The Fanconi anemia/BRCA2 pathway in pancreatic cancer
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CHAPTER 7

IN VITRO AND IN VIVO CROSSLINKER SENSITIVITY: CONTINGENCY UPON FANCONI ANEMIA/BRCA2 STATUS

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Submitted for publication
Crosslinker sensitivity and FA/BRCA2 status

Abstract

BRCA2, FANCC and FANCG gene mutations are present in a subset of pancreatic cancer. Defects in these genes could lead to hypersensitivity to DNA-interstrand crosslinking agents in vitro and a more optimal treatment of pancreatic cancer patients based on the genetic profile of the tumor. Two retrovirally complemented pancreatic cancer cell lines having defects in the Fanconi anemia (FA) pathway, PL11 (FANCC-mutated) and Hs766T (FANCG-mutated), as well as several parental pancreatic cancer cell lines with or without mutations in the FA/BRCA2 pathway, were assayed for in vitro and in vivo sensitivity to various chemotherapeutic agents. A distinct dichotomy of drug responses was observed. FA-defective cancer cells were hypersensitive to the crosslinking agents mitomycin C, cisplatin, chlorambucil and melphalan, but not to 5-fluoro-uracil, gemcitabine, doxorubicin, etoposide, vinblastine, or paclitaxel. Hypersensitivity to crosslinking agents was confirmed in vivo: FANCC-deficient xenografts of PL11 and BRCA2-deficient xenografts of CAPAN1 regressed upon treatment with two different regimens of mitomycin C, whereas FA-proficient xenografts did not. The mitomycin C response comprised cell cycle arrest, apoptosis, and necrosis. Xenografts of PL11 also regressed after a single dose of cyclophosphamide, whereas xenografts of genetically complemented PL11FANCC did not. Mitomycin C or other crosslinking agents as a clinical therapy for pancreatic cancer patients with tumors harboring defects in the FA/BRCA2 pathway should be specifically investigated.

Introduction

In 2004, 31,860 Americans were expected to be diagnosed with pancreatic cancer and 31,270 to die from this disease, making pancreatic cancer the fourth leading cause of cancer death in the United States. The only effective treatment is surgical resection, which is performed in less than 20% of cases. Marginally effective therapies include irradiation, gemcitabine and 5-FU. Regimens combining various chemotherapeutic drugs are being investigated, and a rare complete response is achieved upon treatment with mitomycin C (MMC) or cisplatin-containing regimens. Clearly, new therapeutic approaches are necessary. The emerging field of cancer pharmacogenomics offers an attractive new therapeutic approach, in which treatment is based on the genetic profile of a patient’s cancer. Although successful examples exist, opportunities to translate
this approach into clinic are not often encountered. Proteins encoded by the BRCA2, FANCC and FANCN genes function in the repair of damage caused by DNA-interstrand crosslinking agents. One of these three genes is inactivated in 5-10% of apparently sporadic pancreatic cancers. Although present in only a minority of pancreatic cancers, mutations in the BRCA2 gene and in genes that code for proteins in the proximal FA pathway (collectively referred to as the FA/BRCA2 pathway) could provide a rational target for treatment with chemotherapeutic agents. Non-neoplastic cells taken from Fanconi anemia (FA) patients have a striking hypersensitivity in vivo to interstrand crosslinking agents, such as MMC and cisplatin. This hypersensitivity was confirmed in vivo in pancreatic cancer cells with mutations in the BRCA2, FANCC or FANCN genes, as compared to several FA-proficient pancreatic cancer cell lines. The sensitivity of FA-deficient cancer cells to other chemotherapeutic agents has not yet been assessed. It also remained unknown whether FA-deficient pancreatic cancer cells were hypersensitive to standard doses of crosslinking agents in vivo. In this study, we used retrovirally corrected pancreatic cancer cell lines to study the effect of defects in the FANCC and FANCN genes on the sensitivities to a panel of antineoplastic agents. We find that FA defective pancreatic cancer cells are selectively hypersensitive to DNA-interstrand crosslinking agents in vivo.

