The Fanconi anemia/BRCA2 pathway in pancreatic cancer
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CHAPTER 6

FUNCTIONAL DEFECTS IN THE FANCONI ANEMIA PATHWAY IN PANCREATIC CANCER CELLS

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Abstract
Biallelic BRCA2-mutations can cause Fanconi anemia (FA) and are found in approximately 4-7% of pancreatic cancers. Recently, several sequence changes in FANCC and FANCG were reported in pancreatic cancer. Functional defects in the FA pathway can result in a marked hypersensitivity to DNA-interstrand crosslinking agents, such as mitomycin C. The functional implications of mutations in the upstream FA pathway in cancer have not been fully studied yet; these studies are needed to pave the way for clinical trials of treatment with crosslinking agents of FA-defective cancers. The competence of the proximal FA pathway was screened in 21 pancreatic cancer cell lines by an assay of Fancd2 mono-ubiquitination using a Fancd2 immunoblot. The pancreatic cancer cell lines Hs766T and PL11 were defective in Fancd2 monoubiquitination. In PL11, this defect led to the identification of a large homozygous deletion in FANCC. The FA-defective cell lines Hs766T, PL11 and CAPAN1 were each hypersensitive to the DNA-interstrand crosslinking agent mitomycin C and some to cisplatin, as measured by cell survival assays and G2/M cell cycle arrest. These results support the practical exploration of crosslinking agents for non-Fanconi anemia patients that have tumors defective in the FA pathway.

Introduction
BRCA2 mutations have been shown to be responsible for the Fanconi Anemia (FA) syndrome in a subset of patients: those with FA complementation group D1\textsuperscript{42}. Pancreatic cancers harbor the highest percentages of BRCA2 mutations, present in 4-7% of sporadic pancreatic cancers (all accompanied by loss of the wild-type allele), 12% of familial pancreatic cancer and 17% of families with a strong history of the disease\textsuperscript{38, 59, 144, 145}. Recently, we analyzed a panel of pancreatic cancer xenografts and cell lines for mutations in FANCC and FANCG - essential components of the upstream FA pathway\textsuperscript{44}. We identified several variants, including a homozygous nonsense mutation in FANCG (E105ter), a homozygous frameshift mutation in FANCC, and several amino acid changes\textsuperscript{44}. Brca2 is believed to function downstream of Fancd2 in the FA pathway; the proximal FA pathway and BRCA2 are in this study collectively referred to as the FA pathway. The function of the FA pathway remains to be fully elucidated, but seems to be required for an adequate response to DNA damage as caused by DNA-interstrand crosslinking agents, espe-
cially mitomycin C (MMC) and diepoxybutane: FA cells have an increased sensitivity to MMC.

Pancreatic cancer is diagnosed in more than 50,000 people in the United States each year and remains one of the deadliest forms of cancer, despite surgery or chemotherapeutic treatment. The existence of FA-proficient hosts harboring pancreatic cancers that are defective in the FA pathway could have important implications for clinical treatment: the tumor could be hypersensitive to DNA-interstrand crosslinking agents, whereas the patient would not. Several studies have reported long-term remissions in pancreatic cancer in response to MMC, although the link with FA defects has never been evaluated clinically. The BRCA2-defective cell line CAPAN1 has been shown to be hypersensitive to ionizing radiation (IR) and some chemotherapeutics. There remained unanswered questions that would impair the design of clinical therapeutic trials. The repeated hypersensitivity of FA-deficient nonneoplastic cells to MMC and cisplatin had not been evaluated in cancer cells mutated in the FA pathway, but would be essential for advising the dosing that might be advised in cancer patients.

