

Gene Expression Profiles Induced by High-dose Ionizing Radiation in MDA-MB-231 Triple-negative Breast Cancer Cell Line

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Abstract. *Background/Aim:* Radiation therapy (RT) represents a therapeutic option in breast cancer (BC). Even if a great number of BC patients receive RT, not all of them report benefits, due to radioresistance that gets activated through several factors, such as the hormone receptor status. Herein, we analyzed the gene expression profiles (GEP) induced by RT in triple-negative BC (TNBC) MDA-MB-231, to study signalling networks involved in radioresistance. *Materials and Methods:* GEP of MDA-MB-231 BC cells treated with a high dose of radiation, went through cDNA microarray analysis. In addition, to examine the cellular effects induced by RT, analyses of morphology and clonogenic evaluation were also conducted. *Results:* A descriptive report of GEP and pathways induced by IR is reported from our microarray data. Moreover, the MDA-MB-231 Radioresistant Cell Fraction (RCF) selected, included specific molecules able to drive radioresistance. *Conclusion:* In summary, our data highlight, the RT response of TNBC MDA-MB-231 cell line at a transcriptional level, in terms of activating radioresistance in these cells, as a model of late-stage BC.

Radiation therapy (RT) represents one of the most frequently used therapeutic options in cancer clinical practice, including breast cancer (BC), able to control localized tumor (1). As known, BC represents a highly heterogeneous group of

tumors at both the clinical and molecular level associated with different outcomes for the patients (2).

Today, a great amount of cancer patients receive RT through several modalities, such as internal or external RT, Brachytherapy, intraoperative electron radiation therapy (IOERT), and others (3-5). Although technological advances in radiation delivery have strongly enhanced the tumor killing capacity, the current clinical practice still offers standard RT protocols for patients with cancer in the same anatomical position, without taking into account the molecular characteristics of the tumor histotype.

As described by several groups including our own, IOERT is a RT technique that can destroy residual cancer cells that may be left at the tumor site, immediately following surgical removal of the tumor (6). According to specific eligibility criteria, IOERT may be exclusive (a single radiation dose of 21-23 Gy) or an anticipated boost of 9-12 Gy, followed by conventional RT (7-13).

As recently described by Yadava and Shankara, even if a great number of cancer patients receive RT, not all of them report a therapeutic benefit due to distant metastatic spread and local recurrence, a processes able to induce radioresistance activation (14, 15). Indeed, tumor radiosensitivity depends on many factors, some of which are linked to the clinical state of the patient. For example, the fate of BC disease following ionizing radiation (IR), depends on many factors, such as hormone receptor (HR) status of estrogen (ER), progesterone (PR) and human epidermal growth factor (HER2). Moreover, the balance between radiosensitivity and radioresistance is regulated by different and complex factors including the tumor stem cells which are able to repopulate during the course of the RT schedule, tumor microenvironment such as hypoxia, stromal interaction and variations in the intrinsic sensitivity of cells to radiation, modulation of DNA repair or other cell survival pathways (14-19).

To our knowledge, limited data are reported regarding the IR-induced gene expression changes in relation to BC tumor

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grading (20-24). In this sense, we recently reported IR-induced low grade- and high grade-specific signaling in BC cells (5). This study described the IR-induced genomic biomarkers and gene-signatures of specific breast tumor subtypes, grouped according to HR status. In particular regarding this radiobiological issue, more advanced studies on this topic are needed in order to help personalized biological-driven RT treatment planning alone or in combination with other therapies.

Several authors have reported controversies with respect to the use of RT for patients with triple-negative BC (TNBC) (ER⁻/PR⁻/HER2⁻) (25, 26). TNBC (heterogeneous disease which represents 15%-20% of BC incidences), is associated with an early age at presentation, larger tumor sizes, higher rates of recurrence, more aggressive biology, and poorer prognosis (25, 26). In addition, the absence of hormonal or targeted therapy against TNBC makes it a clinical challenge for oncologists in terms of patient management. However, Yao and colleagues have recently reported a survival advantage of adjuvant RT for TNBC patients, underling the necessity to study and clarify molecular mechanisms active in these cells and how these are involved in regulating cell survival/death balance (24).

Summarizing, as described in the literature, a major obstacle for effective cancer treatment by RT is the development of radioresistance. Thus, a great focus of research should aim to help clinicians understand the molecular features involved in the activation of this process, including the contribution of the specific tumor subtypes in this process. Such studies could better define the most successful treatment plan, including the choice of the best RT modality and schedule in the clinical practice against cancer.

