MicroRNA Analysis of ATM-Deficient Cells Indicate PTEN and CCDN1 as Potential Biomarkers of Radiation Response

Jane Bryant,a Lisa White,a,b Natasha Coen,c Laura Shields,c Brendan McClean,c Aidan D. Meade,a,c Fiona M. Lynga,c and Orla Howea,c,b,1

a Radiation and Environmental Science Centre (RESC), FOCAS Research Institute, b School of Biological and Health Sciences and c School of Physics & Clinical & Optometric Sciences, Technological University Dublin, City Campus, Dublin 8, Ireland; d Department of Clinical Genetics, Division of Cytogenetics, Our Lady’s Children’s Hospital, Crumlin, Dublin 12, Ireland; and e Medical Physics Department, St Luke’s Radiation Oncology Centre, Rathgar, Dublin 6, Ireland

INTRODUCTION

In the last decade, the definition and classification of ionizing radiation biomarkers have been reported through several European Union Framework 7 multidisciplinary consortia such as Multibiodose (2010–2013), Realizing the European Network in Biodosimetry [RENEB (2012–2015)] and Low Dose Research towards Multidisciplinary Integration [DoReMi (2010–2015)], with the multipurpose use of biomarkers for epidemiological and biodosimetry investigations (1–4). These include biomarkers of low-dose radiation exposure and biological response, individual susceptibility and early detection of radiation-induced health effects, of which considerations to the characteristics of a good biomarker and the useful in vitro approaches have been made. Although the DoReMi project was completed in 2015, research has continued under the Multidisciplinary European Low Dose Initiative [Melodi (5)]; and the DoReMi multidisciplinary report (4) was later updated to include novel radiation biomarkers emerging from technical advances in metabolomics and transcriptomics, and to critique the current status of biomarkers (6). A roadmap for the development of biomarkers from discovery to implementation was presented for biomarkers of low-dose exposure and early or late radiation effects. The authors highlighted that the majority of potential biomarkers are in the development stage with only one biomarker that has progressed to the final stages of development with radiation-specific mRNA transcript profiles for FDXR. This gene has been reported in many proposed gene signature panels due to dose-dependent induction in different cell and tissue types (7–10). Furthermore, inter-comparison laboratory or biodosimetry studies have demonstrated that both single genes and gene panels can be used to estimate exposure of...
samples with the same accuracy and sensitivity of established and traditional cytogenetic assays (11, 12).

The DNA damage response (DDR) pathways are potential targets for transcriptional biomarkers of cancer susceptibility and radiation exposure; in particular, the ATM/chk2/p53 pathway responds to radiation-induced double-strand breaks (DSBs), leading to cell cycle arrest or DNA repair. The DSBs are sensed by the MRN complex (MRE11-Rad50-NBS-1) leading to ATM activation, phosphorylation of serine 139 of γ-H2AX and extension around the DSBs, initiating repair protein assembly (13, 14). Consequently, γ-H2AX has been used as a biomarker of DNA damage and repair and for predicting radiosensitivity in individuals (15–17), and has been applied to a wide range of established cell lines, primary cell cultures and peripheral blood lymphocytes, as well as two-dimensional tissue models and tissue sections, as reviewed by Rothkamm et al. (18). The role of ATM, a PI3K-like kinase that is phosphorylated at specific serine/threonine sites when activated, is central to this pathway. Deficiencies in the ATM gene lead to phenotypic elevated radiosensitivity observed in clinical conditions such as ataxia telangiectasia (AT) and AT-like disorders (ATLD) (19–21). After DSBs are sensed, the cell cycle must be halted to allow sufficient time for DNA repair processes, facilitated through ATM-activated Chk2. This leads to p53-mediated inhibition of cyclins and cyclin-dependent kinases, such as cyclin D1 (CCDN1) and CDK4/6 at the G1/S checkpoint (22). Failure to undergo DNA repair may result in permanent cell cycle arrest, enhanced apoptosis or cellular senescence. The PI3K/Akt pathway is also involved in the survival of cells after radiation-induced DNA damage, through overriding of the G2/M cell cycle arrest mechanism; conversely, inhibition of PI3K or Akt, for example through the tumor suppressor PTEN, induces cell apoptosis and therefore elevates cellular radiosensitivity (23–25).

