Fanconi anemia pathway defects in sporadic cancer

Najim Ameziane
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Fanconi anemia pathway defects in sporadic cancer
promotor: prof.dr. H. Joenje

copromotoren: dr. G. Pals
dr. J.P. de Winter
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Chapter 1

General introduction
Introduction

DNA in living organisms is continuously subjected to chemical and physical assaults, of both environmental and endogenous origin. To minimize the damage and its genetic consequences several processes are active within a cell that sense and repair this damage. These processes are diverse in nature and able to manage a variety of DNA damage types. Figure 1 summarizes the types of damage that are commonly inflicted onto the DNA, their sources, and the relevant DNA repair mechanisms that engage in the removal of specific lesions (Hoeijmakers 2001). After unraveling the human genome sequence (Lander et al. 2001; Venter et al. 2001), 150 enzymes have been recognized that could either be functionally linked to the recognition and repair of damaged DNA, or that showed sequence homology to known repair genes in other organisms (Wood et al. 2001; Wood et al. 2005). The list of genes becomes even larger if genes were included that are involved in the control of the cell cycle, DNA replication, and apoptosis, processes that are indirectly involved in stabilization of the genome.

Cancer is a disease that results from uncontrolled cellular proliferation. As growth control of normal cells is tightly regulated by the genome, cancer is considered as a disease of the genes in somatic cells. The capacity of tumor cells to divide, invade, and metastasize requires the acquisition of at least three mutation events (Vogelstein & Kinzler 1993). However, the frequency of spontaneous mutations in healthy individuals is insufficient to explain the lifetime cancer risk (Loeb et al. 2008). Rather, it is thought that the accumulation of mutations is due to an initial defect in a process that maintains genetic stability, which would result in a condition referred to as a “mutator phenotype” that would greatly facilitate subsequent genetic hits. A cascade of mutations and epigenetic alterations may ultimately provide the cell with properties that characterize an aggressive metastasizing tumor (Loeb 1991). Support for the hypothesis is provided by the observation that most tumors are genetically unstable (Lengauer et al. 1998). Furthermore, the majority of the genetic instability disorders are characterized by predisposition to various types of malignancies.

The genes defective in genetic instability disorders code for enzymes that help to maintain genomic integrity and are categorized as “caretaker genes”, a subclass of
tumor suppressor genes also referred to as “stability genes” (Vogelstein & Kinzler 2004). Inactivation of these genes generates a mutator phenotype, which greatly increases the chances of growth regulatory genes to become altered by mutation. Some caretaker enzymes maintain genetic stability by direct interaction with DNA and repair of the damage, while others are involved in control of the cell cycle or activation of specialized machineries that facilitate repair. For example, the nucleotide excision repair system, which is defective in Xeroderma pigmentosum, Cockayne syndrome, and Trichothiodystrophy (Lehmann 2001), plays a direct role in the repair of ultraviolet light-induced DNA damage. On the other hand, for the repair of DNA double strand breaks resulting from ionizing radiation or chemicals, a cascade of reactions is required that delays the cell cycle and recruits repair factors. ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia related), which are defective in ataxia telangiectasia and Seckel syndrome, respectively (Rotman & Shiloh 1998; O'Driscoll et al. 2003), are among the early initiators of the cascade that stimulates cell cycle check points and damage repair via homologous recombination or end joining (Zhou & Elledge 2000; Khanna & Jackson 2001). The enzymes disrupted in the above-mentioned conditions together with those affected in other cancer-prone genetic instability disorders including Bloom syndrome, Werner syndrome, Rothmond Thomson syndrome, Nijmegen breakage syndrome, and Fanconi anemia (FA) (Table 1), appear to be involved in a complex web of interactions to maintain genomic stability (Surralles et al. 2004). Although the proteins found defective in FA patients interact with most of the proteins representing the other instability syndromes, the function of the pathway controlled by FA proteins is still poorly understood.
Figure 1. DNA damaging agents (top), typical consequential lesions (middle), and the relevant repair mechanisms (bottom). IR, ionizing radiation; HU, hydroxyurea; MMC, mitomycin C; HR, homologous recombination; NHEJ, non-homologous end joining. Adapted from Hoeijmakers 2001.
Fanconi anemia

The remainder of this introduction is devoted to the chromosomal instability disease Fanconi anemia (FA). A brief overview is given of the clinical aspects, including diagnosis and treatment, followed by a summary of the cellular phenotype and molecular aspects of the FA pathway. Lastly, the significance of the FA pathway in relation to the development of various malignancies is discussed.

Clinical phenotype

FA is a genetic syndrome named after Guido Fanconi who originally described a family in which three brothers suffered from aplastic anemia and congenital malformations (Fanconi 1927). The disorder is inherited in an autosomal recessive as well as in an X-linked-manner (Meetei et al. 2004a). Although FA is rare, about 5-25 per million live births (Joenje & Patel 2001), it may vary among ethnic populations due to founder effects (Rosendorff et al. 1987; Verlander et al. 1995). Clinical features of FA become manifest as pre-and postnatal growth retardation, organ malformations (head, skin, skeleton, heart, and kidney), progressive bone marrow failure (BMF), and cancer predisposition (Alter et al. 2003; Rosenberg et al. 2008). The average life expectancy of FA patients is 25 years (range 0-50 years), and the main cause of death is due to progressive bone marrow failure that typically starts between 5 and 10 years of age. Other life-threatening complications include malignancies, mainly acute myeloid leukemia and squamous cell carcinoma.
Table 1. Genetic instability syndromes with hypersensitivity to DNA damage and predisposition to cancer

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Clinical presentation</th>
<th>Genes</th>
<th>Critical damaging agents</th>
<th>Cellular process</th>
<th>Type of neoplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ataxia telangiectasia</td>
<td>neurodegeneration, immunodeficiency, premature aging, radiation sensitivity, cancer</td>
<td>ATM</td>
<td>Ionizing radiation</td>
<td>DSB-recognition and repair (NHEJ+HR)</td>
<td>Lymphoma</td>
</tr>
<tr>
<td>AT-like syndromes (ATLD)</td>
<td>cerebellar degeneration, radiation sensitivity, cancer</td>
<td>Mre11, Rad50</td>
<td>Ionizing radiation</td>
<td>DSB-recognition and repair (NHEJ+HR)</td>
<td>Lymphoma</td>
</tr>
<tr>
<td>Primary microcephaly</td>
<td>microcephaly and mental retardation, immunodeficiency, premature aging, cancer</td>
<td>MCPH1, 3, 5, 6</td>
<td>-</td>
<td>Chk1 signalling</td>
<td>Mitosis</td>
</tr>
<tr>
<td>Bloom syndrome</td>
<td></td>
<td>BLM</td>
<td>UV light</td>
<td>Helicase (HDR)</td>
<td>All tumors</td>
</tr>
<tr>
<td>Breast/ovarian cancer susceptibility</td>
<td>Breast and ovarian cancer</td>
<td>BRCA1, BRCA2</td>
<td>Reactive oxygen species, crosslinking agents</td>
<td>Recombinational repair (HDR)</td>
<td>Breast and ovarian cancer</td>
</tr>
<tr>
<td>Cockayne syndrome</td>
<td>dwarfism, mental retardation, UV light sensitivity</td>
<td>ERCC6, 8</td>
<td>UV light</td>
<td>Helicase/Replication-coupled</td>
<td>-</td>
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<tr>
<td>Fanconi anemia</td>
<td>congenital abnormalities, bone-marrow failure, cancer</td>
<td>FANC-A, B, C,</td>
<td>Cross-linking agents, reactive oxygen species</td>
<td>DSB-recognition and homology-directed recombinational repair (HDR)</td>
<td>AML, squamous cell carcinomas</td>
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<td>Hereditary non-polyposis colorectal cancer</td>
<td>Colorectal cancer</td>
<td>MSH2, MSH6, MLH1, PMS1,2</td>
<td>Reactive oxygen species</td>
<td>Mismatch-repair (MMR)</td>
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<tr>
<td>Li-Fraumeni</td>
<td>cancer</td>
<td>p53</td>
<td>Ionizing radiation, chemicals</td>
<td>Cell cycle arrest</td>
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<tr>
<td>Nijmegen Breakage syndrome</td>
<td>microcephaly and mental retardation, immunodeficiency, radiation sensitivity, cancer</td>
<td>NBS1</td>
<td>Ionizing radiation</td>
<td>DSB-recognition and repair (NHEJ+HR)</td>
<td>Lymphoma</td>
</tr>
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<td>Rapadilino syndrome</td>
<td>Congenital malformations</td>
<td>RecQ4</td>
<td>-</td>
<td>Helicase</td>
<td>-</td>
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<tr>
<td>Rothmund-Thompson syndrome</td>
<td>immunodeficiency, premature aging, cancer</td>
<td>RecQ4</td>
<td>Bromodeoxyuridine, UV light</td>
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<td>Seckel syndrome</td>
<td>Growth retardation, microcephaly with mental retardation</td>
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<td>XPB, XPD, TTDA, TTDA1 WRN</td>
<td>UV light</td>
<td>Helicase/replication – coupled repair</td>
<td>Sarcoma</td>
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<td>Werner syndrome</td>
<td>immunodeficiency, cancer</td>
<td>WRN</td>
<td>4-nitroquinoline 1-oxide</td>
<td>Helicase/Exonuclease (NER)</td>
<td>Nucleotide excision repair (NER)</td>
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<td>Xeroderma pigmentosum</td>
<td>UV light sensitivity, skin aging, skin cancer</td>
<td>XPA-XPG</td>
<td>UV light</td>
<td>Nucleotide excision repair (NER)</td>
<td>Skin cancer</td>
</tr>
</tbody>
</table>

Modified from Nevelinget et. al (Neveling et al. 2007a)

DSB, double strand break; HR, homologous recombination; NHEJ, non-homologous end joining;
HDR, homology directed repair, MMR, mismatch repair; NER, nucleotide excision repair.
Diagnosis

The clinical suspicion of FA needs to be confirmed by examining the lymphocytes of the patient for sensitivity to DNA interstrand-cross-linking (ICL) agents, such as diepoxybutane (DEB) or Mitomycin C (MMC), in a chromosomal breakage assay (Auerbach & Wolman 1976; Cervenka et al. 1981). In this test FA cells exhibit dramatically increased levels of chromatid-type aberrations when compared to healthy control cells. However, the test is not always conclusive, since overlapping diseases exist that may also score positive in the test, such as Nijmegen breakage syndrome (Gennery et al. 2004; New et al. 2005). Furthermore, the interpretation of the chromosomal breakage test is sometimes complicated by the occurrence of genetic reversion in a fraction of the lymphocytes, leading to mosaicism in the blood (Lo Ten Foe et al. 1997). The ultimate diagnosis of FA requires a molecular analysis to demonstrate pathogenic mutations in a FA gene. Mutation analysis is strongly recommended as this may allow prenatal and pre-implantation genetic diagnosis and help in establishing the mode of inheritance. A comprehensive screening approach for FA primarily based on mutation screening is described in chapter 2 of this thesis.

Variation in the clinical manifestation of FA has been reported, which is mainly due to genetic heterogeneity based on multiple disease genes and different types of mutations. However, the genetic subtypes D1 (mutated in BRCA2/FANCD1) and N (mutated in PALB2/FANCN) are exceptional in being associated with a distinctly severe clinical phenotype with much poorer prognosis. Within genetic subtypes variation results from differences in severity associated with individual mutations. For example, the splice site mutation c.456+4A>T (formerly denoted as IVS4+4A>T) in the FANCC gene, which accounts for over 80% of all FA patients in Ashkenazi-Jewish people (Whitney et al. 1993), is much more severe than the frame-shift mutation c.67delG (formerly denoted as 322delG) in the same gene, which is relatively prevalent in people of Dutch ancestry. Within subtype A, null mutations are more severe than mutations that allow near-full length protein expression (Faivre et al. 2000). Even within a single family, multiple affected individuals may show marked differences in clinical phenotype, suggesting some effect from modifier genes on the phenotypic manifestation of FA gene defects. Variation has even been reported in
affected identical twins, suggesting that also stochastic effects may play a role (Kwee et al. 1996).

**Treatment**

First-line treatment is based on the immediate life-threatening symptoms, such as critical organ malfunctions, which are treated by surgery. Also some skeletal abnormalities may be treated by surgery to improve the use of parts of the body that are malformed, such as the thumbs and arms. Other life-threatening symptoms in FA patients that need urgent attention are hematological abnormalities that become manifest as failure to form leukocytes, red blood cells, and platelets. Therefore, frequent monitoring is required to detect regression of the blood counts (Butturini et al. 1994). As a short-term treatment of the aplastic anemia, antibiotics are administered to battle infections, while blood transfusions are often necessary to increase the blood count to acceptable levels. Alternatively, hematopoietic growth factors and androgens are used to stimulate the formation of blood cells. However, the effects of these therapies may be poor (Guinan et al. 1994), while the use of androgens pose an increased risk of developing liver adenomas (Touraine et al. 1993).

Anemia in FA patients can be cured by of hematopoietic stem cell transplantation (HSCT). The first-choice donors are human leukocyte antigen-identical (HLA) siblings (Guardiola et al. 1998), which can lead to success rates up to 83% with minimal graft failure (Dufour et al. 2001; Tan et al. 2006; Farzin et al. 2007). In the past decades, unrelated donor transplants in FA have been disappointing because of treatment-related mortality mainly caused by graft failure, graft versus host disease, infections, and organ toxicity related to pre-transplant conditioning regimens. However, following the introduction of adapted conditioning regimens containing fludarabine (Kapelushnik et al. 1997) major improvements of unrelated HSCT in FA have been achieved, with increased survival rates ranging from 53% to 96% (Guardiola et al. 1998; Yabe et al. 2006; Wagner et al. 2007). An attractive alternative approach to treat BMF of patients who lack a histocompatible sibling donor, is based on the ex vivo correction of the genetic FA defect in the bone marrow stem cells using lentiviral carriers to deliver the proficient FA gene (Galimi et al. 2007).
2002). So far, encouraging results have been achieved (Kelly et al. 2007), but the approach is still in the clinical experimental phase.

Due to improved treatment protocols during the last decade, the life expectancy of FA patients has increased from a median age of 20 years to >30 years. However, solid tumors in older patients represent a new major concern. Evidence has been presented that conditioning of FA patients for stem cell transplantation was associated with an increased risk of SCC of the head, neck and oesophagus (Rosenberg et al. 2003). Moreover, a comparative study for cancer risk in FA patients who did or did not receive transplants demonstrated a 4.4-fold higher risk in transplanted patients (Rosenberg et al. 2005). Due to the poor tolerance to radiotherapy and chemotherapy, the management of malignancies in FA patients remains challenging.
Cellular phenotype

In addition to chromosomal breakage, a characteristic feature of FA cells is the prolonged progression through as well as complete arrest within the late S/G2 phase of the cell cycle (Dutrillaux et al. 1982; Akkari et al. 2001), which becomes even more pronounced when the cells are challenged with interstrand crosslink (ICL) agents (Seyschab et al. 1995; Kruyt et al. 1996). Indeed, the accumulation of 4n cells in response to ICLs has been proposed as a convenient alternative method to chromosomal breakage analysis for diagnosing FA patients (Bechtold et al. 2006). This cellular characteristic is also exploited to identify tumor cells that might have a defect in the FA/BRCA pathway (Chapter 8).

Major processes involved in the repair of DNA damage in wild type cells take place in the S and G2 phase (Lau & Pardee 1982), which suggests that FA cells activate a checkpoint to allow for repair of accumulated DNA damage.

The FA pathway

To date 13 FA subtypes have been identified for FA (Joenje & Patel 2001; Wang W. 2007). Each subtype is linked to a defect in a different disease gene (Table 2). The encoded proteins work in concert in a distinct genomic maintenance pathway, called the FA/BRCA pathway (Figure 2). Most of the proteins (FANCA,-B,-C,-E,-F, -G, -L, and –M) form a multiprotein complex that is required for the modification of FANCD2 and FANCI by monoubiquitination. FANCD2 and FANCI form a complex (ID-complex), and are interdependent in terms of stability and monoubiquitination (Smogorzewska et al. 2007). The catalytic subunit of the core complex is FANCL, which contains a PHD domain and has homology with other E3 ubiquitin ligases (Meetei et al. 2004b). Apart from the proteins found defective in FA patients, at least three additional FA-associated proteins, FAAP24, FAAP100 and HES1, have been identified that are considered essential components of the core complex for the modification of FANCD2 and FANCI (Ling et al. 2007; Ciccia et al. 2007; Tremblay et al. 2008). Furthermore, evidence for yet to be identified FA genes is discussed in Chapter 2.
In normal cells, the level of monoubiquitination of the ID-complex is intensified when cells are exposed to DNA damaging agents, such as cross-linking agents, ultraviolet, or ionizing radiation, or when the deoxyribonucleotide triphosphate pool is depleted by hydroxyurea, or when the DNA polymerase $\alpha$ and $\delta$ are inhibited by aphidicolin (Garcia-Higuera et al. 2001; Gregory et al. 2003; Howlett et al. 2005). This step is not influenced by FANCD1 (BRCA2), FANCJ (BRIP1), or FANCN (PALB2), so that these proteins are thought to operate downstream of the ID-complex (Figure 2). Upon modification of the ID-complex, the FANCD2 subunit has been shown to co-localize in nuclear foci with many other proteins, including BRCA1 (Garcia-Higuera et al. 2001), BRCA2 (Wang et al. 2004; Hussain et al. 2004), RAD51 (Taniguchi et al. 2002; Hussain et al. 2004), proliferating cell nuclear antigen (PCNA) (Hussain et al. 2004), and Nijmegen breakage syndrome protein (NBS1) (Nakanishi et al. 2002).

The FA core complex has been purified as part of a larger multiprotein complex that also contained the helicase defective in Bloom syndrome (BLM), replication protein A, and topoisomerase III $\alpha$ (Meetei et al. 2003), proteins that engage in DNA-processing activities. With the identification of FANCM, which contains a helicase and an endonuclease domain, it was suggested that the FA core complex interacts directly with DNA. This interaction could serve as an anchor to flag the sites of DNA damage that may result in the recruitment of specialized repair enzymes through modification of the ID-complex or to process the DNA for subsequent repair (Wang W. 2007).

Also, the downstream proteins have been shown to form stable complexes with DNA-repair proteins. BRCA2 contains BRC-binding domains for RAD51, a RecA ortholog that has been shown to play a major role in homologous recombination (Wong et al. 1997; Kawabata et al. 2005). FANCJ was shown to co-immunoprecipitate with BRCA1, topoisomerase II-binding protein 1 (TOPBP1), and the mismatch repair protein mutL homologue 1 (MLH1) (Greenberg et al. 2006). The link between FA and the mismatch repair system is also suggested by the interaction of FANCJ with the MutL$\alpha$ heterodimer, composed of post-meiotic increased 2 (PMS2) and MLH1 (Peng et al. 2007).

Activation and function of the FA pathway is thought to take place mainly in the S-phase of the cell cycle. Support for this argument is based on cell synchronization studies that showed the monoubiquitination of FANCD2 in early S-
phase and progressing until it is deubiquitinated by USP1 at the exit of S-phase (Taniguchi et al. 2002; Nijman et al. 2005). Furthermore, fluorescent-tagged components of the core complex (FANCA, FANCC, and FANCG) were shown to localize as a complex in foci on chromatin at the beginning of the S-phase, and dissociate from the condensed chromosomes before mitosis (Mi & Kupfer 2005). During S-phase, in response to replication fork arrest, single-stranded DNA is bound by replication protein A (RPA) recruiting ATR and ATRIP, upon which the complex becomes engaged in the phosphorylation of multiple substrates (Zou & Elledge 2003). The main substrate of ATR is checkpoint kinase 1 (CHEK1) (Pichierri & Rosselli 2004). Both ATR and CHEK1 together with ATR activators: RPA, ATRIP, NBS1, and HCLK2 (Zou & Elledge 2003; Stiff et al. 2005; Collis et al. 2007), have been implicated in the activation of the FA/BRCA pathway by facilitating phosphorylation of core complex components, FANCD2, FANCI, BRCA2 and FANCN (Yamashita et al. 1998; Qiao et al. 2004; Ho et al. 2006; Matsuoka et al. 2007; Wang W. et al. 2007).

Besides the involvement in DNA repair and cell cycle control, the FA/BRCA pathway has previously been assigned an important role in the defense against oxidative damage. Joenje et al. have demonstrated an oxygen-dependence of chromosomal breakage in FA cells (Joenje et al. 1981). In another study, FA cells were shown to accumulate in S and G2/M phase when exposed to an elevated oxygen tension (35% O2), while growth and cell cycle transit were normalized when the cells were cultured under a more physiological oxygen level (5% O2) (Schindler & Hoehn 1988). The involvement of FA proteins in the cellular response to oxidative stress was also suggested by an interaction of FANCC with NADPH cytochrome P450 reductase (Kruyt et al. 1998) and glutathione S-transferase (Cumming et al. 2001), enzymes with recognized redox activities. Also FANCG has been found to interact with cytochrome P450 2E1 (Futaki et al. 2002).

Despite extensive work to decipher the function of the FA proteins in response to ICLs and active oxygen species, the exact mechanism by which the FA/BRCA pathway counteracts the (geno) toxic effects from these agents is not fully understood.
Table 2. FA gene characteristics

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome location</th>
<th>exons</th>
<th>Amino acids</th>
<th>Molecular weight</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FANCA</td>
<td>16q24.3</td>
<td>43</td>
<td>1455</td>
<td>163 kD</td>
<td>(Lo Ten Foe et al. 1996b)</td>
</tr>
<tr>
<td>FANCB</td>
<td>Xp22.31</td>
<td>14</td>
<td>859</td>
<td>95 kD</td>
<td>(Meetei et al. 2004a)</td>
</tr>
<tr>
<td>FANCC</td>
<td>9q22.3</td>
<td>14</td>
<td>558</td>
<td>63 kD</td>
<td>(Strathdee et al. 1992)</td>
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<tr>
<td>FANCD1</td>
<td>13q13.1</td>
<td>27</td>
<td>3418</td>
<td>384 kD</td>
<td>(Howlett et al. 2002)</td>
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<td>FANCD2</td>
<td>3p25.3</td>
<td>44</td>
<td>1451</td>
<td>155 kD</td>
<td>(Timmers et al. 2001)</td>
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<tr>
<td>FANCE</td>
<td>6p21.3</td>
<td>10</td>
<td>536</td>
<td>60 kD</td>
<td>(de Winter et al. 2000a)</td>
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<tr>
<td>FANCF</td>
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<td>1</td>
<td>374</td>
<td>42 kD</td>
<td>(de Winter et al. 2000b)</td>
</tr>
<tr>
<td>FANCG</td>
<td>9p13.3</td>
<td>14</td>
<td>622</td>
<td>70 kD</td>
<td>(de Winter et al. 1998)</td>
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<tr>
<td>FANCI</td>
<td>15q26.1</td>
<td>37</td>
<td>1268</td>
<td>140 kD</td>
<td>(Smogorzewska et al. 2007; Dorsman et al. 2007; Sims et al. 2007)</td>
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<td>FANCJ</td>
<td>17q23.2</td>
<td>20</td>
<td>1249</td>
<td>150 kD</td>
<td>(Utman et al. 2005; Levran et al. 2005; Levitus et al. 2005)</td>
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<td>FANCL</td>
<td>2p16.1</td>
<td>14</td>
<td>375</td>
<td>43 kD</td>
<td>(Meetei et al. 2004b)</td>
</tr>
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<td>FANCM</td>
<td>14q21.2</td>
<td>23</td>
<td>2048</td>
<td>250 kD</td>
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<td>FANCN</td>
<td>16p12.1</td>
<td>13</td>
<td>1188</td>
<td>140 kD</td>
<td>(Reid et al. 2007; Xia et al. 2007)</td>
</tr>
</tbody>
</table>
Figure 2. A schematic depiction of the FA/BRCA pathway. Upon DNA replication stress, a multi-protein complex is formed consisting of FANCA, -B, -C, -E, -F, -G, -L, -M (FA core complex), which monoubiquitinates FANCD2 and FANCI, which form the ID complex. After this modification step, whereby FANCL acts as the ubiquitin ligase and UBE2T as the ubiquitin-conjugating enzyme, the ID complex co-localizes with BRCA2 (FANCD1) to sites of DNA damage. HES1, P24, and P100 are proteins that have been found to associate with the core-complex, but no FA patients have so far been identified with mutations in these genes. USP1, ubiquitin specific peptidase 1, is proposed to deubiquitinate FANCD2.
General introduction

*FA gene mutations and cancer*

In view of the role that FA genes seem to play in avoiding deterioration of genetic information, FA gene defects are likely to promote cancer, which is already evident in the population of biallelic mutation carriers (FA patients). The cancer-promoting role of FA gene defects is less straightforward in the populations of heterozygous carriers and in individuals suffering from ‘sporadic’ cancer. The role of FA gene defects in the development of malignancies in these 3 populations is discussed below.