In this sense, the principal aim of this work was to study gene expression profiles (GEP) by cDNA microarray analyses, induced by a high dose of IR used during IOERT schedules in triple negative MDA-MB-231 BC cells and to identify key factors and networks principally involved in the radioresistance process. Here, we report a list of genes and cellular process involved in cellular and molecular responses to high IR doses. We trust that these data could be used to better clarify mechanisms involved in the radioresistance of patients, particularly those with triple negative BC cells.

Materials and Methods

Radiation treatments. Cellular irradiation setup and dose distribution were conducted as previously reported and were performed using the NOVAC7 IOERT system (Sortina IOERT Technologies, Vicenza, Italy) (5, 7, 10, 27). MDA-MB-231 BC cells were irradiated using the following two doses: i) 9 Gy (according to the boost scheme), and ii) 23 Gy (exclusive modality), to the 100% isodose at a rate of 3.2 cGy/pulse.

Cell cultures and clonogenic survival evaluation. The human breast adenocarcinoma MDA-MB-231 cell line, was purchased from the

American Type Culture Collection (ATCC, Manassas, VA, Canada) and cells were cultured according to manufacturer's guidelines, as previously described (5). To detect the cell radiation effect, clonogenic survival assays, according to the method by Franken *et al.*, and morphological evaluations were conducted as previously reported (10, 27).

Whole-genome cDNA microarray expression analysis. GEP of the MDA-MB-231 BC cell line treated with 9 and 23 Gy of IR doses was performed. In addition, we also conducted microarray experiments on the surviving fraction of MDA-MB-231 cells collected 7 days after IOERT treatment, using 9 Gy of IR dose (hereafter named Radioresistant Cell Fraction, RCF). The total RNA was extracted from cells and was evaluated for its concentration and purity, as previously reported (5). cRNA synthesis and labeling were conducted according to the Agilent Two-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Santa Clara, CA, USA), as previously described by our group (5). For this purpose we used Whole Human Genome 4x44K microarrays (Agilent Technologies) that contain all known human genes. Array hybridization, microarray scanning and feature detection, as well as statistical data analysis, background correction, normalization and summary of expression measures were conducted with the Feature Extraction and GeneSpring GX 10.0.2 software (Agilent Technologies), as previously described in detail (7). In addition, the GEPs obtained in this work were also analyzed by pathway analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) network building tool (<https://david.ncifcrf.gov/tools.jsp>). The data discussed here are deposited in the NCBI Gene Expression Omnibus (28) and are accessible through the GEO Series accession number (GSE127789). Microarray data are available in compliance with Minimum Information about a Microarray Experiment standards (29).

Results

Cell survival and morphology. To evaluate MDA-MB-231 cell viability in terms of reproductive capacity, we performed a clonogenic survival assay (10, 27). 23 Gy of IR exposure inhibited the colony-forming ability of cells. On the other hand, 3 weeks following the 9 Gy exposure an RCF was observed (SF=6.4%) (Figure 1). Cell morphology modifications induced by IR, such as membranous and cytoplasmic damages, were starting from 72 hours post-treatment with either dose of 9 or 23 Gy.

Moreover, irradiated MDA-MB-231 cells displayed a large flat shape with evident macroscopic plasma membrane and nucleus alterations, suggesting a typical senescent phenotype (the well-known 'fried egg'), confirmed by SA-b-Gal activity, as previously described (30).

Overview of cDNA microarray gene expression analyses after 9Gy and 23Gy irradiations. Whole human genome Microarray-Based Gene Expression Analyses (Agilent Technologies) were conducted on MDA-MB-231 cells treated with 9 Gy and 23 Gy, delivered by IOERT. MDA-MB-231 untreated cells were used as reference sample for comparing the differential gene expression analyses (Figure 2A).

