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Transcriptomics and Cell Transformation Assay: an Integrated Approach to Evaluate the Effects of Low Dose Ionizing Radiation

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Abstract

Background and aims: Ionizing radiation (IR) are a well-known carcinogenic agent, acting through genotoxic mechanisms. In the last years, great attention has been paid to the effects of IR at low doses and to the non-monotonic dose-response curve for IR exposures. To improve the knowledge of IR-mediated effects and possibly identify biomarkers for IR effects, we combined the Cell Transformation Assay (CTA) with transcriptomics, to correlate cytotoxicity and transformation endpoints with the modulation of gene profiles after IR exposure.

Methods: BALB/c3T3 cells were exposed to ionizing radiation ranging from 0.25Gy and 6Gy. Irradiated cells were seeded for the CTA 20h later. At the same time, RNA was extracted for microarray experiments. The cell clonal survival was significantly increased in 0.25Gy IR exposed cells, while the 3Gy dose strongly inhibited cellular growth. Cell transformation was observed only at the highest dose (3Gy).

Results: Cell's transformation was observed at 1.5, 2 and 3Gy doses. The 0.25Gy dose, which was able to induce an increment of clonal efficiency, did not induce cell transformation. The gene expression profile, which was obtained by comparing cells treated with the highest tested dose of 3Gy with the cells exposed to the lowest, not transforming, dose of 0.25Gy, identified several genes related to mitotic cell cycle and cholesterol biosynthesis.

Conclusion: Our study showed that the up-regulation of genes belonging to the Spindle Assembly Checkpoint and mitosis progression could support the transforming ability of the 3Gy BALB/c3T3 exposed cells, probably through the involvement of genomic instability. Gene transcripts involved into cholesterol biosynthesis appear to be critical, as well. All these transcripts may be regarded as potential biomarkers of IR effects.

Keywords: Ionizing radiation; BALB/c3T3 transformation assay; Transcriptomics; Integrated testing approach; Gene modulation; Genome instability; Cholesterol biosynthesis; Mitochondrial dysfunction

Abbreviations: ACE: Absolute Clonal Efficiency; APC/C-Cdc20: Anaphase Promoting Complex/Cyclosome; AR: Adaptive Response; Aurka: Aurora Kinase A; Calm-1: Calmodulin 1; Cenpa: Centromere Protein A; Cdkn1a; p21: Cyclin-Dependent Kinase Inhibitor 1A; CIN: Chromosome Instability; CTA: Cell Transformation Assay; CVD: Cardiovascular Disease; EASE: Expression Analysis Systematic Explorer analysis; FDPS: Farnesyl Diphosphate Synthase; Gadd45a: DNA-Damage-Inducible 45 Alpha; Gapdh: Glyceraldehyde-3-Phosphate Dehydrogenase; GO: Gene Ontology; Hmgcr: HMG-CoA Reductase; HRS: Hyper-Radiosensitivity; IR: Ionizing Radiation;

IRR: Radioresistance; PBS: Phosphate-Buffered Saline; Pmaip1; Noxa: Phorbol-12-Myristate-13-Acetate-Induced Protein 1; RCE: Relative Clonal Efficiency; SAC: Spindle Assembly Checkpoint; TF: Transformation Frequency

Introduction

Ionizing radiation (IR) represents an important source of both occupational and general population exposure. General population can be exposed through the environment, due to naturally occurring radioactive materials. Additional environmental exposure to IR can caused by the increased utilization of industrial processes requiring radiation sources, or as the consequence of disasters, involving nuclear plants. Medical exposure from diagnostic procedure, using x-rays or nuclear tracers, represents a large source of exposure, especially in developed countries with easy access to high levels of medical care [1,3]. Recent literature reports have risen concern about possible adverse effects related to the increased use of IR for diagnostic and therapeutic procedures [4]. As X- rays and γ-radiation are known to be human carcinogens, IR potential effects on humans have been of concern for over half a century. Several studies on IR-induced damage pointed out at a notable variety of genetic lesions by IR, including DNA double strand breaks, point mutations, chromosomal aberrations, and deletions [5-7]. Cellular oncogenes were shown to be targets for the effects of IR during *in vivo* carcinogenesis and *in vitro* cell transformation [8, 9]. However, the molecular mechanisms sustaining the response to IR, especially at low doses, are not fully understood as yet [10, 11].