Materials and methods

Samples

Pancreatic cancer cell lines MiaPaCa2, AsPc1, Su86.86, CFPAC, CAPAN1 and Hs766T were obtained from ATCC (American Type Culture Collection, Manassas, Virginia). PL11 (Panc3.27) was kindly provided by Dr. E. M. Jaffee (Department of Oncology, Johns Hopkins University) and is also available from ATCC. Cells were grown in conventional tissue medium supplemented with 10% fetal bovine serum, penicillin/streptomycin and L-glutamine. PL11 and Hs766T were infected with retroviral vectors containing the puromycin resistance gene and the cDNA of FANCC or FANCN (respectively) (kind gift of Dr. A. d’Andrea), or infected with only the puromycin resistance gene (empty vector) as previously described. A pooled population of stable clones was selected for with G418 (Invitrogen). Institutional Review Board guidelines of Johns Hopkins University were followed for commercially available cell lines and anonymized patient samples.
**Immunofluorescence and immunohistochemistry**

For Fancd2 immunofluorescence, cells were grown on slides and treated with MMC (Sigma, Saint Louis, Missouri) at 100 nM for 18 hours. Slides were fixed in 2% paraformaldehyde, permeabilized in 0.3% Triton X-100 for 10 minutes, blocked in 1% serum for 20 minutes and labeled using mouse anti-Fancd2 primary antibody (sc20022, Santa Cruz Biotechnology, Santa Cruz, California, diluted 1:1000) and CY3-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). For immunoblots, cells were lysed and boiled, proteins were separated on 3-8% tris-acetate polyacrylamide gels (Invitrogen, Carlsbad, California) and transferred onto a PVDF membrane, blocked for one hour in TBST (tris-buffered saline with Tween-20) 5% milk and incubated overnight with anti-Fancd2 antibody. Binding was detected using Super Signal West Pico chemiluminescence substrate (Pierce Biotechnology, Rockford, Illinois). For immunohistochemistry, xenografts were fixed and labeled using the Envision + kit (Dako Corp., Carpentaria, CA) as previously described. Slides were washed and stained with primary antibody (1:400 dilution for cleaved caspase-3 (Cell Signaling, Beverly, MA)). Positive cells were counted by a blinded study and random sampling of 500 cells per slides.

**Survival assay**

After the appropriate dose range was determined, 1.1 x 10^5 cancer cells per well were incubated in 96-well plates with various concentrations of MMC, doxorubicin, etoposide, vinblastine, cisplatin, paclitaxel, melphalan, 5-FU (Sigma) or gemcitabine (Lilly, Indianapolis, IN). 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 (4-6 days). Medium was replaced by drug-free medium after 72 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 averaged from four identical wells per experiment and converted to a percentage; the wells without drugs were considered as 100 percent. At least six experiments were performed per cell line per concentration on at least three separate occasions.
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Apoptosis assay
For the annexin-V/propidium iodide assay, cells were plated, allowed to adhere overnight, and incubated with 0 or 25 nM MMC for 72 hours. Cells were then harvested, washed with PBS, stained with annexin-V and propidium iodide (Molecular Probes) as indicated by the manufacturer and measured in a flow cytometer. 50μM caspase inhibitor I (Calbiochem) was added to the medium 1h prior to treatment with MMC.

Xenograft establishment and treatment
Pancreatic cancer cell lines were injected subcutaneously in the flank of female athymic nude mice, age 5-9 weeks. Xenografts were established and measured with an electronic caliper every two days; tumor volume was estimated with the formula (width x length²)/2. Xenografts were grown to a size of at least 280 mm³ before treatment was initiated. MMC, cyclophosphamide (Sigma) or gemcitabine were injected intra-peritoneally. In accordance with our institutional guidelines, mice bearing xenografts over 2500 mm³ in size were sacrificed.

