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Functional Defects in the Fanconi Anemia Pathway in Pancreatic Cancer Cells

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Biallelic *BRCA2*-mutations can cause Fanconi anemia and are found in ~7% of pancreatic cancers. Recently, several sequence changes in *FANCC* and *FANCG* were reported in pancreatic cancer. Functional defects in the Fanconi pathway can result in a marked hypersensitivity to interstrand crosslinking agents, such as mitomycin C. The functional implications of mutations in the Fanconi 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 Fanconi-defective cancers. The competence of the proximal Fanconi pathway was screened in 21 pancreatic cancer cell lines by an assay of Fancd2 monoubiquitination 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 first cancer cell line found to be *FANCC*-null. The Fanconi-defective cell lines Hs766T, PL11, and CAPAN1 were hypersensitive to the crosslinking agent mitomycin C and some to *cis*-platin, as measured by cell survival assays and G_2/M cell-cycle arrest. These results support the practical exploration of crosslinking agents for non-Fanconi anemia patients that have tumors defective in the Fanconi pathway. *(Am J Pathol 2004, 165:651–657)*

BRCA2 mutations have been shown to be responsible for a subset of Fanconi anemia (FA) patients of complementation group D1 and perhaps B.[1] Pancreatic cancers harbor the highest percentages of *BRCA2* mutations, present in 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.[2–5] Recently, we analyzed a panel of pancreatic cancer xenografts and cell lines for mutations in *FANCC* and *FANCG*—essential components of the upstream Fanconi pathway.[6] We identified several variants, including a homozygous germline nonsense mutation in *FANCG* (E105ter), a homozygous somatic frameshift deletion in *FANCC*, and several amino acid changes.[6] Brca2 is believed to function downstream of Fancd2 in the Fanconi pathway; the proximal Fanconi pathway and *BRCA2* are in this study collectively referred to as the Fanconi 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 crosslinking agents, especially mitomycin C (MMC) and diepoxybutane: FA cells have an increased sensitivity to MMC. Pancreatic cancer is diagnosed in more than 30,000 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 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.[7–9] The *BRCA2*-defective cell line CAPAN1 has been shown to be hypersensitive to ionizing radiation and some chemotherapeutics.[10–12] There remained unanswered questions that would impair the design of clinical therapeutic trials. The repeated hypersensitivity of Fanconi-deficient nonneoplastic cells to MMC and *cis*-platin had not been evaluated in cancer cells mutated in the Fanconi pathway, but would be essential for estimating 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 *FANCG*. A new Fanconi defect was also identified by functional screening in an additional

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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 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 and Methods

Samples

Pancreatic cancer cell lines MiaPaCa2, BxPC3, Panc-1, AsPC1, Su86.86, CFPAC, CAPAN1, CAPAN2, Hs766T, Hpaf II, Colo357, and Mpanc96 were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and European Collection of Animal Cell Cultures (ECACC) (Salisbury, UK). Pancreatic cancer cell lines Panc 3.27 (PL11), Panc 6.03, Panc 8.13, Panc 2.03, Panc 2.13, Panc 1.28, Panc 4.21, Panc 5.04, PL3, PL5, PL6, and PL13 were kindly provided by Dr. E.M. Jaffee (Department of Oncology, Johns Hopkins University, Baltimore, MD); PL45 was created in our lab.[13] Panc 3.27, Panc 6.03, Panc 8.13, Panc 2.03, Panc 2.13, and PL45 are also available from ATTC. Cells were grown in media supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine. Pancreatic cancer xenografts were established as previously described.[14]

Fancd2 Immunoblots

Equal numbers of cells were grown in six-well plates and treated with or without MMC, 45 nmol/L, for 18 to 24 hours. Cells were lysed, boiled, and loaded on 3 to 8% Tris-acetate polyacrylamide gels (Invitrogen, Carlsbad, CA). Protein was transferred onto a polyvinylidene difluoride membrane and blocked for 1 hour in TBST (Tris-buffered saline, Tween-20) 5% milk. Blots were incubated with mouse anti-Fancd2 antibody (sc20022; Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:1000 and incubated overnight at room temperature. Blots were washed with TBST and incubated with goat anti-mouse horseradish peroxidase. Binding was detected using Super Signal West Pico chemiluminescence substrate (Pierce Biotechnology, Rockford, IL). Defects in Fancd2 monoubiquitination were confirmed with separately prepared lysates.

