Radiation clastogenesis and cell cycle checkpoint function as functional markers of breast cancer risk


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Background: Familial breast cancer is associated with mutations in several genes (BRCA1, BRCA2, p53, ATM) whose protein products protect against radiation-induced genotoxicity. This study tested whether sporadic breast cancer was associated with constitutive radiation hypersensitivity. Methods: Blood lymphocytes and EBV-transformed lymphoblasts from patients with newly diagnosed breast cancer and controls without cancer were evaluated for ionizing radiation (IR)-induced chromosomal aberrations and cell cycle delays. Lymphoblasts from patients with ataxia telangiectasia (AT) and heterozygous AT carriers were tested as positive controls for radiation hypersensitivity. Results: Lymphoblasts from AT patients and AT carriers displayed G2-irradiation, chromosomal hypersensitivity (GICH). Irradiated G2 phase lymphocytes from breast cancer cases and controls displayed 3-fold inter-individual variation in frequencies of chromatid damage. However, the percentage of breast cancer cases with damage frequencies in excess of 2 SD of the control mean (8/102 or 8%) was not significantly elevated compared to controls (2/48 or 4%, \( P = 0.5 \)). Lymphoblasts sampled 24 h after 3 Gy of IR also varied in the ratios of cells with 4N and 2N DNA content (4N/2N ratio), as a measure of cell cycle checkpoint function. 4N/2N ratios in irradiated lymphoblasts were strongly correlated with the fractions of S phase cells in unirradiated control cultures (Pearson’s correlation coefficient, \( r = 0.87 \)). After normalization to S fraction, the radiation-induced increment in the 4N/2N ratio was significantly elevated in AT lymphoblasts but not in lymphoblasts from AT carriers. The fraction of breast cancer cases with reduced checkpoint function (2/45 or 4%) was equal to the control fraction (2/45 or 4%). For breast cancer cases and controls, GICH in primary lymphocytes was not associated with reduced cell cycle checkpoint function in lymphoblasts. Conclusion: Constitutive radiation hypersensitivity in blood lymphocytes and lymphoblasts was not a useful biomarker for identifying women at increased risk of breast cancer.

Background

Mutations in several genes involved in DNA damage responses are known to predispose to development of cancer in breast and other tissues. Inherited mutations in p53 (1–3) and Chk2 (4) are seen in Li–Fraumeni syndrome in which patients display increased risk of soft-tissue sarcomas and breast carcinoma (5,6). p53 is known as the ‘guardian of the genome’ (7) for its required functions in cell cycle checkpoint response (8) and DNA repair (9–11). Chk2 is a checkpoint transducer kinase that phosphorylates p53 and BRCA1 in response to radiation-induced DNA damage (12,13). Recent studies have associated a specific mutation in Chk2 (1100delC) with familial breast cancer (6). BRCA1 and BRCA2 were identified using linkage analysis in familial breast cancer (14,15). BRCA1 protein is expressed in the nucleus in a large complex known as the BRCA1-associated surveillance complex (BASC) (16) and is required for S and G2 checkpoint responses to radiation-induced DNA damage (17,18). BRCA2 also participates in DNA repair (19) and was recently shown to be one of the genes that is mutated in Fanconi’s anemia (20), a familial cancer syndrome with chromosomal instability. Mutations in ATM (ataxia telangiectasia-mutated) have been associated with increased risk of development of lymphoma and breast cancer (21,22). ATM is a checkpoint kinase that phosphorylates a large number of proteins in response to radiation-induced DNA damage, including p53, Chk2, BRCA1 and BRCA2 (23). The above-mentioned studies have demonstrated that inherited susceptibility to development of breast cancer is associated with mutations in DNA repair genes that mitigate or ameliorate the genotoxic effects of DNA damage. The purpose of this study was to determine whether sporadic cases of breast cancer might also be associated with constitutive functional defects in DNA repair.