Results
Pancreatic cancer cells mutated in FANCC or FANCG are hypersensitive to DNA-interstrand crosslinking agents in vitro
In previous work, the pancreatic cancer cell line Hs766T was found to harbor a germline truncating mutation in the FANCG gene⁴⁴; another pancreatic cancer cell line, PL11, was found to have a homozygous deletion of the FANCC gene.⁴⁵ These mutations lead to a defect in Fancd2 monoubiquitination that can be corrected by retroviral complementation with FANCC and FANCG cDNA.⁴⁶. In the current work, we found that Fancd2 nuclear focus formation could be restored by retroviral complementation (Figure 7.1A). Empty-vector-transfected FA-defective cancer cells had an eight-to-ten-fold greater sensitivity to MMC as compared with the appropriately complemented cell lines (Figure 7.1B and Table 7.1). The parental FA-defective cell lines Hs766T and PL11 and the BRCA2-mutated pancreatic cancer cell line CAPAN1 were also hypersensitive to MMC as compared to the FA-proficient pancreatic cancer cell lines Su86.86, CFPAC, AsPc1 and MiaPaCa2 (Figure 7.1C).
To establish whether FA-defective cancer cells were hypersensitive exclusively to MMC and other DNA-interstrand crosslinking agents, the sensitivity of a panel of cell lines to several commonly used drugs from dif-
Figure 7.1 Retroviral correction and MMC-sensitivity of Fanconi-defective cell lines Hs766T (FANCG-mutated) and PL11 (FANCC-mutated)

A Restoration of Fancd2 nuclear focus-formation in response to MMC at 100 nM for 18 hours. B In vitro MMC-sensitivity of Fanconi-defective pancreatic cancer cells PL11EV and Hs766TEV as compared to complemented cells PL11FANCC and Hs766TFANCG. ■ Hs766T, ▲ PL11; interrupted line: empty vector - transfected; solid lines: complemented. C In vitro MMC sensitivity of FA/BRCA2 defective and control pancreatic cancer cell lines: ■ Hs766T, ▲ PL11, ◆ CAPAN1, □ MiaPaCa2, ● CFPAC1, × AsPc1, ◊ Su86.86. Interrupted lines represent FA/BRCA2-defective cell lines. Error bars represent standard error of the mean (SEM). Sensitivity was assayed using PicoGreen (see materials and methods). Cells were assayed 4-6 days after plating.

Different classes of anti-neoplastic agents was determined. Two cell line pairs were utilized: (1) the retrovirally-corrected PL11 cell line (PL11FANCC) and the empty vector-transduced, FANCC-mutated PL11 cell line (PL11EV); and (2) the corrected Hs766T cell line (Hs766TFANCG) and the FANCG-mutated Hs766T cell line transduced with an empty vector (Hs766TEV). Both FANCC- and FANCG-deficient cancer cells were hypersensitive to the DNA-interstrand crosslinking agents cisplatin, chlorambucil and melphalan (Figure 2A-C, Table 1). These results were consistent over multiple experiments. The FA functional status of the cell lines was confirmed periodically throughout the study by Fancd2 immunoblot (data not shown). There was no difference in sensitivity to the microtubule inhibitors.
Table 7.1 Sensitivity of Fanconi anemia deficient cell lines to various chemotherapeutic agents.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Hs766T (FANCG)</th>
<th>PL11 (FANCC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC</td>
<td>7.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>5.1</td>
<td>6.0</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>4.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Melphalan</td>
<td>6.4</td>
<td>5.4</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Etoposide</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

paclitaxel and vinblastine, nor to the topoisomerase inhibitors etoposide and doxorubicin, when comparing the FA-defective cancer cells to their genetically corrected counterparts (Figure 7.2D-G, Table 7.1). Unexpectedly, the complemented cell line PL11_{FANCC} was more sensitive than PL11_{EV} to the two antimetabolites tested, 5-fluorouracil (5-FU) and gemcitabine (Figure 2 H and I); Hs766T_{EV} and Hs766T_{FANCG} were equally sensitive to these drugs.