Retroviral Correction

The retroviral expression vectors pMMP-puro,[15] pMMP-puroFANCC,[16] and pMMP-puroFANCG[17] were generously provided by Dr A. D. d'Andrea (Dana-Farber Cancer Institute, Boston, MA) and were used as previously described.[18]

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, IA).

Survival Studies

Picogreen

Cancer cells (1.2×10^3 per well) were incubated with various concentrations of MMC (range, 0 to 4.5 μmol/L; Sigma, St. Louis, MO) or *cis*-platin (range, 0 to 10 μmol/L; Sigma) in 96-well plates. Cells were incubated for a period of time long enough to allow nontreated cells to reach at least a threefold increase in fluorescence as compared to day 1 (3 to 7 days). Medium was changed every 48 hours. Cells were washed with phosphate-buffered saline (PBS) and lysed in 100 μl of sterile water. After 1 hour, 100 μl of 0.5% Picogreen (Molecular Probes, Eugene, OR) in Tris-ethylenediaminetetraacetic acid 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

Cells (1×10^5) were plated in tissue culture flasks (25 cm^2). The next day, the medium was substituted with MMC-containing medium (range, 0 to 4.5 μmol/L). Cells were counted after three to four population doublings (4 to 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 2 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 minutes, and analyzed using a flow cytometer. A G_2/M arrest was defined as a twofold increase of the percentage of cells in G_2/M, as compared to untreated cells.

Results

FA Pathway Defects Identified by Fancd2 Monoubiquitination

The FA proteins Fanca, Fancc, Fance, Fancf, 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* (*FANCL*), another

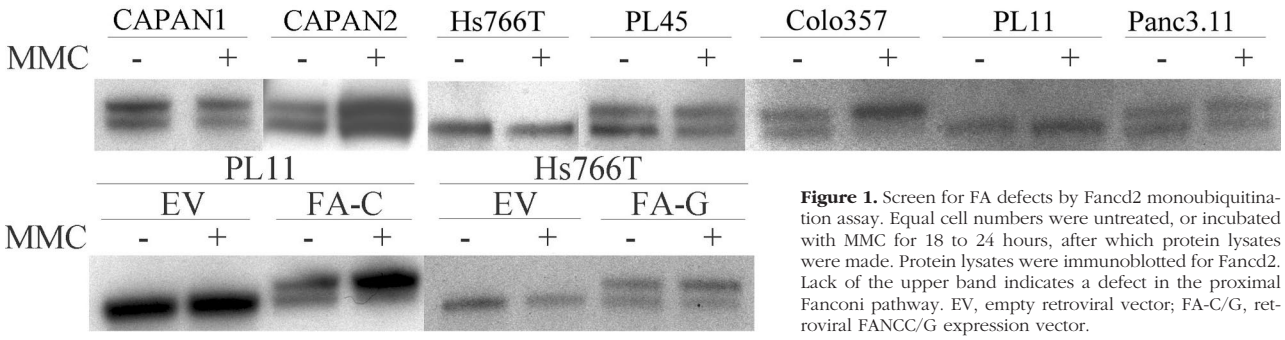

Figure 1. Screen for FA defects by Fancd2 monoubiquitination assay. Equal cell numbers were untreated, or incubated with MMC for 18 to 24 hours, after which protein lysates were made. Protein lysates were immunoblotted for Fancd2. Lack of the upper band indicates a defect in the proximal Fanconi pathway. EV, empty retroviral vector; FA-C/G, retroviral FANCC/G expression vector.

member of the Fanconi nuclear complex, has an important role in Fancd2 monoubiquitination.[19] 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.[20] To assess the pathogenicity of previously described changes in the *FANCC* and *FANCG* genes in pancreatic cancer cell lines,[6] a Fancd2 immunoblot of cells treated with MMC was used to analyze Hs766T (*FANCG*, E105ter), CAPAN1 (*FANCG*, S7F), Su86.86 (*FANCC*, M350V), and CAPAN2 (*FANCC*, E521K, heterozygous) cells (Figure 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.[21] CAPAN1 cells, carrying a mutation in *BRCA2*,[2] were thus found to undergo Fancd2 monoubiquitination (Figure 1).

We next extended our functional test of the FA pathway to a panel of 17 additional pancreatic cancer cell lines (Figure 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 *FANCC* and *FANCG*, the only FA genes proximal to *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 *FANCC*: exons 7 to 14 (Figure 2). This deletion was further analyzed by polymerase chain reaction 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 to a region between 15,057 and 20,846 bp 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 breakpoints of the 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. 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).