In the current study, we survey the frequency with which the FA pathway is functionally defective in pancreatic cancer and whether these defects lead to an increased sensitivity to crosslinking agents. We found the FA pathway, as functionally screened for Fancd2 monoubiquitination in response to MMC, to be defective in Hs766T, a pancreatic cancer cell line carrying a nonsense mutation in FANC G. A FA defect was also identified in an additional pancreatic cancer cell line, PL11, and a genetic cause was uncovered. The FA-defective cell lines Hs766T, PL11 and CAPAN1 had an increased sensitivity to DNA-interstrand crosslinking agents. The importance of these results is underscored by the fact that despite an advanced understanding of many genetic features of pancreatic cancer, no rational therapy specifically targeting genetic defects has been used to date.

Materials & Methods

Samples

Pancreatic cancer cell lines MiaPaCa2, BxPC3, Panc-1, AsPC1, Su86.86, CFPAC, CAPAN1, CAPAN2, Hs766T, HpaF II, Colo357, Mpanc96 were obtained from ATCC (American Type Culture Collection, Manassas, Virginia) and ECACC (European Collection of Animal Cell Cultures).
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Salisbury, UK). Pancreatic cancer cell lines Panc 3.27 (PL11), Panc 6.03, Panc 8.13, Panc 2.03, Panc 2.13, Panc 1.23, Panc 4.21, Panc 5.04, PL5, PL6 and PL13 were kindly provided by Dr. E. M. Jaffe (Department of Oncology, Johns Hopkins University); PL45 was created in our lab60. Panc 3.27, Panc 6.03, Panc 8.13, Panc 2.03, Panc 2.13 and PL45 are also available from ATCC. Cells were grown in media supplemented with 10% fetal bovine serum, penicillin/streptomycin and L-glutamine. Pancreatic cancer xenograft PX191 was established as previously described25.

Fancd2 immunoblots
Equal numbers of cells were grown in 6-well plates and treated with or without MMC, 45 nM, for 18-24 h. Cells were lysed, boiled and loaded on 3-8% tris-acetate polyacrylamide gels (Invitrogen, Carlsbad, California). Protein was transferred onto a PVDF membrane and blocked for one hour in TBST (tris-buffered saline; Tween-20) 5% milk. Blots were incubated with mouse anti-Fancd2 antibody (sc20022, Santa Cruz Biotechnology, Santa Cruz, California), diluted 1:1000 and incubated overnight at room temperature. Blots were washed with TBST and incubated with goat anti-mouse HRP. Binding was detected using Super Signal West Pico chemiluminescence substrate (Pierce Biotechnology, Rockford, Illinois). Defects in Fancd2 monoubiquitination were confirmed with separately prepared lysates.

Retroviral correction
The retroviral expression vectors pMMP-puro149, pMMP-puroFANCC150 and pMMP-puroFANCG151 were generously provided by Dr. A.D. d’Andrea (Dana-Farber Cancer Institute, Boston, MA) and were used as previously described152.

Sequencing and deletion mapping
FANCA, FANCC, FANCD2, FANCE, FANCF and FANCG were sequenced by automated sequencing. Primers for determination of the breakpoints of the homozygous deletion were purchased from IDT DNA (Coralville, Iowa).

Survival studies
Picogreen: 1.2 x 10^5 cancer cells per well were incubated with various concentrations of MMC (Sigma, Saint Louis, Missouri: range 0–4.5 μM) or cisplatin (Sigma: range 0-10 μM) in 96-well plates. Cells were incubated for a period of time long enough to allow non-treated cells to
reach at least a threefold increase in fluorescence as compared to day 1 (3-7 days). Medium was changed every 48 hours. Cells were washed with PBS, and lysed in 100 μL sterile water. After 1 hour, 100 μl 0.5% Picogreen (Molecular Probes, Eugene, Oregon) in tris-EDTA buffer was added to each well. After 45 minutes, wells were read in a fluorometer. Survival was calculated as a percentage; the wells without drugs were considered as 100%. Each experiment was done in duplicate; at least six experiments per cell line per concentration were performed.