**FA-defective cancer cells are hypersensitive to DNA-interstrand crosslinkers in vivo**

To establish whether the observed drug hypersensitivity of FA-deficient cancer cells *in vitro* can be extended to an *in vivo* model, we transplanted various human pancreatic cancer cell lines subcutaneously into athymic nude mice. The xenografts were measured every two days and were grown to a size of at least 280 mm\(^2\) (average = 395 mm\(^2\), SD = 84) before treatment was initiated. In the first treatment regimen, xenografts of the
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**Figure 7.2** Responses of Fanconi-defective vs. proficient pancreatic cancer cells to various chemotherapeutics in vitro. Fanconi-proficient pancreatic cancer cells PL11\textsuperscript{FANCC} and Hs766T\textsuperscript{FANCG} and -deficient PL11\textsuperscript{EV} and Hs766T\textsuperscript{EV} cells were seeded in 96-well plates with various concentrations of drugs, analyzed after 4-6 days for DNA content by PicoGreen assay and results expressed relative to untreated cells. A cisplatin B chlorambucil C melphalan D paclitaxel E vinblastine F etoposide G doxorubicin H gemcitabine I 5-FU. ■ Hs766T (red), ▲ PL11 (blue); interrupted line: empty vector-transfected; solid lines: complemented. Each data point represents at least three experiments done in duplicate, error bars reflect SEM.

pancreatic cancer cell lines Su86.86 (n=11), CFPAC (n=11), MiaPaCa2 (n=11), CAPAN1 (BRCA2-mutated, n=12), PL11 (FANCC-mutated, n=9) and PL11\textsuperscript{FANCC} (n=12) were treated with MMC, 2.5 mg/kg i.p.
every fourth day. Due to differing xenograft growth rates, the two Hs766T cell lines (Hs766T and Hs766T FANCC) could not be analyzed. Xenografts were followed for at least 40 days, up to a maximum of 60 days (Figure 7.3A and data not shown). Xenografts of CAPAN1 and PL11, harboring mutations in the BRCA2 and FANCC genes, respectively, regressed (a decline in volume) soon after treatment was initiated, whereas the FA-proficient xenografts, including the genetically corrected PL11 FANCC, progressed. After several weeks of treatment, growth-inhibition of FA-proficient xenografts became apparent, probably reflecting the additive effect of MMC doses adequate to inhibit neoplastic growth independent of gene status.

Substantial toxicity after treatment was observed with the regimen of repetitive doses of MMC in both the mice with FA-deficient and FA-proficient xenografts. Mice frequently became anemic after approximately 30 days of treatment. The mortality rate with this treatment regimen was 43%, with mice dying on average 51 days (range 45-59) after the treatment was initiated. Death often occurred several days after petechiae initially appeared, eventually progressing to widespread hemorrhage. Separately, we treated a panel of pancreatic cancer xenografts with a single dose of MMC (5 mg/kg) (Figure 7.3, panels B and C). This treatment regimen was generally well tolerated. FA-defective xenografts (PL11 EV) regressed remarkably after a single dose of MMC (Figure 7.3B). In contrast, genetically corrected xenografts (PL11 FANCC) progressed. The PL11 EV xenografts were followed for 60 days after treatment with a single dose of MMC. Through 50 days after treatment, all tumors remained smaller than they were at the initiation of treatment. At sixty days after a single dose of MMC, two tumors had reached a size larger than at the start of treatment: the average size at 60 days was 52% (SEM=22%) of the size at treatment initiation (data not shown). Eight of eleven (75%) CAPAN1 (BRCA2-mutated) xenografts regressed after treatment with a single dose of MMC; a substantial growth inhibition was also seen when all observations were averaged. Two of nine (22%) CFPAC (FA/BRCA2-proficient) xenografts regressed after MMC treatment; the other xenografts progressed almost uninhibited (Figure 3C). PL11 EV xenografts also regressed upon a single-dose treatment with cyclophosphamide (230 mg/kg), another interstrand crosslinking agent, whereas PL11 FANCC xenografts did not (Figure 7.3D). The growth rate of PL11 EV xenografts was slower than the growth rate of PL11 FANCC.
In vivo treatment of FA defective cancer cells with gemcitabine

