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SPANDIDOS PUBLICATIONS

Gene expression profiling of breast cells induced by X-rays and heavy ions

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Abstract. Several genetic aberrations and gene expression changes have been shown to occur when cells are exposed to various types of radiation. The integrity of DNA depends upon several processes that include DNA damage recognition and repair, replication, transcription and cell cycle regulation. Ionizing radiation has many sources, including radon decay from the soil and X-rays from medical practice. Epidemiological evidence indicates a risk for cancer by inducing genetic alterations through DNA damage, and molecular alterations have been reported in epidemiological studies of the A-bomb survivors. A spontaneously immortalized human breast epithelial cell model, MCF-10F, was used to examine the gene expression profiling of breast cells induced by X-ray and heavy ion exposure, by a cDNA expression array of DNA damage and repair genes. This cell line was exposed to 10, 50, 100 and 200 cGy of either X-rays or heavy ions and gene expression profiles were studied. Results indicated that out of a total of 161 genes, 38 were differentially expressed by X-ray treatment and 24 by heavy ion (Fe^{+2}) treatment. Eight genes were common to both treatments and were confirmed by Northern blot analysis: *BRCA1, BIRC2/CIAP1, CENP-E, DDB1, MRE11A, RAD54/ATRX, Wip1* and *XPF/ERCC4*. A number of candidate genes reported here may be useful molecular biomarkers of radiation exposure in breast cells.

Introduction

DNA is vulnerable to a wide variety of agents including exposure to ionizing radiation and heavy ions. Several genetic aberrations and gene expression changes have been shown to occur when cells are exposed to various types of radiation (1). The integrity of DNA depends upon several processes that include DNA damage recognition and repair, replication, transcription and cell cycle regulation (2). Ionizing radiation has many sources, including radon decay from the soil and X-rays from medical practice that induce oxidized bases and breaks in one or both strands of DNA involving several signaling pathway genes (3-6).

Epidemiological evidence indicates a risk for cancer (7) by inducing genetic alterations through DNA damage (8), and the nucleus is the main target for radiation-induced genotoxicity (9). Radiation from high-energy heavy ions (e.g. Fe^{+2} ions) such as those encountered in space, originates mainly from hadrontherapy, atomic explosion and galactic cosmic radiation. Among these, galactic cosmic rays are the main concern for the possible risk of inducing malignancies in astronauts during long-term manned space missions (5-6).

We have reported that X-ray and heavy ion radiation induce changes both at transcriptional and translational level involving multiple cellular pathways at high and low doses of radiation exposure (9,10). Molecular alterations associated with high doses of radiation are available mainly from the epidemiological studies of the A-bomb survivors (11), but the effect of lower doses cannot be detected in epidemiological studies and must be inferred by extrapolation from the high-dose risk. Research programs have developed a scientific basis for risk estimates since the procedure became controversial due to the overestimation of the cancer risks at a low-dose exposure (12). Individuals are constantly exposed to low levels of natural background radiation from cosmic radiation and from naturally occurring radioactive materials in the earth. Certain genes have been found to be altered exclusively by low doses of radiation (13). Consequently, the biological effect of such radiation is a major concern for the general population. Therefore, it is important to assess the risk of short-term exposure to ionizing radiation, originating mainly from X-rays or heavy ions.

Until recently, there were few human cell culture models available for the study of radiation (14,15). To determine the molecular changes associated with the effect of various types of radiation in the breast, the spontaneously immortalized human breast epithelial cell line, MCF-10F, was used in these studies. This cell line has all the morphological characteristics of normal breast epithelial cells (16,17) and it was irradiated with graded doses of X-rays and heavy ions.

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Differential gene expression through cDNA expression array (18) was used to show the expression profiles of many genes, providing clues to the functional role of several genes, including potentially important genes associated with DNA damage and repair (19). Therefore, this study concentrated on identifying the differential expression of early responsive DNA damage and repair genes when cells are exposed to various doses of X-ray and heavy ion radiation.

Materials and methods

Cells. The spontaneously immortalized human breast epithelial cell line MCF-10F (ATCC, Manassas, VA), was used as a control (16,17). It was derived from the mortal human breast epithelial cell line, has a near diploid karyotype, and is of luminal epithelial origin (20). This cell line retains all the characteristics of normal epithelium *in vitro*, including dome formation in confluent cultures, three-dimensional growth in a collagen gel, dependence upon hormones and growth factors, anchorage dependence, non-invasiveness and non-tumorigenicity in nude mice (16,17,20). The experimental cells were used in this study as follows: MCF-10F cells were irradiated with different doses (10-200 cGy) of X-rays and heavy ions (Fe^{+2}). The cells were cultured on Dulbecco's modified Eagle's medium (DMEM)-F12 (1:1) medium supplemented with 10 μg/ml insulin (all from Life Technologies, Grand Island, NY), 5% equine serum (Biofluids Inc., Rockville, MD), 0.5 μg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO) and 0.02 μg/ml epidermal growth factor (Collaborative Research, Bedford, MA) (21). The experiments were performed in a stable population with exponentially growing culture of about 80-90% confluency, and were repeated three times.