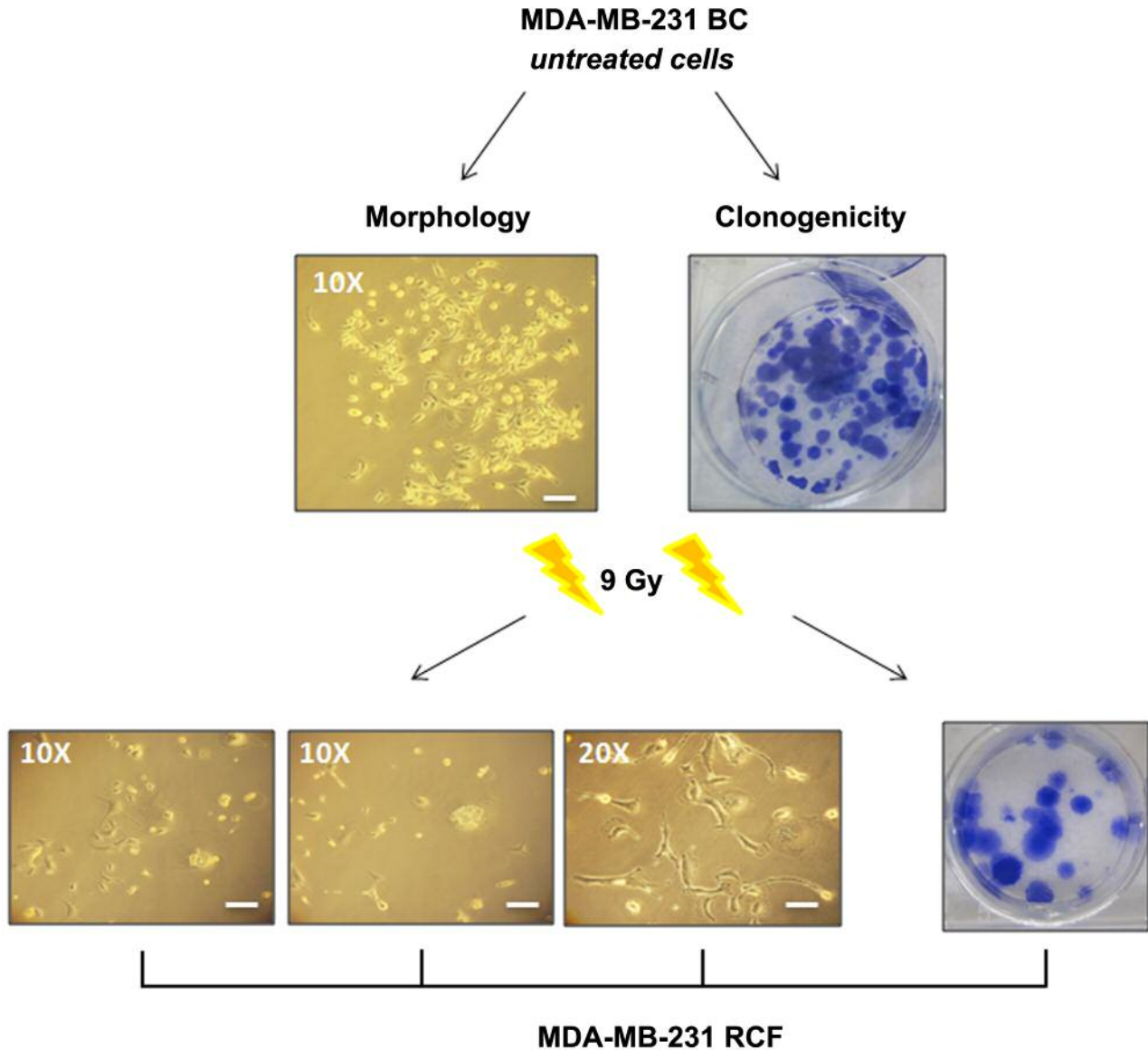


Figure 1. Micrographs of MDA-MB-231 RCF cell line following 9 Gy for morphological and clonogenic survival evaluation. Scale bars: 30 μ m.

A total of 2,127 genes changed their expression levels by 1.5-fold or more (1129 genes were down-regulated and 998 up-regulated) compared to the untreated reference group of MDA-MB-231 cells irradiated with 9 Gy.

Deregulated genes (DEGs) were grouped according to their involvement in specific pathways using DAVID and REACTOME tools (<https://david.ncifcrf.gov/tools.jsp>; <https://reactome.org>). The result of this mapping, shown in Table I highlights the involvement of a set of factors, principally involved in stem cell pluripotency pathway: Hippo, Rap1 and FoxO signaling as well as in cell cycle control.

From the MDA-MB-231 cells irradiated with 23 Gy, 3438 DEGs were selected a difference in their GEP by 1.5-fold or greater compared to the control: 1588 genes were down-regulated and 1850 up-regulated (Figure 2A).

