ARTICLES

Double-Strand Break Repair Deficiency and Radiation Sensitivity in BRCA2 Mutant Cancer Cells

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Background: The protein product of the BRCA2 gene mediates repair of double-strand breaks in DNA. Because a number of cancer therapies exert cytotoxic effects via the initiation of double-strand breaks, cancers comprised of cells carrying BRCA2 gene mutations may be more amenable to treatment with agents that cause such breaks. *Methods:* We identified a human pancreatic adenocarcinoma cell line lacking one copy of the BRCA2 gene and containing a mutation (6174delT) in the remaining copy. *In vitro* and *in vivo* experiments were conducted with this cell line and with other carcinoma cell lines matched for similar genetic mutations, similar differentiation status, and/or similar carcinoma type to examine double-strand break repair, sensitivity to drugs that induce double-strand breaks, and radiation sensitivity. *Results:* BRCA2-defective cells were unable to repair the double-strand DNA breaks induced by ionizing radiation. These cells were also markedly sensitive to mitoxantrone, amsacrine, and etoposide (drugs that induce double-strand breaks) (two-sided P = .002) and to ionizing radiation (two-sided P = .001). Introduction of antisense BRCA2 deoxyribonucleotides into cells possessing normal BRCA2 function led to increased sensitivity to mitoxantrone (two-sided P = .008). Tumors formed by injection of BRCA2-defective cells into nude mice were highly sensitive (>90% tumor size reduction, two-sided P = .002) to both ionizing radiation and mitoxantrone when compared with tumors exhibiting normal BRCA2 function. Histologic analysis of irradiated BRCA2-defective tumors showed a large degree of necrosis compared with that observed for control tumors possessing normal BRCA2 function. *Conclusion:* BRCA2-defective cancer cells are highly sensitive to agents that cause double-strand breaks in DNA. [J Natl Cancer Inst 1998;90: 978–85]

The field of cancer genetics has been greatly advanced by the discovery of genes that affect an individual's susceptibility to developing cancer. It is hoped that identification of cancer gene mutations will eventually lead to individualized, mutation-targeted rational therapies *(1)*. Despite this hope for genotype-driven therapy, few practical advances have been made. Two barriers to genotype-driven therapy exist presently: 1) the unknown function of many cancer susceptibility and tumor suppressor genes and 2) the lack of clear strategies to exploit known or presumed gene functions for targeted therapy.

The cancer predisposition gene, BRCA2, is a good candidate for genotype-driven therapies. Inherited mutations in the BRCA2 gene are responsible for a wide spectrum of familial cancers, including those of the breast, ovary, and prostate *(2–4)*. In addition, the BRCA2 gene has been found to be mutated in approximately 10% of pancreatic cancers studied *(5)*. It has been shown that the carboxy-terminus of the Brca2 protein interacts with the protein product of Rad51, a DNA double-strand break repair gene *(6,7),* and that disruption of this interaction leads to radiation sensitivity in mouse blastocysts *(6,8)*. This finding has led to the hypothesis that the Brca2 protein helps to maintain genomic stability, protecting the cell against damage induced by double-strand DNA breaks *(9)*. Cells that have mutated forms of BRCA2 (gene and protein) appear to be hypersensitive to double-strand DNA breaks. Kinzler and Vogelstein *(9)* have discussed the fact that a number of clinical agents exert their cytotoxic effects through the formation of double-strand DNA breaks, and they have suggested that the effect of BRCA2 on double-strand break repair might be exploited clinically. To examine this hypothesis, we identified a pancreatic cancer cell line with a mutant BRCA2 gene but no copy of the normal BRCA2 gene by showing loss of heterozygosity. These BRCA2-defective cancer cells were compared with cancer cells having the normal BRCA2 gene in a variety of assays. These assays were designed to determine whether a BRCA2 gene deficiency renders the cell incapable of repairing double-strand DNA breaks and to determine whether BRCA2 deficiency led to increased *in vivo* and *in vitro* cytotoxicity by agents that exert their cytotoxic effect via the induction of double-strand DNA breaks.

Materials and Methods

Cell lines and growth conditions. Capan-1 human pancreatic carcinoma, BxPC-3 human pancreatic carcinoma, AsPC-1 human pancreatic carcinoma, Hs766T human pancreatic carcinoma, PA-1 human ovarian carcinoma, and ES-2 human ovarian carcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA). Capan-1 cells were cultured in Iscove's minimal

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See ''Notes'' following ''References.''

essential medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) supplemented with 20% fetal bovine serum (FBS), 5 m*M* glutamine (Sigma Chemical Co., St. Louis, MO), and antibiotics (penicillin G, 100 U/mL; streptomycin sulfate, 100 μg/mL; amphotericin B, 0.25 μg/mL; Sigma Chemical Co.). AsPC-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% FBS, 5 m*M* glutamine, and antibiotics (*see* above). All other cell lines were cultured in DMEM supplemented with 10% FBS, 5 m*M* glutamine, and antibiotics (*see* above).

Polymerase chain reaction analysis and allele-specific oligonucleotides. To obtain cellular DNA, cells were lysed and protein was digested in 10 m*M* Tris–HCl (pH 7.5), 50 m*M* EDTA (pH 8.0), and 1 mg/mL Proteinase K (Life Technologies Inc.) overnight at 55 °C. The sample was then centrifuged at 1400*g* for 10 minutes at 4 °C. The supernatant was then extracted with Tris-equilibrated phenol plus chloroform and the DNA was precipitated in the presence of 70% ethanol and 0.3 *M* sodium acetate (pH 5.5) at –20 °C. Polymerase chain reaction (PCR) amplification of cellular DNA was performed with the BC11-RP and BC11-LP primers (Research Genetics, Huntsville, AL) and the products were resolved on a polyacrylamide sequencing gel as described *(10).* Detection of allele-specific oligonucleotide PCR products by blotting was performed as previously described *(11).* Briefly, cellular DNA was extracted from the cell lines by harvesting the cells in 10 m*M* Tris–HCl (pH 7.5), 50 m*M* EDTA, and 1 mg/mL Proteinase K at 55 °C. DNA was used as template for PCR with primers 6153F (5′-TGGGATTTTTAGCACAGC-3′) and 6391R (5′-AAGCAGATGAATTTACCACA-3′) synthesized by Ransom Hill Bioscience (Ramona, CA) and the products were electrophoresed through a native 1.5% agarose gel and transferred by capillary action to GeneScreen Plus nylon membranes (NEN Life Science Products, Boston, MA). After 1 hour of prehybridization at 42 °C, ³²P-end-labeled oligonucleotides synthesized by Ransom Hill Bioscience corresponding to the normal BRCA2 gene (5′-CACAGCAAG-TGGAAAATCT-3′) and the del6174T mutant BRCA2 gene (5′-CACAG-CAAGGGAAAATCTG-3′) were hybridized to the membranes overnight at 42 °C in 1 *M* NaCl, 10% dextran sulfate, and 1% sodium dodecyl sulfate (SDS). After washing in 0.1 *M* NaCl and 0.1% SDS at 1 °C below the melting temperature of the DNA hybrids, the membranes were subjected to autoradiography with intensifying screens at –70 °C for 4 hours.