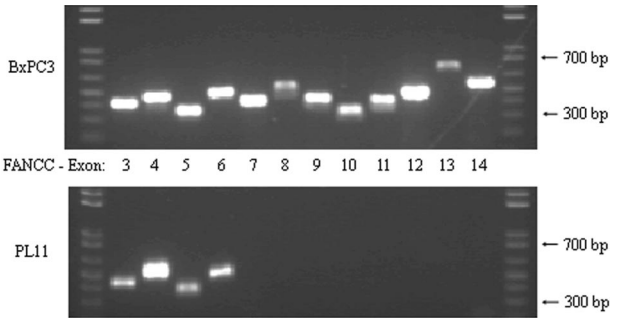

Figure 2. Homozygous deletion of exons 7 to 14 in pancreatic cancer cell line PL11. DNA from pancreatic cancer cell line BxPC3 was used as a control; exons for both samples were amplified in the same polymerase chain reaction plate. Independent reactions were used to confirm the deletion in PL11 and in the parallel xenograft PX192.

To provide additional evidence that *FANCC* and *FANCG* are the mutated genes responsible for the Fanconi 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 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 *cis*-platin, 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 3A). CAPAN1 and PL11 were hypersensitive to *cis*-platin (Figure 3B); Su86.86 and Hs766T were less sensitive than CAPAN1 and PL11, but had an increased sensitivity to *cis*-platin 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 3C).

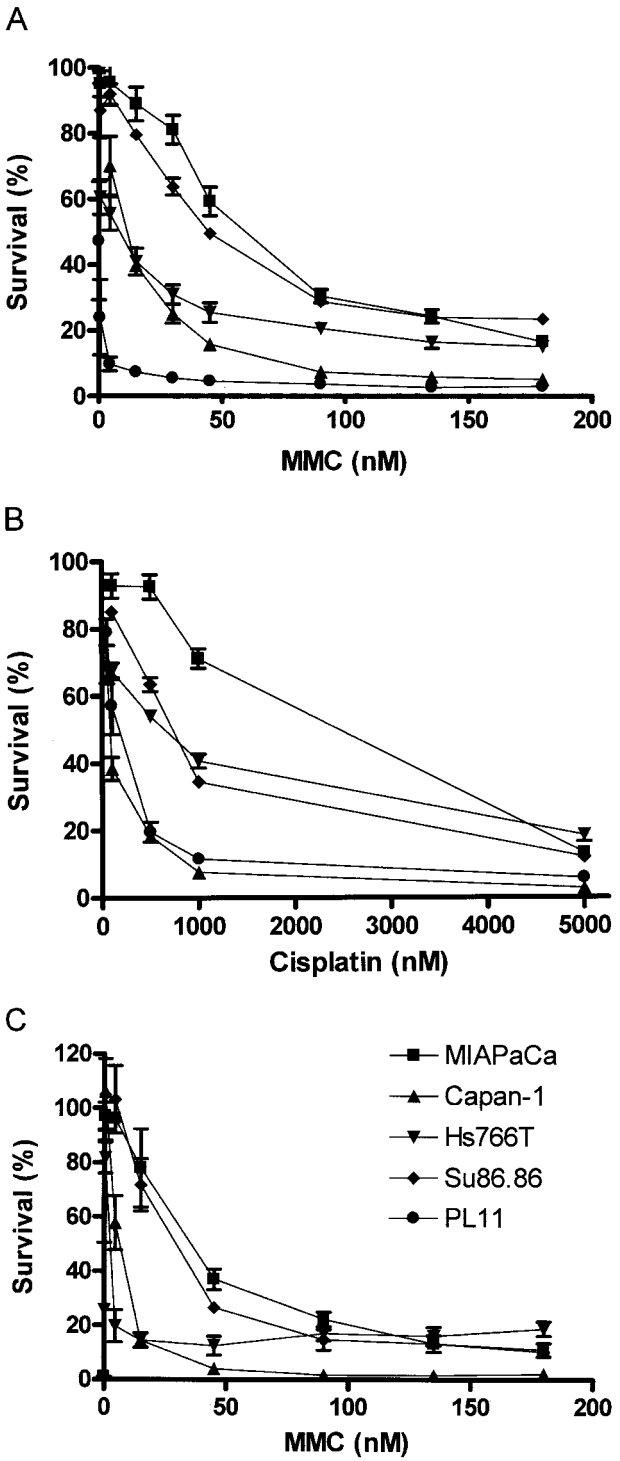

Figure 3. FA-defective cell lines are hypersensitive to crosslinking agents. **A:** MMC sensitivity of pancreatic cancer cell lines as measured by population quantitation using a measurement of total DNA. **B:** *cis*-Platin sensitivity of pancreatic cancer cell lines by DNA quantitation. **C:** MMC sensitivity of pancreatic cancer cell lines as measured by manual cell counts. Legends are consistent throughout **A** to **C**. **Bars**, standard error of the mean.

