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Transcriptional changes in U343 MG-a glioblastoma cell line exposed to ionizing radiation

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Glioblastoma multiforme (GBM) is a highly invasive and radioresistant brain tumor. Aiming to study how glioma cells respond to γ-rays in terms of biological processes involved in cellular responses, we performed experiments at cellular context and gene expression analysis in U343-MG-a GBM cells irradiated with 1 Gy and collected at 6 h post-irradiation. The survival rate was approximately 61% for 1 Gy and was completely reduced at 16 Gy. By performing the microarray technique, 859 cDNA clones were analyzed. The Significance Analysis of Microarray algorithm indicated 196 significant expressed genes (false discovery rate (FDR) = 0.42%): 67 down-regulated and 97 up-regulated genes, which belong to several classes: metabolism, adhesion/cytoskeleton, signal transduction, cell cycle/apoptosis, membrane transport, DNA repair/DNA damage signaling, transcription factor, intracellular signaling, and RNA processing. Differential expression patterns of five selected genes (*HSPA9B*, *INPP5A*, *PIP5K1A*, *FANCG*, and *TPP2*) observed by the microarray analysis were further confirmed by the quantitative real time RT-PCR method, which demonstrated an up-regulation status of those genes. These results indicate a broad spectrum of biological processes (which may reflect the radio-resistance of U343 cells) that were altered in irradiated glioma cells, so as to guarantee cell survival.

Key words: gene expression profiles; glioma cells; ionizing radiation; stress response

Introduction

Glioblastoma multiforme (GBM) is the most common primary tumor affecting the central nervous system in adults. Survival rates for patients with the most aggressive form of GBM range from 9 to 12 months. The classic GBM treatment is surgery to maximal tumor resection; however, total tumor resection is almost impossible due to its infiltrative nature. Therefore, surgery is followed by radiotherapy (total of 60 Gy over 6 weeks), which clearly improves survival rates. Unfortunately, in almost 100% of cases, some radioresistant cells foci persist at primary sites and retake the tumor growth, with a poor prognosis for patients.[1–3] In the last 20 years, chemotherapy has been used in combination with surgery and radiation therapy, predominantly in patients with intermediate-grade gliomas; although this treatment combination produces palliative effects, it does not promote cancer cure.[1] Nowadays, the temozolomide, an alkylating agent which is effective to overcome the blood–brain barrier, represents the successful cytotoxic drug in current use for glioma treatment. The most promising therapeutic strategy to GBM treatment consists in surgery followed by radiotherapy in combination with low-dose daily temozolomide and at least 6 months of adjuvant temozolomide.[4]

Advances in the cDNA microarray methodology allow studying the expression of a large number of genes and, simultaneously, can also provide relevant information for the elucidation of molecular events underlying cellular responses to ionizing radiation (IR). This approach has been used to monitor radiation-responsive genes in different cell types.[5–9] Studies carried out at different conditions and in different cell types indicated that cellular responses to IR consist of an integrated network of

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protein signaling and transcriptionally regulated pathways that differ among cell lines presenting different genetic background.

It is expected that studies on gene expression at large scale may contribute to clarify the mechanism underlying cellular resistance to radiotherapy. In fact, reports about modulation of gene expression in response to IR in tumor cell lines, such as leukemia,[10] lymphoma,[11] cervical[12] and lung cancer[13] have indicated a list of radiation-responsive genes, but quantitative and qualitative differences have been found among different studies.

An important factor to be considered in data interpretation is the genetic background. Otomo, *et al.*[14] reported time-related changes on gene expression profiles displayed by two glioblastoma cell lines presenting different radiosensitivity in response to 6 Gy. Furthermore, it has been demonstrated that different dose levels of γ-rays may display different results in terms of modulated genes and transcript levels. In a study that compared the expression profiles changes induced by ionizing radiation in normal fibroblasts in response to low (2cGy) and high dose (4 Gy), Ding, *et al.*[7] reported that for 2 cGy, fibroblasts preferentially modulated genes related to cell–cell signaling, signal transduction, development and DNA damage responses, whereas for high dose (4 Gy), genes involved with apoptosis and cell proliferation were preferentially modulated. Additionally, genes involved in cytoskeleton organization and cell–cell signaling were found to be responsive only to low dose irradiation.

Similarly, Tachiiri, *et al.*[6] obtained quantitative and qualitative differences in transcript profiles in fibroblast irradiated with low (0.5 Gy) and high dose (50 Gy) and collected at different time points (1 to 72 h).

