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ORIGINAL RESEARCH

Validation of the cell cycle G_2 delay assay in assessing ionizing radiation sensitivity and breast cancer risk

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Abstract: Genetic variations in cell cycle checkpoints and DNA repair genes are associated with prolonged cell cycle G_2 delay following ionizing radiation (IR) treatment and breast cancer risk. However, different studies reported conflicting results examining the association between post-IR cell cycle delay and breast cancer risk utilizing four different parameters: cell cycle G_2 delay index, $\%G_2$–M, G_2/G_0–G_1, and $(G_2/G_0$–$G_1)/S$. Therefore, we evaluated whether different parameters may influence study results using a data set from 118 breast cancer cases and 225 controls as well as lymphoblastoid and breast cancer cell lines with different genetic defects. Our results suggest that cell cycle G_2 delay index may serve as the best parameter in assessing breast cancer risk, genetic regulation of IR-sensitivity, and mutations of ataxia telangiectasia mutated (*ATM*) and *TP53*. Cell cycle delay in 21 lymphoblastoid cell lines derived from *BRCA1* mutation carriers was not different from that in controls. We also showed that IR-induced DNA-damage signaling, as measured by phosphorylation of H2AX on serine 139 (γ-H2AX) was inversely associated with cell cycle G_2 delay index. In summary, the cellular responses to IR are extremely complex; mutations or genetic variations in DNA damage signaling, cell cycle checkpoints, and DNA repair contribute to cell cycle G_2 delay and breast cancer risk. The cell cycle G_2 delay assay characterized in this study may help identify subpopulations with elevated risk of breast cancer or susceptibility to adverse effects in normal tissue following radiotherapy.

Keywords: breast cancer, ionizing radiation sensitivity, cell cycle, G_2 delay, radiotherapy

Introduction

Breast cancer is the most common cancer among American women. In 2008, it is estimated that 182,460 new cases would be diagnosed and 40,480 women would die of breast cancer.[1] Rare germ line mutations in DNA damage/repair response genes, such as *BRCA1*, *BRCA2*, *ATM*, *FANC*, and *CHEK2*, are associated with breast cancer susceptibility and highlight the importance of DNA damage/repair in breast cancer development.[2] Prevalent low-penetrance polymorphisms in cell cycle checkpoint and DNA repair genes and their gene–gene and gene–environment interactions may underlie the etiology of breast cancer. Genetic variations in DNA damage signaling, cell cycle checkpoints, and DNA repair pathways may result in downregulation of damage signaling and repair functions which, when combined with environmental exposures, may result in genomic instability that promotes breast cancer. Accordingly, breast cancer risk may be associated with single nucleotide polymorphisms (SNPs) and mutations in cell cycle checkpoint and DNA repair genes, as well as overall cellular response to genotoxic insults, such as ionizing radiation (IR), the repair of

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which involves the combined action of multiple cell cycle checkpoints and DNA repair pathways.

Cellular exposure to IR induces a myriad of cytotoxic and premutagenic DNA damages, including double-strand breaks and oxidative DNA damage.[3,4] Failure of cell cycle checkpoint and repair pathways to correct DNA damages prior to replication may result in the propagation of deleterious chromosomal aberrations and mutations that decrease genomic stability and lead to transformation. Therefore, assessment of defects in cell cycle checkpoints and DNA repair at the cellular level may allow for identification of individuals at high risk for breast cancer and afford opportunities for surveillance and behavioral intervention. IR sensitivity has been correlated with breast cancer risk.[5–9] Methodologies for assessing IR sensitivity and breast cancer risk include genetic screening, comet assay, G_2-irradiation chromosomal hypersensitivity assay (GICH), G_2-micronucleus assay, and analysis of post-IR cell cycle arrest function in peripheral blood lymphocytes (PBLs).[8,10–13] GICH and the G_2-micronucleus assay, though effective at identifying IR sensitivity, are laborious and frequently utilize subjective analysis. The analysis of cell cycle G_2 delay in PBLs following IR exposure has been used in numerous studies as a probe for genetic variations of cell cycle checkpoint and DNA repair genes that are associated with cancer risk.[8,9,12,14] Such assays are simple, inexpensive, and utilize automated sample analysis. However, sample size and the use of different methods for analysis of radiation-induced changes in cell cycle distribution, including G_2 delay index, %G_2–M, G_2/G_0–G_1, and (G_2/G_0–G_1)/S, may have contributed to conflicting results in population-based studies.[5,8,15,16]

We previously demonstrated an association between breast cancer risk and cell cycle G_2 delay in a breast cancer case-control study.[9] However, a recent study with smaller sample size found no association between breast cancer risk and IR sensitivity when cell cycle arrest was quantified using the parameter (G_2/G_0–G_1)/S.[15] Therefore, we first examined the IR sensitivity data from 118 breast cancer cases and 225 controls reported in a previous study[9] and compared the results obtained using four parameters described in previous studies: G_2 delay index, %G_2–M, G_2/G_0–G_1, and %(G_2/G_0–G_1)/S.[5,8,15,16] Furthermore, we compared these four parameters in 54 lymphoblastoid and three breast cancer cell lines with different genetic defects in cell cycle checkpoint and DNA repair. Lastly, we evaluated the association between phosphorylated histone H2AX, a measure of DNA damage signaling[17] and cell cycle G_2 delay index. This study aims to further characterize the cell cycle G_2 delay assay as a simple and reproducible screening tool for assessing IR sensitivity and breast cancer risk.

