DNA DAMAGE RESPONSE
GENES AND CHROMOSOME
11q21-q24 CANDIDATE TUMOR
SUPPRESSOR GENES IN
BREAST CANCER

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GENES IN BREAST CANCER

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Oulu, Finland
2002

Abstract

As the defects in DNA repair and cell cycle control are known to promote tumorigenesis, a proportion of inherited breast cancers might be attributable to mutations in the genes involved in these functions. In the present study, three such genes, TP53, CHK2 and ATM, which are also associated with known cancer syndromes, were screened for germline mutations in Finnish breast cancer patients.

In combination with our previous results, three TP53 germline mutations, Tyr220Cys, Asn235Ser and Arg248Gln, were detected in 2.6% (3/108) of the breast cancer families. The only observed CHK2 alteration with a putative effect on cancer susceptibility, Ile157Thr, segregated ambiguously with the disease, and was also present in cancer-free controls. The available functional data, however, suggests that the altered CHK2 in some way promote tumorigenesis. Furthermore, compared to the other studied populations, Ile157Thr seems to be markedly enriched in Finland. Thus, the clinical significance of Ile157Thr requires further investigation among Finnish cancer patients.

ATM germline mutations appear to contribute to a small proportion of the hereditary breast cancer risk, as two distinct ATM mutations, Ala2524Pro and 6903insA, were found among three families (1.9%, 3/162) displaying breast cancer. They all originated from the same geographical region as the AT families with the corresponding mutations, possibly referring to a founder effect concerning the distribution of these mutations in the Finnish population.

The genes important for tumorigenesis in sporadic disease might also contribute to familial breast cancer. Therefore, four putative LOH targets genes in chromosome 11q21-q24 were screened for intragenic mutations, and five were analyzed for epigenetic inactivation in sporadic breast tumors. The lack of somatic intragenic mutations in MRE11A, PPP2R1B, CHK1 and TSLC1 led us next to investigate promoter region hypermethylation as a mechanism capable of silencing these genes, as well as the ATM gene. Only TSLC1 demonstrated involvement of CpG island methylation, which was especially prominent in three tumors. This suggests that together with LOH, methylation could result in biallelic inactivation of the TSLC1 gene in breast cancer.

Keywords: breast cancer susceptibility, double-strand break signaling, CpG island methylation
To Paavo
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May 2002, Oulu

Minna Allinen
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>AT</td>
<td>ataxia-telangiectasia</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia-telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ataxia-telangiectasia and Rad3-related</td>
</tr>
<tr>
<td>BASC</td>
<td>BRCA1-associated genome surveillance complex</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell chronic lymphatic leukemia/lymphoma 2 oncogene</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer 1 gene</td>
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<tr>
<td>BRCA2</td>
<td>breast cancer 2 gene</td>
</tr>
<tr>
<td>CC</td>
<td>cell cycle</td>
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<tr>
<td>CDC</td>
<td>cell division cycle -protein</td>
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<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CGH</td>
<td>comparative genomic hybridisation</td>
</tr>
<tr>
<td>CHK1</td>
<td>cell cycle checkpoint kinase 1 gene</td>
</tr>
<tr>
<td>CHK2</td>
<td>cell cycle checkpoint kinase 2 gene</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine-guanine dinucleotide</td>
</tr>
<tr>
<td>CSGE</td>
<td>conformation-sensitive gel electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyl transferase</td>
</tr>
<tr>
<td>DSB</td>
<td>double-strand break</td>
</tr>
<tr>
<td>FHA</td>
<td>forhead associated</td>
</tr>
<tr>
<td>G1</td>
<td>gap between mitosis and the onset of DNA replication</td>
</tr>
<tr>
<td>G2</td>
<td>gap between DNA synthesis and the onset of mitosis</td>
</tr>
<tr>
<td>GGR</td>
<td>global genome repair</td>
</tr>
<tr>
<td>GSTP1</td>
<td>glutathione S-transferase P1 gene</td>
</tr>
<tr>
<td>HNPCC</td>
<td>hereditary nonpolyposis colorectal cancer</td>
</tr>
<tr>
<td>HR</td>
<td>homologous recombination</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LFL</td>
<td>Li-Fraumeni-like</td>
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<tr>
<td>LFS</td>
<td>Li-Fraumeni syndrome</td>
</tr>
</tbody>
</table>
LOH  loss of heterozygosity
MMR  mismatch repair
MLH1  mutL (E.coli) homologue 1 gene
MLH3  mutL (E.coli) homologue 3 gene
MRE11  meiotic recombination protein 11 (S.cerevisiae) homologue
MRE11A  gene for MRE11 protein
MSH2  mutS (E.coli) homologue 2 gene
MSH6  mutS (E.coli) homologue 6 gene
NBS  Nijmegen breakage syndrome
NBS1 (nibrin, p95)  Nijmegen breakage syndrome gene
NER  nucleotide excision repair
NHEJ  non-homologous end-joining
p  short arm of the chromosome
p21 (WAF1, CDKN1A)  cyclin-dependent kinase inhibitor 1A
p53  tumor protein 53
PCR  polymerase chain reaction
PMS2  postmeiotic segregation increased (S.cerevisiae)-like 2 gene
PP2A  serine-theronine protein phosphatase 2A
PPP2R1B  gene encoding the β isoform of the regulatory subunit of serine/threonine protein phosphatase 2A
q  long arm of the chromosome
RAD50  S.cerevisiae RAD50 homolog
RNA  ribonucleic acid
S  DNA synthesis of the cell cycle
TCR  transcription coupled repair
TP53  gene for tumor protein 53
TSG  tumor suppressor gene
TSLC1 (IGSF4)  tumor suppressor in lung cancer 1
UV  ultra violet
List of original articles

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


*The authors contributed equally to the study
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# 1 Introduction

Apart from being the most common malignancy in women worldwide (Parkin *et al*. 1993), breast cancer is also one of the most extensively investigated human cancers. The genetic background of cancer predisposition, tumor initiation, and the malignant transformation ultimately leading to a metastatic disease are all widely addressed topics, and understanding of the genetic defects underlying these fundamental processes is expected to unravel new possibilities for cancer treatment and prevention.

Mutations linked to an inherited cancer predisposition are usually found in tumor suppressor genes (TSG), which often encode proteins involved in cell cycle regulation and the maintenance of genomic integrity. In breast cancer research, a significant leap forward was taken with the identification of the two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* (Miki *et al*. 1994, Wooster *et al*. 1995), which both have important functions in DNA double-strand break (DSB) repair (Liu & West 2002 and refs. therein). By genetic linkage and mutation analysis, it was shown that the lifetime risk of breast cancer is notably elevated in people carrying germline mutations in these genes (Ford *et al*. 1998). Further, mutations in TSGs linked to inherited syndromes with increased cancer predisposition, e.g. *TP53*, *ATM*, *PTEN*, *LKB1* and *AR* (Swift *et al*. 1987, Malkin *et al*. 1990, Srivastava *et al*. 1990, Wooster *et al*. 1992, Lobaccaro *et al*. 1993, Tsou *et al*. 1997, Boardman *et al*. 1998), seem to explain a small additional fraction of inherited breast cancer susceptibility. Despite these findings, however, the genes behind the increased disease susceptibility have remained unidentified in a large proportion of inherited breast cancers.

Subtle sequence variants in low-penetrance genes have been hypothesized to confer a clearly higher attributable risk of breast cancer in the general population than the much more rare mutations in high-penetrance cancer susceptibility genes (Nathanson & Weber 2001). Because genetic linkage studies are expected to identify only moderate or high-penetrance susceptibility genes, other approaches are currently utilized to find and evaluate the contribution of genetic variants in putative low-penetrance genes. These candidates are often chosen for analysis based on their essential biological functions. For instance, case-control studies of several genes involved in a variety of cellular pathways essential to DNA repair and cell cycle control are being performed. Furthermore, genetic alterations which have an effect on cancer risk in *BRCA1* and *BRCA2* mutation carriers
might alone or in combination with alterations in other genes, such as RAD51 and HRAS, for example, slightly increase the risk even in non-carriers (Phelan et al. 1996, Rebbeck et al. 1999, Nathanson & Weber 2001, Levy-Lahad et al. 2001).

Alternatively, genes harboring the defects leading to cancerous cell growth can be uncovered by studying somatic mutations. According to Knudson’s ‘two-hit’ hypothesis, the inactivation of a TSG requires two independent events (Knudson 1971). Loss of heterozygosity (LOH) within a certain narrow chromosomal region is usually recognized as a hallmark of a putative TSG (Cavenee et al. 1983). If one functional allele is inactivated as a result of chromosomal deletion, for example, the other copy can be subsequently silenced by either a genetic or an epigenetic alteration. However, if one of the alleles harbors an inherited mutation, even a single event can be sufficient to unmask the two inactivating alteration and thus result in biallelic silencing of the tumor suppressor gene (Kinzler & Vogelstein 1997, Jones & Laird 1999).

In this study, the focus was on assessing the contribution of germline mutations in selected DSB signaling and DNA repair genes associated with inherited cancer syndromes to breast cancer susceptibility in Finland. Mutation analysis of three such genes, TP53, CHK2 and ATM, was performed both in cancer families and in an appropriate control population. Additionally, in a cohort of sporadic breast cancer patients exhibiting LOH on chromosome 11q21-q24, candidate tumor suppressor genes were analyzed for intragenic alterations as well as promoter region hypermethylation, an epigenetic mechanism of gene silencing.
2 Review of the literature

2.1 Genetic aberrations in cancer

Cancer is a multistage process, and three to seven successive mutations are estimated to be required for the malignant conversion of a normal cell (Miller 1980, Weinberg 1989, Vogelstein & Kinzler 1993, Kinzler & Vogelstein 1996). Thus, as the mutation rate is typically of the order of less than $10^{-6}$ per each cell division, the likelihood of a single cell to accumulate the requisite number of independent mutations to inactivate the cellular defenses against the development of cancer appears to be very low (Loeb 1991). However, there are two general mechanisms that make the series of cancer-promoting mutations more likely: mutations enhancing the cell proliferation rate increase the size of the target population of cells where the next mutation may occur, and mutations affecting the stability of the genome increase the overall mutation rate (Strachan & Reed 1999).

The multistep nature of cancer has been most extensively studied in colorectal cancer (Fearon & Vogelstein 1990, Kinzler & Vogelstein 1996). Pathogenic mutations usually occur in the genes controlling the cell cycle, apoptosis and genomic integrity. Genetic lesions may also influence proteins, which are responsible for cell-to-cell contacts, or the factors needed for tumor expansion and invasion into nearby tissues. Proto-oncogenes and tumor suppressor genes (TSG) compose the two main categories of aberrantly functioning genes in cancer.

The normal activity of a proto-oncogene supports cell proliferation, but a gain of function mutation (e.g. amplification, point mutation, or transposition to an active chromosome domain) in a single allele may result in inappropriate or excessive activity (Park 1998 and refs. therein). Tumor suppressor gene products, on the other hand, are targeted to inhibit events typical of cancerous behavior. They are responsible for controlling the cell cycle progression and the induction of apoptosis and for maintaining genomic integrity by ensuring accurate replication, repair and segregation of the cell’s DNA. Inactivation of both alleles of a TSG may leave some of these crucial regulatory functions uncontrolled, and thus provide the mutated cell with a growth advantage (Fearon 1998 and refs. therein). The germline alterations related to inherited cancer susceptibility are frequently found in tumor suppressor genes, and only on a few
occasions (RET, CDK4 and MET) have they been found in proto-oncogenes (Mulligan et al. 1993, Zuo et al. 1996, Schmidt et al. 1997). Conversely, cancer-associated somatic mutations are found in genes belonging to both categories.

Apart from the sequential tumor progression pathway, an alternative model has also been described (Shibata & Aaltonen 2001). In the new branching model, the final clonal expansion arises from a single founder cell, which is the only survivor among the other dead-end lineages. Therefore, the mutations in the developing tumor fall into two groups: mutations acquired by the final founder cell are present in every cell, whereas mutations arising during clonal expansion are found in only a fraction of malignant cells.

2.2 Clinicopathological profile of breast cancer

About 95% of all breast cancers arise from the epithelium of the mammary gland, including the terminal ducts and lobules. The transition of normal breast epithelium to a malignant tumor occurs in several stages. First, normal cells become hyperplastic, and some of these cells with atypical appearance subsequently go through malignant transformation and give rise to a carcinoma. A noninvasive carcinoma (carcinoma in situ) may eventually develop into an invasive carcinoma with metastatic potential. Ductal (70%) and lobular (about 6%) cancers are the two largest histological subgroups of invasive breast cancer. Rare histological subtypes include medullar, mucinous, papillary and tubular carcinomas, as well as Paget’s disease (Berg & Hutter 1995).

At present, the most powerful factors for assessing the prognosis of breast cancer are tumor size and differentiation (histological type and grade), lymph node stage and vascular invasion (reviewed in Elston et al. 1999). The clinical TNM staging of a tumor is based on the above-mentioned factors, and the stage of a tumor is defined at the time of diagnosis. The 10-year relative survival in patients with stage 0 tumors (carcinoma in situ) is 95% and decreases in each class as follows: stage I (diameter 2 cm or less, axillary nodes not involved), 88%; stage II (diameter between 2 to 5 cm and/or mobile axillary nodes), 66%; stage III (larger than 5 cm and/or fixed axillary nodes), 36%; stage IV (distant metastases), 7% (Bland et al. 1998).

The response to endocrine therapy can be predicted by evaluating the estrogen receptor (ER) and progesterone receptor (PgR) status. While only one-third of unselected breast cancer patients will respond to anti-estrogen therapy, the response rate in ER-positive tumors is approximately 50%. Combined with positive PgR status, almost 80% of patients will respond to endocrine therapy (NIH Consensus Development Conference 1980).

Besides the traditional indicators for assessing prognosis, many molecular markers have been proposed as putative prognostic factors. Among the most promising ones are the urokinase plasminogen activator (uPA) and its inhibitor PAI-1, which participate in apoptosis through matrix degradation (Liotta et al. 1981, Foekens et al. 1994). The prognostic information of uPA seems to be independent of the traditional morphological factors (Duffy et al. 1999). Amplification and overexpression of the ERBB2 proto-oncogene occurs in 10-34% of breast cancers, and generally correlates with a poor
outcome (Ross & Fletcher 1999). These patients with tumors overexpressing ERBB2 can be treated with a therapeutic antibody, herceptin, and as a single agent, the treatment produces a response rate of approximately 15% (Cobleigh et al. 1999). Furthermore, administration of herceptin with chemotherapy results in better response rates that chemotherapy alone (Shaks 1999). In addition, TP53 gene mutations, expression of E-cadherin, epidermal growth factor (EGF), Ki67 and BCL2 as well as the microvessel count have all been shown to possess prognostic importance (Dowsett 1998, Duffy 2001).