Although expression profiles at different dose levels are important to be evaluated, physiological responses should also be analyzed in parallel experiments in order to associate cellular responses with transcript profiles after induction of DNA damage. In the present study, we aimed to evaluate how glioma cells respond to moderate dose levels of γ-rays by performing parallel experiments at cellular context (clonogenic survival, cell cycle kinetics) in addition to gene expression analysis by the microarray method. For the dose of 1 Gy (moderate level), a broad spectrum of cellular processes were found modulated, represented by gene classes associated with metabolism, cell cycle control, stress response, DNA repair, cytoskeleton/cell adhesion, transport, transcription regulation, and signaling transduction. These results indicate the amplitude of cellular responses involving a number of gene categories playing role in the defense mechanism at cellular context under conditions of cell injury provoked by a moderate radiation dose. This panorama reflects the complexity of the cellular genetic cascade controlling responses to genotoxic agents in glioma cells, which may reflect the radio-resistance of this cell line. Other experiments are under way so that to study cellular responses to high dose levels in different cell lines differing in the genetic background.

Materials and methods

Cell line and culture conditions

U343 MG-a cell line was kindly provided by Prof. James T. Rutka, The Arthur and Sonia Labatt Brain Tumour Research Centre, Canada. Cells were cultured at 37 °C in HAM F10/DMEM (1:1) medium (Sigma-Aldrich, St. Louis, MO) supplemented with 15% fetal bovine serum (Cultilab, Campinas, SP, Brazil) in 25 cm^2 culture flasks (Corning, Corning, NY). Cells were fed every 3–4 days and used for the experiments until the 10^{th} passage after thawing.

Cell irradiation

Cell cultures were irradiated with γ-rays from ^{60}Cobalt, at a dose rate of 2.18 Gy/min, using a Gammatron S-80 equipment (Siemens Medical Systems Inc., Iselin, NJ) in the Clinical Hospital (FMRP-USP). The radiation doses varied depending on the biological assay (survival, cell cycle kinetics, and gene expression) and cells were harvested at different time points depending on the assay employed.

Clonogenic assay

300 cells/flask were seeded and cultures were irradiated with 0.25, 0.5, 1, 2, 4, 8, and 16 Gy of γ-rays and incubated at 37 °C for 12 days. After this period, cells were washed in phosphate buffered saline, stained with Giemsa, and cell colonies (presenting >50 cells) were counted. Three independent experiments were performed. The statistical analysis was performed by *one way* analysis of variance with repeated measures.

Cell cycle kinetics

The distribution of cells at different phases of the cell cycle was obtained at 1, 2, 3, 6, 12, and 24 h following irradiation with 0.5 e 1 Gy. Cells were treated with 4,68U trypsin diluted in stock solution (sodium citrate 3.4 mM/Igepal 0.1% v/v/ spermin 1.5 mM/ Tris 0.5 M), followed by inactivation with trypsin 50% w/v plus ribonuclease A 777,6U.[15] The staining procedure was performed with propi-

dium iodide 0.62 mM, spermin 3.3 mM/stock solution). A FACScan (Becton Dickinson, San Jose, CA) was used to score 10,000 cells, and the ModFit LT software (Verity Software House Inc., Sunnyvale, CA) was used to analyze the distribution of cells at different phases of the cell cycle. Three independent experiments were carried out and the data were analyzed by the *Z* test for comparison of proportions.

RNA extraction and cDNA microarrays

Cell cultures were irradiated with 1 Gy, and RNA extraction was performed 6 h after the irradiation. Unirradiated cells were used as a control and were collected at the same time. Total RNA was isolated by using Trizol® Reagent (Invitrogen, Carlsbade, CA) according to the manufacturer's protocol.

About 859 cDNA clones from the integrated molecular analysis of genomes and their expression (IMAGE) consortium were amplified in 96-well plates by polymerase chain reaction (PCR). These PCR products were purified and spotted onto Hybond N+ membranes (Amersham Pharmacia Biotech, Buckinghanshire, UK), using the Generation III Microarrays Spotter device (Amersham Pharmacia Biotech). These clone sets were distributed in the following gene classes, among others: cellular metabolism, signal transduction, physiological responses, macromolecule metabolism, transport, cell cycle/proliferation, cell differentiation, death, cell communication, response to DNA damage, cell adhesion, immune response according to the Gene Ontology classification, biological process-level 3.

Hybridization was carried out with a vector oligonucleotide labeled with $\alpha^{33}P$, using the T4 kinase–labelling kit (Invitrogen, Life Technologies, Carlsbade, CA). Membranes were prehybridized in hybridization mix (5 x saline-sodium citrate (SSC), 5 x Denhardt solution, 0.5% sodium dodecyl sulfate [SDS], and 100 μg/ml of salmon sperm DNA for 24 h at 42 °C) and then hybridized with the oligo-vector probe for 27 h at 42 °C. After hybridization, the filters were washed in 2 × SSC, 0.1% SDS, and exposed to radiation-sensitive imaging plates for 3 days. The hybridization signals were further detected in the Cyclone phosphor image device (Packard Bioscience Co., Meriden, CT).