The top 5 statistically and biologically relevant pathways, deregulated in MDA-MB-231 following 23 Gy, were analyzed by using the DAVID tool, underlining the involvement of the following listed pathways: i) Rap1 signaling, ii) Jak-STAT network, iii) stem cell pluripotency pathways, iv) cell cycle control and v) Tumor Necrosis Factor (TNF) network (Table II). Moreover, we performed Venn diagrams in order to study the number of unique and

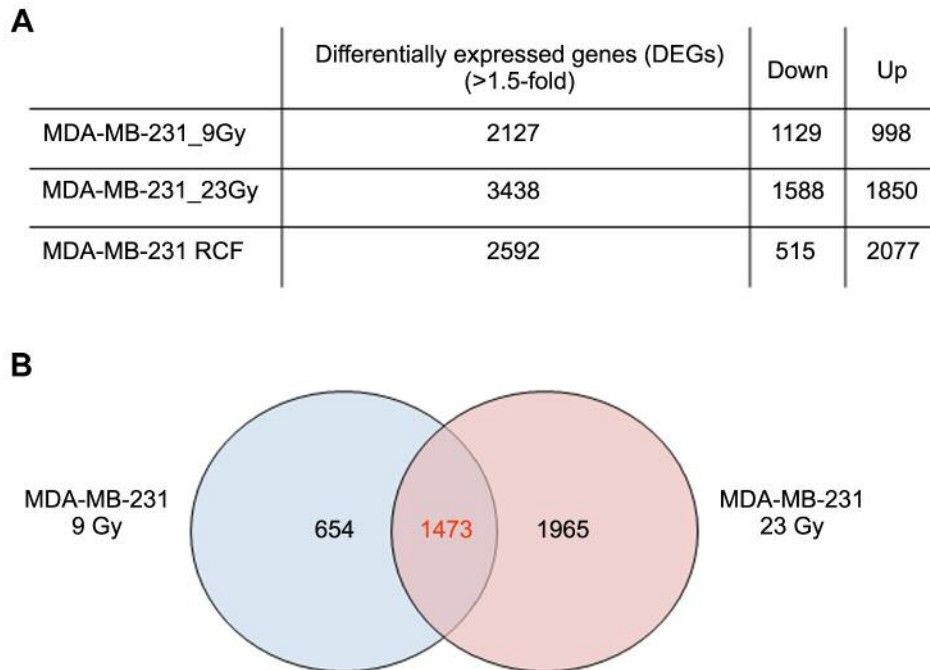


Figure 2. (A) Genes whose expression was significantly altered by 1.5-fold or greater following different irradiation conditions compared to the untreated reference group. (B) Venn diagrams showing the number of unique and shared differentially expressed genes (DEG) of the MDA-MB-231 BC cells, following exposure to doses of 9 and 23 Gy.

shared differentially expressed genes between the cells exposed to IR doses of 9 and 23 Gy (Figure 2B). 1473 genes were found to be common to MDA-MB-231 BC cells exposed to either 9 or 23 Gy (848 were down- and 625 were up-regulated). As shown in Table III, the stem cells pluripotency pathway, Rap1, Jak-STAT and PI3K-Akt signaling, were the top-5 statistically relevant pathways selected using the DAVID tool.

Interestingly, the analysis GO biological processes of all the configurations analyzed in this work, underlined the involvement of multiple cellular processes associated with chromatin assembly, such as nucleosome and telomere organization.

Overview of cDNA microarray gene expression analyses of RCF. As described above, following 9 Gy treatment a surviving MDA-MB-231 RCF (6.4%), was isolated and maintained up to 3-weeks post-IOERT (Figure 1). In order to analyze key genes involved in radioresistant cell response, we performed GEP analysis of MDA-MB-231 RCF samples collected 7 days post IOERT treatment. We used a comparative reference sample (MDA-MB-231 9 Gy-treated cells collected 24 hours post irradiation) in order to study genes specifically and temporally involved in the activation of the radioresistant process in the post irradiation time

window analyzed. Precisely, 2592 genes were found to be deregulated in the MDA-MB-231 RCF pool by 2-fold or more compared to control (515 genes were down-regulated and 2,077 were up-regulated) (Figure 2A).

Also in this case, we performed DAVID analysis in order to analyze the top-5 cellular pathways regulated by the selected gene list, as shown in Table IV. This analysis confirmed once again the involvement of networks, such as TNF, NF-kappa B, Jak-STAT and PI3K-Akt signaling and phagosome pathway. Interestingly, the analysis of GO biological processes performed using DAVID tool, underlined once again the involvement of multiple processes associated with chromatin assembly, possibly driving the activation of radioresistance processes in MDA-MB--231 RCF cells (Figure 3).