Further transcriptomic analyses have shown that micro-RNAs (miR) are promising biomarkers of radiation oncology (26). They are small, non-coding RNA molecules of 19–22 nucleotides that regulate more than 50% of cell protein coding genes and regulate important processes of the DNA damage response such as DNA repair, cell cycle control and apoptosis. It has previously been shown that important genes of these processes (such as CDKN1, SESN1, ATF3, MDM2, PUMA and GADD45A) were upregulated in stimulated T cells in response to radiation with a significant dose- and time-dependent modification of miR expression (specifically, miR-34-5p and miR-182-5p) (27–28).

Given the current published evidence associating the ATM/chk2/p53 pathway with elevated radiosensitivity and potentially regulated by miR, normal and AT radiosensitive lymphoblastoid cell lines were used to measure radiation-induced DNA damage using the classic cytogenetic and DNA damage biomarkers followed by miR screening and identification of gene targets in a multi-biomarker approach. All biomarkers selected for this study were based on the DoReMi multidisciplinary biomarker reports by Pernot et al. (4) and Hall et al. (6), and the recently reported review of the progress made in low-dose health risk research by the DoReMi consortium (29).

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Epstein-Barr immortalized lymphoblastoid cell lines (LCLs), coded C1, 2139, AT2Bi and AT3Bi, were used for this study. C1 and 2139 cell lines were derived from healthy donors and kindly gifted by the Queensland Institute of Medical Research (Brisbane, Australia) and Institut Curie (Paris, France), respectively. The AT2Bi and AT3Bi cell lines were derived from clinically diagnosed ataxia-telangiectasia patients and kindly gifted from the College of Medical and Dental Sciences, University of Birmingham (Birmingham, UK). Both AT2Bi and AT3Bi are known to have defective ataxia telangiectasia-mutated (ATM) protein causing the typical clinical and cellular manifestations of AT including heightened radiosensitivity (30). C1, 2139, AT2Bi and AT3Bi lymphoblast cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Wexford, Ireland) supplemented with 12.5% fetal bovine serum (FBS) and 1% L-Glutamine (Sigma-Aldrich), at 37°C and 5% CO₂. All cell lines were seeded at a density of 2 × 10⁶/ml and passaged once until a density of 1 × 10⁶/ml cells had been reached. Cells were seeded 18 h prior to irradiation, in T25 flasks at a density of 1 × 10⁶ cells/ml (G, chromosomes radiosensitivity assay), 2 × 10⁴ cells in total (growth curves) or 2 × 10⁶/ml (γ-H2AX and molecular experiments) at a final volume of 5 ml per T25 flask (Sarstedt, Numbrecht, Germany).

Irradiation Conditions

Cells were irradiated using a 6-MV photon beam produced by an Elekta Precise Linear Accelerator (LINAC; Stockholm, Sweden) at St. Luke’s Hospital (Dublin, Ireland), operating at a nominal dose rate of 6 Gy/min. The LINAC was calibrated in accordance with the 1990 IPSM code of practice by the Medical Physics Department at St. Luke’s Hospital (31), with 100 monitor units (MU, a measure of “beam on” time) delivered at dose of 1 Gy at 1.4 cm deep in water positioned 100 cm from the source for a 10 × 10 cm² field. To achieve uniform irradiation of flasks, the irradiation conditions were altered from those at calibration. A 30 × 35 cm² field was used to deliver each dose. The flasks were also positioned 10 cm deep in a water-equivalent phantom 90 cm from the source in which 100 MU delivers a dose of 0.812 Gy at 10 cm deep in water for a 10 × 10 cm² field. The number of MU required to deliver each of the doses outlined were corrected for the different scatter conditions present with the larger field size (30 × 35 cm²). Therefore, a correction factor of 1.372 was applied, which is the ratio of the area of a large field to a smaller one. Thus, at 90 cm from the source, 100 MU delivers a dose of 0.9234 Gy (0.812 × 1.1372), and therefore the delivery of 0.05 Gy required 6 MU and 0.5 Gy required 55 MU (MU were rounded up to the nearest whole number, as partial MU could not be delivered on the LINAC). The calculated doses were verified using Gafchromic™ EBT3 film (Ashland Inc., Bridgewater, NJ) and the film was calibrated against a Farmer type ionization chamber using the triple channel dosimetry method (31). The film was scanned using the single scan protocol (32) on an Epson Expression 10000 XL scanner (Plainfield, NJ) with the recommended scanning resolution of 72 dpi in a 48-bit RGB format (31, 33, 34). Glass was placed over the calibration and test film during scanning to minimize ringing artifacts. The film was analyzed using FilmQA™ Pro (Ashland Inc.).
Cell Growth Assay

To determine the effect of radiation on the growth potential of the cells, flask cells were seeded and irradiated as described above. At 5–7 days postirradiation, cells were isolated and counted in duplicate using a Coulter cell counter (Beckman Coulter, Co Clare, Ireland). Total cell numbers were calculated and analyzed with reference to sham-irradiated controls.

