wild-type p53 proteins with DNA.[16, 17] Arg^{273} is especially noteworthy because it takes part in an extended network of p53-DNA interactions. Four of the other *p53* missense mutations target residues Cys^{135}, Arg^{175}, Met^{237} and Arg^{249}, which are thought to stabilize the structure of the DNA binding surface of p53.[16, 17] Only six of the 22 *p53* missense mutations were identified in the β sandwich of the core domain (Val^{157}, Arg^{158}, Ala^{161}, Tyr^{220}, Leu^{265}, Gly^{266}). Of these six residues, only Val^{157} and Tyr^{220} have been thus far reported to be important for the structure of the β sandwich.[16]

Finally, 27 of the 32 *p53* mutations described in the present paper have also been detected in uncultured human cancers, implying that they are relevant for human carcinogenesis. Importantly, 13 of the *p53* mutant breast cancer cell lines have not yet been reported. The publicly

available breast cancer cell lines described here thus provide a useful tool in p53 cancer research, both in functional studies and in *in vitro* studies regarding breast cancer therapy.

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CHAPTER 5

Deleterious *CHEK2* 1100delC and L303X mutants identified among 38 human breast cancer cell lines

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Abstract

The CHEK2 protein plays a major role in the regulation of DNA damage response pathways. Mutations in the *CHEK2* gene, in particular 1100delC, have been associated with increased cancer risks, but the precise function of *CHEK2* mutations in carcinogenesis is not known. Human cancer cell lines with *CHEK2* mutations are therefore of main interest. Here, we have sequenced 38 breast cancer cell lines for mutations in the *CHEK2* gene and identified two cell lines with deleterious *CHEK2* mutations. Cell line UACC812 has a nonsense truncating mutation in the CHEK2 kinase domain (L303X) and cell line SUM102PT has the well-known oncogenic *CHEK2* 1100delC founder mutation. Immunohistochemical analysis revealed that the two *CHEK2* mutant cell lines expressed neither CHEK2 nor P-Thr^{68} CHEK2 proteins, implying abrogation of normal CHEK2 DNA repair functions. Cell lines UACC812 and SUM102PT thus are the first human CHEK2 null cell lines reported and should therefore be a major help in further unraveling the function of *CHEK2* mutations in carcinogenesis.

Introduction

Cell cycle checkpoint kinase 2 (CHEK2, also known as CHK2, MIM604373) is a key regulator of DNA damage response pathways.[1-3] In response to DNA double strand breaks, CHEK2 is activated through ATM-mediated phosphorylations in the N-terminal regulatory domain, specifically at residue Thr[68]. CHEK2 kinase is then fully activated by homodimerisation, intermolecular phosphorylation and subsequent release of active CHEK2 monomers. Activated CHEK2 kinase may control cell cycle arrest through phosphorylation of its substrates CDC25A and CDC25C, promote DNA repair through phosphorylation of BRCA1 and/or regulate apoptosis through phosphorylation of p53.[1, 2]

We and others identified the protein truncating *CHEK2* 1100delC mutation as a moderate-risk breast cancer susceptibility allele.[4-6] We recently have shown that *CHEK2* 1100delC also is a colorectal cancer susceptibility allele (MW and MS, manuscript submitted for publication). Although classified as a moderate-risk cancer allele, the *CHEK2* 1100delC mutation tends to be more prevalent in families with a high-risk cancer inheritance pattern. In addition, the *CHEK2* 1100delC mutation only partially segregates with the cancer phenotype in these families. Together, these observations have suggested a polygenic cancer model in which the *CHEK2* 1100delC mutation acts in concert with another as-yet-unknown cancer susceptibility allele to confer high cancer risks.[4-6] Apart from the *CHEK2* 1100delC mutation, four other allelic variants have convincingly been associated with cancer risks: the non-synonymous I157T and S428F mutations, the IVS2+1G>A splice site mutation and the truncating del5395 mutation.[6] Such a limited *CHEK2* mutation profile renders the gene particularly amenable for a polygenic cancer susceptibility model that involves biological synergy among partners. However, thorough investigation of the biological mechanism of *CHEK*2-related carcinogenesis is hampered by the lack of human cancer cell lines with proven oncogenic *CHEK2* mutations. Here, we report two novel deleterious *CHEK2* mutant cell lines that were identified though an extensive *CHEK2* mutation analysis of 38 human breast cancer cell lines.

Materials and methods

Breast cancer and colon cancer cell lines

The 38 human breast cancer cell lines used in this study are listed in Table 5.1 and have been described previously by Wasielewski *et al.*[7] The SUM102PT cell line is generated in the Ethier laboratory (available at www.asterand.com) and the UACC812 cell line is obtained from American Type Culture Collection (available at www.lgcpromochem-atcc.com). The twelve evaluated human colon cancer cell lines included Caco-2, COLO205, DLD-1, HCT15, HCT116, HT29, NCI-H716, RKO, SW403, SW480, SW620 and SW1116. All colon cancer cell lines were obtained from

American Type Culture Collection. Cell lines DLD-1 and HCT15 were independently established from the same tumor.[8]

Breast cancer families and control population

The *CHEK2* L303X mutation was screened in blood-derived DNA from the youngest diagnosed breast cancer case of 290 breast cancer families from the Southwestern Netherlands. All families had at least two breast cancer cases in first or second-degree relatives of whom at least one was diagnosed before age 60 years. Breast cancer families were ascertained through the Rotterdam Family Cancer Clinic at Erasmus MC. The 171 control cases were spouses of heterozygous cystic fibrosis mutation carriers ascertained through the department of Clinical Genetics at Erasmus MC. DNA of 69 European American and African American control cases were from the Coriell Human Variation Collection and were a kind gift by Dr. André G. Uitterlinden. All breast cancer cases gave informed consent to screen for susceptibility genes, and the medical ethical committee of Erasmus MC approved this study.

CHEK2 mutation analysis

The complete coding sequence, including intron/exon boundaries, of *CHEK2* (ENSG00000183765) was analyzed for genetic alterations. Exons 1 through 8 were amplified from genomic DNA by standard PCR.[7] Amplification of exons 9 through 14 was performed by long-range PCR and the ~9.2 kb product served as template for nested PCRs for each of exons 9 through 14. *CHEK2* amplification products were sequenced using BigDye™ Terminator v3.0 Ready Reaction Cycle mix (Applied Biosystems) and were analyzed on an ABI 3100 capillary sequencer. Unique sequence alterations were confirmed at least once on an independently generated (long-range) PCR product. Primer sequences and reaction conditions are available upon request.

Azacytidine treatment

Promoter hypermethylation was evaluated by treating exponentially growing cells with the demethylating agent 5-aza-2′-deoxycytidine (Sigma). Cells were cultured for three days with daily addition of 10 μM azacytidine. RNA of azacytidine-treated cells was isolated at the fourth day using RNeasy kit (Qiagen). *CHEK2* transcript expression was determined by RT-PCR, using Qiagen OneStep RT-PCR kit and *CHEK2*-specific primers annealing to sequences in exon 1 and exons 10-11.

CHEK2 immunohistochemistry

A cell line tissue microarray (TMA) was constructed by arraying triplicate 1-mm punches of paraffin embedded cell lines in a recipient paraffin wax block, using an automatic tissue micoarray system (Beecher). Normal placenta, colon and kidney tissues were arrayed as controls. Paraffin sections (4-µm) of the cell line TMA were mounted on starfrost glass slides (Knittel Gläser), deparaffinized and dehydrated. Epitopes were retrieved in 0.1M Tris-EDTA pH 9.0 for 30 minutes at 100°C in a microwave oven and slides were blocked with 2% BSA in PBS for 30 minutes at RT. CHEK2 staining was performed for 1 hour at RT with mouse monoclonal antibody NCL-CHK2 (clone DCS 270.1, 1:40; Novocastra Laboratories). Staining of phosphorylated CHEK2 at residue Thr^{68} was done overnight at 4°C with rabbit polyclonal antibody CHK2 phosphoT68 (clone E126, 1:100; Abcam). Isotype-matched mouse monoclonal antibody X0943 (1:40; Dako) and normal rabbit IgG sc-2027 (1:1000; Santa Cruz Biotechnology) were used as negative controls. Positive control antibodies were Vimentin for SUM102PT (clone V9, 1:4500; Dako) and Cytokeratin 8/18 for UACC812 (clone NCL5D3, 1:50; Biogene). Reactions were visualized using the EnVision+® System-HRP (DAB) kit (DakoCytomation) as described.[9]

Results and discussion

CHEK2 mutation analysis in human breast cancer cell lines identifies two deleterious mutants

Sequencing of the *CHEK2* gene identified eight unique sequence alterations among 38 breast cancer cell lines (Table 5.1 and Table 5.2). Six known single nucleotide polymorphisms included five intronic and one synonymous (E84E) sequence alterations that together were identified 48 times, varying from once to 33 times. The two non-synonymous sequence alterations c.908T>A and c.1100delC were each identified once.

The hemizygous *CHEK2* c.908T>A mutation was identified in cell line UACC812, predicting the substitution of a leucine to a termination codon at amino acid position 303 that is located in the kinase domain of the CHEK2 protein (p.L303X; Table 5.1, Table 5.2). The patient from whom UACC812 was derived was first diagnosed with breast carcinoma at age 39 years.[10] The original tumor or blood-derived DNA were however unavailable for analysis, precluding confirmation of the mutation. Although the cancer history of the family was not known and *CHEK2* L303X has never been reported, the early age at diagnosis might be suggestive for a germline mutation. We therefore genotyped the L303X mutation in 290 Dutch familial breast cancer cases and in 171 geographically matched controls, but no positive cases were identified. As *CHEK2* L303X was identified in a cell line of American origin, we also screened 69 European American and African American controls for the mutation, but again none were positive. Together, these

Table 5.1 CHEK2 mutation analysis of 38 human breast cancer cell lines.

Breast cancer cell lines (n=38)	CHEK2 variant *	*CHEK2* protein expression	CHEK2 Thr^{68} protein expression
BT20	wt	+++	+++
BT483	2,7	+++	+++
BT549	7	+++	+++
CAMA-1	7	++	++
DU4475	2,7	+/-	++
EVSA-T	2,7	+++	+++
HCC1937	wt	+++	+++
Hs578T	7	+++	+++
MCF-7	7	+++	+++
MDA-MD-134VI	2,7	+	++
MDA-MD-157	7	+++	++
MDA-MD-175VII	7	+++	+++
MDA-MD-231	7	+/-	+/-
MDA-MD-330	1,7	+/-	+/-
MDA-MD-361	7	++	++
MDA-MD-415	7	+++	+++
MDA-MD-435S	7	++	++
MDA-MD-436	7	+++	+++
MDA-MD-453	7	++	++
MDA-MD-468	7	+	+
MPE600	7	+	+
OCUB-M	2,7	n.d.	+++
SK-BR-3	7	+/-	++
SK-BR-5	wt	+/-	+
SK-BR-7	7	+++	++
SUM52PE	7	+++	+++
SUM102PT	2,6,7	-	-
SUM149PT	1,4,7,8	+/-	+
SUM159PT	7	+++	+++
SUM185PE	2,7	+++	++
SUM190PT	2,7	+++	+++
SUM225CWN	7	+++	++
SUM229PE	7	+	+
SUM1315MO2	7	+++	++
T47D	2,7	+++	+++
UACC-812	3,5	-	-
UACC-893	7	+++	+++
ZR75-1	3	+++	+++

*, Identified *CHEK2* gene variants are detailed in Table 5.2; n.d., not determined; +++, nuclear staining in > 75% of cells; ++, nuclear staining in 50-75% of cells; +, nuclear staining in 25-50% of cells; +/-, nuclear staining in < 25% of cells; -, no nuclear staining detected.

Table 5.2 *CHEK2* sequence variants identified among 38 human breast cancer cell lines.

CHEK2 variant	Location	Nucleotide change *	Predicted protein change †	Type of variant ‡	Number of cell lines
1	Exon 1	c.252A>G	p.E84E	SNP (rs1805129)	2
2	Intron 1	c.319+38insA	-	SNP (rs3841692)	9
3	Intron 5	c.793-127T>C	-	SNP (rs9625541)	2
4	Intron 5	c.793-11G>A	-	SNP (rs5997387)	1
5	Exon 7	c.908T>A	p.L303X	Oncogenic	1
6	Exon 10	c.1100delC	p.T367fsX15	Oncogenic	1
7	Intron 11	c.1375+78C>G	-	SNP (rs5762749)	33
8	Intron 13	c.1542+11T>A	-	SNP (rs17881716)	1

* Numbering of nucleotide changes according *CHEK2* ensemble gene ID ENSG00000183765.
† Frame shift mutation is indicated by the first changed codon and the number of newly encoded codons, including premature termination codon X. ‡ Oncogenic; variant associated with cancer susceptibility [4] or presumed oncogenic as it predicts a premature termination codon (L303X). Both *CHEK2* mutant cell lines did not express CHEK2 and P-Thr^{68} CHEK2 proteins.

results suggest that the L303X mutation is not a common variant among Dutch and American populations. It also is possible that the mutation has been acquired during *in vitro* propagation of UACC812 cells. Be what it may, the homozygous deleterious nature of the L303X mutation renders the UACC812 cell line the first human CHEK2 null cell line.

The heterozygous *CHEK2* c.1100delC mutation was identified in cell line SUM102PT, predicting a shift in the *CHEK2* reading frame with an insertion of 14 new amino acids after codon 367 followed by a termination codon (p.T367fsX15; Table 5.1, Table 5.2). Cell line SUM102PT was established from a patient who was diagnosed with *in situ* ductal breast carcinoma at age 56 years.[11] The family of the patient had a hereditary cancer inheritance pattern, but no details are known. Analysis of blood-derived DNA from the SUM102PT patient confirmed that the *CHEK2* 1100delC mutation was present in her germline. The *CHEK2* 1100delC mutation is indeed a well-known oncogenic founder mutation that has been associated with an increased breast cancer risk.[4-6] Thus, the SUM102PT cell line is the first oncogenic *CHEK2* cell line reported thus far.