It is well known that γ-radiations are able to alter the expression of stress-responsive genes at doses as low as 10 cGy and to induce large-scale changes in gene expression by activating several unexpected signaling pathways [12,15]. Given the complexity of the radiation response, it is evident that conventional approaches pointing at limited number of genes may not be satisfactory. Microarray technology allows the simultaneous analysis of thousands genes in a single experiment, providing new opportunities to understand the molecular events involved in the response to stressors. Modulated genes have been considered as potential biomarkers of IR exposure [16,18]. Studies in radio-sensitive mouse organs or cell lines were performed to identify differentially expressed genes related to IR exposure [19,23]. Even if these studies paved the way for the discovery of new potential IR biomarkers in human peripheral blood cells, the high variability in time or doses of exposure in both experimental and observational studies as well as the heterogeneity of the study design and final outcomes limit the applicability of these results [16,18].

Therefore, the possibility to identify markers of IR exposure or effect is still a challenge [24,25]. In recent years, the scientific community encouraged the use of experimental models based on the integration of *in vitro* methodologies with toxicogenomics technologies. This combined approach may provide information at the cellular and molecular level and promote a shift from *in vivo* costly and time-consuming animal studies to short term *in vitro* assays [26].

The cell transformation assay (CTA) can represent an alternative to animal models, when used in an integrated approach to support the weight of evidence in carcinogenicity evaluation [27, 28]. The cellular processes involved in *in vitro* cell transformation closely resembles *in vivo* carcinogenesis and occur as a result of comprehensive cellular responses to direct and indirect damage to DNA [29]. In particular, the *in vitro* transformation assay in BALB/c3T3 cells a system that benefits from a convenient protocol and high predictability of mammalian carcinogenicity [30-33]. The test is based on the malignant transformation of BALB/c3T3 embryonic mouse fibroblasts, which results in the induction of morphologically aberrant foci, formed by cells that lost contact inhibition and piled up with random orientation [31]. However, this assay alone does not provide information on the molecular mechanisms sustaining the carcinogenesis process, limiting its use in the regulatory context.

We propose here an approach that conjugates toxicogenomic technologies and *in vitro* BALB/c3T3 CTA to identify gene expression profiles supporting IR-induced transformation and possibly discriminate the response to low and high IR doses at the molecular level.

Whilst the integrated experimental approaches concerning the implementation of BALB/c3T3 transformation assay with toxicogenomic has been adopted to examine the effects of chemicals acting through genotoxic or non-genotoxic mechanisms in the carcinogenesis process, no similar studies are available for IR [34,35].

Our aim was to identify specific biological signatures of IR exposure in the BALB/c3T3 model by investigating modulated genes in the early steps of the oncotransformation. To address this issue, the effects of two IR doses, 0.25 and 3Gy, were investigated. Results show a different response according to the dose of exposure. Toxic and transforming effects were observed only in 3Gy exposed cells Differentially expressed genes between 3 and 0.25Gy treated cells are mainly related to the cell cycle network. These results show that the use of integrated testing strategies, including transcription analysis, provide further information on the response associated with different doses of ionizing radiation. The gene list we found would be of possible use in the monitoring of IR exposure.

Materials and Methods

Cells

The original stock of BALB/c3T3 cells, clone A31, was obtained from the American Type Culture Collection, Rockville, MD, USA. Working cultures were expanded from the original cryopreserved stock. Cells were grown in Dulbecco's modified Eagle's medium (D-MEM, Gibco BRL, Glasgow, Scotland) supplemented with 10% New-born Calf Serum (NCS, Gibco BRL). Only sub-confluent cells were used and the target cells were not maintained beyond the third passage after thawing. Cultures were maintained in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C.

Cell irradiation

Exponentially growing BALB/c3T3 cells, were exposed to ^{137}Cs radiation source (IBL 437C, 0.66 MeV, Dose-rate 221c Gy/min), to obtain an exposure range from 0.25 to 6Gy, then maintained in culture for 20 hours before seeding.

