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Utility of DNA Repair Protein Foci for the Detection of Putative BRCA1 Pathway Defects in Breast Cancer Biopsies

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Abstract

The DNA damage response pathway controlled by the breast cancer and Fanconi anemia (FA) genes can be disrupted by genetic or epigenetic mechanisms in breast cancer. Defects in this pathway may render the affected tumors hypersensitive to DNA-damaging agents. The identification of these defects poses a challenge because of the large number of genes involved in the FA/BRCA pathway. Many pathway components form subnuclear repair protein foci upon exposure to ionizing radiation *in vitro*, but it was unknown whether foci can be detected in live cancer tissues. Thus, the goal of this pilot study was to identify pathway defects by using a novel *ex vivo* foci biomarker assay on tumor biopsies. Fresh pretreatment biopsy specimens from patients with locally advanced sporadic breast cancer were irradiated or mock-treated in the laboratory (*ex vivo*). Foci formation of DNA repair proteins BRCA1, FANCD2, and RAD51 was detected by immunofluorescence microscopy. Three out of seven tumors showed intact radiation-induced foci formation, whereas the other four tumors exhibited a defective foci response. Notably, three of the foci-defective tumors were estrogen receptor/progesterone receptor/HER2–negative (triple-negative), a phenotype that has been associated with BRCA1 deficiency. In conclusion, in this pilot study, we report the successful detection of BRCA1, FANCD2, and RAD51 foci in breast cancer biopsies irradiated *ex vivo*. Our approach represents a potentially powerful biomarker assay for the detection of pre-existing and functionally important defects within the complex FA/BRCA pathway, which may ultimately allow us to tailor cancer treatment to the DNA repair profile of individual tumors. (Mol Cancer Res 2009;7(8):1304–9)

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Introduction

The tumor suppressor gene, *BRCA1*, is mutated in up to 50% of cases of familial early-onset breast cancer and in most families with hereditary breast and ovarian cancer. The vast majority of cancers arising in *BRCA1* germ line mutation carriers are of the basal-like subtype (1-3), a genetically distinct group of tumors characterized by markers expressed in normal basal/myoepithelial cells (4). Basal-like cancers only infrequently express estrogen receptor or progesterone receptor, and rarely express HER2 (4), which has been referred to as a triple-negative phenotype. On a cellular level, loss of BRCA1 function leads to multiple abnormalities, including a defect in the homologous recombination pathway of DNA repair (reviewed in refs. 5-7). The homologous recombination defect is associated with a hypersensitivity to many agents that cause DNA double-strand breaks or interfere with DNA replication, such as ionizing radiation (IR) or cisplatin.

Fanconi's anemia (FA) is a rare hereditary childhood disorder characterized by many abnormalities including chromosomal instability and cancer predisposition (reviewed in refs. 8, 9). FA has autosomal or X-linked inheritance caused by mutation in any of 13 known genes (*FANCA* to *N*). The pathways controlled by the FA and BRCA1 proteins are connected on multiple levels including a functional BRCA1-FANCD2 interaction (10). Thus, these proteins are regarded as forming a common "FA/BRCA" pathway. The exact mechanisms by which the FA proteins are involved in the repair of damaged DNA and maintenance of chromosomal stability remain to be elucidated. Accumulating evidence suggests that the FA proteins participate in the maintenance of DNA replication forks, thereby cooperating with BRCA1 to promote homologous recombination (8). Recently, the breast cancer–associated genes *BRIP1* and *PALB2* have been identified as FA genes *FANCJ* and *FANCN*, respectively (11, 12), further strengthening the importance of the FA/BRCA pathway for breast carcinogenesis.

BRCA1 mutations are rarely found in sporadic breast cancer (reviewed in ref. 13), but reduced BRCA1 expression has been reported in a subset of these cancers, particularly in patients with triple-negative tumors (14-17). However, whether reduced BRCA1 protein expression has functional consequences remains unknown. In addition, given the network-like complexity of the FA/BRCA pathway, assessing the expression of individual known proteins is unlikely to reveal the overall incidence of defects that can occur anywhere in the pathway (9).