The two deleterious *CHEK2* mutant cell lines do not express CHEK2 and P-Thr^{68} CHEK2 proteins

To address biological consequences of the identified mutations, we analyzed the 38 breast cancer cell lines as well as the *CHEK2* mutant colorectal cancer cell line HCT15 for CHEK2 and P-Thr^{68} CHEK2 protein expression by immunohistochemistry (Table 5.1, Figure 5.1).[12, 13] All 36 *CHEK2* wild-type breast cancer cell lines showed CHEK2 and P-Thr^{68} CHEK2 protein expression, where the level of CHEK2 protein expression generally was in concordance with the expression

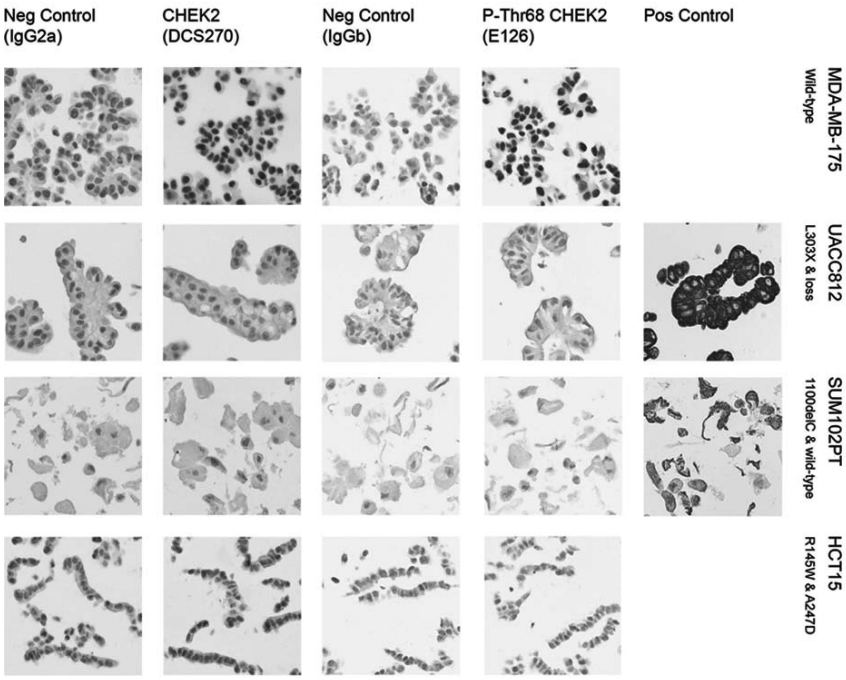

Figure 5.1 Absence of *CHEK2* protein expression in mutant *CHEK2* breast cancer cell lines.
CHEK2 mutant breast cancer cell lines UACC812 and SUM102PT do not express *CHEK2* or P-Thr^{68} CHEK2 protein, in contrast to *CHEK2* wild-type cell line MDA-MB-175. *CHEK2* mutant colorectal cancer cell line HCT15 shows weak CHEK2 and P-Thr^{68} CHEK2 protein expression. Negative control antibodies were isotype-matched for CHEK2 and P-Thr^{68} CHEK2 antibodies. Positive control antibodies were Vimentin for SUM102PT and Cytokeratin 8/18 for UACC812.

level of P-Thr^{68} CHEK2. Cell line HCT15 had weak CHEK2 and P-Thr^{68} CHEK2 protein expression, consistent with its reported impaired CHEK2 function due to bi-allelic *CHEK2* missense mutations (Figure 5.1, Table 5.4).[12, 13] In contrast, neither CHEK2 nor P-Thr^{68} CHEK2 protein expression was detected in the *CHEK2* mutant cell lines SUM102PT and UACC812 (Figure 5.1), implying complete loss of CHEK2 function in these cell lines.

No *CHEK2* promoter hypermethylation in SUM102PT cells

Absence of CHEK2 and P-Thr^{68} CHEK2 protein expression in cell line SUM102PT was somewhat unexpected as this cell line had retained a *CHEK2* wild-type allele. To exclude *CHEK2* promoter hypermethylation as an additional mechanism for *CHEK2* inactivation in cell line SUM102PT, we treated SUM102PT cells with the demethylating agent azacytidine. Treatment with azacytidine did however not upregulate *CHEK2* transcript levels in SUM102PT cells, suggesting that *CHEK2*

promoter hypermethylation is not underlying its decreased transcript expression and absent protein expression in cell line SUM102PT.

CHEK2 and *p53* mutations are mutually exclusive in human cancer cell lines

It is well established that CHEK2 is an upstream activator of p53 proteins but it is as yet unclear whether both proteins also function in the same tumor suppressor pathway.[1, 2] Such functional interaction predicts that *CHEK2* and *p53* mutations occur mutually exclusively in a single tumor. We have evaluated the *CHEK2* and *p53* mutation status in a cohort of 54 human cancer cell lines. This cohort included the here described 38 breast cancer cell lines [7], five previously reported prostate cancer cell lines [14] and 11 unique colon cancer cell lines with known *p53* mutation status [15-17] that we also have analyzed for *CHEK2* mutations (Table 5.3). Forty-two cell lines were *p53* mutant and five cell lines were *CHEK2* mutant. The five *CHEK2* mutant cell lines included the here reported SUM102PT and UACC812 breast cancer cell lines, the colorectal cancer cell lines HCT15 and HCT116 and the prostate cancer cell line LNCaP (Table 5.4). Cell line HCT15 has bi-allelic inactivation of the *CHEK2* gene as it has the R145W mutation in the fork head-associated domain of CHEK2 and the A247D mutation in the kinase domain.[12, 13] Cell lines HCT116 and LNCaP carry the heterozygous missense mutations L355P and T387N in the CHEK2 kinase domain, respectively.[14, 18] The *CHEK2* R145W variant was also found in a Li-Fraumeni patient with a sarcoma and a breast carcinoma but was not detected among 2520 additionally genotyped cases and controls (MW and MS unpublished data).[13, 19] The *CHEK2* A247D mutation was absent in 187 genotyped controls and the L355P and T387N variants have not been further evaluated.[13] The significance of these four mutations in CHEK2-related carcinogenesis is thus still unclear. However, CHEK2 function has been shown to be impaired in cell lines HCT15 and LNCaP, suggesting that the R145W, A247D and T387N *CHEK2* mutations may be oncogenic. Two of five mutant *CHEK2* cell lines also had a heterozygous *p53* sequence alteration (Table 5.4).

Table 5.3 Mutual exclusiveness of *CHEK2* and *p53* mutations among 54 human cancer cell lines.

***CHEK2* and *p53* oncogenic mutations**	**Human cancer cell lines**			
	Breast cancer	**Colorectal cancer**	**Prostate cancer**	**Total**
CHEK2 wild-type and *p53* wild-type	6	1	0	7
CHEK2 wild-type and *p53* mutant	30	8	4	42
CHEK2 mutant and *p53* wild-type	2	1	1	4
CHEK2 mutant and *p53* mutant	0	1	0	1
Total	38	11	5	54

CHEK2 and *p53* mutational status was determined in 54 breast, colorectal and prostate cancer cell lines. Note that only one of 54 cell lines is *CHEK2* and *p53* mutant (P=0.005).

Table 5.4 Overview of identified *CHEK2* variants in human cancer cell lines

Cell line	*CHEK2* mutation	*p53* mutation
Breast cancer		
SUM102PT	T367fsX15	wt
UACC812	L303X	wt
Colorectal cancer		
HCT15/DLD-1	A247D, R145W	S241F
HCT116	L335P	wt
Prostate cancer		
LNCaP	T387N	wt

wt, wild-type; *, *p53* sequence variant reported in reference 15.

Cell line LNCaP carried the silent *p53* P152P polymorphism and cell line HCT15 had the *p53* S241F missense mutation that is likely oncogenic as this residue is crucial for interaction of wild-type p53 proteins with DNA.[15, 20] Cell line HCT15 also was reported to have the *p53* P153A missense mutation.[17] Yet, this mutation could not be confirmed by us nor by others that had sequenced the *p53* gene in HCT15 and/or DLD1.[15] Thus, only one of the 54 human cancer cell lines (HCT15) had oncogenic mutations in both *CHEK2* and *p53* (P=0.005 using Fisher's Exact Test, Table 5.3 and Table 5.4), suggesting that coincident *CHEK2* and *p53* mutant cells do not have a selective survival advantage compared to either *CHEK2* or *p53* mutant cells. Our observation is in concordance with a report on 84 primary prostate cancers that had been screened for mutations in the *CHEK2* and *p53* genes.[14] Those results had not been significant because too few mutant tumors had been identified (11 *CHEK2* mutant and 11 *p53* mutant; P=0.8). However, combining both datasets conclusively shows that mutations in the *CHEK2* and *p53* genes are mutually exclusive (P=0.02). Our analysis thus implies that the abolished CHEK2 and p53 function results in similar effects on carcinogenesis.

In conclusion, we here report two breast cancer cell lines with deleterious *CHEK2* mutations, L303X and 1100delC. Both mutations generate a premature termination codon in the encoded *CHEK2* transcripts leading to loss of CHEK2 and P-Thr^{68} CHEK2 protein expression. These mutation data, together with our previous mutation reports of the *BRCA1*, *E-cadherin*, *p53*, *PIK3CA*, *PTEN* and *RAS* genes, provide a mutation profile of human breast cancer cell lines that will be a useful tool in functional and *in vitro* breast cancer studies.[7, 9, 21, 22]

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CHAPTER 6

MDM2 SNP309 accelerates familial breast carcinogenesis independently of estrogen signaling

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Abstract

A single nucleotide polymorphism (SNP309T>G) in the intronic promoter of *MDM2* was recently found to accelerate carcinogenesis in early-onset cancer cases. This cancer acceleration presumably was due to increased SP1 binding, resulting in enhanced *MDM2* transcriptional activation by estrogens. We evaluated *MDM2* SNP309 in 343 familial breast cancer cases with known mutation status for *CHEK2* 1100delC, *BRCA1* and *BRCA2*. Cancer acceleration was indeed observed in early-onset familial breast cancer cases (diagnosed $\leq$ 51 years), with 16% of cases carrying the *MDM2* SNP309 GG genotype as compared to 4% of late-onset cases (*P*=0.029). The cancer acceleration was even more pronounced in the non-mutant familial breast cancer cases, with 17% of early-onset cases carrying *MDM2* SNP309 GG as compared to 2% of late-onset cases (n=215; *P*=0.015). There was no evidence for an influence of estrogen signaling in the cancer acceleration by *MDM2* SNP309, as there were no differences in the prevalence of *MDM2* SNP309 GG among *CHEK2* 1100delC and *BRCA2* mutant cases (with 90% ER-positive cancers) or *BRCA1* mutant cases (10% ER-positive cancers). Nor did we observe differences in *MDM2* SNP309 frequencies among 75 familial breast cancer cases of our cohort with known ER status. Overall, our data suggest that *MDM2* SNP309 accelerates familial breast carcinogenesis, but that this acceleration is not influenced by estrogen signaling.

Introduction

Recently, Bond et al. reported an intronic polymorphism in the promoter of *MDM2* (SNP309T>G) that resulted in increased *MDM2* transcript and protein levels.[1] Since MDM2 is a negative regulator of p53 [2], it was suggested that *MDM2* SNP309 could counteract p53's tumor suppressor function and thus accelerate carcinogenesis. To test this hypothesis, they screened 66 cancer patients with *p53* germline mutations (Li-Fraumeni syndrome patients; MIM191170) for *MDM2* SNP309. Although the frequency of *MDM2* SNP309 carriers was similar between Li-Fraumeni syndrome (LFS) cancer cases and controls, the median age at cancer diagnosis was 9 years younger in carriers of *MDM2* SNP309 as compared to non-carriers (18 vs. 27 yrs; P=0.03). A similar younger onset was observed for LFS patients with breast cancer, with a median age of 29 years in carriers and 39 years in non-carriers (P=0.01). Bond *et al*. suggested that these earlier cancer onsets reflect an acceleration of carcinogenesis by *MDM2* SNP309.[1]

More recently, Bond *et al*. further substantiated their findings on *MDM2* SNP309 accelerated carcinogenesis by analysis of patients with sporadic forms of LFS-related tumor types.[3] They found that the *MDM2* SNP309 homozygous GG genotype was more frequent in women with an early-onset (i.e. diagnosed before 52 years) of diffuse large B-cell lymphomas (P=0.02), soft-tissue sarcomas (P=0.02) and invasive ductal breast carcinomas (P=0.01), whereas the *MDM2* SNP309 GG frequency was not increased in women with late-onset cancers (diagnosed > 51 years). The authors suggested that the observed cancer acceleration in the younger women may be related to their typically higher hormone levels, particularly estrogens.[3] This hypothesis is conceivable because the *MDM2* SNP309 polymorphism increases the affinity of the transcriptional activator SP1, predicting enhanced *MDM2* transcriptional activation under the influence of estrogens.[1, 3] To further evaluate this hypothesis, Bond *et al*. conducted an additional study on the involvement of *MDM2* SNP309 in colorectal cancer patients.[4] They again found the mutant G-allele to be more pronounced in early-onset female patients as compared to late-onset patients (P<0.01). All together, their data point toward a model in which female hormones play a role in *MDM2* SNP309 associated cancer acceleration.[4]

Here, we have tested this model by screening *MDM2* SNP309 in a large cohort of 343 familial breast cancer cases that all had been screened for mutations in *BRCA1* and *BRCA2* and for the *CHEK2* 1100delC mutation.[5, 6] Importantly, the cohort of familial breast cancer cases includes a high proportion of early-onset breast cancers, allowing detection of age-related influences. Also, the known mutation status of the familial breast cancer cases allows us to evaluate the relevance of estrogen signaling, as approximately 90% of *CHEK2* 1100delC and *BRCA2* breast cancers is known to express the estrogen receptor (ER) in contrast to only 10% of *BRCA1* breast cancers and 60% of non-mutant familial breast cancer cases.[7-9]

Materials and methods

Breast cancer families and control population

The 343 breast cancer families from our cohort originated from the Southwestern Netherlands, and all were collected through the Rotterdam Family Cancer Clinic. A breast cancer family was defined by at least two patients with breast cancer who were first or second-degree relatives, of whom at least one had been diagnosed before the age of 60 years. The 126 control individuals were spouses of heterozygous carriers of cystic fibrosis gene mutations, and were geographically-matched to the familial breast cancer cases.

Of these 343 families, the youngest diagnosed breast cancer case was screened for *MDM2* SNP309. These cases had also been screened for mutations in *BRCA1*, *BRCA2* and the *CHEK2* 1100delC mutation.[5, 6] Eighty-seven cases had a *BRCA1* mutation, 21 cases had a *BRCA2* mutation and 21 cases carried the *CHEK2* 1100delC mutation. One of these mutant cases was double mutant for *BRCA1* and *CHEK2* 1100delC; this case was further classified as a *BRCA1* case in our analyses. The medical ethical committee of the Erasmus University Medical Center approved this study, and all breast cancer cases gave informed consent to screen for susceptibility genes.

Genotyping

The intronic SNP309 in the *MDM2* promoter (rs2279744) was PCR amplified from blood-derived DNA with primers 5'-GCGAGCGGTCACTTTG-3' and 5'-GAAAAGCTGAGTCAACCTGC-3' in the presence of 5% DMSO and 0.5M betaine, as described.[10] The amplified *MDM2* products were sequenced on an ABI 3100 capillary sequencer and analyzed with the Mutation Surveyor V2.51 software, as described.[11]

Statistics

The difference between *MDM2* SNP309 carriers (GG) and non-carriers (TT) was tested by a t-test, with regard to diagnosis $\leq$ 51 and > 51 years, mean age at diagnosis, and ER status. This was done for all familial breast cancer cases together and within four subgroups of germline mutation carriers (*BRCA1*, *BRCA2*, *CHEK2* 1100delC and non-mutant). All statistical analyses were performed with STATA SE version 9.

Results and discussion

The *MDM2* SNP309 polymorphism was detected in 232 (68%) of 343 familial breast cancer cases, including 185 (54%) heterozygous and 47 (14%) homozygous *MDM2* SNP309 carriers

Table 6.1 *MDM2* SNP309T>G frequency and age at diagnosis in familial breast cancer cases.