Cytotoxicity test

In order to calculate the number of cells surviving after radiation treatment, γ-irradiated cells were seeded at a concentration of 250 cells/60mm dish in 5 dishes for each treatment and plates were incubated at 37°C in a 5% CO_2 humidified atmosphere. After 48h, cells were washed with phosphate-buffered saline (PBS) and fresh culture medium was added. Cells were maintained in culture for 10 days, with bi-weekly medium changes, then were fixed with methanol, stained with 10% aqueous Giemsa and scored for colony formation. Only colonies containing more than 50 cells were counted. Untreated BALB/c3T3 cells were carried out as negative controls. Results were expressed as the mean number of colonies/plate (± Standard Error, S.E.), absolute clonal efficiency (ACE.), i.e., the fraction of cells which survived after treatment with respect to the number of seeded cells, and relative clonal efficiency (RCE.), which estimates the percentage of reduction of cell clonal efficiency in treated groups compared to non-exposed control cells.

Cell Transformation Assay

Exponentially growing BALB/c3T3 cells were -irradiated (0.25-3Gy) to estimate the occurrence of transformed foci. Cells were seeded at a

concentration of 3 x 10^4 cells/60 mm dish, 20h after the irradiation, in 10 dishes for each exposure. Plates were incubated at 37°C in a 5% CO_2 humidified atmosphere for 48 h. Then, cells were washed with PBS. Unexposed BALB/c3T3 cells were carried out as negative controls. Cells were maintained in culture for 5 weeks, with bi-weekly medium changes, then were fixed with methanol, stained with 10% aqueous Giemsa and scored for the formation of transformed foci. Only foci considered as positive, i.e., not smaller than 1 mm deeply basophilic, showing a dense layer formation and a random orientation of cells at the edges, were counted. Data was reported as transformation frequency (TF), calculated on the cells that survived after radiation exposure [30].

Microarray: RNA Extraction and Hybridization

Total RNA was isolated 20 h after IR exposure by using TRIzol Reagent (Invitrogen, San Diego, CA, USA) followed by purification with Rneasy affinity column (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. RNA quality was analysed with Agilent Bioanalyzer 2100 (RNA 6000 Nano Lab Chip, Agilent Technologies, Palo Alto, CA, USA).

cDNA was synthesized from 20µg of total RNA and directly labelled with Cy3-dCTP and Cy5-dCTP, following the manufacturer protocol optimized for the use with Agilent oligo microarray Kit. Labelled cDNAs from the control and treatment reactions were combined and purified by QIA quick spin column (Qiagen), then hybridized into the Mouse (V2) oligo microarray slide according to the Agilent 60-mer oligo microarray processing protocol. Slides were scanned with Agilent dual laser Microarray Scanner, including both Cy-3 and Cy-5 channels. Scanned images were analysed by Feature Extraction software 8.1 (Agilent Technologies) to derive raw intensity data used in the next analysis step.

Microarray Experimental Design and Data Analysis

Two doses were chosen to perform microarray experiments on the basis of results from the preliminary cytotoxicity test: the lowest assayed dose 0.25Gy, which fostered the cell clonal efficiency, and the intermediate dose 3Gy, responsible for significant cytotoxic effects, although still compatible with cell survival. We used four slides for each IR-dose to have four technical replicates, two of which were performed in dye-swap. Slide's control and saturated features were filtered out. Filtered raw intensity data sets (median green and red signal) were then log2-transformed and normalized intra-array with Joint-LOWESS algorithm. Transformed data were analysed by MAANOVA (Micro Array Analysis Of Variance) data analysis package of R programming environments. A fixed-effect linear ANOVA model was chosen to fit transformed data (variance due to different sources, including array, dye, gene, variety and their combined effects). We then tested a null hypothesis of no differential expression using F statistics computed on the James-Stein shrinkage estimates of the error variance. To avoid any assumption on error distribution, we computed p values for hypothesis tests via permutation methods (1000 permutations were carried out). The false-discovery rate controlling method was used to correct significance estimate for multiple testing hypothesis. MAANOVA analysis returned a list of 2523 differential expressed genes in the 3Gy sample versus 0.25Gy dose treatment. Gene modulations were calculated as the mean log ratio difference between the two sets of data.