**Homozygous mutation carriers**

All FA patients face a high risk of developing cancer, in particular leukemia and squamous cell carcinomas. In fact, in about 25% of patients with FA who have cancer, the diagnosis of leukemia or a tumor preceded the diagnosis of FA (Alter 2003a). Based on a literature review that included 1300 FA cases, 9% of patients had leukemia, mainly acute myeloid leukemia (AML), 7% had myelodysplastic syndrome (MDS), 5% had solid tumors, and 3% had liver tumors (Alter 2003a). The high incidence of liver tumors can likely be attributed to prolonged androgen treatment (Velazquez & Alter 2004). If FA patients outlive the severe complications of bone marrow failure and leukemia, the cumulative probability of developing a solid tumor is 76% at the age of 45 (Alter 2003a). Similar observations were made in a recent investigation, where a cohort of 181 individuals of mainly German nationality was studied (Rosenberg *et al.* 2003). The most common non-hematological malignancies in FA are squamous cell carcinomas (SCCs) of the head and neck, the vulva, and the cervix (Kutler *et al.* 2003), which occur at a much earlier age in FA patients than observed in the general population.

A marked difference in life expectancy and cancer proneness is observed within FA subtypes. Patients with defective proteins that operate in the downstream part of the FA pathway, especially FANCD1/BRCA2 and FANCN/PALB2, have much more severe congenital abnormalities and a higher risk of malignancies than the other FA subtypes. Patients who carry bi-allelic mutations in *BRCA2* present with an early onset of leukemia with a median age of 2.2 years, compared to 13.4 years in FA patients belonging to the ‘upstream’ complementation groups (Wagner *et al.*).
An assessment of 23 patients in 17 families with bi-allelic \textit{BRCA2} mutations identified 9 cases of various types of brain tumor (6 medulloblastoma, 2 glioblastoma multiforme, and 1 astrocytoma), 5 cases of Wilms tumor, 10 cases of leukemia (7 AML, and 3 acute lymphoblastic leukemia, ALL). In 4 of those cases, multiple cancers were observed. Only one patient reached adulthood without developing cancer, which might be explained by a relatively mild mutation that was homozygously present in this patient (Reid \textit{et al.} 2005). Patients belonging to the FA-N subtype with mutations in the recently identified gene \textit{FANCN} show similar severe clinical characteristics and childhood cancer proneness as patients in the FA-D1 group. Eight FA-N patients have been described that suffered from brain tumors (5 medulloblastoma, 1 neuroblastoma), Wilms tumor (3), AML (2), and one case of kaposiform hemangioendothelioma (Xia \textit{et al.} 2007; Reid \textit{et al.} 2007), a common vascular tumor of infancy (Mueller & Mulliken 1999). The close molecular connection of \textit{BRCA2} with \textit{FANCN} in the DNA repair pathway may explain the overlap in tumor spectrum that is observed between these two patients groups.

**Heterozygous mutation carriers**

Given that FA cells are genetically unstable and are likely to develop into cancer cells, at least in homozygous mutation carriers, it is reasonable to hypothesize that loss of the wild type allele in a somatic cell of heterozygous mutation carrier may initiate a cascade of events leading to cancer, similar to the situation in carriers of a mutant Rb (gate-keeper) gene, who likely develop retinoblastoma upon inactivation of the wild-type allele (Knudson’s two hit model of carcinogenesis (Knudson, Jr. 1971)). In addition, malignancies might arise as a consequence of haplo-insufficiency (Santarosa & Ashworth 2004). A distinct cellular phenotype has been reported for lymphocytes from heterozygous carriers of mutations in \textit{BRCA1} or \textit{BRCA2} (Buchholz \textit{et al.} 2002), suggesting that haplo-insufficiency might represent a carcinogenic mechanism in itself. Alternatively, a dominant-negative characteristic of an altered enzyme could result in the obstruction of the DNA repair process, as described for several tumor suppressors, such as the transcription factor p53 (Ko & Prives 1996). Additional support for the hypothesis of haplo-insufficiency was obtained from experiments where FA heterozygous cells
were shown to accumulate more DNA breaks in response to irradiation than wild type control cells in a comet assay analysis (Djuzenova et al. 2001). In another study, the mean number of DEB-induced chromosomal breaks in FA heterozygous lymphocytes was found to be slightly but significantly higher compared to wild type control cells (0.13±0.06 versus 0.07±0.09) (Pearson et al. 2001), again suggesting that heterozygous FA mutations may be associated with an abnormal cellular phenotype.

The question of whether family members of FA patients, which are obligatory carriers of a single FA gene mutation, would have a higher risk to develop cancer was addressed as early as in 1971. Although initially an increased incidence of cancer in relatives of FA patients was reported (Swift 1971), in a larger study that included 25 families the previous observation could not be confirmed (Swift et al. 1980). Also no convincing evidence for an elevated cancer risk was found in another study describing relatives of 9 FA patients, where 7 cases of diverse cancers were observed, as compared with the 10.4 expected (P>0.05) (Potter et al. 1983). A recent comprehensive and rigorous study was reported by Berwick et al. (2007), which included 784 grandparents and 160 other relatives of FA patients, with a total of 404 heterozygous carriers of mutations in all known FA genes, except FANCL, -M, and –N. The general conclusion of the study was that heterozygous FA gene mutations do not confer an overall increased cancer risk. However, a 1.7-fold higher rate of breast cancer (BC) was observed among carrier grandmothers when compared to non-carriers. The highest standardized increased risk (SIR, 2.4, 95% confidence interval [CI], 1.1–5.2) was found in grandmother carriers with FANCC mutations, of which the IVS4 mutation (c.711+4A>T) was the most predominant (5 out of 8 FANCC mutation carriers). However, in a separate study including 42 Ashkenazi Jewish IVS4 mutation carriers, the alteration did not seem to result in an increased prevalence of malignancies (Baris et al. 2007). The overall results of the study by Berwick et al. (2008) provided family relatives of common FA patients with molecular support for no elevated risk of cancer with respect to the general population, with the possible exception of breast cancer. Furthermore, in a more recent study of 36 families consisting of 575 individuals from the UK, no overall increased cancer risk was observed in FA families as compared to the general population (Tischkowitz et al. 2008a).

An alternative approach to study whether FA gene mutations are associated with an increased risk of cancer, and hence act as tumor suppressor genes, is the
analysis of germ-line FA mutation prevalence in patients with an inherited form of cancer. In approximately 20-25% of families with hereditary breast cancer (BC), the disease can be mainly explained by mutations in \textit{BRCA1} and \textit{BRCA2} genes (Antoniou \textit{et al.} 2001; Wooster & Weber 2003). A smaller fraction of familial BC can be caused by mutations in \textit{TP53} (Lalloo \textit{et al.} 2003), \textit{PTEN} (Ueda \textit{et al.} 1998), \textit{STK11} (Giardiello \textit{et al.} 2000a), \textit{ATM} (Stankovic \textit{et al.} 1998), or \textit{CHEK2} (CHEK2 Breast Cancer Case-Control Consortium (The CHEK2 Breast Cancer Case-Control Consortium 2004). It was hypothesized that at least part of the remaining hereditary BCs might be explained by defects in yet-to-be-identified proteins that interact with proteins encoded by the known predisposition-causing genes. The identification of \textit{BRIP1} (BRCA1-Interacting Protein) (Cantor \textit{et al.} 2001) and \textit{PALB2} (Partner And Localizer of BRCA2) (Xia \textit{et al.} 2006) as the disease-causing genes in the FA-J and FA-N subtypes, respectively, triggered many researchers to investigate whether germ-line mutations in these genes may be associated with familial breast/ovarian cancer. In addition, \textit{FANCD2}, which was found to functionally interact and co-localize with \textit{BRCA1} and \textit{BRCA2} in nuclear foci at sites of DNA damage, is to be considered a candidate for causing predisposition to BC (Garcia-Higuera \textit{et al.} 2001; Wang \textit{et al.} 2004).

Several studies have been conducted to evaluate the possible implication of FA genes in BC susceptibility and other cancers. Studies performed on hereditary cancers are summarized in Table 3. The involvement of an FA gene other than \textit{BRCA2}, in BC susceptibility was first suggested when two germline mutations in \textit{FANCJ} were detected in 65 individuals with early-onset breast cancer of whom the majority had a strong family history (Cantor \textit{et al.} 2001). However, no \textit{FANCJ} mutations were found in a panel of 340 \textit{BRCA1/2}-negative familial or early-onset BC patients in the Swedish population (Luo \textit{et al.} 2002). Absence of germ-line pathogenic alterations in \textit{FANCJ} was also reported in the Finnish familial BC population (214 cases, (Karppinen \textit{et al.} 2003)), in the North American population (21 cases, (Rutter \textit{et al.} 2003)), Australian population (75 cases, (Lewis \textit{et al.} 2005), and in the French Canadian population (96 cases, (Guenard \textit{et al.} 2008). Nonetheless, in a large study that included 1212 cases in the UK, 9 cases were identified that carried truncating mutations in \textit{FANCJ} (Seal \textit{et al.} 2006); the authors estimated that such mutations conferred a relative risk of 2 (95% CI = 1.2-3.2, \(P = 0.012\)). An association between \textit{FANCN} mutations and elevated BC risk has been reported in a similar study
that included nearly 1000 familial BC patients from the UK. Truncating mutations were detected in 10/923 index cases, compared to 0/1084 controls (Rahman et al. 2007). The calculated relative risk for FANCN mutation carriers for BC was 2.3-fold (95% CI = 1.4-3.9, P = 0.0025). After screening for FANCN mutations in 113 Finnish familial BC, a recurrent frameshift mutation, c.1592delT, was found to be present at significantly elevated frequency (Erkko et al. 2007). This specific mutation occurred in 18 cases of 2059 unselected BCs. The contribution of FANCN mutations to BC susceptibility was also reported for the Ashkenazi Jewish population (Tischkowitz et al. 2007), French-Canadian populations (Foulkes et al. 2007), Spanish population (Garcia et al. 2008), as well as for the Chinese population (Cao et al. 2008). However, mutations in FANCN do not seem to contribute to BC risk in the Icelandic population (Gunnarsson et al. 2008). A contribution to familial BC predisposition was also evaluated for the other FA genes, FANCA, FANCC, FANCF, FANCG, and FANCD2 in 88 cases from the UK, but this study did not reveal any disease-causing mutation (Seal et al. 2003). Absence of FANCD2 mutations was also reported in the Australian BC cohort (Lewis et al. 2005). So far there has been only one study investigating the involvement of the X-linked gene FANCB in BC predisposition, but none of the 95 analyzed Spanish cases was associated with mutations in this gene (Garcia et al. 2008).

It is relevant to note that mutation screening of FANCD2 in the above-mentioned studies was performed on genomic DNA. However, such an analysis is flawed as it may overlook certain alterations due to the presence FANCD2 pseudogenes. Therefore, as long as the analysis has not been carried out on cDNA, it seems premature to conclude whether or not FANCD2 is implicated in the predisposition to BC, or to other cancers.

Germ-line mutations in BRCA2 have also been linked to familial and sporadic cancers of the pancreas and the prostate (Swift 1971; Goggins et al. 1996; Ozcelik et al. 1997; Sigurdsson et al. 1997; Kern et al. 2002; Murphy et al. 2002; Tryggvadottir et al. 2007), which suggested the possible role of the other members of the FA/BRCA pathway in the development these tumors. The disruption of the coding sequence of FANCC and FANCG has been reported for a proportion of pancreatic cancers, which were selected for LOH at the 9q22.3 and 9p13 loci, respectively (van der Heijden et al. 2003). Subsequent examination of the FANCC deficient cell line and an additional FANCG mutated cell line demonstrated lack of FANCD2 monoubiquitination and
sensitivity to MMC, which could be restored by transduction of the relevant gene (van der Heijden et al. 2004). Interestingly, the identified FA gene mutations involved early-onset pancreatic cancer cases. Also, truncating FANCC mutations were identified in two early-onset cases after screening of 421 unselected pancreatic cases, which were not found in 658 controls (Couch et al. 2005). In a Finnish study where the FANCN founder mutation c.1592delT was screened in 639 prostate cancer cases (164 familial, and 475 unselected cases), one familial case was found positive (Erkko et al. 2007). Thus mutations in FANCC, FANCG and the FANCN genes seem to have a limited contribution to early-onset pancreatic cancer and familial prostate cancer, respectively.

Altogether, mono-allelic mutations in most of the FA genes coding for the core complex proteins do not seem to have a major role in cancer predisposition, except mutations in FANCC, which were shown to be associated with a slightly elevated risk for cancers of the breast and pancreas. However, in addition to the well-established BC susceptibility gene BRCA2/FANCD1, it appears that a substantial role in the susceptibility to BC may be ascribed to mutations in the downstream FA genes FANCJ, and FANCN. Finally, the most recently identified FA gene, FANCI, which is considered a paralog of FANCD2, has not been tested for its potential connection with cancer in heterozygous carriers. There is also evidence for at least one as yet unidentified downstream FA gene based on a comprehensive mutation screening analysis (Chapter 3). To obtain a comprehensive insight into the role of FA/BRCA pathway defects in familial or early-onset cancer further analysis should include FANCI, FANCD2 (on cDNA) and other yet-to-be-discovered genes acting downstream in the pathway.
Table 3. FA gene mutation analysis in hereditary/familial cancer patients*

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Cases</th>
<th>Region</th>
<th>Gene(s)</th>
<th>Relative risk</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>65</td>
<td>USA</td>
<td>FANCJ</td>
<td>N.C.</td>
<td>(Cantor et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>340</td>
<td>Sweden</td>
<td>FANCJ</td>
<td>Not significant</td>
<td>(Luo et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>UK</td>
<td>FANC-A, -C, -D2, -E, -F, &amp; -G</td>
<td>Not significant</td>
<td>(Seal et al. 2003)</td>
</tr>
<tr>
<td>41, 396</td>
<td>Australia</td>
<td>FANCD2</td>
<td>Not significant</td>
<td></td>
<td>(Lewis et al. 2005)</td>
</tr>
<tr>
<td>83, 253</td>
<td>Australia</td>
<td>FANCJ</td>
<td>Not significant</td>
<td></td>
<td>(Lewis et al. 2005)</td>
</tr>
<tr>
<td>43, 888</td>
<td>Finland</td>
<td>FANCJ</td>
<td>Not significant</td>
<td></td>
<td>(Vahteristo et al. 2006)</td>
</tr>
<tr>
<td>1212</td>
<td>UK</td>
<td>FANCJ</td>
<td>2-fold</td>
<td></td>
<td>(Seal et al. 2006)</td>
</tr>
<tr>
<td>923</td>
<td>UK</td>
<td>FANCN</td>
<td>2.3-fold</td>
<td></td>
<td>(Rahman et al. 2007)</td>
</tr>
<tr>
<td>68</td>
<td>Canada</td>
<td>FANCN</td>
<td>2.3-fold</td>
<td></td>
<td>(Tischkowitz et al. 2007)</td>
</tr>
<tr>
<td>50</td>
<td>Canada</td>
<td>FANCN</td>
<td>3.4-fold</td>
<td></td>
<td>(Foulkes et al. 2007)</td>
</tr>
<tr>
<td>96</td>
<td>Canada</td>
<td>FANCJ</td>
<td>Not significant</td>
<td></td>
<td>(Guenard et al. 2008)</td>
</tr>
<tr>
<td>95, 725</td>
<td>Spain</td>
<td>FANCN</td>
<td>&gt;2-fold</td>
<td></td>
<td>(Garcia et al. 2008)</td>
</tr>
<tr>
<td>360</td>
<td>China#</td>
<td>FANCN</td>
<td>N.C.</td>
<td></td>
<td>(Cao et al. 2008)</td>
</tr>
<tr>
<td>111, 638</td>
<td>Iceland</td>
<td>FANCN</td>
<td>Not significant</td>
<td></td>
<td>(Gunnarsson et al. 2008)</td>
</tr>
<tr>
<td>Breast &amp; ovarian</td>
<td>214</td>
<td>Finland</td>
<td>FANCJ</td>
<td>Not significant</td>
<td>(Karppinen et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>USA</td>
<td>FANCJ</td>
<td>Not significant</td>
<td>(Rutter et al. 2003)</td>
</tr>
<tr>
<td>113, 2059</td>
<td>Finland</td>
<td>FANCN</td>
<td>11-fold, 4-fold</td>
<td></td>
<td>(Erko et al. 2007)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>44</td>
<td>USA</td>
<td>FANCA</td>
<td>Not significant</td>
<td>(Rogers et al. 2004a)</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>USA</td>
<td>FANCC^ &amp; -G</td>
<td>Not significant</td>
<td>(Rogers et al. 2004b)</td>
</tr>
<tr>
<td></td>
<td>421^</td>
<td>USA</td>
<td>FANCC and FANCG</td>
<td>Low for FANCC</td>
<td>(Couch et al. 2005)</td>
</tr>
<tr>
<td>Prostate</td>
<td>95</td>
<td>USA</td>
<td>FANCN</td>
<td>Not significant</td>
<td>(Tischkowitz et al. 2008b)</td>
</tr>
</tbody>
</table>

* BRCA2 is not included

§ screening for specific mutations, not entire gene

& unselected cases

* other types of cancer tested for the specific mutation, 476 colorectal (188a, 288u), 639 prostate (164a, 475u).

^ FANCC exons 2, 3, 7, and 10 not analyzed

# FANCN mutations are responsible for 1% of Chinese women with early onset breast cancer.

E 4 mutation were identified of which 3 were considered germline mutations.

€ 26 Ashkenazi Jewish, 22 Canadian, mixed ethnicity

N.C: not calculated
Sporadic cancer

The term ‘sporadic cancer’ is commonly used to describe malignancies occurring in individuals at relatively old age and without a family history of cancer. However, the distinction from hereditary or ‘familial’ cancer is not absolute, because differences in genetic background may alter the penetrance of hereditary cancers, whereas exposure to similar environments or lifestyles may cause sporadic cancers to mimic familial cancers. In addition, familial/hereditary cancer cases may be misclassified as ‘sporadic’ due to circumstances such as small family size, lack of or inaccurate information on family members, or lack of susceptible gender in the case of sex-associated cancers like prostate or breast cancer (Trepanier et al. 2004). Thus, studies on sporadic malignancies are likely to be ‘contaminated’ with cases that are actually caused by inherited mutations.

Several studies have reported on FA/BRCA pathway inactivation in sporadic malignancies of various origins. One out of 10 AML cell lines analyzed by Xie et al. (Xie et al. 2000) lacked FANCF protein expression and was hypersensitive to MMC. The aberrant expression resulted from hypermethylation of the FANCF promoter region. Resistance to MMC was restored upon transfection with a FANCF-expressing construct (see Chapter 5 for details). A MMC-hypersensitive AML cell line with loss of FANCD2 monoubiquitination was reported in another study (Lensch et al. 2003). However, the MMC sensitivity in that cell line could only partially be rescued by ectopic expression of FANCA, while no evidence was found for inactivation of the FANCA gene by mutations or epigenetic silencing.

Silencing of FANCF was also observed in 2 ovarian cancer cell lines (Taniguchi et al. 2003), which correlated with increased cisplatin sensitivity and lack of FANCD2 modification. In vitro selection for resistance to cisplatin restored FANCF expression, although the FANCF promoter region remained partially methylated. In the same study, analysis of primary material from 19 ovarian tumors showed promoter methylation in 4 cases. Furthermore, promoter methylation of FANCF was observed in 15% of HNSCC, and in 14% of non-small cell lung cancer (Marsit et al. 2004), granulosa cell tumors (Dhillon et al. 2004), breast cancer (Wang et al. 2006; Wei et al. 2008), and bladder carcinoma (Neveling et al. 2007b). Recently, FANCN promoter methylation was found in two primary breast tumors from BRCA2 mutation carriers, four sporadic primary breast tumors, and four sporadic primary ovarian
tumors (Potapova et al. 2008). However, in these investigations primary archival material was used so that the observations could not be validated by functional studies. It should be pointed out, that the original observations by Taniguchi et al. (2003) have been contradicted by a recent study in which not a single case of FANCF silencing could be demonstrated amongst 106 ovarian tumors (Teodoridis et al. 2005), so that the clinical significance of FANCF silencing in sporadic ovarian cancer remains uncertain. Furthermore, FANCF promoter methylation could also not be confirmed for HNSCC (this thesis, chapter 7), where a panel of 22 patients was examined of whom 11 (50%) responded favorably to cisplatin containing treatment. The discrepancy between the results might be explained by the methylation detection method (MSP) used, which is prone to produce false positive results (Chapter 7).

Sporadic cancers in which FA gene inactivation has been documented, are summarized in Table 4. In Chapter 6 of this thesis, evidence for FANCC and FANCL silencing in sporadic acute leukemia is reported, which was associated with MMC sensitivity.
### Table 4. Evidence for FA gene inactivation in “sporadic” malignancies

<table>
<thead>
<tr>
<th>Aberration in tumor</th>
<th>Gene</th>
<th>Type of malignancy#</th>
<th>cases</th>
<th>Functional testing</th>
<th>Detection method(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Methylation</strong></td>
<td><strong>BRCA2</strong></td>
<td>Breast</td>
<td>9/18</td>
<td>-</td>
<td>MSP</td>
<td>(Cucer et al. 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Granulosa</td>
<td>1/25</td>
<td>-</td>
<td>MSP</td>
<td>(Dhillon et al. 2004)</td>
</tr>
<tr>
<td><strong>FANCC</strong></td>
<td>AML</td>
<td>1/143</td>
<td>-</td>
<td>MS-MLPA, BS</td>
<td></td>
<td>Chapter 6</td>
</tr>
<tr>
<td></td>
<td>ALL</td>
<td>3/97</td>
<td>-</td>
<td>MS-MLPA, BS</td>
<td></td>
<td>Chapter 6</td>
</tr>
<tr>
<td><strong>FANCF</strong></td>
<td>AML</td>
<td>1</td>
<td>+</td>
<td>BS, PE</td>
<td></td>
<td>Chapter 5</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>1/41</td>
<td>-</td>
<td>MSP</td>
<td></td>
<td>(Neveling et al. 2007b)</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>1/23</td>
<td>+</td>
<td>MSP, BS, PE</td>
<td></td>
<td>(Neveling et al. 2007b)</td>
</tr>
<tr>
<td></td>
<td>Breast</td>
<td>13/75</td>
<td>-</td>
<td>MSP</td>
<td></td>
<td>(Olopade &amp; Wei 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1/120</td>
<td>-</td>
<td>MSP</td>
<td></td>
<td>(Wei et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Cervical</td>
<td>27/91</td>
<td>-</td>
<td>MSP</td>
<td></td>
<td>(Narayan et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Germ cell</td>
<td>4/70</td>
<td>-</td>
<td>MSP, mRNA</td>
<td></td>
<td>(Koul et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Granulosa</td>
<td>6/25</td>
<td>-</td>
<td>MSP, mRNA</td>
<td></td>
<td>(Dhillon et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>HNSCC</td>
<td>13/89</td>
<td>-</td>
<td>MSP</td>
<td></td>
<td>(Marsit et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>NSCLC</td>
<td>22/158</td>
<td>-</td>
<td>MSP</td>
<td></td>
<td>(Marsit et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>Ovarian</td>
<td>4/19</td>
<td>-</td>
<td>MSP</td>
<td></td>
<td>(Taniguchi et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Ovarian$</td>
<td>5/18</td>
<td>-</td>
<td>MSP, PE, mRNA</td>
<td></td>
<td>(Wang et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>Ovarian</td>
<td>7/53</td>
<td>-</td>
<td>MSP</td>
<td></td>
<td>(Lim et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Ovarian</td>
<td>1/7</td>
<td>-</td>
<td>MSP, PE, mRNA</td>
<td></td>
<td>(Wang et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>Ovarian</td>
<td>2</td>
<td>+</td>
<td>MSP, BS, PE</td>
<td></td>
<td>(Taniguchi et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Ovarian</td>
<td>1/9</td>
<td>-</td>
<td>MSP, mRNA</td>
<td></td>
<td>(Lim et al. 2008)</td>
</tr>
<tr>
<td><strong>FANCL</strong></td>
<td>ALL</td>
<td>1/97</td>
<td>-</td>
<td>MS-MLPA, BS</td>
<td></td>
<td>Chapter 6</td>
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<tr>
<td><strong>PALB2</strong></td>
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<td>BS, mRNA</td>
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<td>(Potapova et al. 2008)</td>
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<tr>
<td></td>
<td>Ovarian</td>
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<td>-</td>
<td>BS, mRNA</td>
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<td>(Potapova et al. 2008)</td>
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<tr>
<td><strong>Mutation</strong></td>
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<td>4/92*</td>
<td>-</td>
<td>PTT, M</td>
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<td><strong>FANCC</strong></td>
<td>Pancreatic$</td>
<td>2/33^</td>
<td>-</td>
<td>M</td>
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<tr>
<td><strong>FANCG</strong></td>
<td>Pancreatic$</td>
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<td>-</td>
<td>M</td>
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<td><strong>BRCA2</strong></td>
<td>Ovarian</td>
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<td>-</td>
<td>mRNA</td>
<td>(Zikan et al. 2007)</td>
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<tr>
<td><strong>BRCA2</strong></td>
<td>Ovarian</td>
<td>12/92*</td>
<td>-</td>
<td>mRNA, MSP</td>
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* Except for the study by Hilton et al, all studies were done on patients without family history of cancer.
# In the studies indicated in Italics only cell lines were used.
^FANCF down regulation was found in three other cell lines without evidence for promoter methylation; all 6 cell lines were hypersensitive to MMC.
*Cohort is composed of familial and sporadic cases (61% based on negative family history). In 12 cases, BRCA2 mRNA was not detectable, of which one case showed BRCA2 promoter methylation.
$ 11 unselected pancreatic cell lines and a selection of 22 tumors was made that showed loss of heterozygosity at 9q22.3 or 9p13 for mutation screening of FANCC or FANCG, respectively. Mutations were found in young-onset pancreatic cancer cases.
*Among the two FANCC mutations, c.839 A>T, p.D195V has previously been demonstrated to be non-pathogenic (Lo Ten Foe et al. 1996a).