Cohorts	*MDM2* SNP309 genotype									*P*-value*
	Non-carriers (TT)			Heterozygous carriers (GT)			Homozygous carriers (GG)			
	No.	(%)	Age at diagnosis (yrs) Mean / Median	No.	(%)	Age at diagnosis (yrs) Mean / Median	No.	(%)	Age at diagnosis (yrs) Mean / Median	
Controls (n=126)	38	(30)		67	(53)		21	(17)		
All breast cancer families (n=343)	111	(32)	44 / 42	185	(54)	45/ 44	47	(14)	43 / 43	0.36
CHEK2 1100delC families (n=20)	4	(20)	39 / 34	15	(75)	48/ 49	1	(5)	n.d.	-
BRCA1 families (n=87)	37	(42)	43 / 40	39	(45)	37/ 35	11	(13)	42 / 43	0.94
BRCA2 families (n=21)	4	(19)	42 / 37	11	(52)	43/ 40	6	(29)	38 / 35	0.63
Non-mutant families (n=215)	66	(31)	45 / 45	120	(56)	47/ 46	29	(13)	44 / 45	0.37

* *P*-value for the difference in mean age at breast cancer diagnosis between homozygous *MDM2* SNP309 carriers (GG) and non-carriers (TT); n.d., not determined, as only a single case diagnosed at 40 years was available

(Table 6.1). In our control cohort, 88 (70%) of 126 cases were positive for *MDM2* SNP309, which was similar to the prevalence among the familial breast cancer cases (*P*=0.73, Table 6.1). *MDM2* SNP309 was detected in 80% of 20 *CHEK2* 1100delC cases, 57% of 87 *BRCA1* mutant cases and 81% of 21 *BRCA2* mutant cases, which again was similar to the control cohort. Thus, the *MDM2* SNP309 frequency was not increased in either cohort of familial breast cancer cases, which is consistent with the reports by Bond *et al.*[1, 3, 4]

We then classified the familial breast cancer cases in those with an early cancer onset and those with a late cancer onset (i.e. diagnosed $\leq$ 51 and > 51 years, similar to Bond *et al.*).[3] Seventy-nine percent of the breast cancer cases had an onset before 52 years, reflecting the selection for a genetic predisposition in the cohort of familial breast cancer cases. Sixteen percent of 271 early-onset familial breast cancer cases carried *MDM2* SNP309 GG whereas this genotype was only present in 4% of 72 late-onset familial breast cancer cases (*P*=0.029, Figure 6.1 part A), suggesting that *MDM2* SNP309 GG is indeed associated with cancer acceleration. Notably, when the familial breast cancer cases were analyzed without prior subdivision by age of cancer onset, as Bond *et al*. had done in their initial report [1], the association with cancer acceleration was not apparent (median age at diagnosis of 43 years for *MDM2* SNP309 GG carriers vs. 42 years for TT carriers; Table 6.1). These different outcomes most likely are related to the non-normal distribution of the ages of cancer onset in the case cohort, reflected by differences between mean and median ages at diagnosis (Table 6.1). In conclusion, our data support ac-

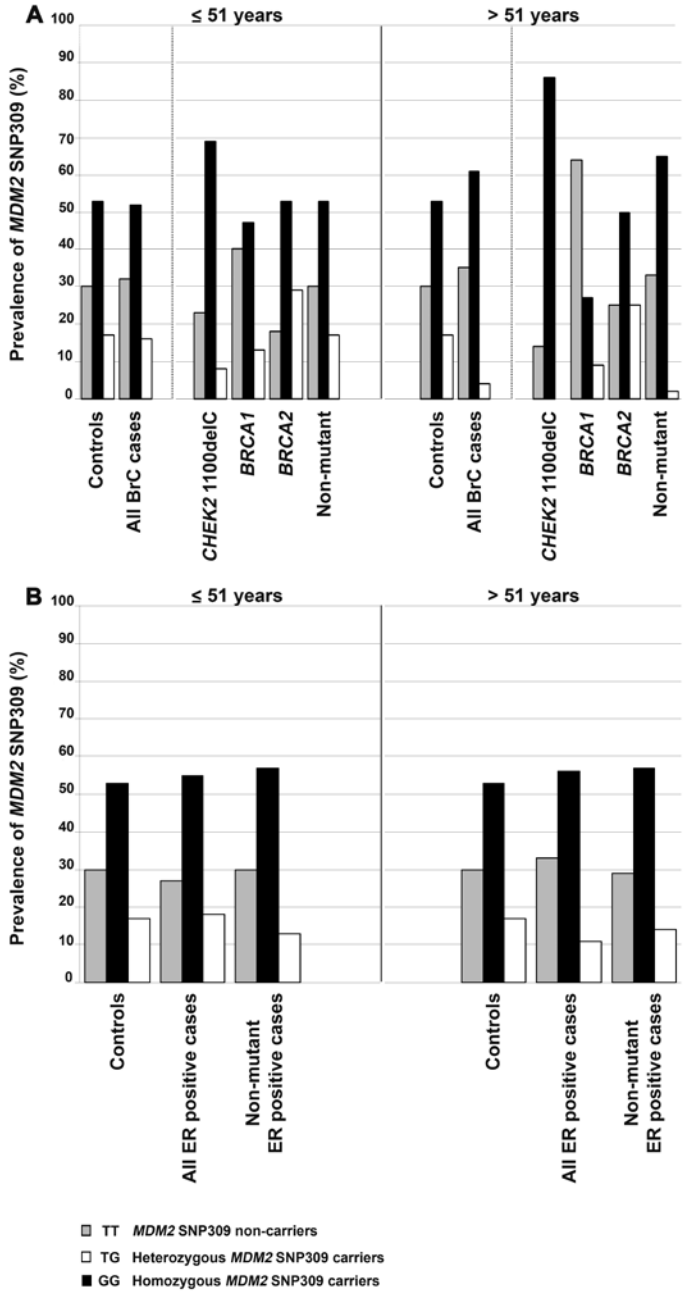

Figure 6.1 *MDM2* SNP309 T>G frequency according to age at diagnosis in familial breast cancer cases. Familial breast cancer cases are classified according (A) age at onset (n=343), arbitrarily set at the average menopausal age of 51 years [3]; or (B) estrogen receptor status of their breast cancers (n=75). Bars depict the frequency of *MDM2* SNP309 carriers in the various groups.

celeration of breast carcinogenesis by *MDM2* SNP309. However, others [12] and we believe that *P*-values of 0.03 demand further confirmation in independent case cohorts.

The effect of estrogen signaling was evaluated by analysis of the familial breast cancer cases according their mutation status (Figure 6.1 part A). The frequency of *MDM2* SNP309 GG carriers was not different in early-onset *CHEK2* 1100delC breast cancer cases as compared to late-onset cases (8% of 13 cases vs. 0% of 7 cases; *P*=0.64). Likewise, there was no difference in *MDM2* SNP309 GG frequency between early-onset and late-onset *BRCA2* breast cancer cases (29% of 17 cases vs. 25% of 4 cases; *P*=0.94). Neither was there a difference in *MDM2* SNP309 GG frequency between early-onset and late-onset *BRCA1* breast cancer cases (13% of 76 cases vs. 9% of 11 cases; *P*=0.32). Yet, we did find an increased frequency of *MDM2* SNP309 GG in the early-onset non-mutant familial breast cancer cases as compared to the late-onset cases (17% of 165 cases vs. 2% of 50 cases; *P*=0.015; Figure 6.1 part A). As the estrogen receptor (ER) is expressed by the vast majority of *CHEK2* 1100delC and *BRCA2* mutant breast cancers and by only a minority of *BRCA1* breast cancers (90% and 10%) [7-9], these results strongly suggest that the acceleration of breast carcinogenesis by *MDM2* SNP309 is not related to estrogen signaling. In fact, the association of *MDM2* SNP309 with cancer acceleration appears restricted to the non-mutant familial breast cancer cases.

To further explore the influence of estrogen signaling, we analyzed all 75 familial breast cancer cases with known ER status from our cohort. Forty-two cases had a positive ER status, of which three carried a *BRCA1* mutation, seven carried a *BRCA2* mutation and two the *CHEK2* 1100delC mutation. Of the 33 ER-negative cases, 15 carried a *BRCA1* mutation and one a *BRCA2* mutation. Thirty-three (79%) ER-positive cases were diagnosed before age 52 years, reflecting the age distribution of our complete cohort of familial breast cancer cases. There was no difference in *MDM2* SNP309 GG frequency between the early-onset ER-positive cases as compared to the late-onset cases (18% of 33 cases vs. 11% of 9 cases; *P*=0.46; Figure 6.1 part B). When the analysis was restricted to the 30 non-mutant ER-positive familial breast cancer cases, we again did not find a difference in frequency of *MDM2* SNP309 GG between the early-onset and late-onset cases (13% of 23 cases vs. 14% of 7 cases; *P*=0.99; Figure 6.1 part B). Although the number of cases in these analyses is small, there again is no evidence for an involvement of estrogen signaling in the observed cancer acceleration by *MDM2* SNP309.

Together, our results suggest that the *MDM2* SNP309 polymorphism accelerates carcinogenesis in early-onset familial breast cancer cases. Yet, our results do not support an involvement of estrogen signaling in cancer acceleration by *MDM2* SNP309. If not estrogens, then what underlies the cancer acceleration of *MDM2* SNP309 in early-onset familial breast cancer? The high population frequency of *MDM2* SNP309 precludes that the polymorphism on its own is a high-risk breast cancer susceptibility allele. The particularly pronounced association of *MDM2* SNP309 with familial breast cancer cases without mutations in *BRCA1*, *BRCA2* or *CHEK2* 1100delC, raises the possibility of interaction with another – as yet unknown – genetic risk

factor for breast cancer. Further analysis of non-mutant familial breast cancer cases with the *MDM2* SNP309 GG genotype may identify this putative breast cancer susceptibility allele.

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CHAPTER 7

Increased *MUTYH* mutation frequency among Dutch families with breast cancer and colorectal cancer

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Abstract

Compound heterozygous and homozygous *MUTYH* mutations predispose for MUTYH-associated polyposis (MAP). The clinical phenotype of MAP is currently characterized by the presence of multiple colorectal adenomas and colorectal carcinoma. We previously found that female MAP patients may also have an increased risk for breast cancer. Here, we have evaluated the involvement of *MUTYH* in breast cancer, by screening of the *MUTYH* Y179C, G396D and P405L founder mutations (previously annotated as Y165C, G382D and P391L) in 154 Dutch families with breast cancer and colorectal cancer patients. Families were classified as polyposis, hereditary non-polyposis colorectal cancer (HNPCC), HNPCC-related[†], hereditary breast and colorectal cancer (HBCC) and non-HBCC breast cancer families. Consistent with previous reports, bi-allelic *MUTYH* mutations were identified among 13% of 15 polyposis families, which was significantly increased compared to the absence of bi-allelic *MUTYH* mutations in the population (P=0.0001). Importantly, six heterozygous *MUTYH* mutations were identified among non-polyposis families with breast and colorectal cancer. These mutations were identified specifically in HNPCC-related and in HBCC breast cancer families (11% of 28 families and 4% of 74 families, respectively; P=0.02 for both groups combined vs. controls). The 11% *MUTYH* frequency among HNPCC-related families was about 5-fold higher than the reported frequencies for HNPCC-related families unselected for the presence of breast cancer patients. Together, our results indicate that heterozygous *MUTYH* mutations are associated with families that include both breast cancer patients and colorectal cancer patients, independent of which tumor type is more prevalent in the family.

[†] During the course of this thesis, the term HNPCC has been replaced by Lynch syndrome, referring to cancer families with a germline oncogenic mutation in one of the mismatch repair (MMR) genes and/or evidence for microsatellite instability (MSI) in one of the cancers. All published chapters in this thesis refer to HNPCC and/or HNPCC-related disease with the first referring to Lynch syndrome families and families that fulfill the revised Amsterdam criteria and the latter referring to non-Lynch syndrome families (and families that do not fulfill the revised Amsterdam criteria). For clarity and consistency, the terms HNPCC and/or HNPCC-related disease have also been used in all unpublished parts of this thesis, including this chapter.

Introduction

Germline mutations in the base excision repair gene *MUTYH* are associated with the recessively inherited colorectal cancer syndrome MUTYH-associated polyposis (MAP) that is characterized by multiple colorectal adenomas and colorectal carcinoma.[1-4] Compound heterozygous and homozygous *MUTYH* mutations have been identified in about 25% of patients with polyposis.[5, 6] Two prevalent *MUTYH* mutations, Y179C and G396D (previously annotated as Y165C and G382D, see materials and methods), account for approximately 75% of the reported mutations in MAP patients thus far.[5] In addition, several other *MUTYH* founder mutations have been identified, including the Dutch *MUTYH* P405L (previously P391L) mutation.[6] Together, the *MUTYH* Y179C, G396D and P405L mutations represent approximately 90% of *MUTYH* mutations reported in Dutch MAP patients.[6, 7]

The clinical phenotype associated with MAP overlaps with the familial adenomatous polyposis (FAP) and attenuated familial adenomatous polyposis (AFAP) phenotypes.[5, 8] The number of colorectal adenomas in MAP patients ranges from five to a few hundreds, generally presenting during the 5th decade of life. Approximately 50% of MAP patients presents with colorectal cancer at the time of diagnosis.[5, 8, 9] In addition, germline *MUTYH* mutations have been identified in HNPCC-related familial colorectal cancer patients.[10-16] Recently, we have identified a relatively high prevalence of breast cancer (18%) among female Dutch bi-allelic *MUTYH* mutation carriers, suggesting that breast cancer may be part of the clinical phenotype associated with MAP.[6] However, a recent study did not detect an association between *MUTYH* mutations and an increased breast cancer risk.[17] Here we have evaluated whether *MUTYH* mutations predispose to breast cancer in the context of familial colorectal cancer, by screening the *MUTYH* Y179C, G396D and P405L founder mutations in 154 Dutch families with breast cancer and colorectal cancer patients.

Materials and methods

Cancer family and population control cohorts

The 154 cancer families included in this study were ascertained by two academic cancer centres in the Southwestern Netherlands: Leiden University Medical Centre (LUMC) in Leiden and Erasmus University Medical Centre (Erasmus MC) in Rotterdam. The LUMC cohort included 84 cancer families with at least one breast cancer patient and at least one colorectal cancer patient within second degree relatives (DGR's). This cohort was selected from XX cancer families registered by either the Dutch Foundation for Detection of Hereditary Tumors (STOET), or by the Clinical Genetic Centres of Leiden, Rotterdam and Nijmegen. The Erasmus MC cohort included 70 breast cancer families with hereditary breast and colorectal cancer (HBCC) as

defined by Meijers-Heijboer *et al*. (reference 18 and see below). The Erasmus MC cohort was selected from 578 breast cancer families registered by the Rotterdam Family Cancer Clinic at Erasmus MC.[18, 19]

The 154 selected breast and colorectal cancer families were classified in six categories (Table 7.1), including families with: 1, polyposis; 2, hereditary non-polyposis colorectal cancer (HNPCC, colorectal cancer families positive for the revised Amsterdam criteria); 3, HNPCC-related (colorectal cancer families negative for the revised Amsterdam criteria); 4, HBCC; 5, non-HBCC breast cancer; and 6, mixed families with breast and colorectal cancer that could not be assigned to any of these five categories. To assign each family to only a single category of families, the six categories were ranked with the highest rank assigned to category 1. Families classified as polyposis families (n=15) when at least one patient in the family was diagnosed with many colorectal adenomas (more than 15) or when at least two patients in the family were diagnosed with an unknown number of colorectal adenomas. Families classified as HNPCC (n=9) when they included at least three relatives with a HNPCC-associated tumor (colorectal, small bowel, endometrial, ureter or renal pelvis) *and* one patient was a first DGR of the other two *and* two successive generations were affected *and* one patient was diagnosed before age 50 years (revised Amsterdam criteria).[20] Families classified as HNPCC-related (n=28) when they did not fulfill the revised Amsterdam criteria but included at least two colorectal cancer patients in first DGR's of whom at least one was diagnosed before age 50 years (n=13) *or* at least three colorectal cancer patients of whom at least one is a first DGR of the other colorectal cancer patients (n=15). Families classified as HBCC families (n=74) when they fulfilled the breast cancer family criterion and in addition included at least one patient with breast and colorectal cancer *or* at least one patient with colorectal cancer diagnosed before age 50 years who is within second DGR of a breast cancer patient *or* at least two colorectal cancer patients of whom at least one is within second DGR of a breast cancer patient.[18] Non-HBCC breast cancer families (n=13) included at least two first or second DGR's with breast cancer of whom at least one was diagnosed before age 60 years. Fifteen families could not be classified according to any of these criteria. On average these fifteen "mixed" families included two colorectal cancer patients and one breast cancer patient.