Complex probes were prepared with 5 μg of total RNA and 8 μg of oligo(dT)25 (to saturate the long polyA tails), heated at 70 °C for 8 min, and cooled to 42 °C. Reverse transcription was performed in a reaction mixture containing 50 mM Tris (tris(hydroxymethyl)aminomethane)–HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 5 units RNase inhibitor, 0.4 mM each of deoxyuridine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate, 240 nM deoxycytidine triphosphate (dCTP), 5 μL of[γ-33P] dCTP, and 400 units Superscript RNAse H free RT (Invitrogen, Life Technologies) for 2 h at 42 °C. After alkali treatment and neutralization, unincorporated nucleotides were removed by purification on a sephadex G-50 column. The hybridization was performed at 65 °C for 48 h. After that, the filters were washed in 0.1 × SSC, 0.1% SDS at 68 °C for 90 min and exposed to imaging plates for 1–3 days, as described for the oligo-vector probe.

Quantitative Real-Time PCR

cDNA clones were reverse-transcribed from the total RNA by using the SuperScript III Reverse Transcriptase (Invitrogen), after decontamination from DNA traces by treatment with Deoxyribonuclease I (Amplification Grade kit, Invitrogen), according to manufacturer's instructions. The following primers sets were used: *TPP2:* 5' GGTGGGCAAGTCTCAGTGAT 3' (F) and 5' ACATCAAAGCGGTTGATTCC 3' (R); *FANCG:* 5' CTGTTCTTCCCTTGGAGCTG 3' (F) and 5' TCTCTAGGCTCCGCTGGATA 3'(R); *HSPA9B:* 5' AATTACTTGGGGCACACAGC 3' (F) and 5' CGAAGCACATTCAGTCCAGA 3' (R); *PIP5K1A:* 5' CCGATGATTACTTGTATTCCCT 3' (F) and 5' CCGCCTCTTTATGTTGGAC 3' (R); and *INPP5A:* 5' CATGATGCTTCCAATCTGGT 3' (F) and 5' GCTGATCAATGATTCTGTCCA 3' (R) (Integrated DNA Technologies, Coralville, IA). β-actin was amplified as an internal control with the following primer set: 5' GATGAGATTGGCATGGCTTT 3' (F) and 5' ATTGTGAACTTTGGGGGATG 3' (R). Real-time quantitative PCR reactions were performed using SYBR Green PCR Master Mix reagents (Applied Biosystems, Foster City, CA) on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). The PCR conditions were: preheating at 50 °C for 2 min, 10 min at 95 °C (denaturation step), followed by 40 cycles at 95 °C for 15 s, and at 60 °C for 1 min. The dissociation curves were set up as follows: 95 °C for 15 s, 60 °C for 20 s, and 95 °C for 15 s. The remaining RNA samples from microarray were used, and all reactions were performed in replicate. Relative fold-changes in gene expression were calculated using the ΔΔCt method.[16]

Data acquisition and gene expression analysis

After hybridization and image acquisition, numerical values were obtained for each spot using the BZScan software.[17] These values of hybridization signals were normalized (using the OpenOfice.org

software spreadsheets) by applying the following steps: 1) Background subtraction; 2) Normalization of the spotted cDNA amount through the oligo-vector labeling values; 3) A correlation-based filtering of array elements, by eliminating the lowest and highest intensity elements presenting low correlation; and 4) Application of a global normalization procedure, which consists on dividing all the individual spot values obtained for one experiment by the median value observed for the whole experiment.[18]

The normalized data was exported to MEV format and analyzed by multiexperiment viewer (MEV) software (http://tm4.org/mev.html), which was used to perform statistical analysis by t test and significance analysis of microarrays (SAM),[19] in order to select significantly modulated genes at a FDR <0.42%. Gene functions were searched by using S.O.U.R.C.E. (http://source.stanford.edu/cgi-bin/source/sourceSearch) and NCBI (http://www.ncbi.nlm.nih.gov).

Results

Irradiation of U343 MG-a cell cultures with γ-rays significantly ($p<0.001$) reduced the cloning efficiency in repeated experiments; whereas 8 Gy decreased the survival rate to ~1% and 16 Gy completely reduced the survival to zero (Figure 1). The survival percentage obtained for the dose of 1 Gy was approximately 61%, and this dose was chosen to analyze gene expression profiles. Analysis of cell cycle kinetics showed a significant ($P < 0.01$) transitory arrest at G2-phase in cells irradiated with 1 Gy, following 6 h after irradiation, with recovering tendency at later time (Figure 2).