Cell counts: 1 x 10^5 cells were plated in tissue culture flasks (25 cm^2). The next day, the medium was substituted with MMC-containing medium (range, 0-4.5 μM). Cells were counted after 3-4 population doublings (4-7 days) using a hemacytometer. Four experiments per cell line per concentration were done.

**Cell cycle analysis**

Cells were cultured in 25 cm^2 flasks and treated with MMC for two hours. Cells were washed with PBS and incubated with normal tissue culture medium for 48 hours. Cells were obtained by trypsinization and resuspended in 3.7% paraformaldehyde in PBS, stained with Hoechst 33258 (Sigma), incubated at 4°C for 10 min and analyzed using a flow cytometer. A “G2/M arrest” was defined as a twofold increase of the percentage of cells in G2/M, as compared to untreated cells.

**Results**

**FA pathway defects identified by Fancd2 monoubiquitination**

The FA proteins Fanca, Fancc, Fance, Fanf and Fancg assemble in a nuclear complex in response to DNA damage from crosslinking agents. This multiprotein complex is required for the monoubiquitination of Fancd2. Recently, evidence has been provided that PHF9 (FANDCL), another member of the FA nuclear complex, has an important role in Fancd2 monoubiquitination. An immunoblot for Fancd2 after MMC treatment normally detects a short (Fancd2-S; 155 kD) and a long (Fancd2-L, mono-ubiquitinated; 162 kD) isoform. The presence of only the short band is indicative of a defect in the upstream FA pathway. To assess the pathogenicity of previously described changes in the FANCC and FANCG genes in pancreatic cancer cell lines, a Fancd2 immunoblot of cells treated with MMC was used to analyze Hs766T (FANCG, E105ter), CAPAN1 (FANCG, S7F), Su86.86 (FANCC, M350V), and CAPAN2.
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**Figure 6.1** Screen for FA defects by Fancd2 monoubiquitination assay. Equal cell numbers were untreated, or incubated with MMC for 18-24 hours, after which protein lysates were made. Protein lysates were immunoblotted for Fancd2. Lack of the upper band indicates a defect in the proximal Fanconi pathway. EV, empty retroviral vector; FA-C/G, retroviral FANCC/G expression vector.

(FANCC, E521K, heterozygous) cells (*Figure 6.1 and data not shown*). Hs766T cells contained only the Fancd2-S isoform, indicating a defect in Fancd2 monoubiquitination. The other cell lines had normal Fancd2 monoubiquitination, indicating that the variants in these cell lines are not null alleles. The Brca2 protein functions downstream of Fancd2 in the FA pathway or in a separate pathway with overlapping functions.\(^4^6\)

CAPAN1 cells, carrying a mutation in \(\text{BRCA2}\), were thus found to undergo Fancd2 monoubiquitination (*Figure 6.1*).

We next extended our functional test of the FA pathway to a panel of 17 additional pancreatic cancer cell lines (*Figure 6.1 and data not shown*). One additional cell line, pancreatic cancer cell line PL11, was defective in Fancd2 monoubiquitination. We next examined PL11 for genetic defects in \(\text{FANCC}\) and \(\text{FANCG}\), the only FA genes proximal to \(\text{BRCA2}\) shown to date to be mutated in cancers in non-FA patients. The pancreatic cancer cell line PL11 had a deletion of eight exons of the \(\text{FANCC}\) gene: exons 7-14 (*Figure 6.2*). This deletion was further analyzed by PCR with additional primer sets: at the 5' end, the breakpoint was found to occur between IVS6+88 and IVS6+1018; at the 3' end the breakpoint was mapped down to a region between 15,057 and 20,846 basepairs downstream from the stop codon. PL11 was derived independently from the same surgically resected cancer as was the xenograft PX192.