Although the markers mentioned above are significant in univariate analysis, many of them lose their prognostic values in multivariate analysis because of internal correlations (Howat et al. 1983, Battaglia et al. 1988). In a more recent approach to evaluate the clinical outcome in breast cancer patients, the genome-wide expression pattern of thousands of genes can be simultaneously assayed by utilizing cDNA microarrays. Classification of tumors based on these gene expression patterns can then be used as a prognostic marker of the clinical outcome (Perou et al. 2000, Sørlie et al. 2001). For instance, a specific gene expression signature associated with poor prognosis was recently identified based on microarray data from 117 breast tumors (van’t Veer et al. 2002).

### 2.3 Hereditary breast cancer predisposition

Breast cancer is recognized as the most prevalent malignancy among females worldwide (Parkin et al. 1993). The number of new cases has been increasing annually, and in 1999, altogether 3585 new breast cancer cases were reported in Finland (Finnish Cancer Registry 2002). However, mortality rates have been declining at the same time. The most prominent and therefore one of the best studied risk factors is a family history of breast cancer. Women who carry either a BRCA1 or a BRCA2 mutation have an estimated 60% lifetime risk of developing breast cancer (Easton et al. 1993, Ford et al. 1998). They also have a high lifetime risk of ovarian cancer, although for BRCA2 carriers, the risk is somewhat lower. In addition to breast and ovarian cancer, an excess of prostate and colon cancers has been observed in families with BRCA1 mutations (Ford et al. 1994). Men carrying a BRCA2 mutation have an estimated 6% lifetime risk of breast cancer, and BRCA2 mutations may also be associated with prostate and pancreatic cancer (Breast Cancer Linkage Consortium 1999). Furthermore, mutations in genes linked to certain inherited cancer-predisposing syndromes are known to increase the risk of breast cancer (Nathanson et al. 2001).

Besides family history, early age of menarche, nulliparity, late age of menopause and benign breast diseases are considered less powerful endogenous cancer risk factors. Of the exogenous risk factors, radiation exposure has clearly been shown to increase the cancer risk. Additional known exogenous risk factors are the use of oral contraceptives, a high-fat diet and high socio-economic status (Couch & Weber 1998 and refs. therein).
2.3.1 Beyond BRCA1 and BRCA2

Environmental factors, such as geographically limited exposure to carcinogens, cultural behavior patterns (e.g., age at first live birth) or socio-economic influences (e.g., dietary routine) undeniably play a role in familial clustering of breast cancer. However, approximately 7% of breast cancers are caused by the inheritance of a germline mutation in a cancer-predisposing gene (Claus et al. 1996). Mutations in the two most important, high-penetrance breast cancer susceptibility genes, BRCA1 and BRCA2 (Miki et al. 1994, Wooster et al. 1995), were found in a majority of breast cancer families with apparent autosomal dominant inheritance of susceptibility to both breast and ovarian cancer (Ford et al. 1998). Among these families, especially the proportion of BRCA1 mutations was strikingly high (52%). However, later studies confirmed the observation that the breast cancer predisposition in a majority of families with less than six cases of female breast cancer and no ovarian cancer is not due to BRCA1 or BRCA2 mutations (Rebbeck et al. 1996, Schubert et al. 1997, Serova et al. 1997, Vehmanen et al. 1997a,b, Huusko et al. 1998). In Finland, most of the breast-ovarian cancer families were found to be BRCA1 or BRCA2 mutation-positive, while only 5-11% of the breast cancer families lacking ovarian cancer carried these mutations (Vehmanen et al. 1997a,b, Huusko et al. 1998, Vahteristo et al. 2001a). Additionally, germline mutations in the TP53, ATM, PTEN, LKB1 and AR genes have been reported to increase the risk of breast cancer (Swift et al. 1987, Malkin et al. 1990, Srivastava et al. 1990, Wooster et al. 1992, Lobaccaro et al. 1993, Tsou et al. 1997, Boardman et al. 1998). The involvement of TP53 and ATM will be discussed in more detail later (see chapters 2.5.1.1 and 2.5.1.2).

Evidently, a residual dominantly inherited risk for breast cancer in addition to the risk due to mutations in BRCA1 and BRCA2 does exist. In addition, there is a substantially increased recessively inherited risk associated with early-onset breast cancer (Cui et al. 2001). Multiple approaches are now being used to identify additional cancer susceptibility genes (Nathanson & Weber 2001). For example, genetic linkage studies are being performed on families with three or more cases of breast cancer. Positive linkage was observed at the chromosome region 8p12-p22 in two German breast cancer families (Seitz et al. 1997), but was not confirmed in a larger study (Rahman et al. 2000). In a study of Finnish, Swedish and Icelandic breast cancer families, another plausible breast cancer susceptibility locus at chromosome 13q21-q22 was identified (Kainu et al. 2000). Somatic deletions detected by comparative genomic hybridization (CGH) were used as the basis of a targeted linkage analysis of 77 families, resulting in a multi-point LOD score of 3.46. This locus (OMIM 605365) has been estimated to explain approximately two thirds of Finnish BRCA1 and BRCA2 mutation-negative breast cancer families. The results obtained in this study were also evaluated in 128 high-risk breast cancer families of Western European ancestry carrying no identified BRCA1 or BRCA2 mutations, but no evidence of linkage was found (Thompson et al. 2002). Thus, the authors of the latter study concluded that if a susceptibility gene does exist at this locus, its contribution to breast cancer susceptibility is likely to be very small or at least geographically limited. However, the characteristic clustering of genetic disease alleles in Finland (Peltonen et al. 1999) and the different ascertainment criteria of the study cohort might explain the
contradictory linkage results and the variation in the observed \textit{BRCA1} and \textit{BRCA2} mutation frequencies in different populations.

As genetic linkage studies have had limited success in identifying new breast cancer susceptibility genes, genome-wide association studies utilizing single nucleotide polymorphisms (SNPs) could provide an efficient tool suitable for automated high-throughput genotyping and identification of disease-associated haplotypes (Irizarry \textit{et al.} 2000, International SNP Map Working Group 2001). Classification of breast cancer families according to the gene expression data from cDNA microarrays (Hedenfalk \textit{et al.} 2001) and screening for altered gene products using oligonucleotide-based exon-specific arrays (Shoemaker \textit{et al.} 2001) might also prove invaluable in the search for new susceptibility genes.

\textbf{2.3.2 Low penetrance candidate genes on the basis of biological plausibility}

Sequence variants or polymorphisms in low-penetrance genes might be associated with a slightly elevated risk for breast cancer. However, due to their frequent occurrence, these alterations might confer a much higher attributable cancer risk in the general population than rare mutations in high-penetrance genes (Nathanson & Weber 2001). Based on the biological plausibility, candidate low-penetrance genes are usually chosen from among the genes in which even a subtle change could have an effect on the biochemical pathways that influence carcinogenesis. For example, the genes involved in carcinogen and steroid hormone metabolism, DNA damage repair and immune surveillance have been hypothesized as candidates for low-penetrance cancer susceptibility genes (Nathanson & Weber 2001). Due to the rare appearance of low-penetrance alleles in cancer families appropriate for genetic linkage studies, either population-based case-control studies or evaluation of low-penetrance genes as modifiers of high-penetrance genes are currently the two most suitable approaches. Based on case-control studies, certain polymorphisms in the \textit{CYP19}, \textit{GSTM1}, \textit{GSTP1} and \textit{TP53} genes seem to be candidates for low-penetrance genes (Dunning \textit{et al.} 1999). Association studies have demonstrated that women carrying a \textit{BRCA1} mutation and an altered form of either \textit{HRAS} or the androgen receptor gene have an increased cancer risk compared to those carrying only the \textit{BRCA1} mutation (Phelan \textit{et al.} 1996, Rebbeck \textit{et al.} 1999). A single nucleotide polymorphism in the \textit{RAD51} gene has also been reported to increase the cancer risk in \textit{BRCA2} carriers (Levy-Lahad \textit{et al.} 2001). Both approaches, however, have their limitations. Case-control studies are generally time-consuming and expensive, and the association studies of modifier genes are often underpowered, lacking an appropriate control population (Nathanson \textit{et al.} 2001, Nathanson & Weber 2001).
2.4 Cellular pathways maintaining genomic integrity

2.4.1 DNA damage repair pathways

Deficiencies in DNA damage signaling and repair pathways are fundamental to the etiology of most human cancers. Throughout its biological life, DNA is exposed to a variety of different damaging agents, which can be of either exogenous or endogenous origin. No single repair process could manage with the great variety of genetic lesions, and multiple, partly overlapping damage repair pathways are therefore required in mammals. The major mechanisms of DNA repair include excision, recombinational and mismatch repair (Figure 1) (Hoeijmakers 2001). Many human syndromes predisposing the patient to cancer are due to defects in these repair pathways (Table 1).

Table 1. Aberrations of the components in cell cycle checkpoint and DNA repair in human tumors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Defect</th>
<th>Hereditary syndrome</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>DSB</td>
<td>Ataxia-telangiectasia</td>
<td>lymphoma, leukemia, breast</td>
</tr>
<tr>
<td>MRE11A</td>
<td>DSB</td>
<td>AT-like disorder</td>
<td>lymphoma</td>
</tr>
<tr>
<td>NBS1</td>
<td>DSB</td>
<td>Nijmegen breakage syndrome</td>
<td>lymphoma</td>
</tr>
<tr>
<td>BRCA1</td>
<td>HR</td>
<td>Familial breast cancer 1</td>
<td>breast, ovarian, prostate, colon</td>
</tr>
<tr>
<td>BRCA2</td>
<td>HR</td>
<td>Familial breast cancer 2</td>
<td>breast (female/male), ovary, prostate, pancreas</td>
</tr>
<tr>
<td>CHK1</td>
<td>CC</td>
<td>NR</td>
<td>colorectal and endometrial cancer</td>
</tr>
<tr>
<td>CHK2</td>
<td>CC</td>
<td>Li-Fraumeni syndrome</td>
<td>breast, lung, colon, urinary bladder, testis</td>
</tr>
<tr>
<td>p53</td>
<td>CC</td>
<td>Li-Fraumeni syndrome</td>
<td>sarcoma, breast, brain, leukemia,</td>
</tr>
<tr>
<td>RECQL2</td>
<td>HR?</td>
<td>Werner syndrome</td>
<td>various cancers</td>
</tr>
<tr>
<td>RECQL3</td>
<td>HR?</td>
<td>Bloom syndrome</td>
<td>leukemia, lymphoma</td>
</tr>
<tr>
<td>RECQL4</td>
<td>HR?</td>
<td>Rothmund-Thomson syndrome</td>
<td>osteosarcoma</td>
</tr>
<tr>
<td>MSH2</td>
<td>MMR</td>
<td>HNPCC</td>
<td>colon, rectum, gastric, endometrium, ovarian, urinary organs</td>
</tr>
<tr>
<td>MSH6</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>MLH3</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>p16</td>
<td>CC</td>
<td>Familial melanoma</td>
<td>melanoma, pancreas</td>
</tr>
<tr>
<td>RB</td>
<td>CC</td>
<td>Familial retinoblastoma</td>
<td>retinoblastoma, osteosarcoma</td>
</tr>
<tr>
<td>CSA, CSB</td>
<td>TCR</td>
<td>Cocayne’s syndrome</td>
<td>Skin</td>
</tr>
<tr>
<td>XPA-XPG</td>
<td>NER</td>
<td>Xeroderma pigmentosum</td>
<td>skin</td>
</tr>
</tbody>
</table>

Abbreviations: CC, cell cycle control; DSB, double-strand break repair; HR, homologous recombination repair; MMR, mismatch repair; NER, nucleotide excision break repair; NR, not reported; TCR, transcription coupled repair. Adapted from Bartek & Lukas (2001), Hoeijmakers (2001), Svejstrup JQ (2002).

Two basic types of excision repair, nucleotide excision repair (NER) and base excision repair (BER), have been described both in bacteria and in mammals (reviewed in Hoeijmakers 1993a,b, Wood 1997). NER acts on a variety of helix-distorting DNA
lesions mostly caused by exogenous sources interfering with base pairing. The most important function of NER in humans is to remove ultra violet (UV)-induced damage from DNA (e.g. pyrimidine dimers). Defects in NER cause an autosomal recessive disease called xeroderma pigmentosum (XP), which makes the patients extremely prone to develop sun-induced skin malignancies. XP is due to mutations in seven different genes, XPA, XPB, XPC, XPD, XPE, XPF and XPG, all of which function in the NER pathway (Hoeijmakers 2001).

Eukaryotic NER includes two major branches, transcription-coupled repair (TCR) and global genome repair (GGR) (de Laat et al. 1999, Tornaletti & Hanawalt 1999). GGR is a slow random process of inspecting the entire genome for injuries, while TCR is highly specific and efficient and concentrates on damage-blocking RNA polymerase II. The two mechanisms differ in substrate specificity and recognition. In GGR, the XPC-HR23B complex recognizes damage located in nontranscribed regions (Sugasawa et al. 2001), whereas the arrest of RNA polymerase II (RNAPII) serves as the recognition signal in TCR. The molecular mechanism of RNAPII displacement is currently unclear, but essential factors, such as the Cocayne’s syndrome proteins CSA, CSB, XPA-binding protein 2 (XAB2), TFIIH and XPG (Svejstrup 2002), have been identified to function in TCR. Subsequently, both in GGR and TCR, an open unwound structure forms around the lesion. This creates specific cutting sites for XPG and ERCC1-XPF nucleases, and the resulting gap is filled in by PCNA-dependent polymerase and sealed by DNA ligase (Wood 1997, de Laat et al. 1999).