Discussion

As recently described by Sosin *et al.*, IOERT demonstrates favorable patient-reported outcomes and low rates of toxicity with adequate local disease control at 2-year follow-up when implemented in early BC (6). However, the radiosensitivity of BC depends on many factors, including a positive or negative HR status (5). In particular, controversial data are available in the literature regarding the radiation responses

Table I. Top-5 molecular pathways of deregulated genes expressed in MDA-MB-231 cells treated with 9 Gy.

	Pathway name	Genes found in GEP list	p-Value	Genes
1	Stem cells pluripotency signaling	23	0.004	FGFR3, IL6ST, WNT3A, OTX1, SMAD5, PAX6, LIFR, FZD4, STAT3, HESX1, IGF1R, WNT7B, ACVR2B, KRAS, RIF1, ID2, WNT11, SKIL, BMPR1B, PIK3R3, FGF2, AKT3, KAT6A
2	Hippo signaling	22	0.02	PRKCZ, NF2, GDF7, WNT3A, PRKCI, TEAD1, WWTR1, FZD4, TCF7L2, LATS2, TGFB2, WNT7B, CCND1, CCND3, ID2, BBC3, CCND2, AMOT, WNT11, BMPR1B, BMP8B, DLG2
3	Rap1 signaling	27	0.04	FGF5, PRKCZ, FGFR3, TLN2, ADORA2A, PGF, KITLG, CTNND1, LPAR3, APBB1IP, IGF1R, KRAS, CNR1, ANGPT1, CALML6, PDGFC, PIK3R3, FGF2, MAP2K6, AKT3, GRIN1, PRKCI, VASP, RGS14, PDGFRB, EFNA5, NGFR
4	FoxO signaling	19	0.04	IL6, PRKAB2, PRKAG2, CCNG2, STAT3, ATM, TGFB2, CCNB1, IGF1R, CCND1, EP300, KRAS, CCND2, CDKN2D, MDM2, CAT, PIK3R3, AGAP2, AKT3
5	Cell cycle	17	0.05	PRKDC, RB1, ATM, MCM5, CDC25B, MCM6, TGFB2, CCNB1, CCND1, EP300, CCND3, CCND2, CDKN2D, MDM2, BUB3, STAG2, STAG1

Table II. Top-5 molecular pathways of deregulated genes expressed in MDA-MB-231 cells treated with 23 Gy.

	Pathway name	Genes found in GEP list	p-Value	Genes
1	Rap1 signaling	32	0.001	FGF18, FGFR1, FGF5, FGFR3, ADCY7, ADORA2A, GNAI1, MRAS, CSF1, KITLG, LPAR3, CTNND1, ITGB3, FGF12, ITGB1, APBB1IP, IGF1R, KRAS, GRIN2B, CNR1, FGF1, EGF, FGF2, AKT3, MAP2K6, GNAO1, MAPK11, DOCK4, ID1, MAPK13, KRIT1, EFNA5
2	Jak-STAT signaling	24	0.001	CSH1, CSF3, IL4, CSF2, IL6, IL2RB, IL2RA1, IL6ST, CREBBP, LIFR, IL24, IL21, IRF9, LIF, CCND1, EP300, IL12A, EPOR, IL2RG, JAK3, MYC, CSF2RA, AKT3, IL22RA2
3	Stem cells pluripotency signaling	22	0.004	BMI1, FGFR1, NANOG, FGFR3, IL6ST, LIFR, MAPK11, ACVR1C, LIF, IGF1R, ACVR2B, KRAS, ID2, MAPK13, ID1, JAK3, BMPR1B, AXIN2, FGF2, MYC, AKT3, KAT6A
4	Cell cycle	18	0.02	E2F1, E2F2, CDC14A, CDC14B, CREBBP, CDC23, CDK6, ESPL1, MCM3, MCM4, ATM, MCM5, MCM6, CCND1, EP300, MDM2, MYC, BUB3
5	TNF signaling	16	0.02	CFLAR, CSF2, IL6, CSF1, MAPK11, CX3CL1, LIF, FOS, MAPK13, JUN, MAP3K8, IL1B, MLKL, FAS, MAP2K6, AKT3

of TNBC cells. In this work we analyzed genetic and cellular pathways deregulated after 9 Gy (IOERT BC ‘boost’ scheme) and 23 Gy (BC exclusive treatment modality) of IR doses, conventionally delivered during the IOERT plans.