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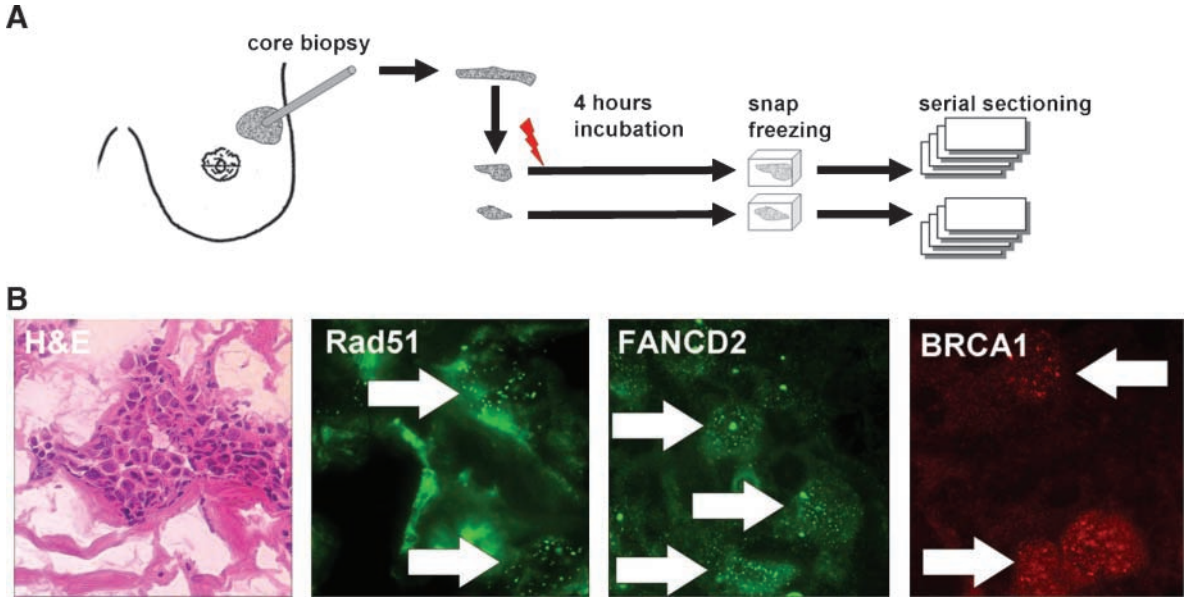

FIGURE 1. Detection of repair protein foci in fresh-frozen breast cancer tissues. **A.** Illustration of the experimental approach. **B.** Representative images illustrating the detection of areas containing viable tumor as well as subnuclear RAD51, FANCD2, and BRCA1 foci.

One important aspect that determines the effectiveness of the cellular response to cytotoxic therapies is the recruitment of pathway components to nuclear regions containing the damaged DNA (8, 9, 18-20). The cytologic manifestation of this nuclear redistribution is the formation of protein foci, which can be visualized by immunofluorescence microscopy. The repair activity of the FA/BRCA network is less dependent on protein expression levels than on the ability to localize these proteins into foci, in order to coordinate and execute repair (10, 21-23). Thus, the ability of the cells to form repair foci may serve as a functional biomarker of the integrity of the FA/BRCA pathway and associated resistance to chemotherapeutics (7, 9). An important feature of the FA/BRCA pathway is that its activity is frequently revealed only when cells are exposed to DNA-damaging agents. In untreated repair-proficient cells, BRCA1 and RAD51 foci may be visible in S phase even