BER removes small chemical alterations of bases, focusing mainly on modifications due to endogenous cellular metabolism (e.g. reactive oxygen species and byproducts of hydrolysis, methylation and deamination) (Lindahl & Wood 1999, Hoeijmakers 2001). In BER, a lesion-specific glycosylase removes the modified bases from DNA, resulting in the generation of an apurinic or apyrimidinic (AP) site. The AP endonuclease then cuts the sugar-phosphate backbone at the position of the missing base. Exonucleases further remove a few nucleotides from the DNA, and the remaining gap is filled by resynthesis and sealed by DNA ligase 3 (Lindahl & Wood 1999). Similarly to NER, there is substantial evidence that TCR can also occur through the BER pathway (Cooper et al. 1997, Nouspikel et al. 1997, Le Page et al. 2000).

For the cell, double-strand breaks (DSB) are probably the most deleterious form of DNA damage and may arise from ionizing radiation (IR), X-rays, free radicals, chemicals, or during replication of single-strand breaks (SSB) (Khanna & Jackson 2001). A single DSB is sufficient to kill a cell if it inactivates an essential gene or triggers apoptosis (Rich et al. 2000). There are two distinct and complementary mechanisms for DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Haber 2000, Karran 2000). When an intact DNA copy is available, HR is preferred. Otherwise, cells utilize the more error-prone NHEJ. It has been proposed that Ku70 acts as a switch between the two DSB pathways (Goedcke et al. 1999). When present, Ku70 destines DSB for NHEJ by binding to DNA ends and attracting other factors, including MRE11. However, the absence of Ku70 allows participation of DNA ends together with MRE11 in the meiotic HR pathway.
In HR, the DNA ends are first resected in the 5’ to 3’ direction by the exonuclease activity of the RAD50/MRE11/NBS1 complex (Paull & Gellert 1998). Assisted by several proteins facilitating the identification and correct positioning with the homologous sister chromatid sequence, the 3’ single-stranded tails invade the DNA double helix of the intact molecule, which is used as a template for DNA polymerase. Following branch migration and ligation, Holliday junctions are resolved by resolvases to yield two intact DNA molecules (Khanna & Jackson 2001). On the contrary, NHEJ does not require an undamaged template: here, the two DNA ends are simply attached together...
using the end-binding Ku70/80 complex and DNA-PKcs, followed by ligation by XRCC4 (Haber 2000, Hoeijmakers 2001, Khanna & Jackson 2001). Mutations in many of the genes involved in DSB detection and repair give rise to many cancer-predisposing syndromes (Table 1).

Mismatch repair (MMR) removes both nucleotides mispaired by DNA polymerases and insertion/deletion loops, which are due to slippage during replication of repetitive sequences or formed during recombination (Harfe & Jinks-Robertson 2000). Initially, the heterodimeric MSH complex recognizes the nucleotide mismatch, subsequently followed by interaction with MLH1/PMS2 and MLH1/MLH3 complexes. Several proteins participate in process of the nucleotide excision and resynthesis. Tumor cells deficient in mismatch repair have much higher mutation frequencies than normal cells (Parsons et al. 1993, Bhattacharyya et al. 1994). In humans, the mechanism involves at least six genes MSH2, MLH1, PMS2, MSH3, MSH6 and MLH3. The defects in these genes (excluding MSH3) result in hereditary nonpolyposis colon cancer (HNPCC) (Hoeijmakers 2001, Peltomäki 2001).

Traditionally, the DNA repair mechanisms have been regarded as separate processes. However, since the different replication complexes share many of the participating proteins, it is possible that these partly similar processes should be regarded as temporary associations of DNA interacting proteins rather than as rigid complexes where the participating proteins are devoted solely to one mission (Cleaver et al. 2001). The various associations of these complexes would occur according to the specific challenges set by DNA damage. An example of such complex is the BRCA1-associated genome surveillance complex (BASC), which contains many known DNA polymerases, repair enzymes and recombination proteins (Wang et al. 2000) (Table 2). All of these proteins possess the ability to bind abnormal DNA structures and might therefore act as sensors for these aberrations (Uchiumi et al. 1996, Alani et al. 1997, Bennett et al. 1999, Marsischky et al. 1999).

It has been proposed that BRCA1 has a central role in BASC (Table 2), acting as a scaffold that both organizes and coordinates the multiple activities required to maintain genomic integrity (Wang et al. 2000). BRCA1 has two C-terminal BRCT (BRCA1 carboxy-terminal repeat) domains, which are important for protein-protein interactions and have been found in many proteins to be linked to DNA damage response checkpoints (Koonin et al. 1996, Bork et al. 1997, Zhang et al. 1998a). BRCA1 has also been found to participate in other regulatory complexes, such as the RNA polymerase II holoenzyme (Scully et al. 1997) and the Swi/Snf chromatin remodeling complexes (Bochar et al. 2000), connecting BRCA1 to transcription regulation and providing a link to IR-induced transcription-coupled repair. BRCA2, on the other hand, regulates both the intracellular localization and the DNA-binding ability of RAD51, which is an essential protein in homologous recombination and DNA repair (Davies et al. 2001).
Table 2. Suggested roles of known proteins and protein complexes in the BRCA1-associated genome surveillance complex (BASC).

<table>
<thead>
<tr>
<th>Protein or subcomplex</th>
<th>DNA damage sensor</th>
<th>DNA repair mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1</td>
<td>scaffold protein</td>
<td>DSB repair</td>
</tr>
<tr>
<td></td>
<td>to organize different DNA damage sensor proteins</td>
<td></td>
</tr>
<tr>
<td>ATM</td>
<td>double-strand break detection</td>
<td>signal transduction of DSB response</td>
</tr>
<tr>
<td>MRE11/RAD50/NBS1</td>
<td>double-strand break detection</td>
<td>DSB repair</td>
</tr>
<tr>
<td>MSH2/MSH6</td>
<td>base-pair mismatches</td>
<td>MMR</td>
</tr>
<tr>
<td></td>
<td>Holliday junctions</td>
<td></td>
</tr>
<tr>
<td>PMS2/MLH1</td>
<td>base-pair mismatches</td>
<td>MMR</td>
</tr>
<tr>
<td>RECQL3</td>
<td>abnormal double-stranded structures</td>
<td>HR?</td>
</tr>
<tr>
<td></td>
<td>during replication</td>
<td></td>
</tr>
<tr>
<td></td>
<td>abnormal telomere repeat sequences</td>
<td></td>
</tr>
<tr>
<td>RF-C complex</td>
<td>abnormal template-primer junctions</td>
<td>NER</td>
</tr>
</tbody>
</table>

2.4.2 Apoptotic pathways

The efficiency of the DNA repair mechanisms, cell type, the oncogenic composition of the cell, extracellular signals, the intensity of the stress conditions, the level of p53 expression and its interactions with other proteins all have an effect on the cell’s response to the existing damage (reviewed in Vogt Sionov & Haupt 1999). Should the damage be irreparable, a defective cell can be sacrificed for the benefit of the organism. Two main apoptotic pathways have been described in mammalian cells, namely the death-receptor pathway and the mitochondrial pathway (Hengartner 2000). Members of the death-receptor superfamily, including CD95, trigger the death-receptor pathway. After DNA damage, p53 is essential for transcriptional upregulation of CD95 (Müller et al. 1997, Müller et al. 1998). In general, signaling of apoptosis by members of the death receptor family includes ligand binding, receptor trimerization and death inducing signaling complex (DISC) formation, recruitment of multiple procaspase-8 molecules, and subsequent autocatalytic cleavage of the procaspase. Active caspase-8 then activates other downstream caspases that cleave cellular substrates, leading to cell destruction (Hengartner 2000).

The mitochondrial pathway, on the other hand, is utilized in response to extracellular signals and internal insults such as DNA damage (Kroemer & Reed 2000, Rich et al. 2000). Apart from its essential role in regulating cell growth, p53 also participates in this cell death pathway. Its function leads to elevated BAX-levels (Miyashita & Reed 1995),
whereas the expression of BCL2 is downregulated through abolished promoter function (Budhram-Mahadeo et al. 1999). Pro- and anti-apoptotic BCL2 family members meet at the surface of mitochondria, where they compete to regulate cytochrome c release (Gross et al. 1999). Together with Apaf-1 and procaspase-9 and possible additional proteins, cytochrome c participates in apoptosome formation (Acehan et al. 2002). The two pathways come together at the level of caspase-3 activation, and the following multiple downstream pathways finally result in organized destruction and removal of the destined cell (Hengartner 2000).

2.5 DNA double-strand break signaling and its relation to cell cycle control

The DNA DSB damage response pathway provides a mechanism for transducing a signal from a sensor, which recognizes the damage, through a transduction cascade to a series of downstream effector molecules. Depending on the severity of the damage, cells may either choose to arrest the cell cycle until the damage is repaired, or if the damage is irreparable, proceed to apoptosis. A schematic presentation of the mammalian DSB signaling pathway is shown in Figure 2.

The two main sensor molecules, ATM and ATR, act in parallel branches at the front line of the DNA damage response pathway. They respond primarily to different types of DNA damage (Gatei et al. 2001). ATM reacts mainly to the DNA-damaging agents that cause DSBs, such as IR, and its downstream targets include CHK2, BRCA1 and p53 (Banin et al. 1998, Canman et al. 1998, Matsuoka et al. 1998, Cortez et al. 1999, Matsuoka et al. 2000). On the other hand, ATR, along with ATRIP (ATR-interacting protein), responds primarily to UV and hydroxyurea (HU)-induced damage, which may potentially interfere with DNA replication (Cortez et al. 2001). ATR regulates CHK1 and BRCA1 (Chen 2000, Liu et al. 2000), but phosphorylation of p53 is also possible (Tibbetts et al. 1999). Due to ATM and ATR, BRCA1 becomes phosphorylated not only at overlapping but also at distinct residues, depending on the type of the DNA lesion (Gatei et al. 2001). Furthermore, the two pathways overlap and often cooperate with each other to ensure efficient repair without delay and to maintain genomic integrity. When one pathway is genetically compromised, they can also function redundantly, although probably less efficiently and with different kinetics (Liu et al. 2000).

Cell cycle arrest is controlled at specific checkpoints. At the G1 and S checkpoints, the quality of the DNA about to be replicated is ensured, whereas at the G2-M, the segregation of damaged chromosomes is prevented. At G1, as a consequence of DNA damage, activated ATM phosphorylates p53 on Ser15, CHK2 on Thr68, and MDM2 on Ser395 (Banin et al. 1998, Canman et al. 1998, Ahn et al. 2000, Matsuoka et al. 2000, Melchionna et al. 2000, Maya et al. 2001). Subsequently, activated CHK2 phosphorylates p53 on Ser20 (Hirao et al. 2000, Shieh et al. 2000). Together, these phosphorylations interfere with p53 binding to MDM2, leading to stabilization and activation of p53 (Chehab et al. 2000). The increased level of p53 transcriptionally induces p21 (also known as CDKN1A and WAF1), which inhibits CDK2-cyclin E causing the cell cycle arrest (Kastan & Lim 2000).
Fig. 2. A schematic representation of the DNA DSB signaling pathway. Phosphorylation sites, when known, are indicated next to each protein. The sites with a lighter background are inhibitory residues, which have to be removed by an upstream effector. The dashed arrows represent unidentified, putative parallel or converging pathways influencing S-phase arrest. Modified from Vogt Sionov & Haupt (1999), Kastan & Lim (2000), Bartek & Lukas (2001), Futaki & Liu (2001), Khanna & Jackson (2001), Taylor & Stark (2001), Kim et al. (2002), Yazdi et al. (2002).

The defective S-phase checkpoint is defined by a phenomenon of persistent DNA synthesis after IR (also called radioresistant DNA synthesis). In contrast to G1 and G2, the S phase can only be delayed, but never permanently blocked. This delay seems to be independent of the p53 and p21 functions (Bartek & Lukas 2001). Instead, a pathway operating through ATM/ATR, CHK2/CHK1 and CDC25A prevents the removal of inhibitory phosphorylations of CDK2 (Mailand et al. 2000, Costanzo et al. 2000, Falck et...
Besides its importance in the S-phase response, this pathway also provides an
alternative route for generating rapid arrest in G1. Activated ATM phosphorylates CHK2
(Matsuoka et al. 2000, Melchionna et al. 2000), which then targets CDC25A phosphatase
for degradation by phosphorylation it on Ser123 (Falck et al. 2001b), and thus prevents
the activation of CDK2. In a parallel S-phase checkpoint pathway (Falck et al. 2002),
ATM phosphorylates NBS1 on Ser343, which is an event required for activation of
phosphorylates SMC1 on Ser957 and Ser966 in an NBS1-dependent manner, and these
phosphorylations are required for S-phase checkpoint activation (Kim et al. 2002, Yazdi
et al. 2002).

In G2, ATM and ATR activate the checkpoint kinases CHK2 and CHK1, respectively
(Matsuoka et al. 2000, Zhao & Piwnica-Worms 2001), but also BRCA1 is essential for
CHK1 activation (Yarden et al. 2002). Subsequently, CHK2 and CHK1 phosphorylate the
protein phosphatase CDC25C on Ser216, which leads to its inactivation and binding by
the 14-3-3σ protein (Peng et al. 1997, Sanchez et al. 1997, Matsuoka et al. 1998). This
binding prevents the removal of an inhibitory phosphate group on Thr14 and Tyr15 of
CDC2 and thus also the cell’s entry to mitosis (Lee et al. 1992, Sebastian et al. 1993).
The G2 checkpoint is also controlled by p53 through transcriptional repression of Cdc2
and cyclin B1. Several other transcriptional targets of p53 can inhibit CDC2: p21 inhibits
CDC2 directly, 14-3-3σ anchors CDC2 in the cytoplasm, where it cannot induce mitosis,
and GADD45 dissociates CDC2 from Cyclin B1 (Taylor & Stark 2001).