DNA repair assays. One million cultured cells were embedded in 100-μL agarose plugs as described *(12–14).* The plugs were then exposed to the indicated dose of ionizing radiation at 4 °C. To allow the cells to repair damaged DNA, the plugs were then covered with 10% FBS-supplemented DMEM in a 37 °C incubator. After DNA repair was allowed to proceed for the indicated time, the plugs were transferred to a solution containing 10 m*M* Tris–HCl (pH 7.4), 20 m*M* NaCl, 50 m*M* EDTA, and 1 mg/mL Proteinase K. After incubation at 50 °C overnight, the plugs were embedded in a 0.7% native agarose gel in Tris acetate buffer and subjected to pulsed-field gel electrophoresis (CHEF system; Bio-Rad Laboratories, Richmond, CA) at 3 V/cm at 14 °C for 48 hours with a pulse time of 45 seconds. The gel was then stained with ethidium bromide and the DNA was visualized with ultraviolet light. Quantitation was performed with use of the Bio-Rad Gel Doc 1000 densitometer.

Radiosensitivity and drug sensitivity analysis. The radiosensitivity assays were performed as previously described *(15).* Briefly, Capan-1 cells, ES-2 cells, PA-1 cells, and BxPC-3 cells were seeded at 1000 cells/plate. Twelve hours later, the cells were exposed to different doses of ionizing radiation (¹³⁷Cs source, 2.35 Gy/min; J. L. Shepard and Associates, San Fernando, CA). The growth medium on the plates was changed every 3 days over a 2-week period. At the end of 2 weeks, the colonies were fixed in absolute ethanol and stained with crystal violet in ethanol. Colonies containing greater than 50 cells were considered a single colony for quantitation purposes. Relative survival refers to the number of colonies at a given dose compared with the number of colonies on an unirradiated plate seeded on the same day. Drug sensitivity tests were performed by seeding cells as single-cell suspensions and then exposing them to log dilutions of each drug for 1 hour at 37 °C. The cells were subsequently washed, reseeded in their appropriate complete growth media (*see* above) for 3 weeks, and stained; colonies were then quantified. The IC₅₀ was then determined as the drug concentration that caused a 50% inhibition of cell proliferation. Relative survival was calculated by standardization to the average survival of the other carcinoma cell lines, performed in triplicate.

Antisense inhibition studies. Modified phosphorothioate deoxyribonucleotides (synthesized by Midland Certified Reagent Company, Midland, TX) were analyzed by gel electrophoresis through denaturing 20% polyacrylamide gels and visualized by ultraviolet shadowing. Each oligonucleotide was shown to be

homogeneous and of appropriate molecular size. These oligonucleotides were further purified by repeated cycles of solubilization and lyophilization and finally resuspended in buffered media as described *(11).* The antisense BRCA2 oligonucleotide was designed to hybridize to the translation initiation site of the human BRCA2 gene (nucleotides 229–245 from GenBank accession number HSU43746). The sequence of the BRCA2 antisense oligonucleotide used in these studies (synthesized by Midland as described above) is: 5′-TTGGATCCAATAGGCAT-3′. The sequence of the complementary sense BRCA2 oligonucleotide is: 5′-ATGCCTATTGGATCCAA-3′. An oligonucleotide with a shuffled sequence containing four mismatched bases compared with the antisense BRCA2 oligonucleotide had the sequence: 5′-TTGGAATCATCAGGCAT-3′. To perform the antisense experiments, BxPC-3 carcinoma cells were grown in DMEM with 10% FBS and oligonucleotides were introduced by transfection with Lipofectin (Life Technologies, Inc.) as previously described *(11).* The inhibition of BRCA2 messenger RNA levels was determined by a ribonuclease (RNase) protection assay *(11).* Briefly, the BRCA2 hybridization probe consists of a fragment of exons 3–8 cloned into pBluescript SK(+) (Stratagene, La Jolla, CA). This plasmid was digested with *Pst* I restriction enzyme and transcribed with T3 polymerase in the presence of [³²P]guanosine triphosphate (GTP). A human glyceraldehyde-3-phosphate dehydrogenase (GADPH) complementary DNA, cloned as a *Sac* I–*Xba* I fragment *(11),* was used as a template to synthesize control hybridization probes. The GADPH hybridization probe was transcribed by use of T7 RNA polymerase in the presence of [³²P]GTP and unlabeled ribonucleotide triphosphates. Total RNA (2 μg) from each sample was hybridized with the ³²P-labeled hybridization probes for the BRCA2 gene and the GADPH gene in 50% formamide at 55 °C for 3 hours. The samples were treated with RNase A and RNase T1 (Life Technologies, Inc.) to degrade the unhybridized probes. The DNAs were recovered by ethanol precipitation and fractionated through an 8% denaturing polyacrylamide gel containing 6 *M* urea. The expected sizes of the RNase-protected fragments were 330 bases (BRCA2) and 140 bases (GADPH), respectively. Drug sensitivities of BxPC-3 cells transfected with sense, antisense, or shuffled oligonucleotide sequence were determined as described above.