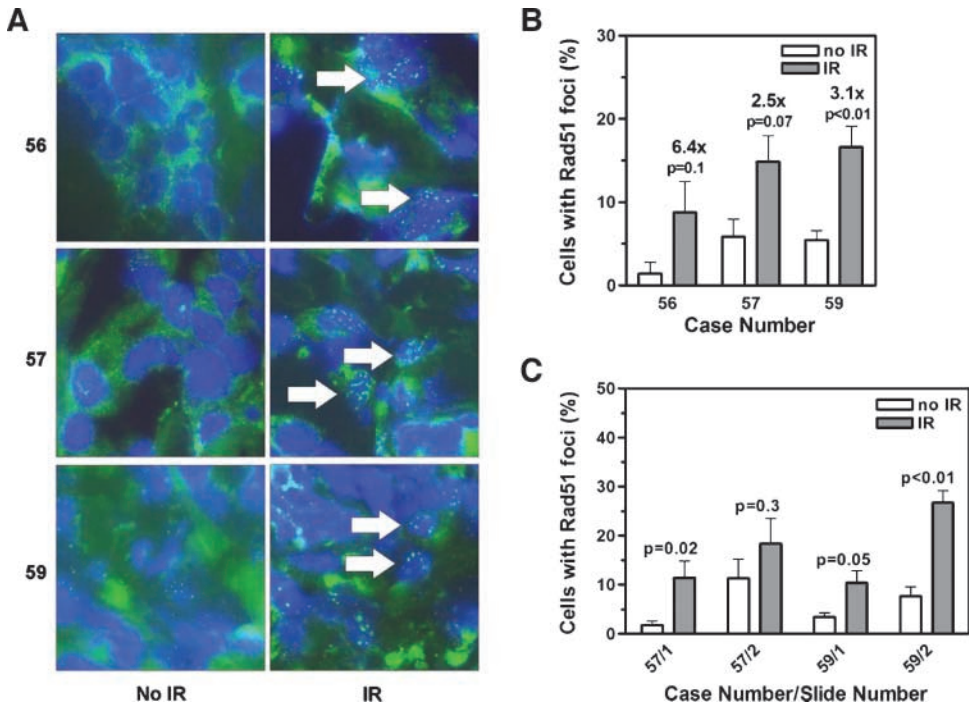

FIGURE 2. **A.** Representative images illustrating the induction of RAD51 foci in three tumors (cases 56, 57, and 59) following exposure of samples to IR *ex vivo*. **B.** Quantification of cells with RAD51 foci. Columns, means; bars, upper SE. Relative fold induction with IR is given. *P* value, unpaired *t* test, two-sided. **C.** RAD51 foci analysis analogous to **B**. Numbers, slide numbers per case.

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in the presence of pathway defects, and the fraction of cells with foci and the number of foci per cell increases postirradiation (22, 24, 25). In contrast, BRCA1-deficient cells have an impaired ability to mount a FANCD2 and RAD51 foci response (10, 22).

Therefore, the purpose of this pilot study was to monitor the activation of the FA/BRCA pathway in sporadic human breast cancers by subjecting biopsy specimens to IR *ex vivo*. We report the successful detection of RAD51, BRCA1, and FANCD2 IR-induced foci (IRIF) in these tissues. The ability of individual tumor cells to form IRIF allowed the classification of tumors as having functional versus nonfunctional BRCA1 status. Our approach represents a potentially powerful functional biomarker assay that may enable us to detect pre-existing and clinically relevant defects within the complex FA/BRCA DNA damage response pathway.

Results

Detection of IRIF in Breast Cancer Cells Ex vivo

For visualization of BRCA1, FANCD2, and RAD51 foci in live tumor tissues, core biopsies from previously untreated breast cancers were subjected to X-irradiation with 8 Gy or mock treatment (Fig. 1A). Samples were incubated in a cell culture incubator for 4 hours to allow for foci formation and then snap-frozen for later analysis. Viable tumors were identified on serial sections by H&E staining (Fig. 1B). Repair protein foci could be readily visualized in individual irradiated cells (Fig. 1B), but were often difficult to detect in the nonirradiated sample from the same tumor (see below). The observed foci are morphologically similar to IRIF seen in human cell lines (22).