Assays have been developed to quantify DNA repair function in white blood cells. One assay known as GICH (for G2-irradiation, chromosomal hypersensitivity) scores radiation-induced chromosomal aberrations (24). In this assay primary cultures of blood lymphocytes are stimulated to proliferate and are treated with a low, subtherapeutic dose of ionizing radiation (IR; 0.25–0.5 Gy) when cells are actively dividing. By sampling mitotic cells during the first hour or two after irradiation, chromatid damage in metaphase spreads is restricted to cells that were irradiated in G2. This assay consistently detects a repair defect in cells from ataxia telangiectasia (AT) patients (25–27) and in most reports heterozygous carriers of disease-associated mutations in ATM...
also display hypersensitivity in comparison to normal controls (24,28–31). When applied to a series of 135 sporadic breast cancer patients, the assay detected hypersensitivity in 42% of cases, a fraction in great excess of that seen in healthy controls (6%) (32,33). Similar observations of increased frequency of radiation hypersensitivity in breast cancer cases were reported by two other laboratories (34,35).

The GICH assay detected a functional deficit in cells from patients with germline mutations in p53 (36), BRCA1 and BRCA2 (37). A similar chromosomal hypersensitivity assay, which uses bleomycin to induce DNA double strand breaks, detected a repair defect in AT cells (38) and identified increased frequencies of chromosomal hypersensitivity in patients with head, neck and lung cancer (39,40). While the GICH assay displays good sensitivity and specificity for detection of DNA repair defects, it is laborious, expensive and subject to the potential for reader bias or subjective error.

A second functional assay uses flow cytometry to score the accumulation of lymphoblasts in the G2 phase of the cell cycle one day after a therapeutic dose of IR (3 Gy) (41). AT cells with defects in the immediate cell cycle checkpoint responses to IR progress through G1, S and G2 with less than normal delays and thereby acquire additional chromosomal damage. Over an extended incubation post-irradiation an alternative checkpoint pathway probably involving the AT- and rad3-related checkpoint kinase, ATR, causes these severely damaged AT cells to accumulate in G2 (42). Not only do AT cells display hypersensitivity to G2 accumulation post-IR, cells from heterozygous carriers of mutant ATM alleles also display enhanced accumulation at a level intermediate between normal controls and AT cells (41). When this checkpoint assay was initially applied in a case/control study of breast cancer, hypersensitivity in the range seen in AT heterozygotes was detected in 22% of cases and 8% of controls (43). A more recent application of the method also reported increased G2 delay in breast cancer cases relative to matched controls (44).

The GICH and cell cycle checkpoint assays are sensitive measures of radiation hypersensitivity in AT lymphocytes and lymphoblasts, and may be useful screening tools for detection of individuals at increased risk of cancer due to inherited defects in DNA repair such as ATM heterozygotes. To assess the utility of these two assays for detection of genetic susceptibility to breast cancer, a hospital-based, case–control study design was employed. Patients with newly diagnosed breast cancer were matched by age and race and hospital patients who did not have cancer. Radiation hypersensitivity in cancer cases and matched controls was assessed by primary cultures of blood lymphocytes and derivative lymphoblasts. The two assays also were compared directly using normal and AT lymphoblasts to determine whether one was superior for detecting radiation hypersensitivity and whether hypersensitivity in sporadic cancer cases was of the same magnitude as recognized in cells with mutations in ATM. While both assays detected radiation hypersensitivity in AT lymphoblasts, and the GICH assay detected hypersensitivity in AT heterozygotes, neither assay detected a significantly increased incidence of hypersensitivity in patients with breast cancer. These results suggest that the association of sporadic breast cancer with chromosomal hypersensitivity may not be as great as previously reported.