The genetically corrected PL11\textsuperscript{FANCC} cells had an unexpected \textit{in vitro} hypersensitivity to gemcitabine, as compared to PL11\textsuperscript{EV}. We extended these studies \textit{in vivo} and observed PL11\textsuperscript{FANCC} to regress measurably for a period after a single dose of gemcitabine (100mg/kg), whereas PL11\textsuperscript{EV} xenografts were not inhibited in growth (Figure 7.3E).

\textbf{Cell-cycle arrest contributes to MMC hypersensitivity}

An arrest in late S- or G2/M-phase of the cell division cycle is known to parallel the MMC hypersensitivity\textsuperscript{156, 158}; the role of apoptosis is less certain in FA-deficient cancer cells. We examined cell cycle progression in Hs766T\textsuperscript{EV}, Hs766T\textsuperscript{FANCG}, PL11\textsuperscript{EV} and PL11\textsuperscript{FANCC} cells at 48 and 72 hours after \textit{in vitro} treatment with 25nM MMC (Figure 7.4A), at which...
Figure 7.4 Cell cycle and apoptosis profiles of Fanconi-deficient and complemented cancer cells. A: Fanconi-deficient and complemented PL11 and Hs766T cells were treated with 25 nM MMC and fixed and analyzed at the indicated time points by flow cytometry. Experiments were performed in duplicate. Representative results are shown. B: Assessment of apoptosis in Hs766T EV, Hs766T FANCG, PL11 EV and PL11 FANCC, 72 hrs after MMC, 25 nM, by annexin V / propidium iodide flow cytometry. Percentages of annexinV-positive cells are corrected for background apoptosis (untreated cells) (see results for SEM).

At 72 hrs, a large percentage of the viable PL11 EV and Hs766T EV cells had a 4N DNA content, reflective of an arrest in late S- or G2/M-phase of the cell cycle. Most PL11 FANCC and Hs766T FANCG cells had a cell cycle distribution approximately equal to untreated cells.
**MMC toxicity is in part attributable to caspase-dependent apoptosis**

Hs766T^{EV}, Hs766T^{FANCC}, PL11^{EV} and PL11^{FANCC} cells were treated *in vitro* with 25 nM MMC for 72 hours; proportions of annexin V (an early marker for apoptosis) - and propidium iodide-stained cells were measured and corrected for the levels of background (untreated) apoptosis (*Figure 7.4B*). Annexin V stained 16.5% of the MMC-treated PL11^{EV} population, but only 4.2% of the PL11^{FANCC} cells (*p* = 0.009); 17.3% of Hs766T^{EV} cells but only 6.4% of Hs766T^{FANCC} cells was positive for annexin V (*p* = 0.013). Likewise, MMC treatment of parental PL11 cells in vitro increased the percentage of annexin V-positive cells, as assayed by flow cytometry assay. PL11 cells treated with MMC were 19.9% (S.E.M. +/− 1.98) positive for annexin V, as compared to untreated PL11 cells at 11.4% (S.E.M. +/− 1.83) positive. The addition of a caspase inhibitor cocktail reduced the apoptosis caused. PL11 cells treated with MMC and caspase inhibitor cocktail were 12.7% positive for annexin V. PL11 cells treated with MMC acquired caspase 2 and 9 cleavage products as assayed by immunoblot (data not shown). Xenografts treated with MMC and then examined by caspase 3 immunohistochemistry (a marker for apoptosis) had a greater increase in apoptosis in PL11 tumors than did FA-corrected tumors at day 7 (*figure 7.5B*).  