X-ray and heavy ion irradiation. MCF-10F cells were plated in 75-cm flasks and incubated at 37˚C in humidified 5% CO_2 with 95% air, in culture medium, and allowed to grow exponentially for 48-72 h until 80-90% confluent. The cells were then exposed to 10, 50, 100 and 200 cGy of 100-kVp X-rays from a Phillips RT-100 at a dose rate of 100 cGy/min in the Medical Department of Brookhaven National Laboratory, and to heavy ion beam (^{56}Fe) of 1 GeV/nucleon at a dose rate of 100 cGy/min at the NASA Space Radiation Laboratory (NSRL) of the same institution. Control and irradiated samples were allowed to grow in 5% CO_2, incubated at 37˚C for 1 h after irradiation.

Flow cytometry. MCF-10F cell lines were harvested 1 h post-irradiation by trypsinization and centrifuged at 200 x g for 10 min. Cell pellets were re-suspended in PBS by vortexing, then fixed by the slow addition of 10 volumes of cold 70% ethanol while vortexing. After at least 24 h at 4˚C, the samples were pelleted by centrifugation, re-suspended by vortexing in a solution of 5 μg/ml propidium iodide/200 μg/ml RNase, and incubated in the dark at room temperature for 15 min. Samples of 30,000 cells each were then analyzed by flow cytometry using a Becton Dickinson FACScalibur instrument to obtain a quantitative measurement of their DNA content. Cell cycle profiles were subsequently determined using ModFit LT software.

Isolation and purification of total RNA and mRNA. Total RNA was isolated from both the control (MCF-10F) and the X-ray- and heavy ion-treated cells with TRIzol reagent (Invitrogen Corp., Long Island, NY). Each sample comprising 500 μg of total RNA was treated with 5 μl of DNAse I (10 U/μl) (Roche Pharmaceuticals, Indianapolis, IN) for 60 min at 37˚C. Then 10X Termination Mix [0.1 M EDTA, pH 8.0 and 1 mg/ml glycogen (Clontech, Palo Alto, CA)] was used to stop the reaction. Each sample was then purified following an established procedure (22). The amount of each purified RNA sample was first measured by a spectrophotometer (required ratio of absorbance reading at 260/280 nm, >1.8) and then electrophorsed on denaturing formaldehyde/agarose/ethidium bromide gel, to check its quality and purity from proteins and free nucleotides. Each sample of 500 μg of purified total RNA was then subjected to $polyA^+$ RNA analysis with the Oligotex mRNA Purification Kit (Qiagen Inc., Valencia, CA). $PolyA^+$ RNA was then purified following an established procedure (22).

cDNA expression array. GE Array Q Series Human DNA Damage and Repair Signaling Pathway cDNA expression array membranes were used in these studies (SuperArray, Bethesda, MD). These arrays are designed to profile the gene expression of a panel of 161 key genes, including 36 genes associated with both DNA damage and repair, 120 genes exclusively for DNA damage and repair (60 each) and 5 standard control genes involved in the DNA damage and repair signaling pathways (23-25). All the DNA damage signaling pathway genes were associated with the *ATR/ATM* signaling network and transcriptional targets of DNA damage responses which include cell cycle arrest, apoptosis, genome stability and pathways. Genes associated directly with DNA repair were linked to direct reversal of damage, base excision repair, nucleotide excision repair, mismatch excision repair, double-strand break repair (homologous recombination and end joining), and rad6-dependent and other genome stability related genes. Each of these genes was amplified by polymerase chain reaction (PCR) with gene-specific primers to generate 200-600 bp products. PCR product (~100 ng of each) was spotted in quadruplicate onto a positively charged membrane. Each GE Array Q series membrane was spotted with a negative control of pUC18 DNA, blanks and housekeeping genes, including *ß-actin*, *GAPDH*, *cyclophilin A* and *ribosomal protein L13A*.

Synthesis of cDNA probes from mRNA. The purified mRNAs were used for the synthesis of cDNA probes with Biotin-16-dUTP (Roche). An annealing mixture was prepared by mixing about 1.0-5.0 μg mRNA with 3 μl buffer A (GE primer mix; SuperArray) and the final volume was adjusted to 10 μl. The mixture was then incubated in a preheated thermal cycler at 70˚C for 3 min. It was then cooled to 42˚C and kept at that temperature for 2 min. Then 10 μl of RT cocktail was prepared by mixing 4 μl of 5X buffer BN [50 μl 10X buffer, with addition of 1 μl 1M DTT and 50 μl 10X dNTP mix (5 mM dATP, dCTP, dGTP and 500 μM dTTP)], 2 μl Biotin-16-UTP, 2 μl RNase free H_2O, 1 μl RNase inhibitor (Promega Corp., Madison, WI) and 1 μl MMLV Reverse Transcriptase (Promega). The RT cocktail was then

at 42˚C for 1 min and slowly mixed with 10 μl of ed annealing mixture. The incubation was continued at 42˚C for 90 min and then labeled cDNA probe was denatured by heating at 94˚C for 5 min, and quickly chilled on ice. In each cell line tested, mRNA was isolated and purified from different passages, and cDNA probes were prepared from each and hybridized to the respective membranes. Experiments using the same mRNA preparation were repeated three times, and measurable, median-normalized expression values of each gene were compared to avoid false positive signals (26).