Tumor sensitivity to ionizing radiation, mitoxantrone, and etoposide. Male nu/nu mice (Harlan Sprague-Dawley Corporation, Indianapolis, IN) were used for tumor studies. Guidelines established by the National Institutes of Health (Bethesda, MD) for the proper and humane use of animals in research were followed. The mice were fed only autoclaved food and water while housed in microisolator cages at all times. In addition, these mice were handled only with gloves. BxPC-3 cells and Capan-1 cells were grown in Iscove's minimal essential medium (Life Technologies, Inc.) with 15% fetal calf serum. Cells were scraped from tissue culture plates immediately prior to injection into the mice and 5×10^6 cells were injected into each animal. The cells were injected into the right quadriceps muscle of each mouse, so that the tumor could be irradiated at high doses but with little whole-body irradiation. After 2–4 weeks (tumor growth 5–8 mm greatest dimension), the tumors were either not irradiated (0 Gy), irradiated with 8 Gy or 10 Gy using a ⁶⁰Co irradiator (Neutron Products Inc., Dickerson, MD) at a dose rate of 2.35 Gy/min, or treated intraperitoneally with mitoxantrone or etoposide (doses of 3 μg/kg or 3 mg/kg respectively). Animals were examined daily and none of the mice showed local radiation damage to skin or soft tissue at the time of radiation. Two weeks later, the mice were killed. The tumor dimensions were measured, tumors were resected, fixed in formalin and embedded into paraffin, and 4-μm sections were produced with a microtome and stained with hematoxylin–eosin.

Statistical analysis. Statistical analysis was performed using SigmaPlot 4.10 software (Jandel Scientific, Corte Madera, CA). *P* values were calculated using the two-sided Student's *t* test (paired or independent, as appropriate). Error bars represent 95% confidence intervals. The survival curves were fitted as previously described *(12).*

Results

To determine whether BRCA2 gene mutation status could be used to define possible treatment approaches, we identified a human cancer cell line that only contains a known cancer-causing BRCA2 allele. We screened 10 cancer cell lines for the most common BRCA2 gene mutation, the 6174delT mutation found in the Ashkenazi Jewish population *(10).* This mutation truncates the carboxyl-terminal half of the Brca2 protein, delet-

ing the RAD51-interacting domain. A pancreatic cancer cell line, Capan-1, was found by PCR analysis to contain this mutation. Three other pancreatic carcinoma cell lines (HS766T, BxPC-3, and AsPC-1) that matched the Capan-1 cell line in tumor type and differentiation state did not contain this mutation (Fig. 1, A). Capan-1 cells also showed loss of heterozygosity at this locus (data not shown). Last, allele-specific oligonucleotides *(11)* were used to show that this cell line does not contain a normal copy of the BRCA2 gene. Genomic DNA from Capan-1 cells hybridized only to the 6174delT oligonucleotide, indicating that a normal copy of the gene was not present (Fig. 1, B). In contrast, three control cell lines showed only the normal allele (Fig. 1, B). Thus, by directly screening for the BRCA2 gene mutation, by looking for loss of heterozygosity, and by employing allele-specific oligonucleotide hybridization to show that the normal BRCA2 gene is not present, we established that Capan-1 cells contained only a mutated BRCA2 gene allele. This allele is of the type known to cause cancer in the Ashkenazi Jewish population and that leads to truncation of the RAD51-interacting domain of Brca2 protein itself.

The proposed role of Brca2 protein in double-strand break repair and data supporting the hypothesis that Brca2 protein has a role in such repair that is relevant to carcinogenesis have been based only on experiments conducted in mice with homologous recombination-induced defects in the BRCA2 gene *(6,8)*. Before we used the Capan-1 cell line to test the hypothesis that human cancers carrying a BRCA2 mutation would be hypersensitive to agents causing double-strand DNA breaks *(9)*, we first wanted to establish that human BRCA2-defective cancer cells were in fact deficient in double-strand DNA break repair. For this reason, DNA repair assays were performed. In these experiments, BxPC-3 cells were used as a control cell line. Like the Capan-1 cell line, the BxPC-3 cell line is a well-differentiated pancreatic adenocarcinoma cell line. BxPC-3 cells also showed loss of heterozygosity at the BRCA2 locus *(1)*, but unlike Capan-1 cells, BxPC-3 cells had one remaining normal BRCA2 allele (data not shown). In addition, the BxPC-3 cell line matches the Capan-1 cell line in the mutation status of a number of other tumor suppressors. Neither cell line contains functional p53, p16, or Rb genes *(1,16,17)*. To perform the DNA repair experiments, equal numbers of Capan-1 cells and BxPC-3 cells were exposed to ionizing radiation and allowed to repair the DNA
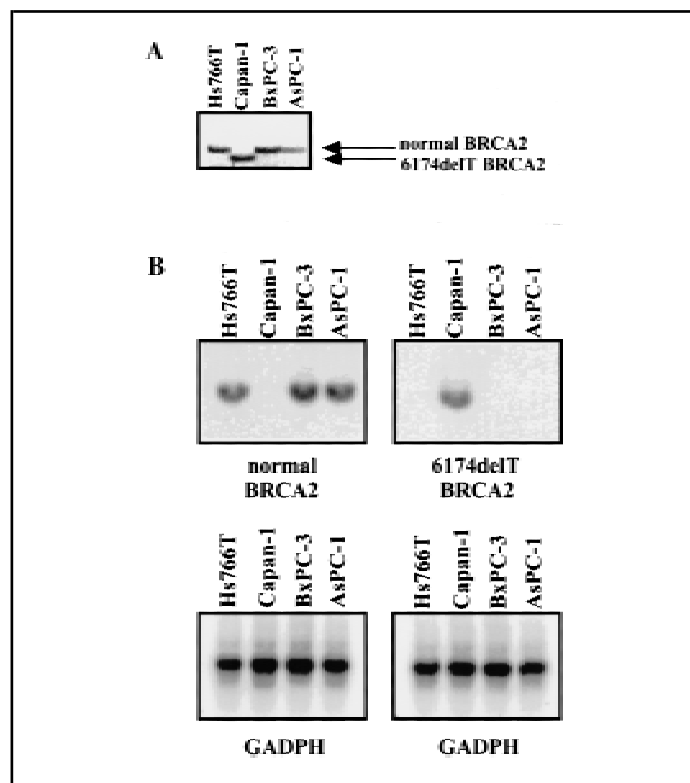
Fig. 1. Capan-1 cells contain only the del6174T mutant BRCA2 gene. **A)** Polymerase chain reaction (PCR) analysis of the BRCA2 gene. Genomic DNA from the four pancreatic carcinoma cell lines (HS766T, Capan-1, BxPC-3, and AsPC-1) was extracted and subjected to PCR analysis *(10)*. The Capan-1 cell line contains a deletion of T at 6174, so the PCR band is 95 base pairs (bp), whereas the three other cell lines contain a normal BRCA2 gene (96 bp). **B)** Allele-specific oligonucleotides *(11)* show that only the mutated allele is present in the Capan-1 cells. Upper panels: blots containing DNA from each cell line were probed either with an oligonucleotide corresponding to the normal BRCA2 (left panel) or an oligonucleotide corresponding to 6174delT BRCA2 (right panel) at a temperature 1 °C below their melting temperature. Lower panels: to show equal DNA loading, the blots were stripped and reprobed with oligonucleotides corresponding to glyceraldehyde-3-phosphate dehydrogenase (GADPH).
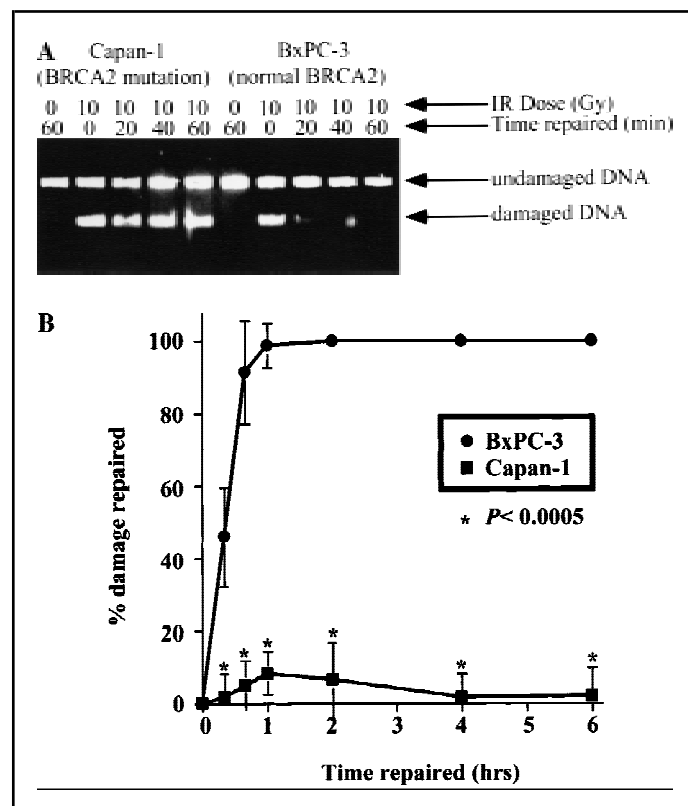
Fig. 2. Capan-1 cells show slower repair of DNA double-strand breaks induced by ionizing radiation. **A)** Equal cell numbers of BxPC-3 cells and Capan-1 cells were embedded in agarose plugs and exposed to 10-Gy ionizing radiation *(12,13)*. Cells were allowed to repair damaged DNA for the indicated amount of time before DNA was extracted and subjected to pulse-field gel electrophoresis. The gel was then stained with ethidium bromide and bands were visualized with UV light. The upper band is undamaged or repaired DNA. The lower band is damaged DNA, as shown by the arrows at the right. **B)** Quantitation of double-strand DNA break repair assays. Each data point was performed in triplicate. Shown is % damage repaired. Error bars represent 95% confidence intervals. Where error bars are not seen, the 95% confidence interval is too small to be visualized on this graph. Asterisks indicate $P<.0005$.