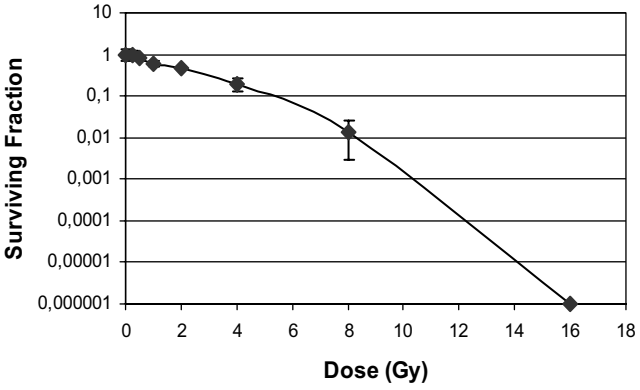

Figure 1 Survival curve obtained by the clonogenic assay in irradiated U343 MG-a GBM cells (pooled data from three experiments).

Gene expression profiles were analyzed for cells collected at 6 h after irradiation (1 Gy). The statistical analysis by the SAM method indicated a list of 196 differentially expressed genes: 67 down-regulated and 97 up-regulated, for FDR <0.42 % (Table 1).

Significantly expressed genes were classified according to biological functions (Figure 3). The most relevant categories (represented by variable number of genes) were related to metabolism

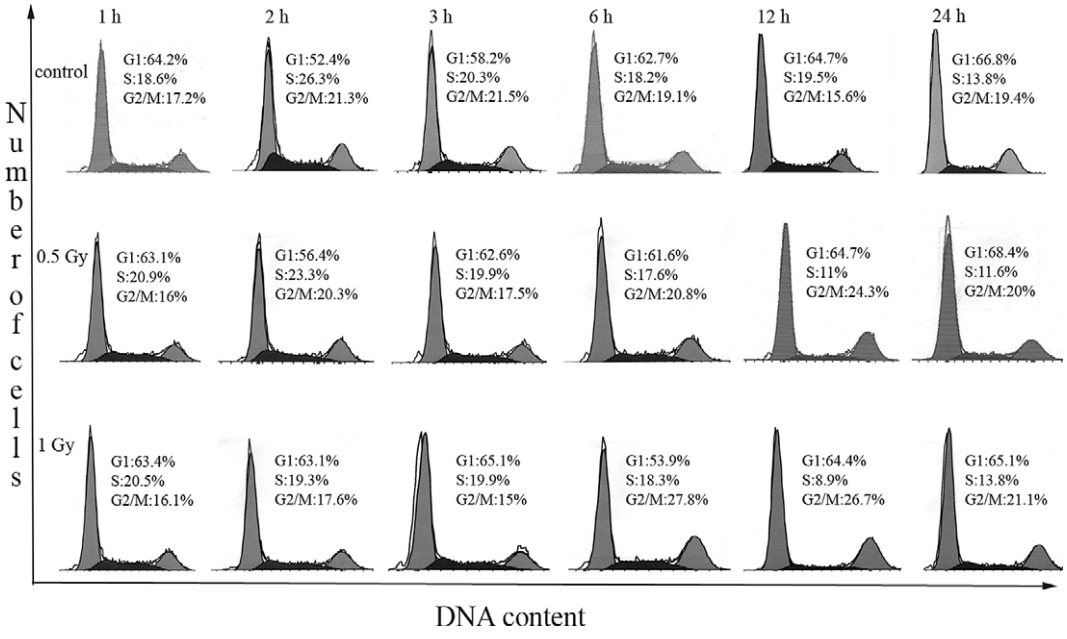

Figure 2 Distribution of cells at different cell cycle phases as analyzed by flow cytometry. The shown results correspond to one of three independent experiments.

