The Fanconi anemia/BRCA2 pathway in pancreatic cancer
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CHAPTER 9

A FUNCTIONAL SCREEN OF THE FANCONI ANEMIA PATHWAY IN CANCER CELLS BY FANCD2 IMMUNOBLOT

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Abstract
The Fanconi anemia (FA) genes BRCA2, FANCC and FANCG are mutated in a subset of pancreatic cancer. Additionally, the FA pathway is inactivated, probably by FANCF promoter methylation, in a subset of ovarian cancers. The competence of the proximal FA pathway was screened by an assay of Fancd2 monoubiquitination in a panel of 35 cancer cell lines: 15 breast, 6 prostate, 8 head and neck, 4 biliary cancers, an astrocytoma and a large cell lung carcinoma. Two (6%) cell lines displayed abnormal Fancd2 monoubiquitination: the head and neck cancer cell line FaDu and the breast cancer cell line UACC812. In UACC812, we found that FANCF was not expressed. In the case of FaDu, no explanation for the abnormal Fancd2 monoubiquitination was found. FaDu had a moderately increased sensitivity to mitomycin C, as compared to two FA proficient head and neck cancer cell lines. Future studies should aim to investigate the involvement of defects in the FA pathway in breast and head and neck cancer.

Introduction
Fanconi anemia (FA) is a rare autosomal recessive disease, characterized by congenital bone deformities, progressive bone marrow failure and a predisposition to hematological malignancy (especially acute myelogenous leukemia) and squamous cell carcinoma of the head and neck, anogenital region, skin and other organs. Mutations in the FA genes FANC-A (65%), FANCC (15%) and FANCG (10%) account for the majority of cases. BRCA2 mutations have been shown to be responsible for a subset of FA patients: complementation group D1 and perhaps B. Mutations in these patients affected both alleles and included at least one hypomorphic mutation per patient, in which some residual function may have remained. Complementation of a FANCD1 cell line with wild-type BRCA2 corrected the cytogenetically measured mitomycin C (MMC) hypersensitivity. FA cells display spontaneous chromosome breakage, greatly enhanced by DNA-interstrand crosslinking agents such as MMC and diepoxybutane. 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. Recently, mutations in the FA genes FANCC and FANCG have been identified in pancreatic cancer. In addition, functional defects in the FA pathway have been found in ovarian cancer, which was attributed to promoter methylation of FANCF.
In the current study, we survey the frequency with which the FA pathway is functionally defective in several types of cancer. We found the FA pathway, as measured by Fancd2 monoubiquitination in response to MMC, to be defective in two cell lines: FaDu, a head and neck squamous cell carcinoma (HNSCC) cell line, and UACC812, a breast cancer cell line. UACC812 did not express FANCf, as measured by RT-PCR. The underlying cause of the defect in FaDu remains to be identified.

**Materials and Methods**

**Samples**

For the FA pathway screen by Fancd2 immunoblot, we included breast and prostate cancer cell lines because of the role of BRCA2 in familial breast cancer and young-onset prostate cancer. Because of the occurrence of HNSCC in a substantial percentage of FA patients who survive until adulthood, we included eight HNSCC cell lines. A number of cancer cell lines were previously tested by the National Cancer Institute for sensitivity to various chemotherapeutic agents (http://dtp.nci.nih.gov); the two cell lines most sensitive to MMC (SW-1088, astrocytoma and NCI-H460, large cell lung cancer) were obtained from ATCC (American Type Culture Collection, Manassas, Virginia) and included in our panel. Breast cancer cell lines MDAMB 175-VII, MDAMB 231, MDAMB 361, MDAMB 456, MDAMB 453, MDAMB 461, MDAMB 468, BT 474, BT 549, ZR75-1, ZR75-30, SKBR3, MCF7, HS578, UACC812; head and neck cancer cell lines Detroit 562, FaDu, SCC-15, SCC-25, Cal27, RPMI-2650, A-253, SW-579; prostate cancer cell lines MDA Pca-2b, DU145, PC3, LNCA P and the pancreatic cancer cell line AsPC1 were obtained from ATCC. Prostate cancer cell lines C4-2B and CWR22Rv1 were kindly provided by Dr. A. M. DeMarzo (Department of Pathology, Johns Hopkins University). Biliary cancer cell lines HUCCT1, GBD1, TFK1 and SNU308 were kindly provided by Dr. A. Maitra (Department of Pathology, Johns Hopkins University). Cells were grown in media supplemented with 10% fetal bovine serum, penicillin/streptomycin and L-glutamine.

**Fancd2 immunoblots**

Equal numbers of cells were grown in 6-well plates and treated with or without MMC, 45 nM, for 18-24 hours, or irradiated with 15 Gy and incubated for 2 hours. 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, 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).