Quantitative Real-Time PCR (qPCR)

An amount of 1µg of total RNA was reverse-transcribed at 50°C for 30′ in 30µl reaction mixture containing SuperScript™ III Reverse Transcriptase, RNaseOUT Recombinant Ribonuclease Inhibitor, oligo(dT) (2.5µM), random hexamers (2.5ng/µl), $MgCl_2$ (10mM), and NTPs (SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR, Invitrogen). Then, 2.5 ng cDNA were amplified with the appropriate primer pair (200nM) by using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), in the same cycling conditions (50°C for 2', 95°C for 8', 45 cycles at 95°C, 15" and at 60°C, 60"). The primers for aurora kinase A (Aurka), calmodulin 1 (Calm-1), centromere protein A (Cenpa), phorbol-12-myristate-13-acetate-induced protein 1 (Pmaip1, Noxa), were designed with Primer 3 software [36], while those for the growth arrest and DNA-damage-inducible 45 alpha (Gadd45a), cyclin-dependent kinase inhibitor 1A (Cdkn1a, p21) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were obtained from the Quantitative PCR Primer Database [37]. Primers sequences were used at a concentration of 200nM. Sequences are listed in Table 1. All amplifications were run at least in triplicate with an iCycler IQ Multicolor Real Time PCR detection system (Biorad, Hemel Hempstead, UK). Reactions were characterized at the time point when the fluorescence signal of the PCR product was first detected (quantification Cycle). Gene target $C_{q\ values}$ were normalized to Gapdh and mRNA levels for each gene calculated as $2^{-\Delta\Delta Cq}$, by using the non-exposed sample as calibrator.

Table 1: Forward and reverse sequences of Quantitative Real-Time PCR primers.

Gene Mus Musculus	Forward Sequence (200nM)	Reverse sequence (200nM)
Gapdh	5'-AATGTGTCCGTCGTGGATCTGA-3'	5'-GATGCCTGCTTCACCACCTTCT-3'
Aurka	5'-TCGGGTTGAATTCACTTTCC-3'	5'-CCGCTAGTGTTAGCCTTTGG-3'
Gadd45a	5'-TGGTGACGAACCCACATTCAT-3'	5'-ACCCACTGATCCATGTAGCGAC-3'
Calm-1	5'-GCTGCTGCTGACCTGTTGTA-3'	5'-TCTTTGTCTGGCCTGCTTTT-3'
Cdkn1a (p21)	5'-CCAGGCCAAGATGGTGTCTT-3'	5'-TGAGAAAGGATCAGCCATTGC-3'
Noxa (Pmaip1)	5'-GGCAGAGCTACCACCTGATGAGT-3'	5'-TCCTCATCCTGCTCTTTTGC-3'
Cenpa	5'-ACACTGCGCAGAAGACAGA -3'	5'-ACACCACGGCTGAACTTCTC-3'

Results

Cytotoxicity test

A preliminary cytotoxicity test was performed, in order to evaluate the effects of a wide range of doses, ranging from 0.25Gy to 6Gy, on the ability of BALB/c3T3 to form colonies and to select the working doses to be used in the CTA. At the lowest tested dose (0.25Gy), the statistically significant increase of the cell clonal efficiency was observed. As the IR dose increased, the clonal efficiency linearly decreased, with the almost complete inhibition of the colony formation at 6Gy of exposure. The calculation of ACE. and RCE. further confirmed this biphasic cell response to the tested IR doses [Table 2].

Transformation assay

Within the tested range of doses (0.25-3Gy), statistically significant increase of the transformation frequency (TF) ($p<0.01$, Poisson test) was observed only at 1.5, 2 and 3Gy doses. The 0.25Gy dose, which was able to induce an increment of clonal efficiency, did not induce cell transformation, as compared to the negative controls. Results are shown in Figure 1.