2.5.1 Double-strand break signaling and DNA repair genes associated
with inherited cancer syndromes

2.5.1.1 Germline TP53 and CHK2 mutations in Li-Fraumeni syndrome

Li-Fraumeni syndrome (LFS) is a rare familial multicancer syndrome involving
sarcomas, breast cancer, brain tumors, leukemia, and adrenocortical tumors (Li &
Fraumeni 1969, Li et al. 1988). The criteria for classic LFS are: a proband with sarcoma
before the age of 45, a first-degree relative with any cancer by age 45, and another first-
or second-degree relative with any cancer before age 45 or sarcoma at any age (Li et al.
1988). In many LFS families, affected members carry a germline TP53 mutation (Malkin
et al. 1990, Varley et al. 1997). Having discovered TP53 mutations in families with a less
severe cancer phenotype, Birch et al. (1994) presented criteria for a somewhat milder
form of LFS, namely the Li-Fraumeni-like syndrome (LFL). In this case, a family would
exhibit a proband with any childhood cancer or sarcoma, brain tumor, or adrenocortical
carcinoma diagnosed before age 45, with one first- or second-degree relative with LFS-
type cancer at any age, and one first- or second-degree relative with any cancer at age 60.
The mutation distribution along the protein-encoding region of the TP53 gene was
investigated by Soussi & Béroud (2001). By reviewing the recent studies, they
demonstrated that the mutations are not only concentrated to the highly conserved regions between the exons 5 and 8, but a considerably high proportion are also seen outside this section of the gene. TP53 differs from the other tumor suppressor genes (e.g. APC and BRCA1) showing a high prevalence of missense mutations (Olivier & Hainaut 2001). In breast tumors, TP53 is the most commonly altered gene with a frequency ranging from 12-60% (Olivier & Hainaut 2001). Breast cancer is also the most frequent type of cancer in patients with inherited TP53 mutations, suggesting that a constitutive TP53 mutation could also predispose to early onset breast cancer. Therefore, the role of TP53 as a breast cancer predisposing gene, even without the LFL/LFS features, has been studied in considerable detail. When the TP53 mutation pattern in families showing excessive cases of breast cancer was compared to all germline mutations, an absence of transversions affecting G bases and an excess of mutations in A:T base pairs was observed (Olivier & Hainaut 2001).

In a fraction of the LFS families lacking alterations in TP53, CHK2 mutations have been found to explain the necessary disease predisposition (Bell et al. 1999, Vahteristo et al. 2001b). Due to its essential functions in DSB signaling and close interactions with p53 and BRCA1 (see chapter 2.5), defects in CHK2 can also be hypothesized to have breast cancer predisposing effects similar to those of the mutations in TP53 and BRCA1.

2.5.1.2 Germline ATM mutations in ataxia-telangiectasia

Ataxia-telangiectasia (AT) is a highly pleiotropic, recessive neurodegenerative disorder resulting from germline mutations in the ATM gene (Savitsky et al. 1995a). AT is characterized by progressive cerebellar ataxia, oculocutaneous telangiectasias, premature aging, hypogonadism, sensitivity to ionizing radiation and immunodeficiency. Furthermore, due to ATM involvement in DSB repair, defects in ATM protein function cause genetic instability and, consequently, an increased risk of cancer. AT patients are especially prone to develop lymphatic and leukemic malignancies, but also breast cancer. Cells derived from AT patients are hypersensitive to DSB-inducing agents (Gatti 1998).

The ATM gene located at 11q22.3 is composed of 66 exons and encodes a 13-kb mRNA (Uziel et al. 1996). ATM is a 370 kDa phosphoprotein, which is ubiquitously expressed. ATM is localized mainly in the nuclei of fibroblasts or lymphoid cells, but also in the cytoplasm, where it appears to be associated with vesicular structures through its interaction with β-adaptin (Chen and Lee 1996, Brown et al. 1997, Watters et al. 1997, Lim et al. 1998). In Purkinje cells and other neurons, however, ATM is predominantly cytoplasmic, and its absence leads to lysosome accumulation (Oka & Takashima 1998, Barlow et al. 2000). It belongs to a family of large proteins identified in various organisms, all of which share a highly conserved carboxy-terminal region showing significant sequence homology to the catalytic domain of phosphatidylinositol-3 kinases (Savitsky et al. 1995b).

In addition to its role in the cellular response to DNA damage (see chapter 2.5), ATM activates a separate radiation signal transduction pathway through the stress-activated protein kinase by interacting with ABL (Baskaran et al. 1997, Shafman et al. 1997).
(Figure 3). Cytoplasmic ATM may also have a role in vesicle and protein transport (Watters et al. 1997, Lim et al. 1998) and in protection against endogenous oxidative damage (Barlow et al. 1999). Based on observations in mice, Atm links to the mitochondrial apoptotic pathway due to its association with Bax (Chong et al. 2000) and is specifically cleaved by caspase-3 in cells induced to undergo apoptosis (Smith et al. 1999). This interferes with the ability of ATM to phosphorylate p53, but does not have an effect on its DNA-binding ability. Since ATM is able to bind DNA ends in vitro, it is possible that cleaved ATM binds to the genomic DNA DSBs generated during the apoptotic process, preventing their repair (Smith et al. 1999). In the central nervous system of mice, Atm participates in genotoxic damage-induced apoptosis, aiming to eliminate genetically damaged neurons (Herzog et al. 1998). ATM also seems to have a role in telomere maintenance and replication (Hande et al. 2001).

**Fig. 3.** The many functions of ATM. Some of the key interacting proteins are shown under the title of the involved function. According to Ángele & Hall (2000), Kastan & Lim (2000).

The observation of intermediate radiosensitivity in cells from AT heterozygotes by Chen et al. (1978) first suggested that these individuals might have an elevated cancer risk. Several years later, it was proposed that chromosomal radiosensitivity could be a marker of a low-penetrance cancer-predisposing gene (Scott et al. 1994, Scott et al. 1999). To validate this hypothesis, the same group demonstrated the heritability of chromosomal radiosensitivity of G2 lymphocytes in families with breast cancer (Roberts et al. 1999). Segregation analysis suggested that a single gene could account for as much as 82% of the variance in radiosensitivity observed between the family members, but the data could also be explained by using an alternative model including another, even rarer
gene defect. In addition, increased sensitivity to radiation-induced oncogenesis has recently been demonstrated in murine models carrying heterozygous Atm mutations (Smilenov et al. 2001, Weil et al. 2001). Despite this observation, radiation therapy still appears to be beneficial for carriers of ATM germline mutations (Su & Swift 2001).

The estimated frequency of ATM germline mutation carriers in Finland is one in 280 (Olsen et al. 2001). In epidemiological studies, the female relatives of AT patients, especially mothers, have been demonstrated to have an increased risk to develop breast cancer, with an estimated relative risk of 3.9 (Swift et al. 1987, Pippard et al. 1988, Swift et al. 1991, Janin et al. 1999, Inskip et al. 1999, Olsen et al. 2001). The risk of any other cancers in obligate mutation carriers is not increased in a similar manner (Geoffroy-Perez et al. 2001). A clear discrepancy remains between the epidemiological data and the observed low frequency of ATM germline mutations in breast cancer patients (Athma et al. 1996, Vorechovský et al. 1996, FitzGerald et al. 1997, Bebb et al. 1999, Izatt et al. 1999, Laake et al. 2000b). Interestingly, a high percentage (8.5%) of ATM truncating mutations was demonstrated among patients with sporadic breast cancer (Broeks et al. 2000). All the studied patients had been exposed to radiation therapy, suggesting that radiation might, indeed, induce the development of breast cancer. On the contrary, truncating mutations were not found in a cohort of breast cancer patients with contralateral tumors who had received radiation therapy for their first tumor (Shafman et al. 2000). Thus, the role of radiation as a trigger of tumorigenesis still warrants further investigation. Furthermore, both studies utilized methods that were incapable of detecting missense mutations.

It has been hypothesized that there are two different populations of heterozygous ATM carriers (Meyn 1999, Khanna 2000), having one allele carrying either a truncating or a missense mutation in combination with a normal ATM allele. In the first group, a truncating mutation acts effectively as a null allele, producing an unstable protein, which is present in the cell in very low amounts. Therefore, carriers with a truncating mutation would have a nearly normal phenotype. Instead, in carriers with a missense mutation, a stable but functionally abnormal protein would be present at normal intracellular levels. These abnormal polypeptides would compete with the normal protein in complex formation, thus interfering with their essential cellular functions and contributing to an increased cancer risk. Thus, to elucidate the significance of the proposed hypothesis, it would be important to determine the frequency of ATM missense variants in the general population in greater detail. It is possible that the reasons why these studies have failed to detect the expected number of cancer-related ATM mutations is due to the choice of technique used in mutation screening as well as in the compositions of the study and control populations (reviewed in Angele & Hall 2000).

2.5.1.3 Germline mutations of MRE11A in ataxia-telangiectasia-like disorder and NBS1 in Nijmegen breakage syndrome

Apart from AT, there are two other phenotypically very similar chromosome breakage syndromes. Germline mutations in MRE11A give rise to an AT-like disorder (ATLD)
(Stewart et al. 1999), whereas defects in NBS1 (nibrin, p95) result in Nijmegen breakage syndrome (NBS) (Carney et al. 1998, Varon et al. 1998). The clinical features of these three syndromes overlap, sharing the traits of cancer predisposition, immunodeficiency, hypersensitivity to radiation as well as chromosomal instability (Hoeijmakers 2001). Additionally, cells derived from affected individuals show very similar phenotypes (Petrini 2000). This can be explained by the interacting roles of these three proteins in the DSB pathway and S-phase checkpoint control (Figure 2). MRE11, RAD50 and NBS1 form a nuclear complex, which includes a manganese-dependent single-stranded DNA endonuclease and 3’ to 5’ exonuclease activities (Carney et al. 1998, Trujillo et al. 1998). Along with BRCA1, ATM, and many other proteins, the MRE11/RAD50/NBS1 complex is a member of BASC, which has been suggested to serve as a sensor of DNA damage and a regulator of the repair process (Wang et al. 2000) (see also 2.4.1). Furthermore, ATM phosphorylates NBS1 on several residues in response to DNA damage (Lim et al. 2000, Wu et al. 2000, Zhao et al. 2000), and a functional MRE11/RAD50/NBS1 complex is required for CHK2 activation (Buscemi et al. 2001). This links the MRE11/RAD50/NBS1 complex to DNA damage recognition and also provides a plausible explanation for the phenotypic similarities between these three chromosome breakage syndromes.

2.6 Somatically occurring alterations in breast cancer

As already discussed in chapter 2.1, cancer results from a series of genetic alterations leading to progressive disordering of the important regulatory cellular mechanisms (Fearon & Vogelstein 1990). A small fraction of breast cancers show inherited mutations in certain susceptibility genes, which thus cause the carrier to have a higher risk of developing breast cancer. For cancer to arise, however, several additional somatic mutations are required. Contrary to hereditary cancer, most tumors are devoid of inherited germline mutations. Despite this fundamental difference in molecular genetics between familial and sporadic tumors, many of the same genes are altered in both forms of the disease. For instance, the TSGs modified in sporadic tumors are also good candidates for susceptibility loci in corresponding familial disease. Moreover, studies of the somatic alterations (e.g. point mutations and other more extensive genetic rearrangements, such as insertions, deletions and amplifications, as well as aberrant DNA methylation) provide clues to the mechanisms that result in genomic instability, which is inherent in cancer cells (Couch & Weber 1998 and refs. therein). Indeed, it has been demonstrated that DSB-initiated chromosomal instability is the major driving force of breast cancer progression. Furthermore, a specific methylator phenotype possibly resulting in MMR deficiency has been observed in colorectal cancer (Toyota et al. 1999). It might be due to the genetic heterogeneity resulting from these defects that different breast tumors also present such a great variety of molecular profiles (Shen et al. 2000).
2.6.1 Loss of heterozygosity and comparative genomic hybridization studies in breast cancer

According to Knudson’s ‘two-hit’ hypothesis (Knudson 1971), inactivation of both alleles of a TSG is required for cancer formation. In the hereditary form of cancer, the other allele has an inherited mutation derived from the affected parent, whereas the other allele is mutated somatically. In sporadic cancer, a somatic mutation targeting each allele is required to completely inactivate a TSG. Inactivating mutations include point mutations, loss of chromosomal material, gene conversion, or mitotic recombination or deletion (Knudson 1978, Cavenee et al. 1983). Therefore, chromosomal regions that frequently exhibit allelic losses are expected to harbor putative tumor suppressor genes (Sato et al. 1990). LOH and CGH analysis are currently the two methods most frequently used to detect chromosomal losses.

Traditional LOH analysis is a PCR-based method in which paired blood and tumor samples are screened with polymorphic microsatellite markers spaced across the region of interest. The possible allelic loss at each marker locus can be detected in tumor tissue compared to normal tissue of the same individual. CGH has been designed to detect amplified and deleted stretches of DNA in tumors (Kallioniemi et al. 1992). This technique has been recently used to identify the loci for the Peutz-Jehgers cancer syndrome and the putative new breast cancer susceptibility locus (Hemminki et al. 1997, Hemminki et al. 1998, Kainu et al. 2000). In principle, CGH provides the means to screen the entire genome for chromosomal imbalances in a single experiment. A mix of differently labeled DNAs from normal and tumor cells is used in competitive fluorescence in situ hybridization. The smallest alteration visible in standard CGH is 5-10 Mb, but by using high-density oligonucleotide arrays instead of metaphase chromosomes, CGH analysis can be significantly improved (Monni et al. 2001). Furthermore, in combination with microarray based expression analysis, CGH provides a powerful approach to identify the putative targets of gene amplification (Monni et al. 2001).

LOH in breast tumors has been observed in multiple chromosomal regions, such as the chromosomes 1p, 1q, 3p, 6q, 7q, 8p, 9p, 9q, 11q, 13q, 16q, 17p, 17q, 18p, 18q and 22q (Bièche & Lidereau 1995, Kerangueven et al. 1997a, O’Connell et al. 1998, Shen et al. 2000). By utilizing CGH, the most frequently deleted regions in breast cancer cell lines were found in the chromosomes 1p, 4p, 8p, 10q, 11q, 18p, 18q, 19p, Xp, Xq (Forozan et al. 2000). The authors also reviewed the existing CGH data from primary breast tumors (altogether 698 samples) and discovered that many of the genetic changes detected in breast cancer cell lines were similar to those found in primary breast cancers. Although several of these chromosomal regions have been designated to contain a putative tumor suppressor gene, the actual number and identity of these genes relevant to breast carcinogenesis is currently unknown.
2.6.2 Chromosome 11q loss of heterozygosity target candidate genes in breast cancer

Chromosome 11q is one of the most common targets for allelic loss in human cancers (Seizinger et al. 1991). In breast cancer, high LOH frequencies have been reported by several investigators (Hampton et al. 1994, Winqvist et al. 1995, Kerangueven et al. 1997a,b, Koreth et al. 1997, Negrini et al. 1995, Laake et al. 1997). Together with CGH studies, in which a loss of chromosome 11q in primary breast tumors has also been seen on a regular basis (Isola et al. 1995, Courjal & Theillet 1997, Kuukasjärvi et al. 1997, Schwendel et al. 1998, Tirkkonen et al. 1998, Roylance et al. 1999), these observations suggest the presence of one or more tumor suppressor genes within this region.