damage over time (Fig. 2). The DNA from these cell lines was prepared and subjected to pulse-field gel electrophoresis and the DNA was visualized by ethidium bromide staining. A representative experiment is shown in Fig. 2, A. Capan-1 cells were found to be unable to repair double-strand DNA damage (the upper band on the gel represents undamaged or repaired DNA and the lower band represents damaged DNA, Fig. 2, A). Results from three independent experiments were quantitated. The data are presented in Fig. 2, B. Little repair occurred in the cells of the BRCA2-deficient line even 6 hours after irradiation (Fig. 2). In contrast, nearly all of the damage to the BxPC-3 cell line was repaired within 40 minutes after irradiation (Fig. 2, A and B). Thus, the BRCA2-defective cell line shows a significantly decreased ability to repair double-strand DNA breaks ($P<.0005$ at all data points).

Because the cancer cell line containing the BRCA2 mutation showed a striking deficiency in double-strand break repair and because agents that cause double-strand DNA breaks are commonly used in cancer chemotherapy, we decided to test the Capan-1 cell line for sensitivity to these agents. As additional controls, two ovarian cancer cell lines (ES-2 and PA-1) that do not show a loss of heterozygosity at the BRCA2 locus (data not shown) were also tested for sensitivity to chemotherapeutic agents. PA-1 cells contain two normal copies of the p16 gene *(18)*, overexpressed normal p53 protein *(19),* and contained one mutant Rb allele *(18),* but the sequences of the p16, p53, and RB genes in the ES-2 cells are not known. Pharmacologic agents were chosen for their ability to induce double-strand DNA breaks and for their use in clinical chemotherapy. Mitoxantrone is a chemotherapeutic agent that causes double-strand DNA breaks directly, whereas both amsacrine and etoposide cause double-strand DNA breaks indirectly via an inhibition of topoisomerase II *(20).* Capan-1 cells are highly sensitive to mitoxantrone, amsacrine, and etoposide and show a threefold to tenfold difference in sensitivity when compared with the control cells (Fig. 3). Paclitaxel and hydroxyurea were used as control treatments to determine differences in the survival of the different cell types. Paclitaxel causes cytotoxicity by an inhibition of microtubule dissociation and hydroxyurea causes cytotoxicity by inhibiting ribonucleotide reductase *(20).* These agents do not cause double-strand DNA breaks *(20).* Capan-1 cells showed similar sensitivity to both paclitaxel and hydroxyurea when compared with the control cells, so BRCA2-defective cancer cells are only hypersensitive to pharmacologic agents that cause double-strand DNA breaks.