Gamma-H2AX Analysis by Flow Cytometry

DNA damage was determined using γ-H2AX analysis and measured by flow cytometry. Cells were fixed at 1 h postirradiation in 2% paraformaldehyde and stored in 70% ethanol at –20°C. To stain, cells were permeabilized using 0.25% Triton-X, followed by blocking with a 4% FBS solution in PBS for 30 min. A primary antibody solution [anti-phospho-histone H2A.X (Ser139), clone JBW301, 1:500; Merck Millipore, Darmstadt, Germany] was added and incubated overnight at 4°C, followed by a 1-h incubation with the secondary antibody [F(ab')- Goat anti-Mouse IgG (H+L), Alexa Fluor®-488, 1:200; Thermo Fisher Scientific Inc., Carlsbad, CA] at room temperature. Cells were washed, counterstained with 1% propidium iodide solution and analyzed using an Accuri™ C6 flow cytometer (BD Biosciences, Oxford, UK). The mean fluorescence of 10,000 cells was calculated using the Accuri C6 Sampler software, with cells stained only with the secondary antibody acting as a negative control for each sample.

G2 Chromosomal Radiosensitivity Assay

The G2 chromosomal radiosensitivity assay, as previously reported for whole blood lymphocytes (35–37), was applied to all 2139, AT2Bi and AT3Bi cells to measure radiation-induced cell cycle checkpoint response by mitotic indices and G2 chromosomal radiosensitivity. The mitotic index (MI) was calculated by counting the ratio of cells in metaphase to all cells on the slide up to 1,000 cells in total for each dose (0 Gy and 0.5 Gy) and cell line. Radiation-induced mitotic inhibition (RIMI) was calculated by subtracting the 0.5 Gy MI from the 0 Gy MI. A G2 radiosensitivity score was assigned to each of the cell lines and radiation dose by calculating the total number of chromosomal aberrations per 100 metaphases scored for each cell line and dose. A radiation-induced G2 score (RIG2) was calculated by subtracting the spontaneous aberrations in the G2 score at 0 Gy from those recorded at 0.5 Gy.

Cytogenetic G-Banding and Karyotyping

Cytogenetic preparations were made from irradiated 2139 and AT (AT2Bi and AT3Bi) LCL according to the G2 chromosomal radiosensitivity assay. For G-banding, the metaphase spreads on glass slides were covered with 30% hydrogen peroxide solution for 1 min followed by a wash with 0.9% NaCl solution. The metaphase preparations were then placed in Trypsin solution for 2 mins, washed with Gurr buffer (pH 8) and then stained in 1 ml of Leishmann-Gurr buffer (1:2) solution for 1 min. The slides were washed with Gurr buffer, then distilled water, and dried before they were mounted with a coverslip using DPX. Each slide was evaluated under the microscope set up for brightfield use, noting conditions of under- or over-banding or staining. A total of 25 metaphases were karyotyped under the microscope and analyzed for chromosomal aberrations.

Fluorescent In Situ Hybridization (FISH)

Cytogenetic preparations (metaphase spreads as above) obtained from irradiated 2139, AT2Bi and AT3Bi LCL were soaked in sodium chloride and sodium citrate buffer (SCC) for 2 min at 37°C, before being applied to/treated with protease solution for 30–40 s at 37°C. Slides were then washed in 1× PBS, dehydrated in an ethanol series (70%, 85% and 100%) for 2 min each at room temperature and air dried before hybridization. Hybridization FISH probes were used to identify deletions or rearrangements in ATM-TP53, particularly for the AT cells (AT2Bi and AT3Bi). Probes for ATM-TP53 were used to confirm the presence of ATM or TP53 gene in all LCL. Conditions such as B-cell chronic lymphocytic leukemia (B-CLL), a malignancy often associated with ataxia-telangiectasia, has shown deletions in the genes of ATM (38, 39) and P53 (40). Probes were mixed according to the manufacturer’s instructions and the required amount was added to each slide. Slides were transferred to a Hybrite machine with the selected hybridization program of 75°C for 2 min and 37°C for 20 h. When hybridized samples were removed, the slides were immersed in wash solution (0.4 × SSC/0.3% NP 40) for 2 min and then transferred into a solution of 2 × SSC/0.1% NP40 for a minimum of 1 min. DAPI (20 μl) was added as a counterstain and coverslips were mounted onto the slides. For FISH microscopy, 100 interphase cells were recorded.