Differential hybridization of cDNA expression array. Each array membrane was pre-wetted with 5 ml of de-ionized water and incubated at 60˚C for 5 min. It was then replaced with 2 ml of pre-warmed (60˚C) GEAprehyb solution (GEAhyb solution with a heat-denatured sheared salmon sperm DNA at a final concentration of 100 μg/ml; SuperArray) and mixed gently for a few seconds. Pre-hybridization was continued at 60˚C for 1-2 h with continuous gentle agitation. About 0.75 ml solution of GEAhyb was prepared by adding the entire volume of denatured cDNA probe into the GEAprehyb solution and maintaining it at 60˚C. Then the GEAprehyb solution was replaced by the GEAhyb solution and hybridization was continued overnight at 60˚C with continuous gentle agitation. Subsequently, array membranes were washed twice in wash solution 1 (2X sodium chloride sodium citrate and 1% sodium dodecyl sulfate) at 60˚C for 15 min each with gentle agitation and then twice with solution 2 (0.1X sodium chloride sodium citrate and 0.5% sodium dodecyl sulfate) at 60˚C for 15 min each with gentle agitation. To assess the reproducibility of the hybridization array assays, pair-wise comparisons between array data sets for each cell line were tested by repeated hybridization and the mRNAs prepared in different lots were analyzed in scatter plots with multiple regression as previously (26). In each case, the expression levels of 95% of the genes had repeated values that were within 2-fold (26).

Chemiluminescence detection of cDNA probes. After discarding the last wash, 2 ml of GEAblocking solution was added to each membrane and incubated for 40 min at room temperature with continuous agitation. Then binding buffer was prepared by diluting alkaline phosphatase-conjugated streptavidin (AP) with 1X buffer F (SuperArray) in a 1:7500 dilution. GEAblocking solution was replaced by 2 ml of binding buffer and incubated for 10 min with continuous but gentle agitation. Each membrane was washed 4 times with 4 ml 1X binding buffer F for 5 min per wash and rinsed twice with 3 ml of rinsing buffer G (SuperArray). The membranes were covered with 1.0 ml of CDP-Star chemiluminescent substrate and incubated at room temperature for 2-5 min. They were then exposed to X-ray film (Kodak BioMax MS Film; Kodak Corp., Rochester, NY) with a corresponding intensifying screen at room temperature, for multiple exposures of 1-5 min.

Quantification of array hybridization. Quantification of hybridization signals on the expression array membranes was carried out by exposing the autoradiographic film in a densitometric scanner (Model 300A; Molecular Dynamics, Sunnyvale, CA). It was then estimated with both the ImageQuant (Molecular Dynamics) and ScanAlyze programs (Eisen Lab). Volume quantification was performed by calculating the volume under the surface created by a three-dimensional plot of pixel locations and pixel values as described (26). All raw signal intensities were corrected for background by subtracting the signal intensity of a negative control or blank. Results were also normalized to that of a housekeeping gene. These corrected, normalized signals could then be used to estimate the relative abundance of particular transcripts. To delineate the potential signal interference between adjacent strong hybridization signals, equal-sized ellipses were drawn around each signal area (hybridization spots) using software (ImageQuant/ScanAlyze) and these were then separately scanned and compared with housekeeping genes so that chances of interference between adjacent strong hybridization signals were minimized. Normalization of the expression levels of different housekeeping genes from multiple autoradiographic exposures between different hybridization experiments was done by taking the average signals of each of the housekeeping genes. Data from high intensity spots were chosen for further use. Median background was subtracted, and signals that were <2.0-fold above background level were considered too low to accurately measure and were omitted from the analysis. Signals for each individual gene were also normalized to the geometric mean of the expression level of that gene across the set of membranes being compared. Mean signals were calculated from quadruplicate measurable spots, or if three of the four spots were measurable. For gene-specific reverse transcription-polymerase chain reaction (RT-PCR) and labeling of gene-specific probes, PCR primers were used (Operon Biotech. Inc., Germantown, MD) to amplify the eight randomly selected genes and human *ß-Actin* (Clontech) as a control amplifier set. Table I shows the base-pair length of the amplified cDNA of the eight genes under study and the sequence of sense and antisense primers used to amplify those cDNAs. To confirm the differential expression of the genes under study, gene-specific probes were generated by gene-specific RT-PCR technique (26). About 0.5-1 μg of purified total RNA was used for first-strand cDNA synthesis with The Reaction Ready First Strand cDNA Synthesis Kit (SuperArray) with oligo (dT)18 and random hexamer primers to reverse transcribe the entire population of RNA in an unbiased manner. Different amounts of cDNAs and varied number of PCR cycles were used to generate gene-specific probes. A linear increase was observed in product generation in all the cases. Based on the findings of these experiments, 100 ng of cDNA was used with 30 cycles of PCR for amplification of the eight genes by RT-PCR with an initial denaturation at 95˚C for 15 min followed by 30 cycles, each cycle comprising denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec with a 5-min final extension at 72˚C. The 15-min step at 95˚C was required to activate the HotStart Taq DNA polymerase (SuperArray).