Materials and methods
Study population
Basal and IR-induced cell cycle distribution data in T lymphocytes were derived from a breast cancer-control study of 118 breast cancer cases and 225 controls as described previously.[9] In brief, cancer-free women were recruited from the Comprehensive Breast Center and the Cancer Assessment and Risk Evaluation program at Georgetown University Medical Center from August 1995 to November 1996 as study controls. Breast cancer cases were recruited from the Breast Cancer Section of the Division of Hematology/Oncology. Each woman completed a self-administered questionnaire requesting information about demographics, medical conditions, and family history (FH) of breast cancer in first-degree relatives. A woman with at least one first-degree relative with breast cancer was considered to have a positive FH. The general eligibility criteria were, (i) English-speaking and able to comprehend informed consent, (ii) no personal history of other cancers, and (iii) at least 18 years of age. Blood samples were taken from all study subjects. Subjects received a detailed description of the study protocol and signed informed consent as approved by the Institutional Review Board of the Georgetown University Medical Center.

Lymphoblastoid and breast cancer cell lines
Epstein–Barr virus-immortalized human lymphoblastoid cell lines from 21 *BRCA1* mutation carriers, 12 controls, and 21 individuals from *ATM* families were obtained from the Coriell Institute for Medical Research (Camden, NJ). Lymphoblastoid cell lines were maintained in RPMI 1640, 15% fetal bovine serum (FBS), at 37°C, 5% CO_2. Human breast cancer cell lines MCF7, BT-20, and HCC1937 were obtained from American Type Culture Collection (Manassas, VA) and maintained in the recommended growth medium at 37°C, 5% CO_2.

Mitogen response and cell cycle G_2 delay assay
The cell cycle distributions and IR-induced G_2 delay data on 118 breast cancer cases and 225 controls were obtained from a previous study.[9] In brief, PBLs were stimulated with phytohemagglutinin-P (PHA-P, Sigma-Aldrich,

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St Louis, MO) for 72 hours prior to irradiation. We reported the cell cycle G_2 delay data but not the mitogen response data.[9] In the current study, we performed the cell cycle delay assay in 54 lymphoblastoid and three breast cancer cell lines using the method as described previously with minor modifications.[9] In brief, lymphoblastoid and breast cancer cell lines were plated 24 hours prior to irradiation at a cell concentration of 0.5×10^6/ml. Irradiation was performed using a Nordion Gammacell ^{137}Cs irradiator at a dose rate of 0.9 Gy/min. Twenty-four hours post-irradiation, cells were harvested and stained with Vindelov's propidium iodide (PI)[18] containing 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 μg/mL RNase A, 0.1% NP-40 (Igepal CA-630; Sigma-Aldrich), and 75 μM PI. Untreated and IR-irradiated cells (3 Gy) were analyzed for cell cycle distribution (10,000 cells per sample) using an LSR dual laser flow cytometer and CellQuest Pro software (BD BioSciences, San Jose, CA).

The cell cycle G_2 delay index was calculated as (% irradiated cells in G_2–M – % control cells in G_2–M)/(% control cells in S phase) $\times$ 100.[9] Other parameters were calculated as follows: %G_2–M (% of irradiated cells in G_2–M),[16] G_2/G_0–G_1 (% irradiated cells in G_2–M)/(% irradiated cells in G_0–G_1) $\times$ 100,[5] (G_2/G_0–G_1)/S, (% irradiated cells in G_2–M)/(% irradiated cells in G_0–G_1)/(% controls cells in S) $\times$ 100.[15] For assay quality control, we performed repeat measures at least twice for 54 lymphoblastoid cell lines until the coefficient of variation (CV) for batch-to-batch variation dropped to less than 30%. For a majority of the samples, the inter-assay CV was under 10% (range 5%–27%). We also established that the intra-individual variation in 23 lymphocyte samples with repeat visits was very low (mean CV at 6%).

Histone H2AX phosphorylation (γ-H2AX) assay

Lymphoblastoid cell lines in culture were centrifuged at $300 \times g$ for 10 min and resuspended in 4°C growth medium (RPMI 1640, 15% FBS, antibiotics) at a concentration of 3.3×10^6 cells/ml. One hundred and fifty microliters (0.5×10^6 cells) of cells was irradiated on ice for 10 min (9 Gy) at a dose rate of 0.9 Gy/min in a ^{137}Cs irradiator. Following irradiation, cells were immediately placed in a 37°C water bath for repair. After repair for up to 4 hours, tubes were placed on ice and 150 μl of cold medium containing 0.2% NP-40 was added to each tube. Tubes were inverted 10x and kept on ice in the dark for one hour to block nonspecific binding sites. 33 μL of a 1:200 solution of γ-H2AX-FITC antibody (16-202A; Millipore, Billerica, MA) in ice cold medium containing 0.1% NP-40 was added to each tube for a final 1:2000 antibody

dilution. Tubes were mixed by inversion 10x and placed on ice for 1 hour in the dark. Cells were transferred to flow cytometry tubes and stained with 3.3 μl of PI (50 μg/mL). PI positive cells were analyzed for relative FITC staining (10,000 cells) using an LSR flow cytometer and fluorescence intensity was quantified using CellQuest Pro software.