Nearly half of the cancer families in the LUMC cohort have been screened for germline *BRCA1* and *BRCA2* mutations because they had a mutation detection chance >10%. A single *BRCA2* mutant family was identified. The LUMC cancer families have also been evaluated for germline mutations in the mismatch repair genes *MLH1* (23 families), *MSH2* (21 families), *MSH6* (19 families) and *PMS2* (5 families), revealing one family with a pathogenic *PMS2* deletion and one family with a pathogenic *MSH2* mutation. All cancer families in the Erasmus MC cohort have been screened for germline *BRCA1* and *BRCA2* mutations, identifying fourteen and three *BRCA1* and *BRCA2* mutant families, respectively. Additionally, more than three-quarters of Erasmus MC cancer families have been screened for germline mutations in the *MLH1*, *MSH2* and *MSH6* genes but no pathogenic mutations were identified (Wasielewski *et al*., manuscript in preparation).

APC mutation analysis has been performed for three LUMC and two Erasmus MC cancer families but no pathogenic germline *APC* mutations were detected.

The 1192 population controls originated mainly from the Southwestern Netherlands and included a hospital-based cohort of 165 spouses of members from breast cancer families, a hospital-based cohort of 254 individuals screened for non-cancer related genetic diseases, and a population-based cohort of 773 randomly-selected blood donors.

The Medical Ethical Review Boards of Leiden University Medical Centre and Erasmus University Medical Centre have approved this study and informed consent had been obtained from all patients.

MUTYH mutation analysis

MUTYH (ENSG00000132781) mutations were annotated according to the most up-to-date *MUTYH* annotation (www.lovd.nl/MUTYH and NM_001128425.1, http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=190358496). The two most common *MUTYH* founder mutations Y179C and G396D as well as the Dutch *MUTYH* P405L founder mutation were screened in blood-derived DNA of the index patient from each cancer family. In the majority of families, the index patient was the breast cancer or colorectal cancer patient with the youngest age at diagnosis for whom DNA was available. *MUTYH* Y179C, G396D and P405L mutation screening included amplification of exons 7 (Y179C) and 13 (G396D and P405L) of the *MUTYH* gene by standard PCR as described.[6, 7, 21] Amplified fragments were subsequently sequenced with BigDye™ Terminator v3.0 or v3.1 Ready Reaction Cycle mix (Applied Biosystems) and analyzed on an ABI-3100 or 3730 capillary sequencer. Analysis of the sequence data was performed using SeqScape software (Applied Biosystems) or Mutation Surveyor™ software (SoftGenetics, LLC). The *MUTYH* Y179C, G396D and P405L mutation frequency in the control cohorts was determined by three mutation-specific Taqman® assays designed by Assays-by-designSM Service (Applied Biosystems). Samples were run on an ABI-7500 Analyzer and allelic discrimination was performed using SDS software (Applied Biosystems). No differences were identified for 238 genotypes that had been screened by Taqman analysis and sequencing, indicating that the sensitivity for detection of *MUTYH* mutations is similar for both techniques. All mutant *MUTYH* Y179C, G396D and P405L samples were confirmed by analysis of an independently generated template. For the six heterozygous *MUTYH* mutation carriers, the entire *MUTYH* coding sequence including exon-intron boundaries has been analyzed for additional genetic alterations. Primer sequences, PCR, Taqman and sequence conditions are available on request.

Statistical analysis

The difference between the *MUTYH* Y179C, G396D and P405L mutation frequency in the various cohorts of cancer patients versus controls was analyzed using Fisher's Exact Test. Odds ratios

(OR) and 95% confidence intervals (CI) were calculated from two-by-two tables. *P*-values of 0.05 or smaller were considered significant. All statistical analyses were performed with STATA statistical package, release 10 (STATA Corp, College Station, TX).

Results and discussion

Bi-allelic *MUTYH* inactivation in polyposis families with breast and colorectal cancer

Genotyping of the *MUTYH* Y179C, G396D and P405L founder mutations in 154 families with breast and colorectal cancer identified eight *MUTYH* mutant families, including two families with compound heterozygous index patients and six families with heterozygous index patients (Table 7.1). In addition, we detected the pathogenic *MUTYH* founder mutation (p.Glu410GlyfsX43, previously p.E396fsX437; one family) and two intronic polymorphisms (c.504+35G>A, previously c.462+35G>A; 29 families and c.1187-27C>T, previously c.1145-27C>T; three families).[22]

The two *MUTYH* families with compound heterozygous index patients were both polyposis families, with family 20090 carrying the *MUTYH* Y179C and P405L mutations and family 53119 carrying the *MUTYH* Y179C and G396D mutations (Table 7.1 and Table 7.2). Both index patients were diagnosed with colorectal cancer and colorectal adenomas. For family 20090, DNA of two first-degree affected family members was available for additional *MUTYH* genotyping, revealing that the mother of the index patient (diagnosed with colorectal cancer at age 81 years) was a heterozygous *MUTYH* Y179C carrier and a brother of the index patient (diagnosed with colorectal cancer at age 40 years) was a compound heterozygous *MUTYH* Y179C and P405L carrier. With regard to family 53119, one first-degree affected family member was available for additional *MUTYH* genotyping, revealing that a sister of the index patient (diagnosed with breast cancer at age 63 years) carried a heterozygous *MUTYH* Y179C mutation (Table 7.2). Together, 2 of 15 (13.3%) polyposis families carried compound heterozygous pathogenic *MUTYH* mutations as compared to none of 1192 population controls (*P*=0.0001; Table 7.1). Previous reports had identified homozygous and compound heterozygous *MUTYH* mutations in approximately 10% of FAP patients and in 20-25% of AFAP patients.[3, 6, 23] Our results are in line with these reports and hence confirm the reported association of bi-allelic *MUTYH* mutations with a polyposis phenotype.

Heterozygous *MUTYH* mutations in non-polyposis families with breast and colorectal cancer

Heterozygous *MUTYH* mutations were identified among 3 of 28 (10.7%) HNPCC-related families, including two families with index patients carrying the *MUTYH* G396D mutation and one family carrying *MUTYH* P405L (Table 7.1 and Table 7.2). Of these three HNPCC-related families, family

Table 7.1 Prevalence of *MUTYH* Y179C, G396D and P405L mutations among 154 Dutch families with breast and colorectal cancer.

Classification of families with breast and colorectal cancer			*MUTYH* +/ Total tested (%)	*P*-value (OR; 95% CI)
Controls			23/1192 (1.9)	
1. Polyposis families			2/15† (13.3)	0.0001[1] (52.7; 54.0-159.2)
2. HNPCC families			0/9	n.s.[2]
3. HNPCC-related families			3/28 (10.7)	0.02[2]
	I)	2x CRC 1st DGR+ 1CRC<50 y	1/13	(5.6; 1.8-17.4)
	II)	3x CRC 1st DGR+ 1st DGR others	2/15	
4. HBCC families			3/74 (4.1)	n.s. [2]
5. Non-HBCC breast cancer families			0/13	n.s. [2]
6. Other, mixed families			0/15	n.s. [2]

Cancer families were selected for the presence of both breast cancer and colorectal cancer patients. Cancer families were classified and ranked according to six categories, including polyposis, HNPCC, HNPCC-related, HBCC, non-HBCC breast cancer, and mixed families. BRC; breast cancer CRC, colorectal cancer; DGR, degree relative; HBCC, hereditary breast and colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; *MUTYH*+, number of *MUTYH* Y179C, G396D and P405L mutations; y, years of age at diagnosis; n.s., not significant; 1, *P*-value for cancer patients with compound heterozygous *MUTYH* mutations versus controls with compound heterozygous *MUTYH* mutations, using Fisher's Exact Test; 2, *P*-values for cancer patients with heterozygous *MUTYH* mutations versus controls with heterozygous *MUTYH* mutations, using Fisher's Exact Test; OR, odds ratio from two-by-two tables with 95% confidence intervals; †, both families were compound heterozygous *MUTYH* mutant.

50483 classified as HNPCC-related because it included two colorectal cancer patients in first DGR's of whom one was diagnosed before age 50 years, whereas families 10075 and 54058 included at least three colorectal cancer patients of whom at least one was a first DGR of the other colorectal cancer patients. DNA of non-index cases from two families (10075 and 50483) was available for additional genotyping. Yet, no *MUTYH* mutations were identified among the three breast cancer patients and the single patient with breast cancer and vulva melanoma (Table 7.2). The 10.7% heterozygous *MUTYH* mutation frequency among HNPCC-related families was significantly increased compared to the 1.9% heterozygous *MUTYH* mutation frequency among controls (Table 7.1, *P*=0.02) and was also higher than the absence of mutations among the nine HNPCC families. Thus far, seven studies have evaluated *MUTYH* in familial colorectal

Table 7.2 Cancer histories of *MUTYH* mutant families.

Family Id	*MUTYH* mutation(s)		Index	Family history of cancer		
(Criterion)				First DGRs	Second DGRs	Third DGRs
20090	Y179C	P405L	CRC:50	Mother CRC:81	4 CRC:62/77/79/83	1 BRC
(Polyposis)			>20 pol:55	(Y179C +/-)	1 other:<55	1 BRC+pol:52
				1 sib CRC:40		1 BRC+ 2 other:<51
				(Y179C +/- and P405L +/-)		3 other:<56/63
				2 sib pol		
53119	Y179C	G396D	CRC+pol:46	Mother other		
(Polyposis)				3 sibs BRC:40/<50/63		
				(1 Y179C +/-)		
				1 sib BRC+3x CRC+pol:57		
50483	G396D	-	CRC+pol:62	1 sib CRC:40	2 BRC:49/<60	
(HNPCC-related)				1 sib other+BRC:63+76	(1 G396D -/-)	
				3 sib other:<50/<50/79	1 other:35	
10075	P405L	-	BRC:42	Father CRC:<80	2 BRC:33/56	3 BRC:43/49
(HNPCC-related)				1 sib CRC:69	1 BRC+other:56+58	(2x P405L -/-)
					(P405L -/-)	
					2 CRC:<77/86	
54058	G396D	-	CRC:54	Mother CRC:90	1 CRC:<71	
(HNPCC-related)				1 sib BRC:<57	1 other	
15138	P405L	-	BRC:41	Father CRC:52	1 BRC:70	3 BRC:45/51/60
(HBCC)					1 CRC:<72	
					1 other:85	
57466	Y179C	-	BRC:43	2 sibs BRC:44/46	2 other:66/<70	
(HBCC)				(1 Y179C -/-)		
				1 sib CRC:26		
59168	G396D	-	BRC:35	Mother BRC:48	1 CRC:49	1 BRC
(HBCC)					1 other:69	

Detailed are the affected relatives up to the third degree from the main cancer-prone branch of the *MUTYH* mutant cancer families. Two families carried compound heterozygous *MUTYH* mutations and heterozygous *MUTYH* mutations were identified in six additional families. The number of relatives with a particular tumor type is indicated before the tumor type, with the age at diagnosis after the semicolon, when known. Genetic screening results for *MUTYH* Y179C, G396D and P405L in non-index cases are indicated between brackets. Genotypes are indicated as +/- and -/- for heterozygous and non-mutation carriers, respectively. BRC, breast cancer; CRC, colorectal cancer; Pol, adenomatous polyp(s) in the colorectum; other, cancer other than CRC or BRC; HBCC, hereditary breast and colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; DGR, degree relative; y, years of age at diagnosis.

Table 7.3 Prevalence of *MUTYH* Y179C, G396D and P405L mutations among 139 Dutch non-polyposis families with breast cancer and colorectal cancer.

Classification of families	*MUTYH* +/ Total tested (%)
Families with CRC patients > BRC patients	1/15 (6.7)
Families with CRC patients = BRC patients	0/44
Families with CRC patients < BRC patients	5/80 (6.3)

Cancer families were classified into three categories, including families with at least two more colorectal cancer patients than breast cancer patients (CRC patients > BRC patients), families with equal numbers (plus or minus one) of colorectal cancer patients and breast cancer patients (CRC patients = BRC patients), and families with at least two more breast cancer patients than colorectal cancer patients (CRC patients < BRC patients). *MUTYH*+, number of *MUTYH* Y179C, G396D and P405L mutations.

cancer patients who did not fulfill the revised Amsterdam and/or Bethesda criteria, together including 866 cases.[10-16] The heterozygous *MUTYH* mutation frequencies ranged from 0% to 5.7%, with an average *MUTYH* mutation frequency of 2.3% (20/866). Thus, the reported *MUTYH* mutation frequency among such colorectal cancer families is about 5-fold lower than we have observed among our HNPCC-related families (2.3% versus 10.7%). In contrast to the earlier reported HNPCC-related families, we have selected HNPCC-related families that included at least one breast cancer patient. It is plausible that the dissimilarity in *MUTYH* mutation frequency reflects this difference in selection criteria, suggesting that women from heterozygous *MUTYH* mutation families that classify as HNPCC-related are at increased risk for breast cancer.

The *MUTYH* Y179C, G396D and P405L mutations were each identified once among the 74 HBCC families (Table 7.1 and Table 7.2). Two of the *MUTYH* HBCC families (57466 and 59168) classified as HBCC families because they included a colorectal cancer patient diagnosed before age 50 years. HBCC family 15138 included two colorectal cancer patients of whom one was a first DGR of a breast cancer patient. DNA of non-index cases was available only for family 57466, revealing that a sister of the index patient (diagnosed with breast cancer at age 46 years) did not carry the *MUTYH* Y179C mutation (Table 7.2). Together, 3 of 74 (4.1%) HBCC families carried heterozygous *MUTYH* mutations, which was higher but not significantly different from the 1.9% population frequency (Table 7.1). However, it is important to realize that our classification method by ranking allowed assignment of families to only a single category. As a result, families that fulfilled the criteria for both HNPCC-related and HBCC were classified as HNPCC-related families. Indeed, 17 of 28 HNPCC-related families (61%) also met the HBCC criteria. Moreover, two of these 17 families carried *MUTYH* mutations. In total, 5 of 95 (5.3%) HBCC families were heterozygous *MUTYH* mutant which was increased compared to controls (P=0.05), suggesting that *MUTYH* mutations also associate with HBCC. To further evaluate the involvement of heterozygous *MUTYH* mutations with HBCC, we re-classified all non-polyposis families according

to the number of breast cancer and colorectal cancer patients in the family, resulting in three categories with more, equal, or less colorectal cancer than breast cancer patients (Table 7.3). This analysis showed that the *MUTYH* mutation frequency is increased among families with more colorectal cancer than breast cancer patients (6.7%) as also among families with more breast cancer than colorectal cancer patients (6.3%), and that these frequencies were rather similar. Together, our analyses suggest that heterozygous *MUTYH* mutations are associated with a breast and colorectal cancer phenotype, specifically in families that are classified as HNPCC-related and HBCC. Importantly, the families may present with more colorectal cancer than breast cancer patients or vice versa. More research is required not only to confirm our results in larger series but also to more precisely define the clinical criteria for the *MUTYH*-related breast and colorectal cancer phenotype.