The presence of a crucial tumor suppressor gene is further supported by the observation that introduction of a normal chromosome 11 can reverse the tumorigenic and metastatic potential of MCF-7 and MDA-MB-435 breast cancer cell lines, respectively (Negrini et al. 1994, Phillips et al. 1996). In addition, transfer of the normal chromosome 11 suppresses tumorigenicity in Wilms’ tumor, lung, rhabdomyosarcoma, cervical, and melanoma cell lines (Weissman et al. 1987, Koi et al. 1989, Oshimura et al. 1990, Satoh et al. 1993, Robertson et al. 1996). Certain chromosome 11q22-q23 fragments represented by YAC clones are also known to significantly restrain in vivo tumor formation (Murakami et al. 1998, Koreth et al. 1999).

In breast tumors, there are three commonly deleted regions in chromosome 11q. The most proximal region maps to 11q22.3-q23.1 (Hampton et al. 1994, Negrini et al. 1995, Kerangueven et al. 1997b, Koreth et al. 1997, Laake et al. 1997), and contains for instance the genes ATM, DDX10, PPP2R1B and SDHD (Savitsky et al. 1995a, Savitsky et al. 1996, Wang et al. 1998, Baysal et al. 2000). The second region, 11q23.2-q23.3 (Negrini et al. 1995, Kerangueven et al. 1997b, Launonen et al. 1999), harbors genes such as DDX6, TSLC1 and ALL1 (Lu & Yunis 1992, Baffa et al. 1995, Kuramochi et al. 2001). The most distal region is situated at 11q24 (Negrini et al. 1995, Kerangueven et al. 1997b). In addition to CHK1, this region contains a putative TSG called ST14 (Cao et al. 1997, Zhang et al. 1998b) and also the genes PIG8 and P53AIP1, which are both regulated by p53 (Polyak et al. 1997, Oda et al. 2000).

2.6.2.1 Genes related to DNA repair

Besides ATM (11q22.3) and MRE11A (11q21) (see sections 2.5.1.2 and 2.5.1.3), CHK1 (11q24.2) is another candidate gene in chromosome 11q21-q24 directly related to cell cycle regulation and DNA repair. CHK1 is a protein kinase, which acts to integrate signals from ATM and ATR (Flaggs et al. 1997, Sanchez et al. 1997) (see also 2.5). Of the two sensors, ATR is the main regulator of CHK1 in response to DNA damage, probably by phosphorylating Ser345 (Liu et al. 2000, Lopez-Girona et al. 2001). CHK1 phosphorylates and inhibits both CDC25A and CDC25C in response to DNA damage, thus assisting the arrest at several stages of the cell cycle (Sanchez et al. 1997, Falck et al. )
Somatic mutations in both ATR and CHK1 have been observed in gastric tumors with microsatellite instability (MSI) (Menoyo et al. 2001). CHK1 mutations associated with a high degree of MSI were also found in colorectal and endometrial tumors (Bertoni et al. 1999).

2.6.2.2 Other candidate genes

PPP2R1B (11q23.1) (Wang et al. 1998) and TSLC1 (11q23.2) (Kuramochi et al. 2001) are other candidate genes on 11q21-q24 that have been linked to tumorigenesis. The protein phosphatase 2A (PP2A) is one of the major cellular serine-threonine phosphatases (Wera & Hemmings 1995), and it is involved in pathways related to cellular metabolism, DNA replication, transcription, translation, cell-cycle progression, RNA splicing and several other processes (Janssens & Goris 2001, Wera & Hemmings 1995). It is a heterotrimeric holoenzyme generated by the association of a 36 kDa catalytic subunit and a 65 kDa structural subunit A with variable regulatory subunits. The regulatory subunit forms the scaffold of the holoenzyme and exists in two isoforms, α and β, which share 86% amino acid identity (Hemmings et al. 1990). These α and β isoforms are encoded by different genes, PPP2R1A and PPP2R1B, respectively.

In early G2, PP2A inhibits the complete CDC25C phosphorylation and activation, thus preventing the activation of CDC2-cyclin B1 complex and entry to mitosis (Janssens & Goris 2001). It is also required to keep the CDC2-cyclin B1 in inactive form by positively regulating the activity of the kinase necessary to phosphorylate CDC2 on Tyr15 (Kinoshita et al. 1993, Dunphy 1994). In addition, PP2A may have a role in the exit from mitosis by participating in cyclin B destruction and by activating specific mitotic substrates of the activated CDC2-cyclin B complex (Janssens & Goris 2001). Furthermore, PP2A might also function in apoptosis, as suggested by its interactions with caspase-3 and BCL2 (Deng et al. 1998, Santoro et al. 1998, Ruvolo et al. 1999). It has also been shown to inhibit nuclear telomerase activity in breast cancer cells (Li et al. 1997). Interestingly, cancer-associated mutations in both isoforms of the regulatory subunit, PPP2R1A and PPP2R1B, have been identified (Wang et al. 1998, Calin et al. 2000).

TSLC1 (also called IGSF4) is transcribed into a 1.6- or 4.4-kb mRNA and encodes a ubiquitously expressed transmembrane glycoprotein of 442 amino acids (Gomyo et al. 1999), which localizes to perinuclear and plasma membranes. TSLC1 contains three immunoglobulin-like C2-type domains, one transmembrane domain and a short cytoplasmic domain. The extracellular domain shows significant homology to those of immunoglobulin superfamily proteins, including NCAM1 and NCAM2, as well as to mouse IGSF-B12 (Gomyo et al. 1999). Also, the cytoplasmic domain is highly homologous with that of glycoporphin C, a protein required for anchoring protein 4.1 in
the red blood cells (Marfatia et al. 1995). Thus, TSLC1 might play an important role in the formation of certain cell-to-cell or cell-to-substrate junctions (Kuramochi et al. 2001, Fukuhara et al. 2001). Also, based on their structural homology, two TSLC1 family members, TSLL1 and TSLL2, have recently been isolated (Fukuhara et al. 2001). Unlike TSLC1, however, these two genes are expressed in several specific tissues, most abundantly in the prostate, brain and kidney, and to a lesser extent in other tissues.

TSLC1 resides in presumably the most important 100-kb subsegment of the larger 700-kb genomic region shown to suppress completely the tumorigenicity of the human non-small-cell lung cancer (NSCLC) cell line A549 (Murakami et al. 1998). Truncation of the cytoplasmic domain of TSLC1 in a primary NSCLC tumor suggests that this domain is important for tumor suppression activity (Kuramochi et al. 2001). However, mutational inactivation of TSLC1 was found to be rare in tumors exhibiting LOH, but instead, frequent downregulation of TSLC1 expression through promoter region hypermethylation was observed (Kuramochi et al. 2001).

2.6.3 Alternative mechanisms of gene inactivation

In addition to qualitative alterations, which modify genes structurally, gene function can also be disrupted quantitatively through epigenetic alterations by changing the level of gene expression. For example, the altered intensity of DNA methylation on some important regulatory regions can affect the gene function. Following the change in methylation level, MeCP2 (methyl-CpG-binding protein 2) attaches to methylated DNA in a sequence independent fashion recruiting a complex, which contains a transcriptional co-repressor and a histone deacetylase (Nan et al. 1998, Jones et al. 1998). Subsequently, two possible mechanisms have been hypothesized for histone deacetylation to cause changes in chromatin structure. It could either allow ionic interactions between the positively charged amino-terminal histone tails and the negatively charged DNA backbone, which interferes with the binding of transcription factors to their specific recognition sequences, or lead to chromatin compaction through favoring interactions between adjacent nucleosomes (Bestor 1998). Eventually, these modifications lead to altered level of gene expression.

The methylation patterns of normal and cancerous cells differ from each other in many ways. Of the known mammalian DNA methyltransferases (DNMT), DNMT1 is generally responsible for maintenance of methylation, whereas de novo methylation is due to DNMT3a and DNMT3b. In malignant cells, the normal activities of these methyltransferases are dysregulated (Okano et al. 1999, Yokochi & Robertson 2002). Due to altered activity of the DNMT enzymes, both global hypomethylation and intense hypermethylation at normally unmethylated regions are observed in tumor cells (Baylin & Herman 2000, Esteller 2000). The amount of methylated CpGs decreases with age, and the rate of loss of these residues appears to be inversely related to the life span (Wilson et al. 1987). As the correct methylation pattern is essential for proper gene expression and the maintenance of genomic integrity, as a result of hypomethylation, the risk of accumulating cancer-related expression changes also increases. For example, in
demethylated somatic cells, the loss of DNMT1 changes the expression of several genes indicated in cell cycle control and signal transduction and also results in p53-dependent apoptosis (Jackson-Grusby et al. 2001). Indications of global hypomethylation have been reported in both hereditary and sporadic breast tumors (Esteller et al. 2001b).

Local hypermethylation is the most widely studied altered methylation pattern. Silencing of gene transcription is associated with gains of DNA methylation in normally unmethylated gene promoter regions (Jones & Laird 1999, Baylin & Herman 2000). In contrast to the genome in general, which is scarce of CpGs, approximately half of the gene promoters contain a stretch of DNA rich in CpG dinucleotides. These CpG islands are unmethylated in normal cells, apart from the imprinted genes and the inactive X chromosome in women. Their unmethylated state allows the expression of the adjacent genes in the presence of appropriate transcription factors. In cancerous cells, however, several of these CpG islands become hypermethylated, and as a consequence, shut down the expression of the corresponding gene.

In addition to CpG methylation, another site of methylation C\textsuperscript{5m}C(A/T)GG, which has previously been reported only in retroviral gene silencing after viral integration into the mammalian genome (Lorincz et al. 2000), has now also been found to control promoter activity in mammalian cells (Malone et al. 2001). However, whether the C\textsuperscript{5m}C(A/T)GG marks genetic elements for transcriptional silencing similarly to CpGs, or whether they protect CpG islands from CG methylation needs to be investigated in more detail (Lorincz & Groudine 2001).

To date, in various neoplasias several aberrantly methylated genes have been reported, including many of the genes associated with DNA repair: MLH1, MGMT, BRCA1 and GSTP1 (Lee et al. 1994, Dobrovic & Simpfendorfer 1997, Herman et al. 1998, Esteller et al. 1999, Esteller et al. 2000). Promoter hypermethylation seems to be a key feature of all major tumor types (Esteller et al. 2001a), and although many tumors share the same changes for some particular genes, unique profiles do exist for different tumor types. These profiles will provide important knowledge of the critical cancer-related cellular pathways and thus facilitate the development of molecular detection strategies for different cancer types.
3 Outlines of the present study

Despite active efforts, the existence of additional major high-penetration cancer susceptibility genes with similar harmful effects in diverse geographical populations worldwide as has been observed for BRCA1 and BRCA2 remains questionable. As the defects in DNA repair and cell cycle control are known to promote carcinogenesis, a proportion of inherited breast cancers might be attributable to mutations in the genes involved in these mechanisms. Therefore, we wanted to assess the role of germline mutations in some of the central genes in the DNA damage response pathway, which are also associated with familial cancer predisposing syndromes. Furthermore, the tracking of genes important for tumorigenesis in sporadic disease might also open up new perspectives into familial breast cancer. Against this background, the specific aims of this study were to:

1. Investigate the contribution of germline mutations in the two known genes associated with the Li-Fraumeni syndrome, TP53 and CHK2, to breast cancer predisposition in Finland.

2. Examine the prevalence of the ATM germline mutations previously detected in Finnish ataxia-telangiectasia families among breast cancer patients.

3. Evaluate the role of putative target genes of allelic loss in chromosome 11q21-q24, and along with intragenic mutations, also to investigate CpG island hypermethylation as a mechanism of gene expression silencing.
4 Materials and methods

4.1 Patients

4.1.1 Studies I-II

One hundred and eight Finnish BRCA1 and BRCA2 mutation-negative breast cancer families were analyzed for TP53 mutations. Geographically, 79 families came from the Oulu University Hospital District, 13 from the Tampere University Hospital District and 16 from the Helsinki University Hospital District. For CHK2 mutations analysis, only the families from the Oulu University Hospital District were selected. The inclusion criteria were one or more of the following: 1) at least three (two in combination with other selection criteria) cases of breast cancer in first- or second-degree relatives, 2) early disease onset (≤35 years alone, or <45 in combination with other inclusion criteria), 3) bilateral breast cancer, or 4) multiple tumours including breast cancer in the same individual. A proportion of the studied families also fulfilled the criteria for Li-Fraumeni or Li-Fraumeni-like syndromes (Birch et al. 1994, Eng et al. 1997) (Table 3).

4.1.2 Study III

A total of 215 breast cancer patients from 162 families from Central and Northern Finland were chosen for ATM mutation screening. All families had indications of moderate to high genetic susceptibility to breast cancer, basically fulfilling the same selection criteria than in studies I and II. Most of these patients had previously been excluded for BRCA1, BRCA2 and TP53 mutations by using extensive PCR-based screening methods (Huusko et al. 1998, Huusko et al. 1999, study I). Additionally, 85 sporadic breast cancer cases from the Oulu area were included in the study. Reference
Blood samples from 200 geographically matched controls were used to validate the observations from the two test groups.

Table 3. Summary of the breast cancer families’ phenotypes (I,II)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Number of families</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TP53 study</strong></td>
<td></td>
</tr>
<tr>
<td>All studied families</td>
<td>108</td>
</tr>
<tr>
<td>Families with implications of hereditary breast cancer</td>
<td>75</td>
</tr>
<tr>
<td>Breast cancer/LFL families</td>
<td>32</td>
</tr>
<tr>
<td>Breast cancer/LFS families</td>
<td>1</td>
</tr>
<tr>
<td><strong>CHK2 study</strong></td>
<td></td>
</tr>
<tr>
<td>All studied families</td>
<td>79</td>
</tr>
<tr>
<td>Families with implications of hereditary breast cancer</td>
<td>58</td>
</tr>
<tr>
<td>Breast cancer/LFL families</td>
<td>20</td>
</tr>
<tr>
<td>Breast cancer/LFS families</td>
<td>1</td>
</tr>
</tbody>
</table>

4.1.3 Study IV

Breast tumor DNAs from 31 sporadic cases were selected to elucidate the role of suitable candidate genes in chromosome 11q21-q24. All patients showed loss of heterozygosity (LOH) in chromosomal region 11q23 (Laake et al. 1999, Launonen et al. 1999). In three patients, LOH had been detected only distally from the ATM locus on 11q (marker APOC3), but in the remaining 28 cases LOH occurred both in the ATM region and at least with one additional microsatellite marker (D11S1819, D11S1778, D11S2179, D11S1294, D11S1818, D11S927).