Northern blot analysis. About 500 μg of total RNA was treated with 5 μl of DNAse I (10 units/μl; Roche) for 60 min at 37˚C. The RNA was then extracted and precipitated using 7.5 M ammonium acetate, pH 5.2 (24). A sample of 0.5-1 μg of total RNA was then used for first-strand cDNA synthesis

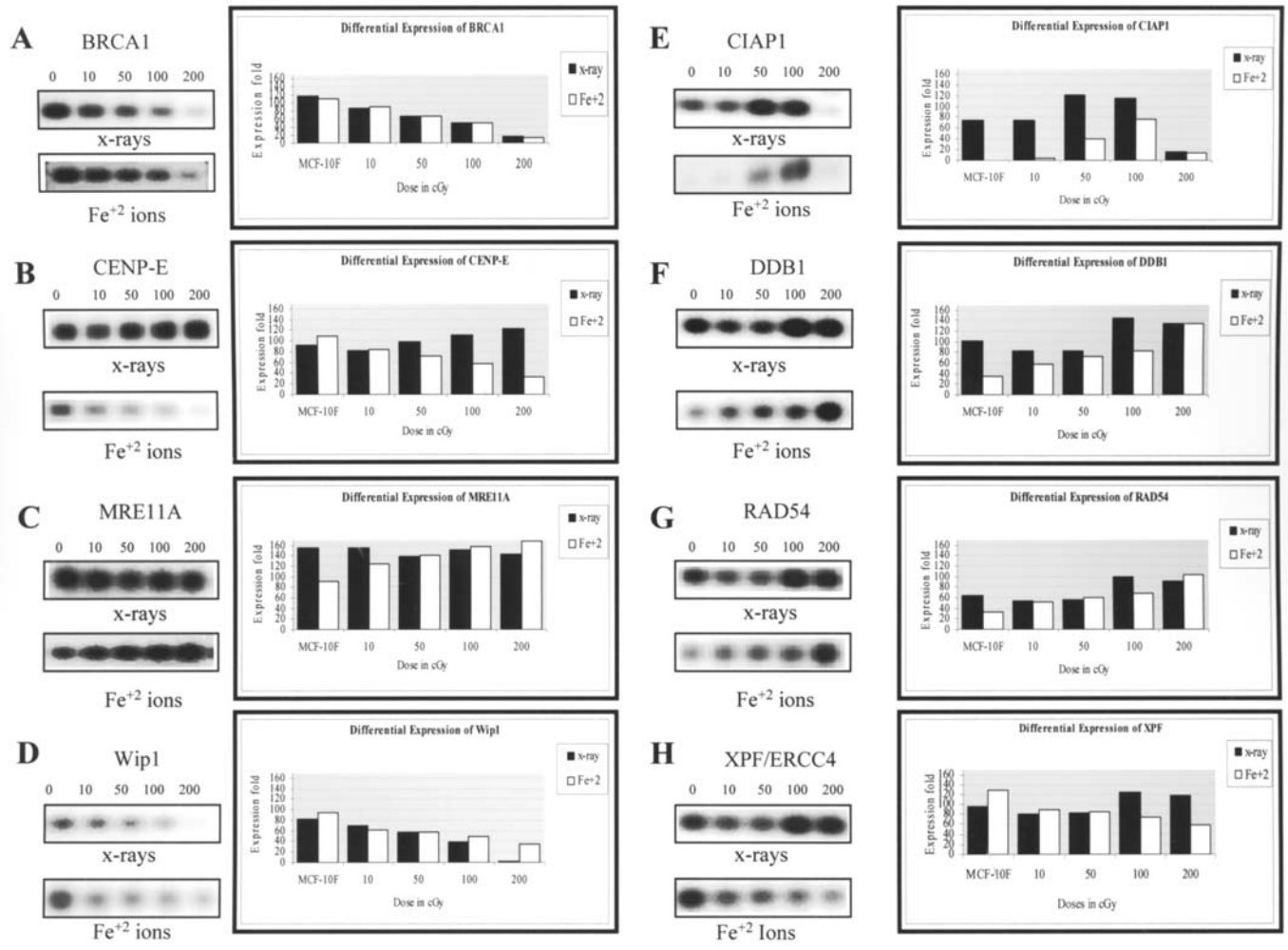

Figure 1. Gene expression studies on X-ray and Fe^{+2} ion treatment with doses of 0, 10, 50, 100 and 200 cGy on the MCF-10F cell line. Quantitative estimation and gene-specific RT-PCR analysis of the amplified fragments of eight genes (*BRCA1*, *CENP-E*, *MRE11A*, *Wip1*, *CIAP1*, *DDB1*, *RAD54*, *XPF/ERCC4*) identified by differential hybridization of DNA damage/repair cDNA expression array. In each case, *ß-actin* was used as a housekeeping control gene (not shown).

by using the Advantage® RT-for-PCR Kit (Clontech) using oligo (dT)18 and random hexamer primers. Approximately 100 ng of the first-strand cDNA synthesis product was used to perform RT-PCR reactions using gene-specific primers as mentioned above. The PCR-amplified products were then labeled using respective primers and Biotin-16-UTP as well as the RT cocktail, as before, to generate the probes, and were then used for Northern hybridization analysis. In addition, 1 μg of mRNA was electrophoresed in a 1% (w/v) agarose-formaldehyde gel and transferred to a nylon membrane (Hybond-N, Amersham-Pharmacia Biotech, Piscataway, NJ). RNA transfer was confirmed by visualization of ethidium bromide-stained RNA under UV light. The blots were UV cross-linked and stored at 4˚C until hybridization. Human ß-actin control amplifier set probe was also used in Northern hybridization to confirm similar expression in all samples. The blot was then exposed to Kodak X-OMAT AR film at -80˚C for 24 h. The intensity was assessed by densitometric scanning (Molecular Dynamics) (26).