MicroRNA Expression

An expression panel of 752 miR was performed on 2139, AT2Bi and AT3Bi cell lines (Exqxon, Vedbaek, Denmark), in accordance with company protocols. Briefly, RNA (50 ng) was reverse transcribed and cdNA assayed in 10 µl PCR reactions (miRCURY LNA™ universal RT microRNA PCR, Polyadenylation and cdNA Synthesis Kit; ExiLENT SYBR® Green Master Mix). The amplification was performed in a LightCycler® 480 Real Time PCR System (Roche Life Science, Penzberg, Germany) in 384-well plates. Melting curve and Cq values were analyzed using Roche LC software. Cq values were calculated as the second derivative, with values greater than 37 omitted from further analysis. All data were normalized to the average of assays detected in all samples (average – assay Cq).

Gene Expression

Irradiated LCL were analyzed for selected PTEN and CCND1 gene expression by Real Time PCR (RT-PCR). RNA was extracted from cells using the phenol-chloroform method and concentration measured using NanoDrop (Maestrogen, Las Vegas, NV). CDNA was synthesized using the q-script cDNA kit (QuantaBio, Beverly, MA), according to manufacturer’s instructions. Primers for Tubulin, PTEN and CCND1 were designed (Table 1) and synthesized (Sigma-Aldrich), and reactions were performed in duplicate in 96-well plates (Applied Biosystems, Carlsbad, CA). Each reaction was composed of 10 μl SYBR Green with low ROX, (Kapa Biosystems, London, UK), 1 μl of forward and reverse primers, 6 μl PCR grade water and 2 μl cdNA. Non-template controls replaced cdNA with 2 μl PCR grade water. Reactions were run for 45 cycles on an AB 7500 fast PCR cycler (Applied Biosystems).

Statistical Analysis

All statistical analyses were performed using Microsoft® Excel® versions 2010–2016 (Redmond, WA). Mean and standard deviations were calculated, and significance was determined using paired or unpaired t tests of each radiation dose relative to its 0 Gy control, for each individual cell line, as appropriate.

RESULTS

Cell Growth Assay for Monitoring Cellular Viability

All cell lines were 0.05 and 0.5 Gy irradiated and cultured for 5 days to measure growth potential. Percentage growth was calculated relative to the sham-irradiated control after 5 days in culture, and counted using a Coulter Counter (Fig. 1A). After 5 days in culture the control 2139 cells indicated a linear dose response for each low dose (0.05 and 0.5 Gy)
compared to the 0 Gy control (Fig. 1A). Similarly, the AT cells (AT2Bi and AT3Bi) indicated a dose response for 0.5 Gy but not 0.05 Gy. This was expected, since we had previously reported differential molecular mechanisms of apoptosis for 0.05 Gy compared to 0.5 Gy between 1 h and 24 h direct irradiation (41). The additional control cell line C1 did not show a radiation dose response comparative to the 2139 control cells.

Gamma-H2AX Biomarker of DNA Damage Response

All cell lines were 0.05 and 0.5 Gy irradiated and fixed for γ-H2AX analysis through flow cytometry, as shown in Fig. 1B. Percentage positive cells were calculated, and normalized to the sham-irradiated control of each cell line. Since the cytogenetic biomarker of radiosensitivity (G2 chromosomal radiosensitivity) did not discriminate G2 radiosensitivity between the control 2139 and AT cells (AT2Bi and AT3Bi), the γ-H2AX assay was employed to measure radiation-induced DSBs in all cells. An additional control cell (C1) with functional ATM similar to 2139 was also analyzed. Fluorescent foci are equal to the number of radiation-induced DSBs. Figure 1B shows γ-H2AX positive cells in the 4 LCLs at 1 h postirradiation. A modest increase in γ-H2AX positive cells was evident in the AT3Bi cell line to 1.5-fold of the 0 Gy control, however, this was not significant (P > 0.05). Irradiation of the 2139 and AT2Bi cell line decreased γ-H2AX levels below that of the sham-irradiated cells, however, this was not significant (P > 0.1). There was no dose dependent response in any cell line tested (Fig. 1B). This assay was also performed at later timepoints with no observable trends between the cell lines and doses (data not shown).