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CHAPTER 8

General discussion

General discussion

In 2002, our research group and others had identified the *CHEK2* 1100delC mutation as a moderate-risk breast cancer susceptibility allele.[1, 2] In this thesis, we have shown that the *CHEK2* 1100delC mutation is also associated with an increased risk for male breast cancer and for hereditary non-polyposis colorectal cancer (HNPCC) and HNPCC-related disease† (chapters 2 and 3). Evaluation of *CHEK2* 1100delC mutation-positive breast cancer families and colorectal cancer families had suggested the presence of an additional putative cancer susceptibility allele or alleles in *CHEK2* 1100delC families. It has been hypothesized that *CHEK2* 1100delC and these putative cancer susceptibility allele(s) may function in biologically related pathways in the polygenic CHEK2 cancer model.[3]

Analyses of the *p53* and *CHEK2* genes in human cancer cell lines had suggested that inactivation of p53 or CHEK2 results in abrogation of the same function that suppresses carcinogenesis (chapters 4 and 5). Yet, in contrast to CHEK2 inactivation, inactivation of p53 reportedly results in more severe cancer phenotypes in humans and mice. We therefore hypothesized that the *MDM2* SNP309 allele, which was reported to attenuate p53 function, may act in concert with *CHEK2* 1100delC in the polygenic CHEK2 cancer model. However, genotyping of *MDM2* SNP309 in familial *CHEK2* 1100delC breast cancer cases showed that *MDM2* SNP309 is not of major relevance in the polygenic CHEK2 cancer model (chapter 6).

Our observation that *CHEK2* 1100delC specifically associated with HNPCC/HNPCC-related colorectal cancer had raised the hypothesis that mutations in known colorectal cancer genes may also be involved in *CHEK2*-related colorectal cancer susceptibility. Mutation analysis of the colorectal cancer susceptibility gene *MUTYH* in hereditary breast and colorectal cancer (HBCC) families with known *CHEK2* 1100delC mutation status however showed that *MUTYH* mutations do not play a major role in the polygenic CHEK2 cancer model (chapter 7).

The polygenic CHEK2 cancer model

It was estimated that women carrying the *CHEK2* 1100delC mutation had an approximately 2-fold increased breast cancer risk compared to women negative for this mutation (i.e., odds ratio (OR)=2.3).[1, 4] Although this classified the *CHEK2* 1100delC mutation as a moderate-risk breast cancer susceptibility allele, the mutation was particularly prevalent in families with a

† During the course of this thesis, the term HNPCC has been replaced by Lynch syndrome, referring to cancer families with a germline oncogenic mutation in one of the mismatch repair (MMR) genes and/or evidence for microsatellite instability (MSI) in one of the cancers. All published chapters in this thesis refer to HNPCC and/or HNPCC-related disease with the first referring to Lynch syndrome families and families that fulfill the revised Amsterdam criteria and the latter referring to non-Lynch syndrome families (and families that do not fulfill the revised Amsterdam criteria). For clarity and consistency, the terms HNPCC and/or HNPCC-related disease have also been used in all unpublished parts of this thesis, including this discussion.

high-risk breast cancer inheritance pattern. Also, the *CHEK2* 1100delC genotype segregated incompletely with the breast cancer phenotype in the high-risk *CHEK2* 1100delC families.[1-3, 5] Together, these two peculiarities had suggested the presence of at least one additional putative breast cancer allele in the *CHEK2* 1100delC breast cancer families. In such a polygenic CHEK2 cancer model, women carrying only the *CHEK2* 1100delC mutation will have an approximately 2-fold increased breast cancer risk. Women carrying only the putative breast cancer allele will have the breast cancer risk that is conferred by this allele. Yet, when women carry both *CHEK2* 1100delC and the putative breast cancer allele, the breast cancer risks associated with these two alleles together confer higher risks. Modelling based on *CHEK2* 1100delC breast cancer families had shown that the data were most compatible with a simple multiplicative model in which the breast cancer risk conferred by *CHEK2* 1100delC and the additional putative breast cancer allele(s) are independent.[1, 4]

Major questions regarding the polygenic CHEK2 cancer model include: what are the cancer risks of the other risk alleles involved in the model (cancer risks associated with breast cancer, colorectal cancer and/or other cancers); how many putative risk alleles are present in the general population; and how many are present within a single family? Linkage analysis of the high-risk EUR60 breast cancer family had identified the *CHEK2* gene as a breast cancer susceptibility gene.[1] Linkage analysis of the EUR60 family also provided insight in the cancer risks of the additional risk alleles involved in the polygenic CHEK2 cancer model. If the *CHEK2* 1100delC mutation were not conferring the highest cancer risk in the EUR60 family, then the linkage analysis would have pointed to the chromosomal region of another risk allele involved in the polygenic CHEK2 cancer model (i.e., the allele with the highest risk). Given that the *CHEK2* 1100delC mutation is associated with an OR of 2.3 for breast cancer [4], it can thus be inferred that the other, currently unknown, putative cancer allele(s) are associated with an OR of 2.3 at best.

Clues on the number of putative risk alleles in the polygenic CHEK2 cancer model that are present in the general population are provided by comparison of our Dutch *CHEK2* 1100delC data with the Finnish *CHEK2* 1100delC data. Three reasons have induced comparison of these two datasets. First, in both countries *CHEK2* 1100delC has been genotyped in large sample cohorts therewith providing the statistical power required to show associations. Second, the prevalence of *CHEK2* 1100delC in population controls, unselected breast cancer cases and familial non-*BRCA1/BRCA2* breast cancer cases was similar in both countries (Figure 8.1). And third, in both countries the *CHEK2* 1100delC genotype segregated incompletely with the breast cancer phenotype in *CHEK2* 1100delC breast cancer families. Hence, the Finnish *CHEK2* 1100delC breast cancer data and our Dutch data may be considered replicates.[2-4]

Interestingly, however, this appears not to be the case for the association of *CHEK2* 1100delC with colorectal cancer. Our data show an approximately 4-fold increased *CHEK2* 1100delC prevalence among Dutch HNPCC/HNPCC-related colorectal cancer cases whereas this frequency was not increased among Finnish HNPCC/HNPCC-related colorectal cancer cases (Figure 8.1; chapter

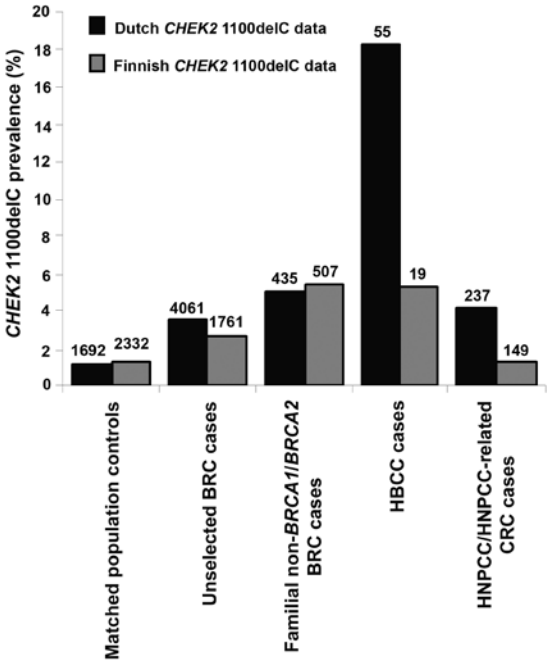

Figure 8.1 Prevalence of the *CHEK2* 1100delC mutation in cancer cases and controls from the Netherlands and Finland.

Data are derived from chapters 2 and 3 of this thesis and from seven reports that have evaluated *CHEK2* 1100delC in Dutch and Finnish cancer cases and controls.[1-4, 6-8] The numbers above the bars indicate the number of samples genotyped for *CHEK2* 1100delC. The familial non-*BRCA1/BRCA2* breast cancer cases include HBCC breast cancer cases. BRC, breast cancer; CRC, colorectal cancer; HBCC, hereditary breast and colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; non-*BRCA1/BRCA2*, *BRCA1* and *BRCA2* mutation-negative.

3).[7] As the colorectal cancer incidence is similar in both countries, the difference in *CHEK2* 1100delC-associated colorectal cancer risk suggests that at least one putative colorectal cancer allele is present in the Dutch polygenic CHEK2 cancer model but not in the Finnish model. If one assumes a substantial number of such putative colorectal cancer alleles to be present in the Dutch population, it becomes very unlikely that none of these alleles would have been present in Finland. It therefore appears that only a few putative colorectal cancer alleles may be present in the Dutch population.

Assumptions on the number of additional putative risk alleles within a single *CHEK2* 1100delC family are guided by the observation that *CHEK2* 1100delC is particularly prevalent in families with a high-risk breast cancer inheritance pattern. A recent meta-analysis has estimated an OR of 4.8 for breast cancer risk in familial *CHEK2* 1100delC mutation carriers.[9] Given that *CHEK2* 1100delC appears to operate in a multiplicative polygenic model [1, 4], it can be calculated how many additional putative risk alleles may be involved in a single *CHEK2* 1100delC family. If one

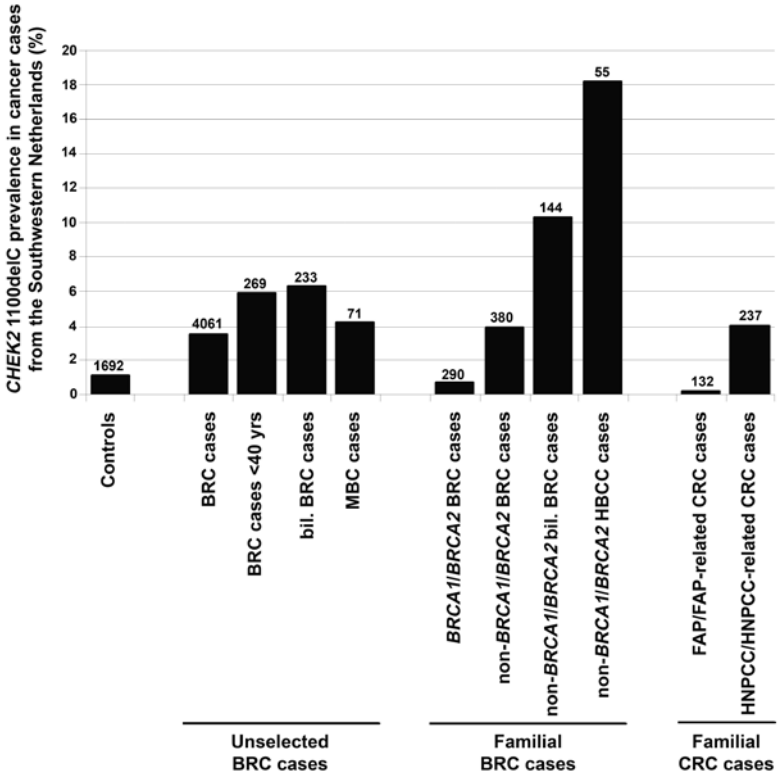

Figure 8.2 Prevalence of *CHEK2* 1100delC in cancer cases and controls from the Southwestern Netherlands. Data are derived from chapters 2 and 3 of this thesis and from five reports that have evaluated *CHEK2* 1100delC in cancer cases and controls from the Southwestern Netherlands.[1, 3, 4, 8, 12] The numbers above the bars indicate the number of samples genotyped for *CHEK2* 1100delC. The unselected BRC cases are excluded for breast cancer cases diagnosed before age 40 years and the non-*BRCA1/BRCA2* familial breast cancer cases are excluded for breast cancer cases with the HBCC phenotype. BRC, breast cancer; bil. BRC, bilateral breast cancer; CRC, colorectal cancer; FAP, familial adenomatous polyposis; HBCC, hereditary breast and colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; MBC, male breast cancer; non-*BRCA1/BRCA2*, *BRCA1* and *BRCA2* mutation-negative; <40 years, breast cancer diagnosed before age 40 years.

assumes that the additional putative risk alleles each are associated with a moderate-risk OR of about 2.3 and the combined OR of *CHEK2* 1100delC and these alleles is 4.8, then only one moderate-risk allele in addition to *CHEK2* 1100delC can be postulated within a single family (i.e., $2.3^{x}=4.8 \rightarrow x=1.9$, meaning two risk alleles including *CHEK2* 1100delC). The number of additional risk alleles within a *CHEK2* 1100delC family is larger when each additional risk allele is associated with substantially lower breast cancer risks than *CHEK2* 1100delC, for example, in the range of the recently identified low-risk alleles.[10] If one assumes an average OR of 1.3 for low-risk alleles [10], then three additional low-risk alleles can be postulated in a single

CHEK2 1100delC family (i.e., 2.3 * 1.3^{x} =4.8 › x=2.8, meaning three risk alleles excluding *CHEK2* 1100delC). For a very high-risk breast cancer family, such as the EUR60 family with an inheritance pattern comparable to that of *BRCA1* and estimated OR of 13 [11], two moderate-risk or seven low-risk additional risk alleles can be postulated. Yet, the EUR60 family is exceptional and the number of additional risk alleles postulated within an average *CHEK2* 1100delC cancer family is more likely restricted to one moderate-risk allele or three low-risk alleles.

The data in this thesis have suggested the presence of at least one additional colorectal cancer allele in the polygenic CHEK2 cancer model, in addition to the previously postulated breast cancer allele. As *CHEK2* 1100delC in Finland is associated with familial breast cancer but not with familial colorectal cancer, the additional breast cancer and colorectal cancer alleles must represent two different entities (Figure 8.1; Chapter 3).[7] The almost 5-fold increased *CHEK2* 1100delC frequency observed among Dutch hereditary breast and colorectal cancer (HBCC) families compared to breast cancer only and colorectal cancer only families (18% vs. 4% each, Figure 8.2; chapter 3) [3], also supports a polygenic CHEK2 cancer model with at least two additional cancer susceptibility alleles. The dramatically increased *CHEK2* 1100delC prevalence among the HBCC families suggests that the combined breast and colorectal cancer phenotype results from a strong concerted action of *CHEK2* 1100delC with the putative breast cancer allele as well as the putative colorectal cancer allele. As the putative colorectal cancer allele appears to be absent in the Finnish population, the prevalence of HBCC families in the Finnish population is anticipated to be low. Indeed, only approximately 4% of Finnish familial non-*BRCA1/BRCA2* breast cancer families classified as HBCC families compared to nearly 13% of our Dutch non-*BRCA1/BRCA2* breast cancer families. Similarly, the *CHEK2* 1100delC frequency in Finnish HBCC cases is also not increased (Figure 8.1).[3, 7] It thus appears that within an average Dutch HBCC family, the *CHEK2* 1100delC mutation may act in concert with a maximum of three additional cancer susceptibility alleles (assuming OR's varying from about 1.3 to at most 2.3), of which at least one is a breast cancer allele and at least one a colorectal cancer allele.

MDM2 SNP309 and the polygenic CHEK2 cancer model

Comprehension of CHEK2-associated cancer pathways may provide clues regarding the nature of the currently unknown additional cancer susceptibility alleles involved in the polygenic CHEK2 cancer model. Mutation analysis of the *p53* and *CHEK2* genes in human cancer cell lines has shown that mutations in these two genes occur mutually exclusive (chapters 4 and 5), suggesting that *CHEK2* and *p53* mutations abrogate similar functions involved in suppression of cancer development.[13, 14] Yet, it had been reported that CHEK2-deficient cells are resistant to p53-mediated apoptosis whereas p53-deficient cells are also defective in p53-mediated G1-phase cell cycle arrest.[13-19] Concordantly, it had been reported that mice deficient in *Trp53* or *Chk2* (the murine homologues of human *p53* and *CHEK2*) have different cancer phenotypes in that *Trp53*-deficient mice are prone to develop tumors whereas *Chk2*-deficient mice do not have

a cancer phenotype.[15, 16, 18-20] Similarly, germline *p53* mutations in humans predispose to the Li-Fraumeni cancer syndrome which is associated with significantly increased risks for different cancer types, whereas germline *CHEK2* mutations confer only moderately increased cancer risks.[1, 21-23] These differences in cancer phenotypes in both humans and mice suggest that abrogation of p53-mediated G1-phase cell cycle arrest provides a surplus in the development of cancer. Therefore, we hypothesized that a putative risk allele in the polygenic CHEK2 cancer model may function within p53-mediated G1-phase cell cycle arrest. In this context, a SNP in the promoter of the *MDM2* gene (*MDM2* SNP309) had been of particular interest. *MDM2* SNP309 was reported to increase the affinity of the transcriptional activator SP1 to the *MDM2* promoter, resulting in enhanced MDM2 transcript and protein expression.[24] As *MDM2* is a negative regulator of p53, it had been suggested that *MDM2* SNP309 counteracts p53 function and thereby accelerates carcinogenesis.[24] Additionally, it had been suggested that the attenuation of p53 function by *MDM2* SNP309 was enhanced in tumors that express the estrogen receptor because *MDM2* SNP309 increases the affinity of the MDM2 promoter for SP1 which is a cotranscriptional activator for the estrogen receptor.[25] As approximately 90% of *CHEK2* 1100delC breast tumors express the estrogen receptor, we hypothesized that *MDM2* SNP309 might be a putative risk allele in the polygenic CHEK2 cancer model. Yet, we found that the *MDM2* SNP309 allele frequencies were not significantly different between 20 *CHEK2* 1100delC familial breast cancer cases and 126 geographically-matched controls that were analyzed (chapter 6). To improve statistical power, we since have screened *MDM2* SNP309 in 65 unselected *CHEK2* 1100delC breast tumors, but again found similar *MDM2* SNP309 allele frequencies between unselected breast cancer cases and controls (M. Wasielewski unpublished data). These results thus suggest that *MDM2* SNP309 is not of major importance in the polygenic CHEK2 cancer model.