Cell viability showed that 9 Gy treatment did not totally inhibit cell growth and proliferation of the MDA-MB-231 BC cell line, which generated RCF (SF=6.4%). On the contrary, radiation treatment using 23 Gy of IR dose was able to inhibit cell clonogenic activity (undetectable colonies) (30). IR-induced morphological changes, that were similar at either dose. Irradiated MDA-MB-231 cells displayed a large flat cell shape with evident macroscopic plasma membrane and nuclear alterations, suggesting a

typical senescent phenotype, confirmed by SA-b-Gal activity and linked with a senescence-associated secretory phenotype (SASP), as previously reported (30).

As suggested by GO analyses, both irradiation conditions created conspicuous changes in DNA structure assembly and in nucleosome and telomere organization, and consequently in gene expression regulation.

In particular, following 9 Gy IR exposure, MDA-MB-231 BC cells changed the expression levels in a large set of genes controlling the stem cell pluripotency pathway: Hippo, Rap1 and FoxO signaling as well as cell cycle regulators. In particular, DEGs belonging to the selected pathways, are listed in Table I.

Table III. Top-5 molecular pathways of differentially expressed genes shared between MDA-MB-231 BC cells exposed to 9 and 23 Gy.

	Pathway name	Genes found in GEP list	p-Value	Genes
1	Stem cells pluripotency signaling	18	0.01	FGFR3, IL6ST, OTX1, SMAD5, PAX6, LIFR, STAT3, IGF1R, ACVR2B, KRAS, ID2, RIF1, SKIL, BMPR1B, PIK3R3, FGF2, AKT3, KAT6A
2	Cancer	13	0.01	HSP90AA1, RB1, TCF7L2, IGF1R, CCND1, HSP90B1, EP300, KRAS, MDM2, PDGFRB, PDGFC, PIK3R3, AKT3
3	Rap1 signaling	22	0.03	FGF5, FGFR3, TLN2, ADORA2A, PRKCI, KITLG, LPAR3, CTNND1, APBB1IP, RGS14, VASP, IGF1R, KRAS, CNR1, PDGFRB, PDGFC, EFNA5, NGFR, PIK3R3, FGF2, MAP2K6, AKT3
4	Jak-STAT signaling	16	0.04	CSH1, IL4, CSF2, IL6, IL22RA1, IL6ST, LIFR, STAT3, PTPN11, CCND1, STAT4, EP300, PIK3R3, CSF2RA, AKT3, IL22RA2
5	PI3K-Akt signaling	30	0.05	CSH1, PHLPP1, FGF5, FGFR3, PPP2R5A, PPP2R5C, TLR2, KITLG, LPAR3, IGF1R, KRAS, PDGFC, MYB, PIK3R3, FGF2, COL11A1, AKT3, IL4, IL6, HSP90AA1, PKN2, NR4A1, COL4A6, COL4A5, HSP90B1, CCND1, PDGFRB, MDM2, EFNA5, NGFR

Table IV. Top-5 molecular pathways of deregulated gene datasets in MDA-MB-231 RCF cells.

	Pathway name	Genes found in GEP list	p-Value	Genes
1	TNF signaling	31	0.00001	CXCL1, TRAF1, CSF2, CCL2, TNF, PTGS2, CXCL3, CSF1, CXCL2, NFKBIA, CX3CL1, MMP3, CCL5, CXCL10, VCAM1, FOS, NOD2, CCL20, MAP3K8, BCL3, IL1B, ICAM1, IL6, CEBPB, CREB5, MAPK10, BIRC3, JUNB, TAB3, RIPK3, TNFAIP3
2	Phagosome	38	0.00001	HLA-DQB1, HLA-DRB1, C3, HLA-DRB3, TLR4, ATP6V1G2, C1R, ITGB3, HLA-DMB, TAP1, HLA-DRB4, HLA-DRB5, HLA-DPB1, FCGR3A, HLA-DOA, PLA2R1, ATP6V0D2, MRC1, OLR1, NCF2, NCF4, HLA-A, HLA-C, COLEC11, HLA-B, CTSS, HLA-E, HLA-G, HLA-F, ATP6V1C2, LAMP2, CD36, ATP6V1E2, HLA-DPA1, ATP6V0A4, CD14, DYNC112, HLA-DRA
3	NF-kappa B signaling	25	0.00002	TRAF1, ICAM1, IL1R1, TNF, PTGS2, LY96, BCL2A1, RELB, CXCL8, NFKBIA, TNFSF14, TLR4, BIRC3, TAB3, VCAM1, CARD11, TNFSF13B, PLCG2, ZAP70, IL1B, LBP, ERC1, TNFAIP3, LTBB, CD14
4	Jak-STAT signaling	25	0.03	CSF3, CSF2, OSMR, IL6ST, LEPR, IL13, IL7R, IFNL1, STAT4, IL23A, IL2RG, PRL, MYC, IL2RB, IL6, IL7, LIFR, IL24, IL6R, STAT3, IL20, OSM, PRLR, CCND2, JAK3
5	PI3K-Akt signaling	50	0.04	FGF18, EFNA1, FGF9, FGF14, OSMR, FASLG, TLR4, COL11A2, PRL, MYC, COL11A1, ANGPT4, PRKCA, HSP90AA1, IL6R, BCL2L11, OSM, LPAR5, CCND2, COL1A2, RELN, COL24A1, CSF3, CSF1, COL3A1, KIT, ITGB3, IL7R, KRAS, ITGB8, TEK, IL2RG, EGF, PPP2R2D, SPP1, COL4A3, IL2RB, IL6, TNXB, IL7, ITGA1, CREB5, COL4A6, GNGT1, LAMA1, GNGT2, PRLR, ITGA6, ITGA7, JAK3