Materials and methods

Patient population

Women with newly diagnosed invasive primary breast cancer at UNC Hospitals were recruited as cases from the outpatient Breast Center clinic. Cases were required to be recruited after diagnosis but prior to receiving any radiation therapy or systemic therapy such as chemotherapy. Women with BRCA1 or BRCA2 mutations were excluded. Age- and race-matched control subjects were recruited from UNC non-malignancy outpatient clinics. Approximately half of the cases had a matched control subject. A cohort of unmatched cases was also included. Neither case nor control subjects could have had prior malignancy or have received immunosuppressive drugs. Each study participant provided 25 ml of whole blood into heparinized tubes, which was transferred during the same working day (preferably within 2–3 h) to the UNC Lineberger Comprehensive Cancer Center Tissue Culture Core Facility for processing and Epstein–Barr virus (EBV) immortalization. All samples were coded with a de-identified study number. Laboratory investigators were blinded to case and control status. All subjects provided informed consent prior to study entry. This study was approved and monitored by the University of North Carolina at Chapel Hill School of Medicine Committee on the Protection of Human Subjects.

Establishment of lymphoblastoid cell lines

The heparinized blood sample was mixed with RPMI human blood culture medium with 24 h of receipt and within 24 h of initiating the culture, the T cell mitogen, phytohemagglutinin (Invitrogen) was added at 1:100 dilution. Blood cultures were incubated at 37°C in a humidified atmosphere of 5% CO2. Another aliquot of blood was subjected to Ficoll/Hypaque gradient processing to obtain the lymphocyte buffy coat, which was washed once with buffer then incubated at 10^7 cells/ml in RPMI containing 15% fetal calf serum (FCS) and 2 mM L-glutamine. An inoculum of filtered medium from EBV-infected human lymphocytes was added to cultures. Cell numbers were monitored over time and when cultures had established continuous growth 14–21 days after initiation, aliquots were cryopreserved.

In addition to the lymphoblastoid lines generated in the UNC Lineberger Comprehensive Cancer Center Tissue Culture Facility from cases and controls, cell lines for testing and determination of assay parameters were purchased from the Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research. Cell lines from the repository included GM02254A, GM03659A, GM03714A and GM01815 from apparently normal donors, GM03188A, GM03334A, GM09579 and GM02781B from mothers of AT patients, and respectively GM03189D, GM03332C, GM09582 and GM02782B from their children with AT. All lymphoblastoid cell lines were grown in a humidified atmosphere of 5% CO2 at 37°C in RPMI medium supplemented with 2 mM L-glutamine, 15% FCS, 100 μg/ml streptomycin and 100 U/ml of penicillin.

GICH assay

Whole blood cultures were assayed for G2-irradiation chromosomal hypersensitivity beginning 72 h after addition of phytohemagglutinin. Generally, for screening purposes, blood cultures were irradiated with 27 cGy of 137Cs gamma rays from a Gammacell 40 apparatus (MDS Nordion) at a dose rate of 0.94 cGy/min. Transport to and from the irradiation facility was done in an insulated box with cultures surrounded by 20% C14/C12 water. Thirty minutes after irradiation, colcemid was added to cultures at 50 ng/ml. After an additional 60 min incubation, cultures were terminated for cytogenetic preparation of metaphases according to standard methods (45). Giemsa-stained metaphases were scored by light microscopy for chromatid breaks and gaps according to criterion established by Hsu et al. (46). EBV-transformed lymphoblastoid lines in logarithmic growth were irradiated and processed similarly. Two methods of enumeration of chromosomal aberrations in irradiated cells were compared. The first method, direct counting, involved counting chromatid breaks and gaps on 50 or 100 metaphases/cell line. In the second method, break frequency estimation, the percentage of 50 metaphases without a chromatid break or gap was determined, and the Poisson formula was used to estimate the frequencies of breaks and gaps.