Because Rad51 was originally identified as a key protein in the recombination repair of ionizing radiation-induced double-strand DNA breaks, Capan-1 cells were also tested for radiation sensitivity using the colony-forming assay *(15).* The results of a representative experiment are shown in Fig. 4, A. Significantly fewer colonies were observed for Capan-1 cells irradiated with as little as 1 Gy ionizing radiation, and this survival is quantitated in Fig. 4, B. At all doses of radiation, survival of the Capan-1 cell line is statistically lower than the survival of the control cell lines ($P<.002$ at all data points). Survival curves have been used to measure relative sensitivities of cell lines to ionizing radiation and to examine DNA repair capacity. It has been demonstrated *(14,21)* that the most appropriate analysis for distinguishing differences in radiosensitivity is obtained from
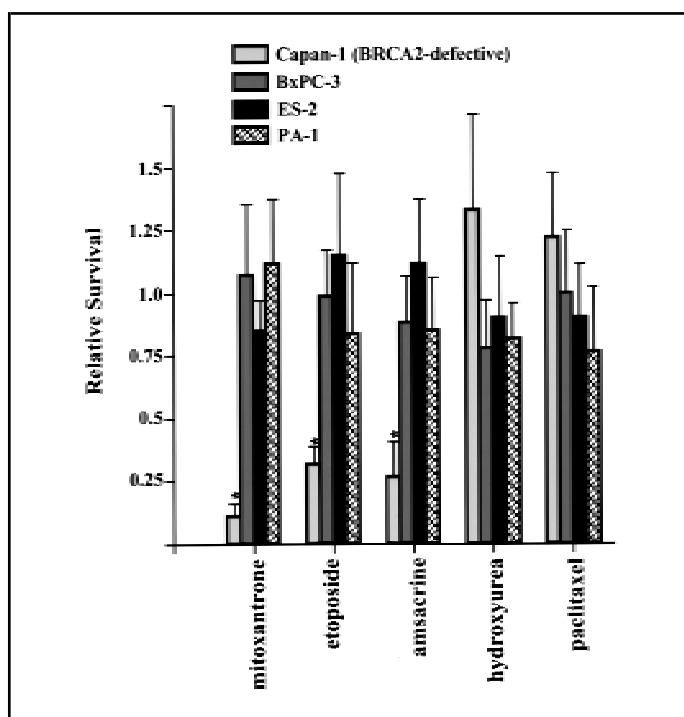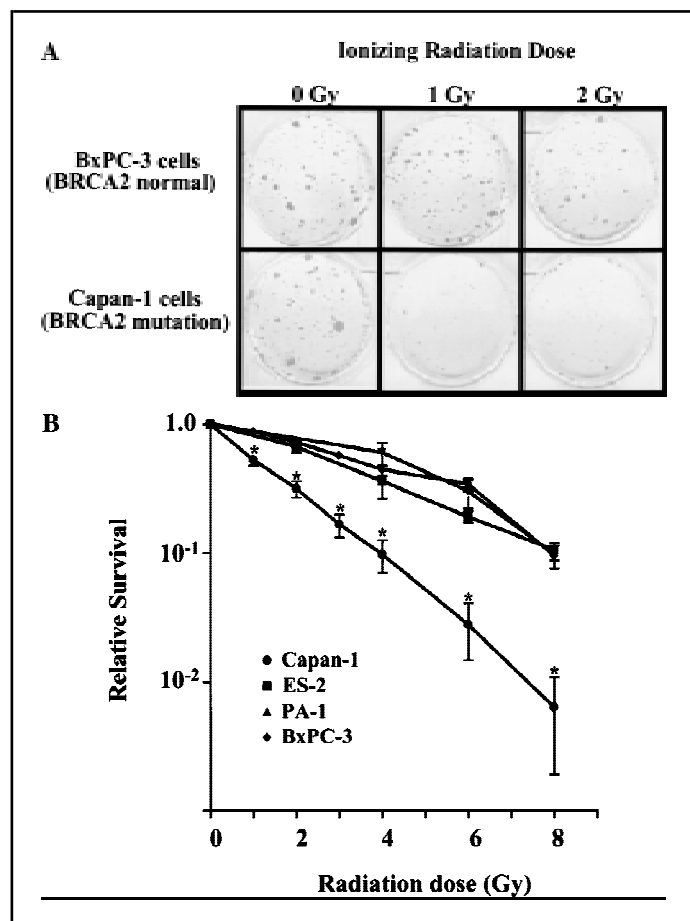

Fig. 3. Relative survival of human tumor cells after exposure to agents that cause double-strand DNA breaks. Cells were seeded at 1000 cells/plate and assayed for colony formation and IC$_{50}$ (inhibitory concentration of drug which produces 50% growth inhibition) values after exposure to varying doses of each drug (*see* ''Materials and Methods''). Data are presented in terms of relative survival in comparison with untreated cells. Error bars represent 95% confidence intervals. IC$_{50}$'s are as follows: mitoxantrone—Capan-1 = 0.0025 μM, BxPC-3 = 0.02 μM, ES-2 = 0.027 μM, and PA-1 = 0.028 μM. Etoposide (VP16)—Capan-1 = 0.25 μM, BxPC-3 = 0.8 μM, ES-2 = 0.9 μM, and PA-1 = 0.65 μM. Amsacrine—Capan-1 = 0.12 μM, BxPC-3 = 0.36 μM, ES-2 = 0.46 μM, and PA-1 = 0.3 μM. Paclitaxel—Capan-1 = 1.22 μM, BxPC-3 = 1.00 μM, ES-2 = 0.90 μM, and PA-1 = 0.77 μM. Hydroxyurea—Capan-1 = 1.32 μM, BxPC-3 = 0.79 μM, ES-2 = 0.90 μM, and PA-1 = 0.82 μM. P values were calculated by use of the two-sided Student's *t* test (independent) and ranged from a high of 2.5×10^{-3} to a low of 7.3×10^{-4} when Capan-1 cell survival was compared with the control cell lines (BxPC-3, ES-2, and PA-1) following treatment with mitoxantrone, etoposide, or amsacrine. There were no statistically significant survival differences between the Capan-1 cells and the control cells in the presence of paclitaxel or hydroxyurea, agents that do not cause DNA breaks. Asterisk shows significance at the $P = .002$ level.

the initial portion of the survival curve and by fitting the data to a linear quadratic model:

$$\ln S = -\alpha D - \beta D^2,$$

where S represents cell survival and D represents radiation dose in Gy. The α coefficient represents the rate of cell killing produced by a single particle traversing a cell and predominates at low doses of radiation where the probability of multiple particles interacting with the cell is low. The β coefficient represents the rate of cell killing produced when more than one particle traverses a cell and is generally taken to represent a cell's ability to repair damaged DNA. The survival data presented in Fig. 4, B, were analyzed using this linear quadratic model (Table 1). The β coefficient calculated for the Capan-1 cells over 2 decades of survival was zero. Therefore, this curve can be described as a strict exponential function. Typically, cell lines that can repair sublethal damage exhibit a β coefficient greater than zero. Sublethal damage repair is damage that can be repaired between