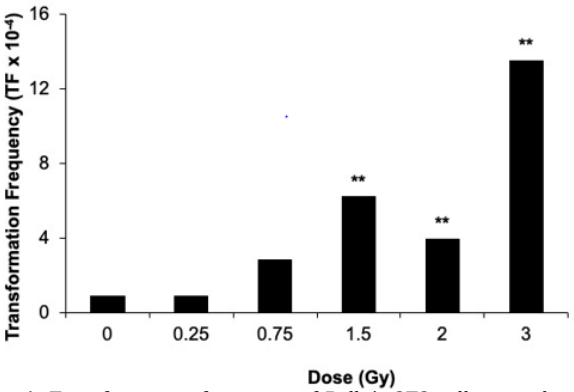

Figure 1: Transformation frequency of Balb/c 3T3 cells at each tested dose. **p-value < 0.01, by the Poisson test after comparison with untreated cells.

Microarray Biological Interpretation

To investigate the biological processes that resulted from the list of differentially expressed genes between 3 and 0.25Gy IR exposures, we used the Expression Analysis Systematic Explorer (EASE) analysis [38]. EASE is a powerful tool for the interpretation of microarray data, that gives gene ontology (GO) annotations of gene lists with statistics adjusted for multiple comparisons using a conservative variant of Fisher exact probability (EASE score), that penalizes the significance of categories supported by few genes and favors more robust biological themes [39]. We selected the main biological processes, according to an EASE score threshold of 0.01, corrected for multiple comparisons with the Bonferroni method, (p-value < 0.01). The results are summarized in Table 3. We obtained several categories within biological processes sustained by the list of up-modulated genes, but not by the down-expressed genes.

Table 2: Mean number of colonies, Absolute Clonal Efficiency (ACE) and Relative Clonal Efficiency (RCE) of BALB/c 3T3 cells after IR exposure.

IR Exposure (Gy)	Mean N. of Colonies ± SE[a]	ACE	RCE
0	30.35 ± 1.47	0.1214	1
0.25	86.26 ± 4.77[b]	0.3450[c]	2.84[d]
0.5	42.39 ± 2.35[b]	0.1696[c]	1.40[d]
1	41.60 ± 2.63[b]	0.1664[c]	1.37[d]
1.5	33.05 ± 2.33	0.1322	1.09
2	17.30 ± 1.61[b]	0.0692[c]	0.57[d]
3	10.30 ± 0.92[b]	0.0412[c]	0.34[d]
4	3.70 ± 0.77[b]	0.0148[c]	0.12[d]
6	0.70 ± 0.21[b]	0.0028[c]	0.02[d]

[a]Data are reported as a mean of five replicates.
[b]Significantly different (p<0.01) from controls at the t-Student test.
[c]Significantly different (p<0.01) from controls at the z-test.
[d]Significantly different (p<0.01) from controls at the Chi-square test.

Table 3: Main biological processes returned by EASE analysis of genes ratios obtained by direct comparison between 3Gy and 0.25Gy irradiation.

Gene Category	Child Terms	EASE Score	Bonferroni	Gene Symbol
Mitotic Cell Cycle		1.08E-08	1.89E-05	ANAPC5, BIRC5, BUB1B, BUB3, CCNA2, CCNB2, CDC20, CDC45L, CDK4, CHAF1A, CHEK1, CKS1B, DCTN1, DCTN2, DNM2, DUT, EXO1, FZR1, GMNN, HMGB1, HNRPL, INCENP, KHDRBS1, KIF22, KIF23, KIF2C, KPNA2, MAD2L1, MCM2, MCM3, MCM5, MCM6, MCM7, NEK2, NFIC, NFIX, POLD4, POLE, POLE2, PPARBP, PPP1R9B, PPP2R1A, PTTG1, RFC5, RPA2, SSSCA1, TOP2A, UBE2D3
Steroid Biosynthesis		2.66E-06	0.00467	DHCR24, DHCR7, DIA1, EBP, FDPS, FDXR, HMGCR, HMGCS1, HMGCS2, HSD17B7, IDI1, LSS, MVD, OPRS1, TM7SF2, TSTA3
Sterol Metabolism		4.49E-06	0.00786	DHCR24, DHCR7, DIA1, EBP, FDPS, FDXR, HDLBP, HMGCR, HMGCS1, HMGCS2, IDI1, MVD, OPRS1, PCSK9, SREBF2, TM7SF2
	sterol biosynthesis	9.69E-08	0.00017	DHCR24, DHCR7, DIA1, EBP, FDPS, HMGCR, HMGCS1, HMGCS2, IDI1, MVD, OPRS1, TM7SF2
	cholesterol biosynthesis	1.03E-07	0.00018	DHCR24, DHCR7, DIA1, EBP, FDPS, HMGCR, HMGCS1, HMGCS2, IDI1, MVD, TM7SF2