Cytogenetic Biomarkers of Radiation Response

The G2 chromosomal radiosensitivity assay was used as a cytogenetic biomarker of low-dose radiation-induced effects in the control 2139 and AT (AT2Bi and AT3Bi) lymphoblastoid cell lines. Assessment of mitotic indices (MI) through the G2 chromosomal radiosensitivity assay is a good indicator of cell cycle checkpoint response to ionizing radiation, whereby radiation-induced mitotic inhibition (RIMI) is the calculated difference between 0.5 Gy and 0 Gy MI. The normal expected MI for the G2 chromosomal radiosensitivity varies between 2–5%, whereas the RIMI can be varied depending on cellular response to radiation. All cell lines presented MI within the expected ranges for 0 Gy (Fig. 2A); however, RIMI was more pronounced in 2139 (1.2) compared to AT2Bi (–0.3) and AT3Bi (0.6). This indicated that the control 2139 cells had superior cell cycle checkpoint efficacy compared to the AT cells, probably due to functional ATM. All cell lines had elevated G2 chromosomal aberrations when 0.5 Gy irradiated compared to their nonirradiated counterpart (Fig. 2B). Interestingly, the control 2139 cell line had similar radiation-induced G2 chromosomal radiosensitivity RIG2 (203 aberrations/100 metaphases) as the two AT cell lines AT2Bi and AT3Bi (134 and 183 aberrations/100 metaphases, respectively), which indicated that although checkpoint response by MI appeared to be functional compared to the AT cells, radiation-induced chromosomal damage was similar to the AT cells. This finding merited further cytogenetic investigation, performed in collaboration with the Genetics Department of Our Lady’s Children’s Hospital, Crumlin (Dublin, Ireland). Cytogenetic karyotyping using the G-banding technique was performed on the 2139 and AT cells (AT2Bi and AT3Bi) and followed up with FISH.
analysis using an ATM/TP53 probe. Cytogenetic analysis of the 2139 cells surprisingly showed a loss of a sex chromosome in all of the cells analyzed (Fig. 3), with no other single cell or recurrent aberrations detected. The loss of a sex chromosome is associated with the constitutional diagnosis of Turner’s syndrome in females. FISH analysis using ATM (11q22)/TP53 (17p13.1) probe set presented two copies of each ATM and P53 in each cell line with no detectable deletions, numerical aberrations or translocations at these loci in the 100 interphase cells analyzed.

MicroRNA Biomarkers of Radiation Response

MicroRNA (miR) analysis was performed on the control (2139) and two AT (AT2Bi and AT3Bi) cell lines, to generate miR expression profiles and elucidate the efficacy of miR as a biomarker of radiation response, compared to the cytogenetic and DNA damage biomarkers shown in Figs. 1–3. The 2145 cell line was not considered in the analysis or further studies because it reached senescence in the period during miRNA testing. The Fig. 4A heatmap shows the most highly expressed miR in the cell profiles, which were then further analyzed to determine their increase or decrease in cells deficient in ATM relative to the mean of all cell lines (Fig. 4B and C).

While all three cell lines showed differences in overall miR expression profiles, there were common patterns between the two AT cell lines, which differed from ATM-expressing 2139 cells (Fig. 4A). MiR424-5p presented the most marked differential expression between 2139 and both AT cell lines, with a 3.8-fold decrease in normally responding cells, and a 1.9-fold increase in ATM-deficient cells (Fig. 4B). MiR618 also decreased in normally responding cells by 2.5-fold relative to the mean, while expression increased in both AT cell lines (Fig. 4B). Conversely, miR335-3p increased in ATM-expressing cells by 3.4-fold, with a decrease in both AT cell lines by an average of 1.7-fold relative to the mean (Fig. 4C).