Cell cycle checkpoint assay

EBV-transformed lymphoblasts were recovered from cryopreservation and after outgrowth for one week in RPMI medium with 15% FCS and 2 mM L-glutamine, a series of subcultures were established. Each day for three consecutive days, two cultures were established with cells at 50,000/ml. One day after establishment, proliferating cultures were treated with 3 Gy of IR or were sham-treated as controls, with the same transport to and from the irradiation facility as the irradiated cultures. They were then incubated for 24 h before cell harvest for flow cytometric evaluation of DNA content. Cells
were sedimented and washed in phosphate-buffered saline, then fixed in 70% ethanol. Fixed cells were mixed with 10 μg/ml RNase and 25 μg/ml propidium iodide, then analyzed for DNA content using a FACScan (Becton Dickinson) flow cytometer. Histograms of DNA content were analyzed by the Modfit commercial software program to determine fractions of cells in G1/2N (2N DNA content), S (>2N and <4N DNA content) and G2/M (4N DNA content). As suggested by Lavin et al. (41) the 4N/2N ratio was determined by dividing the fraction of cells in G2/M by the fraction in G1/2N. This 4N/2N ratio was determined for irradiated cells and sham-treated controls as an index of checkpoint-dependent cell cycle delays. Each analysis of DNA content was done on three consecutive days and mean values were determined.

Statistical methods

Fisher’s exact test was used to test for differences in proportions (or percentages) between cases and controls (i.e. the proportion of observations scoring >2 SD cutoff for radiation-induced chromatid damage and cell cycle checkpoint function). Wilcoxon rank-sum tests (using Van der Waerden normal scores) were used for group comparisons of continuous variables (such as chromatid breaks and gaps per cell by normal versus AT cell lines). Pearson’s correlation coefficients were used to evaluate correlations between continuous variables. A method known as the simple order analysis (47) was used to examine order-restricted properties of ATM homozygotes, ATM heterozygotes and presumptive wild-type homozygotes. In the nonparametric version of this analysis, the null hypothesis is that the mean of the ranks is the same in all three of the groups. The alternative hypothesis is that the mean of the ranks in the ATM homozygotes is strictly greater than the mean of the ranks in the wild-type homozygotes. Therefore, a significant P-value in this test gives evidence for this ordering. Statistical analyses were performed with SAS statistical software, Versions 8.2 and 9.1, SAS Institute, Cary, NC.

Results

Validation of GICH

The GICH assay consistently demonstrates chromosomal hypersensitivity in cells from AT patients and usually, but not always (48), in carriers of mutant ATM alleles. Lymphoblast lines from AT patients, carriers of mutant ATM alleles, and normal individuals were obtained from the Coriell Institute and were used to validate the assay. Control experiments indicated that the AT cells did not express detectable levels of full length ATM by western immunoblot analysis (not shown) and all AT lines displayed a significant defect in radiation-induced G2 checkpoint function (49). In the GICH assay, proliferating cells were treated with a low dose of IR, then incubated with colcemid between 30 and 90 min after irradiation, at which point cells were harvested for preparation of metaphase spreads. Metaphases were scored for chromosomal damage, which consisted virtually exclusively of chromatid breaks and gaps. All of the lines displayed a very low background of chromatid damage. Increasing doses of IR induced increasing frequencies of chromatid breaks and gaps (Figure 1A). The two AT lines had greater frequencies of chromatid damage than the two normal lines. After 27 cGy the break frequencies in the pooled AT lines were significantly greater than those recorded in the two pooled normal lines (P = 0.001). As shown in Figure 1B, the two methods of enumeration, direct counting and break frequency estimation, gave equivalent results (Pearson’s correlation coefficient, r = 0.97).

Chromatid damage was determined using the Poisson formula for 12 lymphoblastoid lines after treatment with 27 cGy IR. When four AT lymphoblast lines were compared with four normal lymphoblasts, all AT lines displayed radiation-induced chromatid aberrations significantly in excess of values seen in the normal lymphoblast lines (Figure 1C). Four lymphoblast lines from heterozygous carriers of mutant ATM alleles also displayed frequencies of chromatid breaks and gaps in excess of three of the normal controls. One control line was scored in the range of the AT carriers. The mean frequency of chromatid breaks and gaps in irradiated AT cells was about twice that scored in the normal controls (Figure 1D). The mean frequency of chromatid damage in the AT carriers was ~1.5 times that seen in controls. The order-restricted difference among the three groups was highly significant (P < 0.0001). The validation studies using lymphoblastoid lines indicated that GICH was a sensitive assay for detecting hypersensitivity in cells from AT patients and ATM carriers.