Fig. 4. Relative survival of human tumor cells after exposure to ionizing radiation. **A)** Sensitivity of Capan-1 cells to ionizing radiation. Cells were seeded at 1000 cells/plate and exposed to different doses of ionizing radiation. The cells received fresh media every 3 days for 2 weeks as described in ''Materials and Methods.'' Surviving colonies were then stained with crystal violet and counted. A representative experiment is shown for Capan-1 cells and BxPC-3 (control) cells receiving 0, 1, or 2 Gy of radiation. Upper panels: BxPC-3 cells showing lower sensitivity with increasing doses of ionizing radiation. Lower panels: Capan-1 cells showing higher sensitivity with increasing doses of ionizing radiation. **B)** Relative survival of human tumor cells after exposure to ionizing radiation. Relative survival was calculated relative to unirradiated cells plated at the same cell number and allowed to grow for the same number of days. Data presented are from six separate experiments. The survival of Capan-1 cells relative to each of the control cell lines (ES-2, PA-1, and BxPC-3) at each dose was compared and P values were calculated using the two-sided Student's t test (independent), and those values range from a low of 6.3×10^{-8} to a high of 1.4×10^{-3}, with all points showing significance at the $P = .001$ level (asterisk).

clinical radiation fractions. The fact that Capan-1 cells show a β coefficient of zero implies that these cells cannot repair sublethal damage, and this inability raises the possibility that radiation treatment could be useful in treating patients with BRCA2 mutations.

Because these sensitivity studies were not performed in genetically identical cell lines, we wanted to establish that the sensitivity to double-strand DNA breaks seen in the BRCA2-defective cells was in fact due to the mutant Brca2 protein. We therefore used antisense technology to inhibit BRCA2 gene expression in the BxPC-3 cell line. BxPC-3 cells were transfected with sense, antisense, and shuffled phosphorothioate oligonucleotides (*see* ''Materials and Methods''). Because reliable BRCA2 antisera are not available, we performed ribonuclease protection

assays to determine the level of BRCA2 gene expression. Cells transfected with antisense BRCA2 oligonucleotides showed substantially lower levels of BRCA2 messenger RNA than those cells transfected with either the sense or the shuffled oligonucleotide (Fig. 5, A, lanes 3–6). Similarly, mitoxantrone exposure had no effect on the degree that BRCA2 messenger RNA levels were inhibited (Fig. 5, A, lanes 7–10). To show that decreased BRCA2 gene expression correlated with increased sensitivity to double-strand DNA breaks, survival was assayed in the presence of mitoxantrone. The BxPC3 cells treated with antisense BRCA2 oligonucleotides were significantly more sensitive to mitoxantrone than those cells treated with a sense BRCA2 oligonucleotide or a shuffled BRCA2 oligonucleotide (Fig. 5, B; $P<.01$). Because antisense inhibition of BRCA2 messenger RNA levels in cells with normal BRCA2 genes mimics the effect seen in BRCA2-defective cancer cells, it is likely that the sensitivity of Capan-1 cells to agents causing double-strand DNA breaks is due to a mutant BRCA2 gene.

To test whether this sensitivity to double-strand DNA breaks would be applicable to *in vivo* solid tumors, the BRCA2-defective cells were injected into the thigh muscle of nude mice. The thigh was chosen so that radiation exposure could be localized and whole-body exposure could be minimized. As a control, a pancreatic adenocarcinoma cell line expressing the normal BRCA2 gene (BxPC-3) was used to generate tumors. After appropriate tumor growth (5–8 mm greatest dimension), the tumors were left unirradiated (0 Gy) or irradiated with 8 or 10 Gy of ionizing radiation. At these doses of radiation, there was no local skin sloughing. In addition, we did not observe any whole-body radiation toxicity effects. At the indicated time points after irradiation, the tumor volumes were measured. The BRCA2-defective tumors showed a statistically significant decrease in tumor volume (Fig. 6, A). At 10 Gy, the tumor volume had decreased 93% in the BRCA2-defective tumors, while there was no decrease in tumor volume in the control cell line (Fig. 6, A). In addition, histologic analysis of the irradiated BRCA2-defective tumors showed a large degree of necrosis, while the control tumors showed no necrosis (data not shown, available on request). This finding was surprising because at the doses chosen, we predicted that tumor growth would be slowed but would not cause necrosis. A computer-based survey of the literature indicated that the usual dose of ionizing radiation needed to cause necrosis in human cancer xenografts is approximately 60–70 Gy *(22–24)*. It has also been reported that the doses of ionizing radiation necessary to control 50% of soft tissue xenografts range from approximately 25 Gy to 66 Gy *(22–24)*. The BRCA2-defective xenografts exhibited areas of necrosis after

**Table 1.** Dose–response parameters for irradiated cell lines

Cell line	$\alpha(Gy^{-1})$*	$\beta(Gy^{-2})$†
Capan-1	0.81	0
BxPC-3	0.16	0.0325
PA-1	0.10	0.0325
ES-2	0.19	0.0415

*Coefficient represents the rate of cell killing produced by a single particle traversing a cell since the $\ln S = -\alpha D - \beta D^2$ where S is survival and D is dose in Gy.