Analysis of Differentially Expressed MicroRNA Reveals Common Gene Targets

A panel of targets for the most differentially expressed miR was compiled through a systematic literature search, with emphasis on genes with roles in DNA damage response and repair. ATM is an integral part of this machinery and it was hypothesized that its deficiency in AT cell lines would be reflected in an increase or decrease in expression of a panel of miR. The mean expression of miR in all three cell lines (2139, AT2Bi and AT3Bi) was calculated and each individual cell line subtracted from the mean. MiR that were consistent in expression between both AT cell lines and different from the control cells were included, with the targets for those miR also detailed. As shown in Table 2, the predominant DNA repair-associated genes identified as targets of miR increased or decreased in AT cells, including the tumor suppressor phosphatase and tensin homolog (PTEN) and the G1/S cell-cycle checkpoint gene cyclin D1 (CCND1). These genes were both found to be directly and indirectly regulated by the miR of interest.

Gene Biomarkers of Radiation Response

Gene expression analysis of PTEN and CCND1 was performed on normal (C1 and 2139) and AT (AT2Bi and AT3Bi) cell lines. RT-PCR was performed on cDNA isolated from all cell lines to investigate the expression of miR target genes were 0, 0.05 and 0.5 Gy irradiated. Fold increase of genes was calculated using the 2^ΔΔCt method, relative to 0 Gy controls, and an expression was recorded over a value of 1 (y-axis). As shown in Fig. 5, the expressions of PTEN (Fig. 5A) and CCND1 (Fig. 5B) were elevated after 0.05 Gy irradiation relative to 0 Gy in 2139 and AT cells. Normally responding C1 cells showed a modest increase in expression of both genes in response to radiation, however, the relative increase did not exceed 2.2-fold (CCND1, 0.5 Gy). The highest increase in PTEN expression was observed in AT3Bi cells, with a 35-fold increase relative to sham-irradiated cells, although this was not significant (Fig. 5A). The largest increase observed in CCND1 expression was seen in 2139 cells, with a 6.4-fold increase over sham-irradiated cells. The AT cell lines

FIG. 2. Control (2139) and AT (AT2Bi and AT3Bi) LCLs received 0 Gy or 0.5 Gy irradiation in G2 chromosomal radiosensitivity assay for (panel A) mitotic index and (panel B) G2 score. Data shown represent three independent experiments. Mean ± SD. **P < 0.01, ***P < 0.005.
showed a more modest increase of 2.6- (AT2Bi) and 4.7-fold (AT3B) (Fig. 5B). However, due to inter-experimental variation, these fold changes were not significant.

### DISCUSSION

Many advances have been made in low-dose-radiation research throughout this decade and through the multidisciplinary European Union DoReMi Consortium (2010–2015), which arose from the original recommendations made by the High-Level Expert Group (HLEG) on low-dose-radiation-risk research (29). In particular, it was recognized that there was an urgent need for biomarkers of low-dose-radiation exposure, individual susceptibility and the effects of radiation damage (early and late), which have since been characterized by members of the consortium (4, 6). Our group was also involved in a part of DoReMi for investigating the use of Raman spectroscopy as a novel tool and biomarker of individual radiation sensitivity. Raman spectra can be generated from patient samples to produce a unique low-dose radiation-induced biochemical profile (57, 58). To validate and consolidate the Raman spectral analysis, the G2 chromosomal radiosensitivity assay was used as a cytogenetic biomarker of radiosensitivity because it was routinely performed in our laboratory for different cohorts of patient lymphocytes and cell lines (35–37). More recently, our group has employed the use of γ-H2AX as a biomarker of DNA damage and individual radiosensitivity because it can yield quantitative results through flow cytometry with parallel qualitative confocal imaging, which is more time-efficient than cytogenetics. Previously reported studies have shown increased γ-H2AX foci increased with increasing radiation dose in lymphoblastoid cell lines (18). Herein we applied both cytogenetic and γ-H2AX biomarkers to assess the radiation sensitivity of normal (C1 and 2139) and clinically characterized AT (AT2Bi and AT3Bi) lymphoblastoid cell lines. Lymphoblastoid cell lines (LCLs) are T lymphocytes immortalized with Epstein-Barr virus and they were selected because parallel studies on whole blood lymphocytes from cohorts of patients were being performed at the same time; therefore, biomarker studies were limited. Although LCLs are not directly comparable to responses recorded in whole blood lymphocytes, they were advantageous for conducting the additional biomarker studies reported within. Similarly, the low doses selected for the experiments were based on the parallel blood studies that were conducted. It was surprising that the G2 chromosomal radiosensitivity scores in the AT cell lines were not significantly elevated compared to the control 2139 cells, although cell cycle checkpoint efficacy observed by mitotic indices (MI) and the calculated radiation-induced mitotic

| Cell line | Karyotype | Comments |
|-----------|-----------|----------|
| 2139      | 45,X[50]  | Loss of a sex chromosome in all cells (Turners syndrome) |
| AT2Bi     | 45,X,−X[9]/46,XX[39] | Monosomy X in 9 out of 50 cells |
| AT3Bi     | 45,XX,add(14)(q32),add(15)(p13)[25] | Addition of material of unknown origin to the long arm of chromosome 14 and to the short arm of chromosome 15 |