Abbreviations: BS, bisulphite sequencing. M, DNA sequencing. MSP, methylation specific PCR. mRNA, undetectable messenger RNA. PE, protein undetectable by Western blotting. PTT, protein truncating test. HNSCC, squamous cell carcinoma of the head and neck region. NSCLC, non-small cell lung cancer.
Outline of the thesis

The overall aim of this thesis is to examine the possible occurrence and significance of FA/BRCA pathway defects in sporadic cancer. Part of the work has focused on expanding our knowledge of the pathway through diagnostic mutation detection and gene identification procedures. In Chapter 2 a comprehensive mutation screening approach, in combination with functional assays, was used to determine the molecular defects in a group of 80 unselected FA cases. This study resulted in the identification of unclassifiable patients who were likely to represent new complementation groups, with defects in both the upstream and downstream parts of the FA/BRCA pathway. In one of the latter patients the gene could be identified as \textit{PALB2/FANCN}, as described in Chapter 3, which extended the number of genes acting in the pathway to thirteen. Examination of large genetic rearrangements of \textit{PALB2/FANCN} in familial breast cancer was performed in Chapter 4, but did not reveal the involvement of such aberrations in breast cancer predisposition. To explore the occurrence of FA pathway defects in sporadic cancer, the types of malignancy that are most commonly observed in FA patients were examined: leukemia and squamous cell carcinoma (SCC). Evidence for FA pathway inactivation by gene silencing was observed in AML (Chapter 5) and ALL (Chapter 6), albeit in a minority of the cases examined. In contrast to previous studies no \textit{FANCF} promoter methylation was observed in a panel of head-and-neck SCC. A possible explanation for this discrepancy is explored in Chapter 7. In Chapter 8 various types of SCCs were screened for FA protein defects by various procedures, which led to the discovery of deficiencies in BRCA1 and -2 protein, again in a minority of the cases. The strength of our approach was that we used cell lines instead of primary tumor material (in contrast to most published studies), which enabled us to carry out functional experiments to confirm results from the DNA and expression studies. In Chapter 9 the results of our experiments are discussed from the perspective of their potential utility in the personalized treatment of cancer patients.
References


General introduction


General introduction


Chapter 2

Genetic subtyping of Fanconi anemia by comprehensive mutation screening

Najim Ameziane
Abdellatif Errami
France Léveillé
Chantal Fontaine
Yne Waterham
Rosalina M.L. van Spaendonk
Johan P. de Winter
Gerard Pals
Hans Joenje

Human mutation
This chapter was published in slightly modified form
Abstract

Fanconi anemia (FA) is a recessively inherited syndrome with predisposition to bone marrow failure and malignancies. Hypersensitivity to cross-linking agents is a cellular feature used to confirm the diagnosis. The mode of inheritance is autosomal recessive (12 subtypes) as well as X-linked (1 subtype). Most genetic subtypes have initially been defined as ‘complementation groups’ by cell fusion studies. Here we report a comprehensive genetic subtyping approach for FA that is primarily based on mutation screening, supplemented by protein expression analysis and by functional assays to test for pathogenicity of unclassified variants. Of 80 FA cases analyzed, 73 (91%) were successfully subtyped. In total, 92 distinct mutations were detected, of which 56 were novel (40 in \textit{FANCA}, 8 in \textit{FANCC}, 2 in \textit{FANCD1}, 3 in \textit{FANCE}, 1 in \textit{FANCF}, and 3 in \textit{FANCG}). All known complementation groups were represented, except D2, J, L, and M. Three patients could not be classified because proliferating cell cultures from the probands were lacking. In cell lines from the remaining 4 patients immunoblotting was used to determine their capacity to monoubiquitinate FANCD2. In one case FANCD2 monoubiquitination was normal, indicating a defect downstream. In the remaining 3 cases monoubiquitination was not detectable, indicating a defect upstream. In the latter 4 patients, pathogenic mutations in a known FA gene may have been missed, or these patients might represent novel genetic subtypes. We conclude that direct mutation screening allows a molecular diagnosis of FA in the vast majority of patients, even in cases where growing cells from affected individuals are unavailable. Proliferating cell lines are required in a minority (<15%) of the patients, to allow testing for FANCD2 ubiquitination status and sequencing of \textit{FANCD2} using cDNA, to avoid interference from pseudogenes.
**Introduction**

Fanconi anemia (FA, MIM# 227650) is an inherited bone marrow failure and cancer predisposition disorder with both autosomal recessive and X-linked modes of inheritance (Taniguchi & D'Andrea 2006; Levitus et al. 2006). Since clinical manifestations of FA are diverse and variable and since FA patients require special treatment, clinical suspicion should be confirmed at the cellular level, e.g. by a cross-linking agent-induced chromosomal breakage test. Thirteen complementation groups (genetic subtypes) have been distinguished (A, MIM#607139; B, MIM#300515; C, MIM#227645; D1, MIM#605724; D2, MIM#227646; E, MIM#600901; F, MIM#603467; G, MIM#602956; I, MIM#609053; J, MIM#605882; L, MIM#608111; M, MIM#609644; N, MIM#610355). The largest complementation group is A (65%), and together with FA-B, -C, -E, -F, and –G accounts for over 90% of all FA cases (Levitus et al. 2006).

Most FA proteins (FANCA, -B, -C, -E, -F, -G, -L, and –M) assemble into a multiprotein complex that activates the small form of FANCD2 (FANCD2-S) into the mono-ubiquitinated larger form (FANCD2-L). The recently identified FANCI/KIAA1794 protein is also monoubiquitinated and appears to form a complex with FANCD2 (Xia et al. 2007; Sims et al. 2007; Dorsman et al. 2007; Smogorzewska et al. 2007). BRCA2/FANCD1 (Howlett et al. 2002), BRIP1/FANCJ (Levitus et al. 2005), and PALB2/FANCN (Xia et al. 2007; Reid et al. 2007) are so far the only known components of the pathway that function downstream of the FANCD2 modification step. Although a positive cytogenetic test result is highly indicative for FA, molecular analysis is still required to demonstrate pathogenic mutations in a FA gene. This is essential for adequate clinical management, as this establishes the mode of inheritance, helps to assess prognosis and allows to exclude diseases with overlapping clinical symptoms.

Retrovirus-mediated complementation of the cellular defect is an established method to classify FA patients according to genetic subtype, as a prelude for mutation analysis (Chandra et al. 2005; Casado AJ. et al. 2007). A prerequisite for such an assay is that growing, cross-linker sensitive, cells from the patient are available. Since these may not always be obtainable, the applicability of complementation tests
for genetic subtyping is limited. In addition, for some of the subtypes, such as B, D1, L, M, and N, complementing constructs are not yet available, leaving patients of these subtypes functionally unclassifiable. For such patients it is unclear whether they belong to one of the rarer known groups or represent a novel subtype. To avoid some of these drawbacks we developed a comprehensive classification strategy primarily based on direct mutation screening. Here we evaluate the results obtained from 80 FA patients.
Results and discussion

The mutation screening strategy adopted for FA subtyping involved the following steps (Figure 1). (1) Screening of \textit{FANCA} for deletions by MLPA, in parallel with sequencing the entire \textit{FANCA} coding region; (2) – in case of a family with only male patient(s) – mutation screening of \textit{FANCB} by MLPA and direct sequencing; (3) Screening of \textit{FANCC}, \textit{-E, -F, and –G}, simultaneously, by DHPLC and sequencing of aberrant fragments. Up to this point a (provisional) molecular diagnosis was obtained for >85% of the patients. For the remaining patients, further analysis was carried out provided that growing cells from the proband were available. Western blotting was used to visualize whether two FANCD2 isoforms were present at normal levels. [In cases where both FANCD2-S and -L bands appear to be absent or very weak, FANCD2 should be sequenced, from cDNA; however, no such cases were encountered in the patient group reported here.] When only the short (non-ubiquitinated) isoform of FANCD2 was present, \textit{FANCI}, \textit{FANCL}, and \textit{FANCM} were sequenced. If no mutations were detected, this was taken as evidence for a novel subtype with a defect upstream in the FA pathway. When both FANCD2 isoforms were present at normal levels, \textit{BRCA2/FANCD1} was screened by MLPA, denaturing gradient gel electrophoresis (DGGE), and sequencing. If negative, \textit{BRIP1/FANCJ} and \textit{PALB2/FANCN} were sequenced. If negative again, the patient was considered to be mutated in a novel FA gene acting downstream of FANCD2 ubiquitination, to be confirmed by additional research (Figure 1).

As summarized in Table 1 and Figure 2, detection of sequence alterations allowed the classification of 73 patients (91%) as belonging to one of the following 8 groups: A (48), B (2), C (9), D1 (2), E (3), F (1), G (6), I (1) and N (1) (Figure 3). In the autosomal FA genes biallelic mutations were detected, except in \textit{FANCA} (4x), \textit{FANCD1} (1x), \textit{FANCE} (1x), \textit{FANCF} (1x), and \textit{FANCI} (1x), where extensive additional efforts would be required to identify the second mutation. Out of the 93 distinct sequence alterations found, 56 (60%) have not been reported before.
Figure 1. Scheme for the molecular diagnosis of patients who tested positive for FA in a chromosomal breakage test. For the majority of FA patients (86%) a diagnosis is reached based on the analysis of genomic DNA from the patients themselves or their parents (above dotted line). For the remaining patients proliferating cell cultures are needed to allow for Western blot analysis of FANCD2 and sequencing of FANCD2 at the level of cDNA. *, FANCB is sequenced if the patient is a male. #, FANCD2 transcript is sequenced to detect splice aberrations.
Table 1. Results from screening 80 FA patients for sequence variants in the 13 known FA genes

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<th>Mutation 2</th>
<th>Effect 1</th>
<th>Effect 2</th>
<th>Reference</th>
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Genetic subtyping of Fanconi anemia
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*a Description of the mutations is based on cDNA sequence, +1 corresponding to the A of the ATG translation initiation codon of the reference sequence. NF, mutation not found; N, new mutation, not reported before; A, altered splicing of pre-mRNA; B, micro-deletion/insertion, out-of-frame; b, micro-deletion/insertion, in-frame; D, exon(s) deletion, out-of-frame; d, exon(s) absent by MLPA, breakpoints unknown. Some of the novel missense mutations were functionally tested and found pathogenic (boldface); the status of the two underlined variants was uncertain, because some functional activity was present in a transfection assay, suggesting that these mutations might be hypomorphs.

Figure 2. Mutations in FANC-A, -B, -C, -D1, -E, -F, -G, -I, and -N. The open reading frames with their exon structures are drawn roughly to scale. Most mutations occur in FANCA, a large proportion of which are deletions (horizontal bars), as detected by MLPA. Polymorphic variants are not indicated.
Most novel mutations were considered pathogenic because of their severity, such as frameshifts and deletions. For missense mutations the pathogenic consequences are often uncertain. Some of these unclassified variants were tested for pathogenicity by assessing their ability to complement the cellular FA defect in a deficient cell line. Of 6 FANCA missense variants tested, 4 failed to completely restore MMC resistance and were thus considered pathogenic. Two variants (c.3746T>C / p.L1249P and c.4199G>A / p.R1400H in FANCA) exhibited a level of activity similar to wild type, suggesting non-pathogenicity of these mutations. However, we cannot exclude that these mutations are hypomorphic and that overexpression has compensated for potential instability of the mutant protein.

**Figure 3.** Genetic subtyping of 80 unselected FA patients. Seventy three patients were assigned to 9 subtypes: A: 58; B: 2; C: 9; D1: 2; E: 3; F: 1; G: 6; I: 1; N: 1. In this cohort no patients were found in groups D2, J, L, and M. Of the remaining 7 patients, 3 were unclassifiable because available material was unsuitable for further analysis. Cell lines from the remaining four patients were analyzed for the presence of monoubiquitinated FANCD2, which was detected in only one case. The latter 4 patients might represent novel complementation group(s).

In 7 patients we failed to find relevant sequence alterations in any of the known FA genes. For 3 of these, only genomic DNA was available, so that analysis at the
protein level was not possible. These patients could thus not be classified. From the remaining 4 patients cultured cells were used to examine their FANCD2 ubiquitination status by Western blotting. Three patients expressed only non-ubiquitinated FANCD2, suggesting a defect upstream in the FA/BRCA pathway. These patients may thus represent (a) novel complementation group(s). The remaining patient expressed both FANCD2 isoforms, indicating a defect downstream. No sequence alterations in FANCD1, -J, or -N were found, suggesting the possible existence of a novel FA protein that functions downstream in the FA/BRCA pathway. However, the possibility that pathogenic mutations in a known FA gene were missed can not be excluded completely. The ultimate diagnosis of the four unclassified patients therefore remains uncertain until pathogenic mutations have been demonstrated in their disease-causing genes.

The mutation screening strategy presented here proved to be efficient in the molecular diagnosis of FA patients. Of the 73 successfully diagnosed patients 69 (94%) were classified by analysis of genomic DNA from the patients. Six of the variants identified (6%) required functional testing to assess their pathogenicity. In seven patients mutations were found in only one allele (3 in FANCA, 1 in FANCD1, 1 in FANCE, 1 in FANCF, and 1 in FANCI). Mutations on the second allele had either been missed or were absent due to reverse mosaicism, a relatively common event in FA (Waisfisz et al. 1999). For these cases complementation testing is required to confirm whether the identified sequence variants reside in the disease-causing genes.

Complementation group FA-A is by far the largest group, accounting for about 65% of the FA cases (Faivre et al. 2000). Because of the high frequency of large deletions encountered in FANCA (Morgan et al. 1999) deletion screening by MLPA as an initial step in the mutation screening approach is highly efficient, as this allows the classification of approximately 25% of unselected FA patients. MLPA did not only detect large deletions but also detected some micro-deletions, such as the relatively common micro-deletion c.3788-3790delTCT, which is observed in approximately 5% of the all FA-A patients (Levran et al. 1997). A novel micro-deletion c.3021-3027del7 as well as the previously described mutation c.4069-4082del15 (Levran et al. 2005) were also detected. Moreover, the missense mutation c.2807A>G (p.E936Q) apparently interfered with the amplification of the exon 29-specific probe and was thus detected as an aberration by MLPA. Further examination of this substitution on
the cDNA level showed aberrant splicing, resulting in skipping of exon 29. Examination of cDNA is sometimes essential to determine the pathogenicity of certain variants. For example, c.2632G>C in FANCA has been reported as nonpathogenic (Wijker et al. 1999). However, cDNA analysis demonstrated a 33-bp deletion of exon 28 sequence, so that this mutation is probably pathogenic by causing aberrant splicing.

We conclude that our comprehensive mutation screening approach offers a highly effective method for the ultimate diagnosis of the great majority of FA patients. In a minority of cases functional tests, such as retroviral complementation or transfection, are required to help assess the pathogenic status of novel unclassified missense variants. With an ever expanding database of already characterized sequence variants the requirement for additional functional tests will gradually diminish.

Materials and Methods

Patients, cells, and DNA. Patients were referred for mutation screening, after the FA diagnosis had been established by a chromosomal breakage test. Genomic DNA was obtained from blood, skin, B lymphoblastoid cell lines (LCLs), or fibroblasts, according to standard protocols. Where applicable, cDNA was synthesized from total RNA isolated from cycloheximide-treated cells using Superscript™ II RNase H reverse transcriptase (Invitrogen), and Oligo dT priming (Roche) according to standard protocols. This study was approved by the VUmc Institutional Review Board.

MLPA Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was used to detect deletions or amplifications in the FANCA, FANCB, FANCD1, and FANCN genes. MLPA is a rapid quantitative method for the detection of deletion/amplification of up to 40 specific DNA fragments in a single PCR reaction (Schouten et al. 2002). The MLPA kits, developed in collaboration with the manufacturer (MRC-Holland BV, Amsterdam) detect aberrations in FANCA, FANCB, FANCD1, and FANCN; details are available at http://www.mrc-holland.com. Briefly, target DNA was denatured for 5 min at 98 °C, 3 µl of the probe mix was added, after which the mixture was heated for
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1 min at 98 °C and incubated at 60 °C overnight (16 hr); after addition of ligase the mixture was incubated at 54 °C for 15 min. Ligase was subsequently inactivated at 98 °C for 5 min. Next, 10 µl was transferred to PCR mix containing PCR buffer, deoxynucleotide triphosphates, Taq polymerase, and one unlabeled and one carboxyfluorescein-labeled PCR primer, which are complementary to the universal primer sequence. The PCR reaction was carried out for 33 cycles (30 s at 95 °C, 30 s at 60 °C, and 60 s at 72 °C). The fragments were analyzed on an ABI model 310 capillary sequencer (Applied Biosystems) using genescan-TAMRA 500 size standard (Applied Biosystems). Description of the deletions was limited to the affected exons. No attempts were made to determine the exact breakpoints of deletions at the genomic DNA level.

**PCR.** Genomic fragments representing all exons of FANCA, –B, –C, –E, –F, –G, and –J, (GenBank accession numbers: NM_000135.2, NM_152633.2, NM_000136.2, NM_021922.2, NM_022725.2, NM_004629.1, NM_032043.1, respectively) were amplified by PCR. Primers were designed to include flanking intronic regions and to generate amplicons ranging between 200 and 450 bp, ensuring optimal results when analyzed by Denaturing High-Performance Liquid Chromatography (dHPLC).

FANCD1, -D2, -I, -L, and –M were analyzed from cDNA, in cases where a cell line was available (GenBank accession numbers: NM_000059.3, NM_033084.3, EF469766.1, NM_018062.2, NM_020937.1, respectively). Experimental details are available upon request.

**Mutation screening by DHPLC.** PCR products were separated, after a denaturation at 98 °C and a gradual renaturation at a final temperature of 37 °C, by DHPLC on the WAVE HT system (Transgenomics, USA) using an acetonitrile gradient (Rischewski & Schneppenheim 2001). Analysis was at three column temperatures according to predictions by the Wavemaker software (Transgenomics, USA).

**Sequencing and description of mutations.** Independent PCR fragments, derived from genomic DNA or cDNA, were sequenced using the BigDye Terminator Cycle Sequencing V3.1 Ready Reaction kit (Applied Biosystems), and analyzed on an ABI PRISM 3100 Genetic Analyzer. The same primer sets were used for PCR and sequencing. With the exception of large deletions (see MLPA) all sequence variants
were described according to current nomenclature rules (http://www.hgvs.org/mutnomen) using the following GenBank reference sequences: FANCA, NM_000135.2; FANCB, NM_152633.2; FANCC, NM_000136.2; FANCD1, NM_000059.2; FANCE, NM_021922.2; FANCF, NM_022725.2; FANCG, NM_004629.1; FANCI, NM_018193.2; FANCN, NM_024675.3.

Construction of mutants. The complete wild type cDNAs of Flag-tagged FANCA and HA-tagged FANCE were cloned into the episomal expression vector pMEP4 (Invitrogen). The FANCA mutations, Leu210Arg and Leu274Pro were generated by site-directed mutagenesis using the QuickChangeII mutagenesis kit (Clontech) on a KpnI – KpnI fragment from pMEP4 FANCA-Flag subcloned into pBluescript. The primers used for Leu210Arg were: forward, 5' CTGTGGGATCGTGGCGCTTCAGGAATCTGTG 3', reverse, 3' GACACCTAGACCCGAAGTCTCCTAGACATCTGTG 5'. The primers used for Leu274Pro were: forward, 5' GTACTGCAGAGGATGCCGATTTTTGCACTTGACGC 3', reverse 3' CATGACGTCTCCTACGGCTAAAAACGTGAACTGCG 5'. After mutagenesis and sequencing, the mutant KpnI – KpnI fragment was cloned into pMEP4 FANCA-Flag. The FANCA mutations, Leu660Pro and Glu869Pro were generated by site-directed mutagenesis using the QuickChangeII mutagenesis kit on a KpnI – SalI fragment from pMEP4 FANCA-Flag subcloned into pBluescript. The primers used for Leu660Pro were: forward, 5' GCACTGGGAGAGCCTAGAGCCTCCATGACAG 3', reverse 3' CGTGACCCTCTCAGGCCTAAAAACGTGAACTGCG 5'. The primers used for Glu869Pro were: forward, 5' CTCCAGGGCTTATATTAAAAAGTTTTCCGTCTCAGTTCGAAGTTCG 3', reverse 3' GAGGTCCTAAATAATTTTTCTAAAGGCAAGGGTACCAAGTTCG 5'. After mutagenesis and sequencing the mutant KpnI – SalI fragment was extended at the 5’ and 3’ ends with wild type FANCA sequence and cloned into pMEP4 to generate mutant FANCA-Flag constructs. The FANCA mutations Leu1249Pro, Pro1324Leu, and Arg1400His were obtained by digestion of cDNA derived from patient cell lines with SalI and HindIII and subsequently cloned in pBluescript. The 5’ ends of the inserts were then extended with a SalI - HindIII fragment from pDR FANCA-Flag. The construct was digested with KpnI – HindIII and cloned in pMEP4 FANCA-Flag. Mutated insert cDNAs were verified by DNA sequencing.
**Functional tests.** Some missense variants were tested for pathogenic status by transfection, essentially as described (Lo *et al.* 1996a). Briefly, lymphoblastoid cell lines derived from FA patients lacking functional FANCA protein were used for functional complementation to assess the pathogenicity of mutations in FANCA. Cells were cultured at 37 °C in RPMI1640 medium (Gibco) containing 10% Fetal Bovine Serum (FBS, Gibco) and supplemented with 200 µg/ml hygromycin, for transfected cells. A suspension of 5 million cells in 0.8 µl serum-free RPMI1640 were transfected by electroporation using BTX’s Electro Square Porator ECM830 at 900V with a pulse length of 333 µs (3 pulses with an interval of 100 ms). The cells were then transferred to flasks containing 8 ml RPMI1640 with 10% FBS and cultured for 2 days before they were cultured on selection medium.

**Western blotting.** The antibodies used for detection of FANCD2 were affinity-purified rabbit polyclonal antibodies raised against FANCD2 mouse monoclonal antibody (FI17, Santa Cruz Biotechnologies, Santa Cruz, California, USA). For direct Western blot, cells were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, supplemented with proteinase inhibitors aprotinin, pepstatin, leupeptin and pefablock). Protein lysates corresponding to 500,000 cells or the indicated amounts of protein, as determined with a Bio-Rad (Hercules, CA) protein assay, were denatured at 70 °C and loaded on a 3-8% NuPAGE Tris-Acetate gradient gel (Invitrogen, Carlsbad, California, USA). After electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, Massachusetts, USA). The membrane was blocked with 5% fat-free milk in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and incubated with rabbit polyclonal or mouse monoclonal antibodies against FANCD2 (1:500) overnight. To visualize FANCD2, blots were incubated with horseradish peroxidase-labelled secondary antibodies (Dako Diagnostics, Glostrup, Denmark) and detected using the ECL Western blot analysis system (Amersham Pharmacia Biotech, UK). Detection of other FA proteins by direct Western blot has been described elsewhere (de Winter *et al.* 2000).

**Cell fusion.** Polyethylene glycol (PEG)-induced fusions were carried out as described (Joenje *et al.* 1995), using as fusion partners the hygromycin (H) or G418/neomycin.
(N) marked cell line EUFA592, representing the complementation group I. The cell fusion procedure entailed mixing \( 2 \times 10^7 \) lymphoblasts from each fusion partner. After centrifugation (250 \( \times \) g for 5 minutes) and resuspension in serum-free medium, the cells were divided into 4 equal portions for 4 parallel fusion experiments. After centrifugation, cell fusion was induced by gentle resuspension of the cells in 1 ml of a solution containing 50\% PEG (type 1000; Merck, Darmstadt, Germany) in serum-free medium. After 1 minute, the samples were carefully mixed with 4 ml of serum-free medium, followed 2 minutes later by another 4 ml of serum-free medium, after which the cells were left at room temperature for 20 minutes. The cells were then washed once by centrifugation at 111 \( \times \) g and resuspended in 10 ml of complete medium. After recovery at 37 °C for 48 hours, selection medium was added. In successful experiments outgrowth of the hybrids typically occurred after 2-5 weeks. Success rates were typically 80\%-90\%.

*Mitomycin C-induced growth inhibition test.* The growth-inhibiting effect of mitomycin C (MMC) was assessed by growing the transfected cells in the presence of various concentrations of MMC over a period of time that allowed cells without addition of drug to undergo at least three population doublings, as described previously (Joenje et al. 1995). IC\(_{50}\) values are defined as the concentration of MMC causing 50\% inhibition of growth.