Analysis of this xenograft showed the same homozygous deletion (data not shown), proving unequivocally that this homozygous deletion must have been present in the original tumor and did not occur in cell culture.
Figure 6.2 Homozygous deletion of exons 7-14 in pancreatic cancer cell line PL11. DNA from pancreatic cancer cell line BxPC3 was used as a control; exons for both samples were amplified in the same PCR plate. Independent reactions were used to confirm the deletion in PL11 and in the parallel xenograft PX192.

A heterozygous polymorphism was encountered between exons 7 and 8 in normal DNA taken from the same patient, indicating that the deletion was somatic, with a larger deletion of the other allele (data not shown). To provide additional evidence that FANCC and FANCG are the mutated genes responsible for the FA defects observed in PL11 and Hs766T, respectively, we retrovirally transduced PL11 with either FANCC or an empty vector (EV), and transduced Hs766T with either FANCG or EV. In PL11, Fancd2 monoubiquitination was restored by transduction with FANCC, but not with EV. Likewise, in Hs766T Fancd2 monoubiquitination was restored by expression of FANCG, but not by EV (Figure 6.1).

**FA defects are associated with increased cytotoxicity by crosslinking agents**

The FA-defective pancreatic cancer cell lines Hs766T (FANCG-mutated), PL11 (FANCC-mutated) and CAPAN1 (BRCA2-mutated) and FA-proficient cell lines Su86.86 and MiaPaCa2 were treated with various concentrations of either MMC or cisplatin, and incubated in 96-well plates. Relative cell numbers were determined by measurement of double stranded DNA content using Picogreen; wells containing no compound were used as
controls. The FA-defective cell lines Hs766T, PL11 and CAPAN1 had an increased sensitivity to MMC, as compared to MiaPaCa2 and Su86.86 (Figure 6.3A). CAPAN1 and PL11 were hypersensitive to cisplatin (Figure 6.3B); Su86.86 and Hs766T were less sensitive than CAPAN1 and PL11, but had an increased sensitivity to cisplatin as compared to MiaPaCa2. To confirm the results obtained with the Picogreen assay, we also assessed sensitivity to MMC of the cell lines MiaPaCa2, Su86.86, CAPAN1 and Hs766T with manual (hemacytometer) cell counts. This assay confirmed their hypersensitivity to MMC (Figure 6.3C).

**G2/M cell cycle arrest by low-dose DNA-interstrand crosslinking agents in FA-defective cancer cells**

The methods used to investigate cell “survival” upon treatment with MMC and cisplatin integrate the influence of cell death, slow growth and the occurrence of a cell cycle arrest. To determine the potential independent contribution of an arrest and to confirm this MMC hypersensitivity in a separate system, we analyzed cell cycle distributions of DNA content after MMC treatment. Six pancreatic cancer cell lines (BxPC3, MiaPaCa2, Su86.86, PL11, Hs766T and CAPAN1) were analyzed 48 hours after MMC treatment for 2 hours; a G2/M arrest was defined as a twofold increase of the fraction of cells containing 4N DNA content as compared to untreated cells (Figure 6.4). FA-defective cancer cells had a striking difference as compared to FA-proficient cells in the concentration of MMC that caused a G2/M arrest. Hs766T arrested in G2/M at a MMC
concentration of 100 nM, PL11 at 100 nM and CAPAN1 at 200 nM, whereas control pancreatic cancer cell lines MiaPaCa2, Su86.86 and BxPC3 arrested at MMC concentrations as high as 2 mM. These results further established the hypersensitivity of FA-defective cancer cells to DNA-interstrand crosslinking agents.

**Mutational screen of FANCA, C, D2, E, F, G**

The FA genes FANCA, FANCC, FANCD2, FANCE, FANCF and FANCG were sequenced in 24 xenografted nonfamilial pancreaticobiliary adenocarcinomas. No additional mutations were detected.