**FIG. 3.** G-banding karyotype report on 2139 and AT (AT2Bi and AT3Bi) LCLs reveal loss of sex chromosome X in 2139 cells (bottom left side) and two copies of ATM and TP53 in all cells (bottom right side).
inhibition (RIMI) appeared to be superior in the 2139 cells compared to both AT cells. This would be expected if ATM is functional in the normal 2139 cells, as ATM transduces the radiation-induced DNA damage signal through a serine/threonine phosphorylation cascade. AT2Bi and AT3Bi cells were derived from clinically characterized AT patients, and cellular features of radiosensitivity were previously established through the colony-forming cell survival and chromosomal assays in which both AT cell lines showed similar spontaneous chromosomal aberration rates. However, clinical and cellular heterogeneity was reported between the cell types (30). Given this reported heterogeneity between AT2Bi and AT3Bi, and the unexpected G2 chromosomal radiosensitivity response between the AT cells and 2139, a further cytogenetic analysis incorporating G-banding with karyotyping and fluorescent in situ hybridization (FISH) using a dual ATM/TP53 probe set was performed. FISH was included in the analysis as TP53 is directly signaled by ATM phosphorylation and deletions of TP53 have been previously reported in 17% of B-cell...
leucocytic leukemia (B-CLL) (40). Deletions in ATM in ataxia-telangiectasia patients have been long associated with malignancies such as leukemia and lymphomas (38, 39), and in particular, older AT patients. Since both AT2Bi and AT3Bi were derived from a 36- and 15-year-old AT patient, respectively, the cytogenetic FISH analysis of ATM and TP53 was warranted. Two copies each of ATM and TP53 were detected in the control 2139 and AT cells (AT2Bi and AT3Bi) in the specific cells that were analyzed, and therefore, no specific deletion was detected. There is well-documented evidence of the heterogeneity in AT mutation types which leads to defective ATM (59–61), and a significant proportion are attributed to missense mutations, which would not be detectable at the cytogenetic level and would require molecular characterization. However, given the established presence of both copies of ATM by FISH in all cell lines, knowledge of the mutation type was not required. Nevertheless, the G-banding karyotyping analysis led to a surprising incidental finding in the control 2139 cells. The absence of an X-chromosome was evident and is characteristic of Turner’s syndrome. There are conflicting reports of chromosomal radiosensitivity levels in Turner syndrome cells. In one published study, five patients with the 45,X karyotype compared to nine controls who were X-ray irradiated (200 rads) demonstrated chromosomal aberrations similar to the controls, indicating the X-monosomy does not influence radiation-induced chromosomal aberrations (62). However, another published study demonstrated elevated levels of chromosomal radiosensitivity after receiving 3 Gy irradiation in two comparative Turner’s syndrome variants (45,X complement and 46,XX gonadal dysgenesis) that were compared to age- and sex-matched controls (63). There is limited evidence in the literature to support either hypothesis. In light of this cytogenetic incidental finding, an additional control lymphoblastoid cell line (C1) was later incorporated as an additional control to 2139 where possible.

| Target   | Ref(s) |
|----------|--------|
| FOXO1    | (42)   |
| PSEN1    | (43)   |
| FOXO1    | (44)   |
| TCF7L2   | (45)   |
| PSEN1    | (46)   |
| PI3K/Akt | (47)   |
| PSEN1    | (48)   |
| AGO-2    | (49)   |
| CCND1    | (50, 51)|
| PSEN1    | (52, 53)|
| PSEN1    | (54)   |
| PSEN1    | (55)   |
| CCND1    | (56)   |