Results

A human DNA damage and repair signaling pathway cDNA expression array designed to profile the gene expression of a panel of 161 key genes was used in the present study. The aim of this study was to identify the differential expression of responsive genes in MCF-10F cells exposed to 10, 50, 100 and 200 cGy of both X-rays and heavy ions, after 1 h of irradiation. The genes present in the array were associated with DNA damage and repair signaling, the DNA damage pathway and the DNA repair pathway. Five genes were used as standard controls. All the DNA damage signaling pathway genes found in these arrays were associated with the *ATR/ATM* signaling network and transcriptional targets of DNA damage responses which include cell cycle arrest, apoptosis and genome stability and repair pathways; these genes are associated directly with DNA repair and linked to direct reversal of damage, base excision repair, nucleotide excision repair, mismatch excision repair, double-strand break repair (homologous recombination and end joining), and rad6-dependent and other genome stability related genes.

Of the 161 genes found in the array, 46 were altered by X-rays and heavy ion exposure (30 and 16 respectively). Furthermore, another eight genes were common to both treatments, four were associated with both DNA damage and repair and four were involved exclusively with DNA damage. Examination of the gene expression profiling of cells exposed to X-rays and heavy ions showed that there

Primers of eight differentially expressed genes and control *β-actin* selected for gene-specific RT-PCR analysis.

Gene symbol	GeneBank accession no. /UniGene no.	[a]PCR product (base pairs) (*Homo sapiens*)	Locus	Primer Sequence (5' to 3' direction) 1, forward; 1', reverse	Important functions of the gene
BRCA1	L18209	222	17q21	1-GTACCTTGATTTCGTATTC 1'-GACTCTACTACCTTTACCC	Tumor suppression
BIRC2/ CIAP 1	Hs.503704	144	11q22	1-AAGCACCAAAGACAATTCGG 1'-GCTTGTTATGCATCATTTCAGG	Apoptosis inhibition
CENP-E	G19562	144	4q24-q25	1-AACAGGAGAGGGGATTTAAAGG 1'-GGTGGAGGATGACGTTCG	Chromosome movement and spindle elongation
DDB1	Hs.290758	267	11q12-q13	1-ACTCAGAAACTAACAATTCA 1'-TTATTTAGATTGGCAGTGTA	Nucleotide-excision repair mechanism
MRE11A	G22564	102	11q21	1-CTTGTCAGGATACTTTAGTGACCA 1'-AGCTGTGGGCCACATCAG	Homologous recombination maintenance of telomere length and DSB repair
RAD54/ ATRX	Hs.533526	121	Xq13.1-q21.1	1-GTCTAGCTGCAAACACCAAGG 1'-TCACTTAACAGGTGTGGGCA	Chromatin remodeling associated with α-thalassemia syndrome
Wip1	Hs.286073	138	17q23.2	1-AGCAATCTTCCAGATGTCTGG 1'-ACCTGGAAGTTAAAAGCATTGA	Negatively regulates p38 MAP kinase activity in a p53-dependent manner
XPF/ ERCC4	Hs.460019	187	16p13.3-p13.11	1-GAACATCGCAGAATTAGCAGC 1'-GAGCCGCTGAAAAGTACAGG	Involved in nucleotide excision repair
ACTB	Hs.288061	125	Multiple loci 2q21.1	1-AAAGACCTGTACGCCAACA 1'-GGAGCAATGATCTTGATCTTC	ß-actin

[a]Length of cDNA product amplified by gene-specific RT-PCR analysis.

were eight genes common to both types of radiation. Primers for these genes (Table I) were selected for validation by gene-specific RT-PCR analysis as seen in Fig. 1A-H. Profiling of genes associated with DNA damage and repair induced by X-rays (Table II) and heavy ions (Fe^{+2}) (Table III) was performed.

Among these eight genes, the tumor suppression *Breast Cancer 1* (*BRCA1*), early onset, gene was down-regulated by X-rays from 2.4 with 50 cGy, to 2.0 with 100 cGy, and disappeared with 200 cGy as seen in Table II; it was also down-regulated by heavy ions from 2.9 with 10 cGy, to 2.3 with 50 cGy, and 2.0 with 100 cGy, as seen in Table III. This effect was corroborated by molecular studies as seen in Fig. 1A, that show that both types of radiation decreased seven times in comparison to the control MCF-10F cells. The *centromere protein E* (*CENP-E*) was up-regulated by X-ray exposure from 2.9-fold with 50 cGy, to 4.8 with 100 cGy (Table II) and corroborated as seen in Fig. 1B. However, there was down-regulation by heavy ions from 3.0 to 2.1 with 10 and 50 cGy, respectively (Table III), and this was corroborated by molecular studies (Fig. 1B).

The DNA repair protein *MRE11A* gene expression was down-regulated by X-rays from 4.4 to 3.2 and 2.8 with 50, 100 and 200 cGy, respectively (Table II). There were not significant differences in gene validation (Fig. 1C). There was down-regulation by the effect of heavy ions from 5.0 with 10 cGy, to 4.2 with 50 cGy, to 2.3 with 100 cGy, and 2.0 with 200 cGy (Table III). Results were not corroborated (Fig. 1C). The Mg-dependent *1Wip1* expression was down regulated by X-ray radiation from 2.6 to 2.1 with 50 and 100 cGy respectively (Table II), and by heavy ions from 4.0 to 3.8, 3.4 and 2.2 with 10, 50, 100 and 200 cGy, respectively (Table III). These results were corroborated with both types of radiation (Fig. 1D).