Statistical analysis

To compare cell cycle distribution in PHA-activated T lymphocytes and cell cycle delay in breast cancer cases and controls, a one-way ANOVA, stratified by breast cancer family history, was performed using SPSS software (version 15.0; SPSS, Inc., Chicago, Illinois). We also performed Bonferroni post hoc pair wise comparisons. The means and standard deviations (SD) were presented. For quality control, CV (SD/mean) was calculated for intra-individual variations of repeat visits in 23 subjects and assay batch-to-batch variations of 54 lymphoblastoid cell lines.

Results

PHA response and IR sensitivity and breast cancer risk

In Table 1, the cellular response to PHA in PBLs from 118 breast cancer cases was significantly lower compared to that in 225 controls based on the percentage of cells in S phase of the cell cycle (24.8% vs 28.5%; $p < 0.05$). After 3 Gy of IR treatment, breast cancer cases have a significantly higher percentage of cells in G_0–G_1 phase (63.3% vs 59.0%, $p < 0.05$) and lower percentage of cells in S phase (17.7% vs 21.2%; $p < 0.05$). In Table 2, we used four different parameters for measuring IR-induced cell cycle delay: G_2 delay index, %G_2–M, G_2/G_0–G_1, and (G_2/G_0–G_1)/S. The G_2 delay index was the only parameter that showed significant increases in IR-induced cell cycle delay in breast cancer cases compared to controls (36.0 vs 31.4; $p < 0.01$). The other three parameters did not yield useful findings; the G_2/G_0–G_1 ratio even showed a significantly lower value in breast cancer cases compared to controls (30.8 vs 34.7; $p < 0.05$).

Cell cycle distribution and IR sensitivity in lymphoblastoid cell lines

In Table 3A, lymphoblastoid cell lines used in this study had similar cell cycle distributions without IR treatment. With IR treatment, cell lines from *ATM* mutation carriers, particularly five AT patients, had a lower percentage of cells in the G_0–G_1 phase (38.7% vs 56.0%, Table 3B) or obligate *ATM* mutation carriers (38.7% vs 53.5%, Table 3B) and a higher percentage

Table 1 Cell cycle distribution in breast cancer cases and controls

(A) Without IR treatment	Control				Case			
Family history	(N)	% G_0–G_1	% S	% G_2–M	(N)	% G_0–G_1	% S	% G_2–M
None	87	60.7 ± 7.1	28.4 ± 5.7	10.9 ± 2.1	61	63.8 ± 6.8[a]	25.5 ± 5.7[a]	10.7 ± 2.4
Mother or sister	69	60.3 ± 5.8	29.0 ± 4.7	10.7 ± 1.9	11	65.9 ± 7.5[a]	24.1 ± 5.2[a]	10.0 ± 2.8
Mother and sister	13	60.6 ± 7.5	28.0 ± 5.7	11.4 ± 2.9	3	70.8 ± 4.4[a]	21.4 ± 5.9	7.8 ± 2.0
Missing	56	60.9 ± 4.8	28.0 ± 3.9	11.0 ± 1.9	43	66.0 ± 5.4[a]	24.1 ± 4.3[a]	9.8 ± 2.0[a]
Total	225	60.6 ± 6.2	28.5 ± 5.0	10.9 ± 2.1	118	64.9 ± 6.4[a]	24.8 ± 5.2[a]	10.3 ± 2.2[a]
(B) With IR treatment								
None	87	59.1 ± 8.0	21.1 ± 5.6	19.8 ± 4.1	61	62.4 ± 7.0[a]	18.2 ± 5.3[a]	19.4 ± 3.3
Mother or sister	69	59.1 ± 7.9	21.4 ± 5.4	19.5 ± 4.2	11	62.9 ± 7.3	19.5 ± 4.4	17.5 ± 4.3
Mother and sister	13	57.0 ± 6.2	21.3 ± 5.5	21.7 ± 4.3	3	67.3 ± 2.6[a]	13.8 ± 2.2[a]	18.9 ± 2.6
Missing	56	59.3 ± 6.1	20.9 ± 4.3	19.8 ± 3.9	43	64.4 ± 6.3[a]	16.9 ± 4.3[a]	18.7 ± 3.4
Total	225	59.0 ± 7.4	21.2 ± 5.2	19.8 ± 4.1	118	63.3 ± 6.7[a]	17.7 ± 4.9[a]	19.0 ± 3.4

Notes: [a]$p < 0.05$, pair-wise comparison, cases vs controls.
Abbreviation: IR, ionizing radiation.

of cells in the G_2–M phase compared to controls (55.6% vs 35.2%, Table 3B), *BRCA* mutation carriers (55.6% vs 37.0%, Table 3B), or obligate *ATM* mutation carriers (55.6% vs 41.7%, Table 3B). In Table 4, the mean cell cycle delay following IR treatment in 21 heterozygous *BRCA1* mutation carriers was not significantly different from that in controls using all four parameters. Although all four parameters were able to detect significantly higher cell cycle G_2 delay in AT patients compared to each of the three other groups ($p < 0.05$), only G_2 delay index and (G_2/G_0–G_1)/S detected significant higher cell cycle delay in obligate *ATM* mutation carriers (Table 4C) compared to controls (Table 4A) or cell lines with *BRCA1* mutations (Table 4B).