The most significant GO term that distinguished the 3Gy exposure from the lowest dose treatment was *mitotic cell cycle*, GO: A0000278, together with its parent category *cell cycle* (GO: A0007049, (EASE score <0.01, and p-value < 0.01). This term was enriched by genes involved in the spindle assembly checkpoint (SAC), like Bub1b, Bub3 and Mad2l1, that have the role of preventing premature separation of sister chromatids and mitotic exit throughout a metaphase block. Other important genes belonging to this gene category were Kif22, Kif23 and Kif2c, which encode for KIF proteins, a superfamily of microtubule-based molecular motors involved in intracellular organelle transport and chromosomes movement during cell division. Moreover, Kif2c seems to be required to coordinate the onset of sister centromere separation during anaphase chromosome segregation.

Mitotic cell cycle GO term was also supported by several genes specifically attributed to *S phase of mitotic cell cycle*, (GO: A0000084), (Dut, Mcm2, Mcm 3, Mcm 5, Mcm 6, Mcm 7, Nfic, Nfix, and Top2a) and *M phase of mitotic cell cycle*, (GO: A0000087), (Anapc5, Ccna2, Ccnb2, Cdc20, Fzr1 and Pttg1) showing an EASE score 0.01, but a p-value >0.05. All these genes seem to promote cell cycle progression, and some are overexpressed in tumors.

Within the biological processes significantly modulated by 3Gy versus 0.25Gy dose treatments, EASE also identified gene networks for *biosynthesis* (GO: A0009058, EASE score <0.01 and p <0.01), with its child GO terms *sterol metabolism,* (GO: A0016125), *steroid biosynthesis,* (GO: A000669), and *sterol biosynthesis,* (GO: A0016126), which clearly converge on *cholesterol biosynthesis*, (GO: A0006695). Indeed, all the genes belonging to this GO term codify for enzymes that have a key role in catalyzing the most important steps of cholesterol formation and operate both in the pre-squalenic phase (Idi1, Fdps, Hmgcr, Hmgcs1, Hmgcs2, MVD), and in the squalenic phase (Dhcr24, Dhcr7, Ebp). The up-modulation of HMG-CoA reductase (Hmgcr), which represents the rate-limiting enzyme for cholesterol synthesis, was also found.

Full details of the roles of all genes mentioned in this article can be conveniently obtained via the hyperlinks to NCBI Entrez in the Gene Section [40].

Quantitative RT-PCR Validation

Real-Time PCR analysis was performed on selected modulated genes (Aurka, Cenpa, Calm1, Gadd45a, Pmaip1, Cdkn1a) returned from MAANOVA analysis of microarray data. The validation was performed across each sample tested in our study by using biological replicates. Indeed, cells were exposed to each IR dose used in microarray experiments and the RNA was re-extracted and tested in Real-Time PCR.

The relative gene expression was determined by comparing the mRNA level obtained from cells exposed to 3Gy and 0.25Gy with the mRNA level of the untreated cells, by calculating the 2-ΔΔCq, where ΔCq= Cq gene-Cq GAPDH and ΔΔCq: ΔCq exposed - ΔCq control. We used a ΔΔCq t-test to establish the statistical significance of each comparison between exposed and not exposed cells. Statistically significant (p<0.05) Real-Time PCR expression data were substantially consistent with microarray results [Table 4].