IR Induces RAD51 Foci Formation Irrespective of Sectioning

Three out of seven irradiated tumor tissues clearly showed an increased percentage of cell nuclei containing RAD51 foci when compared with the nonirradiated controls (Fig. 2A). The number of cells containing RAD51 foci ranged from 9% to 17%, whereas the fraction of positive cells was significantly lower in the nonirradiated samples (range, 1-6%; Fig. 2B). To assess the effect of potential intratumoral heterogeneity, we also compared sections from more distant locations within each biopsy, which is illustrated in Fig. 2C. For example, in tumor no. 57, there was some heterogeneity as the induction of foci was less pronounced in section no. 2 (1.7-fold) compared with section no. 1 (5.5-fold). This was due to a higher background of RAD51 foci in the nonirradiated sample. Specifically, in one of the captured high-power images, the percentage of positive cells was high at 28%, likely reflecting a particularly proliferative group of tumor cells in that location, as spontaneous RAD51 foci are known to form in S phase. In another tumor (no. 59), there was less variation in the nonirradiated sample, with the percentage of RAD51-positive cells ranging from 0% to 8% (mean of 3.5%, *section no. 1*) and 0% to 11% (7.1%, *section no. 2*) for each 100× image. The corresponding induction of foci was ~3-fold to 3.5-fold. Together, these data indicate that IR-induced RAD51 foci formation can be reliably detected and semiquantified using one to two sections from tumor tissues irradiated *ex vivo*.

Correlation of RAD51 IRIF with BRCA1 and FANCD2 IRIF

The ability of cells to form RAD51 IRIF is dependent on intact BRCA1 function (22). We, therefore, expected that the same tumors which showed RAD51 IRIF would also show IR-inducible BRCA1 foci formation. In the three tumors with intact RAD51 foci response, BRCA1 foci were largely absent in nonirradiated samples (0-3.7%; Fig. 3A) but could be detected in the treated samples at frequencies of 6% to 14% (Fig. 3B). The relative induction of BRCA1 foci by IR was of similar magnitude as for RAD51. In addition, the formation of FANCD2 IRIF paralleled the induction of BRCA1 and RAD51 foci in tumor nos. 56 and 59. However, in tumor no. 57, no FANCD2 IRIF were observed as the percentage of cells with foci was almost identical in the untreated and irradiated samples (~6-7%). The underlying mechanism was not known because the formation of RAD51 IRIF indicated normal BRCA1 function and the presence of spontaneous FANCD2 foci suggested that the upstream nuclear FANC core complex was intact.

Concordant Lack of RAD51, BRCA1, and FANCD2 IRIF

In the remaining four tumors, no RAD51 IRIF could be observed (Fig. 4A). Of note was the variation in the mean fraction of RAD51-positive cells, ranging from 6% to 22% among the four samples, which likely is a reflection of tumor heterogeneity. We therefore analyzed additional sections to exclude the

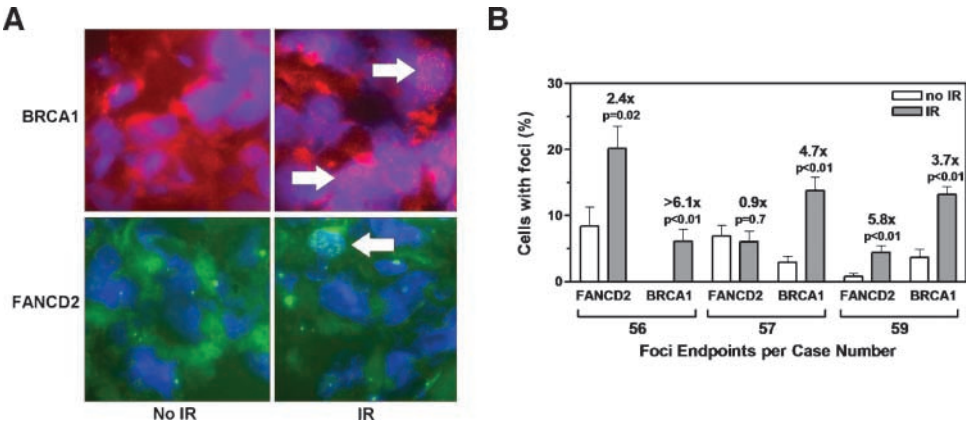

FIGURE 3. **A.** Representative images as in Fig. 2A. **B.** Quantitative analysis of foci as in Fig. 2B.