**Morphology of MMC-treated FA-deficient xenografts**

FA-proficient and FA-deficient xenografts were treated with MMC, harvested, and processed for routine histopathologic examination (*Figure 7.5*). Specifically, PL11^{EV} and PL11^{FANCC} xenografts were treated with a single dose of MMC (5 mg/kg) and harvested at day 0, 3 and 7 (each, *n* = 4). Xenografts of PL11^{EV} and PL11^{FANCC} treated with MMC had islands with morphologically atypical cells. These atypical cells had pleomorphic nuclei, a prominent cytoplasm, and large nucleoli (*Figure 7.5A*). Areas with atypical cells were larger and more prevalent at seven days after treatment as compared to the appearance at day three. All tumors examined had some individual necrotic cells, but only the FA-deficient PL11^{EV} xenografts had confluent areas of necrosis (*Figure 7.5A*), which increased from approximately 10% at three days after treatment to 50-40% of the tumor as assessed in histologic slides at day seven. After a single dose of MMC, xenografts regressed for approximately 40-50 days, after which some of the xenografts started to regain size. Four xenografts were excised 40 days after a single dose of MMC: within the tumors were bands and large areas of fibrosis with islands of viable cancer cells (*Figure 7.5B*).
Figure 7.5 Morphology of MMC-treated xenografts A Histopathology of PL11-FANC and PL11EV xenografts 0, 3 and 7 days after treatment with a single dose of MMC. Necrosis was noted in the PL11EV xenografts as early as day 3 and was quite extensive by day 7 (right half of the image). B PL11EV xenografts. Left panel: histopathology at day 40 after a single dose of MMC; middle panel: activated caspase 3, day 0; right panel: activated caspase 3, day 7. By day 40 only scattered neoplastic glands remained embedded in a densely fibrotic stroma. Xenografts labeled for cleaved caspase 3 were evaluated by counting of positive cells on day 7 (PL11EV tumors: 8.25%, SEM +/- 1.6 compared to PL11FANC tumors: 3.9%, SEM +/-1.0%). C Xenografts 30 days after treatment with MMC, 2.5 mg/kg every 4 days. Dramatic nuclear enlargement and pleomorphism was present in all three cell lines 30 days after treatment. Numerous individual necrotic cells were present in PL11 tumors.

The histologic effects of repeated dosing were also surveyed. Xenografts of CAPAN1, PL11 and Su86.86, treated once every 4 days with 2.5 mg/kg MMC for 28 days, were harvested at day 30. The FA/BRCA2-deficient CAPAN1 and PL11 cells had large areas of dramatically altered cells thirty days after treatment (Figure 5C). These cells were characterized by a greatly increased cell mass, syncytial change and large polymorphic and hyperchromatic nuclei with abnormal mitotic figures and prominent nucleoli. Morphological alterations were much less pronounced in the FA/BRCA2-proficient Su86.86 xenografts (Figure 7.5C).