Discussion

In this work, we integrated the *in vitro* BALB/c3T3 CTA with a global gene expression approach to obtain further information on the molecular events sustaining dose-related cytotoxic and transforming processes induced by IR. We focused on two IR doses, 0.25Gy and 3Gy, which led to completely different outcomes in the BALB/c3T3 cell system. Indeed, a significant increase in RCE and the absence of cell transformation was found in cells exposed at 0.25Gy. Conversely, in response to the 3Gy dose treatment, cell growth was reduced by about 40% while 15-fold increase of TF was observed. These data substantially confirmed the transforming ability of high-dose IR on BALB/c3T3 cells as previously reported by other authors [41,42]. Unlike the well-defined response to the high-dose irradiations, the biological effects at low doses are considerably more complex. Mammalian cells have been reported to show low-dose hyper-radiosensitivity (HRS) at doses below 20-30cGy [43,44], while increased radioresistance (IRR), was observed at doses comprised in the 20-70cGy range *in vitro* [43]. HRS is related to increased cell death, due to the failure of cell machinery to activate the early G2/M checkpoint and, consequently, DNA repair. Conversely, IRR is associated with increased clonal survival, following the induction of a minimum damage, which is able to activate a protective response [43]. The lowest tested dose in our model (0.25Gy) is included in the range of doses, which can induce either HRS or IRR. Cell survival at this dose was increased by almost 3 times with respect to non-irradiated cell survival. This result is consistent with possible not monotone dose-response relationship (NMDR), often related to hormetic effects, which have been described to occur at this level of IR doses and ascribed to an adaptive response (AR) [42].

Table 4: Real-Time PCR validation of six modulated genes detected by microarray. Underlined modulations indicate the statistically significant values obtained in Real-Time PCR that confirm microarray results.

Genes	Real-Time PCR	
	$2^{-\Delta\Delta Cq}$	
	0.25 Gy/NE	3 Gy/NE
Aurka	0.60*	1.04
Cenpa	0.63**	2.70**
Calm1	1.52**	2.30**
Gadd45a	0.95	0.24**
Cdkn1a (p21)	2.52**	3.02**
Noxa (Pmaip1)	1.39	2.88**

NE = not exposed.

*p-value <0. 05 at the t- test after comparison between exposed cells/non-exposed cells in Real-Time PCR.

**p-value <0.01 at the t-test after comparison between exposed cells/non-exposed cells in Real-Time PCR.

The AR is commonly known as a dose-related phenomenon characterized by the ability of pre-exposure at low doses to sustain the cells response to subsequent exposure to radiation high doses. However, an AR can also be observed after a single low dose exposure, not followed by exposure to high doses, triggered by substances released in the culture medium [43]. Some authors have demonstrated that even spontaneous cell transformation can be reduced by low doses of radiation, due to the increase of clonal survival [45,46]. It was previous reported that the rate of neoplastic transformation in non-tumorigenic Hela x human skin fibroblasts, treated with IR doses between 100-300mGy, was at the same level as that of the untreated cells [46]. This observation supports our results.

To have a better comprehension of the different outcomes elicited by the exposure to 0.25Gy and 3Gy IR doses, microarray experiments were performed. The list of statistically different transcripts obtained from MAANOVA analysis of the 3Gy vs 0.25Gy dose treatments underwent EASE biological interpretation.

One of the most significant biological processes belongs to the gene category of mitotic cell cycle, which was supported by several genes related to the spindle assembly checkpoint (SAC) or to mitotic progression. SAC is the major cell cycle control mechanism in mitosis, ensuring the accurate chromosome segregation by delaying anaphase, until all chromosomes have properly attached to spindle microtubules.