GICH in breast cancer cases and controls

Primary cultures of peripheral blood lymphocytes from 102 breast cancer cases (48 matched, 54 unmatched) and 48 controls were used for assay of GICH. The characteristics of the study groups are shown in Table 1. The first 56 lymphocyte preparations that were analyzed, consisting of cases and controls, were scored by direct count of chromatid breaks and gaps in 100 irradiated cells. Mean frequencies of breaks+gaps in the sample of 100 metaphases were quite similar to frequencies determined in the first 50 metaphases sampled (data not shown). Therefore, 50 metaphases were scored in subsequent analyses. Comparison of break+gap frequencies that were obtained by direct count with those estimated using the Poisson formula indicated both methods yielded similar values (Pearson’s correlation coefficient, r = 0.97), as already illustrated with lymphoblastoid lines (Figure 1B). Based on the greater speed in scoring samples, the Poisson formula was then used routinely for scoring chromatid damage in irradiated cells. Irradiated lymphocytes from cases and controls displayed substantial (~4-fold) variation in the frequencies of chromatid damage (Figure 2). The mean frequency of damage per cell in controls was 1.02 (SD = 0.27); values in excess of 2 SD of this mean (i.e. >1.56) were considered to be indicative of radiation hypersensitivity. Of the control samples 2/48 (4%) scored above the cutoff. Among cases with breast cancer, the mean frequency of chromatid damage per cell was 1.07 (SD = 0.32), and 8/102 (8%) patient’s lymphocytes displayed frequencies of chromatid damage in the hypersensitive range. This frequency of radiation hypersensitivity among all cases was, therefore, about twice that determined for the controls, although this difference in percentages was not statistically significant (P = 0.50). Controls were matched to a subset of cases by age and race. A comparison of 48 cases with their controls also failed to identify significantly enhanced hypersensitivity in the cases, although in the matched subset of cases the frequency of hypersensitivity was 6/48, (12%). This level of chromosomal hypersensitivity in matched cases, three times in excess of the control level (which was 2/48 or 4%), was not statistically significant (P = 0.27).

Cell cycle checkpoint function in breast cancer cases and controls

Radiation hypersensitivity in breast cancer was also reported using a method that quantified radiation-induced cell cycle delays in EBV-transformed lymphoblasts (43). A total of 45 matched case and control lymphoblasts were assayed for radiation-induced cell cycle delay to see whether this...
alternative method might detect an excess of radiation hypersensitivity in cases. Figure 3A shows a comparison between normal and AT lymphoblasts to illustrate the method for determining checkpoint-dependent cell cycle delays. In comparison to the control cell lines AT cells displayed greater accumulation with 4N DNA content and greater reduction in 2N DNA content 24 h after treatment with 3 Gy IR. Consequently, the 4N/2N ratio in irradiated AT cells was substantially greater than that seen in irradiated normal controls.

There was substantial inter-sample variation in the 4N/2N ratio in irradiated lymphoblasts from the breast cancer cases and controls (Figure 3B) and this variation was correlated with variation in the fraction of S phase cells in the un-irradiated controls (Pearson’s correlation coefficient, \( r = 0.87 \)). Proliferative cultures of human lymphoblasts displayed substantial inter-individual variation in S phase fraction, with values ranging from 16 to 55% in un-irradiated controls. Intra-individual

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**Table I.** Characteristics of the study groups

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**Fig. 1.** G2-irradiation, chromosomal hypersensitivity in ATM carrier and AT lymphoblastoid lines. (A) Dose–response for chromatid damage in IR-treated normal (GM03714A, squares; GM02254A, diamonds) and AT (GM03320C, circles; GM03189D, triangles) lymphoblasts. Results show the mean ± SD from three separate experiments using the GICH assay. (B) Frequencies of chromatid breaks+gaps determined by direct count in Figure 1A were compared with frequencies estimated by Poisson statistics from the percentage of undamaged metaphases. Pearson’s correlation coefficient was \( r = 0.97 \). (C) Normal, ATM carrier and AT lymphoblasts were analyzed by GICH assay after 27 cGy IR. (D) Mean frequencies (±SD) of IR-induced chromatid damage were determined for groups of normal, ATM carrier and AT lymphoblastoid cell lines (\( n = 8 \) for each). Normal lymphoblasts were assumed to have two wild-type ATM alleles. The order-restricted difference among the three groups was highly significant (\( P < 0.0001 \)).