Function	*Gene symbol*	*IMAGE (ID)*	*Gene name*	*?Fold-? ?change?*
Metabolism	CYP4X1	41391	Likely ortholog of rat cytochrome P450 4X1	23,39
	QDPR	23776	Quinoid dihydropteridine reductase	21,95
	PIP5K1A	143470	?Phosphatidylinositol-??4-phosphate? ?5-??kinase,? type I, alpha	3,66
	ALDOC	23831	Aldolase C, ?fructose-??bisphosphate?	3,65
	ME3	40879	Malic enzyme 3, ?NADP(+)-??dependent,? mitochondrial	3,53
	MGC5566	53119	Hypothetical protein MGC5566	2,96
	SIAT8B	23738	ST8 ?alpha-??N-acetyl-neuraminide? ?alpha-? ?2,8-sialyltransferase? 2	2,76
	INPP5D	150304	Inositol ?polyphosphate-??5-phosphatase,? 40 kDa	2,48
	PI4KII	24718	Phosphatidylinositol ?4-??kinase? type II	1,89
	PPP1R3D	251801	Protein phosphatase 1, regulatory subunit 3D	1,86
	INPP5A	190709	Inositol ?polyphosphate-??5-phosphatase,? 145 kDa	1,76
	CKMT1A	178425	Creatine kinase, mitochondrial 1A	1,61
	HMGCL	39796	?3-??hydroxymethyl-3-methylglutaryl-Coenzyme? A lyase (hydroxymethylglutaricaciduria)	−2,11
	PRPS2	146194	Phosphoribosyl pyrophosphate synthetase 2	−2,55
	ZADH1	22381	?Zinc-??binding? alcohol dehydrogenase, domain containing 1	−2,59
Adhesion/ cytoskeleton	ROBO2	53319	Prokineticin 2	3,89
	DIXDC1	24760	DIX domain containing 1	2,53
	SVIL	23676	Supervillin	2,16
	SDK1	24533	Sidekick homolog 1, ?cell-??adhesion? molecule (chicken)	2,08
	ARHGAP22	23762	Rho ?GTPase-??activating? protein 22	−1,68
	ENAH	39813	Enabled homolog (Drosophila)	−1,75
	FARP2	40316	*Homo sapiens* mRNA; cDNA DKFZp686H1039 (from clone DKFZp686H1039)	−1,80
	EPHB6	172982	EphB6	−1,91
	PTPRK	146123	Protein tyrosine phosphatase, receptor type, K	−1,94
	STK35	52085	Serine/threonine kinase 35	−2,09
	CHN2	25029	Chimerin (chimaerin) 2	−2,22
Signal transduction	DTNA	23529	Dystrobrevin, alpha	6,95
	TAS2R14	23514	Taste receptor, type 2, member 14	2,42
	SPRY2	40262	Sprouty homolog 2 (Drosophila)	2,08
	GNB1	24300	Guanine nucleotide–binding protein (G protein), beta polypeptide 1	1,93
	EPHA3	190902	EphA3	1,82
	DTX1	52120	Deltex homolog 1 (Drosophila)	1,77
	DGKD	24952		1,41

(continued)

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			Endoplasmic reticulum thioredoxin superfamily member, 18 kDa	
	GNAZ	40773	Guanine nucleotide–binding protein (G protein), alpha z polypeptide	−1,68
	MAPK8IP2	23959	?Mitogen-??activated? protein kinase 8 interacting protein 2	−1,72
	CAMK1	52629	?Calcium/calmodulin-??dependent? protein kinase I	−1,95
	DOK5	25664	Docking protein 5	−2,06
Apoptosis	DNASE	150099	Deoxyribonuclease II, lysosomal	1,84
	CUGBP2	26051	CUG triplet repeat, ?RNA-??binding? protein 2	1,71
	MAGEH1	267829	?APR-??1? protein	−1,51
	PTMA	181796	Prothymosin, alpha (gene sequence 28)	−1,56
	CYCS	40017	Cytochrome c, somatic	−1,68
	BAG4	51921	?BCL2-??associated? athanogene 4	−1,80
	BIRC6	51966	Baculoviral IAP ?repeat-??containing? 6 (apollon)	−1,94
	BCL2L10	40052	?BCL2-??like? 10 (apoptosis facilitator)	−2,33
	EI24	24988	Etoposide induced ?2.4? mRNA	−3,09
Membrane transport	RYR2	53099	Ryanodine receptor 2 (cardiac)	5,91
	CLCN4	23809	Chloride channel 4	3,66
	ATP2B2	40674	ATPase, Ca^{++} transporting, plasma membrane 2	2,28
	MCART1	40063	Mitochondrial carrier triple repeat 1	−1,54
	TCN1	25294	Transcobalamin I (vitamin B12 binding protein, R binder family)	−2,04
	TM4SF11	38915	Transmembrane 4 superfamily member 11 (plasmolipin)	−2,10
	TTYH1	41103	Tweety homolog 1 (Drosophila)	−2,19
	DKFZp761D221	25396	Hypothetical protein DKFZp761D221	−2,35
	ABCB8	167041	?ATP-??binding? cassette, ?sub-??family? B (MDR/TAP), member 8	−6,44
DNA repair/DNA damage signaling	H2AFX	1130993	H2A histone family, member X	8,66
	DDB1	4823153	?Damage-??specific? ?DNA-??binding? protein 1, 127 kDa	4,30
	FRAP1	145917	?FK506-??binding? protein ?12-??rapamycin? associated protein 1	3,18
	BRCA1	1333778	Breast cancer 1, early onset	2,52
	ERCC8	231305	Cockayne syndrome 1 (classical)	1,68
	FANCG	1705194	Fanconi anemia, complementation group G	1,55
	FEN1	49950	Flap ?structure-??specific? endonuclease 1	−3,23
	DDB2	1471825	?Damage-??specific? ?DNA-??binding? protein 2, 48 kDa	−1,51
Cell cycle	CABLES2	50389	Cdk5 and Abl enzyme substrate 2	2,56