The *BIRC2/CIAP1* gene was up-regulated by X-rays from 4.4 to 4.8, with 50 and 100 cGy respectively (Table II) and by heavy ions from 2.2 to 2.8, with 10 and 50 cGy respectively (Table III). Results were corroborated for both types of radiation (Fig. 1E). The *damaged DNA-binding protein 1* (*DDB1*) gene was up-regulated by X-rays from 2.4 with 50 cGy, to 2.9 with 100 cGy, and 3.1 with 200 cGy (Table II); these results were corroborated by molecular studies (Fig. 1F). However, there was up-regulation by heavy ions from 5.4 with 10 cGy, to 3.7 with 50, to 2.6 with 100, and 2.1 with 200 cGy (Table III). Molecular studies did not corroborate these results (Fig. 1F).

The *Rad54/ATRX* gene showed no difference in expression with X-ray exposure, with all the doses used. However, there was an up-regulation by heavy ions from 2.1 to 2.9 with 10 and 50 cGy, respectively (Table III). Results were not corro-

Table II. Profiling of genes associated with DNA damage and repair induced by X-rays.

Gene name	10 cGy	50 cGy	100 cGy	200 cGy
Tumor necrosis factor (*TNFA*)	2.185	2.678		
Ubiquitin-conj enzyme E2A (*RAD6A*)	6.086	4.163		
Excision repair cross comp. (*XPF/ERCC4*)	2.050	2.144	2.645	
Exostoses (multiple) 1 (*EXT1*)	5.401	3.324	5.847	
Protein Kinase, DNA-activated (*DNA-PK*)	2.102	2.525	3.101	2.219
X-ray repair complem defective (*XRCC3*)	2.276	3.532	2.419	2.634
DUTP pyrophosphatase (*DUT*)	2.150	2.530	2.662	2.036
Postmeiotic segregation increase-2 (*PMS6*)	2.687	3.409	2.276	5.265
RecQ protein-like 5 (*RECQL5*)	2.183	2.725	2.045	2.595
Replication protein A1 (*RPA1*), 70 kDa	2.300	3.174	2.556	3.594
Replication protein A2 (*RPA2*), 32 kDa	2.404	2.597	3.614	3.516
X-ray repair complem defective (*KU80*)	3.119	3.025	3.289	3.472
BCL2-antagonist/killer 1 (*Bak*)		2.527	3.024	
BCL2-associated X protein (*Bax*)		2.273	3.003	
B-cell CLL/lymphoma 6 (*Bcl-6*)		2.856	2.162	
Baculoviral IAP repeat-contain 2 (*CIAP1*)		4.404	4.891	
CDP-diacylglycerol synthase (*CDS1*)		3.814	2.044	
Centromere protein E (*CENP-E*), 312 kDa		2.878	4.807	
Leucine-rich and death domain contain (*PIDD*)		2.305	2.284	
Protein phosphatase 1D, Mg dependent (*Wip1*)		2.599	2.113	
APEX endonuclease 2 (*APEXL2*)		2.162	2.322	
Ataxia telangiectasia mutated (*ATM*)		2.801	2.378	
Breast cancer 1 (*BRCA1*), early onset		2.454	2.098	
Prostate cancer antigen-1 (*DEPC-1*)		2.506	2.880	
MutS homolog 6 (*MSH6*), (*E. coli*)		2.087	2.447	
RAD23 homolog A (*HHR23A*), (*S. cerevisiae*)		2.210	2.017	
Cyclin-dependent kinase 4 (*Cdk4*)		4.421	5.409	2.323
RAD51 homolog (*RAD51*)		2.010	2.106	2.447
Replication protein A3 (*RPA3*), 14 kDa		2.202	2.103	2.072
ADP-ribosyltransferase (*ADPRTL2*)		2.431	2.442	2.473
Damage-sp DNA bind protein 1 (*DDB1*), 127 kDa		2.429	2.950	3.151
DNA glycosylase hFPG2 (*FLJ10858*)		2.672	2.455	2.302
Gen transcription factor IIH (*GTF2H1*)		2.366	3.126	2.744
MRE11 meiotic recobtn. 11 homolog (*MRE11A*)		4.386	3.197	2.795
Post meiotic segr increased 2 (*PMS2L9*) (*PMS2L3*)		3.805	4.276	5.265
Ubiquitin-conj enzyme E2 (*MMS2*)		2.693	2.541	2.427
X-ray repair complem defective (*XRCC4*)		2.485	2.184	2.070
α-thalassemia, mental retdn synd (*RAD54*)			2.103	2.097

borated for both types of radiation (Fig. 1G). The *excision repair cross complementing protein* (*XPF/ERCC4*) gene was up-regulated by X-rays from 2.0 with 10 cGy, to 2.1 with 50 cGy, and to 2.6 with 100 cGy (Table II); this was corroborated as seen in Fig. 1H. However, it showed no difference in expression with heavy ion treatment at any of the doses used (Table III). There was a down-regulation with every dose of heavy ions; however, there was not significant difference.