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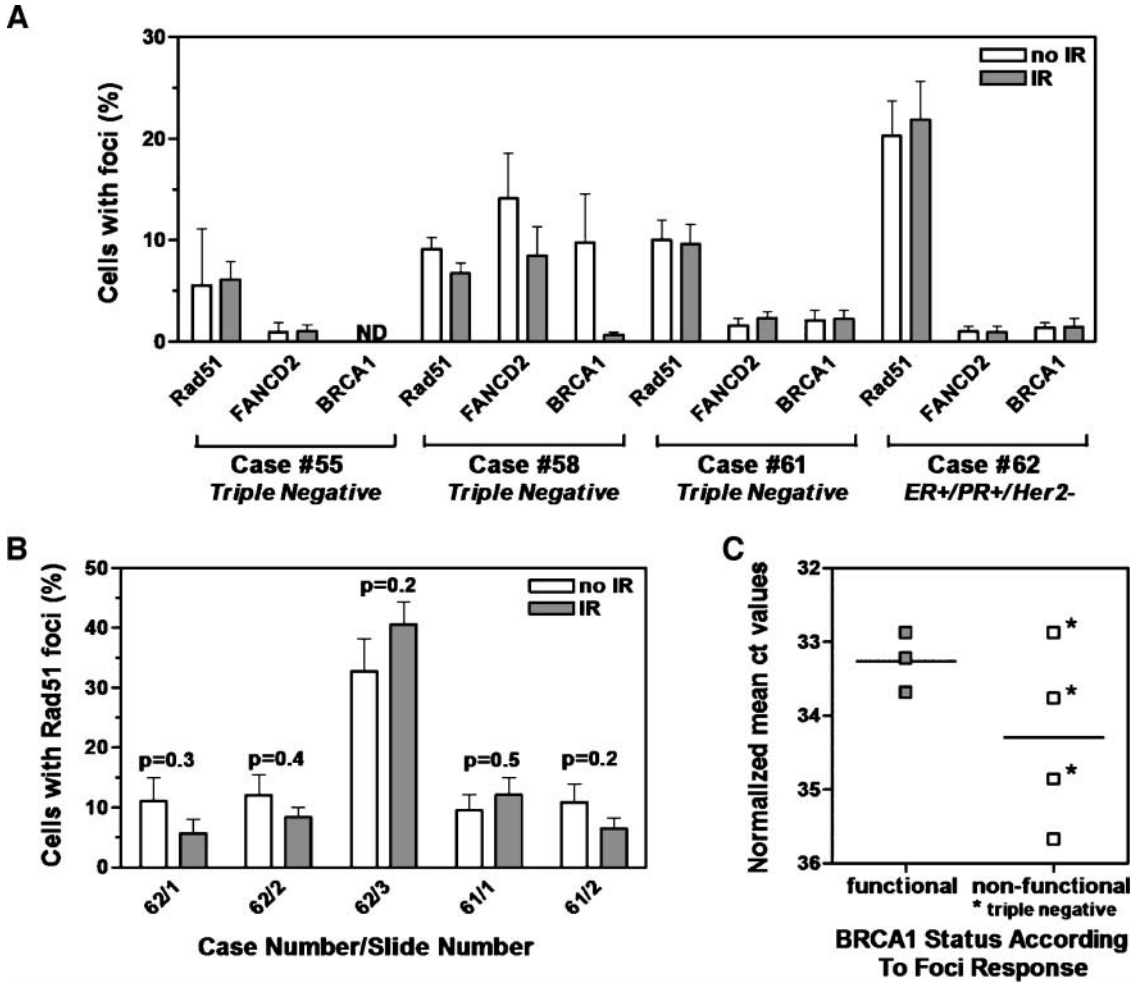

FIGURE 4. Identification of BRCA1-deficient tumors. **A.** Quantification of RAD51, FANCD2, and BRCA1 foci as in Fig. 2B. The hormone receptor status for each of the four tumors also indicated. **B.** Quantification of RAD51 foci over several slides as in Fig. 2C. **C.** Relative BRCA1 expression by quantitative real-time PCR is plotted against tumor classification based on foci analysis. Lines, the logarithmic mean.