Cell cycle distribution and IR sensitivity in breast cancer cell lines

Three breast cancer cell lines with different genetic backgrounds were evaluated for their response to IR. As shown in Table 5, without IR treatment, a higher percentage of cells from BT-20 and HCC1937 cell lines were present in the G_2–M phase of the cell cycle compared to MCF7 cell line (31.9% and 32.1% vs 16.6%). Post-IR treatment, these two *TP53*-mutant lines, BT-20 and HCC1937, showed significantly higher cell cycle delay compared to that of MCF7 using all four parameters. The results suggest that *TP53* mutations may influence IR-induced cell cycle delay (Table 5). However, G_2 delay index showed the maximal 20.7-fold difference in cell cycle delay between MCF7 and HCC1937 cell lines compared to 3.2-, 10.0-, and 8.9-fold differences using %G_2–M, G_2/G_0–G_1, and (G_2/G_0–G_1)/S, respectively.

γ-H2AX induction in irradiated lymphoblastoid cell lines

H2AX phosphorylation kinetics differed significantly in irradiated lymphoblastoid cell lines from controls, obligate *ATM* mutation carriers, and AT patients (Figure 1). Control

Table 2 Ionizing radiation-induced cell cycle delay in breast cancer cases and controls

Family history	(N)		G_2 delay index		%G_2–M		G_2/G_0–G_1		(G_2/G_0–G_1)/S	
	Control	Case	Control	Case	Control	Case	Control	Case	Control	Case
None	87	61	31.6 ± 11.5	35.1 ± 12.8	19.8 ± 4.1	19.4 ± 3.3	34.7 ± 10.9	31.9 ± 7.9	1.21 ± 0.3	1.26 ± 0.2
Mother or sister	69	11	30.1 ± 10.7	31.8 ± 12.3	19.5 ± 4.2	17.5 ± 4.3	34.3 ± 10.7	29.0 ± 10.7	1.17 ± 0.3	1.18 ± 0.2
Mother and sister	13	3	38.3 ± 17.8	51.4 ± 12.3	21.7 ± 4.3	18.9 ± 2.6	38.8 ± 9.77	28.2 ± 4.7	1.40 ± 0.3	1.34 ± 0.2
Missing	56	43	30.9 ± 10.5	37.2 ± 13.4[a]	19.8 ± 3.9	18.7 ± 3.4	34.2 ± 9.72	29.8 ± 8.1[a]	1.22 ± 0.3	1.24 ± 0.3
Total	**225**	**118**	**31.4 ± 11.5**	**36.0 ± 13.1[a]**	**19.8 ± 4.1**	**19.0 ± 3.4**	**34.7 ± 10.5**	**30.8 ± 8.2[a]**	**1.21 ± 0.3**	**1.25 ± 0.2**

Notes: [a]$p < 0.001$, pair-wise comparison, cases vs controls.

Table 3 Cell cycle distribution in lymphoblastoid cell lines

(A) Without IR treatment	N	%G_0–G_1	% S[a]	%G_2–M
Controls	12	60.8 ± 6.3	29.6 ± 5.9[b]	9.6 ± 1.0
BRCA mutation carriers	21	60.1 ± 5.4	29.0 ± 4.9	10.8 ± 1.6
ATM mutation carriers	16	63.7 ± 5.2	24.8 ± 4.9	11.6 ± 5.0
AT patients	5	60.7 ± 2.9	26.5 ± 3.0	12.8 ± 0.9
(B) With IR treatment		%G_0–G_1[a]	% S[a]	%G_2–M[a]
Controls	12	56.0 ± 6.3[b]	8.9 ± 1.8[b,e]	35.2 ± 5.9[b]
BRCA mutation carriers	21	55.9 ± 8.2[c]	7.1 ± 2.1[f]	37.0 ± 7.3[c]
ATM mutation carriers	16	53.5 ± 7.9[d]	4.8 ± 1.6	41.7 ± 7.0[d]
AT patients	5	38.7 ± 5.9	5.7 ± 0.3	55.6 ± 5.8

Notes: [a]$p < 0.05$, ANOVA test for differences among four cell lines; [b]$p < 0.05$, Bonferroni post hoc pair-wise comparison, cell lines from controls compared to AT patients; [c]$p < 0.05$, Bonferroni post hoc pair-wise comparison, cell lines form *BRCA1* mutation carriers compared to AT patients; [d]$p < 0.05$, Bonferroni post hoc pair-wise comparison, cell lines from *ATM* mutation carriers compared to AT patients; [e]$p < 0.05$, Bonferroni post hoc pair-wise comparison, cell lines from controls compared to *ATM* mutation carriers; [f]$p < 0.05$, Bonferroni post hoc pair-wise comparison, cell lines from *BRCA1* mutation carriers compared to *ATM* mutation carriers.
Abbreviation: IR, ionizing radiation.

cells had the most rapid and largest increase in γ-H2AX level following IR exposure. Cells from controls and obligate *ATM* mutation carriers reached maximal γ-H2AX induction within 30 min post-irradiation. However, cells from AT patients had a slower and lower γ-H2AX induction ($p < 0.05$ at 15, 30, and 60 min) which peaked two hours after irradiation. γ-H2AX induction in cells from obligate *ATM* mutation carriers followed kinetics similar to controls, but the maximum γ-H2AX induction level was intermediate between that observed in controls and AT patients. At four hours post-IR treatment, all three groups of cells showed similar levels of γ-H2AX.