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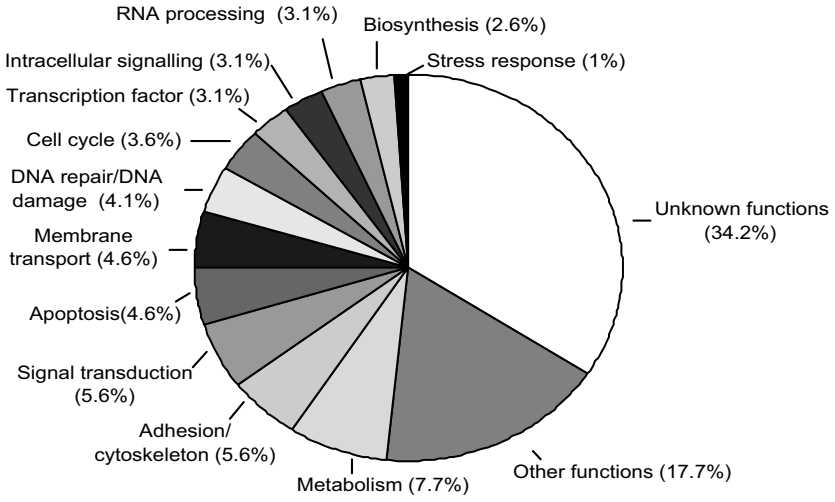

Figure 3 Distribution of functional categories for differentially expressed genes in U343 MG-a cells analyzed at 6 h post-irradiation (1 Gy).

(7.7%), adhesion/cytoskeleton (5.6%), signal transduction (5.6%), apoptosis (4.6%), membrane transport (4.6%), DNA repair/DNA damage signaling (4.1%), cell cycle/transcription regulation (3.6%), intracellular signaling (3.1%), RNA processing (3.1%), biosynthesis (2.6%), and stress response (1%). However, functions for 67 genes (34.2%) are still unknown.

Variations in fold-change values observed among gene categories associated to biological functions were calculated from the expression levels displayed by significant genes listed in Table 1. The relative expression and standard errors were plotted for each category (Figure 4). Higher fold-changes were observed for up-regulated genes playing roles in metabolism, transport, DNA repair/DNA damage sensing, RNA processing, biosynthesis, and stress response. Almost all of these gene categories also presented significant down-regulated genes.

Five genes (*HSPA9B*, *INPP5A*, *PIP5K1A*, *FANCG*, and *TPP2*) were chosen to evaluate the transcriptional expression by the quantitative real-time PCR (qPCR) method, using the same RNA samples as those employed in the microarray method (Figure 5).

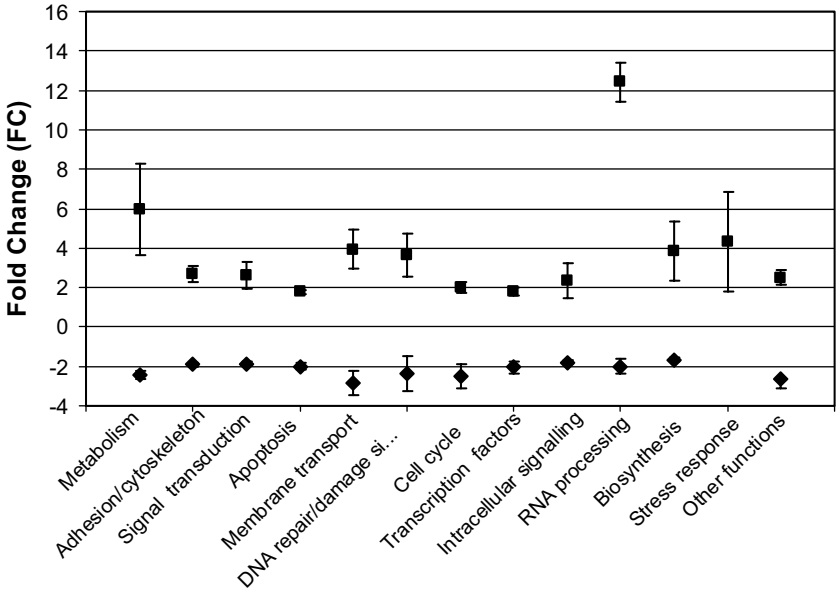

Figure 4 Variations of positive and negative fold-change values (mean ± SE) calculated for differentially expressed genes distributed in different biological functions. Each value of FC was calculated relatively to the respective control value.