The mechanism responsible for SAC silencing is not completely understood. It seems to be the consequence of the occupancy of all kinetochores by microtubules and, probably, of the tension from bipolar attachment [47,48]. Among the key components and regulators of SAC, Bub1b, Bub3 and Mad2l1 were overexpressed in the 3Gy vs 0.25Gy data set. The products of these genes, in particular Bub1b, inhibit the protein ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C-Cdc20), by targeting its coactivator protein Cdc20 for degradation, until all chromosomes are correctly attached to all microtubules [48,51], Bub1b is also the core SAC protein responsible for the recruitment of other SAC components for assembling to kinetocores [51]. Defects in the SAC machinery or its silencing, and the consequent chromosome miss-aggregation, cause cell death in most cases, and also malignant transformation [52]. Mad2l and Bub1b were found to be overexpressed in several cancers. The association of their overexpression with tumorigenesis and chromosome instability (CIN) was supported by the increasing non-disjunction events and aneuploidy [53,54]. These genes have been also considered prognostic markers of tumor progression [55]. Beside these SAC genes, the 3Gy vs 0.25Gy data set also showed the up-regulation of Cdc20, whose degradation is the initial response to SAC activation. Without this early step in the checkpoint establishment, consisting, in Cdc20 deregulation, the existing levels of CDC20 could reach the threshold for APC/C activity causing cells to progress through mitosis [56,58]. Moreover, CDC20 overexpression was found to induce mice tumorigenesis and is associated with human cancer [59,61]. Therefore, in our model, CDC20 may abrogate SAC, possibly leading to CIN, which may represent the critical step in the TF increase observed after 3Gy IR exposure, compared to the lowest dose. This hypothesis is further confirmed by the overexpression of Ccna2 and Ccnb2 genes, which was detected in our study, and was reported to be associated with CIN in colorectal cancer cell lines [62,63].

IR is not only associated with cancer. Studies on high-dose exposures in Japanese population, from the A-bomb follow-out, showed that IR are equally responsible for both cancer and cardiovascular disease (CVD). Moreover, IR doses between 0.5 and 2Gy were associated with an elevated risk of stroke and heart disease, comparable with the risk of cancer, and equally contributing to one third of deaths due to irradiation [64]. Some studies showed the role of IR low doses in stroke and CVD, as reported by Zielinski et al. [65] who analyzed a Canadian cohort exposed to a range of doses between 5-400mSv [65]. Cancer radiotherapy has been associated with cardiovascular diseases, especially in patients affected with breast and lung cancers [66]. Indeed, the effects of radiotherapy in cancer cells are mediated by the generation of reactive oxygen species (ROS), which activate p53-mediated apoptosis. Heart damage has been ascribed to the high production of ROS by mitochondria in cardiomyocytes and endothelial cells after radiation treatment, leading to mitochondrial dysfunction [66]. The activation of NOXA-mediated pathway has been described as an essential event in IR-mediated mitochondrial dysfunction [67]. NOXA is one of the genes modulated in our model, supporting the hypothesis that NOXA activation represents an early key event in the response to IR-induced damage and that our model is able to highlight molecular mechanisms related to adverse outcomes. Indeed, the epidemiological findings alone, without any further information of the key events at the cellular and molecular level, are not sufficient to give a clear evidence for a direct association between the exposure to IR low doses and the adverse outcome of CVD or heart disease [68,69]. Cardiovascular disease due to IR exposure was associated with hypercholesterolemia and specifically to an increase of low-density lipoprotein cholesterol [70,72]. Interestingly, our results showed the modulation of different GO categories converging on cholesterol biosynthesis in the 3Gy vs 0.25Gy data set. The overexpression of HMG-CoA Reductase, the rate limiting enzyme in the synthesis of total cholesterol, pointed at the accumulation of cholesterol together with other genes like the farnesyl diphosphate synthase (FDPS). FDPS, catalyzes the sequential condensation of three 5-carbon isoprene units to form farnesyl pyrophosphate, and the squalenic phase transcripts Dhcr7, Dhcr24 and Ebp, whose defects are associated with syndromes characterized by cholesterol deficiency [73,75].

Beside the role of hypercholesterolemia in sustaining CVD, cholesterol may also play a role in cancer. Indeed, since cholesterol is an essential component of lipid rafts, a more elevated amount of this molecule is likely going to alter raft-dependent signaling in tumors cells, favoring cell survival mechanisms [76]. It can be speculated that cholesterol biosynthesis may be the target of the IR and play a role in different outcomes according to the dose of exposure.

In conclusion, we suggest that the cytotoxicity test and the cell transformation assay on BALB/c3T3 cells have the potential to screen low and high IR exposures and represent an interesting model system for the evaluation of specific biomarkers of effect, when integrated with –omics technologies. Indeed, the use of transcriptional profiling highlighted a set of gene transcripts specifically triggered by the 3Gy vs 0.25Gy dose and defined specific biological processes that could discriminate the response to high doses of IR compared to low levels of exposure. Moreover, the results are not limited only to the carcinogenesis process but could interestingly include other pathologies.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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