**Acknowledgement**

We are indebted to Martin Rooimans, Jürgen Steltenpool, and Ron van Schooten for excellent technical assistance, and Josephine Dorsman for critically reading the manuscript.
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References


Chapter 3

Fanconi anemia is associated with a defect in the BRCA2 partner PALB2

Bing Xia*
Josephine C Dorsman*
Najim Ameziane
Yne de Vries
Martin A Rooimans
Qing Sheng
Gerard Pals
Abdellatif Errami
Eliane Gluckman
Julian Llera
Weidong Wang
David M Livingston
Hans Joenje
Johan P de Winter

* Authors contributed equally to this work

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Abstract

The Fanconi anemia and BRCA networks are considered interconnected, as BRCA2 gene defects have been discovered in individuals with Fanconi anemia subtype D1. Here we show that a defect in the BRCA2-interacting protein PALB2 is associated with Fanconi anemia in an individual with a new subtype. PALB2-deficient cells showed hypersensitivity to cross-linking agents and lacked chromatin-bound BRCA2; these defects were corrected upon ectopic expression of PALB2 or by spontaneous reversion.
Fanconi anemia is associated with a defect in *PALB2*

**Introduction**

Fanconi anemia is a recessive chromosomal instability syndrome with both autosomal and X-linked inheritance. Clinical features are diverse and include progressive bone marrow dysfunction and an extremely elevated cancer risk. Cells derived from individuals with Fanconi anemia are hypersensitive to growth inhibition and chromosomal breakage induced by DNA cross-linking agents such as mitomycin C (MMC) and diepoxybutane. Eleven distinct genes have been identified that, when defective, can cause Fanconi anemia, and all Fanconi anemia gene products are thought to function in a common network of genome maintenance (Taniguchi & D'Andrea 2006; Levitus *et al.* 2006). At least eight Fanconi anemia proteins form a nuclear ‘core complex’ that catalyzes the activation of the Fanconi anemia D2 protein (FANCD2) by monoubiquitination. BRCA2 (also known as FANCD1) and BRIP1 (also known as FANCJ) function downstream and/or independently of this modification step (Howlett *et al.* 2002; Levitus *et al.* 2005).

This study began with a search for a gene defect in an unclassified individual with Fanconi anemia, EUFA1341, who showed normal monoubiquitination of FANCD2 and in whom we did not detect pathogenic sequence alterations in BRCA2 and FANCJ. As no further Fanconi anemia subtypes are known to operate downstream of FANCD2 activation, we suspected this individual of representing a new Fanconi anemia complementation group, designated subtype N. PALB2 (partner and localizer of BRCA2) is a recently discovered protein that interacts with BRCA2 and is important in determining the localization and stability of BRCA2 in the nucleus (Xia *et al.* 2006). HeLa cells in which PALB2 expression is reduced by short interfering RNA show an increased sensitivity to MMC, a hallmark of Fanconi anemia (Xia *et al.* 2006). Therefore, we considered PALB2 a candidate for the protein defective in EUFA1341.
Results and discussion

As an initial test for a possible PALB2 defect, we visualized PALB2 by protein blotting using antibodies raised against a central region of PALB2 (amino acids 601–880). In contrast to cells from individuals with Fanconi anemia subtype D1 or subtype I and in contrast to control cells, we did not detect full-length PALB2 in EUFA1341 lymphoblasts and fibroblasts (Figure 1a). The amount of BRCA2 in EUFA1341 lymphoblasts was much lower than in control cells (Figure 1b), consistent with the previous observation that PALB2 promotes BRCA2 stability in the nucleus (Xia et al. 2006). The lack of full-length PALB2 protein and the reduced amount of BRCA2 suggested the existence of sequence alterations in the gene encoding PALB2. Notably, in a phenotypically reverted (MMC-resistant) subline of EUFA1341 lymphoblasts (designated ‘EUFA1341(R)’), we found a normal amount of BRCA2 without the reappearance of full-length PALB2 (Figure 1b).
Fanconi anemia is associated with a defect in PALB2

Figure 1 Phenotype of PALB2-deficient cells from an individual with Fanconi anemia is corrected by ectopic expression of PALB2. (a) Immunoblotting with an antibody against residues 601-880 of PALB2 demonstrated the absence of full-length PALB2 from whole-cell extracts of lymphoblasts and fibroblasts of individual EUFA1341. (b) The amount of BRCA2 is low in EUFA1341 lymphoblasts and normal in reverted EUFA1341(R) lymphoblasts. (c) EUFA1341 fibroblasts stably transduced with empty pOZC vector or pOZC-PALB2 were analyzed for PALB2, BRCA2 and Rad51 expression by immunoblotting whole-cell extract (WCE). (d) The same cells as in c were fractionated into S100 (soluble, containing cytoplasmic and nucleoplasmic proteins) and P100 (pellet, containing chromatin, nuclear matrix and insoluble proteins). Unlike in WCE and S100, amounts of BRCA2 and Rad51
Four lines of evidence showed that a loss of PALB2 function caused Fanconi anemia in individual EUFA1341. First, although total BRCA2 was not reduced in EUFA1341 fibroblasts (Figure 1c), the protein was mislocalized, being grossly depleted from the nuclear pellet (P100) fraction (Figure 1d). Consequently, Rad51 focus formation induced by MMC treatment was impaired in these fibroblasts (Figure 1e). Second, introduction of wild-type PALB2 into these cells normalized (i) the association of BRCA2 with the chromatin/nuclear matrix fraction (Figure 1d), (ii) the ability to form Rad51 foci (Figure 1e) and (iii) the sensitivity to MMC (Figure 1f). Third, we found pathogenic mutations in the gene encoding PALB2 in EUFA1341. Sequencing of genomic DNA as well as cDNA from EUFA1341 uncovered an apparently homozygous or hemizygous nonsense mutation in exon 4, leading to the amino acid change Y551X (primers are listed in Table 1). As we detected this mutation only in the mother of this individual, EUFA1341 was probably compound heterozygous for a deletion on the paternal allele in the region of the exon 4 mutation. This was confirmed by multiplex ligation-dependent probe amplification (MLPA) analysis (Schouten et al. 2002) (Figure 2). Fourth, cDNA sequencing and MLPA analysis of the reverted cells uncovered a secondary sequence alteration that restored part of the PALB2 ORF and could explain recovery of PALB2 activity (Figure 2). The corrective alteration deleted the premature stop-containing exon 4 from the mRNA, resulting in an in-frame fusion of exons 3 and 5 (Figure 2a). Subsequent genomic sequencing showed a 5,962-bp deletion between Alu repeats in introns 3 and 4 of the maternal allele that was probably generated by spontaneous Alu-mediated recombination (Bazer & Deininger 2002). Although genetic reversion of somatic cells has been attributed to several distinct genetic mechanisms (Waisfisz et al. 1999), the above-noted results indicate that Alu-mediated recombination in cis can be added to...
Fanconi anemia is associated with a defect in PALB2

this list. Given the role of PALB2 in efficient homologous recombination (Xia et al. 2006), it remains to be determined whether Alu-mediated recombination is enhanced in PALB2-deficient cells (for example, by promoting single-strand annealing as a compensatory event for reduced homologous recombination).

Figure 2. Deletion screening by MLPA. The left three panels show the analysis of genomic DNA isolated from control, EUFA1341 and reverted EUFA1341(R) cells. The control probe (C) recognizing DNA sequences on 1p22 served as the reference to which the PALB2 peaks, indicated with numbers 2 to 6, were compared. In EUFA1341 cells, one copy of exons 2, 3, 4, 5 and 6 was lost, whereas in EUFA1341(R) cells, the second copy of exon 4 was also lost. The right two panels show the same analysis for genomic DNA from the mother and the father isolated from whole blood. The maternal alleles did not display deletions, as expected, whereas the paternal DNA displayed the loss of one copy of exons 2 to 6. The horizontal line indicates the height of the control peak and is included to facilitate the comparison with the PALB2 peaks. Arrows indicate lost exons. Extension of the MLPA kit with probes for the additional exons revealed that in EUFA1341 a deletion of exon 1 till 10 is present.
Figure 2. PALB2 protein analysis in EUFA1341 and EUFA1341(R) lymphoblasts. (a) We detected a premature stop (1802T-A, Y551X) in the cDNA and genomic DNA from EUFA1341 and found an in-frame deletion of exon 4 in cDNA from EUFA1341(R). This cDNA encodes a variant of PALB2 (PALB2D4) in which residues 1–70 are fused to residues 562–1186. (b) Whole-cell extracts of HSC93 (wild-type), EUFA1341 and EUFA1341(R) lymphoblasts were immunoprecipitated with antibodies to PALB2 residues 1–200 (lanes 1–3 and 7–9) or 601–880 (lanes 4–6 and 10–12) and analyzed by protein blotting with antibodies against BRCA2 (lanes 1–12), PALB2 residues 1–200 (lanes 1–6) or 601–880 (lanes 7–12). Antibody a1–200 immunoprecipitated a protein ('X'; lane 2) in EUFA1341 cells that was detected when the same antibody was used for protein blotting, but BRCA2 was not coimmunoprecipitated. Both a1–200 and a601–880 immunoprecipitated a slightly larger protein ('Y'; lanes 3, 6, 9 and 12) in EUFA1341(R). This protein reacted with both antibodies in protein blots and coimmunoprecipitated BRCA2. In addition, a601–880 recognized protein 'Z' (lane 11) in EUFA1341 cells. (c) We loaded the same immunoprecipitates as in lanes 2 and 12 of b along with recombinant PALB2 (Y551X) or PALB2D4, generated in HEK293T cells. These recombinant proteins migrated at the same position as proteins X and Y. The
Fanconi anemia is associated with a defect in \textit{PALB2} identity of protein Z is unknown. (d) HEK293T cells were transfected with the indicated plasmids. Immunoprecipitation with anti-Flag M2 agarose beads showed that recombinant PALB2D4 efficiently binds BRCA2.

Further studies showed the existence of truncated PALB2 proteins in primary and reverted EUFA1341 cells. Using immunoprecipitation and protein blotting, we detected truncated PALB2 lacking residues encoded by exon 4 (protein ‘Y’) in EUFA1341(R) cells, whereas a protein comprising only the first 550 residues of PALB2 (protein ‘X’) was present in primary EUFA1341 cells (Figure 2b, c). In contrast to the truncated protein in the primary cell line, the truncated PALB2 protein from the reverted cells did interact with BRCA2 (Figure 2b, d), which provides an explanation of why BRCA2 levels were restored in these cells. This protein also re-established MMC-induced Rad51 focus formation (Figure 1e) and corrected the MMC-hypersensitive phenotype of EUFA1341 cells (Figure 1f). Our results show that a shortened PALB2 protein with a large internal deletion is functional, whereas the N-terminal 550 residues of PALB2 cannot function alone. These data link mutations in PALB2 to the new Fanconi anemia subtype N and justify FANCN as an alias for this gene. Like many other individuals with Fanconi anemia, individual EUFA1341 presented with skin, thumb, heart and kidney abnormalities and growth retardation (Methods). In addition, this individual developed anemia at a very early age and died at the age of 2 from an aggressive, rare type of endothelial cancer, a kaposiform hemangioendothelioma (Mueller & Mulliken 1999; Chang 2003). Similar to Fanconi anemia associated with biallelic mutations in BRCA2, Fanconi anemia caused by mutations in PALB2 might represent an extreme variant of this disorder, with respect to the severity of the clinical phenotype, time of anemia onset and cancer susceptibility (Alter \textit{et al.} 2007). As PALB2 is critical for the function of BRCA2 in DNA repair and tumor suppression (Xia \textit{et al.} 2006), it could, in principle, also be a tumor suppressor protein. Several family members of individual EUFA1341 indeed developed tumors, and some of these tumors fall into the recently proposed BRCA2 tumor spectrum (Figure 3) (Lorenzo & Hemminki 2004; Friedenson 2005). Further studies are necessary to assess the potential role of PALB2 mutations in sporadic and familial (childhood) cancer in non–Fanconi anemia populations.
Figure 3. Pedigree of patient EUFA1341 and cancer cases in the family (including age at diagnosis) Individuals II:3 and III:2 are carriers for the non-sense mutation Y551X, individuals II:2, III:1 and IV:2 are carriers for the large genomic deletion. Material from individuals I:1, I:2, I:3, I:4, I:5, I:6, I:7, I:8 and II:1 was not available.
Fanconi anemia is associated with a defect in PALB2

Materials and methods

Affected individual EUFA1341. The female patient was born after an uneventful pregnancy (40 wks), with low birth weight (1820 g; 27 days of incubator) and a length of 42 cm. Congenital abnormalities: microcephaly (head circumference 28.5 cm), hypertelorism, short neck, heart defect, long bone-less thumb on the right hand, hypoplastic thumb on the left hand, imperforate anus, ectopic right kidney, café-au-lait spots, epicanthus. The patient developed aplastic anemia at 2 and died at 2 years and 4 months from a kaposiform hemangioendothelioma in the left retroocular cavity that had invaded the brain. Cytogenetic investigation of stimulated T-lymphocytes revealed a normal 46, XX karyotype. Spontaneous chromatid breaks were observed in approximately 50% of the cells (0.9 breaks per cell, on average). Breakage was increased by adding diepoxybutane or mitomycin C at 100 ng/ml. This resulted in >10 break events per cell in 100% of the cells examined, thereby confirming an FA diagnosis.

Cell lines. All Epstein Barr virus-immortalized B cell lines from blood samples and SV40-transformed fibroblasts from skin biopsies were established according to standard procedures. The research was carried out after approval by the Institutional Review board of the VU Medical Center that adhered to local ethical standards, and was initiated only after the relevant informed consents had been obtained. Lymphoblastoid cells were propagated in RPMI supplemented with 10 % fetal calf serum (FCS), while fibroblasts were grown in a mixture of F10 (HAM) and Dulbecco's modified Eagle's medium (DMEM) (1:1) containing 10 % FCS. HEK293T cells were grown in DMEM supplemented with 10% FCS. To generate EUFA1341 cells stably expressing PALB2, PALB2 mutants or control cells, fibroblasts were infected with amphotropic pOZC-PALB2, pOZC-PALB2 (Y551X), pOZC-PALB2Δ4 or pOZC retrovirus. After 72 hours, cells were selected using paramagnetic M-450 Dynabeads (Dynal Biotech) coated with anti-IL-2 receptor antibody (Upstate, #05-170).

Plasmids and transient transfections. The retroviral PALB2 cDNA vector, pOZC-PALB2, has been described (Xia et al. 2006). To generate an expression vector encoding PALB2 (Y551X), codon 551 was replaced with a nonsense codon in pOZC-
PALB2 by site-directed mutagenesis using the QuickChange (Stratagene) method. To construct pOZC-PALB2Δ4, exon 4-deleted PALB2 cDNA was first obtained by PCR and then cloned into pOZC. By omitting or including a stop codon at the end of the PALB2 coding sequence (before the C-terminal FLAG-HA tags), both tagged and untagged versions were generated. Transient transfections were carried out in 6-well plates using FuGENE 6 (Roche) for 30 hours.

Western blotting and immunoprecipitation. Polyclonal PALB2 antibodies, α1-200 and α601-880, have been described (Xia et al. 2006). BRCA2 and Rad51 antibodies were purchased from Calbiochem (Ab-1, #OP95) and BD Pharmingen (#551922), respectively. Anti-α-tubulin, anti-FLAG M2 and M2-agarose beads were purchased from Sigma. For initial PALB2 detection (Figure 1), whole cell extracts of lymphoblasts and fibroblasts were prepared in lysis buffer (50 mM Tris-HCl, pH7.4, 450 mM NaCl, and 1% Triton X-100 supplemented with protease and phosphatase inhibitors) and the supernatants (each derived from 500,000 cells) were analysed by Western blotting. Cell fractionation was carried out as described (Xia et al. 2006). In all other experiments, cell extracts were prepared with NETN420 (Xia et al. 2006). Immunoprecipitation was performed overnight at 4ºC. Tris-Acetate (3-8%) gels (Figure 1) and Tris-Glycine (4-12%) gels (Figure 2) from Invitrogen were used for protein separation.

Immunofluorescence. Fibroblasts were treated with 2.4 µg/ml MMC for 1 hour, and the medium was then replaced. Cells were fixed 12 hr after MMC treatment, and Rad51 focus formation was analyzed by immunofluorescence using the aforementioned polyclonal Rad51 antibody (BD Pharmingen, #551922). Immunostaining was performed as described (Xia et al. 2006).

Mitomycin C sensitivity assay. Fibroblast lines were seeded at 1,000 cells per well in 96-well plates. Four hours later, MMC-containing medium was added to achieve the desired final MMC concentrations. Cells were incubated with the drug for 5 days, and their survival rates were measured using a CellTiter Glo kit (Promega) following the manufacturer’s instructions.
Fanconi anemia is associated with a defect in \textit{PALB2}

\textit{Multiplex ligation-dependent probe amplification (MLPA) analysis}. To detect deletions in exon 2, 3, 4, 5, and 6 of the PALB2 gene, synthetic oligonucleotide probes were designed according to standard procedures of MRC-Holland (http://www.mrc-holland.com) (Schouten et al. 2002; Stern et al. 2004). Sequences of the probes are available on request. Annealing and ligation of the probes were performed as described (Schouten et al. 2002). The ligation products were amplified by the addition of 5 µl of the aforementioned ligation mixture to 20 µl of PCR mixture containing PCR buffer, dNTPs, SALSA polymerase and PCR primers (one unlabeled and one D4-labeled) at 60°C as described (Schouten et al. 2002). PCR products were analyzed in an ABI PRISM 310 fragment analyzer (Applied Biosystems), and the data were analyzed using GeneScan analysis software Version 3.7 (Applied Biosystems).

\textit{Amplification of PALB2 sequences}. Total RNA was isolated from cycloheximide-treated lymphoblastoid cell lines using a total RNA isolation kit (Macherey-Nagel) followed by cDNA synthesis using 10 pmol oligo-dT20 primers and Superscript II RT polymerase (Invitrogen). Genomic DNA was isolated from whole blood or lymphoblastoid cell lines with a Blood mini kit (Qiagen). The PCR reactions for amplification of PALB2 cDNA and genomic fragments were carried out using Platinum Taq polymerase (Invitrogen). Sequences of the primers are depicted in Table 1. To determine the deletion in the reverted EUFA1341 cell line, long-range PCR was performed on genomic DNA using the Elongase system (Invitrogen). DNA sequence analysis. PCR fragments were treated with Shrimp Alkaline Phosphatase and Exonuclease I for 30 min at 37°C and 15 min at 80°C according to the manufacturer’s instructions (Amersham Biosciences). Sequencing reactions were carried out using 10 pmol of primer and the Big Dye terminator cycle sequencing kit (Applied Biosystems) in the Gene Amp PCR system 9700 (Applied Biosystems). Samples were analyzed in an ABI 3100 DNA analyzer (Applied Biosystems).
Table 1: Primers used for mutation screening

### Primers for PALB2 coding exons

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer Sequence 5’-3’</th>
<th>Reverse</th>
<th>Amplicon size</th>
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<tr>
<td>1</td>
<td>ACAGCGCGGCTCCTCTTTAG</td>
<td>ATACTGCTGCCCTCGGACTG</td>
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<tr>
<td>2+3</td>
<td>GTAGATTGTATGGAACAGTCTACT</td>
<td>GTCTATTGCTAGTCAATTATCTTCACAC</td>
<td>511</td>
</tr>
<tr>
<td>4A</td>
<td>TCTGCTCTGATGAAATGCTCACTGA</td>
<td>GTCTATTGCTAGAATGTCAACACC</td>
<td>645</td>
</tr>
<tr>
<td>4B</td>
<td>CAGATTCTCCAGAACCACTTTACAGA</td>
<td>TTTCTGCAAGAGAGGAGGGTT</td>
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</tr>
<tr>
<td>4C</td>
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</tr>
<tr>
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<tr>
<td>7</td>
<td>GCTCTTTCTTACCCTCATAAGA</td>
<td>TGGGTATATGGGTGCTCACTATACCA</td>
<td>492</td>
</tr>
<tr>
<td>8</td>
<td>CTTGTCACAGAATACAAAAGAGATGTGA</td>
<td>TAGGTATACCTGACTTTAAAACCA</td>
<td>270</td>
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<td>9</td>
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<td>TTIAATTGTTTTGATGATGATCTGAA</td>
<td>AAATTTATATGCCCATTTTGAGTTAT</td>
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### Primers for PALB2 cDNA

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<tr>
<th>Position*</th>
<th>Primer Sequence 5’-3’</th>
<th>Reverse</th>
<th>Amplicon size</th>
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<td>GGTCCACTGACCCTGTTGGGAA</td>
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<td>160-795</td>
<td>AACAAGATTTTGTTGTCAGGGAGG</td>
<td>GATGGTCTCAAGGCTGACTACTAC</td>
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<tr>
<td>671-1257</td>
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<td>GCCATATTGATGCGCTGGG</td>
<td>587</td>
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<td>1116-1797</td>
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<td>CCAATTGAAGGGCTGCTGGG</td>
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<tr>
<td>1620-2185</td>
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<td>GGGAAAGACGATGCAACCATG</td>
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</tr>
<tr>
<td>2071-2571</td>
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<td>CCAATTTGATGCGCTGGG</td>
<td>501</td>
</tr>
<tr>
<td>2431-3334</td>
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<td>CCAAGTACAGTGCTGTGCTTACATC</td>
<td>678</td>
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</table>

*Position relative to the translation start has been indicated
Fanconi anemia is associated with a defect in \textit{PALB2}

\section*{Acknowledgements}

We thank the family of individual EUFA1341 for contributing to this study, M. Makiya and C. de Leon Lucero for help in the collection of clinical data and J. Steltenpool and A. Oostra for expert technical assistance. The members of the Livingston laboratory are grateful to A. D’Andrea and his colleagues at the Dana Farber Cancer Institute for a number of useful discussions and for Fanconi anemia-related reagents. This study was financially supported by the Netherlands Organization for Health Research and Development (J.P.d.W. and N.A.); by grants, including a breast cancer SPORE, from the National Cancer Institute (D.M.L.); by the Breast Cancer Program of US Army Medical Research and Materiel Command (B.X.); by the Intramural Research Program of the National Institute on Aging, US National Institutes of Health (W.W.) and by the Fanconi Anemia Research Fund (H.J.)
Chapter 3

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Chapter 4

Lack of large genomic deletions/amplifications in \textit{BRIP1, PALB2}, and \textit{FANCD2} genes in \textit{BRCA1/2} negative familial breast cancer

Najim Ameziane
Ans M.W. van den Ouweland
Muriel Adank
Rijmond N.C.P. Vijzelaar
Abdellatif Errami
Josephine Dorsman
Hans Joenje
Hanne Meijers-Heijboer
Quinten Waisfisz

Manuscript in preparation
Abstract

Pathogenic sequence alterations in the Fanconi anemia genes BRIP1/FANCJ and PALB2/FANCN have been implicated in the predisposition to breast cancer. However, in most of the published studies genetic alterations such as large deletions/amplifications may have been missed by the sequencing methods used. We used multiplex ligation-dependent probe amplification (MLPA) to analyze 734 cases of non-BRCA1/2 familial breast cancer patients from the Dutch population for such genetic alterations in BRIP1/FANCJ, PALB2/FANCN, and FANCD2. No aberrations were detected, suggesting that in this population large deletion/amplification within these genes do not significantly contribute to breast cancer predisposition in familial cases.
Introduction

Breast cancer (BC) is the most common malignancy in women worldwide. It is estimated that 5-10% of all BCs may be caused by germline mutations in breast cancer susceptibility genes (Rahman & Scott 2007; Oldenburg et al. 2007). BRCA1 and BRCA2 are the major known BC susceptibility genes accounting for approximately 16% of the familial BC cases (Anglian Breast Cancer Study Group 2000). Other BC susceptibility genes include ATM (Renwick et al. 2006), PTEN (Lynch et al. 1997), CASP8 (Cox et al. 2007), CHEK2 (The CHEK2 Breast Cancer Case-Control Consortium 2004), LKB1/STK11 (Giardiello et al. 2000), TGFβ1 (Dunning et al. 2003) and TP53 (Malkin et al. 1990). However to date, the majority of familial BCs cannot be attributed to mutations in one of known susceptibility gene(s).

Fanconi anemia (FA) is a recessively inherited chromosomal instability syndrome with autosomal or X-linked mode of inheritance, and is characterized by an increased susceptibility to several forms of malignancies (Joenje & Patel 2001; Alter 2003). The disease is caused by mutations in one of the 13 genes so far identified. The FA gene products interact in a common pathway whereby most of the proteins (FANCA,-B,-C,-E,-F,-G,-L, and -M) form a multiprotein complex that is required for the monoubiquitination of FANCD2 and FANCI. However, this modification step is not influenced by FANCD1 (BRCA2), FANCJ (BRIP1), or FANCN (PALB2), and hence these proteins are thought to act downstream of this process. FANCD2 and FANCI form a protein complex (ID complex), which translocates to DNA damage sites where it co-localizes with the downstream FA proteins, BRCA2/FANCD1, BRIP1/FANCJ, and PALB2/FANCN and other proteins that are involved in the recognition and repair of DNA damage, such as BRCA1, ATM, NBS, and RAD51 (Wang 2007).

The discovery of the breast cancer susceptibility gene BRCA2 as the gene defective in the Fanconi anemia (FA)-D1 complementation group (Howlett et al. 2002), the identification of BRIP1 (BRCA1 Interacting Protein) (Bridge et al. 2005; Levran et al. 2005; Levitus et al. 2005) and PALB2 (Partner And Localizer of BRCA2) (Reid et al. 2007; Xia et al. 2007) as the genes responsible for the FA-J and FA-N complementation groups, respectively, triggered many to investigate whether heterozygous carriers for a mutation in one of the FA genes are predisposed to breast cancer. To date, only mutation in the “downstream” FA genes have been
found to significantly elevate the risk of developing breast cancer. Heterozygous mutations in \textit{BRIP1}/\textit{FANCJ} and \textit{PALB2}/\textit{FANCN} appear to increase the risk 2- and 2.3-fold, respectively (Seal \textit{et al.} 2006; Rahman \textit{et al.} 2007). Risk assessment has been based on sequence alteration screening analysis of these genes in familial breast cancer (FBC) cases lacking mutations in \textit{BRCA1}/\textit{2}. However, pathogenic germline mutations in \textit{BRIP1}/\textit{FANCJ} are very rare as exemplified by the observation that these were not detected in several studies using FBC cases from the Swedish (Luo \textit{et al.} 2002), Finnish (Karppinen \textit{et al.} 2003), North American (Rutter \textit{et al.} 2003), Australian (Lewis \textit{et al.} 2005), and (French-) Canadian (Guenard \textit{et al.} 2008) populations. In addition, mutations in \textit{PALB2}/\textit{FANCN} do not seem to contribute to BC risk in the population of Iceland (Gunnarsson \textit{et al.} 2008).