†Coefficient represents the rate of cell killing produced by multiple particles.
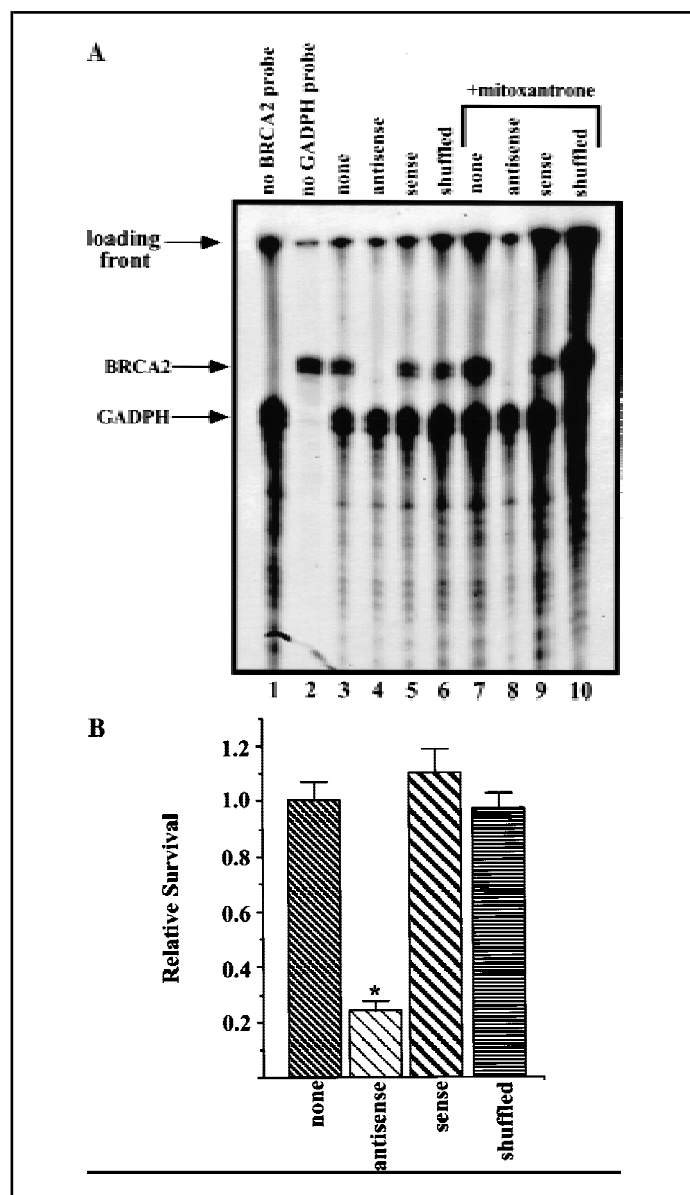
Fig. 5. Antisense inhibition of BRCA2 messenger RNA levels and increased sensitivity of BxPC-3 cells to double-strand DNA breaks. **A)** Nuclease protection assay analyzing extent of BRCA2 gene inhibition by antisense deoxyribonucleotides. BxPC-3 cells (expressing normal BRCA2) were transfected with antisense (lanes 4 and 8), sense (lanes 5 and 9), or shuffled (lanes 6 and 10) BRCA2 antisense single-stranded DNA sequences. Cells that received no exogenous DNA are shown in lanes 3 and 7. Nuclease protection assays were then performed to determine BRCA2 messenger RNA levels following transfection with each single-stranded DNA. In addition, cells were also transfected in the presence of mitoxantrone to determine if mitoxantrone affected antisense oligonucleotide-mediated inhibition of BRCA2 messenger RNA levels (lanes 6–10). The upper band represents BRCA2 messenger RNA. To control for equivalent RNA loading, the levels of glyceraldehyde-3-phosphate dehydrogenase (GADPH) messenger RNA were also measured (lower band). *See* ''Materials and Methods'' for sequence of oligonucleotides and methods for nuclease protection assay. **B)** Relative survival of cells treated with antisense BRCA2 deoxyribonucleotides plus mitoxantrone. BxPC-3 cells transfected with antisense, sense, or shuffled BRCA2 deoxyribonucleotides sequences or untransfected BxPC-3 cells were exposed to mitoxantrone, and cell survival was determined. Results are presented as relative survival. Asterisk indicates statistically significant differences (two-sided *P*<.01; Student's *t* test) between antisense deoxyribonucleotide-treated cells and either sense or shuffled deoxyribonucleotide-treated cells.
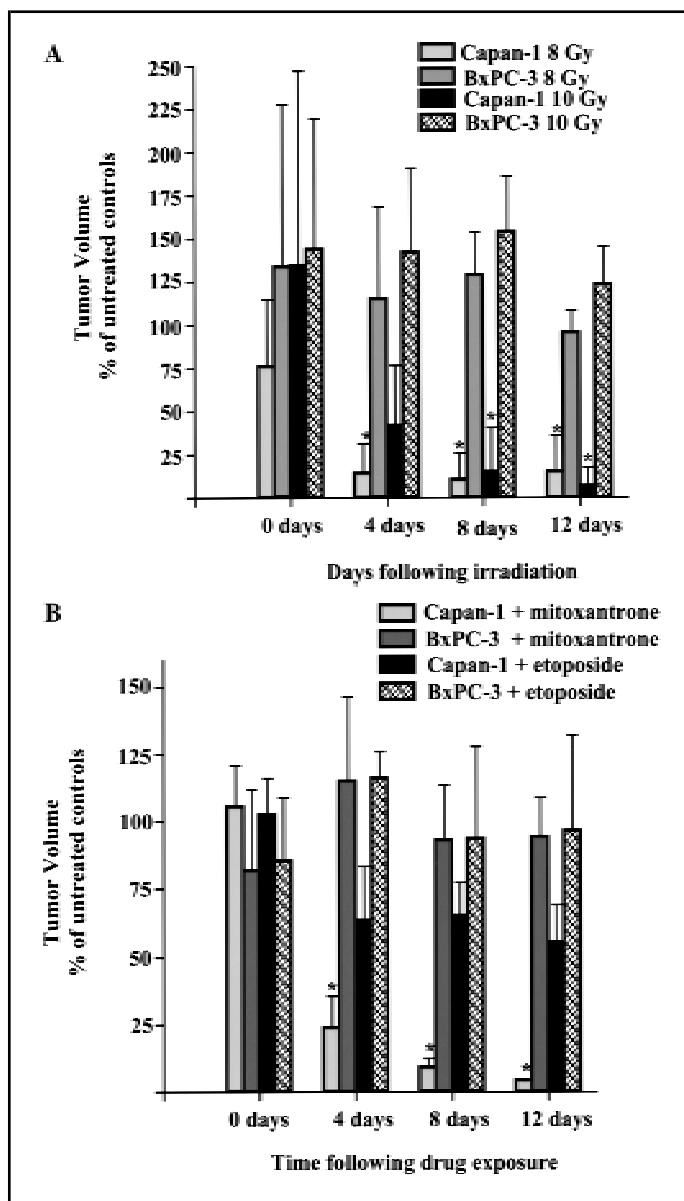
Fig. 6. Volumes of BRCA2-defective and BRCA2-normal tumors on treatment with agents that cause double-strand DNA breaks. **A)** Tumor volumes following exposure to ionizing radiation (8 and 10 Gy) compared with unirradiated tumors. N = 5 for each experimental group. The error bars represent 95% confidence intervals. Early in tumor growth (day 0), error is larger due to the fact that the tumors were smaller. *P* values were calculated by comparing the tumor volume of the Capan-1 xenografts with that of the BxPC-3 xenografts via the two-sided Student's *t* test (paired). Those values are as follows: 8-Gy radiation dose—0 days ($P = .18$); 4 days ($P = 4.6 \times 10^{-3}$); 8 days ($P = 1.2 \times 10^{-4}$); and 12 days ($P = 2.4 \times 10^{-3}$). 10-Gy radiation dose—0 days ($P = .86$); 4 days ($P = 9.2 \times 10^{-5}$); 8 days ($P = 3.6 \times 10^{-7}$); and 12 days ($P = 1.6 \times 10^{-5}$). Asterisk shows significance at all data points at $P = .002$. **B)** Tumor volumes on exposure to mitoxantrone and etoposide when compared with untreated tumors. N = 5 in each experimental group. Error bars represent 95% confidence intervals. Early in tumor growth (day 0), error is larger due to the fact that the tumors are smaller. *P* values were calculated by comparing the tumor volume of the Capan-1 xenografts with the BxPC-3 xenografts via the Student's *t* test (paired). Values are as follows: mitoxantrone, 0 days ($P = .13$); 4 days ($P = 2.9 \times 10^{-3}$); 8 days ($P = 5.5 \times 10^{-4}$); and 12 days ($P = 8.9 \times 10^{-5}$). Etoposide, 0 days ($P = .21$); 4 days ($P = 3.9 \times 10^{-3}$); 8 days ($P = .087$); and 12 days ($P = .075$). Asterisk shows significance at all data points at $P = .002$.