4.2 DNA extraction

Extractions of DNA from blood lymphocytes and tissue material derived from breast cancer patients as well as from control blood samples (I, II, III, IV) were performed using standard phenol-chloroform methods.
4.3 Mutation analysis

4.3.1 Conformation-sensitive gel electrophoresis (CSGE) (I-III)

Conformation-sensitive gel electrophoresis (CSGE) was the key method utilized in mutation screening to identify germline alterations in the studied genes. CSGE analysis is based on the assumption that even a single base mismatch can produce a conformational change in the DNA double helix (Ganguly et al. 1993). When a PCR product containing a mismatch is first denatured and then allowed to reanneal, the randomly forming homo- and heteroduplexes can be distinguished by their differential migration on a mildly denaturing polyacrylamide gel.

Each exon and the corresponding splice sites were PCR-amplified under specifically optimized reaction conditions. In general, reactions were carried out in a total volume of 10 µl, containing 50 ng of genomic DNA as a template, 1 x PCR buffer (1.5-2.5 mM MgCl₂), dNTPs (200 µM each), 20 pmol of each primer and 0.5 U of AmpliTaqGold polymerase (Applied Biosystems). The conditions for PCR were: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 50-65 °C for 1 min, extension at 72 °C for 1 min 30 s; and final extension at 72 °C for 10 min. Following the amplification, the samples were subsequently denaturated at 98 °C for 5 min, and kept at 68 °C for 30 min to allow DNA heteroduplex formation. The samples were then mixed with 6 µl of 10 x loading buffer (containing 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol) and loaded onto a 10% CSGE gel (containing 25% polyacrylamide, 99:1 ratio of acrylamide to 1,4-bis(acryloyl)piperazine, 10% ethylene glycol, 15% formamide and 0.5% Tris-Taurine-EDTA-buffer) and electrophoresed at 400 V for 16-22 h, depending on the length of the analyzed fragment. After electrophoresis, the gel was stained on the glass plate in ethidium bromide, visualized in UV light and photographed.

4.3.2 DNA sequencing (I-IV)

Samples with putative alterations detected in CSGE were re-amplified with the same set of oligonucleotides, but in a larger reaction volume of 30 µl. The PCR product was purified with the QIAquick PCR purification Kit (Qiagen). For sequencing reactions, the SequiTherm EXCEL™II DNA Sequencing Kit-LC (Epicentre Technologies) was utilized. Basically, a mix of 100-200 fmole of purified PCR product, sequencing buffer, IRD-labeled primers (2 pmole each) and 5 U of EXCEL II Polymerase was prepared to a total volume of 17 µl. Into four tubes containing 2 µl of each termination mix, 4 µl of reaction mix was added. The reaction conditions were: intial denaturation at 92 °C for 2 min; 30 cycles of denaturation at 92 °C for 30 s, annealing at 50-65 °C for 30 s, extension at 72 °C for 30 s. The samples were then mixed with 3 µl of loading buffer included in the SequiTherm EXCEL™II DNA Sequencing Kit-LC (Epicentre Technologies) and
denatured at 96 °C for 3 min prior to electrophoresis, which was performed with the Li-Cor IR² 4200-S DNA Analysis system (Li-Cor Inc.). Of each sample, an aliquot of 0.5-1 µl was loaded onto a 6% Long Ranger gel and run at 1500 V for 7-9 hours, depending on the length of the analyzed fragment. The data were analyzed using the AlignIR™ and BaseImagIR™ softwares (Li-Cor Inc.).

4.4 Methylation analysis (IV)

In sodium bisulfite sequencing technique, the unmethylated cytosines are first converted to uracils by sodium bisulfite under conditions in which methylated cytosines remain nonreactive (Frommer et al. 1992). When the modified DNA is subsequently PCR-amplified and sequenced, all the uracil and thymine residues amplify as thymine. On the contrary, the methylated cytosine residues amplify as cytosine, and can be therefore discriminated from the unmethylated cytosines.

Sodium bisulfite treatment was performed as described by Clark et al. (1994). First, 2 µg of tumor DNA was denatured with 0.3 M NaOH (37 °C, 15 min), reacted with freshly prepared 3.6 M sodium bisulfite and 1 mM hydroquinone (55 °C for 14 h) and desalted by using a Wizard DNA Clean Up kit (Promega) according to manufacturer’s instructions. Subsequently, 3 N NaOH was added to a final concentration of 0.3 N to desulphonate DNA. Finally, bisulfite-treated DNA was ethanol-precipitated, dried and eluted with TE buffer prior to PCR.

The gene regions of interest were PCR-amplified using bisulfite-treated DNA as a template. The products were ran on 1% agarose gel, excised and purified with a QIAquick Gel Extraction Kit (Qiagen), and cloned into the pGEM-T-easy vector (Promega). The vectors were transformed into chemically competent E.coli TOP10F’ cells (Invitrogen) following a standard transformation protocol. The transformed cells were grown overnight on agar plates containing ampicillin, IPTG and X-gal. Based on the blue/white selection, at least ten positive recombinants were inoculated into 3 ml of LB broth containing ampicillin and grown overnight with vigorous shaking. Bacteria were harvested and plasmids extracted by using the Wizard®PlusMiniprep Kit (Promega).

DNA sequencing of the clones was performed in essentially a similar way as described above, except that M13 primers were used to amplify the cloned fragments. Also, in addition to utilizing commercial software for data analysis, the methylation status of each analyzed fragment was evaluated by at least two investigators.

4.5 Statistical analysis (II, IV)

The observed differences in mutation frequencies between the studied samples from breast cancer patients and the control samples were analyzed in a Bayesian framework (II) (Gelman et al. 1995). Unlike the Chi-square test, the Bayesian approach provides the probabilities for the presented hypothesis to be both true and false. Furthermore, in the
Bayesian model, none of the expected values are fixed, which results in a more plausible statistical estimate. The probability model was set up by assuming that the number of mutations follows the Poisson distribution with a mean $\lambda_i=\theta_iN_i$, given the number of individuals is $N_i$ and the mutation frequency is $\theta_i$. Also, $\theta_i$ was assumed to follow Beta (1,1)=Unif (0,1) distribution. Formally:

$$
\begin{align*}
  x_i | N_i, \theta_i &\sim \text{Poisson}(\theta_iN_i) \\
  \theta_i &\sim \text{Beta}(1,1)
\end{align*}
$$

The comparisons of mutation frequencies between the different groups were performed by calculating the ratio of the frequencies, $R_{i,j}=\theta_i/\theta_j$. The posterior distributions of the model parameters were obtained by Monte Carlo Markov Chain simulation, which was carried out with the WinBUGS 1.3 software. Furthermore, for $H_0$ (estimating how well the frequency observed in one group equals that in the reference group), traditional Chi-square test calculations were performed using $p=0.01$ as cut-off value for statistical significance.

The Chi-square test for linear trend and Fisher’s two-tailed exact test were used for the statistical evaluation of association between methylation and clinical variables (IV).
5 Results

5.1 Analysis of germline mutations

The mutation analysis of TP53, CHK2 and ATM, which are all central genes in DNA DSB response pathway, was performed to investigate the possibility that germline mutations in these genes could explain at least a fraction of inherited breast cancer susceptibility in families lacking mutations in BRCA1 and BRCA2 genes.

5.1.1 TP53 (I)

Among the 108 breast cancer families studied, one new family with a TP53 mutation, Arg248Gln, was identified. In addition, a silent variant (Arg213Arg) and several intronic polymorphisms were detected. The family carrying Arg248Gln showed a strong family history of breast cancer (Table 4), but also matched the criteria for LFL. Together with the previously identified two families (Huusko et al. 1999), 2.8% (3/108) of the studied breast cancer families were found to carry a TP53 mutation, all of them also meeting the criteria for LFS or LFL.

5.1.2 CHK2 (II)

When screening for CHK2 mutations, the only alteration of interest seen in the exonic regions of the gene was Ile157Thr. It was observed in 8.9% (7/79) of the studied breast cancer families (Table 4). Four of these seven families met the criteria for LFL. However, in two of the mutation-positive families, where suitable multiple DNA specimens were available for analysis, the mutation segregated ambiguously with the cancer phenotype.
Furthermore, Ile157Thr was found in 6.5% (13/200) of the anonymous cancer-free blood donors and 3.9% (10/259) of the unselected breast cancer cases.

Table 4. Germline sequence alterations in the p53, CHK2 and ATM genes in Finnish families with breast cancer (I-III)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>aa change</th>
<th>Status</th>
<th>Family ID</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>659A&gt;G</td>
<td>Tyr220Cys</td>
<td>M</td>
<td>020</td>
<td>Bil Br(36) + Os(13), Br, Cpp(4), Lu, Pan, Ov(19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>703A&gt;G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>743G&gt;A</td>
</tr>
<tr>
<td>CHK2</td>
<td>470T&gt;C</td>
<td>Ile157Thr</td>
<td>U</td>
<td>1</td>
<td>Br(51), Br, Br</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>Br(64), Br(65), Ov</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>Bil Br(40), Br(80), Br(46), Br(54), Sk(42), Csu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>Br(36), Br(54), Csu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>Br(47), Br(50), Br(80), Br(89), Sto</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>Br(50), Br(54), Br(50), Br(70), Leu(41), Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>Br(40), Br(49), Br(64), Br(52), Br(72)</td>
</tr>
<tr>
<td>ATM</td>
<td>133C&gt;T</td>
<td>Arg45Trp</td>
<td>P</td>
<td>001</td>
<td>Bil Br(45)</td>
</tr>
<tr>
<td></td>
<td>146C&gt;G</td>
<td>Ser49Cys</td>
<td>P</td>
<td>002</td>
<td>Br(48), Br(58), Br(60), Pan</td>
</tr>
<tr>
<td></td>
<td>752G&gt;C</td>
<td>Ala2524Pro</td>
<td>M</td>
<td>003</td>
<td>Br(50), Br(59), Br(51), Th(47), Pro(59), Br(71), Sto, Lu (55), Csu</td>
</tr>
<tr>
<td></td>
<td>6903insA</td>
<td>loss of 685</td>
<td>M</td>
<td>004</td>
<td>Br(50), Br(47) + Sar(18), Br(40), St, Br, Pan(40), Br(54), Sto, Pan, Br(45), Br(37), Br(37), Br, Csu, Th(55)</td>
</tr>
</tbody>
</table>

‘M, disease associated mutation; U, disease-association currently unconfirmed; P, polymorphism.

‘Bil Br, bilateral breast; Br, breast; Bo, bone; Bt, brain; Cpp, choroids plexus papilloma; Csu, cancer site unknown; Leu, leukemia; Li, liver; Lu, lung; Os, osteosarcoma; Ov, ovary; Pan, pancreas; Pro, prostat; Sa, sarcoma; Sk, skin; Sto, stomach; Th, thyroid; Ep, ependymoma.

‘Age at diagnosis is given in brackets when known. The + sign indicates more than one primary tumor in the same individual.

‘Reported in Huusko et al. (1999)

‘BRCA2 mutation carrier

The hypothesis that the mutation frequency among hereditary breast cancer patients would be different from the mutation frequency in a reference group was tested using the Bayesian model. The minimum value to prove that the observed incidence was higher than expected was 0.99, and none of the calculated probabilities for mutation frequencies reached this value. The obtained values were 0.78 (breast cancer families vs. cancer-free
blood donors), 0.11 (cancer-free blood donors vs. unselected breast cancer cases), and 0.96 (breast cancer families vs. unselected breast cancer cases). To estimate how well the frequency observed in one group equals that in a reference group, traditional Chi-square test calculations were made. The obtained values were 0.72 (p=0.395), 2.96 (p=0.085) and 5.53 (p=0.019), respectively, being thus statistically insignificant.

Due to the presence of homologous copies of the 3’ part of the \textit{CHK2} gene (Sodha \textit{et al.} 2000), atypical banding in CSGE was observed for the exons 10-14. As the CSGE analysis is based on homo- and heteroduplex formation between wild-type and mutated alleles, based on the appearance of new heteroduplex bands, it is possible to detect more than one kind of mismatch in a given PCR product (Körrkö \textit{et al.} 1998). Therefore, instead of a single band (e.g. homoduplex) indicating a lack of mutation, genomic loci co-amplified in PCR with the exons 10-14 in CSGE analysis resulted in additional bands (e.g. one or more heteroduplexes). For that reason, the screening for samples displaying a banding pattern different from the others in CSGE provided some leads as to whether the analyzed exons contained sequence alterations or not. The banding patterns for the exons 10-14, however, were similar in all the screened DNA samples (data not shown).

\subsection*{5.1.3 ATM (III)}

Two of the eight \textit{ATM} germline mutations previously identified in Finnish \textit{AT} families were found among the studied cohort of breast cancer patients with a hereditary disease background (Table 4). 7522G>C was identified in two women with breast cancer belonging to separate cancer families (003 and 005). The other alteration, insertion of adenine (6903insA), was found in three sisters who all had breast cancer. Neither 6903insA nor 7522G>C was seen among the studied sporadic breast cancer cases or the control samples, indicating that these two \textit{ATM} mutations in addition to being instrumental to \textit{AT}, could also be related to a hereditary predisposition to breast cancer. Altogether, putative disease-associated \textit{ATM} mutations were detected in 1.9\% (3/162) of the studied families displaying breast cancer.

Other sequence variants, two in exon 5 (133C>T and 146C>G) and one in the intronic region between the exons 62 and 63, were all regarded as polymorphisms. 133C>T leads to an amino acid substitution Arg45Trp, and it was observed in a woman with bilateral breast cancer at age 45. The second exon 5 variant, 146C>G, results in Ser49Cys and was detected in a woman diagnosed with breast cancer at age 60. She had two sisters with breast cancer at ages 48 and 58, but neither of them carried the alteration. We did not observe either of these alterations in the sporadic breast cancer group or the healthy controls. Neither Arg45Trp nor Ser49Cys reside in a known functional \textit{ATM} domain, and are thus less likely to interfere with \textit{ATM} kinase function. The third sequence alteration, IVS62+8A>C, was detected in 2.3\% (5/215) of the familial breast cancer patients, 2.4\% (2/85) of the sporadic breast cancer cases and 2.0\% (4/200) of the controls.
5.2 Analysis of somatic aberrations in chromosome 11q21-q24 (IV)

Based on the previous LOH findings (Laake et al. 1999, Launonen et al. 1999), the role of putative target genes of allelic loss in chromosome 11q21-q24 was evaluated. The location of the markers used previously for the LOH analysis (D11S1819, D11S1778, D11S2179, D11S1294, D11S1818, D11S927, APOC3) and the studied LOH candidate target genes are shown in Figure 4. For MRE11A, CHK1, PPP2R1B and TSLC1 genes, both the presence of intragenic mutations and the level of CpG island hypermethylation were determined. In addition, the ATM gene was included to the methylation analysis.