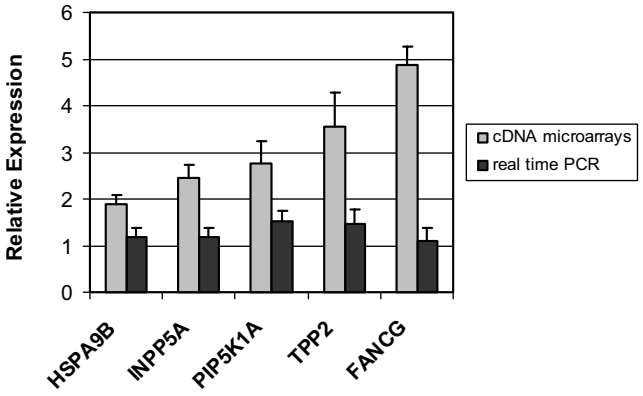

Figure 5 Gene expression levels displayed by five genes (*HSPA9B*, *INPP5A*, *PIP5K1A*, *FANCG and TPP2*) as determined by qPCR and the microarray methods, using the same RNA samples.

These genes were selected according to their functions and/or potential to give relevant information about responses of GBM cells to irradiation. For example, metabolism of phosphatidylinositol is a functional category especially modulated in this study; the *INPP5A* and *PIP5K1A* genes were not previously associated with radiation responses, and they are interesting to be studied by qRT-PCR. *HSPA9B* was recently identified as a gene involved in radioresistance, but there is little information about it, whereas *TPP2* may be involved in apoptosis resistance;[20] and *FANCG2* has a well-recognized function in DNA repair. The up-regulated status of those genes was confirmed, as previously indicated by the array method, in spite of some variation in expression levels.

Discussion

Although radiotherapy has widely been used for the treatment of patients with glioblastoma, the intrinsic radioresistance of these tumors remains a critical problem in the management of such patients.[1,2] The characterization of differences in gene expression profiles displayed by glioma cells irradiated *in vitro* may provide information about molecular mechanisms involved in radioresistance. In the present study, a moderate radiation dose of 1 Gy induced significant alterations in gene expression profiles. For cells irradiated with this dose, the survival fraction was 61%, as determined by the clonogenic assay. At this condition, mild alterations in cell cycle kinetics were observed in irradiated cells, and apoptosis and necrosis induction was not observed following 24–72 h post-irradiation (data not shown). It has been reported that even high radiation doses (5 Gy) did not cause apoptosis induction in U343 GBM cells,[21] and this result has also been demonstrated in our laboratory (data not shown).

Gene expression profiles were analyzed at 6 h following irradiation with 1 Gy. A major functional category of modulated genes were involved with metabolism, especially the metabolism of phosphatidylinositol, which was represented by 4 up-regulated (*INPP5A*, *INPP5D*, *PIP5K1A*, *PI4KII*) genes. The phosphoinositide pathway is a second messenger signaling system regulated in response to a variety of stimuli, including DNA damage.[22] The activation of some genes can be associated with cell resistance to apoptotic stimuli. For example, Jurkat cells expressing *INPP5D* are more resistant to H_2O_2-induced apoptosis.[23] *PIP5K1A* may also contribute to the inhibition of apoptosis by activating phosphatidylinositol-4,5-bisphosphate (PIP_2), a potent inhibitor of caspases 8, 9, and 3, or by preventing cell detachment through the promotion of stress actin fibers.[24,25] On the other hand, the role of inositol phosphates related with DNA repair in mammalian cells has been suggested, as early changes in the *in-vitro* PIP_2 phosphorylation in isolated nuclei of murine erythroleukaemia cells were found to transiently precede the marked DNA synthesis occurring after irradiation.[26] The up-regulation of *INPP5A* and *PIP5K1A* were also demonstrated by the real time RT-PCR method.

Irradiation of U343 MG-a also showed down-regulated (*FARP2*, *PTPRK*, *EPHB6*, *ENAH*, *CHN2*, *STK35*, and *ARGHAP22*) and up-regulated (*SDK1*, *ROBO2*, and *SVIL*) genes, whose functions were related to cytoskeleton and cell adhesion. Expression alterations for these gene classes have been associated with the maintenance of cell viability, leading to acquired chemo- and radio-resistance.[27,28] *PTPRK* is a critical regulator of proliferative epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian) and antiproliferative transforming growth factor-β (TGF-β) pathways. In breast cancer cells, *PTPRK* is required for the increased effects of TGF-β in controlling cell adhesion and inhibition of proliferation, while its down-regulation in human keratinocytes allows hyperactivation of EGFR.[29,30]

EPHB6 can regulate cell adhesion and migration, and induces Fas-mediated apoptosis in Jurkat cells.[31,32] *SVIL* is a member of the gelsolin superfamily, which have roles in several cellular processes, including cell motility and apoptosis control.[33] Malignant astrocytic gliomas are highly invasive tumors, and the process of cell adhesion is strongly related to their ability to infiltrate into the normal parenchyma.[34] The present results demonstrate

that pathways related to cell adhesion, which are constitutively activated in glioma cells, can be modulated following radiation exposure, and probably, this may contribute to survival after genotoxic insult.