possibility that heterogeneity in spontaneous foci formation could have masked the effects of IR (Fig. 4B). However, no significant IR-mediated induction of foci formation was revealed on any of the sections, with relative foci induction compared with untreated samples fluctuating at approximately 1 (range, 0.5-1.2). Defective RAD51 foci formation suggested a defect in BRCA1 function, which should also lead to a failure of damage-induced but not spontaneous FANCD2 foci formation. Indeed, in all four cases, IR was insufficient to trigger the formation of additional FANCD2 or BRCA1 foci above background frequencies (Fig. 4A). BRCA1 expression is often impaired in breast cancers, which could have functional consequences. Thus, when we analyzed *BRCA1* gene expression by real-time PCR, two of the four tumors classified as potentially BRCA1 dysfunctional in light of the abrogated foci response (nos. 58 and 62) also showed much lower expression levels than the three tumors classified as having functional BRCA1 status (nos. 56, 57, and 59; Fig. 4C). The other two tumors with defective foci response (nos. 55 and 61) showed BRCA1 expression levels overlapping with those seen in the IRIF-proficient tumors. Importantly, three of the four tumors classified as BRCA1-deficient (nos. 55, 58, and 61) but none of the BRCA1-proficient tumors were triple-negative (Fig. 4C).

Discussion

We report here, for the first time, the detection of BRCA1, FANCD2, and RAD51 foci in human breast cancer tissues. Strikingly, foci formation could be induced by exposing fresh biopsy samples to IR *ex vivo*. The extent of foci induction, i.e., <30% of cells scored positive, was less than observed for tumor cell lines (21, 22). This is likely related to the more homogeneous nature of cell populations that have been passaged numerous times. Furthermore, the development of replication-associated repair protein foci is related to the growth fraction within the population of tumor cells, which is lower in tumor tissues than in cell lines.

Importantly, based on our foci analysis, we found a putative BRCA1 defect in four out of seven tumors. This defect was characterized not only by an inability to form BRCA1 IRIF

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but also by an impairment of the downstream components FANCD2 and RAD51, as was predicted from cell line studies (10, 22). In a fifth case (no. 57), BRCA1 and RAD51 foci formation was intact and FANCD2 foci were present at baseline, but FANCD2 foci did not increase in number further with IR. The underlying genetic or epigenetic defect in this tumor remains to be identified. Although a statistical analysis of our small study population is naturally limited, it is assuring to note that three of the four BRCA1-deficient tumors are triple-negative, as would be predicted. These data also suggest that functional BRCA1 deficiency may occur in non–triple-negative tumors as well, a notion that is supported by preliminary results from ongoing large-scale cell line screens in our laboratories.[4] Conversely, not all triple-negative sporadic breast cancers will be BRCA1-defective (14). It remains to be established whether BRCA1 defects, as defined by our foci assay, are predictors of clinical sensitivity to chemotherapeutics such as platinum compounds or topoisomerase II inhibitors (26, 27). Although our pilot study is too small to address this question, the observed radiographic responses to neoadjuvant doxorubicin in patients with BRCA1 dysfunctional cancers are entirely consistent with this hypothesis.[5]

Characterizing the activity of the cellular DNA damage response to cytotoxic agents poses a challenge due to the spatiotemporal complexity of the response network (18-21). Identifying functional nodal points, such as BRCA1, FANCD2, or RAD51, offers an attractive approach to characterize the activity of the network. The advantage of using foci as biomarkers is that they can detect repair defects due to various mechanisms such as epigenetic events or gene mutations. Moreover, they provide a global measurement of network function without needing to know the identities of all the components, many of which are still unknown (9). As such, measuring BRCA1 foci formation should be more informative than monitoring BRCA1 mRNA or protein expression levels. Although in Fig. 4, two tumors with relatively low BRCA1 expression levels also had a foci formation defect, two other tumors revealed DNA repair defects based on foci analysis despite having BRCA1 expression levels that were similar to the BRCA1-proficient tumors.

In conclusion, our study suggests that DNA repair foci can be used to detect functionally relevant BRCA1 defects in sporadic human breast cancers. Confirmatory studies with larger patient populations and automated foci scoring are required. Whether BRCA1 dysfunction translates into chemosensitivity or radiosensitivity of the affected tumor is an important topic of ongoing and future investigations.