Cell-cycle analysis for X-ray and heavy ion-treated MCF-10F cell lines was performed to determine the cell-cycle distribution of the culture during gene expression study (Table IV). The G_1-, G_2- and S-phase cell populations after 1 h of irradiation, showed no significant changes in the cell-cycle distribution at different stages of this study. Thus 34-38% of the cells were in S phase after 1 h of irradiation with 0-200 cGy of both X-rays and heavy ions, indicating the uniformity or homogeneous nature of the cell culture used.

Discussion

Various molecular biological techniques have been used to identify the genetic changes involved in the effects of radiation on breast cells (8,10). Among these, expression array technology has become an important tool for the

Profiling of genes associated with DNA damage and repair induced by Fe^{+2} ions.

Gene name	10 cGy	50 cGy	100 cGy	200 cGy
Baculoviral IAP repeat-contain 2 (*CIAP1*)	2.214	2.879		
α-thalassemia, mental retdn synd (*RAD54*)	2.196	2.931		
Centromere protein E (*CENP-E*), 312 kDa	3.000	2.123		
Breast cancer 1 (*BRCA1*), early onset	2.905	2.362	2.006	
PMS2 postmeiootic seg increased 2 (*PMS2*)	3.504	2.926	2.108	
Protein tyr phosphatase (*LPAP*)	4.008	3.530	2.258	
Purine-rich element bind protein A (*PURA*)	3.831	3.556	2.582	
RAD17 homolog (*RAD17*) (*S. pombe*)	8.016	2.348	7.025	
CHK1 checkpt homolog (*S. pombe*) (*Chk1*)	4.407	2.973	2.866	2.435
Damage-sp DNA bind protein 1 (*DDB1*)	5.473	3.693	2.657	2.167
Excision repair cross comp. (*XPF/ERCC4*)	2.070	2.158	2.188	2.195
Legumain (*LGMN*)	6.872	7.171	7.670	3.736
MRE11 meiotic recobtn. 11 homolog (*MRE11A*)	5.071	4.225	2.376	2.081
Protein phosphatase 1D, Mg dependent (*Wip1*)	4.095	3.827	3.419	2.285
MutL homolog 3 (*MLH3*), (*E. coli*)	2.506	2.403	2.245	2.287
MutS homolog 3 (*MSH3*), (*E. coli*)	5.598	4.673	4.242	2.716
RAD51-like 1 (*RAD51B*), (*S. cerevisiae*)		3.091	2.826	2.025
RAD54-like 1 (*RAD54L*), (*S. cerevisiae*)		4.656	4.271	4.863
Telomeric repeat binding factor (*TERF-1*)		3.369	4.021	4.328
Topoisomerase (DNA) III beta (*TOP3B*)		2.077	3.896	5.473
X-ray repair complem defective (*XRCC1*)		4.461	4.021	3.790
RAD51-like 3 (*RAD51D*), (*S. cerevisiae*)			2.010	2.619
Apoptotic protease act factor (*Apaf-1*)			2.001	2.048
Proliferating cell nuclear antigen (*PCNA*)			2.025	3.550

Table IV. Cell cycle studies on X-ray and Fe^{+2} ion treatment with different dose exposures, on the MCF-10F cell line.

	Ion source											
	X-rays						Fe^{+2} ions					
Dose (cGy)	Diploid (%)	G_1 (%)	G_2 (%)	S (%)	G_2/G_1	% CV	Diploid (%)	G_1 (%)	G_2 (%)	S (%)	G_2/G_1	% CV
0	100.00	76.76	6.67	16.58	2.10	11.53	100.00	42.93	21.79	35.28	1.92	6.10
10	100.00	62.25	15.18	22.57	1.85	6.88	100.00	42.79	19.44	37.77	1.91	6.14
50	100.00	77.77	13.40	8.82	1.90	19.55	100.00	40.63	24.90	34.47	1.96	6.11
100	100.00	64.25	12.70	23.05	1.85	8.68	100.00	42.67	23.92	33.41	1.97	6.02
200	100.00	69.26	12.52	18.22	2.10	16.82	100.00	39.43	22.12	38.45	1.95	5.97

identification of differentially expressed genes in complex regulatory pathways (27). These pathways may result in cell cycle arrest, apoptosis and DNA damage and repair. Therefore, to obtain more precise information, the present study analyzed radiation-induced DNA damage and repair genes with respect to low doses of X-ray and heavy ion treatment.

Out of a total of 161 genes, 30 were altered by X-ray exposure and 16 were altered by heavy ion exposure. Of these, eight genes were common to both treatments, four were associated with both DNA damage and repair and four were involved exclusively with DNA damage. Among these eight genes, *BRCA1*, located in chromosome 17 was down-regulated with 50-200 cGy of X-rays and from 10 to 100 cGy of heavy ion radiation. This gene is phosphorylated as a response to various DNA damaging agents by kinases, such as *CHEK2*, *ATM* and *ATR*, which results in changes in its protein-protein interactions and expression of various target genes (28-30). It is known that this gene encodes a large nuclear protein (220 kDa) that is involved in DNA damage

signaling, DNA repair, growth inhibition and transcriptional regulation (28). The *ataxia telangiectasia mutated*, *ATM* gene was down-regulated from 2.8 with 50 cGy, to 2.4 with 100 cGy, and disappeared with 200 cGy X-ray exposure. However, heavy ion exposure did not have any effect on this gene.