MUTYH mutations and the polygenic CHEK2 cancer model

In 2003, our research group had shown that the prevalence of *CHEK2* 1100delC was almost 5-fold increased among breast cancer families with hereditary breast and colorectal cancer (HBCC) compared to non-HBCC breast cancer families.[3] This observation suggested that *CHEK2* 1100delC carriers may also be predisposed to develop colorectal cancer. In chapter 3 of this thesis, we have shown that the frequency of *CHEK2* 1100delC was approximately 4-fold increased among HNPCC/HNPCC-related colorectal cancer cases as compared to matched population controls, suggesting that *CHEK2* 1100delC is indeed also a colorectal cancer susceptibility allele. This observation suggested that mutations in genes that predispose to colorectal cancer may also be putative cancer susceptibility alleles involved in the polygenic CHEK2 cancer model. Recently, germline mutations in the base-excision repair gene *MUTYH* have been associated with the recessive colorectal cancer syndrome MUTYH-associated polyposis (MAP).[26] The clinical phenotype associated with MAP overlaps with the familial adenomatous polyposis (FAP) and attenuated familial adenomatous polyposis (AFAP) phenotypes.[27, 28] In

addition, *MUTYH* mutations have been identified in HNPCC-related familial colorectal cancer patients.[29-33] Interestingly, it had also been suggested that women with *MUTYH* mutations are at increased risk of breast cancer.[34] We therefore hypothesized that mutations in the *MUTYH* gene might be involved in polygenic *CHEK2* 1100delC colorectal cancer susceptibility. Screening of the three most common Dutch *MUTYH* mutations, *MUTYH* Y179C, G396D and P405L, in 154 families with breast cancer and colorectal cancer showed that the *MUTYH* mutation frequency among the 95 HBCC families included in this cohort was significantly increased compared to the 1,192 controls (5.3% vs. 1.9%, *P*=0.05; chapter 7), suggesting an association of *MUTYH* mutations with the HBCC phenotype. However, only one of the 70 HBCC families that had been evaluated for *CHEK2* 1100delC and *MUTYH* carried mutations in both genes, indicating that the *MUTYH* Y179C, G396D and P405L mutations do not play a major role in the polygenic CHEK2 cancer model.

Genetic testing for the *CHEK2* 1100delC mutation

Although *CHEK2* 1100delC is firmly established as a breast cancer susceptibility allele, it is an ongoing matter of debate whether or not genetic testing for *CHEK2* 1100delC should be implemented into routine clinical management of breast cancer families. The recent guideline of the national breast cancer organisation of the Netherlands (NABON) for follow-up of breast cancer patients only discriminates between women carrying a germline *BRCA1/BRCA2* mutation and women not having a germline *BRCA1/BRCA2* mutation (Supplementary Tables S1.1 and S1.2 in chapter 10). Hence, follow-up of patients with a family history of breast cancer and/or ovarian cancer but without a germline *BRCA1/BRCA2* mutation is currently similar to the follow-up of so-called sporadic breast cancer patients that do not have such a family history. Therefore, follow-up of familial breast cancer patients carrying the *CHEK2* 1100delC mutation is the same as follow-up of sporadic breast cancer patients. After breast conserving therapy, this follow-up generally consists of annual clinical (breast) examination and annual mammography until age 60 years, followed by annual clinical (breast) examination and biannual mammography until age 75 years (Supplementary Tables S1.1 and S1.2 in chapter 10).[35] Yet, several studies have shown that *CHEK2* 1100delC breast cancer patients have a substantial increased risk for bilateral breast cancer, and a worse breast cancer-free and metastasis-free survival.[2, 8, 36-38] In fact, it has been suggested that the bilateral breast cancer risk of *CHEK2* 1100delC breast cancer patients is as high as or even higher as the risk reported for *BRCA1/BRCA2*-associated breast cancer patients.[2, 8, 37-40] Hence, I propose to incorporate *CHEK2* 1100delC genetic testing for breast cancer patients with a family history of breast cancer as these patients could potentially benefit from intensified and prolonged surveillance. I then also propose to prolong and intensify the surveillance of familial *CHEK2* 1100delC breast cancer patients by offering annual clinical (breast) examination and annual mammography up to age 75 years. In this way, more knowledge regarding the clinical outcome of *CHEK2* 1100delC breast cancer patients will

also be generated and follow-up of *CHEK2* 1100delC breast cancer patients should be optimised accordingly.

Genetic testing of *CHEK2* 1100delC in familial breast cancer patients will inevitably identify patients who carry the mutation. Consequently, this will raise the question if as-yet-unaffected women in a *CHEK2* 1100delC family should also be tested for the *CHEK2* 1100delC mutation. Currently, no unambiguous breast cancer risk can be estimated for unaffected *CHEK2* 1100delC mutation carriers nor for non-carriers from a breast cancer family with the *CHEK2* 1100delC mutation, because the identity of the additional putative risk alleles in the polygenic CHEK2 cancer model are for the time being unknown. The cancer risk for unaffected women from *CHEK2* 1100delC families varies from a breast cancer odds ratio of 2.3 when *CHEK2* 1100delC is the sole risk allele inherited to an odds ratio of 4.8 when other additional risk alleles are inherited (references 4 and 9, and also see the section the polygenic CHEK2 cancer model). Thus, cancer risks vary within *CHEK2* 1100delC families, depending on whether a woman has inherited *CHEK2* 1100delC plus the putative breast cancer allele(s) or only *CHEK2* 1100delC or only the putative breast cancer risk allele(s). Therefore, I believe that presymptomatic genetic testing for *CHEK2* 1100delC is not yet warranted to guide the counselling and clinical management of unaffected women with a family history of breast cancer. Until the identification of the additional risk alleles involved in the polygenic CHEK2 cancer model allows more accurate *CHEK2* 1100delC breast cancer risk estimates, I advise to survey unaffected women according to the cancer histories of their families as is formulated in the NABON screening guidelines (www.oncoline.nl; NABON richtlijn borstkanker and Supplementary Table S1.3 in chapter 10).

In our previous work, we have shown that *CHEK2* 1100delC is particularly prevalent in hereditary breast and colorectal cancer (HBCC) families.[3] In addition, we have provided strong evidence that *CHEK2* 1100delC is a risk allele for HNPCC/HNPCC-related colorectal cancer (chapter 3). It could thus be argued that (presymptomatic) *CHEK2* 1100delC genetic screening may have implications for the surveillance strategies of HBCC breast cancer families and colorectal cancer families. However, thus far only few other studies have evaluated the involvement of *CHEK2* 1100delC in HBCC and/or familial colorectal cancer. Although there was some suggestion that *CHEK2* 1100delC may indeed be associated with HBCC and/or familial colorectal cancer in other countries, none of the studies had the statistical power to confirm our findings. More research regarding the role of *CHEK2* 1100delC in HBCC and familial colorectal cancer is therefore needed. Until then, clinical surveillance of HBCC and colorectal cancer families should be guided by family cancer histories and screening strategies should be adapted accordingly.

Future perspectives

Comprehension of the polygenic CHEK2 cancer model has been aided by characterization of the cancer phenotypes associated with *CHEK2* 1100delC. It would therefore be important to extend the knowledge of *CHEK2* 1100delC-associated cancer phenotypes. For example, *CHEK2* 1100delC

confers an increased risk for prostate cancer in the Finnish and Polish population. Yet, no data on *CHEK2* 1100delC and prostate cancer are available for the Dutch population. In addition, it may be worthwhile to perform mutation analysis of the complete *CHEK2* gene in various cancer types as these kinds of analyses may reveal other oncogenic *CHEK2* mutations. It would also be of interest to see if these *CHEK2* mutations would partake in a similar polygenic CHEK2 cancer model as suggested for the *CHEK2* 1100delC mutation.

Obviously, the most challenging and most clinically-relevant research proposal would involve identification of the putative risk alleles in the polygenic CHEK2 cancer model. In contrast to the unknown number of putative breast cancer risk alleles in the Dutch population, the data in this thesis suggest the presence of only a few putative colorectal cancer risk alleles. The identification of a putative colorectal cancer risk allele may therefore be more straightforward. Linkage analysis in high-risk HNPCC/HNPCC-related colorectal cancer families and high-risk HBCC breast cancer families may be used to identify these alleles. The families included in this linkage analysis should however be carefully selected to increase the likelihood of identification of a putative colorectal cancer risk allele. In the ideal situation only large *CHEK2* 1100delC families without germline mutations in other cancer susceptibility alleles should be included. Another approach involves combined analysis of genome-wide gene expression and SNP genotypes in *CHEK2* 1100delC and non-*CHEK2* 1100delC colorectal cancer tumors. This approach may identify biological pathways or genomic alterations that specifically associate with *CHEK2* 1100delC and therefore provide clues regarding the nature of the additional colorectal cancer risk alleles involved in the polygenic CHEK2 cancer model. As a putative colorectal cancer risk allele is present in the Dutch population but unlikely among the Finnish population, it appears that this putative colorectal cancer risk allele represents a Dutch founder allele. It would therefore be important to include only Dutch colorectal cancer cases and tumors in the suggested studies. Once a putative colorectal cancer allele is identified in the Dutch setting, it will be of interest to analyze this allele in other populations including Finland. Although the putative breast cancer alleles in the polygenic CHEK2 cancer model may be more difficult to identify, it might be worthwhile to investigate some of the genes associated with the *CHEK2* gene signature that our research group has generated by gene expression profiling of *CHEK2* 1100delC breast tumors.[41] An interesting candidate from this gene signature is the *RECQL5* gene that encodes a protein that functions in the maintenance of genomic stability. Germline mutations in RecQ family members have been identified in genomic instability syndromes that are characterized by an increased predisposition to cancer.[42, 43] It would therefore be interesting to investigate if germline alterations in the *RECQL5* gene are more common in *CHEK2* 1100delC families compared to non-*CHEK2* 1100delC families.

In conclusion, more accurate knowledge of *CHEK2* 1100delC associated cancer risks is required to optimize the clinical management of breast and/or colorectal cancer patients and unaffected relatives from *CHEK2* 1100delC families. Therefore, future CHEK2 research should focus at identification of the putative additional risk alleles in the polygenic CHEK2 cancer

model. In addition, it is necessary to study the biological pathways associated with the CHEK2 protein. Unraveling of these mechanisms will aid the identification of therapeutic targets and subsequently the development of drugs for treatment of *CHEK2*-associated cancers.

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CHAPTER 9

Summary
Samenvatting

Summary

Approximately 15-25% of breast cancers are identified in women with a family history of breast cancer. Yet, germline mutations in the currently known breast cancer susceptibility genes account for only one-third of familial breast cancer cases. In 2002, our research group had identified the *CHEK2* 1100delC mutation as a breast cancer susceptibility allele. It was estimated that this mutation confers an approximately 2-fold increased breast cancer risk for female *CHEK2* 1100delC carriers. Although this 2-fold increased breast cancer risk had classified the *CHEK2* 1100delC mutation as a moderate-risk breast cancer susceptibility allele, the mutation typically was more prevalent among breast cancer families with a high-risk breast cancer inheritance pattern. Also, the *CHEK2* 1100delC mutation did not completely segregate with the breast cancer phenotype in the high-risk breast cancer families. Together, these observations suggested the presence of additional cancer susceptibility alleles in *CHEK2* 1100delC families.

This thesis has focused on three topics related to the *CHEK2* gene and in particular the *CHEK2* 1100delC mutation: analysis of the CHEK2-p53 tumor suppressor pathway by mutation analysis of both genes in human breast cancer cell lines; evaluation of the association of *CHEK2* 1100delC with male breast cancer and colorectal cancer; and identification of genes involved in the polygenic CHEK2 cancer model by using a candidate gene approach.

Chapter 1 provides a general overview of the genetics of familial breast cancer, familial colorectal cancer, and of various molecular and clinical aspects of the *CHEK2* gene. In addition, the aims of this thesis are highlighted.

Part I of the results includes two studies that have focused on the cancer phenotype of *CHEK2* 1100delC. Apart from female breast cancer, the initial *CHEK2* 1100delC identification study had suggested that *CHEK2* 1100delC might also confer a risk for male breast cancer (MBC). In **chapter 2**, the association of *CHEK2* 1100delC with MBC has been evaluated by genotyping of *CHEK2* 1100delC in 94 Dutch MBC cases, including 23 familial and 71 unselected MBC cases. The prevalence of *CHEK2* 1100delC among unselected MBC cases was significantly higher than the Dutch *CHEK2* 1100delC population frequency (4.2% vs. 1.1%, respectively; P=0.05). These results thus suggested that, in the Netherlands, *CHEK2* 1100delC is associated with MBC. Previously, our research group had shown that the *CHEK2* 1100delC mutation was particularly prevalent among breast cancer families with hereditary breast and colorectal cancer (HBCC). The study described in **chapter 3** has investigated whether *CHEK2* 1100delC is also associated with an increased risk for familial colorectal cancer independent of its breast cancer risk. For this purpose, 369 familial colorectal cancer cases have been screened for *CHEK2* 1100delC. This cohort included 237 hereditary non-polyposis colorectal cancer (HNPCC)

and HNPCC-related colorectal cancer cases† and 132 familial adenomatous polyposis (FAP) and FAP-related colorectal cancer cases. None of the FAP/FAP-related colorectal cancer cases carried the *CHEK2* 1100delC mutation. In contrast, the *CHEK2* 1100delC mutation was identified in 4.2% of HNPCC/HNPCC-related colorectal cancer cases, which was significantly increased compared to the Dutch *CHEK2* 1100delC population frequency (P=0.002). These results thus showed that *CHEK2* 1100delC, in addition to increased risks for male and female breast cancer, also was associated with an increased risk for HNPCC/HNPCC-related colorectal cancer. As previously reported for familial breast cancer cases, the *CHEK2* 1100delC mutation was particularly prevalent among colorectal cancer families with a high-risk colorectal cancer inheritance pattern. The mutation also did not segregate completely with the colorectal cancer phenotype in these families. Similar to the current ideas on polygenic *CHEK2* 1100delC breast cancer susceptibility, our results thus suggested that *CHEK2* 1100delC also partakes in polygenic colorectal cancer susceptibility.