**Fig. 2.** GICH assay on lymphocytes from breast cancer cases and controls. Frequencies of IR-induced chromatid breaks + gaps were determined for 48 control and 102 case lymphocyte cultures. The mean frequency of breaks+gaps in control lymphocytes is shown with the solid horizontal line and the horizontal dashed line shows 2 SD above this mean.
variation was much less with S phase fractions varying by 15% of the mean in triplicate determinations. Therefore, to quantify cellular response to irradiation, the increment in the 4N/2N ratio that was induced by irradiation was normalized to the fraction of S phase cells in the un-irradiated control culture. A mean normalized 4N/2N ratio was determined for the control patient samples and an upper cutoff calculated by adding two standard deviations of this mean. Of the matched control samples 2/45 (4%) displayed a response to IR in excess of 2 SD of the mean, while 2/45 (4%) samples from breast cancer patients were in excess of the cutoff. Therefore, the frequency of radiation hypersensitivity in breast cancer cases and controls as determined using the flow cytometric method was the same.

Fig. 3. Cell cycle checkpoint function in human lymphoblasts. (A) Normal (GM11233) and AT (GM01223) lymphoblastoid lines were treated with 3 Gy IR or sham-treated, then incubated for 24 h before harvest for analysis of DNA content by flow cytometry. 4N/2N ratios were determined using Modfit software. (B) IR-induced changes in the 4N/2N ratio are correlated with S phase fraction in sham-treated controls. A total of 48 lymphoblastoid cell lines from breast cancer patients and 48 lines from matched controls were assayed for IR-induced changes in the 4N/2N ratio and the results were combined. 4N/2N ratios in IR-treated (diamonds) and sham-treated cultures (squares) are plotted against the percent of S phase cells in sham-treated cultures. The best-fit line determined by least-squares analysis is shown for the IR-treated samples. Pearson’s correlation coefficient was $r = 0.87$. 

Radiation clastogenesis and cell cycle checkpoint function
The correlation between S phase fraction and increment of the 4N/2N ratio induced by irradiation had not been noted previously in a study of hypersensitivity in AT heterozygotes (41) nor in a breast cancer case/control study (43). When AT and AT carrier lymphoblasts were analyzed using the flow cytometric assay, the same correlation between S phase fraction and 4N/2N ratio in IR-treated samples was observed (Figure 4A). While the irradiated AT carrier lymphoblasts displayed 4N/2N ratios in the range of normal lymphoblasts and the control lymphoblasts in the case–control study (Figure 3B), irradiated AT lymphoblasts displayed values consistently outside this range. The normalization procedure was applied to AT and AT carrier lines to test whether the assay could detect hypersensitivity associated with mutations in ATM. While all the AT cell lines displayed significantly elevated normalized ratios in comparison to the control lymphoblasts from the breast cancer case/control study, none of the AT carrier lines displayed hypersensitivity in excess of the control cutoff value (Figure 4B).

Lack of correlation between GICH and cell cycle checkpoint function in breast cancer cases and controls

A total of 80 samples from the case–control study were analyzed using both assays for radiation hypersensitivity. There was no apparent correlation between the two assays (Pearson’s correlation coefficient was \( r = 0.09 \), see Figure 5). Samples that were hypersensitive in one assay were not hypersensitive in the other. Moreover, among 64 cases who received radiation therapy after surgical excision of their cancer, four displayed clinical hypersensitivity to radiation, with moist desquamation in three and pain in the fourth. None of these patients displayed radiation hypersensitivity in their peripheral blood lymphocytes by GICH assay or in EBV-transformed lymphoblasts by flow cytometric assay.