Discussion

Using data derived from a relatively large breast cancer case-control study and cell lines with different genetic defects, our current results support previous findings that prolonged cell cycle G_2 delay in response to IR may serve as a sensitive biomarker for assessing IR sensitivity and breast cancer predisposition. Some of the conflicting results in the literature may be related to: (1) study sample size and related statistical power, (2) PHA-induced PBLs vs lymphoblastoid cell lines, and (3) parameters used for interpreting the study results.[5,9,15,16]

Another interesting finding in this study is that response of PBLs to PHA is lower in breast cancer cases compared to that in controls. Several earlier studies reported that PBLs from cancer patients are less responsive to mitogenic stimulation by PHA and other plant lectins compared to healthy controls.[19–20] Our current data clearly demonstrate that PHA-stimulated PBLs from breast cancer cases had a significantly higher percentage of cells in the G_0–G_1 and a lower percentage of cells in the S phase compared to controls. PHA-induced

proliferation of PBLs derived from patients with breast cancer may reflect tumor load and be a good clinical predictor for the further course of the disease.[21] Antitumor T lymphocytes play a pivotal role in immune surveillance of cancer cells. Therefore, PHA-stimulated T lymphocyte cell cycle distribution may also serve as an immune function marker for cancer risk. Our observation is also consistent with the results from a recent study showing that genetic polymorphisms in cytotoxic T-lymphocyte antigen 4 influenced T-cell activation and modified the susceptibility to breast and other cancers.[22]

Breast cancer case-control differences in PBL response to PHA have an impact on studies of IR-induced cell cycle delay. Since only cycling cells are arrested in G_2–M following irradiation, PBLs with lower percentages of cycling cells would produce a lower percentage of G_2-arrested cells, subsequently influencing study results. Therefore, we introduced the G_2 delay index to normalize the results with the percentage of cells in S phase without IR treatment.[8,9] The importance of adjusting for PHA response is clearly demonstrated in Table 2, where significant prolonged cell cycle delay in breast cancer cases was observed only when G_2 delay index was used. Neither %G_2–M nor (G_2/G_0–G_1)/S showed a case-control difference in cell cycle delay. When G_2/G_0–G_1 was used, we even observed a lower cell cycle delay response in breast cancer cases compared to that in controls.

PBLs and lymphoblastoid cell lines from *BRCA1* and *ATM* mutation carriers have previously been analyzed for associations between IR-induced cell cycle delay and mutation status.[12,15,16,23] Mutations in *BRCA1* or *ATM* are risk factors for breast and ovarian cancers.[2,24] The results from recent studies suggest that BRCA1 plays critical roles in

Table 4 Ionizing radiation-induced cell cycle delay in lymphoblastoid cell lines

Repository	G_2 delay	%G_2–M	G_2/G_1	(G_2/G_1)/S
(A) Controls (n = 12)				
GM14807	100 ± 5	41 ± 5	81 ± 20	2.5 ± 0.3
GM14548	96 ± 15	37 ± 8	69 ± 24	2.4 ± 0.5
GM14453	95 ± 11	35 ± 7	64 ± 18	2.4 ± 0.5
GM01990	95 ± 11	34 ± 6	61 ± 19	2.3 ± 0.5
GM10924	88 ± 10	40 ± 5	81 ± 15	2.3 ± 0.2
GM14448	87 ± 16	39 ± 4	74 ± 16	2.2 ± 0.2
GM14476	85 ± 9	31 ± 8	53 ± 19	1.9 ± 0.4
GM14452	84 ± 16	40 ± 5	84 ± 20	2.4 ± 0.4
AG09387	84 ± 11	43 ± 4	93 ± 14	2.4 ± 0.5
GM14673	83 ± 23	27 ± 11	47 ± 24	2.0 ± 0.5
GM01814	75 ± 15	31 ± 4	53 ± 11	1.8 ± 0.1
GM13079	72 ± 12	24 ± 5	36 ± 12	2.0 ± 0.5
Mean ± SD	87.0 ± 8.5[a,b]	35.2 ± 5.9[b]	66.2 ± 17.1[b]	2.2 ± 0.2[a,b]
(B) *BRCA1* mutation carriers (n = 21)				
GM13713	120 ± 17	45 ± 3	89 ± 13	3.3 ± 0.2
GM15993	116 ± 9	52 ± 7	135 ± 42	3.8 ± 0.8
GM14637	103 ± 10	35 ± 3	58 ± 8	2.3 ± 0.2
GM13715	100 ± 23	42 ± 5	86 ± 17	2.8 ± 0.8
GM13712	99 ± 6	45 ± 5	100 ± 21	3.2 ± 0.4
GM13714	97 ± 25	35 ± 6	64 ± 17	2.4 ± 0.6
GM13707	95 ± 15	40 ± 5	71 ± 15	2.5 ± 0.7
GM13710	95 ± 13	48 ± 6	112 ± 27	3.1 ± 0.6
GM14636	92 ± 15	34 ± 5	59 ± 13	2.1 ± 0.3
GM15232	90 ± 6	47 ± 5	113± 32	2.7 ± 0.4
GM14093	89 ± 15	32 ± 5	52 ± 14	2.0 ± 0.5
GM16105	89 ± 12	38 ± 7	72 ± 22	2.3 ± 0.5
GM14097	88 ± 18	34 ± 6	61 ± 17	2.2 ± 0.4
GM14638	88 ± 12	35 ± 6	63 ± 18	2.2 ± 0.3
GM14092	82 ± 9	34 ± 1	56 ± 3	1.9 ± 0.2
GM14094	80 ± 10	29 ± 8	45 ± 18	1.9 ± 0.3
GM14091	80 ± 9	27 ± 2	40 ± 5	2.1 ± 0.4
GM13705	78 ± 19	36 ± 6	68 ± 18	2.2 ± 0.5
GM14096	78 ± 9	36 ± 4	70 ± 15	2.0 ± 0.3
GM13709	71 ± 13	28 ± 4	43 ± 8	1.7 ± 0.3
GM14090	61 ± 10	25 ± 5	37 ± 10	1.6 ± 0.3
Mean ± SD	90.0 ± 13.9[c,d]	37.0 ± 7.3[d]	71.1 ± 26.2[d]	2.4 ± 0.6[c,d]
(C) *ATM* mutation obligate carriers (n =16)				
GM09579	162 ± 32	49 ± 4	105 ± 16	4.4 ± 1.0
GM00736	160 ± 15	48 ± 12	106 ± 54	4.5 ± 1.6
GM02781	155 ± 20	32 ± 1	49 ± 2	3.5 ± 0.3
GM02782	134 ± 12	46 ± 25	129 ± 122	4.6 ± 2.3
GM09583	130 ± 23	45 ± 3	94 ± 12	3.4 ± 0.7