The studies in **part II of the results** describe mutation screens of *p53* and *CHEK2* in human breast cancer cell lines. In **chapter 4**, 41 human breast cancer cell lines have been analyzed for mutations in the coding sequence of the *p53* gene. Truncating and missense *p53* mutations were identified in 10 and 22 breast cancer cell lines, respectively. Hence, *p53* alterations that predict changes in the encoded p53 protein were identified in 78% of human breast cancer cell lines. The mutation analysis of the *CHEK2* gene in 38 of these breast cancer cell lines is described in **chapter 5**. Two *CHEK2* mutant breast cancer cell lines were identified, including one cell line that carried the oncogenic *CHEK2* 1100delC founder mutation that we had identified as a cancer susceptibility allele. Immunohistochemical analysis showed that both *CHEK2* mutant cell lines expressed neither CHEK2 nor P-Thr^{68} CHEK2 proteins, suggesting abrogation of CHEK2 function in both cell lines. As CHEK2 and p53 function in the same biological pathway, it had been anticipated that coincident *p53* and *CHEK2* mutations do not provide a selective survival advantage compared to either *CHEK2* or *p53* mutant cells. To address this, *CHEK2* and *p53* mutation status were evaluated in 54 human cancer cell lines. Forty-two *p53* and five *CHEK2* mutant cancer cell lines were identified. Yet, only a single cell line carried oncogenic mutations in both *p53* and *CHEK2*. This analysis thus showed that mutations in *p53* and *CHEK2* occur mutually exclusive (P=0.005), suggesting that both proteins indeed operate in the same biological pathway that suppresses carcinogenesis.

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† During the course of this thesis, the term HNPCC has been replaced by Lynch syndrome, referring to cancer families with a germline oncogenic mutation in one of the mismatch repair (MMR) genes and/or evidence for microsatellite instability (MSI) in one of the cancers. All published chapters in this thesis refer to HNPCC and/or HNPCC-related disease with the first referring to Lynch syndrome families and families that fulfill the revised Amsterdam criteria and the latter referring to non-Lynch syndrome families (and families that do not fulfill the revised Amsterdam criteria). For clarity and consistency, the terms HNPCC and/or HNPCC-related disease have also been used in all unpublished parts of this thesis, including this summary.

Part III of the results includes two candidate gene studies that aimed at identifying genes involved in the polygenic CHEK2 cancer model. Previous studies had suggested that a single nucleotide polymorphism in the *MDM2* gene (*MDM2* SNP309) may accelerate breast carcinogenesis by attenuation of the p53 tumor suppressor pathway, particularly under the influence of estrogens. As MDM2 functions within the CHEK2-p53 pathway and the majority of *CHEK2* 1100delC breast tumors express the estrogen receptor, we hypothesized that *MDM2* SNP309 may partake in the polygenic *CHEK2* cancer model. In **chapter 6**, this hypothesis has been investigated by analysis of *MDM2* SNP309 in 343 familial breast cancer cases. No significant differences in *MDM2* SNP309 genotype frequencies among familial *CHEK2* 1100delC breast cancer cases and matched-population controls were detected, suggesting that *MDM2* SNP309 is not of major importance in the polygenic *CHEK2* cancer model. Our data did however suggest that *MDM2* SNP309 may indeed accelerate breast carcinogenesis. Yet, in contrast to previous studies, this *MDM2* SNP309 associated breast cancer acceleration appeared to be independent of estrogen signaling. In chapter 3, we showed that *CHEK2* 1100delC is a colorectal cancer susceptibility allele. This observation suggested that mutations in genes that predispose to a colorectal cancer phenotype are also putative cancer susceptibility alleles involved in polygenic *CHEK2* 1100delC colorectal cancer susceptibility. A particularly interesting colorectal cancer susceptibility gene was *MUTYH* as it was previously reported that women with *MUTYH* mutations might also be at increased risk for breast cancer. In **chapter 7**, we therefore have genotyped the three most common Dutch *MUTYH* mutations (*MUTYH* Y179C, G396D and P405L) in 154 families with breast cancer and colorectal cancer. Families were classified as polyposis, HNPCC, HNPCC-related, HBCC, and non-HBCC breast cancer families. *MUTYH* mutations were more frequently identified among HNPCC-related and HBCC families compared to controls (10.7% and 4.1% versus 1.9%). Interestingly, the *MUTYH* frequency among HNPCC-related families was about 5-fold higher than the reported frequencies for HNPCC-related families unselected for the presence of breast cancer patients. Together, these results suggested that *MUTYH* mutations associate with cancer families that are characterized by the presence of breast cancer patients and colorectal cancer patients. The *CHEK2* 1100delC mutation status had been determined for 70 of the 154 families with breast cancer and colorectal cancer. Only a single family was mutant for *CHEK2* 1100delC and *MUTYH*, suggesting that *MUTYH* mutations do not play a major role in the polygenic CHEK2 cancer model.

The general discussion in **chapter 8** provides a model for polygenic CHEK2 cancer susceptibility, addresses the used candidate gene approaches, discusses the use of genetic *CHEK2* 1100delC testing in a clinical setting and provides perspectives on future CHEK2 research.

Samenvatting

Ongeveer 15 tot 25 procent van alle borstkankers wordt gediagnosticeerd in vrouwen uit een familie waarin borstkanker vaak voorkomt. Echter, kiembaanmutaties in de tot nu toe bekende borstkankergenen verklaren slechts circa éénderde van alle familiaire borstkankerpatiënten. In 2002 heeft onze onderzoeksgroep de 1100delC mutatie in het *CHEK2* gen ontdekt als een risico-allel dat bijdraagt aan het ontstaan van borstkanker. Onderzoek heeft aangetoond dat vrouwen die draagster zijn van de *CHEK2* 1100delC mutatie een tweemaal hoger risico hebben op het ontstaan van borstkanker dan vrouwen die geen draagster zijn van deze mutatie. Dit tweemaal verhoogde borstkankerrisico classificeerde de *CHEK2* 1100delC mutatie als een risico-allel dat een matig verhoogd risico geeft op borstkanker. Toch werd de mutatie zeer vaak aangetroffen in borstkankerfamilies met een hoogrisico erfelijk patroon van borstkanker. In deze hoogrisico *CHEK2* 1100delC borstkankerfamilies werd bovendien een incomplete koppeling tussen de *CHEK2* 1100delC mutatie en borstkanker waargenomen. Deze twee observaties hebben tot de hypothese geleid dat andere, tot nu toe nog onbekende risico-allelen aanwezig zijn in *CHEK2* 1100delC families.

Dit proefschrift heeft drie onderwerpen betreffende het *CHEK2* gen en in het bijzonder de *CHEK2* 1100delC mutatie onderzocht: het CHEK2-p53 signaalpad door middel van mutatie-analyse van beide genen in humane borstkankercellijnen; het risico van de *CHEK2* 1100delC mutatie op het ontstaan van mannelijk borstkanker en darmkanker; en identificatie van genen betrokken bij het polygene CHEK2 kankermodel met behulp van kandidaatgenanalyses.

Hoofdstuk 1 omvat een algemene introductie in de genetica van familiair borstkanker, familiair darmkanker, en de verscheidene moleculaire en klinische aspecten met betrekking tot het *CHEK2* gen. Tevens worden de doelstellingen van dit proefschrift besproken.

Deel I van de resultaten beschrijft twee studies waarin de associatie tussen de *CHEK2* 1100delC mutatie en mannelijk borstkanker (MBK) en darmkanker is onderzocht. In de originele *CHEK2* 1100delC identificatie studie werd gesuggereerd dat, behalve vrouwelijk borstkanker, *CHEK2* 1100delC ook een risico-allel was voor MBK. In **hoofdstuk 2** is de associatie tussen de *CHEK2* 1100delC mutatie en MBK bepaald door middel van *CHEK2* 1100delC genotypering in 94 Nederlandse MBK patiënten. Deze serie patiënten omvatte 23 MBK patiënten met een familiaire achtergrond van borstkanker en 71 MBK patiënten die niet geselecteerd waren op het voorkomen van borstkanker in hun familie. De *CHEK2* 1100delC prevalentie was, in vergelijking met de Nederlandse *CHEK2* 1100delC populatiefrequentie, significant hoger in de groep van ongeselecteerde MBK patiënten (1.1% vs. 4.2%, respectievelijk; P=0.05). De conclusie van deze studie is dat, in Nederland, de *CHEK2* 1100delC mutatie een bijdrage kan geven op het ontstaan van mannelijk borstkanker. Uit eerdere studies binnen onze onderzoeksgroep was gebleken dat de *CHEK2* 1100delC mutatie veelvuldig voorkwam in borstkankerfamilies met een erfelijk borst- en darmkanker patroon. De studie beschreven in **hoofdstuk 3** heeft onderzocht of de *CHEK2* 1100delC mutatie een verhoogd risico geeft op het voorkomen van familiair darmkanker

onafhankelijk van het risico van dit allel voor borstkanker. In deze studie zijn 369 familiaire darmkankerpatiënten zonder een familiegeschiedenis van borstkanker gescreend voor de *CHEK2* 1100delC mutatie. Deze serie omvatte 237 hereditair non-polyposis colorectaal carcinoom (HNPCC) and HNPCC-gerelateerde darmkankerpatiënten† en 132 familiaire adenomateuze polyposis (FAP) en FAP-gerelateerde darmkankerpatiënten. De *CHEK2* 1100delC mutatie werd niet gedetecteerd in de FAP/FAP-gerelateerde darmkankerpatiënten. In tegenstelling, 4.2% van de HNPCC/HNPCC-gerelateerde darmkankerpatiënten was drager van de *CHEK2* 1100delC mutatie. De *CHEK2* 1100delC frequentie in deze groep van darmkankerpatiënten was significant verschillend van de Nederlandse *CHEK2* 1100delC populatiefrequentie (P=0.002). Deze resultaten laten dus zien dat de *CHEK2* 1100delC mutatie, behalve een verhoogd risico op mannelijk en vrouwelijk borstkanker, ook een verhoogd risico geeft op HNPCC/HNPCC-gerelateerde darmkanker. Zoals eerder ook opgemerkt voor familiair borstkanker, werd de *CHEK2* 1100delC mutatie vaker aangetroffen in darmkankerfamilies met een hoogrisico erfelijk patroon voor darmkanker. Tevens was de koppeling tussen de *CHEK2* 1100delC mutatie en darmkanker incompleet in deze darmkankerfamilies. Vergelijkbaar met de huidige ideeën over het polygene *CHEK2* 1100delC borstkankermodel, laten onze resultaten zien dat *CHEK2* 1100delC ook betrokken is bij een polygeen darmkankermodel.

De studies in **deel II van de resultaten** beschrijven de mutatie analyse van de *p53* en *CHEK2* genen in humane borstkankercellijnen. In **hoofdstuk 4** werden 41 borstkankercellijnen onderzocht op mutaties in de coderende sequentie van het *p53* gen. In tien cellijnen werd een *p53* mutatie aangetroffen die een verkort p53 eiwit voorspellen. In 22 andere cellijnen werd een *p53* mutatie aangetroffen waardoor slechts een enkel p53 aminozuur zou veranderen. In totaal werden er dus *p53* mutaties geïdentificeerd in 78% van de onderzochte borstkankercellijnen. De mutatieanalyse van het *CHEK2* gen in 38 van deze borstkankercellijnen is beschreven in **hoofdstuk 5**. Twee borstkankercellijnen met een *CHEK2* mutatie werden geïdentificeerd, inclusief een cellijn met de door ons met borstkanker geassocieerde *CHEK2* 1100delC mutatie. Immunohistochemische analyse liet zien dat beide cellijnen met een *CHEK2* mutatie geen CHEK2 eiwit tot expressie brachten. Tevens was er geen P-Thr^{68} CHEK2 eiwitexpressie. Op basis van deze resultaten kan verondersteld worden dat de geïdentificeerde *CHEK2* mutaties verlies van CHEK2 functie tot gevolg hebben. CHEK2 en p53 functioneren binnen hetzelfde signaalpad,

† Gedurende dit promotieonderzoek is de term HNPCC gewijzigd in Lynch syndroom. Lynch syndroom verwijst naar kankerfamilies waarin een kiembaanmutatie voorkomt in één van de mismatch repair (MMR) genen en/of microsatellietinstabiliteit (MSI) in één van de tumoren. In de gepubliceerde studies in dit proefschrift wordt gesproken van HNPCC en/of HNPCC-gerelateerde darmkankerfamilies. In de context van dit proefschrift verwijzen HNPCC darmkankerfamilies naar darmkankerfamilies met het Lynch syndroom en darmkankerfamilies welke voldoen aan de herziene Amsterdam criteria. HNPCC-gerelateerde darmkankerfamilies verwijzen naar niet-Lynch syndroom darmkankerfamilies en darmkankerfamilies die niet voldoen aan de herziene Amsterdam criteria. Duidelijkheidshalve is de term HNPCC en/of HNPCC-gerelateerde darmkanker ook gebruikt in alle niet-gepubliceerde studies in dit proefschrift, inclusief deze samenvatting.

waardoor gepostuleerd kan worden dat inactivatie van zowel het *CHEK2* als *p53* gen geen selectief overlevingsvoordeel zal opleveren ten opzichte van inactivatie van slechts één van beide genen. Om deze hypothese te onderzoeken is de mutatiestatus van *CHEK2* en *p53* geanalyseerd in 54 humane kankercellijnen. Mutaties in de *CHEK2* en *p53* genen werden geïdentificeerd in 5 en 42 cellijnen. Opvallend genoeg werd er slechts één enkele cellijn geïdentificeerd met zowel een mutatie in het *CHEK2* als het *p53* gen (P=0.005). Dit resultaat suggereert dat beide genen inderdaad coderen voor eiwitten die een rol spelen in hetzelfde signaalpad dat normaliter de ontwikkeling van kanker remt.

Deel III van de resultaten bevat twee studies waarin een kandidaatgenanalyse werd gebruikt om genen te identificeren die betrokken kunnen zijn bij het polygene CHEK2 kankermodel. Recent gepubliceerde studies hadden gesuggereerd dat een genetische verandering in het *MDM2* gen, genaamd *MDM2* SNP309, de functie van het p53 eiwit aantast waardoor het ontstaan van borstkanker wordt versneld. Deze afname van p53 functie door *MDM2* SNP309 zou sterker zijn in de aanwezigheid van oestrogenen. Omdat het MDM2 eiwit functioneert in het CHEK2-p53 signaalpad en omdat de meerderheid van *CHEK2* 1100delC borsttumoren de oestrogeenreceptor tot expressie brengt, werd de hypothese opgesteld dat *MDM2* SNP309 een rol speelt in het polygene CHEK2 kankermodel. Deze hypothese is onderzocht in **hoofdstuk 6**. Dit hoofdstuk beschrijft de analyse van *MDM2* SNP309 in 343 familiaire borstkankerpatiënten. Er werden geen significante verschillen aangetoond tussen de *MDM2* SNP309 frequentie in de populatie en de *MDM2* SNP309 frequentie in *CHEK2* 1100delC familiaire borstkankerpatiënten. Op basis van deze resultaten werd geconcludeerd dat *MDM2* SNP309 niet van groot belang kan zijn in het polygene CHEK2 kankermodel. Onze data suggereerde verder dat *MDM2* SNP309 het ontstaan van borstkanker inderdaad kan versnellen maar, in tegenstelling tot wat eerder werd beweerd, was deze versnelling onafhankelijk van het al dan niet aanwezig zijn van oestrogenen. In hoofdstuk 3 van dit proefschrift hebben we aangetoond dat *CHEK2* 1100delC een risico-allel is voor darmkanker. Het specifieke samengaan van *CHEK2* 1100delC met darmkanker zou kunnen betekenen dat genen die betrokken zijn bij erfelijk darmkanker ook kandidaatgenen zijn voor het polygene CHEK2 darmkankermodel. Het darmkanker gen *MUTYH* is in dit geval zeer interessant omdat mutaties in dit gen ook geassocieerd werden met een verhoogd borstkankerrisico. In **hoofdstuk 7** is deze hypothese onderzocht door de frequentie van de drie meest voorkomende *MUTYH* mutaties in Nederland (*MUTYH* Y179C, G396D and P405L) te bepalen in 154 families waarin zowel erfelijk borstkanker als darmkanker voorkomt. Op basis van de erfelijke kankerpatronen in deze families werden de families geclassificeerd als FAP-gerelateerde darmkankerfamilies *of* HNPCC darmkankerfamilies *of* HNPCC-gerelateerde darmkankerfamilies *of* borstkankerfamilies met een erfelijk borst- en darmkanker patroon (HBCC) *of* niet-HBCC borstkankerfamilies. Een verhoogde *MUTYH* mutatiefrequentie ten opzichte van de controle populatie werd aangetroffen in HNPCC-gerelateerde darmkankerfamilies en HBCC borstkankerfamilies (1.9% versus 10.7% en 4.1%; respectievelijk). Opvallend was dat de in de literatuur beschreven *MUTYH* mutatiefrequentie in HNPCC-gerelateerde darmkankerfamilies zonder preselectie voor borstkanker ongeveer vijf

keer zo laag was dan de door ons gevonden *MUTYH* mutatiefrequentie. Deze resultaten suggereerden dat *MUTYH* mutaties associëren met families waarin zowel borstkanker als darmkanker voorkomt. De *CHEK2* 1100delC mutatiestatus was bekend voor 70 van de 154 families met HBCC. In slechts één van deze 70 families werd zowel een mutatie in het *MUTYH* gen als de *CHEK2* 1100delC mutatie aangetroffen. Hieruit kan worden geconcludeerd dat mutaties in het *MUTYH* gen geen belangrijke rol spelen in het polygene CHEK2 kankermodel.