Discussion

Although it is clear that inherited defects in DNA damage response predispose to development of breast cancer, the question of whether sporadic cases of breast cancer are associated with constitutive defects in DNA damage response remains unanswered. The GICH assay recognized hypersensitivity to induced DNA damage in AT carriers and the cell cycle checkpoint assay clearly identified hypersensitivity in AT cells. Neither assay detected a significant difference in the frequency of hypersensitivity between breast cancer cases and controls. While the data reported here estimate a 2-fold enhanced risk of breast cancer among women with GICH, the count difference was small and not statistically significant. The fact that two independent assays yielded similar low estimates of hypersensitivity in cases suggests that the constitutive defects in DNA repair detected here are unlikely to be substantial contributors to breast cancer risk.

AT carriers are estimated to represent \(~0.5–2\%\) of the population (50). They are estimated to have a 4-fold increased risk of breast cancer, hence may account for \(~8\%\) of all breast cancer patients versus controls (51). Based on the results presented here, if defects in DNA repair genes other than ATM contribute to sporadic breast cancer, these genes do not manifest as constitutive hypersensitivity to IR-induced DNA damage.

Several GICH studies have reported a high frequency of chromosomal hypersensitivity in breast cancer (32–35). The largest study by Roberts et al. (32) examined 135 apparently sporadic cases before treatment at a regional hospital in the United Kingdom. The control group in this study was
composed of normal healthy donors who were not matched by age, sex or race to the breast cancer cases. The cutoff for hypersensitivity in cases was based on the lowest level of induced chromatid damage seen among lymphocyte preparations from AT carriers. This study was designed to determine the frequency of cases that displayed hypersensitivity in the range of AT carriers, and 42% of breast cancer cases were scored in this range. A much greater fraction of cases displayed the hypersensitivity trait (42%) than was predicted based on the estimated contribution of AT heterozygotes to breast cancer incidence rates (8%). The 6% frequency of the trait in healthy controls also was in excess of the estimated frequency of AT carriers (0.5–2%). These observations suggested that an allele (or alleles) other than ATM contributes to expression of the trait of G2-irradiation hypersensitivity. The hypersensitivity trait displayed a Mendelian inheritance pattern, i.e. ~60% of primary relatives were concordant, and modeling of the data suggested two contributing alleles, one major and one minor (32).

A second study that reported a high frequency of hypersensitivity among breast cancer cases examined 11 cases with a family history of cancer and 12 cases without (35). This study also used the hypersensitivity of AT carriers as the cutoff for detection of the trait in cases. Ninety percent (10/11) of apparently familial cases displayed hypersensitivity in the range of AT carriers, while only 50% (6/12) of non-familial cases displayed hypersensitivity. In the current study, the lowest value seen for AT carriers was above the threshold (control mean + 2 SD), confirming that the GICH assay detects hypermutability stemming from AT mutations. A third study compared GICH in 19 women with no family history of cancer for three generations, 14 cases with breast cancer before therapy, and 19 first degree relatives of the cases not being matched to an appropriate control group or by small sample sizes (including the present study) (52).

There is present no clear explanation for the substantial variance between the frequencies of hypersensitivity reported in this study and the previous studies. One consideration is the question of reader bias since radiation-induced chromatid damage is a subjective cytogenetic assessment. In the current study samples were coded with a simple accession number that provided the reader no information as to case or group status. No mention was made in the Patel et al. (33) study of the reader sample identity. In the Parshad et al. (35) study, cases were apparently undergoing treatment for their cancer, and no mention was made of blinding the reader, raising the question of exposure to DNA-damaging agents such as radiation or chemotherapy in the cases in addition to reader bias. In the Scott et al. (32) study it also appears that sample identity was known to the reader. However, in a follow-up study to test whether hypersensitivity displayed a Mendelian inheritance pattern, samples were coded for blinded scoring (32).