The *CENP-E* gene, located in chromosome 4 was up-regulated from 50 to 100 cGy of X-ray exposure and down-regulated by heavy ion exposure. It is a kinesin-like motor protein localized on the kinetochore of the chromosome. This gene is required for the efficient capture and attachment of spindle microtubules by kinetochores, a necessary step in chromosome alignment during pro-metaphase. Any functional disruption of *CENP-E* results in the appearance of unaligned chromosomes at metaphase (31,32).

The *Mre11A* gene, located in chromosome 11, showed down-regulation from 50 to 200 cGy of X-ray radiation and down-regulation from 10 to 200 cGy of heavy ion radiation. These changes may indicate mutations in the genes that encode components of this complex resulting in DNA damage sensitivity, and genomic instability. The *Mre11A*, *Nbs1* and *Rad50* repair proteins are closely related and form a complex (M-N-R complex) that is essential in maintaining DNA integrity by functioning in double-strand break repair and telomere maintenance (33,34). Previously, abnormal activity of this complex was thought to be related to DNA repair deficiency. However, recent studies revealed that it has a more specific role in checkpoint signaling and DNA replication (33). It has been shown that *BRCA1* is also important for the cellular responses to DNA damage that are mediated by this complex (33). Others have found similar cellular responses to DNA damage by ionizing radiation, mediated by the *hRad50-hMre11-p95 complex* and down-regulation of *BRCA1* (30) that corroborated with the present finding of the downward regulation of expression of this gene at different doses of X-ray and heavy ion radiation.

Another important alteration found in this study is the down-regulation of the *Wip1* gene, located in chromosome 17 by X-ray exposure at doses of 50-100 cGy and down-regulation by heavy ion exposure at doses of 10-200 cGy. It has been shown to be homologous with type 2C protein phosphatases and to be induced by ionizing radiation (35). The p53-induced oncogenic *Wip1* also interacts with uracil DNA glycosylase and suppresses *base excision repair* (*BER*) (36). However, inactivation of Wip1 phosphatase may inhibit mammary tumorigenesis through *p38 MAPK*-mediated activation of the $p^{16Ink4a}$-$p19^{Arf}$ pathway (37,38).

Differential expression of *BIRC2/CIAP1* gene, located in chromosome 11 indicated up-regulation at doses from 50 to 100 cGy of X-rays and at the same doses of heavy ions. The present study indicated that *BIRC2/CIAP1* induced changes in the apoptosis process. This gene belongs to a family of highly conserved anti-apoptotic proteins first identified in baculovirus and later in eukaryotic species from yeast to mammals (39,40). It has been reported that *BIRC2/CIAP1* is an inhibitor of apoptosis and overexpressed through 11q22 amplification in cell lines derived from esophageal squamous cell carcinomas (41). It has also been associated with the resistance of these cell lines to drug-induced apoptosis (41). In esophageal radio-resistant cancer cell lines, this gene is up-regulated during radiation therapy, which may provide a new insight into the mechanisms of radio resistance and effective radiation therapy (42).

The *DDB1* gene, located in chromosome 11 was up-regulated at 50- to 200-cGy doses of X-rays and at the same doses of heavy ions. The function of *DDB1* in damaged-DNA recognition is not well understood. It is believed to be involved in DNA repair, and it has been linked to the repair deficiency disease xeroderma pigmentosum (43). It has been postulated that the repair-protein function of *DDBs* may be associated with the alteration of chromatin conformation to enhance repair at the damaged sites (44). It also exhibits transcriptional activity by binding at the activation domain of *E2F1* (45).

The *Rad54/ATRX* is a chromatin remodeling gene associated with α-thalasemia, mental retardation syndrome. It showed no difference in expression with X-ray and heavy ion exposure with all the doses used. It has been reported to have a specific role in both DNA recombination and repair mechanisms. It has been mapped to chromosome 1p32 in a region of frequent loss of heterozygosity in breast tumors (33,46). Mutations of this gene have also been found in various human disorders (47). *XPF/ERCC4*, a gene involved in nucleotide excision repair was also found to be up-regulated by X-ray exposure at doses of 10-100 cGy. However, it showed no difference in expression with heavy ions, with any dose used. This protein is mainly responsible for removing UV-C photoproducts and bulky adducts from DNA (48). It is assigned to chromosome 16p13.3-p13.11 and it is implicated in complementation group F of the human disorder, xeroderma pigmentosum.

Molecular biomarkers for clinical radio-resistance have been considered by several authors, who analyzed the consecutive mRNA expression of DNA repair-related genes by conducting a pilot study in prostate cancer patients receiving radiotherapy (49). Others studied low-dose irradiation and found that the transcript profiles of human lymphoblastoid cells were altered, including genes associated with cytogenetic radioadaptive response (50). Wand *et al* (51) identified differentially transcribed genes in human lymphoblastoid cells irradiated with 0.5 Gy of γ-ray and the involvement of the low dose radiation inducible *CHD6* gene in cell proliferation and radiosensitivity. However, others indicated that lymphoblastoid cell lines, differing in p53 status showed clear differences in basal gene expression but minor changes after irradiation (52). On the other hand, others reported a genomic as a window on radiation stress signalling (53).

In conclusion, the gene expression altered by X-ray and heavy ion exposure found in these studies was associated with DNA damage. Therefore, these genes could be useful as broad-spectrum biomarkers to detect the effects of a variety of changes induced by radiation in breast cells.

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