The Hippo signaling is a novel tumor suppressor pathway that regulates cell proliferation and stem cell state. Hippo also controls organ size by inhibiting cell proliferation and promoting apoptosis (31). In turn, as described by Saeg and Anbalagan, the deregulation of Hippo signaling is believed to be responsible for the formation of Cancer Stem Cells (CSCs) in various types of cancer, including BC (32-33). The proliferation of CSCs is known to be involved with activation of radioresistance. In MDA-MB-231 9 Gy cells, Hippo

signaling was inhibited, as highlighted by the down-regulation of several factors that are part of this network, such as neurofibromin 2 (NF2 also known as Merlin), large tumor suppressor kinase 2 (LATS2), WW domain containing transcription regulator 1 (WWTR1) and TEA domain family member 1 (TEAD1) (32,34-35). Despite extensive studies, the mechanisms by which breast tumors become radioresistant are not fully understood. Tumor heterogeneity is a key product of CSCs and a key feature of therapy resistance. In particular,

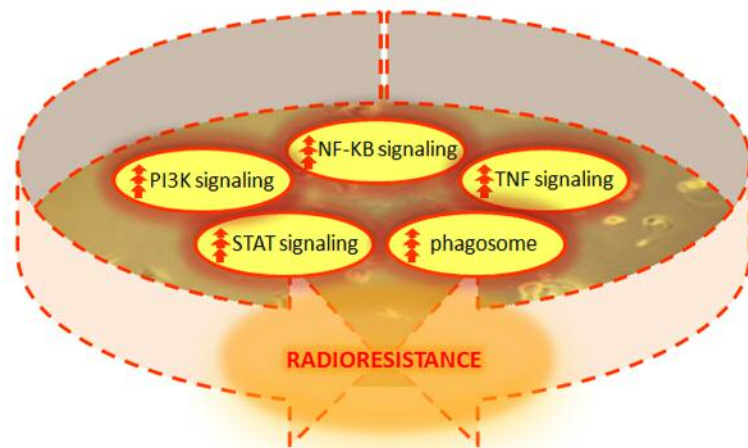


Figure 3. Schematic of the master regulators and signaling pathways strictly involved in the development of the radioresistance phenotype observed in MDA-MB-231 BC cells.

following irradiation CSCs up-regulate IGF (insulin like growth factor) type 1 receptor (up-regulated also in MDA-MB-231 9 Gy) and increase the IGF1 secretion. In the resting G_0 phase of the cell cycle, this expression pattern inhibits PI3K-AKT signaling and activates FoxO, resulting in cell cycle deregulation and stimulation of self-renewal (32, 34-36). Our data here confirm these findings as FoxO signaling and cell cycle key regulators (such as RB1, ATM, CDC25B, CCNB1, CCND1, EP300, CCND3, CCND2, CDKN2D genes and others listed in Table I), were deregulated in MDA-MB-231 9 Gy (Table I). Finally, Rap1 pathway was included in the top 5 biologically relevant pathways activated in MDA-MB-231 BC cells following either irradiation dose. Interestingly, Rap1 gene encodes a protein that is part of a complex involved in the regulation of telomere length and possibly in the activation of senescence (37).