The highly conserved heat-shock proteins, which mainly act by protecting cells from stress effects, are activated under stress stimuli. Two heat-shock genes, *HSPA9B* and *HSPB1*, were up-regulated in irradiated U343 MG-a. Both genes are related with apoptosis induction[35] and also radioresistance. *HSPA9B* gene was also found induced by the qPCR method. It was demonstrated that the overexpression of *HSPB1* activates the *Bcl2* gene, causing a delay in cell growth and radioresistance to murine fibroblasts.[36] Regarding the *HSPA9B* gene, it was demonstrated that the level of transcripts for this gene remained consistently elevated several hours after low-dose irradiation (0.25 Gy), and this was observed only in radioresistant human cancer cell line, MCF-7.[36,37]

Cellular responses to DNA damage include checkpoint activation, and this process triggers DNA repair pathways or apoptosis, depending on the extent of DNA damage, among other factors that may influence the general response. In the present study, at conditions of blockage at G2-phase, U343 MG-a cells collected following 6 h irradiation showed a series of modulated cell cycle control genes (*CDC23*, *CHES1*, *CABLES2*, *CDK2AP1*, *PIN1*, *CCND2*, and *CCNG2*). *CHES1* was initially associated with G2-checkpoint in yeast, but recently, it has been described as differentially modulated in cancer cells.[38,39] In human cells, the product of this gene interacts with ski-interacting protein (SKIP), a well-conserved transcriptional adaptor protein that mediates multiple signaling pathways involved in the control of cell proliferation and differentiation, but its role in cell cycle regulation is unknown.[40] PIN-1 is an important regulator of cell proliferation and DNA-replication checkpoint. In response to DNA damage, it promotes activation of the mitotic form of Cdc25 and interacts with p53 to regulates the function of many proteins involved in cell cycle control and apoptosis.[41,42]

Otomo, *et al.* (2004)[14] analyzed transcriptional changes after irradiation (6 Gy) of a radioresistant glioblastoma cell line (U87MG) compared to the radiosensitive cell line (A172); the authors found several DNA repair genes, which can be potentially determinant of radioresistance in U87MG cells. At our experimental conditions, we also observed modulation of DNA repair genes (*FANCG*, *DDB1*, *DDB2*, *BRCA1*, *ERCC8*, *H2AX*, and *FEN1*) but in response to a moderate dose of 1 Gy. *BRCA1* and *H2AX* can be involved in both repair processes and cell cycle checkpoints (G2-phase arrest) in response to radiation, and under association with *FANCG*, these genes also play a role in homologous recombination repair (HRR).[43–46]

According to Golding, *et al.*,[47] HRR may substantially contribute to DSB repair in glioma cells. A preferential activation of genes participating in HRR is also consistent with the hypothesis that cells are preferentially activating mechanisms that ensure cell survival. However, other types of repair processes, such as nucleotide excision repair (NER) and base excision repair (BER), are also activated in irradiated glioma cells, as indicated by the induction of *ERCC8 (CKN1)*, *DDB1*, and *FEN1*.

Although 1 Gy irradiation did not cause apoptosis induction, gene expression analysis indicated an upregulation of apoptosis-related genes (*CUGBP2* and *DNASE*) and a downregulation of antiapoptotic genes (*BAG4*, *BIRC6*, *BCL2L10*, and *PTMA*). There is evidence that putative death effector genes are often induced irrespective of the outcome of the cellular response, which seems to be regulated at a later step.[48] These results confirm the requirement of a balance between pro-apoptotic and anti-apoptotic signals besides a complex network of signaling pathways following cell irradiation.

In conclusion, the present data showed that a moderate level of radiation dose activated several processes (including G2/M checkpoint) in glioma cells, in order to guarantee cell survival. In parallel, several biological processes were also transcriptionally modulated, represented by gene classes associated with DNA damage/stress responses, such as metabolism, cell cycle control, DNA repair, cytoskeleton, cell adhesion, and transport. These results indicated a wide amplitude of cellular responses involving a number of gene categories, which play roles in cellular defense mechanisms under conditions of cell injury provoked by irradiation with 1 Gy. This panorama reflects the complexity of the cellular genetic cascade controlling responses to genotoxic agents. Studies with high radiation doses are under way, aiming to clarify the mechanism of cellular responses at conditions of high induction of cell death.

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