The γ-H2AX biomarker was utilized to measure the radiation-induced DNA damage response in all cell lines (C1, 2139, AT2Bi and AT3Bi). ATM phosphorylation of the variant histone H2AX on serine 139 (γ-H2AX) localizes as discrete nuclear foci quantifiable by immunofluorescence of which a one-to-one correlation between radiation-induced DSBs and γ-H2AX foci can be recorded. It has been shown that formation of these foci is the recognition step for the non-homologous end joining (NHEJ) DNA repair pathway (15–17). No significant differences between the cell lines in γ-H2AX positivity were observed. A study on 40 human cell lines representing eight different syndromes to detect a quantitative correlation of cellular radiosensitivity with various biomarkers, including γ-H2AX, reported that the radiation-induced γ-H2AX foci did not predict moderate radiation sensitivities (64). Similarly, γ-H2AX foci in T lymphocytes derived from patients receiving radiotherapy for gynecological cancer did not correlate with late radiotoxicity; however, the same authors reported a linear dose response with gamma radiation for whole blood and isolated T lymphocytes (65). In a recent critical review of the functional assays for individual radiosensitivity, it was determined that γ-H2AX immunofluorescence alone was not sufficient to predict radiosensitive cases and that other cytogenetic biomarkers or cell survival bioassays are too time consuming to predict
radiosensitivity in routine clinical use (66). This calls attention to the need for further molecular biomarkers.

Given the overall poor correlation of radiosensitivity with the cytogenetic and γ-H2AX biomarkers in our lymphoblastoid cell lines, a genetic approach was favored, but with complementarity to the previous chromosome and DNA damage biomarkers, with a focus on the ATM/chk2/p53 pathway with other DNA damage and repair mechanisms. A microRNA (miR) expression panel of 752 miR was performed on the control (2139) and AT (AT2Bi and AT3Bi) cell lines, and a panel of gene targets for the most differentially expressed miR was compiled, with an emphasis on DNA damage response genes to align with our chromosome and DNA damage biomarkers related to the ATM/chk2/p53 signaling pathway. One of the limitations of this study was the reliance of only one control (2139) cell line, which was due to the high cost associated with the microRNA experiment. Upregulated microRNAs of miR-152-3p (42), miR-24-5p (46) and miR-92-15p (48) indicated that PTEN was a potential target. In addition, all downregulated miR indicated both PTEN and CCND1 genes as potential targets. The expressions of both PTEN and CCND1 genes were analyzed in all cell lines and were shown to be upregulated, expressed at the lower radiation dose of 0.05 Gy. Interestingly, the C1 control showed no significant expression of PTEN compared to 2139, AT2Bi and AT3Bi, with a dose-dependent expression profile for CCND1. PTEN negatively regulates the PI3-kinase/Akt pathway and has been associated with radiosensitivity and impaired DSB repair in lung and prostate cancer cells (67, 68). Other published studies have reported that PTEN mutations lead to radioresistant phenotypes in glioblastoma (GBM) (67) with resistance mechanisms mediated by phosphorylation of PTEN on tyrosine 240 (pY240-PTEN), leading to DNA repair through Rad51 (69). CCND1, the regulatory subunit of cyclin-dependent kinases (CDK), phosphorylates and inactivates retinoblastoma (RB) protein to promote cell cycle progression in the G1/S stage, and is directly signaled through the ATM/chk2/p53 pathway. Both potential biomarkers are related to the DNA damage and repair mechanisms induced by ionizing radiation and warrant further investigation and validation with more radiation doses, cell lines or biological models.

CONCLUSION

There was an unexpectedly poor correlation observed between the control 2139 cell line with the AT (AT2Bi and AT3Bi) cell lines using cytogenetic and γ-H2AX biomarkers, most likely due to the underlying cytogenetic abnormality identified in the control 2139 cells. However, this is not withstand the fact that these biomarkers have proved invaluable for other associated studies performed at our institute (35–37, 58). When a genetic approach analyzing miR and their gene targets was taken, a better comparison could be made between the control 2139 and AT cells. This miR analysis indicated potential genetic biomarkers of radiosensitivity and provides mechanistic insights into the low-dose radiation response, particularly for 0.05 Gy. Despite the speed at which molecular work can be conducted with the provision of additional mechanistic information of radiation response, it is important not to overlook the traditional, more time-consuming methods of cytogenetics and cell survival. These assays remain hugely informative and reliable, and they are supported by decades of work in radiation research; conversely, molecular technologies are advancing at a rapid rate with far less validation. When undertaking a molecular study on radiosensitivity biomarkers, we suggest a multi-biomarker approach to include optimized traditional methods, with considerations given to the biological model, dose dependence and scale of the study.

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