**Discussion**

In recent years, the emerging field of targeted chemotherapeutics, in particular the targeting of specific genetic defects in cancer, has received much attention. Yet, practiced examples of such therapies are not often encountered. Defects in the FA pathway may provide a vulnerable target for therapeutics, specifically using the DNA-interstrand crosslinking agents. The hypersensitivity of cells taken from FA patients to crosslinking agents and to ionizing radiation already suggests this utility. Although tumors that develop in FA patients cannot easily be treated with these therapies because of toxicity, FA-defective tumors in individuals who carry no mutation or only one (recessive) mutation in FA genes may offer a highly augmented therapeutic response to crosslinking agents, fortuitously with little anticipated toxicity to the patient. Pancreatic cancer, diagnosed in more than 30,000 people in the United States yearly, is one of the most aggressive forms of cancer, leading to death in an overwhelming majority of patients within a few years despite surgery and/or chemotherapeutic treatment. Several lines of evidence suggest the use of combinations of chemotherapy containing MMC and other crosslinking agents to be beneficial for pancreatic cancer patients. Although a significant increase in survival is usually not found, occasional complete and long-term remissions are reported. These reports have not incorporated the genetic testing of these patients, but a gene defect in BRCA2, FANCC, FANCG or another gene in the FA pathway could in theory cause a therapeutically useful hypersensitivity, providing an “Achilles’ heel” in a subset of pancreatic cancers. Perhaps the first link between (pancreatic) cancer and FA was observed as early as 1976 in a Scottish family: a consanguineous pedi-
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Figure 6.4 FA-defective cancer cell lines arrest in G2/M 48 hours after low concentrations of MMC. Cells were treated with various concentrations of MMC for 2 hours, and incubated without MMC for 48 hours, after which the cell cycle was analyzed using a flow cytometer.

Degree was described in which one person had FA, and obligate mutation carriers displayed multiple occurrences of pancreatic and other cancers. Approximately 4-7% of sporadic pancreatic cancers carry mutations in BRCA2, accompanied by LOH. The pancreatic cancer cell line CAPAN1 is derived from such a tumor. To date, one convincing somatic mutation in the FANCC gene, accompanied by LOH and resulting in truncation of the carboxyl-terminus of the protein, has been reported in a pancreatic cancer xenograft. We here show that the pancreatic cancer cell line PL11 has a related defect, a homozygous deletion of exons 7-14 of FANCC and a functional defect in the FA pathway as measured by Fancd2 monoubiquitination. The defect in Fancd2 monoubiquitination
could be corrected by retroviral transduction with FANCC. PL11 was derived from a pancreatic cancer patient who died from her disease at age 65. In FANCC, one germline mutation (E103ter) has been reported, which we show in this study to be pathogenic, resulting in a defect in Fancd2 monoubiquitination in cancer cells that could be corrected by retroviral transduction with FANCC. Assays for FA function offer the potential to screen patients’ tumors for genetic defects by cytological methods or immunohistochemistry. Here, we demonstrate the first successful functional screen that led to the identification of a genetic FA defect in a patient’s cancer.

In addition to the mutations in FANCC and FANCG reported previously, we sequenced FANCA, C, D2, E, F and G in 24 pancreatic cancer xenografts. No additional mutations were found. Combining the different methods of screening for defects in FANCC and FANCG (by Fancd2 immunoblot and by direct sequencing), we have now analyzed 70 tumors for FANCC and 72 tumors for FANCG. Three convincing mutations were found: two in FANCC (3%) and one in FANCG (1%). Combined with a BRCA2 mutation rate of 4-7%, the FA pathway could be defective in as much as 7-10% of pancreatic cancer. Larger studies should establish the exact percentage of defects in the FA pathway in pancreatic cancer.

The FA-defective cell lines CAPAN1, PL11 and Hs766T are all hypersensitive to MMC, as compared to other pancreatic cancer cell lines. These findings may provide an explanation for the anecdotal observations that a subset of pancreatic cancers is highly sensitive to MMC-containing regimens125. One could envision that pancreatic cancers might be genetically tested for defects in the pathways that repair DNA-interstrand crosslinks, such as the FA pathway. Patients with a defect in one of the repair pathways could then be treated rationally with DNA-interstrand crosslinking agents, possibly at a much lower dose than is customary.