The cell cycle checkpoint assay uses flow cytometry to score cellular responses to DNA damage and is thus prone to reader bias. A commercial software package was used to analyze DNA histograms. Care was taken to minimize intra-sample variation by repeating the full protocol on each sample on three successive days. Even with this care to minimize variation in the assay it was apparent that lymphoblast preparations displayed significant variation in proliferative activity as evidenced by the fraction of S phase cells, which varied between 16 and 55%. The greater the fraction of cycling cells, the greater the fraction that can be arrested by cell cycle checkpoints, and the radiation-induced increment of the 4N/2N ratio was strongly correlated with S phase fraction in sham-treated controls. This correlation had not been noted in the initial studies by Lavin et al. (41) although mention was made of the need to sample cells that are actively transiting through the cell cycle. Even with normalization, AT cells displayed a 4N/2N ratio that was significantly greater than that seen in control and case lymphoblasts. The mean normalized 4N/2N ratio in irradiated AT cells (0.09) was three times the mean normalized 4N/2N ratio in the control lymphoblasts (0.03). Thus the results confirmed that inactivation of ATM function causes AT cells to experience greater than normal accumulation in G2 and greater than normal diminution in G1 h after 3 Gy of IR.

Although the cell cycle checkpoint assay displayed a dynamic range at least as great as the chromatid damage assay, it did not reliably identify AT heterozygotes. This result was inconsistent with that of Lavin et al. (41) showing hypersensitivity in AT carriers. Lavin et al. (43) also reported hypersensitivity in 8% of controls and 22% of breast cancer cases. In the current analysis using the checkpoint assay, radiation hypersensitivity was not observed in breast cancer cases versus controls. Given, the significant association between S phase fraction in un-irradiated controls and the 4N/2N ratio in irradiated cells, checkpoint data that are not normalized to the fraction of cycling cells may not reliably assess functional capacity. Hu et al. (53) normalized radiation-induced G2 delay in lymphoblasts to the S phase fraction in un-irradiated control cell preparations and detected an association between G2 delay and expression of two polymorphic DNA repair gene alleles. The codon 399 Arg/Arg variant in XRCC1 and the codon 148 Glu/Glu variant in APE1 were associated with significantly increased G2 delay in comparison to the wild-type alleles. It remains to be determined whether these variant DNA repair gene alleles enhance risk of development of cancer.

In summary this study showed that constitutive defects in DNA damage response were uncommon among women with sporadic breast cancer. Although defective DNA damage response was detected in cancer cases and controls, the frequency of the trait approximated the estimated frequency of AT heterozygotes. Development of a facile means to reliably identify such women remains a pressing need.

Acknowledgements

Authors contributions: W.K.K. designed the experiments, supervised their execution and prepared manuscript drafts; L.F. performed the cytogenetic analysis of IR-induced chromatid breaks and gaps in primary cultures of blood lymphocytes and lymphoblastoid lines; S.E.O. supervised the establishment of primary blood lymphocyte cultures and transformation of lymphoblasts with Epstein–Barr virus; D.A.S. managed the execution of experiments; M.A.L. performed the flow cytometric analysis of cell cycle checkpoint response to IR-induced DNA damage; H.D.M. performed the
cytogenetic analysis of IR-induced chromosomal aberrations in AT and AT carrier lymphoblastoid lines; L.R.S. managed the collection of informed consent and blood from cancer cases and controls; D.T.M. performed biostatistical analyses; R.C.M. and M.C.S. contributed to the experimental design, analysis of data and preparation of manuscript drafts; and L.A.C. supervised the human studies component of the project and contributed to the preparation of manuscript drafts. This work was supported in part by the Breast Cancer Research Foundation and PHS grants CA13143, Breast Cancer spore grant CA16086, ES10126, CA58223, and RR00046. The authors would like to thank Dr Bahadur Singh for his helpful comments.

Conflict of Interest Statement: None declared.

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Received February 7, 2006; revised May 11, 2006; accepted May 27, 2006.