(Continued)

Table 4 (*Continued*)

Repository	G_2 delay	%G_2–M	G_2/G_1	$(G_2/G_1)/S$
GM03383	127 ± 11	39 ± 4	66 ± 13	2.8 ± 0.1
GM03188	125 ± 34	46 ± 5	97 ± 22	3.6 ± 1.7
GM09580	124 ± 16	47 ± 2	103 ± 11	3.3 ± 0.4
GM02783	122 ± 70	46 ± 7	105 ± 36	4.3 ± 0.9
GM03325	118 ± 10	48 ± 1	104 ± 4	3.2 ± 0.1
GM03187	116 ± 14	40 ± 1	71 ± 3	2.7 ± 0.3
GM03324	115 ± 16	40 ± 5	73 ± 17	2.8 ± 0.5
GM03323	107 ± 17	40 ± 3	75 ± 10	2.6 ± 0.4
GM03334	97 ± 12	30 ± 4	46 ± 10	2.2 ± 0.5
GM03333	84 ± 19	44 ± 9	90 ± 40	6.8 ± 5.6
GM03380	77 ± 16	26 ±1	37 ± 3	1.6 ± 0.4
Mean ± SD	122.1 ± 24.2[e]	41.7 ± 7.0[e]	84.4 ± 25.8[e]	3.5 ± 1.2[e]
(D) AT patients (n = 5)				
GM09581	200 ± 10	61 ± 2	179 ± 7	7.7 ± 0.5
GM09582	164 ± 14	60 ± 0.2	173 ± 3	5.9 ± 0.4
GM00719	159 ± 27	59 ± 8	178 ± 66	5.9 ± 0.6
GM03332	148 ± 17	48 ± 23	136 ± 122	5.1 ± 2.9
GM03189	146 ± 30	50 ± 3	114 ± 9	4.6 ± 1.7
Mean ± SD	163.3 ± 21.9[c]	55.6 ± 5.8[c]	155.9 ± 29.4[c]	5.8 ± 1.2[c]

Notes: [a]$p < 0.05$, controls vs *ATM* mutation carriers; [b]$p < 0.05$, controls vs AT patients; [c]$p < 0.05$, *BRCA1* vs *ATM* mutation carriers; [d]$p < 0.05$, *BRCA1* mutation carriers vs AT patients; [e]$p < 0.05$, *ATM* mutation carriers vs AT patients.

DNA repair, recombination, checkpoint control of the cell cycle, transcription, and S- and G_2-phase checkpoints in response to IR.[25–27] We compared the IR-induced G_2 delay in lymphoblastoid cell lines from controls and *BRCA1* carriers using G_2 delay index and three other parameters (Table 4). None of the methods could distinguish a difference in *BRCA1* heterozygote mutation carriers compared to controls. Our findings are consistent with a recent study that showed normal post-IR cell cycle kinetics in *BRCA1* mutation carriers[23] and suggest that one functional *BRCA1* allele may be sufficient for its normal post-IR checkpoint function.