The analysis of the top-5 pathway deregulated genes in MDA-MB-231 treated with 23 Gy, confirmed the deregulation of Rap1, and of pathways affecting stem cells pluripotency and cell cycle signaling following radiation exposure. Interestingly, two out of the five pathways selected were specifically deregulated following 23 Gy exposure: Jak-STAT and TNF networks. JAK-STAT signaling plays a key role in regulating the immune responses to IR. The STAT proteins (STAT1 and STAT3 were up-regulated in MDA-MB-231 23Gy) are considered to be important for cell viability in response to different stimuli, including IR (38-40), and play a significant role in tumor development. Interestingly, Khodarev NN *et al.*, have shown that a radiation-resistant phenotype could be induced by ectopic STAT over-expression, while its down-regulation could significantly increase the radiosensitivity of renal carcinoma

cell lines (38, 41, 42). Thus, the role of JAK-STAT in acquired radioresistance seems to be based not only on its transcriptional regulation, but also on its expression.

Several groups have highlighted the key role of TNF signaling in cancer, including BC. Tumour Necrosis Factor alpha (TNF- α), is a well-known pro-inflammatory cytokine often up-regulated following radiation and has been implicated in the development of fibrosis induced by IR (38, 43). In addition, TNF- α is both an inducer and a target of NF-kB, one of the most important transcription factors involved in immunological responses induced by radiation, and thus a master regulator of radiosensitivity/radioresistance cell balance (38). In summary, Jak-STAT and TNF pathways are strictly involved in immunological responses to radiation, as reported by the literature and by our research group (30, 38, 44). Here, we confirmed their key role in cell radiation response to high IR doses, such as those delivered by IOERT with 23 Gy, according to the exclusive BC RT schedule.

Finally, in order to analyze the number of unique and shared differentially expressed genes of the MDA-MB-231 cells exposed to IR doses of 9 and 23 Gy, we performed Venn diagrams showing a conspicuous amount of genes deregulated following either type of high-dose irradiation. From these, we then listed the top-5 biologically relevant pathways regulated by the 473 genes common to MDA-MB-231 BC cells exposed to either 9 or 23 Gy, underling once again the relevant role in cell radiation response of some of the above mentioned cellular signaling.

Finally, in order to analyze key genes involved in the radioresistant cell responses observed in the MDA-MB-231 RCF samples, we performed GEP analysis. Precisely, 2592 genes were found to be deregulated in MDA-MB-231 RCF by

2-fold or greater compared to control. Even in this case, the top GO biological process were involved in chromatin assembly able to regulate gene expression changes and probably able to drive the activation of radioresistance process in MDA-MB-231 RCF cells. In addition, the top five biological relevant pathways, confirm the involvement of the networks above described and following listed: TNF, NF-kappa B, Jak-STAT, PI3K-Akt signaling and phagosome regulations. In MDA-MB-231 RCF cells, the regulation of phagosome could be involved in radiation-induced autophagy known to be able to enhance radioresistance and leads to activation of the survival pathway, as recently observed (45). In particular, the master regulators of these pathways were up-regulated as displayed in Figure 3, and to our opinion strictly involved in the development of radioresistance phenotype observed in MDA-MB-231 RCF cells, after high radiation dose exposure.

In our opinion, this RCF gene list, needs more investigation in order to clarify the key roles of these factors in driving cell survival after the exposure of high IR doses and potentially used in targeted therapy intervention in tandem with RT schedules.

To date, targeted therapies against these molecules is under investigation and is supported by encouraging data in the literature (also in TNBC patients), however, these need to be further supported and clarified by radiobiology approach to evaluate their synergistic behavior in tandem with RT schedules in order to optimize BC care (46-48).

IOERT demonstrates favorable BC patient outcomes and low rates of toxicity with adequate control of local disease (6). However, a limited number of studies describe the molecular basis of radioresistance activation following RT, especially in the triple negative BC subtype (7, 10, 27, 49). Herein, we report a list of genes and cellular processes involved in cellular and molecular responses to high IR doses, delivered during IOERT schedules, and highlighting key factors involved in radioresistance activation. We trust that these data could be used to better clarify mechanisms involved in the radioresistance process, particularly in triple-negative BC cells in order to optimize RT successful plans in tandem with targeted therapies.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

All Authors participated in the conception and elaboration of the stud. In particular, GR performed the irradiation setups, and, together with VB, LM, FPC and GIF performed the IR cell treatments. LM, RM and GP carried out the cell cultures and the cell survival assays. VB performed the microarray experiments and the gene expression analyses. All authors were involved in the interpretation of the findings and approved the final content of the manuscript.

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