Sequencing and RT-PCR
FANCC and FANCG were sequenced as previously described\(^4\). FANCA was sequenced using automated sequencing. mRNA was extracted using the RNeasy Mini Kit (Qiagen Inc., Valencia, California). Primers for expression of FANCF were used as described by Taniguchi et al.\(^1\). Expression of FANCA, FANCC and FANCG was determined on cDNA by the following primers: 5'-TGTGCTGAAGCATCTGATG and 5'-GTTGAAAGGCAATCATG (FANCA); 5'-TAAACAAAGAAACACAGAATT and 5'-CTTGAGGCAAGGTATC (FANCC); 5'-GGATCAGGGCCAGATAT and 5'-GGTCAATTGGGAGCATCTAATTC (FANCG). All primers were purchased from IDT DNA (Coralville, Iowa). Since the FANCF gene consists of only one exon, primers could potentially amplify off of contaminating genomic DNA. Therefore, mRNA was treated for 48 hours with DNAse (Roche Applied Science; Indianapolis, Indiana). During first-strand cDNA synthesis (Superscript II Reverse Transcriptase; Invitrogen; Carlsbad, California) a sample without reverse transcriptase was prepared to be used as a control for contaminating genomic DNA. DNA gels were run using sodium boric acid as the conductive medium as described by Brody et al.\(^1\).5.

Retroviral correction
The retroviral expression vectors pMMP-puro\(^1\), pMMP-puroFANCA\(^1\), pMMP-puroFANCC\(^1\), pMMP-puroFANCF\(^1\) and pMMP-puroFANCG\(^1\) were generously provided by Dr A. D. d’Andrea and were used as previously described\(^1\).

Survival studies
1.2 x 10\(^5\) cancer cells per well were incubated with various concentrations of MMC (Sigma, Saint Louis, Missouri: range 0–4.5 \(\mu\)M) in 96-well plates. Cells were incubated for a period of time long enough to allow
**Figure 9.1** Screen for Fanconi Anemia defects by Fancd2 monoubiquitination assay. Equal cell numbers were unthreated, or incubated with MMC for 18-20 hours, or irradiated with 15 Gy and incubated for 2 hours, after which protein lysates were made. Protein lysates were immunoblotted for Fancd2. FaDu + A, FaDu + C, FaDu + F, FaDu + G: lysates of FaDu retrovirally transduced with FANCA, FANCC, FANCF and FANCG, respectively.

Non-treated cells to reach at least a threefold increase in fluorescence as compared to day 1 (3-4 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 percent. Each experiment was done in duplicate; at least six experiments per cell line per concentration were performed.

**Results**

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**),

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A functional screen of the Fanconi anemia pathway in cancer cells

![Functional screen of the Fanconi anemia pathway in cancer cells](image)

**Figure 9.2** RT-PCR for FANCA and FANCF: lack of FANCF expression in UACC812. As a control for contaminating genomic DNA, Reverse Transcriptase was left out of the reaction where indicated. Pancreatic cancer cell line ASPC was used as a positive control for FANCF.

Another member of the FA nuclear complex, has an important role in Fancd2 monoubiquitination\(^{45}\). 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\(^{155}\). We utilized this functional test of the FA pathway and applied it to a panel of 21 pancreatic cancer cell lines\(^{48}\). Using this test, we were able to show two cell lines to be defective in Fancd2 monoubiquitination: one cell line (Hs766T) which was previously described to contain a germline nonsense mutation in FANCG\(^{44}\) and one cell line (PL11) which was subsequently shown to contain a large homozygous deletion in FANCC. In the present study, we extended this screen of the upstream FA pathway to a panel of 35 cancer cell lines: 15 breast cancer cell lines, 6 prostate cancer cell lines, 8 HNSCC cell lines, 4 biliary cancer cell lines, a glioma cell line and a lung cancer cell line (Figure 9.1 and data not shown). Two cell lines were found to be defective in Fancd2 monoubiquitination: the HNSCC cancer cell line FaDu, and the breast cancer cell line UACC812 (Figure 9.1). Both defects were confirmed with separately prepared lysates of cell lines reordered from ATTC. In the case of FaDu, a faint shadow above the short Fancd2 isoform was seen on each immunoblot, although a monoubiquitinated band was never seen. Several additional Fancd2 immunoblots, including immunoblots on lysates after irradiation (Figure 9.1) and on an aliquot separately purchased gave the same results.
We next examined DNA prepared from FaDu and UACC812 for genetic defects in FANCA, FANCC and FANCG; no mutations were found. The mRNA encoded by the FANCA, FANCC and FANCG gene were normally expressed, as assayed by RT-PCR, in both UACC812 and FaDu (Figure 9.2 and data not shown). FANCF was normally expressed in FaDu but was not expressed, or expressed in an amount below the level of detection in UACC812 (Figure 9.2). Next, we retrovirally transduced FaDu with FANCA, FANCC, FANCF and FANCG cDNA. None of these expression vectors could complement the defect in Fancd2 monoubiquitination (Figure 9.1). The head and neck cancer cell lines FaDu, Detroit 562 and A-253 were treated with various concentrations of MMC 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 HNSCC cell line FaDu was more sensitive to MMC as compared to the FA-proficient HNSCC cell lines A-253 and Detroit 562 (Figure 9.3). Unfortunately, a slow growth rate of the breast cancer cell line UACC812 precluded retroviral transduction with the various FA cDNAs and an assessment of MMC sensitivity.