The FA-proficient cell lines BxPC3, Su86.86 and MiaPaCa2 arrest in G2/M only at high doses of MMC (2 μM), whereas the FA-defective cell lines Hs766T, PL11 and CAPAN1 arrest in G2/M after a dose of MMC that was 10 times lower or less (100-200 nM), providing additional evidence of the MMC hypersensitivity of FA-defective cancer cell lines. In a study by Heinrich et al., cell cycle changes in FANCC-defective FA lymphoblasts and FA-proficient cells were described in response to various concentrations of MMC. Similar degrees of G2/M arrest after equitoxic doses of MMC were found: normal cells show the same G2/M
arrest as FA-defective cells do, only at a much higher dose\textsuperscript{156}. We saw the same pattern in pancreatic cancer cells. Thus, the arrest at a relatively low dose of MMC in FA-defective cells probably reflects the increased level of damage caused by low level MMC in the absence of the FA-pathway\textsuperscript{157}, rather than a failure of major checkpoints. Akkarı et al.\textsuperscript{158} compared the response of FA lymphoblasts and retrovirally corrected controls to DNA-interstrand crosslinks induced by psoralen-UVA. FA lymphoblasts were able to recover from substantial amounts of DNA-interstrand crosslinks, only after a threefold to fivefold longer growth arrest than in corrected isogeneic controls. The difference in duration of growth arrest was attributed by the authors to a decreased rate of DNA-interstrand crosslink removal. It is unclear whether the cell lines assayed in our study resume the cell cycle normally after an initial arrest, or proceed to apoptosis after a short G2/M arrest.

The degree of MMC hypersensitivity of the FA-deficient cancer cells is more moderate than was suggested in previous studies of nonneoplastic FA cells. This realization is perhaps critical for the planning of clinical trials. It also emphasizes the importance of studies of cancer cells when attempting to extrapolate from basic studies to clinical environments. Additionally, hypersensitivity to cisplatin was relatively moderate in Hs766T. Other genetic defects may alter the sensitivity in these cell lines, although no genetic defect that could account for the observed variability in chemosensitivity in these cell lines is known to us. For example, all cell lines assessed for MMC and cisplatin sensitivity in this study, except for PL11, have been analyzed for mutations in TP53 (p53) and CDKN2A (p16); all were mutated in both genes.

Although pancreatic cancer remains the only form of cancer (in non-FA patients) known to harbor upstream FA pathway mutations to date, mutations in this pathway are unlikely to be restricted to cancers of the pancreas. Two ovarian cancer cell lines were recently shown to be defective in the FA pathway, which was attributed to FANCF-methylation\textsuperscript{159}. Epidemiological studies so far have not found relatives of FA patients to be at an increased risk for cancer. This could be explained by a low penetrance of mutations in the upstream FA pathway. Also, an increased cancer risk for individuals with mutations in one of the FA genes could be missed due to heterogeneity among the patient populations studied. FANCA is the most commonly mutated gene in the general population. Therefore, low penetrance mutations in FANCC and FANCG, contributing to the development of less common forms of cancer, such as cancer
of the pancreas, could be missed in epidemiological studies. More extensive studies of cancer incidence in FA mutation carriers are needed. These studies should aim to look at carriers of mutations in different complementation groups separately, and with adequate numbers to achieve statistical power.

An early detection of defects in the FA pathway in pancreatic cancer could perhaps lead to a better treatment for some patients and a better assessment of risk for family members. The results presented in this study provide pivotal support for the clinical investigation of the possibility to identify patients with pancreatic cancers defective in the FA pathway and to attempt successful treatment of these patients with DNA-interstrand crosslinking agents.

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