Furthermore, \textit{FANCD2} mutations, have been suggested to play a role in the development of breast cancer based on observations of Fancd2 knockout mice, which demonstrated a high incidence of epithelial tumors, including mammary and ovarian tumors (Houghtaling \textit{et al.} 2003). However, in humans a significant contribution of \textit{FANCD2} mutations to FBC could not be established (Seal \textit{et al.} 2003; Lewis \textit{et al.} 2005).

Interestingly, one of the eight FA-N patients described in literature carried a pathogenic nucleotide substitution on one allele and a large genomic deletion encompassing exon 1 through 10 of \textit{PALB2}/\textit{FANCN} on the other allele (figure 1). The latter aberration is not detectable by standard sequencing methods applied for \textit{PALB2}/\textit{FANCN} mutation screening in most studies published to date (Erkko \textit{et al.} 2007; Rahman \textit{et al.} 2007; Garcia \textit{et al.} 2008; Cao \textit{et al.} 2008; Gunnarsson \textit{et al.} 2008; Tischkowitz \textit{et al.} 2008). Hence, we hypothesized that heterozygous large deletion/amplification mutations in the downstream FA genes (\textit{FANCJ} and \textit{FANCN}) and \textit{FANCD2} may contribute to the risk of breast cancer in familial cases. In this study we tested this hypothesis by analyzing gDNA of a large cohort of non-\textit{BRCA1}/\textit{2} FBC patients for large genomic alterations in \textit{FANCD2}, \textit{BRIP1}/\textit{FANCJ}, and \textit{PALB2}/\textit{FANCN} using Multiplex Ligation-dependent Probe Amplification (MLPA).
Results and discussion

In the current study, we analyzed a total of 734 Dutch BRCA1/2 negative familial breast cancer (FBC) patients for large genomic deletions/amplifications in FANCD2, BRIP1/FANCJ, and PALB2/FANCN using MLPA. We failed to detect deletions or amplifications in any of these genes. Absence of PALB2/FANCN large deletions/amplifications was also demonstrated in two previous studies where 600 FBC cases of French-Canadian ancestry (Foulkes et al. 2007) and 61 index patients of Finnish breast and/or ovarian cancer families (Pylkas et al. 2008) were analyzed. The MLPA kits used for the analysis contained probes targeted against all the exons of BRIP1/FANCJ and PALB2/FANCN. Due to the presence of FANCD2 pseudogenes, only a number of FANCD2 exons could be analyzed (see materials and methods section) by MLPA.

Large deletions encompassing multiple exons will be detected by the used MLPA kits, but subtle deletions/amplifications in the intronic region and of FANCD2 exons of which no probes were present in the MLPA kit could have been missed. In addition, genetic rearrangements such as inversions are not detectable by MLPA. However, our results show that large genetic deletions/amplifications of multiple exons of the analyzed genes are unlikely to contribute significantly to the BC risk in the Dutch population.

As the only patient so far described with a large deletion in PALB2 is of Argentinian nationality, this mutation might be derived from a founder within this particular population and it might be of interest to test FBC cases from this population for this large deletion. Alternatively, large deletion mutations in PALB2 may be very rare and the analysis of an even larger cohort of FBCs may be necessary to determine the contribution of such aberrations to breast cancer risk.
**Figure 1.** MLPA analysis. A large deletion spanning exon 1 through 10 of the *PALB2* gene in the FA-N patient EUFA1341 was used as a positive control (Xia et al. 2007). Signals of capillary electrophoresis patterns of wild type DNA (light) and EUFA1341 DNA (dark) are depicted whereby the Probes detecting *PALB2* exons are indicated at the bottom. The arrows indicate the heterozygously deleted exons.
Materials and methods

Familial breast cancer patients

BRCA1/2 negative breast cancer (BC) patients were included in this study who met the criteria described previously (Verhoog et al. 2001). Briefly, women were selected for the study when a single patients within a family was affected by breast cancer before the age of 40, or if affected by breast and ovarian cancer (OC), or if two first- or second degree relatives were affected by BC (one of them diagnosed before the age of 45), or if two first- or second degree relatives of whom one affected by OC and the other affected by BC before the age of 50, or if two first- or second degree relatives were affected by BC before the age of 50, or if two first- or second degree relatives were affected by either BC or OC.

MLPA

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was used to detect deletions or amplifications in the \textit{FANCD2}, \textit{BRIP1/FANCJ}, and \textit{PALB2/FANCN} genes. MLPA is a rapid quantitative method for the detection of deletion/amplification of up to 40 specific DNA fragments in a single PCR reaction (Schouten et al. 2002). The SALSA MLPA kits, P057 and P240 (MRC-Holland BV, Amsterdam) were used for the detection of exon insertions/deletions in \textit{FANCD2} (exons: 1, 2, 4, 9, 12, 17, 19, 23, 28, 32, 35, 41, 43) and \textit{PALB2/FANCN} (all exons), and \textit{BRIP1/FANCJ} (all exons), respectively; details are available at http://www.mrc-holland.com. Briefly, target DNA was denatured for 5 min at 98 °C, 3 µl of the probe mix was added, after which the mixture was heated for 1 min at 98 °C and incubated at 60 °C overnight (16 hr); after addition of ligase, the mixture was incubated at 54 °C for 15 min. Ligase was subsequently inactivated at 98 °C for 5 min. Next, 10 µl was transferred to PCR mix containing PCR buffer, deoxynucleotide triphosphates, Taq polymerase, and one unlabeled and one carboxyfluorescein-labeled PCR primer, which are complementary to the universal primer sequence. The PCR reaction was carried out for 33 cycles (30 s at 95 °C, 30 s at 60 °C, and 60 s at 72 °C). The fragments were analyzed on an ABI model 3730 capillary sequencer (Applied Biosystems) using genescan-LIZ500 size standard (Applied Biosystems). GeneMarker 1.6 software (SoftGenetics) was used for fragment analysis.
DNA obtained from EUFA1341, containing a heterozygous deletion of exon 1 through exon 10 in *PALB2/FANCN* (Xia *et al.* 2007) was used as a positive control for the *PALB2/FANCN* MLPA analysis (Figure 1).
References


Lack of large genetic aberrations in BRIP1, PALB2, and FANCD2 in familial BC


Chapter 5

Bi-allelic silencing of the Fanconi anemia gene
FANCF in acute myeloid leukemia

Marc Tischkowitz
Najim Ameziane
Quinten Waisfisz
Johan P. De Winter
Richard Harris
Toshiba Taniguchi
Alan D’Andrea
Shirley V. Hodgson
Christopher G. Mathew
Hans Joenje

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Abstract

Fanconi anemia (FA) is a chromosomal instability disorder associated with a high risk of acute myeloid leukemia (AML). Previous work has shown that the AML cell line CHRF-288, derived from a sporadic AML-M7 patient, does not express FANCF protein and exhibits a cellular FA phenotype. We show that this phenotype is corrected by a FANCF-expressing plasmid and that the absence of FANCF protein is explained by hypermethylation of the promoter region of the FANCF gene. As FANCF is localized in a hot-spot region for somatic hypermethylation (11p15), FANCF silencing might be an early step in sporadic carcinogenesis, including leukemogenesis.
**Introduction**

Fanconi anemia (FA) is a recessively inherited syndrome characterized by excessive chromosomal breakage, both spontaneous and induced by DNA cross-linking agents such as mitomycin C (MMC). The disease is associated with a high risk of various malignancies, most notably acute myeloid leukemia (AML) (D’Andrea & Grompe 2003). FA is genetically heterogeneous, comprising at least eight complementation groups. The genes FANCA, FANCC, FANCD2, FANCE, FANCF and FANCG are defective in the corresponding complementation groups (Joenje & Patel 2001), while the breast cancer susceptibility gene, BRCA2, is mutated in FA-D1 patients (Howlett et al. 2002). All FA proteins, along with the BRCA1 protein, are thought to function in an integrated pathway that maintains genomic stability. Consequently, even a single defective component would disrupt the pathway leading to chromosomal breaks and gross rearrangements.

We hypothesized that inactivation of the FA/BRCA pathway might be an early step in the development of sporadic malignancies, in particular those that also occur frequently in FA, such as AML. We previously reported (Xie et al. 2000) that the AML cell line CHRF-288 (originally described by Witte et al, 1986) lacked the FANCF protein and was hypersensitive to MMC. We now show that the cellular phenotype of CHRF-288 cells was corrected upon introduction of a FANCF-containing expression plasmid and present evidence that FANCF in CHRF-288 is inactivated by epigenetic silencing.

**Results and discussion**

The cellular phenotype of CHRF-288 cells was corrected upon transfection with the FANCF expression plasmid. Growth inhibition by MMC was restored to a level similar to that observed in wild type cells (Fig 1). Furthermore, after transfection of CHRF-288 cells the spontaneous and MMC-induced chromosomal breakage decreased from 0.6 and 3.9 to 0.1 and 1.6 breaks/cell, respectively (results not shown). At the molecular level, CHRF-288 had no detectable FANCF protein and FANCF antibodies
failed to co-precipitate FANCA protein, similar to what is observed in lymphoblasts from an FA complementation group F patient. However, CHRF-288 cells transfected with a plasmid expressing FANCF clearly had (exogenous) FANCF that co-precipitated with FANCA, indicating a correction at the level of FA protein complex formation.

Sequencing of the entire FANCF coding region in CHRF-288 cells did not identify any mutation. We therefore tested for gene silencing by methylation. Extensive methylation of the upstream sequence was detected, which was unmethylated in blood and bone marrow from healthy individuals and another AML cell line, ML-2. The 38 CpG dinucleotides that were studied represent 75% of all CpG sites in the predicted CpG island. Eight plasmid clones from CHRF-288 were sequenced and methylation was scored for each CpG site (Fig 2). The average frequency of CpG dinucleotide

![Figure 1](image)

(A) FANCF protein expression in stably transfected cell lines. HSC93 and EUFA698 are lymphoblastoid cell lines from a healthy control and an FA-F patient, respectively. CHRF-288, AML cell line. FANCF protein was not detected in EUFA698 and CHRF-288, but was clearly present after transfection with a FANCF-expressing plasmid. After immunoprecipitation with anti-FANCF antibodies, both FANCA and FANCF were co-precipitated in wild type and complemented cells. (B) Functional correction of CHRF-288 cells shown by MMC-induced growth inhibition. Cells were cultured in the presence of MMC for 5 d and evaluated for growth. HSC93, wild type lymphoblasts; CHRF-288 +F, CHRF-288 cells transfected with
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FANCF-containing expression plasmid.

**Figure 2.** Methylation of the CpG island in the FANCF promoter region, demonstrated by bisulphite sequencing. Methylation status at known CpG dinucleotide positions in eight plasmids cloned from CHRF-288 and two normal control samples. Closed circles indicate methylated CpGs.

Methylation in this region was 75% with 16/38 CpG dinucleotides being methylated in all clones analyzed. Genotyping of DNA from the parents with closely linked polymorphic markers confirmed the presence of both FANCF alleles in the patient’s cell line (results not shown). Furthermore, the promoter sequence in the mother’s DNA was unmethylated, indicating that the methylation of the maternal allele in CHRF-288 cells was not inherited but had occurred de novo. The possibility that the methylation arose during establishment of the CHRF-288 cell line rather than in the primary tumor cannot be excluded. We then investigated sporadic cases of adult AML, but did not find any further examples of methylation of FANCF (36 patients examined). However, FANCF silencing by hypermethylation has been reported in a subset of ovarian tumor cell lines and in four of 19 primary ovarian tumors (Taniguchi et al. 2003), suggesting that this phenomenon may be of more general significance. FANCF silencing might be considered a candidate-mechanism underlying the state of genomic instability that is assumed to be rate-limiting in the origin of many types of malignancy. FANCF is localized in a chromosomal region known to be a hot spot for gene silencing through methylation in a variety of malignancies (de Bustros et al. 1988; Schwienbacher et al. 2000; Rush et al. 2001). Although FANCF silencing in CHRF-288 cells seems stable, demethylation may cause re-activation of FANCF expression. Indeed, Taniguchi et al (2003) showed in an ovarian tumor cell line that the cellular FA phenotype associated with epigenetic FANCF silencing could revert in
vitro as a result of demethylation. The possibility of reversion complicates the assessment of tumors in which FANCF methylation has played a critical role. Although it will be difficult to prove such a course of events for FA pathway-dependent carcinogenesis, our data and those of Taniguchi et al (2003) suggest that FANCF gene silencing may contribute to genomic instability in two very different types of malignancy.

Materials and methods

Patient cell lines and DNA samples. The lymphoblast cell line EUFA698 was derived from a FA type F patient (de Winter et al. 2000). The cell line CHRF-288 was derived from the leukemic blasts of a patient with megakaryoblastic leukemia (Witte et al. 1986); genomic DNA isolated from blood samples from the parents and a healthy sibling were analyzed to ensure presence of both FANCF alleles in the AML cell line. Written consent was obtained from the individuals involved or their legal representatives to carry out the studies reported here.

FANCF expression. FANCF protein was immunoprecipitated from whole cell extracts with guinea-pig antiserum against GST-FANCF1-245 and visualized by Western blotting with rabbit antiserum against GST-FANCF1-374. Co-precipitated FANCA was detected with rabbit FANCA antiserum 89. Blots were re-probed with anti-flag monoclonal M2 (Sigma, Zwijndrecht, The Netherlands) to show flag-tagged FANCF. FA-F lymphoblasts (EUFA698) were stably transfected with FANCF in expression vector pCMV-sport6; CHRF-288 cells were stably transfected with FANCF-flag in pCEP-4. Functional correction of CHRF-288 cells was analyzed by MMC-induced growth inhibition, as described by Joenje et al (Joenje et al. 1995). Parallel cultures were evaluated for spontaneous and MMC-induced chromosomal breakage.

FANCF methylation analysis. The sodium bisulphite conversion technique (Clark et al. 1994), was used to analyze the methylation status of the FANCF CpG island. Following sodium bisulphite conversion of genomic DNA, polymerase chain reactions to amplify the FANCF CpG island sequences were performed (details available on request). The amplicon (469 bp in length and predicted to contain 38 CpG
dinucleotides) was analyzed using an ABI 310 Genetic Analyzer (Applied Biosystems, Warrington, UK). Further analysis, to determine the degree of methylation, was performed by generating plasmid clones using a TopoTA cloning kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions and sequencing as above.

Acknowledgements

We thank Reinhard Stöger for help with the methylation assay, Martin Rooimans and Anneke Oostra for expert technical assistance, and Fré Arwert for helpful comments. This study was supported by Cancer Research UK, the Dutch Cancer Society, and the Netherlands Organization for Scientific Research (N.W.O.).
References


Chapter 6

Hypermethylation of the \textit{FANCC} and \textit{FANCL} promoter regions in sporadic acute leukemia

Najim Ameziane*
Corine J. Hess*
Gerrit Jan Schuurhuis
Abdellatif Errami
Fedor Denkers
Gert-Jan L. Kaspers
Jaqueline Cloos
Hans Joenje
Dirk Reinhardt
Gert J. Ossenkoppele
Michel C. Zwaan
Quinten Waisfisz

* Authors contributed equally to this work

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Abstract

Objective: Inactivation of the FA-BRCA pathway results in chromosomal instability. Fanconi anemia (FA) patients have an inherited defect in this pathway and are strongly predisposed to the development of acute myeloid leukemia (AML). Studies in sporadic cancers have shown promoter methylation of the FANCF gene in a significant proportion of various solid tumors. However, only a single leukemic case with methylation of one of the FA-BRCA genes has been described to date, i.e. methylation of FANCF in cell line CHRF-288. We investigated the presence of aberrant methylation in 11 FA-BRCA genes in sporadic cases of leukemia.

Methods: We analyzed promoter methylation in 143 AML bone marrow samples and 97 acute lymphoblastic leukemia (ALL) samples using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). Samples with aberrant methylation were further analyzed by bisulfite sequencing and tested for mitomycin C sensitivity using Colony Forming Units assays.

Results: MS-MLPA showed promoter methylation of FANCC in one AML and three ALL samples, while FANCL was found methylated in one ALL sample. Bisulphite sequencing of promoter regions confirmed hypermethylation in all cases. In addition, samples with hypermethylation of either FANCC or FANCL appeared more sensitive towards mitomycin C in Colony Forming Units assays, compared to controls.

Conclusion: Hypermethylation of promoter regions from FA-BRCA genes does occur in sporadic leukemia, albeit infrequently. Hypermethylation was found to result in hypersensitivity towards DNA cross-linking agents, a hallmark of the FA cellular phenotype, suggesting that these samples displayed chromosomal instability. This instability may have contributed to the occurrence of the leukemia. In addition, this is the first report to describe hypermethylation of FANCC and FANCL. This warrants the investigation of multiple FA-BRCA genes in other malignancies.
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Introduction

The FA-BRCA pathway is thought to be essential for specific DNA repair processes (Niedernhofer et al. 2005; Levitus et al. 2006). Cells, which are defective for one of the FA-BRCA genes, display genomic instability and are hypersensitive to DNA cross-linking agents. This cellular phenotype is associated with increased cancer risk as observed in patients with Fanconi anemia (FA) [OMIM#227650]. These patients are characterized by a diversity of clinical symptoms including an increased risk to develop malignancies in particular myelodysplastic syndrome and acute myeloid leukemia (AML), but also solid tumors. In the past years a number of papers have reported on the potential role of acquired disturbances in the FA-pathway in sporadic cancers (Lyakhovich & Surralles 2006; Taniguchi & D'Andrea 2006). Hypermethylation of the FANCF promoter has been described to occur in various tumor types such as those of the ovaries (Olopade & Wei 2003), cervix (Narayan et al. 2004), lungs and oral cavity (Marsit et al. 2004). Also in AML the occurrence of inherited and somatic abnormalities in the FA-BRCA genes has been studied (Condie et al. 2002; Lensch et al. 2003; Tischkowitz et al. 2003; Tischkowitz et al. 2004). Only in a small proportion of cases data was provided showing that these abnormalities were indeed associated with functional inactivation of the FA-BRCA pathway. In an adult AML sample functional abnormality was suggested but in this case the molecular mechanism remained unidentified (Lensch et al. 2003). FANCF was found hypermethylated in the leukemic CHRF-288 cell line resulting in hypersensitivity towards DNA cross-linking agents (Tischkowitz et al. 2003). However, 36 additional AML patient samples appeared to be negative for FANCF hypermethylation suggesting that this is not a common event in leukemia. This was recently substantiated by Meyers et al., who found no evidence for hypermethylation of either the FANCF or FANCB gene in a total of 33 AML and 48 acute lymphoblastic leukemia (ALL) samples (Meyer et al. 2006). Here we report results from a study exploring aberrant methylation of 11 FA-BRCA genes (FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCJ/BRIP1, FANCL and FANCM) in sporadic acute leukemia, using the recently described Methylation Specific (MS)-MLPA (Nygren et al. 2005) technique.
Results

*MS-MLPA*

Using MS-MLPA with probes directed against 11 FA-BRCA genes we first analyzed 119 unselected adult AML bone marrow samples and 20 pediatric AML samples selected on the basis of an FA-AML like karyotype (complex rearrangements see materials and methods). Aberrant promoter methylation of the FANCC gene was detected in a single adult patient with biphenotypic AML (Fig. 1). Methylation was also present in the relapse sample from this patient. To test whether FA-BRCA gene methylation was associated with the specific leukemic phenotype, four additional biphenotypic AML samples, 15 adult ALL samples and 82 pediatric ALL samples were analyzed. One adult ALL sample showed FANCL promoter methylation, while FANCC promoter methylation was found in three pediatric ALL samples. All four patients had been diagnosed as having BCP-ALL with a hyperdiploid phenotype. In the biphenotypic subgroup we did not detect additional methylated cases.

![Figure 1](image-url)

**Figure 1.** Detection of aberrant methylation by MS-MLPA. MS-MLPA products were analyzed by capillary electrophoresis (CE). Profiles in red correspond with the undigested samples and blue with digested samples. MS-MLPA profiles are from (a) control genomic DNA without aberrant methylation, (b) SssI CpG methyltransferase treated control genomic DNA, and (c-e) cases with aberrant methylation at the region of interest.
Hypermethylation of *FANCC* and *FANCL* in sporadic acute leukemia

DNA showing methylation of all target sequences, (c) genomic DNA derived from the CHRF-288 cell line, which shows *FANCF* promoter methylation (blue peaks indicated by arrows), (d) genomic DNA from an AML sample without methylation and (e) genomic DNA from an AML sample showing *FANCC* promoter methylation (blue peaks indicated by arrows).

*Sodium bisulphite sequencing*

Sodium bisulphite sequencing confirmed hypermethylation in all cases (Fig. 2). Analysis of the FANCC promoter region from the adult biphenotypic AML sample (patient 1) at diagnosis and the corresponding relapse, showed equally dense methylation patterns. Similarly, FANCC hypermethylation was observed in the two pediatric ALL samples (patients 3 and 4). A third sample showed only partial methylation of the FANCC promoter region (patient 2). The methylation status of FANCL was determined in the adult ALL sample (patient 5) and showed dense methylation (Fig. 2b). No methylation of these genes was found in genomic DNA from control CD34+ cells.

*Mitomycin C sensitivity*

A CFU assay was performed to evaluate whether hypermethylation was associated with increased sensitivity towards mitomycin C (MMC), a hallmark of cells defective in the FA-BRCA pathway. From two pediatric ALL samples with FANCC hypermethylation no colonies were obtained in the CFU assay. The other four methylated samples were on average 6.9-fold more sensitive to MMC (median IC50 4.6 nM) than controls (median IC50 32.1 nM), see Fig. 3, an extent of hypersensitivity commonly observed in cells carrying biallelic FA gene defects. This suggests that the observed hypermethylation in these samples indeed is associated with an FA-like cellular phenotype of DNA cross-linker sensitivity.
Figure 2. Bisulphite sequence analysis of the FANCC and FANCL promoter regions in primary leukemic samples. (a) Upper part: schematic representation of the FANCC gene’s promoter region. The 33 assessed CpGs are situated upstream of exon 1, between the arrows; the translation start is in exon 2 (not shown). In the lower parts of the figure each row represents an individually cloned and sequenced allele following sodium bisulphite DNA modification, black and open circles representing methylated and unmethylated CpGs, respectively. Sequence data for a number of CpGs were ambiguous, indicated by an x. Arrows indicate sequences complementary to the primers used (patient 1, adult diagnosed with biphenotypic AML, patients 2, 3 and 4, pediatric patients diagnosed with ALL). (b) Methylation status of the 23 assessed CpGs in the promoter region of the FANCL gene, represented as described above. The translation start of the FANCL gene is located in the first exon (patient 5, adult diagnosed with ALL).
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**Figure 3.** Mitomycin C (MMC) sensitivity in Colony Forming Units (CFU) assay. Number of colonies scored following a 7-day culture in Methocult culture medium with increasing concentrations (5, 10 and 50 nM) of MMC, expressed as percentage of the number of scored colonies in the untreated (0 nM MMC) fraction. Open symbols, patients with FA-BRCA gene promoter methylation (patient 1, biphenotypic AML, *FANCC* methylated; patient 3, pediatric ALL, *FANCC* methylated; patient 5, adult ALL, *FANCL* methylated). Black symbols, controls (control 1, biphenotypic adult AML, control 2, primary adult AML, control 3, primary pediatric ALL, controls 4 and 5, primary hyperdiploid adult ALL).

**Discussion**

This is the first report showing epigenetic alterations of the *FANCC* and *FANCL* promoter regions in malignant cells. Furthermore, we show here for the first time evidence for FA-BRCA gene hypermethylation in primary sporadic leukemia.
samples. We had anticipated to enrich for AML samples carrying a defect in the FA-BRCA pathway by selecting pediatric AML samples with cytogenetic abnormalities that are frequently found in FA patients (Kutler et al. 2003; Tischkowitz & Dokal 2004). However, no aberrant FA-BRCA gene methylation was detected in these samples using MS-MLPA for the detection of methylation in 11 FA-BRCA genes. In a larger series of sporadic leukemia samples, a relative higher incidence of FA-BRCA gene methylation was observed in ALL samples (4/97) compared to AML samples (1/143). This is somewhat surprising, since ALL is rarely observed in FA-patients (Kutler et al. 2003) except for the FA subtype associated with a defect in the FANCD1/BRCA2 gene (Alter et al. 2007). If methylation of the FA-BRCA genes is causally involved in the occurrence of sporadic leukemia our data suggest that this accounts for a small proportion of these cases. On the other hand the presented data may be an underestimate due to a number of reasons. First, MS-MLPA detects the presence of methylation only for a limited number of CpGs. Samples which are methylated but in which these specific CpGs are not will be missed. Second, silencing of the FA-BRCA pathway is predicted to be only necessary temporarily for the accumulation of tumorigenic alterations. Loss of FANCF methylation in vitro has been shown to occur (Taniguchi et al. 2003). Third, we have analyzed 11 of the 13 genes known to cause Fanconi anemia. Not included were the recently identified FANCN/PALB2 (Reid et al. 2007; Xia et al. 2007) and FANCI (Smogorzewska et al. 2007; Sims et al. 2007; Dorsman et al. 2007) genes.