Discussion

Recent years have brought an impressive increase in the understanding of the genetic basis of pancreatic cancer, but so far it has not been possible to translate this improved knowledge of the genetic alterations in pancreatic cancer into significant advancements in clinical treatment. In this study, we show that pancreatic cancer cells having defects in the FA/BRCA2 pathway are remarkably sensitive to interstrand crosslinking agents, both in culture and as xenografts in mice. The hypersensitivity of FA-defective cancer cells to interstrand crosslinking agents echoes the observed hypersensitivity of nonneoplastic FA cells. The current study demonstrates for the first time that the hypersensitivity of pancreatic cancer cells having defects in the FA/BRCA2 pathway to crosslinking agents can be extended to the in vivo setting: FA-defective xenografts PL11 and CAPAN1 regressed after a variety of treatment schedules of MMC, whereas FA-proficient xenografts did not. These
findings lay the foundation for a more rational and efficacious treatment for a subset of pancreatic cancer patients, namely those with a carcinoma defective in the FA/BRCAC2 pathway who thus might more likely respond to the use of DNA-crosslinking agents.
FA cells have an exaggerated arrest in the late S or G2/M cell cycle compartment in response to low doses of MMC. A report by Akkari et al. suggested that MMC could induce long-term but reversible cell cycle arrest in nonneoplastic FA-deficient cells in the absence of significant cell death. In a recent study, we found FA-defective pancreatic cancer cells to arrest in late S- or G2/M-phase 48 hours after an approximately tenfold lower pulsed dose of MMC than needed for FA-proficient cancer cells. To determine whether treated xenografts remained in cell cycle arrest, eventually resumed cycling, or underwent apoptosis, we performed immunohistochemistry on resected tumors. The tumor regression observed here in vivo argued (almost by definition) for a significant contribution from apoptosis and necrosis. Unexpectedly, the complemented PL11\(^{FANCC}\) cells were three- to fourfold more sensitive to gemcitabine and were also more sensitive to 5-FU, as compared to the paired FA-defective PL11\(^{FUV}\) cells. This hypersensitivity of PL11\(^{FANCC}\) cells to gemcitabine was also seen in vivo. An explanation is not readily available; further studies will be needed to validate whether this FANCC-induced hypersensitivity is unique to this cell line. If this were a general finding, it would argue against using a combination of MMC and gemcitabine in a clinical trial investigating the connection between FA defects and sensitivity to MMC or other crosslinking agents. (Unfortunately, Hs766T cell lines were difficult to grow as comparable xenografts and could not be used for in vivo studies.) Follow-up studies are currently limited by a lack of genetically appropriate cancer cell lines. These results suggest that DNA-interstrand crosslinking agents, particularly MMC or cisplatin, could be selectively used on patients with cancers harboring mutations in one of the members of the FA/BRCA2 pathway. Since our results suggest that hypersensitivity of FA/BRCA2 defective tumors applies to all crosslinking agents, treatment options could also include other effective crosslinkers that may be tolerated better, such as oxaliplatin. Patients could be screened for germline mutations in BRCA2 or, if an appropriate test could be developed, tumor specimens screened for somatic mutations in FANCC or for other FA pathway defects. To provide preliminary evidence supporting this principle, six anonymous coded blood samples of pancreatic cancer patients that had experienced a good clinical response to a combination regimen of MMC and 5-FU were analyzed for BRCA2 mutations or deletions. Patient samples were handled anonymously according to guidelines of our institutional review board. One deleterious mutation was found.
6174delT, resulting in a frameshift that would produce a premature truncation of protein translation and misreading of the coding sequence\textsuperscript{52}. One patient had a splice-site variant, IVS25 +3A>T; the significance of this mutation has not been established (MSH, WHI, SEK, unpublished data). The majority of mutations in the upstream FA pathway in pancreatic cancer is likely to be somatic\textsuperscript{161} and would be missed by gene testing of germline DNA from blood samples. Because tumor tissue was not available, we were unable to screen for mutations in \textit{FANCC}, \textit{FANCG} or \textit{BRCA2} in these patients’ tumors.

One could envision that in the treatment of pancreatic cancer, after surgical excision (which most readily permits the full genetic analysis envisioned), MMC (or another DNA-interstrand crosslinking agent) could be a curatively intended adjuvant treatment used selectively in the instance of tumor defects in the FA/BRCA2 pathway. In the meantime, the known germline origin of most pancreatic cancer \textit{BRCA2} defects and the ready availability of reliable and rapid \textit{BRCA2} testing allows for the design of directed studies in either resected or unresectable cases of pancreatic cancer.