treatment with only 8 Gy of radiation and less than 8 Gy was needed to control 50% of tumors (Fig. 6, A). Thus, the data show that these BRCA2-defective xenograft tumors were sensitive to radiation treatments when compared with xenograft tumors matched for tumor type and differentiation status that do not have the same BRCA2 genetic defect.

If the cytotoxicity of ionizing radiation was due to the induction of double-strand DNA breaks in the BRCA2 mutant cancer cells, then chemotherapeutic agents that induce cytotoxicity via double-strand DNA breaks should have similar effects on nude mouse tumor xenografts. Tumors from BRCA2-defective cells and BRCA2-normal cells were again generated in nude mice. After appropriate tumor growth (5–8 mm greatest dimension), the mice were treated intraperitoneally with either mitoxantrone (3 mg/kg) or etoposide (3 mg/kg). Tumors grown from BRCA2-defective cells (Capan-1) were extremely sensitive to mitoxantrone with a 96% overall tumor reduction (Fig. 6, B). Etoposide showed an intermediate effect with a 45% tumor reduction (Fig. 6, B). Tumors grown from BRCA2-normal cells (BxPC-3) showed no reduction in size upon treatment with either mitoxantrone or etoposide (Fig. 6, B). Thus, pancreatic cancer cells containing a BRCA2 gene mutation were highly sensitive to cancer treatments whose efficacy is based on the induction of double-strand DNA breaks.

Discussion

We have employed BRCA2-defective pancreatic cancer cells to show that the BRCA2 hereditary cancer genotype may predict defective double-strand break repair and a corresponding increased sensitivity to radiation and certain chemotherapeutic drugs. Our data indicate that the BRCA2-defective Capan-1 cells, which contain only a mutant BRCA2 gene (the 6174delT Ashkenazi mutation in the BRCA2 gene), have a marked defect in double-strand break repair and radiation hypersensitivity both *in vitro* and *in vivo* using nude mouse xenografts. Nude mouse tumors initiated by the injection of these BRCA2-defective cells were also shown to be highly sensitive (>90% tumor size reduction) to doses of ionizing radiation and mitoxantrone that did not affect control tumors.

Previous studies (6,7) have elucidated a role for Brca2 protein in DNA repair mechanisms. The Brca2 protein interacts with Rad51 protein, which is required for DNA double-strand break repair, and studies employing transgenic mice have shown that animals with hypomorphic BRCA2 genes are unable to repair double-strand DNA breaks (8,25). However, because Brca2 protein is such a large molecule (350 kd), it is unlikely that the interaction with Rad51 is the only protein–protein interaction of Brca2 protein or that Brca2 protein function is limited only to double-strand DNA break repair. In addition, because the mouse BRCA2 genes and human BRCA2 genes show only 57% nucleotide sequence identity (3,6), it was not certain that human Brca2 protein and mouse Brca2 protein would have similar double-strand break repair effects or that the effect of double-strand break repair on genomic instability would be relevant for human carcinogenesis. Our results indicate that human cancer cells containing a mutation in the BRCA2 gene that deletes the Rad51-interacting region (6,8) are defective in double-strand break repair. This further substantiates the fact that the genomic instability first seen in mice expressing hypomorphic BRCA2 (25) may be a precipitating cause of carcinogenesis in individuals carrying BRCA2 mutations. Last, our results suggest that the BRCA2 mutant tumor genotype may predict response to specific therapies, providing a new paradigm for genotype-based tumor treatment.

Because pancreatic cancer cells have numerous mutations in important genes, including p53, p16, and ras (16,17), it is perhaps surprising that a BRCA2 mutation produces this marked alteration in sensitivity to ionizing radiation and to mitoxantrone. Our results showed that antisense inhibition of BRCA2 increased double-strand DNA break sensitivity (Fig. 5). This observation has been supported by the data of others showing that gene transfer of a normal BRCA2 gene into Capan-1 cells reverses sensitivity to methyl methanesulfonate (26). These data suggest that a mutated BRCA2 gene may be the key element affecting DNA repair even in the midst of multiple mutations of other genes. It has been proposed that the majority of cancers may result from impaired DNA repair leading to genomic instability (9). This proposal implies that agents that put stress on repair pathways might provide a selective means to kill tumor cells, while sparing normal cells. This idea conflicts with prior clinical experience indicating that most advanced tumors are not inherently radiation sensitive. However, the data presented in this study show that cancer cells with mutations in the p53, the p16 (also known as CDKN2A), and the RB1 genes remain quite radiation resistant unless a BRCA2 gene mutation is present.

Although the results of this study must be confirmed by analysis of additional BRCA2-defective cancers before treatment strategies may be implemented, it suggests an intriguing new strategy for breast cancer genetic screening. Present strategies for cancer genetic screening test asymptomatic high-risk populations to identify mutation carriers. We propose that BRCA2 genetic screening might ultimately be performed as a predictor of response to specific therapeutic approaches in patients who already have cancer and should not be restricted to risk assessment screening.

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Notes