Fig. 4. Locations of the studied genes and the utilized microsatellite markers in chromosome 11q21-q24. Adapted from http://www.ncbi.nlm.nih.gov/genome/guide/HsChr11.shtml and http://genome.ucsc.edu.
5.2.1 Mutation analysis

In the four analyzed genes, MRE11A, CHK1, PPP2R1B and TSLC1, only one putative cancer-associated mutation was found. However, this missense mutation, Lys354Arg in PPP2R1B exon 9, was also present in the corresponding normal tissue sample, but not in any of the 200 controls. Both lysine and arginine are hydrophilic basic amino acids, and very similar in structure. Therefore, despite the conserved position of this residue in the HEAT (huntingtin-elongation-A subunit-TOR) repeat 9, the presence of either of the two amino acids appears to result in an equally functional protein (Groves et al. 1999).

5.2.2 Methylation analysis

All analyzed CpGs of MRE11A, CHK1, PPP2R1B, and ATM were found to be unmethylated. For TSLC1, however, a high proportion of the tumors (33%, 10/30) displayed multiple methylated cytosines in at least one of the analyzed clones. Especially three of these tumors showed considerably elevated levels of methylation at the six studied CpG sites (Figure 5). The number of clones with methylated cytosines inversely correlated with the percentage of LOH. In normal breast tissue and blood lymphocytes, these CpGs were unmethylated. TSLC1 methylation was not related to any clinicopathological characteristics of the tumors.

Fig. 5. TSLC1 promoter methylation status in tumors from three breast cancer patients. The corresponding LOH percentage is shown next to the case number.
6 Discussion

6.1 Germline mutations in Finnish breast cancer patients

6.1.1 TP53 and CHK2 (I, II)

Earlier studies on Finnish hereditary breast cancer patients (Vehmanen et al. 1997a,b, Huusko et al. 1998) have implied a smaller contribution of BRCA1 and BRCA2 mutations in Finland compared with other Western European populations. Therefore, one possible way to track additional inherited genetic changes resulting in a predisposition to breast cancer was to investigate whether germline mutations in the LFS and LFL-associated genes, TP53 and CHK2, could be found in breast cancer families. Breast cancer is the most prominent type of cancer in these related cancer syndromes (Kleihues et al. 1997), implying that germline mutations in the same genes might also predispose to the early onset breast cancer, even in families lacking the other typical cancer signatures of LFS.

In our initial study, the exons 5-8 of the TP53 gene were screened in seven families, two of them exhibiting a LFS and five a LFL phenotype (Huusko et al. 1999). Two TP53 missense mutations were detected, Tyr220Cys in an LFS family, and Asn235Ser in an LFL family. Subsequently, we screened a cohort of 108 Finnish breast cancer families for TP53 mutations. In this more detailed follow-up study, we included the entire coding region (exons 2-11) and also the flanking intronic regions. As a result, one additional TP53 mutation, Arg248Gln, was detected in a breast cancer family that also exhibited the typical features of LFL. Arg248Gln appears to be the most frequently found mutation in LFS (Shibata et al. 1996).

Two diverse classes of mutations have been distinguished based on in vitro assays and the three-dimensional structure of the p53 protein (Cho et al. 1994). Class I mutations alter the amino acids directly involved in interactions between DNA and proteins. They maintain the wild-type conformation, but do not bind the HSP70 heat-shock protein (Hinds et al. 1990, Ory et al. 1994). On the contrary, the conformations resulting from class II mutations have been altered due to the genetic change, and they bind HSP70...
intensively. Arg248Gln belongs to class I, and structurally it resides in a highly conserved region, L3, which is essential for p53 DNA-binding functions (Cho et al. 1994). In clinical studies, breast cancer patients with somatic mutations in the L2/L3 domain displayed shorter survival (Børresen et al. 1995, Berns et al. 1998, Gentile et al. 1999) as well as a poorer response to tamoxifen (Berns et al. 2000) and doxorubicin (Aas et al. 1996, Geisler et al. 2001) than patients with other types of mutations or wild-type TP53.

The proportion of TP53 mutations (2.6%, 3/108) derived from our two studies is consistent with the earlier data (Børresen et al. 1992, Sidransky et al. 1992). Furthermore, TP53 mutations occur mainly at specific mutation-prone regions of the conserved parts of the gene. In a small proportion of breast cancer patients, the increased risk of inherited breast cancer can be explained by heterozygous germline TP53 mutations. Apart from breast cancer, these mutation-positive families usually also exhibit other cancer types characteristic of LFS and LFL.

Recently, CHK2 germline mutations were identified in LFS families lacking mutations in TP53. As Bell et al. (1999) originally screened four LFS and eighteen LFL cases and found three distinct CHK2 alterations (1100delC, Ile157Thr and 1422delT) in three of the studied families (13.6%, 3/22), a similar incidence of CHK2 mutations was initially expected among the 21 LFL and LFS families included in our study. However, the first of the mutations detected by Bell et al. (1999), 1422delT, was subsequently shown to be an artifact due to homologous duplications of the 3’ end of the genomic sequence (exons 10-14) (Sodha et al. 2000). The second mutation, 1100delC, occurred in a classical LFS family, but as the population prevalence of this alteration was demonstrated to be about 1%, it is not likely to be a high-penetration allele for LFS susceptibility (The CHEK2-Breast Cancer Consortium 2002). In the same study, however, 1100delC was shown to result in a twofold increase of breast cancer risk in women and tenfold increase of risk in men (The CHEK2-Breast Cancer Consortium 2002). The third mutation, Ile157Thr, was seen in a single individual with three primary tumors (breast, melanoma and lung), but without a reported family history of cancer. This phenotype is therefore only suggestive of LFS or LFL (Birch et al. 1994, Eng et al. 1997, Vahteristo et al. 2001b).

Among Finnish breast cancer families exhibiting a LFS or LFL phenotype, the only detected alteration within the protein-encoding region of the gene was Ile157Thr. Seven breast cancer families (8.9%, 7/79) were found to carry this missense mutation, and four of these families also met the criteria for LFL. However, the alteration was also present in the studied controls (6.5%, 13/200) as well as in unselected breast cancer cases (3.9%, 10/259). To conclusively exclude the presence of additional mutations in the exons 10-14, our negative result should be confirmed by long-range PCR based screening method developed for these exons (Sodha et al. 2002).

In Ile157Thr, the altered isoleucine is located within the forkhead-associated (FHA) domain, which is a highly conserved 60-amino acid protein interaction domain essential for the activation of the CHK2 yeast homolog Rad53 in response to DNA damage (Sun et al. 1998). The CHK2 protein carrying the Ile157Thr change has similar kinase activity, expression levels and subcellular localization as endogenous CHK2 (Wu et al. 2001). In addition, similarly to wild-type CHK2, the mutant protein is activated following gamma radiation. However, it was later shown that CHK2 forms a protein-protein complex with p53, whose abundance at the S-phase checkpoint increases upon IR exposure. This interaction requires an intact FHA domain of CHK2 as well as successful tetramerization
of p53 (Falck et al. 2001a, Lee & Chung 2001). Despite the fact that the CHK2 carrying Ile157Thr was able to undergo an activatory mobility shift upon IR, it was not able to bind p53 in vivo, and its in vitro kinase activity remained low after IR. Also, the defective CHK2 protein was unable to bind and phosphorylate CDC25A (Falck et al. 2001b).

It has been suggested that parallel mutations in cell cycle checkpoint genes TP53 and CHK2 might provide some additional selective advantage to the affected tumor cells (Falck et al. 2001a). A colon cancer cell line, HCT-15, concomitantly lacks functions of both CHK2 and p53 (Falck et al. 2001a). Additionally, a simultaneous mutation in CHK2 and TP53 has been reported in a patient with small-cell lung cancer (Haruki et al. 2000). Furthermore, cancer-associated CHK2 mutations, 1100delC and Arg145Trp, showed loss of the wild-type allele in primary tumors, whereas Ile157Thr and Arg3Trp did not (Lee et al. 2001). On the contrary, a breast tumor harboring Ile157Thr demonstrated mutational inactivation of both TP53 alleles, which was not the situation for the 1100delC and Arg145Trp cases (Lee et al. 2001). CHK2 might also have similar effects with other mutated genes, as Vahteristo et al. (2001b) reported a CHK2 mutation carrier harboring a germline mutation in the MSH6 gene.

In addition to LFS and breast cancer, the contribution of germline and somatic CHK2 defects has been studied in a variety of cancers (Bell et al. 1999, Bougeard et al. 2001, Lee et al. 2001, Vahteristo et al. 2001b). Yet, only a few mutations have been detected. The absence of germline mutations has earlier been reported in gastric cancer (Kimura et al. 2000). Infrequent somatic CHK2 mutations have been found in acute myeloid leukemia, non-Hodgkin’s lymphoma, small-cell lung cancer and osteosarcoma, all of which are cancer types associated with the LFS phenotype (Haruki et al. 2000, Hofmann et al. 2001, Tavor et al. 2001, Miller et al. 2002). On the other hand, no somatic mutations were found in malignant gliomas (Ino et al. 2000).

Altogether, we identified Ile157Thr in seven Finnish breast cancer families. Due to a high frequency in the studied controls as well as in unselected breast cancer cases and the ambiguous segregation of the mutation in two of the mutation-positive families, our results suggest that Ile157Thr would not, by itself, be a mutation resulting in predisposition to cancer. The functional evidence, however, suggests that Ile157Thr disturbs the protein function by interfering with the FHA domain binding ability. Therefore, it seems likely that the altered CHK2 might promote cancer formation, possibly in combination with mutations in other relevant genes consistent with the polygenic susceptibility model recently presented by Pharoah et al. (2002). Consequently, Ile157Thr variant might be a low-penetrance allele conferring susceptibility to breast cancer similarly to 1100delC (The CHEK2-Breast Cancer Consortium 2002). Compared to other studied populations, Ile157Thr seems to be enriched in the Finnish population (Bell et al. 1999, Lee et al. 2001, Vahteristo et al. 2001b). Thus, the clinical significance of Ile157Thr requires further investigations among Finnish cancer patients. Additional genes besides TP53 and CHK2 are likely to account for the cancer susceptibility observed in the remaining LFS and LFL families.
The increased risk of breast cancer in ATM carriers has been demonstrated in many studies (Swift et al. 1987, Pippard et al. 1988, Athma et al. 1996, Stancovic et al. 1998, Inskip et al. 1999, Janin et al. 1999, Olsen et al. 2001). However, contradictory data also exist (Vorechovsky et al. 1996, FiztGerald et al. 1997, Bay et al. 1998, Chen et al. 1998), and the true nature of these observations has thus remained elusive. As recurrent ATM mutations have been reported in several countries and within many different ethnic groups (Gilad et al. 1996, Ejima & Sasaki 1998, Laake et al. 1998, Sasaki et al. 1998, Stancovic et al. 1998, Telatar et al. 1998), we anticipated that the previously identified ‘Finnish’ ATM germline mutations would also be the most likely mutations prevalent in Finnish breast cancer patients, provided that there is an association between ATM germline mutations and susceptibility to breast cancer. In three studied families with breast cancer (1.9%, 3/162), we detected two germline alterations potentially relating to breast cancer susceptibility, Ala2524Pro and 6903insA. Both of these detected mutations had previously been found in AT families, segregating with the disease, and were thus known for their pathogenic nature (Laake et al. 2000a). Also, the Finnish AT families displaying the Ala2524Pro or 6903insA alteration showed excessive cases of breast and other cancers.

Earlier, Stancovic et al. (1998) described two AT families in which a heterozygous missense mutation, Val2424Gly (7271T<G), was found to associate with an increased risk of breast cancer. Interestingly, the AT patients carrying the homozygous mutation exhibited a milder phenotype compared to the typical AT. Along with Val2424Gly, another ATM mutation, IVS10-6T<G, was suggested to associate with an increased cancer risk in patients with early-onset breast cancer who had been exposed to low-dose ionizing radiation and had survived for more than five years after being diagnosed with breast cancer (Broeks et al. 2000, Dörk et al. 2001). Subsequently, the relation of these two dominant-negative mutations with respect to the increased breast cancer risk was confirmed in a large population-based case-control study (Chenevix-Trench et al. 2002). The penetrance estimate of these mutations corresponded to an overall 15.7-fold risk for the development of breast cancer averaged up to age 70. As most studies have concentrated on sporadic breast cancer cases instead of cancer families and utilized screening methods preferentially detecting protein-truncating mutations (Angele & Hall 2000), there might be more ATM germline mutations with similar effects yet to be discovered. Interestingly, less common ATM missense substitutions seem to be enriched among breast cancer patients (Dörk et al. 2001, Teraoka et al. 2001), supporting the hypothesis of two diverse populations of ATM heterozygotes (Khanna 2000, Meyn 1999), of which the combination of a wild-type allele and an allele containing a missense mutation would be more prone to cause cancer predisposition.

In addition, other lines of evidence support the role of ATM as a tumor suppressor gene. First, germline ATM mutations have been discovered in breast cancer patients carrying a parallel mutation in BRCA1 or BRCA2 (Teraoka et al. 2001). The frequency of ATM mutations in this group was somewhat elevated compared to the control group, but did not differ significantly when compared to the overall case group, which involved both BRCA1 and BRCA2 mutation carriers and non-carriers. Second, an ATM variant,
Asp1853Asn, was found to modulate the penetrance of MLH1 and MSH2 germline mutations (Maillet et al. 2000). The MLH1 or MSH2 mutation carriers, who concurrently carried the Asp1853Asn variant, had an eightfold risk of developing colorectal and other HNPCC-related cancers (Maillet et al. 2000). Along with this mutation, the Arg1054Pro substitution has been proposed to be a genetic modifier of breast cancer penetrance (Larson et al. 1998). Finally, as women developing early-onset bilateral breast cancer are likely to harbor germline mutations in certain susceptibility genes (Tsuda & Hirohashi 1995, Kollias et al. 2000), loss of the same allele at a specific chromosomal site could suggest a common origin of the two malignancies observed in one individual. Kollias et al. (2000) demonstrated concordant LOH at D11S1778, which maps to the ATM locus, in 25% of the informative bilateral breast cancer cases, which further supports the role of ATM as a TSG in this malignancy.