G_2/M Cell-Cycle Arrest by Low-Dose Crosslinking Agents in FA-Defective Cancer Cells

The methods used to investigate cell survival on treatment with MMC and *cis*-platin 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 G_2/M arrest was defined as a twofold increase of the fraction of cells containing 4N DNA content as compared to untreated cells (Figure 4). Fanconi-defective cancer cells had a striking difference as compared to Fanconi-proficient cells in the concentration of MMC that caused a G_2/M arrest. Hs766T arrested in G_2/M at a MMC concentration of 100 nmol/L, PL11 at 100 nmol/L, and CAPAN1 at 200 nmol/L, whereas control pancreatic cancer cell lines MiaPaCa2, Su86.86, and BxPC3 arrested at MMC concentrations as high as 2 μmol/L. These results further established the hypersensitivity of FA-defective cancer cells to crosslinking agents.

Mutational Screen of FANCA, C, D2, E, F, and G

The Fanconi genes *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, and *FANCG* were sequenced in 24 xenografted nonfamilial pancreatic cancers. 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,[22] specifically using the interstrand DNA-crosslinking agents. The hypersensitivity of cells taken from FA patients to crosslinking agents and to ionizing radiation already suggests this utility.[23,24] 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.[22,25,26]

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.[7–9] 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 hyper-