An association between mutations in the *ATM* gene and IR hypersensitivity has been demonstrated in numerous studies as summarized in a recent review.[28] Early studies identified that AT cells are defective in immediate cell cycle checkpoints in response to IR, but a later ATM-independent response causes these irradiated cells to accumulate in G_2 relative to normal cells.[12,29] Therefore, we also evaluated the four parameters in lymphoblast cell lines derived from AT patients and obligate *ATM* mutation carriers. All four parameters showed significantly higher cell cycle delay in cell from AT patients. In contrast to a previous study,[15] we also showed significantly

higher cell cycle delay in cells from obligate *ATM* mutation carriers compared to that in controls using G_2 delay index and $(G_2/G_0–G_1)/S$. The difference in study results may be related to a larger sample size of our study with 16 *ATM* mutation carriers and 12 controls compared to the previous study with four *ATM* mutation carriers and four controls.[15]

We further investigated the association between G_2 delay index and IR-induced H2AX phosphorylation in seven lymphoblastoid cell lines (Figure 1). In response to DNA double-strand strand breaks induced by IR or other genotoxic agents, H2AX is rapidly phosphorylated at Ser-139 (γ-H2AX) by ATM and other kinases, including ATR and DNA PK.[30] γ-H2AX is immediately localized to DSB sites where it is believed to recruit additional factors required for completion of DNA repair.[31] The formation and disappearance of γ-H2AX foci in a nucleus are proportional to the induction and repair of double-strand breaks, respectively.[32] The result of DNA double-strand breaks measured by γ-H2AX levels is in agreement with data obtained using the comet assay.[33] Thus, the kinetics of γ-H2AX formation may be a useful surrogate for DNA double-strand break induction and repair. The established role of ATM kinase in post-IR double-strand

Table 5 Cell cycle distribution and mitotic delay in breast cancer cell lines

Cell line	Without IR Treatment			With IR treatment			G$_2$ delay index[a]	%G$_2$–M[a]	G$_2$/G$_0$–G$_1$[a]	(G$_2$/G$_0$–G$_1$)/S[a]
	%G$_0$–G$_1$[a]	% S[a]	%G$_2$–M[a]	%G$_0$–G$_1$[a]	% S[a]	%G$_2$–M[a]				
MCF7[b]	63.4 ± 5.6[f]	18.8 ± 2.7[e]	16.6 ± 2.1[e,f]	68.2 ± 3.2[e,f]	11.2 ± 1.1[f]	17.5 ± 1.4[e,f]	5.6 ± 10.8[e,f]	17.5 ± 1.4[e,f]	25.8 ± 2.7[e,f]	1.4 ± 0.1[e,f]
BT-20[c]	52.2 ± 15.6	13.9 ± 1.4[g]	31.9 ± 14.6	41.6 ± 15.3[g]	11.8 ± 1.0[g]	45.2 ± 15.6	95.6 ± 14.0[g]	45.2 ± 15.6	146.7 ± 117.4[g]	10.3 ± 7.8
HCC1937[d]	45.4 ± 6.1	20.7 ± 1.6	32.1 ± 4.0	22.3 ± 3.2	18.6 ± 1.3	56.0 ± 1.9	116.2 ± 18.2	56.0 ± 1.9	257.4 ± 49.7	12.4 ± 1.9
				Fold difference			20.7	3.2	10.0	8.9

Notes: [a]$p<0.05$, ANOVA test for differences among three cell lines; [b]MCF7 cells with wild-type *BRCA1* and wild-type *p53*; [c]BT-20 cells with wild-type *BRCA1* and mutant *p53*; [d]HCC1937 cells with mutant *BRCA1* and mutant *p53*; [e]$p < 0.05$, Bonferroni post hoc pair-wise comparison, MCF7 compared to BT-20 cell line; [f]$p < 0.05$, Bonferroni post hoc pair-wise comparison, MCF7 compared to HCC1937 cell line; [g]$p < 0.05$, Bonferroni post hoc pair-wise comparison, BT20 compared to HCC1937 cell line.
Abbreviation: IR, ionizing radiation.

break repair and checkpoint function underlies the DNA repair deficient- and IR-sensitive phenotype observed in AT patients.[34] Following IR exposure, cells from AT patients had slower and lower levels of γ-H2AX induction than those in obligate *ATM* carriers and controls (Figure 1). In addition to genetic variations in DNA repair, the results from the current study also demonstrate that defective DNA-damage signaling is associated with prolonged cell cycle G$_2$ delay.

Analysis of IR-induced cell cycle delay in breast cancer cell lines utilizing the four parameters further supports the advantage of using the G$_2$ delay index parameter. In three breast cancer cell lines, the G$_2$ delay index was able to detect the largest fold difference between the MCF7 and the HCC1937 cell lines (Table 5). Although both MCF7 and BT-20 cells express wild-type *BRCA1*,[35] they exhibit very different IR responses. The difference may be related to *TP53* mutation (AAG→ CAG; *K132Q*) in BT-20 cells[36] since p53 plays critical role in checkpoint regulation.[37] In HCC1937 cells, the presence of mutations in both *BRCA1* (*5382C*) and *TP53* (CGA→TGA; *R306X*)[36] genes may contribute to its having the highest cell cycle delay among the three breast cancer cell lines.