Discussion

Recently, the first mutations in non-BRCA2 FA genes in somatic solid tumors were detected in pancreatic cancer\(^{44}\). Also, defects in the proximal FA pathway were detected in ovarian cancer, which were attributed to promotor methylation of the FANCF gene\(^{159}\). Both defects led to an abrogation of Fancd2 monoubiquitination, as measured by a Fancd2 immunoblot. The occurrence of FA defective tumors in non-FA patients, either by somatic inactivation (mutation or changes in promotor methylation), or by the inheritance of a germline mutation combined by somatic mutation or loss of the second allele, may have important clinical consequences. Realizing the striking MMC hypersensitivity of cells taken from FA patients, these tumors may provide a widened therapeutic window for the use of DNA-interstrand crosslinking agents. In the case of tumors with loss of FANCF expression by promotor methylation, the occurrence of resistance to crosslinking agents could perhaps be explained by demethylation\(^{49,159}\). Also, knowledge of the frequency of germline FA mutations in somatic tumors could help better estimate individual cancer risk\(^{129}\).
We applied a functional test of the FA pathway by Fancd2 monoubiquitination to screen a panel of human cancer cell lines, including breast, head and neck, prostate and biliary cancer. A head and neck cancer cell line, FaDu, and a breast cancer cell line, UACC812, were defective in Fancd2 monoubiquitination. In the case of UACC812, expression of FANCF could not be detected by RT-PCR, which could be caused by promoter methylation.\(^{159}\) Mutations in the \textit{BRCA2} gene are associated with a substantially increased risk of breast cancer; \textit{BRCA2} gene mutations can also cause FA when both alleles are mutated. In a screen of 88 \textit{BRCA1/2}-negative familial breast cancers, no pathogenic mutations were found in the FA genes \textit{FANCA, FANCC, FANCD2, FANCE, FANCF} and \textit{FANCG}.\(^{181}\) However, sporadic breast cancers could still have defects in the FA pathway that are caused by a lack of \textit{FANCF} expression, just like ovarian cancers.

The HNSCC cell line FaDu was established in 1968 from a hypopharyngeal tumor from a Hindu patient (data from ATTC). Interestingly and perhaps relatedly, FA patients who survive to adulthood have a highly increased incidence of HNSCC. Kutler \textit{et al.}\(^{177}\) found 19 (3\%) cases of HNSCC in their database of 754 FA patients, a much higher percentage than expected (standardized incidence ratio: 500). Although it is possible that the HNSCC cancer cell line FaDu came from a FA patient, FaDu is derived from a 56-year old patient, whereas the HNSCC patients in

\textbf{Figure 9.3} MMC sensitivity of HNSCC cell lines as measured by population quantitation using a measurement of total DNA.
the study by Kutler et al. had a median age of 51 years (range 15 to 49). Furthermore, the typical HNSCC in FA patients in the study by Kutler et al. occurred in the oral cavity (68%). These data make it less likely that FaDu is derived from a FA patient. In the present study we did not find FANCA, FANCC or FANCG gene mutations in FaDu, nor did we find alterations in the expression of FANCA, FANCC, FANCF or FANCG. Retroviral overexpression of the FA genes FANCA, FANCC, FANCF and FANCG could not correct the defect in the FA pathway, as measured by Fancd2 monoubiquitination. A mutation in another gene in the pathway, perhaps in a gene that remains to be discovered, could have caused the defect in the FA pathway observed in these cancer cells. Also, we cannot exclude the possibility that the observed defect was an artifact of the screen used. This screening method is relatively new, and does not necessarily have 100% specificity. Although there was some ambiguity in the interpretation of the result for this cell line, the observation was consistent in over six separately prepared lysate pairs, and was distinctly different from Fancd2 monoubiquitination observed in nondefective cell lines. FaDu had a slightly higher sensitivity to MMC as compared to two other HNSCC cell lines. Although still interesting, this sensitivity is not as marked as expected from studies of cells taken from FA patients and from similar studies in pancreatic cancer. Thus, whereas the Fancd2 monoubiquitination is distinctly different from FA proficient cancer cell lines, the moderate MMC hypersensitivity and the fact that no explanation for the defect in Fancd2 monoubiquitination could be found, do not fit a perfect scenario of a FA defective tumor. A more extensive study of the FA pathway in sporadic head and neck cancer is warranted.

Overall, 6% of the cell lines investigated in this study have an abnormal Fancd2 monoubiquitination, indicating a defect in the FA pathway. These results suggest that FA defects, and probably related hypersensitivity to crosslinking agents, extend beyond the previously demonstrated defects in pancreatic cancer (two out of 22 cell lines studied - 9%) and ovarian cancer (two out of 25 cell lines studied, 8%) Future studies should investigate the possibility that defects in the FA pathway may be present, perhaps at a low percentage, in many different tumor types. These studies could lead to a better treatment of a subset of cancer patients, namely those with tumors defective in the FA pathway.
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