In conclusion, FA-BRCA gene hypermethylation is observed in a small portion of primary sporadic acute leukemia samples. These samples appeared hypersensitive to DNA cross-linking agents. The data suggests that methylation of these genes resulted in chromosomal instability which may have contributed to the accumulation of oncogenic alterations eventually leading to leukemia. Since the role of cross-linking agents in the treatment of leukemias is limited in general, determining FA-BRCA gene hypermethylation may have clinical consequences, as such leukemias are predicted to be particularly sensitive to regimens containing the cross-linking agents cyclophosphamide, cisplatin, or busulfan.
Materials and methods

Sample characteristics. Samples were obtained from the cell banks of the VU medical center, the AML-BFM Study Group and the DCOG. Snap frozen cell pellets of bone marrow samples obtained from newly diagnosed adult AML patients (n = 119, median blast cell percentage was 66%, range 4–97%) together with 15 adult ALL (B-cell precursor (BCP)-ALL; n = 10, T-cell ALL; n = 5, median blast cell percentage of adult ALL samples was 85%, range 10–99%) samples were randomly selected. Additionally, 20 pediatric AML samples with a complex karyotype, defined as 2 or more random aberrations, or loss of chromosome 5 or 7, or the long arms of these chromosomes, were studied, as well as 4 biphenotypic leukemic samples. For the analysis of 82 newly diagnosed pediatric ALL patients (BCPALL; n = 67, T-cell ALL; n = 15, median blast cell percentage of pediatric ALL samples was 94%, range 16–100%) cytospin slides were selected at random. All specimens were collected with informed consent according to institutional guidelines and in accordance with the Helsinki Declaration of 1975. Control CD34+ cells were derived from healthy volunteers.

2.2. DNA isolation and treatment Genomic DNA from cell pellets was isolated using QIAamp® DNA Blood Minikit (Qiagen, Valencia, CA, USA) according to the manufacturer’s recommendations. DNA from cytospin slides was isolated using phenol, chloroform and isoamylalcohol (PCI; 25:24:1). Methylated DNA was obtained by treating control genomic DNA with SssI CpG methyltransferase (M.SssI, New England Biolabs) according to the manufacturer’s recommendations.

MS-MLPA. MS-MLPA reagents were kindly provided by MRC Holland, Amsterdam, The Netherlands (www.mlpa.com). Target DNA was diluted in TE buffer (10 mM Tris-HCl, pH 8.5, 1 mM EDTA) to a concentration of 100 ng/µl in a total volume of 5 µl and denatured for 10 min at 98°C. MS-MLPA was performed as previously described (Nygren et al. 2005). Probes were designed such that these were targeted to the CpG islands within the promoter regions of 11 FA-BRCA genes and contained a recognition site for the methylation-sensitive restriction enzyme HhaI. The probe mix contained single probes directed to FANCA, FANCD2, FANCG, two probes directed to different sites of FANCB, FANCC, FANCD1/BRCA2, FANCE, FANCJ/BRIP1, FANCL and FANCM and three probes directed to different sites of FANCF.
addition to the 11 FA-BRCA genes, probes were included for BRCA1, ATM, MLH1, XPA, WRN, BLM and NBS1. For quantification of the levels of methylation seven control probes lacking HhaI sites were also included. Probe sequences are shown in Table 1.

### Table 1

FA-BRCA probe mix

<table>
<thead>
<tr>
<th>Size (bp)</th>
<th>Gene</th>
<th>Chr pos</th>
<th>Probe 1</th>
<th>Probe 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td>FLJ22301*</td>
<td>01q44</td>
<td>GTGGAACCTGCGGACAGCTACC</td>
<td>CGTGAACACGCGCAATGTCGTTCCGCTTTAGAGAACCCGACATG</td>
</tr>
<tr>
<td>136</td>
<td>FANCN</td>
<td>11p15</td>
<td>GCAGCGGACGGCGGACAGCTGTTATCCGGGTGCGCCGCATG</td>
<td>CACCAGACACACAGCTTGTTCCGCTTTTGGGTTCCGCTTTGACCCGACCATG</td>
</tr>
<tr>
<td>142</td>
<td>BRCA1</td>
<td>17q21</td>
<td>CCCTCTCTTGGGGTGGGAAAGCCGCT</td>
<td>AAAGCCGACGGCGCAATGTCGTTCCGCTTTTGGGTTCCGCTTTGACCCGACCATG</td>
</tr>
<tr>
<td>148</td>
<td>BRCA2</td>
<td>13q12.3</td>
<td>CGGGTTAGTGGTGGTGGTAGTGGGGGACTTCCAGGCT</td>
<td>GTCTTCCGCAGTCCCAGTCCAGCGTGGCATG</td>
</tr>
</tbody>
</table>
| 154      | MLH1*    | 03p22.1 | CGAGGGAGGGCTGAAGTTGATTCAGATCCAGTCTTACGTCAAGGACTG | AGAACTAGGAGGGAAGGCTGGATCTGCGGCT |}

Indicated are the size of the expected PCR product in base pairs (bp), corresponding genes, chromosomal location, and probe sequences. In the probe sequences HhaI recognition sites are underlined. Control probes for genes lacking HhaI sites are indicated by asterisks.

**Sodium bisulphite sequencing.** One µg of genomic DNA was converted by sodium...
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Bisulphite using the EZ DNA Methylation Gold kit™ (ZYMO Research Corporation, Orange, CA) to confirm the aberrant methylation status. Bisulphite-treated genomic DNA was amplified by PCR using primers FANCC-F 5’-
TTTTATATTTTGAATAGAATATGGAAGAAG- 3’ and FANCC-R 5’-
CAATACATTCTAAAACCTAACTAAC- 3’ for FANCC, or FANCL-F 5’-
TTAGTTTTGTGGATTTGAGGGTAAT-3’ and FANCL-R 5’-
TAAAACAAAAACAAAAACACTAAC- 3’ for FANCL. PCR products were subcloned, isolated and analyzed by sequencing.

**CFU assay and mitomycin C (MMC) sensitivity.** To determine the number of Colony-Forming Units present in the total bone marrow of AML and ALL patients, samples were plated in duplicate in Methocult culture medium (Stemcell Technologies Inc., Vancouver, BC) at a concentration of 40,000, 100,000 and 400,000 cells/well. Colonies were counted after 7 days (37°C, 5% CO2, full humidity). MMC sensitivity was assessed by adding various concentrations of MMC (0, 5, 10 and 50 nM) to the cultures. Colonies were expressed as mean values from duplicate cultures and denoted as a percentage of the number of colonies in the wells without MMC.
References


Hypermethylation of *FANCC* and *FANCL* in sporadic acute leukemia


Chapter 7

No evidence for *FANCF* gene silencing in head-and-neck squamous cell carcinomas

Najim Ameziane*
Fei Chen*
C René Leemans
Ruud H Brakenhoff
Hans Joenje

* Authors contributed equally to this work

Cellular Oncology

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Abstract

Hypermethylation of the Fanconi anemia gene, \textit{FANCF}, has been reported to occur in an appreciable proportion of malignancies, including 15\% of head-and-neck squamous cell carcinomas. This feature is claimed to be a useful tumor marker predicting a favorable response to cisplatin-based treatment protocols, since cells with a defective FA/BRCA pathway are hypersensitive to cross-linking agents, such as cisplatin. We examined the promoter methylation status of \textit{FANCF} in paraffin-embedded oral tumor material from a cohort of 22 patients, of whom 11 (50\%) had responded favorably to a cisplatin-containing treatment regimen. No evidence for silencing of \textit{FANCF} was obtained in any of these tumors. This result is in clear contradiction with published data. We provide evidence that the methylation-specific PCR method as used routinely to probe for \textit{FANCF} methylation is flawed and liable to produce false-positive results. We suggest that the discrepancy between our results and those previously reported may be partly explained by this effect and that the occurrence of \textit{FANCF} silencing in head-and-neck cancer may in fact be less frequent than suggested, if existing at all.
No evidence for FANCF silencing in head-and-neck squamous cell carcinoma

To the Editor,

Fanconi anemia (FA) is a recessively inherited genetic disorder with a strong predisposition to malignancies, in particular acute myeloid leukemia (AML) and squamous cell carcinoma (SCC) (Joenje & Patel 2001; Alter 2003). Thirteen FA genes are known today (FANCA, -B, -C, D1/BRCA2, -D2, -E, -F, -G, -I, -J/BRIP1, -L, -M, and –N/PALB2) (Levitus et al. 2006; Meijer 2007; Dorsman et al. 2007; Wang 2007), whose products act in the ‘FA/BRCA pathway’ to protect the genome against spontaneous as well as cross-linker-induced chromosomal breakage and rearrangements. Genomic instability is thought to be the underlying cause of cancer predisposition in FA patients as well as in other chromosomal instability and premature-ageing diseases (Hoehn et al. 2008). It has been hypothesized that FA gene defects might also play a role in sporadic cancer (Jacquemont & Taniguchi 2006; Meijer 2007). In sporadic malignancies, loss of DNA stability and tumor suppressor gene function is often based on (a combination of) DNA sequence alterations, deletions, and promoter methylation. For FANCF, methylation of the promoter sequence has been reported for a proportion of several types of cancer, including leukemia (Tischkowitz et al. 2003; Hess et al. 2008) and cancer of the breast (Olopade & Wei 2003; Wei et al. 2008), ovary (Taniguchi et al. 2003; Dhillon et al. 2004; Wang et al. 2006; Lim et al. 2008), bladder (Neveling et al. 2007), cervix (Narayan et al. 2004), testis (Koul et al. 2004), lung (Marsit et al. 2004), and head-and-neck (Marsit et al. 2004). All of these studies were based on a methylation-specific PCR assay (‘MSP’), described in detail by Taniguchi et al. (Taniguchi et al. 2003) and discussed in more general terms by Derks et al. (Derks et al. 2004).
Using the recently developed method of ‘methylation-specific multiplex ligase-mediated probe amplification’ or ‘MS-MLPA’ (Nygren et al. 2005) we carried out a survey of FANCF promoter methylation in a panel of 25 cell lines derived from squamous cell carcinomas of various origins including the head-and-neck. We were unable to ascertain any convincing example of FANCF silencing, except in CHRF-288 leukemia cells, which were used as positive controls (Tischkowitz et al. 2003).

Because cell lines, due to culturing-artifacts, may not in every respect represent the initial tumor, we analyzed archival formalin-fixed paraffin-embedded HNSCC tumor material to retrospectively examine the possible relevance of FANCF silencing for tumor response to treatment regimens containing cisplatin. Twenty-two tumors from patients treated with cisplatin-containing chemoradiation were analyzed using MSP. Eleven tumors had shown sensitivity towards this treatment regimen; this subgroup was expected to be enriched for cases with FANCF methylation. However, no FANCF promoter methylation was observed in any of the 22 tumors, while formalin-fixed paraffin-embedded CHRF-288 cells, which served as a positive control, clearly showed methylation (Figure 1). Our results represent a marked difference from those previously reported (0 positives out of 22 tested versus the reported 13 positives out of 89 tested; p< 0.07, 2-tailed Fisher’s exact test).
No evidence for FANCF silencing in head-and-neck squamous cell carcinoma

Figure 1. Bisulphite concentration-dependent variation in results obtained by MSP. MSP carried out on DNA isolated from FANCF-methylated CHRF-288 cells, and from unmethylated HSC93 wild type control cells (both formalin-fixed and paraffin embedded), after treatment with various concentrations of sodium bisulphite. An amplification product was generated with the M primers using methylated DNA even when the bisulphite concentration was down to 10% of the prescribed concentration (EZ DNA methylation kit, Zymo research). Using unmethylated DNA as a template, the PCR does not generate an amplification product when reducing the bisulphite concentration to 50%. Unmethylated DNA treated with 10% and 25% of the prescribed concentrations of sodium bisulphite, however, appeared to produce a PCR product with the M primers that will be interpreted as methylation-positive, but in fact is false-positive and due to suboptimal bisulphite treatment of the DNA.

We next analyzed FANCF promoter methylation using a MS-MLPA test (MRC-Holland, Amsterdam, The Netherlands). This test has the advantage over MSP that methylation is determined at multiple promoter sites simultaneously, while at the
same time deletions can be observed. However, this screen did not reveal any case of FANCF promoter methylation or homozygous deletion. Thus, so far our results have failed to provide evidence for FANCF inactivation underlying a favorable response to cisplatin-containing treatment regimens of head-and-neck SCCs.

The discrepancy between our results and those published earlier may be explained, at least in part, by experimental details that could critically influence the results to be obtained with MSP (see Derks et al. 2004 for a review of the MSP method).

First, Marsit et al. (2004), who followed the PCR conditions as described by Taniguchi et al. (2003), used 44 PCR cycles to amplify the (bisulphite-modified) promoter sequences, whereas we used 34 cycles, which is in compliance with Derks et al. (2004), who recommend not to exceed 35 cycles. The use of too many cycles may carry a risk of producing false-positive amplification products.

Second, since the yields of microdissected material may be low and highly variable, we used carrier DNA to standardize the amount of template for bisulphite treatment and to facilitate precipitation, as recommended (Derks et al. 2004). Whether carrier DNA has been used in the reported studies has not been specified.

Third, bisulphite is known to be chemically unstable and may thus be a variable component of the reaction mixture if not used freshly-made. The MSP assay essentially relies on the conversion of unmethylated cytosine residues into uracil by sodium bisulphite treatment and the use of primers that specifically amplify either unmethylated (U) or methylated (M) DNA. However, DNA isolated from formalin-fixed paraffin-embedded specimens is often of relatively poor quality, which might interfere with efficient bisulphite modification of unmethylated cytosines. We hypothesized that incomplete conversion of cytosine bases could cause false-positive results by PCR.
amplification, as most amplification protocols allow few mismatches in the annealing primers. We mimicked suboptimal conditions by applying a range of bisulphite concentrations and determined the amplification of methylated and unmethylated promoter region of \textit{FANCF} using primers identical to those used in published studies (Taniguchi \textit{et al.} 2003; Marsit \textit{et al.} 2004). A concentration-dependent result of the PCR reaction was observed for the amplification of methylated and unmethylated DNA with M and U primers, respectively (Figure 1). Remarkably, a PCR product was generated with unmethylated DNA using M primers at bisulphite concentrations that were 10\% and 25\% of the prescribed concentration. Moreover, when 10\% of bisulphite was used for conversion, only the M- and no U-PCR product was generated with unmethylated control DNA, in which case the sample was to be scored as fully methylated for \textit{FANCF}.

Our results revealed that the method as often applied in the assessment of \textit{FANCF} promoter methylation, is liable to produce false-positive results when suboptimal conditions are used. Suboptimal conditions might exist particularly when using archival formalin-fixed paraffin-embedded material. We suggest that the discrepancy between our results and those reported by Marsit \textit{et al.} (Marsit \textit{et al.} 2004) may be partly explained by this effect and that the occurrence of \textit{FANCF} silencing in head-and-neck cancer may in fact be less frequent than suggested, if existing at all.

In conclusion, the molecular basis underlying a favorable response of HNSCC patients to cisplatin treatment is not related to silencing of \textit{FANCF}. In addition, the reported MSP-results that have led to conclusions regarding \textit{FANCF} promoter methylation in tumor samples of various origins need to be considered with great caution in view of the possibility for the MSP assay to produce false-positive results.
Acknowledgements

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No evidence for FANCF silencing in head-and-neck squamous cell carcinoma

References


Chapter 8

Evidence for FA/BRCA pathway impairment in a proportion of squamous cell carcinomas

Najim Ameziane*
Fei Chen*
Quinten Waisfisz
Anneke B. Oostra
Seija Grennan
Kejian Zhang
Bing Xia
René C. Leemans
Gerard Pals
Johan P de Winter
Ruud H Brakenhoff
Hans Joenje

* Authors contributed equally to this work

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Abstract

Given that Fanconi anemia (FA) patients are strongly predisposed to develop squamous cell carcinomas, we addressed the question of whether squamous cell carcinomas (SCCs) occurring in the general population may be characterized by an impairment of the FA/BRCA pathway. We examined this pathway in 25 cell lines derived from head-and-neck (15), vulva (7), and cervix (3) SCCs. Western blotting was used to probe for (1) monoubiquitinated FANCD2, and (2) FA proteins known to act downstream of FANCD2 monoubiquitination: BRCA2/FANCD1, BRIP1/FANCJ, and PALB2/FANCN; BRCA1, a known FANCD2 interactor, was also included. All cell lines appeared to be able to modify FANCD2 by monoubiquitination, indicating that the upstream part of the FA pathway was intact. However, the downstream-acting protein BRCA2 appeared undetectable in one HNSCC and one vulva carcinoma cell line, while BRCA1 was undetectable in two additional cell lines. These four cell lines were hyper-responsive to MMC-induced arrest in the G2 phase of the cell cycle, a phenotype consistent with a deficiency in the FA/BRCA pathway. We then screened the remaining 21 cell lines and found 12 more that showed a similar hypersensitive response. Thus, amongst 25 SCC cell lines, 16 (64%) responded in a FA-like fashion. In 4 of these the FA phenotype was associated with BRCA1 or BRCA2 deficiency, but in 12 the molecular defect remains undefined. We speculate that deficiencies of as yet unidentified FA proteins acting downstream in the pathway may be responsible for their abnormal response.
Introduction

Fanconi anemia (FA) is a recessively inherited genetic disorder with diverse clinical features, including developmental anomalies, progressive bone marrow failure and a strong predisposition to malignancies, in particular acute myeloid leukemia (AML) and squamous cell carcinoma (SCC) (Joenje & Patel 2001; Alter 2003). Cells derived from FA patients are hypersensitive to DNA cross-linking agents, such as mitomycin C (MMC) and cisplatin. To date, 13 FA complementation groups (FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, and -N) have been defined and the corresponding disease genes have been cloned (Joenje & Patel 2001; Wang 2007). The proteins encoded by these genes act in concert to support a complex genome maintenance pathway that is required for error-free DNA replication, especially in the presence of DNA damage due to cross-linking agents. Apart from the proteins found defective in FA patients at least three additional FA-associated proteins, FAAP24, FAAP100, and HES1, have been identified that are considered essential for the FA pathway to function, even though thus far no FA patients are known with pathogenic mutations in these genes (Ciccia et al. 2007; Ling et al. 2007; Tremblay et al. 2008). A key reaction in the FA pathway is the monoubiquitination of FANCD2 and FANCI, which together form a complex (I-D2) (Smogorzewska et al. 2007). Proteins acting upstream of this step are FANCA, -B, -C, -E, -F, -G, -L, -M, FAAP24, FAAP100, and HES1, which are assembled in a multiprotein ‘core complex’. Proteins acting downstream of the I-D2 complex are FANCD1/BRCA2 (Howlett et al. 2002), FANCN/PALB2 (Xia et al. 2006; Reid et al. 2007), and FANCJ/BRIP1 (Levran et al. 2005; Levitus et al. 2005; Bridge et al. 2005). BRCA1, which is known to associate with FANCJ and FANCD2, and which when defective generates a FA-like cellular phenotype, is likely to play a role in the pathway as well. The existence of at least one additional as yet unidentified downstream-acting gene has been implicated on the basis of a recent comprehensive mutation screening study (Ameziane et al. 2008).

FA patients have an approximately 500-fold increased risk of developing SCC (Kutler et al. 2003; Alter 2003), most notably tumors of the oral cavity and gynecological tumors (Carvalho et al. 2002), which occur several decades earlier in FA patients than in the general population. Tumor cell lines derived from oral tumors obtained
from FA patients were found indistinguishable from those derived from sporadic tumor patients in showing highly complex karyotypes with a high proportion of whole-arm translocations (Hermsen et al. 2001) in addition to numerous gains and losses that also characterize sporadic HNSCC (van Zeeburg et al. 2005).

We hypothesized that (a proportion of) sporadic SCCs may originate from precursor cells with an acquired defect in the FA/BRCA pathway due to somatic inactivation, such as gene silencing or mutation. Indeed, previous studies have reported FA/BRCA pathway inactivation due to gene silencing in sporadic malignancies of various origins. Silencing of FANCF has been reported in ovarian cancer (Taniguchi et al. 2003), and in one megakaryocytic AML cell line (Tischkowitz et al. 2003), while FANCC and FANCL promoter methylation has been reported in five other cases of sporadic leukemia (Hess et al. 2008). Promoter methylation was apparently associated with a functional FA/BRCA pathway defect as demonstrated by cellular hypersensitivity to MMC and, in some cases of FANCF silencing, by correction of the FA phenotype by ectopic expression of FANCF. In addition, hypermethylation of FANCF has been reported for a proportion of primary material from head-and-neck and lung tumors (Marsit et al. 2004), granulosa cell tumors (Dhillon et al. 2004), breast tumors (Wei et al. 2008), and bladder carcinoma (Neveling et al. 2007). However, in these studies paraffin-embedded material was used, which is not suitable for examining the functional consequences of the observations.

To ascertain the possible occurrence of FA/BRCA pathway defects in sporadic cancer we chose to study solid tumors that are known to occur frequently in FA patients: SCCs from the oral cavity and vulva/cervix. Instead of archival material we used cell lines established from such tumors, enabling us to correlate molecular findings with cellular phenotypes. Cell lines after being established from head-and-neck tumors have been shown to faithfully reproduce critical properties of the primary tumor material (Martin et al. 2008). To characterize our tumor cell lines we utilized protein analysis by Western blotting, mRNA expression analysis, promoter hypermethylation and deletion analysis, and mutation screening. Tests for functional FA/BRCA pathway defects, applied in a subset of cases, included MMC-induced growth inhibition, cell cycle arrest, and chromosomal breakage analysis.
Results

Twenty five SCC cell lines from sporadic cancer patients (15 head-and-neck and 10 gynecological; Table 1) were examined for FA/BRCA pathway defects. Three HNSCC cell lines derived from FA patients were included as controls. The cell lines were examined for FANCD2, BRIP1/FANCJ, PALB2/FANCN, and BRCA2/FANCD1 protein, by Western blotting. In contrast to the FA patient-derived tumor cell lines EUFA1131-T (FA-C), EUFA1365-T (FA-A), and EUFA974-T (FA-A), which expressed only the short form of FANCD2 (FANCD2-S), the gene-corrected EUFA1131-T (+FANCC) as well as all 25 sporadic SCC cell lines displayed the ubiquitinated form, FANCD2-L, in addition to the short form. This indicated that in all sporadic tumor cell lines studied the components functioning upstream of FANCD2 ubiquitination were intact (Figure 1).

**Figure 1.** FANCD2 and BRCA2 protein expression. Whole-cell extracts were analyzed by direct Western blotting, using mouse monoclonal FANCD2 antibody and rabbit polyclonal BRCA2 antibody (Ab-2), which recognizes the amino acids between 3245 and 3418 of
human BRCA2. (a) The monoubiquitinated larger form of FANCD2 (FANCD-L) is detectable in sporadic HNSCC cell lines but lacking in the FA patient tumor-derived negative controls EUFA1131-T (FA-C), EUFA1365-T (FA-A), and EUFA974-T (FA-A). BRCA2 protein was undetectable in 1 sporadic HNSCC cell line, VU147-T. WT, wild type; D1, BRCA2-negative control; D2, FANCD2-negative control; A, FA-A; C, FA-C. (b) BRCA2 protein is detectable in all vulva and cervix tumor cell lines except in SCV-6, while all cell lines possess both forms of FANCD2. (c) BRCA2 protein was undetectable in cell lines SCV-6 and VU147-T with a rabbit BRCA2 antibody (Ab-2, upper panel), raised against the at C-terminus of the protein. After stripping and re-probing with a mouse BRCA2 antibody that recognized amino acids 1651-1821 (Ab-1, lower panel), a weak band running slightly faster than full-length BRCA2 was detected in the VU147-T cells.

**BRCA2 deficiency**

In most sporadic HNSCC cell lines also downstream components appeared normal; examples of BRCA2 blots are shown in Figure 1a. However, in cell line VU147-T BRCA2 was undetectable, using a rabbit polyclonal BRCA2 antibody raised against the C-terminus of the protein (Ab-2). A similar observation was made in the gynecological cancer cell lines, where all cell lines appeared normal except for one vulva tumor cell line, SCV-6, in which we were unable to detect BRCA2 protein by the Ab-2 antibody (Figure 1b). Results from the Western blots for FANCD2, BRIP1/FANCJ, PALB2/FANCN, and BRCA2/FANCD1 are summarized in Table 1. Since most reported BRCA2 mutations result in a truncated protein (Tavtigian et al. 1996) a mouse BRCA2 antibody (Ab-1) raised against the middle part of the BRCA2 protein was used to detect potentially C-terminal truncated BRCA2 protein in SCV-6 and VU147-T. As shown in Figure 1c, BRCA2 was – as before – undetectable in SCV-6 and VU147-T using Ab-2. However, after stripping the blot and re-probing with Ab-1, a weak band was detected in VU147-T suggesting a truncated BRCA2 protein. Su et al. (Su et al. 1998) reported that the detection of BRCA2 is improved when samples were denatured at lower temperature, 55 °C. However, these conditions did not improve the ability of antibody Ab-1 to detect wild-type BRCA2 in HSC93 cells, nor the truncated protein in VU147-T cells. In SCV-6 cells, BRCA2 remained undetectable (Figure 2). Detection of BRCA2 was neither improved by pretreating the cells with MMC, although this treatment increased the ratio FANCD2-L/-S, as reported before in other cell types.
Figure 2. Detection of BRCA2 protein under different conditions. (a) Sixty microgram (µg) aliquots of protein from whole cell lysates were analyzed using a mouse monoclonal antibody (Ab-1) following a denaturation temperature of 55 °C next to the routine 70 °C. BRCA2 protein was still undetectable in SCV-6, although there was indication of improved detection in wild type HSC93 cells and truncated BRCA2 protein was detected in VU147-T. FANCD2 is shown on the same blot. (b) Cells were treated with the indicated concentrations of MMC and whole cell extracts from the same number of cells were analyzed by direct Western blotting using a rabbit BRCA2 antibody. BRCA2 protein remained undetectable in SCV-6, whereas FANCD2-L levels were elevated after MMC treatment. WT, wild type; D1, FA-D1.