Materials and Methods

Study Population

We obtained pretreatment biopsies from eight patients with breast cancer (cases 55-62), consecutively enrolled into the Dana-Farber/Harvard Cancer Center Protocol 99-278, Neoadjuvant chemotherapy in palpable breast cancer: evaluation of physiologic, radiological and molecular markers in predicting response (A.G. Taghian, principal investigator). The study was approved by the Institutional Review Board and written informed consent was obtained from every patient.

Ex vivo *Treatment*

Fresh core biopsies were transported in chilled complete cell culture medium to the laboratory and processed within 1 to 1.5 hours post-biopsy. Biopsy tissues were arbitrarily divided into two samples and subjected to 8 Gy X-irradiation (Siemens Stabilipan 2 X-ray generator operated at 250 kVp and 12 mA, dose rate of 2.08 Gy/min) or mock treatment. Samples were incubated in humidified cell culture incubators at 37°C and 5% CO_2 and snap-frozen in optimal cutting temperature compound (Sigma-Aldrich) after 4 h. Viable tumors were identified on serial cryosections by H&E staining. One specimen (no. 60) was of poor quality and was not analyzed further.

Immunofluorescence Microscopy

Adjacent cryoslides were analyzed for subnuclear foci formation by immunofluorescence microscopy (Olympus BX51). Slides were fixed in 100% methanol at −20°C for 10 min and immersed in acetone for 30 s, followed by permeabilization with 0.5% Triton X-100 in PBS for 15 min. After blocking in 3% bovine serum albumin and 0.2% Triton X-100 in PBS, rabbit polyclonal anti-RAD51 (Oncogene), mouse monoclonal anti-BRCA1 (Oncogene), and rabbit polyclonal anti-FANCD2 (Novus) were used at appropriate dilutions of 1:150, 1:50, and 1:200 to 1:400, respectively. Incubation with the primary antibodies was carried out at 37°C for at least 2 h. Slides were then washed with PBS and incubated with Alexa-488 or Alexa-568 (Molecular Probes) or FITC-conjugated (Pierce) secondary antibodies at 37°C for 50 min. Slides were washed and counterstained with 1 μg/mL of 4′,6-diamidino-2-phenylindole (Sigma-Aldrich). Slides were mounted in antifade reagent (Bio-Rad). The specificity of the antibodies was confirmed by monitoring increased foci formation in irradiated versus untreated control cell lines (data not shown). In order to semiquantify foci formation, each tumor sample was analyzed by capturing at least eight random 100× images on one to three sections. On each high-power image, typically 20 to 100 nuclei were scored. Only nuclei that could be clearly discerned were counted. A nucleus was scored as positive if it contained at least two foci. Tumor nuclei were identified by 4′,6-diamidino-2-phenylindole staining (data not shown). If 4′,6-diamidino-2-phenylindole was unable to distinguish between individual tumor nuclei due to the underlying tissue architecture, these cells were censored and not included in the count.

Real-time PCR

Slides were subjected to laser capture microdissection and real-time quantitative PCR (28). TaqMan real-time PCR was done using an ABI 7900HT (Applied Biosystems) as previously described (29). Each gene was analyzed in triplicate and the relative standard curve method was used for linear regression analysis of unknown samples in which β-actin levels served as an internal normalizing control. The sequences of the PCR primer pair and the fluorogenic TAMRA and MGB probes (5′ to 3′), for BRCA1 and β-actin, respectively, were as follows: FP,

[4] Willers, Powell et al., unpublished data.
[5] Taghian et al., unpublished data.

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GGCTATGCAAGGGTCCCTTAA; RP, TGAATCAGCATCTTGCTCAATTG; Probe, VIC-TCTCCCTTGGAAATCTGCCATGAGCA-TAMRA; FP, CTTCCTGGGCATGGAGTCC; RP, ACGTCACACTTCATGATGGAGTT; Probe, VIC-ATCCACGAAACTAC.

Disclosure of Potential Conflicts of Interest

Dr. Simon Powell is a consultant and serves on the advisory board of DNAR, Inc.

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