In several studies, the association between IR sensitivity in patient PBLs and acute radiotherapy (RT) toxicity has been inconclusive.[6,7,16,38] Some breast cancer patients experience RT-induced acute adverse skin reactions of varying severity.[39] In addition, RT-treated patients may also develop telangiectasia and fibrosis as late effects.[40] Accordingly, there is increasing interest in the development of predictive tests for RT-induced adverse reactions. However, several studies have reported conflicting results utilizing chromosomal aberration analysis in predicting acute RT reactions.[37,41,42] IR-induced DNA damages activate checkpoints that delay cell cycle progression to facilitate DNA repair. However, continued proliferation after DNA damage in IR-irradiated cells has been well documented. The results from a recent study estimated that cells with about 10 to 20 double-strand breaks are released from a G$_2$ checkpoint delay and enter mitosis.[43] Although these cells continue to divide for one to two cell cycles; the unrepaired damaged DNA eventually results in rapid apoptosis, senescence, a permanent cell cycle arrest, or mitotic catastrophe.[44] Therefore, our validation of the cell cycle G$_2$ delay assay suggests that it may serve as a simple screening tool to probe for combined genetic defects and variations in cell cycle checkpoint regulation (eg, *ATM* and *TP53* mutations) and DNA repair (eg, *BRCA1* mutations and DNA repair SNPs) in assessing radio-sensitivity and cancer risk.

The major strengths of our study are: (1) large sample size of the case-control study and cell lines, (2) stringent laboratory
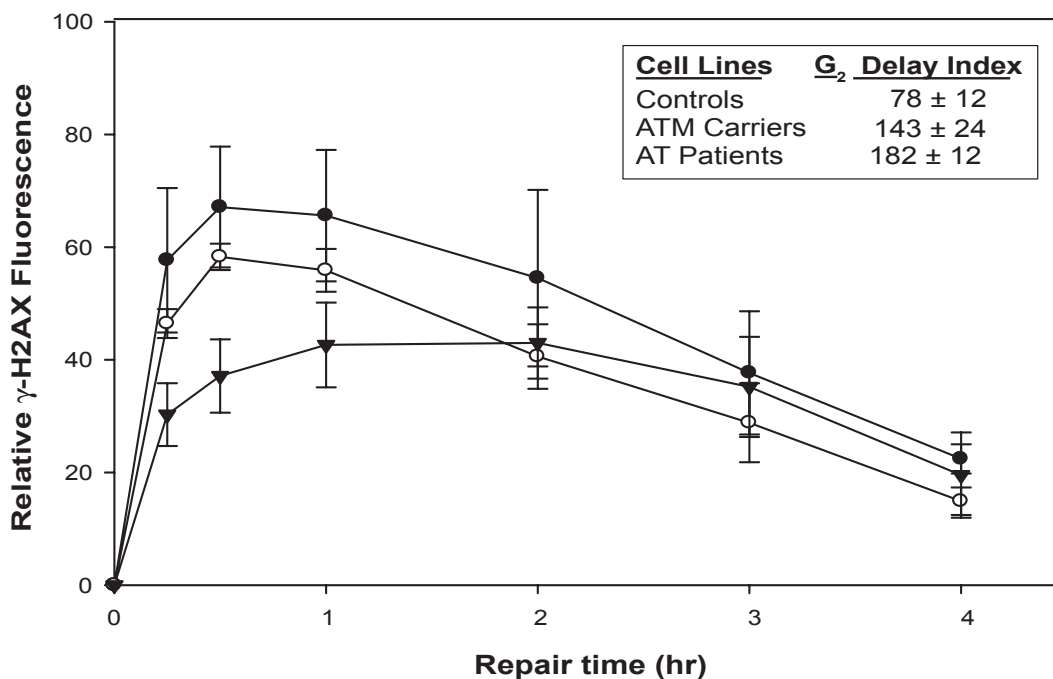
Figure 1 γ-H2AX staining in irradiated lymphoblastoid cell lines. Induced γ-H2AX fluorescence in lymphoblastoid cell lines stained with γ-H2AX-FITC antibody at the indicated time interval following exposure to 9 Gy IR. Closed circles, average γ-H2AX fluorescence of three control lymphoblastoid cell lines GM10924, GM01814, and GM13079. Open circles, average γ-H2AX fluorescence of two *ATM* carrier lymphoblastoid cell lines GM09579 and GM09580. Closed triangles, average γ-H2AX fluorescence of two AT patient lymphoblastoid cell lines GM09581 and GM09582. Inset: G$_2$ delay index of corresponding lymphoblast groups assayed for γ-H2AX. All values are means of three independent experiments with standard deviation.

assay quality control with adequate batch-to-batch assay variations, (3) validation of the G$_2$ delay index as the best parameter for testing IR sensitivity in a case-control study and among cell lines with different genetic defects, and (4) availability of genomic DNA for future testing of genotype–phenotype association studies. However, our study has several limitations. First, the current study uses a dataset from a previous study. Future studies with a larger sample size and a different case-control population will be required to confirm our study findings and to evaluate the genetic regulation of cell cycle delay in IR sensitivity and breast cancer risk. Second, viable cells are required for performing the cell cycle assay, which may limit its application in population-based studies of breast-cancer risk assessment and tumor response. Therefore, we are currently evaluating whether the newly developed antibody-based γ-H2AX assay may overcome this limitation.

In summary, the cellular responses to IR-induced DNA damage are complex. The cell cycle G$_2$ delay assay may serve as a probe for genetic defects/variations in cell cycle checkpoint regulation and DNA repair in assessing IR sensitivity and breast cancer risk. However, there is a need to evaluate future genotype-phenotype relationships in cellular IR responses and compare results from other functional DNA damage and repair phenotype assays in order to establish the utility of the cell cycle G$_2$ delay assay in the assessment of breast-cancer risk and prediction of RT response.

Acknowledgments

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