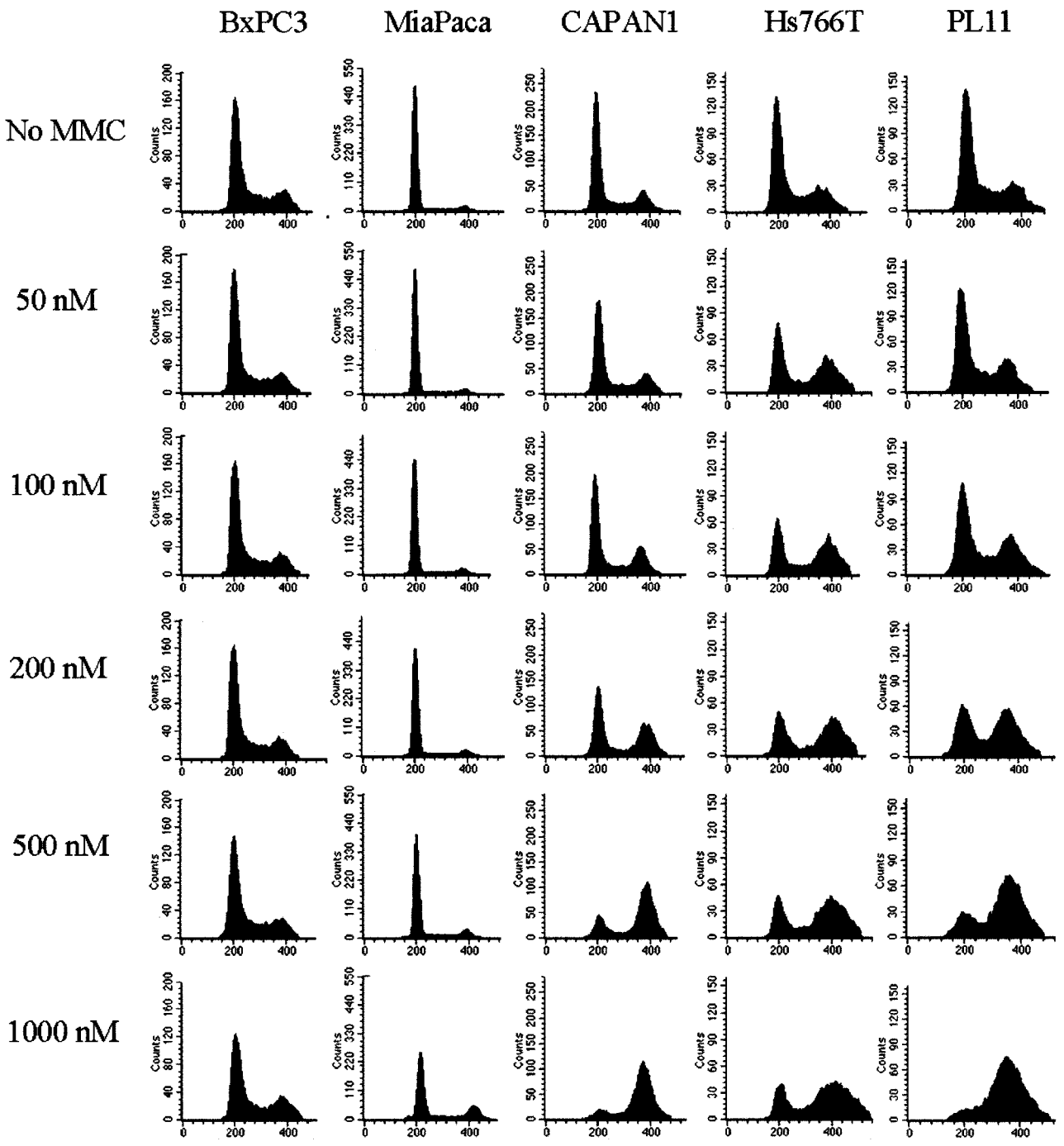

Figure 4. FA-defective cancer cell lines arrest in G_2/M 48 hours after low concentrations of MMC. Cells were treated with various concentrations of MMC for 2 hours, and incubated without MMC for 48 hours, after which the cell cycle was analyzed using a flow cytometer.

sensitivity, 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 pedigree was described in which one person had FA, and obligate mutation carriers displayed multiple occurrences of pancreatic and other cancers.[27] Approximately seven percent of sporadic pancreatic cancers carry mutations in *BRCA2*, accompanied by loss of heterozygosity.[2] The pancreatic cancer cell line CAPAN1 is derived from such a tumor. To date, one convincing somatic mutation in the *FANCC* gene, accompanied by loss of heterozygosity and resulting in truncation of the carboxyl-terminus of the protein, has been reported in a pancreatic cancer xenograft.[6] We here show that the commercially available pancreatic cancer cell line PL11 has a related defect, a homozygous deletion of exons 7 to 14 of *FANCC* and a functional defect in the FA pathway as measured by Fancd2 mo-

noubiquitination. The defect in Fancd2 monoubiquitination in PL11 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 *FANCG*, one germline mutation (E105ter) has been reported,[6] 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 *FANCG*. Assays for Fanconi 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,[6] 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 7%,[2,4] the FA pathway could be defective in as much as 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 regimens.[26] One could envision that pancreatic cancers might be genetically tested for defects in the pathways that repair interstrand crosslinks, such as the FA pathway. Patients with a defect in one of the repair pathways could then be treated rationally with crosslinking agents, possibly at a much lower dose than is customary.

The FA-proficient cell lines BxPC3, Su86.86, and MiaPaCa2 arrest in G_2/M only at high doses of MMC (2 μmol/L), whereas the FA-defective cell lines Hs766T, PL11, and CAPAN1 arrest in G_2/M after a dose of MMC that was 10 times lower or less (100 to 200 nmol/L), providing additional evidence of the MMC hypersensitivity of FA-defective cancer cell lines. In a study by Heinrich and colleagues,[28] cell-cycle changes in *FANCC*-defective FA lymphoblasts and FA-proficient cells were described in response to various concentrations of MMC. Similar degrees of G_2/M arrest after equitoxic doses of MMC were found: normal cells show the same G_2/M arrest as FA-defective cells do, only at a much higher dose. 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,[29] rather than a failure of major checkpoints. Akkari and colleagues[30] compared the response of FA lymphoblasts and retrovirally corrected controls to interstrand DNA crosslinks induced by psoralen-UVA. FA lymphoblasts were able to recover from substantial amounts of interstrand crosslink damage, although only after a threefold to fivefold longer growth arrest than in corrected isogeneic controls. This difference in duration of growth arrest was attributed by the authors to a decreased rate of 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 G_2/M arrest.

The degree of MMC hypersensitivity of the Fanconi-deficient cancer cells is more moderate than was suggested in previous studies of nonneoplastic Fanconi 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 *cis*-platin 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 *cis*-platin sensitivity in this study, except for PL11, have been analyzed for mutations in *TP53* (p53) and *CDKN2A* (p16); all were mutated in both genes.[31,32]

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.[33] 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 Fanconi pathway. Also, an increased cancer risk for individuals with mutations in one of the FA genes could be missed because of 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 Fanconi pathway and to attempt successful treatment of these patients with DNA crosslinking agents.

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