De algemene discussie in **hoofdstuk 8** is gewijd aan een interpretatie van het polygene CHEK2 kankermodel. Verder worden de resultaten van de toegepaste kandidaatgenanalyses behandeld. Tenslotte wordt in dit hoofdstuk besproken of de *CHEK2* 1100delC mutatie getest zou moeten worden in klinische centra en hoe het onderzoek met betrekking tot het *CHEK2* gen kan worden voortgezet.

CHAPTER 10

Appendices

List of abbreviations

Supplementary tables

List of publications

PhD portfolio

Dankwoord

List of abbreviations

14-3-3σ	Stratifin
AFAP	Attenuated familial adenomatous polyposis
APC	Adenomatous polyposis coli
A-T	Ataxia-telangiectasia
ATLD	Ataxia-telangiectasia-like disorder
ATM	Ataxia telangiectasia mutated
BASC	BRCA1-associated genome surveillance complex
BCAC	Breast cancer association consortium
BER	Base excision repair
BRCA1	Breast cancer 1, early-onset
BRCA2	Breast cancer 2, early-onset, also called FAND1
BRCT	BRCA1 C-terminus
BRIP1	BRCA1 interacting protein C-terminal helicase 1, also called FANCJ or FA-J
BSA	Bovine serum albumin
CAPP	Colorectal adenoma/carcinoma prevention programme
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CBO	Centraal begeleidings orgaan
CD	Cowden disease
CDC25A	Cell division cycle 25 homolog A (S. pombe)
CDC25C	Cell division cycle 25 homolog C (S. pombe)
Cdk1	Cell division cycle 2, G1 to S and G2 to M
Cdk2	Cyclin-dependent kinase 2
CHEK2	CHK2 checkpoint homolog (S. pombe), also called Chk2
CHRPE	Congenital hypertrophy of retinal pigment epithelium
CI	Confidence interval
CIMBA	Consortium of investigators of modifiers of BRCA1/2
CYP17	Cytochrome P450, family 17, subfamily A, polypeptide 1
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E2F1	E2F transcription factor 1
E-cadherin	Cadherin 1, type 1, E-cadherin (epithelial)
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian), also called NEU or HER2
ERGO	Erasmus Rotterdam gezondheid onderzoek
FA	Fanconi anemia
FA-A	Fanconi anemia, complementation group A, also called FANCA
FA-B	Fanconi anemia, complementation group B, also called FANCB
FA-C	Fanconi anemia, complementation group C, also called FANCC
FA-D1	Fanconi anemia, complementation group D1, also called FANCD1 or BRCA2
FA-D2	Fanconi anemia, complementation group D2, also called FAND2
FA-E	Fanconi anemia, complementation group E, also called FANCE
FA-F	Fanconi anemia, complementation group F, also called FANCF
FA-G	Fanconi anemia, complementation group G, also called FANCG
FA-I	Fanconi anemia, complementation group I, also called FANCI
FA-J	Fanconi anemia, complementation group J, also called FANCJ or BRIP1
FA-L	Fanconi anemia, complementation group L, also called FANCL
FA-M	Fanconi anemia, complementation group M, also called FANCM
FA-N	Fanconi anemia, complementation group N, also called FANCN or PALB2
FAP	Familial adenomatous polyposis
FGFR2	Fibroblast growth factor receptor 2
FHA	Fork head-associated
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GWA	Genome wide association
HBC	Hereditary breast cancer
HBCC	Hereditary breast and colorectal cancer
HBOC	Hereditary breast and ovarian cancer
HCl	Hydrochloric acid
HNPCC	Hereditary non-polyposis colorectal cancer
HOC	Hereditary ovarian cancer
HPRT	Hypoxanthine phosphoribosyltransferase 1
IgG	Immunoglobuline G
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LFS	Li-Fraumeni syndrome
LOD	Logarithm of odds
LOH	Loss of heterozygosity
LSP1	Lymphocyte-specific protein 1
MAP	MUTYH-associated polyposis
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MDM2	Mdm2 p53 binding protein homolog (mouse)
MKK4	Mitogen-activated protein kinase kinase 4, also called MAP2K4
MLH1	MutL homolog 1 (E. coli)
MMR	Mismatch repair
MRE11	MRE11 meiotic recombination 11 homolog (S. cerevisiae)
MRN	MRE11-RAD50-NBS1
MRPS30	Mitochondrial ribosomal protein S30
MSH2	MutS homolog 2 (E. coli)
MSH6	MutS homolog 6 (E. coli)
MSI	Microsatellite instability
MUTYH	MutY homolog (E. coli)
NABON	Nationaal borstkanker overleg nederland
NBS1	Nibrin
NLS	Nuclear localisation signal
OR	Odds ratio
P	P-value
p21	Cyclin-dependent kinase inhibitor 1A
p53	Tumor protein p53, also called TP53
PALB2	Partner and localizer of BRCA2, also called FANCN
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PJS	Peutz-Jeghers syndrome
PML	Promyelocytic leukemia
PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)
PR	Progesterone receptor, also called PgR
PTEN	Phosphatase and tensin homolog
RAD50	RAD50 homolog (S. cerevisiae)
RAD51	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)
RB1	Retinoblastoma 1
RecQL5	RecQ protein-like 5
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SP1	Sp1 transcription factor
SQ/TQ	Serine-glutamine/threonine-glutamine
STK11	Serine/threonine kinase 11
STOET	Stichting opsporing erfelijke tumoren
TMA	Tissue microarray
TNM	Tumor node metastases
TNRC9	TOX high mobility group box family member 3
Tris	Tris(hydroxymethyl)aminomethane
VEGF	Vascular endothelial growth factor

Supplementary tables

Table S1.1 Follow-up after primary breast cancer treatment, as from end of radiation and/or chemotherapy, for patients without *BRCA1/BRCA2* mutation.

Years after primary breast cancer treatment		Patients without germline *BRCA1/BRCA2* mutation		
		C(B)E	**Mammography**	**MRI**
0-1	(all ages)	Once every 3 months	Yearly	-
1-2	(all ages)	Once every 6 months	Yearly	-
2-5	(all ages)	Yearly	Yearly	-
>5	(age ≤ 60 years)	Yearly	Yearly	-
>5	(age 61-75 years)	Yearly	Once every 2 years*	-
>5	(age >75 years)	Consider ending follow-up		

Table adapted from www.oncoline.nl; NABON richtlijn borstkanker. C(B)E, clinical (breast) examination; MRI, magnetic resonance imaging; *, mammographic screening is performed within the national breast cancer screening programme.

Table S1.2 Follow-up after primary breast cancer treatment, as from end of radiation and/or chemotherapy, for patients with *BRCA1/BRCA2* mutation.

Years after primary breast cancer treatment		Patients with germline *BRCA1/BRCA2* mutation		
		C(B)E	**Mammography**	**MRI**
0-1	(all ages)	Once every 3 months	Yearly	Yearly
1-2	(all ages)	Once every 6 months	Yearly	Yearly
2-5	(all ages)	Yearly	Yearly	Yearly
>5	(age ≤ 60 years)	Yearly	Yearly	Yearly
5-10	(age 61-75 years)	Yearly	Yearly	-
>10	(age 61-75 years)	Yearly	Once every 2 years*	-
>5	(age >75 years)	Consider ending follow-up		

Table adapted from www.oncoline.nl; NABON richtlijn borstkanker. C(B)E, clinical (breast) examination; MRI, magnetic resonance imaging; *, mammographic screening is performed within the national breast cancer screening programme.

Table S1.3 Screening schedules for unaffected women with an increased risk for breast cancer based on the cancer history of their family.

Relative breast cancer risk	Age	Screening		
		C(B)E	Mammography	MRI
2-3	40-50	-	Yearly	-
	50-75	-	Once every two years*	-
	>75		Screening not advised	
3-4	35-60	Yearly	Yearly	-
	60-75	-	Once every two years*	-
	>75		Screening not advised	
6-8	25-60	Yearly	Yearly	Yearly
	60-75	-	Once every two years*	-
	>75		Screening not advised	

Table adapted from www.oncoline.nl; NABON richtlijn borstkanker. C(B)E, clinical (breast) examination; MRI, magnetic resonance imaging; *, mammographic screening is performed within the national breast cancer screening programme.

List of publications

Publications included in this thesis

Marijke Wasielewski, Astrid A. Out, Joyce Vermeulen, Maartje Nielsen, Ans van den Ouweland, Carli M.J. Tops, Marjan M. Weiss, Jan G.M. Klijn, Peter Devilee, Frederik J. Hes, and Mieke Schutte. Increased *MUTYH* mutation frequency among Dutch families with breast cancer and colorectal cancer.
Submitted for publication

Marijke Wasielewski, Michael A. den Bakker, Ans van den Ouweland, Marion E. Meijer-van Gelder, Henk Portengen, Jan G.M. Klijn, Hanne Meijers-Heijboer, John A. Foekens, and Mieke Schutte. *CHEK2* 1100delC and male breast cancer in the Netherlands.
Breast Cancer Research and Treatment DOI: 10.1007/s10549-008-0162-7

Marijke Wasielewski, Pejman Hanifi-Moghaddam, Antoinette Hollestelle, Sofia D. Merajver, Ans van den Ouweland, Jan G.M. Klijn, Stephen P. Ethier, and Mieke Schutte. Deleterious *CHEK2* 1100delC and L303X mutants identified among 38 human breast cancer cell lines.
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Jord H.A. Nagel, Justine K. Peeters, Marcel Smid, Anieta M. Sieuwerts, **Marijke Wasielewski**, Vanja de Weerd, Anita M.A.C. Trapman-Jansen, Ans van den Ouweland, Henk Portengen, Hennie Brüggenwirth, Wilfred van Ijken, Jan G.M. Klijn, Peter J. van der Spek, John A. Foekens, John W.M. Martens, Mieke Schutte, and Hanne Meijers-Heijboer. Gene expression profiling assigns CHEK2 1100delC breast cancers to the luminal intrinsic subtypes.
Submitted for publication

Jord H.A. Nagel, Antoinette Hollestelle, Marcel Smid, Suzanne Lam, Fons Elstrodt, **Marijke Wasielewski**, Ser Sue Ng, Pim J. French, Justine K. Peeters, Marieke J. Roozendaal, Muhammad Riaz, Ellen C. Zwarthoff, Amina Teunisse, Peter J. van der Spek, Jan G.M. Klijn, Stephen P. Ethier, Hans Clevers, Aart G. Jochemsen, Michael A. den Bakker, John A. Foekens, John W.M. Martens, and Mieke Schutte. Distinct gene mutation profiles among luminal and basel type breast cancer cell lines.
Submitted for publication

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Nat Genet 2002; 31: 55-59

PhD portfolio

A summary of PhD training and teaching activities

Name PhD student:	Marijke Wasielewski
Erasmus MC department:	Medical Oncology
Research school:	Postgraduate school Molecular Medicine
PhD period:	February 2005- March 2009
Promotor:	Prof. dr. J.G.M. Klijn
Copromotor:	Dr. M. Schutte

1. PhD training

	Year	Workload
General academic skills		
- English biomedical writing and communication	2005	3.0 ECTS
Research skills		
- Microsoft Access	2002	0.9 ECTS
- Classical methods for data-analysis	2005	5.7 ECTS
In-depth courses		
- Biomedical research techniques III	2004	1.5 ECTS
- Cellular responses to DNA damage	2004	1.5 ECTS
- In vivo imaging- from cell to organism	2005	1.5 ECTS
- Molecular medicine	2006	1.2 ECTS
- Basic and translational oncology	2006	1.5 ECTS
- Comparative gene function analysis- from yeast to man	2006	1.5 ECTS
Presentations		
- Annual oral presentation at the Josephine Nefkens Institute (JNI) Scientific Lab meetings	2005-2008	1.2 ECTS
- Oral presentation at the annual Tumor Cell Biology meeting of the Dutch Cancer Society in Lunteren	2007	0.3 ECTS
- Poster presentation at the annual joint Medisch Genetisch Centrum (MGC)- Cancer Research UK Graduate Student Conference in Luik, Belgium	2005	0.3 ECTS
- Poster presentation at the Nature CNÏO Conference Oncogenes and Human Cancer: the next 25 years in Madrid, Spain	2007	0.3 ECTS

	Year	Workload
International conferences		
- Annual joint MGC- Cancer Research UK graduate student conference in Leuven, Belgium	2004	0.9 ECTS
- Annual joint MGC- Cancer Research UK graduate student conference in Luik, Belgium	2005	0.9 ECTS
- Annual joint MGC- Cancer Research UK graduate student conference in Oxford, United Kingdom	2006	0.9 ECTS
- Nature CNÏO Conference Oncogenes and Human Cancer: the next 25 years in Madrid, Spain	2007	1.2 ECTS
Seminars and workshops		
- Monthly JNI Oncology lectures	2005-2008	0.9 ECTS
- Annual Molecular Medicine Day in Rotterdam	2005	0.3 ECTS
- Annual MGC day in Rotterdam	2005	0.3 ECTS
- Annual Tumor Cell Biology meeting of the Dutch Cancer Society in Lunteren	2005	0.6 ECTS
- Genetica retraite in Kerkrade	2006	0.6 ECTS
- Master class with Prof.dr. Mary-Claire King	2006	0.3 ECTS
- Annual Tumor Cell Biology meeting of the Dutch Cancer Society in Lunteren	2007	0.6 ECTS
- Symposium: New developments in hereditary cancer of the Stichting Opsporing Erfelijke Tumoren (STOET)	2008	0.3 ECTS

2. Teaching activities

Supervising practicals and bachelor's thesis		
- Internship of master student Joyce Vermeulen Thesis: Mutation analysis of the *human MutS Homolog 6* (*MSH6*) gene in familial breast cancer October 2007- May 2008	2007-2008	8.6 ECTS

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