Genetic analysis of BRCA2 deficiency in VU147-T and SCV-6 cells

VU147-T. Analysis of genomic DNA for copy number alterations by MLPA showed a heterozygous deletion of all BRCA2 exons (data not shown). A normal quantity normally sized BRCA2 transcript was detected by semiquantitative PCR of exon 27 (Figure 3), suggesting that BRCA2 expression was normal at the mRNA level. Mutation screening on cDNA revealed a hemizygous nonsense mutation at 10204A>T (p.K3326X), which results in deletion of 93 C-terminal amino acids. This sequence change is known as an unclassified variant or polymorphic stop (Mazoyer et al. 1996), and was evaluated as nonpathogenic using a mouse embryonic stem cell-based functional assay (Kuznetsov et al. 2007). However, this variant has
recently been associated with an increased risk of esophageal squamous cell carcinoma in the Turkmen population of Iran (Akbari et al. 2008), suggesting that the mutation may have pathogenic consequences.

SCV-6. Deletion analysis by MLPA was carried out on genomic DNA from SCV-6. We detected a heterozygous deletion of all \textit{BRCA2} exons that also encompassed the promoter region (data not shown). RT-PCR of exon 27 indicated a normally sized \textit{BRCA2} transcript (Figure 3), which was present at a normal level as based on real-time PCR (results not shown). To screen the second allele for mutations, the open reading frame and the 5'-untranslated region were sequenced. However, no pathogenic alterations were found.

\textbf{Figure 3.} \textit{BRCA2} transcript in SCV-6 and VU147-T cells. PCR was carried out after \textit{BRCA2} cDNA was synthesized (+, with reverse transcriptase; −, without reverse transcriptase).

HSC93, wild type human lymphoblasts. 396 bp, PCR product corresponding to exon 27 of \textit{BRCA2}.

\textbf{MMC sensitivity associated with \textit{BRCA2} deficiency}

Although the protein analysis suggested a deficiency in the levels of \textit{BRCA2} in VU147-T and SCV-6 cells, results of the genetic analysis did not necessarily imply complete loss of function. Cells derived from FA-D1 patients, who carry biallelic mutations in \textit{BRCA2} that cause a severe form of the disease, are known to be characterized by excessive chromosomal breakage and cell cycle delay in G2 when treated with MMC (Godthelp et al. 2006). We examined the cellular phenotype of VU147-T and SCV-6 cells by assessing MMC-induced growth inhibition, cell cycle delay, and chromosomal breakage. All three tests indicated a cellular phenotype in VU147-T and SCV-6 cells that was very similar to that of the FA tumor cell lines (Figure 4). These results indicated that the apparent absence of \textit{BRCA2} protein in
SCV-6 and the hemizygous truncating mutation with loss of the second allele in VU147-T are associated with a FA-like cellular phenotype. However, proof for a direct causal relationship would require correction of the phenotype by ectopic expression of BRCA2, which has thus far been technically unfeasible in our hands.
Figure 4. FA-like phenotype in SCV-6 and VU147-T cells. (a) MMC-induced growth inhibition in the BRCA2-deficient cell lines SCV-6 and VU147-T. The cell lines exhibit ~5-fold lower IC\textsubscript{50} values than the gene-corrected EUFA1131-T+FANCC cells, similar to the uncorrected EUFA1131-T cells. (b) Cell cycle analysis after treatment with 50 or 100 nM MMC for 72 h shows excessive accumulation of cells in the G2/M phase in EUFA1131-T, VU147-T and SCV-6 cells compared to EUFA1131-T+FANCC. (c) Chromosomal breakage analysis. Elevated levels of chromosomal breakage is observed for EUFA1131-T, SCV-6, and VU147-T cells, when compared to the gene-corrected EUFA1131-T+FANCC control and the HNSCC cell line VU120-T. BRCA1-deficient HCC1937 cells were also included as MMC-hypersensitive controls. P-values for 2-sample Chi\textsuperscript{2} tests were <0.001, <0.001, and <0.002 for the differences between EUFA1131-T, SCV-6, and VU147-T versus EUFA1131-T+FANCC, respectively.
BRCA1 deficiency
Burkitt and Ljungman (Burkitt & Ljungman 2007) recently reported a FA-like phenotype in a subset of cisplatin-sensitive HNSCC cell lines, which was shown to be associated with deficient BRCA1 protein. Screening our cell lines for BRCA1 protein by Western blotting revealed that the protein was undetectable in a HNSCC cell line, VU96-T, and a vulva tumor cell line, ME-180 (data not shown). These 3 cell lines were also found to exhibit hypersensitivity to MMC-induced cell cycle arrest in G2 (Table 1). However, after sequencing genomic DNA in these cell lines no pathogenic BRCA1 mutations could be detected.

Functional screen for a FA-like phenotype
Since the downstream part of the FA/BRCA pathway also depends on as yet unidentified proteins, we tested all remaining cell lines for MMC-induced cell cycle arrest. A MMC concentration was used that caused a strong accumulation in the G2 phase of the cell cycle in EUFA-T (FA-C) cells, but hardly affected their gene-corrected counterparts (EUFA-T+FANCC). By defining a FA-like response as the accumulation of >25% of cells in the G2 phase after 72 h exposure to 50 nM MMC, we observed that as many as 16 out of 25 SCC cell lines exhibited a FA-like response, which included the 4 cell lines that lacked detectable BRCA2 or BRCA1 protein. Thus, in 12 cell lines the FA-like phenotype remains unexplained. These cell lines might be defective in as yet unidentified genes that play a role in the FA/BRCA pathway.
<table>
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<td>EUFA1131-T (FA-C)</td>
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<td>Oral</td>
<td>+</td>
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<td>(van Zeeburg et al. 2005d)</td>
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<td>EUFA1131-T (+FANCC)</td>
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<td>n.d.</td>
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</tr>
<tr>
<td>EUFA1365-T (FA-A)</td>
<td>M</td>
<td>Oral</td>
<td>+</td>
<td>--</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+</td>
<td>n.d.</td>
<td>--</td>
<td>(van Zeeburg et al. 2005c)</td>
</tr>
</tbody>
</table>

Abbreviations: FoM, floor of the mouth; ToBase, base of the tongue; TrigRe, trigonum retromolare; n.m., neck metastasis; U, unspecified. \(^a\) +, >25%, -, <25% of the cells accumulated in the G2/M phase of the cell cycle after 3-d exposure to MMC at 50 nM (examples are shown in Figure 4). \(^b\) +, present; --, undetectable.
Discussion

In the present study 25 cell lines from sporadic tumor types that frequently occur in FA patients were screened by Western blotting for FANCD2 monoubiquitination and expression of BRCA2/FANCD1, BRIP1/FANCJ, PALB2/FANCN, and BRCA1. All cell lines were apparently capable of modifying FANCD2 by monoubiquitination, indicating that the upstream part of the FA pathway was functionally intact. However, a deficiency was observed in the downstream-acting BRCA2 protein in SCV-6 (vulva SCC) and VU147-T (HNSCC) cell lines. As expected for BRCA2-negative cells, SCV-6 cells showed a dramatic increase in MMC-induced G2 phase arrest, chromosomal breakage, and growth inhibition, when compared to both wild type and other vulva-derived cell lines that expressed full-length BRCA2 protein. VU147-T cells, which expressed a truncated BRCA2 protein, displayed a moderate MMC sensitivity in the chromosomal breakage assay. Further analysis of these two BRCA2-deficient cell lines showed that BRCA2 mRNA was present at a level comparable to other SCC cell lines. Sequencing of the BRCA2 cDNA identified a hemizygous nonsense mutation 10204A>T in VU147-T, a mutation that has been associated with predisposition for esophageal squamous cell carcinoma in the Turkmen population of Iran (Akbari et al. 2008). Our results thus raise the question whether this mutation might also predispose to head-and-neck cancer, although this may hold true only for certain ethnic backgrounds, since no such predisposition has been observed in European and North American BRCA2 mutation carriers (1999). The molecular cause underlying the apparently aberrant BRCA2 expression in a subset of our cell lines is still unclear. Although a heterozygous BRCA2 deletion in SCV-6 was observed, no other (epi)genetic aberrations were detected that could explain the absence of the BRCA2 protein in these cells. This observation suggests that additional mechanisms, besides genetic and epigenetic alterations, might abrogate BRCA2 protein expression or stability.

Similar observations were made for BRCA1, which appeared undetectable by Western blotting in an oral tumor cell line (VU96-T) and a cervix SCC cell line (ME-180). This abnormality was also associated with a hyper-responsiveness to MMC-induced cell cycle arrest. Our findings concur with results recently reported by Burkitt and Ljungman (Burkitt & Ljungman 2007), who demonstrated BRCA1 deficiency to
be responsible for cisplatin sensitivity in a subset of HNSCC cell lines, although the authors did not report results from sequence analysis of the \textit{BRCA1} locus. We did sequence the gene in our 2 BRCA1-deficient cell lines, but failed to find any pathogenic alterations. Thus, a molecular explanation of the apparent absence of BRCA1 protein is still lacking.

A striking result from our study is that as many as 16 out of 25 cell lines exhibited a FA-like phenotype, as assessed by MMC-induced cell cycle arrest, while only 4 of these cases could be correlated with a deficiency in BRCA1 or -2. In the remaining 12 cell lines the underlying molecular mechanism is still to be elucidated. The mechanism may be based on deficiencies in as yet unidentified proteins acting downstream in the FA/BRC\textit{A} or –related pathways.

In conclusion, we found that 16 out of the 25 SCC cell lines studied exhibited a cellular phenotype consistent with a defective downstream component of the FA/BRC\textit{A} pathway. However, in only 4 cell lines could the MMC-hypersensitive phenotype be associated with a demonstrable molecular abnormality in BRCA1 or -2, although further work is required to provide final proof for the association actually representing a causal relationship. For the remaining 12 cell lines no clue about the molecular basis underlying their FA-like phenotype is as yet available. In spite of this, our findings may have clinical significance. Bifunctional alkylating agents such as cisplatin in combination with inhibitors of the poly(ADP)ribose polymerase might offer a therapeutic option for treating tumors with a defective FA/BRC\textit{A} pathway (Evers \textit{et al}. 2008). Immunohistochemical staining for BRCA1 and -2 may help to recognize patients who might benefit from such treatment.

\textbf{Materials and methods}

\textit{Tumor cell lines}. Twenty five cell lines derived from sporadic tumor patients were studied (Table 1). Fifteen HNSCC and 10 vulva/cervix tumor cell lines were from the Departments of Clinical Genetics and Otolaryngology/Head-Neck surgery, VU Medical Center, Amsterdam, and the Department of Obstetrics and Gynecology, Turku University Hospital, respectively. The following cell lines were used as controls in various experimental settings. Three HNSCC cell lines derived from FA patient oral
tumor biopsies were used as prototype FA-like tumor cells: EUFA-T (FA-C), VU1365-T (FA-A), and VU974 (FA-A). Genetically corrected EUFA-T cells (EUFA-T + FANCC), obtained by transduction with a phoenix retroviral construct containing FANCC-GFP, were included as prototype for non-FA-like tumor cells. Human EBV-immortalized lymphoblasts were derived from a healthy individual (HSC93) and from FA patients: EUFA1289 (FA-D2) and EUFA579 (FA-D1/BRCA2). AML cell line CHRF-288, which contains a silenced FANCF gene due to promoter hypermethylation, was used as a positive control for methylation analysis (Tischkowitz et al. 2003). HCC1937 tumor cells, obtained from the American Type Culture Collection, were included as BRCA1-deficient controls.

**Antibodies and Western blot.** FANCD2\(^{(1-272)}\) mouse monoclonal antibodies were used for the detection of FANCD2 (Santa Cruz Biotechnologies, Santa Cruz, California, USA). BACH1 rabbit antibodies were used to detect FANCJ. PALB2\(^{1-200}\) rabbit antibodies were used to detect FANCN. BRCA1 mouse monoclonal antibody was used to detect BRCA1 (Santa Cruz Biotechnology, California, USA). BRCA2\(^{(1651-1821)}\) (Ab-1) and BRCA2\(^{(3245-3418)}\) (Ab-2) are mouse monoclonal and rabbit polyclonal antibodies, respectively (Merk Biosciences, Germany). For direct Western blot, cells from tumor cell lines were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, supplemented with proteinase inhibitors aprotinin, pepstatin, leupeptin and pefablock). Protein lysates corresponding to 500,000 cells or the indicated amounts of protein, as determined with a Bio-Rad (Hercules, CA) protein assay, were denatured at 70 °C and/or 55 °C and loaded on a 3%-8% NuPAGE Tris-Acetate gradient gel (Invitrogen, Carlsbad, California, USA). After electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, Massachusetts, USA). The membrane was blocked with 5% fat-free milk in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and incubated with primary antibodies against FANCD2 (1:500) and BRCA2 (1:50) overnight. To visualize FANCD2 and BRCA2, blots were incubated with horseradish peroxidase-labelled secondary antibodies (Dako Diagnostics, Glostrup, Denmark) and detected using the ECL Western blot analysis system (Amersham Pharmacia Biotech, UK). Detection of other FA proteins by direct Western blot has been described elsewhere (de Winter et al. 2000).
RT-PCR and real-time RT-PCR. Total cellular RNA was isolated using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. cDNA synthesis was performed with Superscript II reverse transcriptase (Life Technology, Gaithersburg, MD), parallel to controls from which reverse transcriptase was omitted. The sequences of primers used to amplify exon 27 were as follows: **BRCA2** (sense), 5’-TTCAGCCACCAAGGAGT-3’; **BRCA2** (anti-sense), 5’-GGCGACAATAAATTATTGAC-3’. Semi-quantitative PCR was performed as follows: 94 °C for 2 minutes; 94 °C, 1 minute; 55 °C, 1 minute; 72 °C, 1 minute, for 35 cycles, and extended at 72 °C for a final 7 minutes.

Real-time RT–PCR was performed using LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Germany) with a Light Cycler apparatus. Each capillary contained a total reaction volume of 20 µl after the addition of primers (final concentration: 0.25 pM), MgCl2 (final concentration: 3.25 mM) and template cDNA (2 µl of each) to the Master mix. PCR reaction was performed as: 94 °C for 5 min, then denaturation (95 °C for 0 s), annealing (59 °C for 10 s) and extension (72 °C for 12 s) for 60 cycles. After completion of the PCR amplification, a melting curve analysis was performed. The house keeping gene beta-2-microglobulin (B2M) was amplified at the same time and used as an internal control. Standard curves for BRCA2 and B2M were made using cDNA of HSC93 wild type lymphoblasts with 5-step dilutions as template. Calculation of ratios between target (BRCA2) and reference (B2M) genes according to their PCR efficiencies and crossing points has been described previously (Pfaffl et al. 2002). PCR products of BRCA2 and B2M were analyzed on a 1.2% agarose gel.

MLPA analysis and cDNA sequencing. Genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Multiplex ligation-dependent probe amplification (MLPA), which detects gene deletions and amplifications, was performed as previously described (Hogervorst et al. 2003). Following reverse transcription, PCR reactions to amplify overlapping fragments of **BRCA2** cDNA were performed. PCR products were purified and cycle sequencing performed using the ABI Big Dye cycle sequencing kit v3.1 according to the manufacturer's instructions, and products were analyzed on an ABI3100 machine for sequencing.
Mitomycin C-induced growth inhibition test. The growth-inhibiting effect of mitomycin C (MMC) was assessed by growing the cells in the presence of various concentrations of MMC over a period of time that allowed cells without added drug to undergo at least three population doublings. IC₅₀ values are defined as the concentration of MMC causing 50% inhibition of growth.

Flow cytometric analysis. Cells (150,000-200,000) were exposed for 72 h at 37 °C to MMC at 50 nM or 100 nM. DNA was stained with Hoechst, after which the cell cycle distribution was analyzed by flow cytometry using a Particle Analysing System (PAS) (Partec, Germany).

Chromosomal breakage analysis. Spontaneous and MMC-induced chromatid-type aberrations were scored by visual inspection of Giemsa-stained metaphase spreads, as described previously (Joenje et al. 1981).

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Chapter 9

General discussion
General discussion

Several studies, including this thesis, have demonstrated the occurrence of a disrupted FA/BRCA pathway in a proportion of hereditary and sporadic malignancies. Patients with such malignancies might benefit from specific individualized treatment, designed to exploit the exquisite sensitivity of FA-like tumors to treatment with cross-linking agents. Below, I will review the actual occurrence of FA-like malignancies in non-FA individuals, the methods of identification, and some of the various personalized treatment options. I will also briefly touch upon the phenomenon of resistance to chemotherapy, which is often observed after initial successful application of specific agents.

The important difference between familial/inherited and sporadic cancer in the present context is that in the inherited form a germ-line mutation in a DNA caretaker gene is present which facilitates the accumulation of oncogenic DNA alterations resulting in a high risk of cancer at a relatively young age. In the eventually arising tumor, the predisposing gene is typically found inactive, the unaffected allele often being deleted by ‘loss of heterozygosity’. The spontaneous mutation rate of an average gene is in the order of $10^{-6}$ to $10^{-8}$ per cell per division. With some $10^9$ cells per gram tissue, heterozygous spontaneous mutations are likely to occur in most tissues. However, in the absence of a heterozygous germ-line mutation, the likelihood for both alleles to be hit by a mutation in a single cell would seem extremely small. Therefore, if inactivation of a caretaker gene were to play an important role in the genesis of a sporadic tumor, this inactivation is not likely to result from biallelic DNA sequence alterations, but rather from epigenetic gene silencing, which is a frequently observed phenomenon in tumor cells.
Inherited cancer

Genes acting upstream in the pathway

Carriership of a mutation in one of the ‘upstream’ FA genes does not appear to appreciably contribute to cancer susceptibility. If a cancer-predisposing effect were to result from heterozygous mutations in any of the upstream FA genes one might expect to observe a significant proportion of sporadic malignancies carrying mutated $\textit{FANCB}$, which is X-linked and therefore present as only one active copy in a cell. Although comprehensive surveys have not been carried out so far, a recent screen in 95 familial breast cancers has failed to record any case of $\textit{FANCB}$ mutation (Garcia et al. 2008). The fact that FA patients, who are homozygous for an ‘upstream’ defect, have an extremely high risk to develop malignancies seems to be at variance with the absence of an increased cancer risk in heterozygous carriers. A possible explanation may be that loss of the wild type allele as an early event in a somatic cell of a heterozygous carrier individual would create a situation in which an FA-defective cell exists in a non-FA tissue; in such a situation this cell may have little chance to survive due to its intrinsic growth disadvantage. Support for this argument is provided by the observation of female $\textit{FANCB}$ mutation carriers, who demonstrated complete skewed X-inactivation towards the mutated allele (Meetei et al. 2004). Whether germline mutations in the ‘central’ genes $\textit{FANCI}$ and $\textit{FANCD2}$ are associated with increased cancer risk still remains to be examined in detail.

Genes acting downstream in the pathway

In contrast to mutations in the genes acting upstream in the FA pathway heterozygous germ-line mutations in the downstream-acting genes $\textit{BRCA1}$ and $\textit{BRCA2}$ are well known to impose an increased cancer risk, in particular breast and ovarian cancer. Heterozygous germ-line mutations in the remaining ‘downstream’ FA genes $\textit{FANCJ}$ and $\textit{FANCN}$ also confer an elevated risk for the development of breast cancer (Table 3), although to a lesser extent than mutations in $\textit{BRCA1}$ and -2. In this case the homozygously affected cells resulting from inactivation of the wild type allele for some reason may have better survival chances than cells with an upstream defect. Alternatively, haploinsufficiency may be the conductive force towards cancer progression in $\textit{BRCA}$ heterozygously affected cells, consistent with their caretaker characteristics. In contrast to the widely accepted hypothesis that loss of the wild-
type BRCA allele occurs in the development of breast cancer (Smith et al. 1992), it has recently been demonstrated that BRCA LOH is not required for BRCA-linked breast tumorigenesis (King et al. 2007). Either which way these genes are involved in tumorigenesis, it should encourage the search for additional FA genes that act downstream in the pathway, which may be relevant for both hereditary and sporadic cancer.

**Sporadic cancer**

*FA pathway defects due to gene silencing*

Abrogation of the FA/BRCA pathway through epigenetic inactivation of FA genes has been demonstrated in a proportion of sporadic malignancies (Table 4, Introduction; Chapters 5 and 6). However, the proportion of sporadic malignancies with methylated FANCF promoter sequences is likely to be overestimated due to methodological flaws in the procedure that has been used to assess the promoter methylation status, as demonstrated in Chapter 7. Methylation of FANCF, FANCC and FANCL seems firmly established in sporadic leukemias, albeit at a low frequency, in the order of 5-10%. That promoter methylation is indeed associated with gene silencing has only been demonstrated for one AML (Chapter 5), one ovarian carcinoma cell line (Taniguchi et al. 2003), and one bladder cell line (Neveling et al. 2007). For the remaining cases this validation was not obtained, even though the cells’ phenotype was consistent with the genes actually being inactive. Interestingly, FANCF promoter methylation has also been observed in two squamous cell carcinoma cell lines (2 out of 25, Chapter 8), which showed normal FANCD2 monoubiquitination, suggesting proficient FANCF expression. Whether FANCF promoter methylation is a coincidental event due to the localization of FANCF to a hypermethylation hotspot region (11p15) or a specific carcinogenesis promoting step causing genomic instability through the disruption of the FA/BRCA pathway, remains a subject of debate.

The relevance for FA gene methylation screening in leukemic patients to predict tumor response to treatment may possibly be determined in a retrospective study using a large cohort of patients that either responded favorably or poor to cross-linking agents containing regimens.
Yet-to-be-identified molecular defects

Based on direct mutation screening and protein analysis of DNA and cell lines obtained from unclassified FA patients, evidence for new FA subtypes, with both defects in the upstream and downstream parts of the FA/BRCA pathway has been presented in Chapter 2. Furthermore, in Chapter 8, the prevalence of FA/BRCA pathway deficiency was assayed in sporadic squamous cell carcinoma cell lines established from tumors that occur frequently in FA patients. The monoubiquitinated form of FANCD2 was present in all cell lines suggesting an intact FA core- and ID-complex. However, deficient protein expression of BRCA2 was found in two cell lines. As expected for BRCA2 deficient cells, these cell lines were sensitive to MMC in terms of growth inhibition, cell cycle arrest, and chromosomal breakage. Analysis of the other cell lines demonstrated abnormal expression of BRCA1 in 3 instances, which appeared to associate with hyper-responsiveness to MMC-induced cell cycle arrest. However, no pathogenic genetic mutations or promoter methylation were found that could explain the absence of the BRCA1 and 2 proteins in these cell lines. This observation suggested the involvement of additional mechanisms in addition to mutations and promoter hypermethylation that might abrogate protein expression or stability. These findings are in agreement with results recently reported by Burkitt and Ljungman (Burkitt & Ljungman 2007) that demonstrated BRCA1 deficiency in a subset of cisplatin-sensitive HNSCC cell lines, where the molecular nature of this apparent defect could also not be explained.

The aberrant levels of BRCA1/2 proteins might be explained by abnormal expression of interacting proteins that sustain BRCA1/2 stability, such as BRCA1-associated RING domain 1 (BARD1) (Wu et al. 1996) for BRCA1, and the protein defective in ‘deleted in spilt hand/spilt foot’ (DSS1) (Li et al. 2006), FANCN (Xia et al. 2006), and the inhibitor EMSY (Hughes-Davies et al. 2003) for BRCA2. However, normal levels of PALB2 protein were found and no mutation could be detected in DSS1, (and EMSY amplification remains to be analyzed). Alternatively, BRCA2 silencing could result from other epigenetic silencing mechanisms like histone H3 lysine 27 trimethylation, which can occur independent of promoter DNA methylation (Kondo et al. 2008), or inhibition of BRCA2 mRNA translation or mRNA degradation through microRNAs (Bartel 2004).
Interestingly, the majority of the analyzed cell lines that exhibited a FA/BRCA-like phenotype as assessed by MMC-induced cell cycle arrest, could not be explained by an apparent defect in BRCA1 or BRCA2, suggesting deficiencies in as yet unidentified proteins acting downstream in the FA/BRCA or related pathways. Hence, these cell lines may serve as cloning vehicles for the identification of novel players in FA/BRCA pathway.