In conclusion, heterozygous ATM germline mutations seem to contribute in some extent to breast cancer predisposition in Finland. Interestingly, the cancer families carrying ATM germline mutations originated from the same geographical region as the AT families displaying corresponding mutations. Although this implies the possibility of a founder effect concerning the distribution of Ala2524Pro and 6903insA, due to the circular nature of the argument followed by the inclusion of only the mutations previously identified in Finnish AT families, a more extensive study will be needed to validate this preliminary lead.

### 6.2 Somatic alterations in chromosome 11q (IV)

The presence of one or more tumor suppressor genes in the long arm of chromosome 11 has been established on several occasions. Apart from the available LOH and CGH data, there are many functional transfection studies that further verify these observations (Negrini et al. 1994, Koreth et al. 1999). However, despite the efforts to hunt down the target genes contributing to malignant transformation and growth of tumor cells, these genes still remain to be identified. Therefore, we assessed the possible role of some of the most interesting candidate genes as putative targets in chromosome 11q21-q24 by analyzing tumor DNAs for gene inactivation through both genetic and epigenetic alterations.

First, for the MRE11A and CHK1 genes, which are both involved in DNA DSB signaling, a comprehensive mutation analysis was performed. In the absence of somatic mutations, the two other candidate genes, PPP2R1B and TSLC1, were subsequently included in the study. The lack of somatic mutations in the coding region and splice junctions of all these genes supports the prevailing view of only a low frequency of intragenic cancer-associated alterations. A small number of mutations in MRE11A and PPP2R1B have been previously reported (Wang et al. 1998, Calin et al. 2000, Takagi et al. 2000, Fukuda et al. 2001). CHK1 mutations seem to be absent in most of the studied tumor types (Bell et al. 1999, Semba et al. 2000, Vahteristo et al. 2001b), with the exception of two studies in which frameshift CHK1 mutations in MSI-positive gastric, colon and endometrial tumors were observed (Bertoni et al. 1999, Menoyo et al. 2001).
Furthermore, only two inactivating TSLC1 mutations were detected among 161 primary tumors and cell lines (Kuramochi et al. 2001).

Because all previous studies on MRE11A, CHK1, and PPP2R1B inactivation or dysfunction have concentrated on finding mutations within the protein-encoding region, the possible influence of promoter region CpG methylation of these putative TSGs in 11q21-q24 was subsequently evaluated. As it was known from previous studies (Rodriguez et al. 2002) that tumors presenting LOH at the ATM locus do not show significant involvement of mutations in the corresponding wild-type allele, the ATM gene was also included in the methylation study that was performed. Of the selected candidate genes, the studied regions of MRE11A, CHK1, PPP2R1B, and ATM were all found to be unmethylated. However, three tumors showed relatively high methylation levels at the analyzed CpG sites of the TSLC1 gene. In these cases, methylation occurred in up to 45% of the analyzed clones. This percentage is likely to reflect the presence of contaminating stromal and other non-malignant cells in the sample, as well as heterogeneity within the tumor. Thus, the percentage of methylation in a pure tumor specimen might be considerably higher. The assumption was supported by the results of the LOH analyses, which showed traces of contaminating cells in all the three tumors (Laake et al. 1999, Launonen et al. 1999). Additionally, the average percentage of methylation inversely correlated with the percentage of contamination of non-malignant cells. Among the other analyzed breast tumors, seven showed elevated TSLC1 CpG island methylation, but to a lesser extent than the three major cases. This might reflect even more increased tumor heterogeneity. The inherent cellular heterogeneity of tissue material affecting the results can be avoided by using microdissection to achieve purer and more homogenous tissue samples, in which very small amounts of tumor cells are sufficient to reliably determine the methylation status (Kerjean et al. 2001).

TSLC1 was identified by Gomyo et al. (1999) as a target gene for 11q23.2 deletions, and it is contained in a 700-kb genomic region that completely suppresses tumorigenicity of the A549 human NSCLC cell line (Murakami et al. 1998). By functional complementation, it has been determined that most of this suppressive activity localizes to a 100-kb chromosomal subregion (Murakami et al. 1998). Kuramochi et al. (2001) demonstrated that TSLC1 silencing is a frequent event in multiple human cancers, and typically occurs due to promoter region hypermethylation. In addition to TSLC1, the same 100-kb subregion harbors, for example, the ALL1, EVA1, TMPRSS4 and IL10RA genes (UCSC database, http://genome.ucsc.edu). All these genes encode proteins that might promote tumorigenesis (Tan et al. 1993, Baffa et al. 1995, Guttinger et al. 1998, Wallrapp et al. 2000).

Apparently, based on recent knowledge, Knudson’s two-hit hypothesis could now be modified to include epigenetic mechanisms as an additional means to bring about the required two hits for TSG inactivation. Transcriptional silencing through methylation may occur in different combinations with the other known inactivating mechanisms (e.g. intragenic mutations and loss of chromosomal material) (Jones & Laird 1999). First, hypermethylation together with LOH has been shown to result in the silencing of many genes. For instance, BRCA1, in which mutations are rarely found in sporadic breast cancers, was found to be hypermethylated in both breast and ovarian tumors informative for LOH (Esteller et al. 2000). In breast cancer, both CDH13 and FHIT are silenced by a combination of LOH and methylation (Toyooka et al. 2001, Zöchbauer-Müller et al.
Second, two transcriptional pathways per se can make up the two hits required for gene silencing. This is the case with RARβ2, where both biallelic epigenetic inactivation and epigenetic modification combined with LOH occur in breast cancer (Yang et al. 2001). Also, in invasive ductal carcinoma (IDC), methylation and increased expression of the negative regulator Snail affecting CDH1 expression were found to result in gene inactivation (Cheng et al. 2001). Interestingly, in IDC, methylation of CDH1 rarely occurs in combination with LOH, whereas in invasive lobular carcinoma (ILC), all the three mechanisms take place in different combinations (Cheng et al. 2001, Droufakou et al. 2001). Third, according to Esteller et al. (2001b), CpG island promoter hypermethylation can also be considered a mechanism to accomplish ‘second hits’ for the inactivation of tumor suppressor genes in breast cancer families. In this study, aberrant methylation was never observed in a tumor exhibiting LOH, indicating that in tumors of hereditary origin, methylation does not coincide with allelic loss. On the contrary, if both alleles were present in the tumor and one carried a germline mutation, promoter methylation was frequently found to accomplish the second hit leading to biallelic inactivation of gene expression. Other genes in which hypermethylation has been shown to correlate with a loss of gene expression in breast cancer include RASSF1A, (Burbee et al. 2001), NES1 (Li et al. 2001), CDKN2A (Esteller et al. 2001a), GSTP1 (Esteller et al. 2001a), APC (Jin et al. 2001), SRBC (Xu et al. 2001) and HIC1 (Nicoll et al. 2001).

In human cancers, some methylation patterns are shared by the different tumors types, but some genes are altered as a group in a tumor type-specific manner, probably stressing the importance of the methylation process similarly to the loss of mismatch repair function (Esteller et al. 2001a). Both MMR and methylation might influence both critical and non-critical loci, and subsequently, only those interfering with genes essential for genomic integrity would confer selective advantage to tumor growth (Toyota et al. 1999, Baylin et al. 2000, Costello et al. 2000, Esteller et al. 2001a).

Taken together, in addition to non-small-cell lung cancer, hepatocellular carcinoma, and pancreatic cancer, TSLC1 promoter hypermethylation has now also been detected in primary breast cancer. Together with LOH of the other allele, this event could result in gene silencing in at least in 10% (3/30) of breast cancer cases. At present, however, it seems evident that TSLC1 is not the only target gene harbored in this chromosomal region important for tumor-suppressor activity. Therefore, supportive studies on breast cancer cell lines and microdissected tissue material will be carried out to evaluate further the role of TSLC1 promoter region CpG hypermethylation in this malignancy.
Future directions

Given that all the three studied DSB response genes, \textit{TP53}, \textit{CHK2} and \textit{ATM}, display at least some pathogenic germline mutations in breast cancer patients with familial breast cancer, other DNA damage response genes, such as \textit{NBS1}, \textit{MRE11A}, \textit{BACH1}, \textit{BARD1} and \textit{RAD51} (see chapter 2.5.1.3 and Shinohara \textit{et al.} 1993, Petrini \textit{et al.} 1995, Wu \textit{et al.} 1996, Carney \textit{et al.} 1998, Varon \textit{et al.} 1998, Cantor \textit{et al.} 2001), are also good candidates to harbor mutations related to breast cancer susceptibility. For instance, two germline mutations in \textit{BACH1} have been detected in early-onset breast cancer patients, one of which had a background of familial cancer (Cantor \textit{et al.} 2001). Somatic \textit{BARD1} mutations in combination with loss of the wild-type allele have been reported in breast, ovarian and endometrial tumors (Thai \textit{et al.} 1998). Recently, also germline \textit{BARD1} mutations were discovered in three breast-ovarian cancer families, however the effect of these alterations on protein function remains to be determined (Ghimenti \textit{et al.} 2002).

In contrast to the actual pathogenic mutations, certain variants of these genes might only have a subtle effect on cancer predisposition by themselves. In some cases, however, in combination with other constitutive alterations in genes belonging to the same or some other supplementary biochemical pathway, their cumulative effect could be sufficient to explain the increased breast cancer risk. As a result, less somatic alterations would be required for the malignant transformation of a cell. Consequently, screening for germline mutations in only one or two such genes at a time might not be a sufficiently powerful approach to provide the desired information about the contribution of DSB-related genes to breast cancer. Instead, simultaneous assessments of several genes in large sample sets, combined with high-throughput expression studies, would be required to gain more insight into the putative cumulative effects of rare variants in DNA damage response genes.

Obviously, at present there are also other concurrent efforts to elucidate factors involved in the inherited breast cancer susceptibility in the Finnish population. First, some of the \textit{BRCA1} and \textit{BRCA2} mutation-negative families also included to this study have been selected for genetic linkage studies initiated to identify additional high-penetrance susceptibility genes. As has already been successfully demonstrated (Hedenfalk \textit{et al.} 2001), microarray-based expression analysis will provide an efficient way to cluster breast cancer families into certain smaller subgroups based on the
molecular profiles of the studied tumors. This will greatly facilitate the selection of a more homogenous study population for genetic linkage analysis. Second, as the contribution of certain ATM mutations to breast cancer risk was recently confirmed in a large case-control association study (Chenevix-Trench et al. 2002), our intention is to perform a survey among Finnish breast cancer patients to see whether any of the ATM mutations found in Finnish AT patients have similar effects in our population. Third, due to the availability of the human genome sequence, it will be possible to perform high-throughput somatic mutation searches to trace genes frequently altered in cancer. Consequently, a subset of these genes is also likely to carry cancer-predisposing germline mutations, thus providing a list of candidates to be screened in cancer families and case-control studies (Nathanson et al. 2001).

Finally, as the contribution of CpG island hypermethylation to tumorigenesis has been well established (Jones & Laird 1999, Baylin & Herman 2000), it will be interesting to further investigate the role of epigenetic cancer-associated changes in breast cancer. For example, we have expanded our study of TSLC1 to a larger cohort of breast cancer patients in order to gain more fundamental understanding of the role of this gene in tumorigenesis. Furthermore, new techniques are needed to more efficiently study the phenomenon. For this purpose, a microarray-based strategy to investigate genome-wide CpG island hypermethylation patterns has recently been developed (Huang et al. 1999, Yan et al. 2001). This method does not only offer an alternative to cDNA microarrays for molecular classification of tumors, but may also have diagnostic potential, as it could be utilized to predict patients’ responsiveness to demethylating agents (Yan et al. 2001).
In this study, we investigated the roles of three putative breast cancer predisposing genes \((TP53, CHK2\) and \(ATM\)\), which are known to be essential in the pathways for cellular signaling and the maintenance of genomic integrity. Germline mutations in these genes are also associated with known cancer syndromes, frequently displaying breast cancer as a major phenotype. Furthermore, to gain more insight into the chromosome 11q21-q24 LOH targets, four candidate genes were screened for intragenic mutations, and five were analyzed for epigenetic inactivation. The observations and conclusions based on the results are:

1. In combination with a previous study (Huusko et al. 1999), \(TP53\) germline mutations were detected in 2.6% (3/108) of the breast cancer families, all showing characteristics of LFS or LFL. The only detected \(CHK2\) alteration with a putative effect on cancer susceptibility (Ile157Thr) segregated ambiguously with the disease, and it was also present in cancer-free controls. The available functional data, however, suggests that Ile157Thr disturbs the protein function by interfering with the FHA domain-binding ability, and it therefore seems likely that the altered \(CHK2\) in some way promotes cancer formation. Furthermore, compared to the other studied populations, Ile157Thr appears to be markedly enriched in the Finnish population. Thus, the clinical significance of Ile157Thr requires further investigation among Finnish cancer patients.

2. \(ATM\) germline mutations appear to contribute to a small proportion of the hereditary breast cancer risk, as two distinct \(ATM\) mutations were found among the three families (1.9%, 3/162) displaying breast cancer. They all originated from the same geographical region as the \(AT\) families with the corresponding mutations, providing a preliminary suggestion of a possible founder effect concerning the distribution of these mutations in the Finnish population.

3. The low frequency or absence of somatic intragenic mutations observed among sporadic breast tumors in the putative LOH target genes \((MRE11A, PPP2R1B, CHK1, TSLC1)\) residing in chromosome 11q21-q24 led us to assess the role of
promoter region hypermethylation as a mechanism capable of silencing these genes, as well as the \textit{ATM} gene. Of the five genes studied, only \textit{TSLC1} demonstrated involvement of CpG island methylation, which was especially prominent in three tumors. Altogether, one third of the analyzed tumors showed some evidence of \textit{TSLC1} promoter methylation. This suggests that together with LOH, methylation could result in biallelic inactivation of the \textit{TSLC1} gene in breast cancer.
9 References


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