FANCF promoter methylation has been reported for various types of malignancy suggesting inactivation of FA/BRCA pathway. The observation triggered many to support the proposition for an adapted adjuvant therapy treatment with cisplatin for tumors that harbor FANCF promoter methylation. Promoter methylation of FANCF is also reported in Chapter 8 for two squamous carcinoma cell lines, but did not seem to associate with gene silencing as FANCD2 monoubiquitination in these cells was normal. The partial methylation may suggest an early event resulting in the disruption of FA/BRCA pathway creating a mutator phenotype, followed by demethylation which would give a growth advantage to FANCF re-expressing cells during tumor development. Therefore, in order to exploit the FANCF promoter methylation as a potential predictor of tumor response to ICL agents, it is critical to ensure whether the methylation status is correlated with silencing. The clinical significance of FANCF promoter methylation for HNSCC patients that received cisplatin treatment is explored in Chapter 7, but did not demonstrate any predictive value. Moreover, a discrepancy in frequency of FANCF promoter methylation was observed between the data presented in Chapter 7 and that reported by Marsit et. al (2004), which might be explained by the used detection methods that may produce false-positive results (see chapter 7 for more detail).

Identification of FA/BRCA tumors

The generation of tools that could identify a functionally defective FA/BRCA pathway and hence may distinguish an ICL agent-sensitive tumor may demonstrate valuable implications for a more personalized therapy. The apparent disruption of the FA/BRCA pathway through BRCA1 and -2 impairment in cell lines may represent the condition of a proportion of tumors in situ. Furthermore, in Chapter 8 evidence for novel ‘downstream’ FA/BRCA key proteins has been shown based on hyper-responsiveness to ICL agents. Therefore, immunohistochemical staining for BRCA1
and -2 and for yet to be identified proteins may help identify ICL agent-sensitive tumors.

Recently, an assay has been demonstrated to measure the functional integrity of the FA/BRCA pathway in core biopsy samples from cancer patients. The technique is based on the evaluation of FANCD2, BRCA1, and RAD51 foci formation after irradiating the cells ex vivo (Powell & Kachnic 2008). This detection method may also be applicable on circulating tumor cells, isolated from the blood stream (Nagrath et al. 2007).

A defect in any of the 8 FA core complex members or a component of the ID-complex results in the failure to monoubiquitinate FANCD2 and FANCI at the lysine residues K561 and K523, respectively. The ability to specifically detect these modified forms might be exploited for the identification of a disrupted FA/BRCA pathway caused by a defective core- or ID-complex. Indeed, ongoing attempts are aimed to generate antibodies that distinctively detect the monoubiquitinated form of FANCD2 using branched peptide epitopes consisting of parts of FANCD2 protein flanking the K561 amino acid and a part of the ubiquitin molecule. The recently identified FANCD2 paralog, FANCI, may also be considered for the development of such specific antibodies.

_Treatment strategies for ‘FA/BRCA’ malignancies_

Chemotherapy treatment containing the ICL agent cisplatin or its derivatives, such as carboplatin and oxaliplatin has been used for various types of malignancies, including head and neck, testicular, breast, ovarian, cervical, lung, colorectal and lymphid tumors. The treated patients demonstrate dissimilar responses, while these chemotherapeutics can have severe side effects. Identifying patients that may optimally benefit from this specific treatment and excluding patients who may be subjected to ineffective treatment management with unnecessary burden is therefore very important. A higher response rate to platinum-based chemotherapy has been demonstrated in Jewish BRCA1/2 mutation carriers with ovarian cancer compared to non-hereditary ovarian cancer patients (Cass et al. 2003). In addition, patients with low/intermediate levels of BRCA1 mRNA were shown to have significantly improved overall survival following treatment with platinum-based chemotherapy as compared to patients with high levels of BRCA1 mRNA (Quinn et al. 2007). The observed sensitivity of BRCA1/2 cancer patients to ICL agents may also apply for cancer
patients who are carriers of mutations in other FA genes beside FANCD1/BRCA2. Retrospective examination of tumor biopsies from cancer patients who were subjected to treatment regimens containing ICLs may provide answers for this question.

**Synthetic lethality**

Synthetic lethality is explained as the occurrence of fatality in a cell when two genes or pathways are disrupted whereas inactivation of the individual gene or pathway has no effect on viability (Dobzhansky 1946). Recently, a synthetic lethal interaction has been described for the FA/BRCA pathway and ATM, where FA/BRCA deficient pancreatic cell lines were shown to be more sensitive to ATM inhibition as compared to their isogenic controls (Kennedy et al. 2007). Furthermore, Poly-ADP Ribose Polymerase (PARP) inhibitors, which have been shown to abrogate the base excision repair pathway (BER, involved in DNA single strand break repair), have been shown to cause selective cell killing in FA/BRCA deficient cells with defects in the FANCA, -C, -D1, and -D2 (Farmer et al. 2005; Bryant et al. 2005; McCabe et al. 2006). In the context of cancer treatment of familial BRCA1/2 and sporadic cases, with a disrupted FA/BRCA pathway, these small molecules may have major therapeutic implications as the use of sub-lethal doses of the drug kills the malignant cells with a defective FA/BRCA pathway, leaving the normal cells unharmed. Indeed, PARP inhibitors are currently being tested in clinical trials on breast and ovarian cancer of BRCA mutation carriers. Preliminary data from phase I trials with ovarian cancer patients carrying BRCA1/2 mutations showed promising results in terms of anti-tumor activity based on radiological and biochemical observations (Yap et al. 2007). Provided that a positive safety and efficacy of the described treatment approach is obtained for hereditary BRCA1/2 cancers, the approach might also be beneficial to sporadic tumors with a functionally impaired FA/BRCA pathway.

Recently, several compounds have been identified to elicit a strong growth inhibition in FA/BRCA-deficient cells compared to their isogenic controls (Gallmeier et al. 2007). These compounds seemed to function in a mechanistically distinct manner as compared to ICLs and showed a synergistic effect when combined with ICLs. These observations warrant further investigation of these attractive candidate agents for the treatment of FA/BRCA-deficient tumors.
Acquired resistance in ‘FA/BRCA tumors’

Although tumors treated with platinum-based chemotherapy initially have a high response, most of them eventually become resistant, as is documented for ovarian tumors (Agarwal & Kaye 2003). Several mechanisms of resistance can occur including, changes in cellular uptake and efflux of the drug, increased detoxification, alteration of the function or quantity of target, and increased repair of damaged DNA. Recently, resistant clones from a pancreatic cell line, which carries the protein-truncating c.6174delT frameshift mutation, were developed by culturing the cells in media containing cisplatin or PARP inhibitors (Sakai et al. 2008; Edwards et al. 2008). Resistance to both DNA damaging agents was found to result from intragenic mutations that restored the BRCA2 open reading frame (ORF), leading to expression of functional proteins. Examination of recurrent cancers from BRCA2-mutated ovarian carcinoma patients who received either cisplatin or carboplatin treatment demonstrated secondary BRCA2 ORF-restoring mutations (Sasaki & Tonomura 1973; Edwards et al. 2008). Similar observations were made with recurrent BRCA1-mutated ovarian cancers that were treated with platinum compounds, where four of the six recurrent platinum-resistant tumors had developed secondary genetic changes producing functional BRCA1 proteins (Swisher et al. 2008). Altogether, secondary mutations that restore the BRCA1/2 function suggest a major mechanism by which tumors acquire resistance to platinum-based chemotherapy.

Spontaneous genetic alterations compensating for inherited disease-causing mutation have been described to occur in hematopoietic cells of FA patients belonging to subtypes FA-A and –C (Lo Ten Foe et al. 1997; Waisfisz et al. 1999; Gregory, Jr. et al. 2001; Gross et al. 2002; Hirschhorn 2003; Hamanoue et al. 2006), -D1 (Ikeda et al. 2003), and in a lymphoblastoid cell line established from a FA-N patient (Chapter 3). In FA patients, these reversion events, which might be the consequence of genetic instability, may provide proliferative advantage of the reverted cell lineages leading to improved bone marrow function. However, in the context of FA/BRCA-defective tumors in non-FA patients, the mutator phenotype as a result of the inability to maintain genomic stability may give rise to ICL and PARP inhibitor resistant subclones in the tumor due to increased single strand annealing (SSA) that compensates for the defective homologous recombination process (Jonnalagadda et al. 2005). In such cases, inhibition of the SSA process, e.g. by interfering with RAD52 may circumvent resistance. So far, secondary mutations as a
mechanism of resistance has only been demonstrated for tumors that carried frameshift mutations in \textit{BRCA1} and -2, suggesting additional mechanisms of resistance in tumors with no such mutations.

\textit{Alternative approaches}

Given that tumor resistance to DNA damaging agents can occur through reactivation of the FA/BRC\textit{A} pathway, approaches that specifically target the pathway may partly provide a solution to the problem. Small molecule inhibitors of the FA/BRC\textit{A} pathway have been identified to sensitize breast and ovarian tumor cells to cisplatin, although their exact mechanism of action remains to be elucidated (Chirnomas \textit{et al.} 2006). Among the potent inhibitors, the natural compound curcumin, was identified and is currently investigated for its sensitizing effect to cisplatin in animal models. Alternatively, targeting the FA/BRC\textit{A} pathway in tumor cells could be achieved with viral delivery of dominant negative genes coding for enzyme components of the FA/BRC\textit{A} pathway (Ferrer \textit{et al.} 2004). This approach has been explored using an adenovirus over-expressing a FANCA variant, which disrupted the FANCD2-monoubiquitination ability in NSCLC cell lines. However, so far, moderate sensitization effects were reported (2 to 3 fold) mainly due to low infection efficiency. An improved bioengineering of viruses that target the tumor cells efficiently and specifically, which deliver more potent inhibitors of the FA/BRC\textit{A} pathway may hold the answer for successful cancer gene therapy.

Understanding the complex biological system of cancer is a prerequisite to ensure appropriate diagnosis, treatment and possibly prevention of the disease. The study of chromosomal instability disorders, among which FA, have increased our knowledge of mechanisms partly underlying cancer development, and at the same time provided us with possible treatment approaches that may enhance our chances in the battle against cancer. The goal of this thesis was to contribute to the effort of finding biomarkers, which may identify a subtype of tumors that may benefit from individualized treatment strategies. Evidence for additional key players in the FA/BRC\textit{A} pathway has been presented and their identification may have valuable implications for both better understanding of the pathway as well as new targets for advanced treatment strategies.
References


Chapter 10

Summary/Samenvatting
Summary

Cancer is characterized by uncontrolled cell growth and is caused by alterations in genes that regulate the cell cycle, apoptosis and communication between cells. These alterations are essentially due to misrepaired DNA damage, which may result from DNA replication errors as well as from chemically reactive agents produced by both endogenous and exogenous mechanisms. Yet, the spontaneous mutation rate in healthy individuals is unable to explain the life-time cancer risk. This is because cells are equipped with sophisticated molecular systems that are highly efficient in maintaining the integrity of the genome and keeping the mutation rate low. Failure of these systems to protect the genome increases the mutational load and increases the chance for growth-controlling genes to become dysregulated. That is precisely the case in individuals who suffer from genetic instability disorders, of which Fanconi anemia (FA) is a well-known example.

FA is a rare recessively inherited disorder characterized by diverse clinical symptoms, including organ malformations, bone marrow failure and increased cancer risk. To date, 13 genes have been identified that, when mutated, can cause FA. Among these genes, the breast/ovarian cancer susceptibility gene, BRCA2, has been found defective in a subgroup of FA patients. The proteins encoded by the FA genes appear to function in a distinct biochemical pathway, the ‘FA/BRCA pathway’, which acts to stabilize the genome. A defect in this pathway is not lethal, but has profound consequences, both at the cellular and clinical levels. Cells with an FA/BRCA pathway defect exhibit an extremely high sensitivity to DNA cross-linking agents, such as the chemotherapeutic agents cisplatin and mitomycin C (MMC).

The purpose of this thesis was two-fold. First, to expand our knowledge of the pathway by searching for novel genes that participate in the pathway (Chapters 2-4). Second, to explore the possible occurrence and significance of FA/BRCA pathway defects in sporadic cancer (Chapters 5-8).

In Chapter 2 a comprehensive genetic subtyping approach for FA is presented that is primarily based on mutation screening, supplemented by protein expression analysis and by functional assays to test for pathogenicity of unclassified variants. A total of 80 unselected FA patients were analyzed, of which 73 could be successfully
subtyped. Ninety-two distinct mutations were detected, of which 56 were novel. All known genetic subtypes were represented, except D2, J, L, and M. Four patients could not be assigned to any of the known subtypes; these patients may represent novel subtypes. One of these patients had a defect downstream of the FANCD2 monoubiquitination, whereas the remaining 3 were defective upstream of this step. The conclusion from this study was that direct mutation screening, in combination with functional tests, allows a molecular diagnosis of FA in the vast majority of patients. The patients that were unclassifiable by this approach likely represent novel genetic subtypes for which the underlying gene defect remains to be identified.

Chapter 3 reports that for one of these patients the defect could be traced to a gene that was known to encode PALB2, a protein that is essential for the stabilization and localization of BRCA2. This subtype, which was named FA-N, is characterized by relatively severe clinical symptoms and extreme susceptibility to childhood cancers, similar to the FA-D1 subtype (mutated in \textit{BRCA2}). Furthermore, as reported by others, individuals who carry a heterozygous mutation in this gene appear to have an increased risk to develop breast cancer. In Chapter 4, the occurrence of large \textit{PALB2} deletions, such as identified in the FA-N patient described in Chapter 3, was assessed in a large cohort (734) of familial breast cancer (FBC) patients but did not reveal the involvement of such aberrations in BC risk.

The extreme proneness of FA patients to develop malignancies such as acute myeloid leukemia (AML) and squamous cell carcinoma of the head and neck region, suggested the involvement of the FA/BRCA deficiency as an underlying mechanism in a proportion of these ‘sporadic’ malignancies in the general population. In Chapter 5, a functional disruption of the FA/BRCA pathway in a leukemic cell line was demonstrated to occur through promoter methylation of the \textit{FANCF} gene, which codes for an essential component of the pathway. In contrast to gene-disrupting mutations, promoter methylation is another mechanism by which genes can be inactivated and is frequently observed for other genes in various types of malignancies. These \textit{FANCF} deficient cells were hypersensitive to MMC, while reintroduction of the active \textit{FANCF} gene into these cells restored resistance to MMC. Promoter methylation of two other FA genes, \textit{FANCC} and \textit{FANCL}, in cells from sporadic leukemia patients (AML and ALL) is described in Chapter 6. In these cases the methylation was also correlated with hypersensitivity to MMC. To investigate the possible predictive value of \textit{FANCF} promoter methylation in head-and-neck
squamous cell carcinoma patients towards treatment strategies containing cisplatin, a panel of 22 patients of whom 11 responded favorably to the treatment were assessed for FANCF methylation in Chapter 7. In contrast to previous reports, promoter methylation was absent in all patients, which might partially be explained by the used detection method that is prone to produce false-positive results.

In an attempt to assess the involvement of the FA/BRCA pathway defects in sporadic cancer, 25 cell lines derived from tumors that occur frequently in FA patients were evaluated for their FA/BRCA pathway status (Chapter 8). Interestingly, as many as 65% of the cell lines demonstrated FA-like behavior in terms of sensitivity to MMC, suggesting an abrogated FA/BRCA pathway. All cell lines appeared to be able to monoubiquitinate FANCD2, suggesting a defect downstream of this step. However, only in a small portion of the MMC-sensitive cell lines could this phenotype be correlated with molecular defects: BRCA2 protein was undetectable in two cell lines, while BRCA1 appeared to be absent in 3 cell lines. The molecular basis for these aberrations was unclear, since we were unable to find sequence alterations in these genes that could explain the protein deficiencies. However, inactivation of the FA/BRCA pathway in the remaining “FA-like cell lines” could not be attributed to any of the known FA proteins, suggesting the involvement of (a) novel FA protein(s). Further analysis of these cell lines may result in the identification of new important player(s) in the FA/BRCA pathway. These possible new FA genes may turn out to be frequent targets that are inactivated in the oncogenesis process and may thus also serve as predictors for cancer treatment response.

In conclusion, the work presented in this thesis has contributed to the identification of a new breast cancer susceptibility gene, FANCN. Furthermore, evidence for novel FA genes yet to be identified has been presented. Identifying these genes will enhance our understanding of the FA/BRCA pathway. Given that inactivation of the FA/BRCA pathway renders cells hypersensitive to specific chemotherapeutic agents, identification of malignancies with such a defect may pinpoint patients that could benefit from adapted treatment strategies that exploit the Achilles heel of such malignancies: hypersensitivity to cross-linking agents.
Samenvatting

Het hoofdkenmerk van kanker is ongebreidelde groei, die is ontstaan als gevolg van veranderingen in genen die een rol spelen bij de regulatie van de celcyclus, celdood en communicatie tussen cellen. Die veranderingen zijn veroorzaakt door het falen van DNA schadeherstelprocessen, die geacht worden beschadigingen in het erfelijk materiaal te repareren. DNA schade wordt bij voortdurend aangebracht, als gevolg van bijvoorbeeld straling en chemische mutagentia. Spontane mutaties in het DNA komen weinig voor, omdat de cel er alles aan doet om de correcte genetische informatie te behouden, waarbij gebruik wordt gemaakt van ingewikkelde maar zeer efficiënte mechanismen die schade kunnen herstellen. Als zo’n mechanisme niet meer goed functioneert, kunnen mutaties zich ophopen en lopen de belangrijke genen die normale celgroei moeten garanderen gevaar om defect te raken, met als gevolg een verhoogd risico op het ontstaan van kanker. Deze situatie is van toepassing op patiënten die lijden aan een 'genetisch instabiliteitssyndroom', zoals Fanconi anemie (FA).


Het doel van deze studie was twee-voudig. Allereerst is getracht de kennis van het FA/BRCA pad uit te breiden door op zoek te gaan naar nieuwe genen, die deel uitmaken van dit pad (hoofdstukken 2-3). Daarnaast is onderzocht of en in hoeverre defecten in het FA/BRCA pad voorkomen bij sporadische gevallen van kanker (hoofdstukken 4-8).
Hoofdstuk 2 beschrijft een uitgebreide genetische analyse van 80 FA patiënten, primair gebaseerd op mutatiedetectie en aangevuld met een functionele analyse van de gevonden missense mutaties. Die analyse was nodig om onderscheid te maken tussen ongeclassificeerde varianten en echte ziekteverwekkende mutaties. Van de 80 patiënten konden 73 eenduidig worden geclassificeerd als behorend tot een van de bekende subtypes. In totaal zijn 92 mutaties gevonden, waarvan er 56 nog niet eerder waren gerapporteerd. Alle bekende subtypes werden aangetroffen, behalve D2, J, L en M. Vier patiënten konden niet aan één van de bekende subtypes worden toegerekend; deze patiënten zouden nieuwe subtypes kunnen vertegenwoordigen.

Eén van deze patiënten had een defect downstream van de FANCD2 monoubiquitinering, terwijl de overige 3 patiënten upstream van deze stap defect waren. De conclusie van deze studie was, dat mutatie detectie in combinatie met functionele testen een geschikte methode is om het moleculaire defect bij de overgrote meerderheid van FA patiënten te achterhalen. De patiënten die door deze methode niet konden worden geclassificeerd, vertegenwoordigen waarschijnlijk nieuwe genetische subtypes van de ziekte, waarvoor het onderliggende gendefect nog moet worden gevonden. Zoals beschreven in hoofdstuk 3, is dit gelukt voor één van de patiënten, bij wie een defect in PALB2 kon worden vastgesteld. Dit eiwit was al bekend als een partner eiwit van BRCA2 dat zorg draagt voor stabilisatie en correcte lokalisatie van BRCA2 in de cel. Patiënten behorend tot dit nieuwe genetische subtype, FA-N, lijden aan een relatief ernstige vorm van FA, waarbij sprake is van AML en solide tumoren op de vroege kinderleeftijd, hetgeen ook wordt waargenomen bij patiënten in de D1 groep, die een defect hebben in BRCA2. Uit het werk van anderen is bekend, dat draagsters van een mutatie in PALB2/FANCN een verhoogd risico hebben op het krijgen van borstkanker. In hoofdstuk 4 is getracht grote genetische deleties in het PALB2 gen, zoals die gevonden is in de FA-N patient welke beschreven is in hoofdstuk 3, te onderzoeken op prevalentie in familiaire borstkanker gevallen. Geen enkel geval van de 734 geanalyseerd patiënten bleek zulke mutaties te hebben wat suggereert dat dergelijke gen afwijkingen geen verklaring bieden voor de erfelijke vorm van borstkanker.

Het extreem hoge risico voor FA patiënten om maligniteiten te ontwikkelen zoals AML en plaveiselcel carcinomen, leidde tot de hypothese dat een defect in het FA/BRCA pad wellicht ook een rol zou kunnen spelen bij het ontstaan van dit type maligniteiten in de algemene populatie. Hiervan bleek in een aantal gevallen sprake

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te zijn. In hoofdstuk 5 wordt een cellijn afgeleid uit een sporadische AML beschreven met een defect in het FA/BRCA pad. Dit defect berustte op een deficiëntie van het FANCF eiwit. Deze deficiënte was terug te voeren op downregulatie van het FANCF gen als gevolg van promoter methylering. Naast inactivering van genen door schrijffouten in de genetische code, kan inactivering ook optreden door het ‘op slot zetten’ van een gen middels methylering van de deoxycytidines in de promoter sequentie. Een dergelijke vorm van gen-inactivering wordt in tumorcellen veelvuldig aangetroffen en kan dus in bepaalde gevallen ook leiden tot uitschakeling van het FANCF gen. De cellen waarin dit fenomeen werd aangetroffen waren als gevolg hiervan hypergevoelig voor MMC, zoals ook bij cellen met een mutatie in een FA gen wordt waargenomen. Zoals mocht worden verwacht, kon deze gevoeligheid worden gecorrigeerd door introductie van een plasmide dat het normale FANCF eiwit tot expressie bracht. Uitbreiding van dit onderzoek leidde tot de ontdekking, beschreven in hoofdstuk 6, dat inactivering door promoter methylering ook bij andere FA genen wordt waargenomen in sporadische leukemieën. Zo werd FANCC methylering gevonden in 1 AML en 3 ALL gevallen, en 1 geval van FANCL methylering in een ALL. In al deze gevallen was er sprake van hypergevoeligheid voor MMC. De mogelijk voorspellende rol van FANCF promoter methylering in hoofd-hals- tumor patiënten op behandeling die cisplatin bevat is onderzocht in hoofdstuk 7. In een groep van 22 patiënten met een dergelijke aandoening, waarvan de helft een goed response heeft gegeven op de behandeling, bleek geen enkele FANCF promoter methylering te hebben in tegenstelling tot eerdere studies. Een mogelijke verklaring kan liggen in de gebruikte methylerings detectie methode die gevoelig is voor het opleveren van vals-positieve resultaten.

Het onderzoek werd uitgebreid naar 25 cellijnen afgeleid van solide tumoren, die bij FA patiënten veelvuldig voorkomen: plaveiselcel carcinomen in de mondholte en de vulva/cervix. Een verrassend groot aantal (16/25; 65%) bleek hypergevoelig te zijn voor MMC. Echter, in slechts een minderheid kon deze gevoeligheid gecorrigeerd worden met een FA/BRCA defect, en wel in het downstream gedeelte van het FA/BRCA pad. In twee gevallen kon het eiwit BRCA2 niet gedetecteerd worden, terwijl in drie gevallen het eiwit BRCA1 leek te ontbreken. Het moleculaire defect dat aan deze afwijkingen ten grondslag ligt kon echter niet worden achterhaald. Bij alle 16 MMC-gevoelige tumorcellijnen werd een normale FANCD2 monoubiquitinering vastgesteld, zodat er geen sprake was van een defect upstream
in het FA/BRCA pad. Bij elf van de gevoelige cellijnen kon het (downstream) defect niet worden opgehelderd. Verder onderzoek zal moeten uitwijzen of hier sprake is van een defect in nieuwe componenten van het FA/BRCA pad.

Samenvattend kan gezegd worden dat het werk gepresenteerd in dit proefschrift heeft bijgedragen tot het identificeren van een nieuw gen (FANCN), dat participeert in het FA/BRCA pad van genoomprotectie, terwijl aanwijzingen zijn verkregen voor het bestaan van nog niet geïdentificeerde FA genen. Het vinden van die genen zal ons inzicht in het FA/BRCA pad kunnen verdiepen. Bovendien kunnen die genen nieuwe aanknopingspunten bieden voor betere diagnostiek en behandeling van kankerpatiënten.
List of publications

Kalkers NF, Ameziane N, Bot JC, Minneboo A, Polman CH, Barkhof F.


Curriculum vitae

Najim Ameziane is geboren op 5 september 1974 te Beni Ammart, Marokko. In 1995 behaalde hij zijn VWO diploma aan de Rijksscholengemeenschap 'De Drie Waarden' te Schoonhoven. Na een half jaar scheikunde te hebben gestudeerd aan de Universiteit van Utrecht is hij van studie veranderd en heeft hij zijn bul behaald voor de studie medische biologie aan de Vrije Universiteit te Amsterdam in 2001. In dat zelfde jaar is hij in contact gekomen met Prof.dr. Joenje bij wie hij de interesse voor de oncogenetica heeft ontwikkeld.

In 2002 heeft hij bij de sectie DNA en eiwit diagnostiek van de afdeling klinische genetica, onder supervisie van dr. Pals, de moleculaire diagnostiek voor de ziekte, Fanconi anemie, opgezet.

In 2004 heeft hij een beurs gehonoreerd gekregen van de Nederlandse organisatie voor wetenschappelijk onderzoek (NWO) om onderzoek te verrichten naar de rol van Fanconi anemie eiwitten in kanker waarvan het resultaat beschreven is in voorgaande pagina's.
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