Evaluation of Biological Effect in Breast Cell Irradiated with Dose 2 Gy in Radiotherapy

Leslie Pereira (leslie@ird.gov.br)
Institute of Radio Protection and Dose Measuring: Instituto de Radioprotecao e Dosimetria
https://orcid.org/0000-0002-6055-836X

Antonio Gilcler F. Lima
Rio de Janeiro State University: Universidade do Estado do Rio de Janeiro

Marcella T. Ferreira
Rio de Janeiro State University: Universidade do Estado do Rio de Janeiro

Camila Salata
Brazilian Nuclear Energy Commission: Comissao Nacional de Energia Nuclear

Samara C Ferreira-Machado
Rio de Janeiro State University: Universidade do Estado do Rio de Janeiro

Veronica Morandi
Rio de Janeiro State University: Universidade do Estado do Rio de Janeiro

Luis Alexandre Gonçalves Magalhães
Rio de Janeiro State University: Universidade do Estado do Rio de Janeiro

Research Article

Keywords: 2 Gy, radiotherapy, breast cell, γH2AX, transendothelial migration

Posted Date: December 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1112329/v1

Abstract

Introduction: Breast cancer (BC) is the most common female malignancy worldwide. For the definitive treatment of MC, radiotherapy can be used, as an important component, and uses ionizing radiation (IR). Studies reveal the potential capacity of IR to promote metastasis. The clinical response of BC to radiotherapy is related to radiosensitivity and resistance of irradiated cells, which is associated with clonogenic activity and sensitivity to radiation. Unsuccessful treatment increases the risk of local and distant recurrence.

Methodology: Three breast cell lines (MCF-10A, MCF-7, and MDA-MB-231) were irradiated with 2 Gy and after 72 hours following markers were evaluated: E-cadherin, fibronectin, vimentin, and Snail. The processes of invasion, degradation of MMP2 and MMP9, and transendothelial migration were then assessed. Double-strand DNA breaks (DSBs), apoptosis, and colony formation were quantified.

Result: The detection of γH2AX histone of irradiated cells showed that MCF-10A non-tumor cell is more radiosensitive while the MDA-MB-231 tumor cell is more radioresistant. The dose 2 Gy altered the formation of colonies to any of the cell lines. Tumorigenic cells exhibited a markedly increase in apoptosis, 24 h after irradiation while MCF-10A cells only after 72 h. A single dose of 2 Gy does not induce changes in the cellular microenvironment that lead to changes in the mesenchymal epithelium in breast BC.

Conclusion: A dose of 2 Gy induces apoptosis and consequently an alteration in cell survival. However, a single dose of 2 Gy does not induce changes in the cellular microenvironment that lead to changes in the mesenchymal epithelium.

Introduction

Breast cancer (BC) is the most common female malignancy worldwide [1], it is a heterogeneous disease, which includes a wide variety of histological subtypes and a diversity of clinical behaviors and patient outcomes [2, 3]. For the definitive treatment of BC, radiotherapy can be used, which uses ionizing radiation (IR). The IR interacts with living tissues, causing cell damage, mutations with possible carcinogenic effects, or leading to cell death. Studies, in vitro, reveal the potential capacity of IR to promote metastasis but do not consider the high level of cell death induced by these doses [4, 5].

The objective of radiotherapy is to make tumorigenic cells lose their clonogenicity, preserving the functions of normal tissues. The clinical response of BC to radiotherapy is related to the clonogenic activity and the sensitivity to radiation, which depends on the radiosensitivity and resistance of the irradiated cells [6]. Radiotherapy is generally used to eradicate the microscopic tumor foci left after tumor resection. Currently, after primary surgery, women undergo treatment receiving 50 Gy, that is, in 25 fractions of 2 Gy, for 5 weeks [7]. Unsuccessful treatment of these microscopic tumor foci increases the risk of local and distant recurrence [8].

Several investigations have tried to identify potential mechanisms by which IR may be facilitating cell invasion and metastasis. One of the processes is the Mesenchymal Epithelial Transition (EMT), which is related to induced tumor progression [9]. The characteristics of EMT involve loss of epithelial characteristics such as loss of polarity and intercellular adhesion (E-cadherin), decreased expression of epithelial markers, and the acquisition of a mesenchymal phenotype increased expression of mesenchymal markers (vimentin and N-cadherin) result in increased motility [10]. Studies have shown that breast tumor recurrence after radiotherapy is a feature of EMT [11]. Several in vitro studies have shown that single doses of IR ranging from 1 to 3 Gy can induce EMT in multiple tumor cell lines, increasing tumor cell invasiveness and resistance to radiotherapy treatment by some patients [12].

This study aimed to investigate the biological effects in response to exposure of non-cancer and cancer breast cells to radiation beam established in the international protocol [7] used in radiotherapy (2 Gy) in the fractional treatment of BC. The techniques used to monitor and compare the response after exposure to IR were immunofluorescence to label γH2AX foci, apoptosis, and clonogenic assay. EMT was evaluated through the expression of its markers (E-cadherin, N-cadherin, vimentin, and snail) and characteristics (individual invasion, metalloproteinase degradation, and transendothelial migration) in three human mammary cell lines: non-cancer (MCF-10A); cancer cell in an intermediate stage of neoplastic transformation, with little aggressive profile and low metastatic potential, estrogen-positive adenocarcinoma (MCF-7) and; highly invasive and metastatic potential profile of cancer cells, triple-negative adenocarcinoma (MDA-MB-231).

Material And Methods

Cell culture

The three human breast epithelial cell lines MCF-10A, MCF-7, MDA-MB-231 were acquired from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The MCF-10A cell line was cultured in high glucose DMEM (GIBCO) supplemented with 10% fetal horse serum (FHS, Sigma-Aldrich/Merck) and the following additives: 10 µg/mL insulin (Sigma-Aldrich/Merck), 0.5 µg/mL hydrocortisone (Sigma-Aldrich/Merck), 20 µg/mL epidermal growth factor (EGF, Sigma-Aldrich), and 1% penicillin–streptomycin (Sigma-Aldrich). The MCF-7 and MDA-MB-231 cell lines were cultured in RPMI medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin–streptomycin (Sigma-Aldrich/Merck). The cells were kept in an incubator at 37°C and 5% CO_2. The cells are from the ATCC (American Type Culture Collection, Manassas, VA, USA) and kindly provided by the group of Dra. Verônica Morandi (LabAngio/UERJ, Rio de Janeiro, Brazil). All strains were identified for genotypic authentication through the search for STRs profiles (Short Tandem Repeats), by the DNA Diagnostic Laboratory (LDD/UERJ).

Human Umbilical Vein Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained as previously described [Jaffe et al, 1973]. Endothelial cells were used at passage 3. Umbilical cords were collected according to ethical guidelines, upon the informed consent given by the donors and after approbation by Ethics Committee of the Municipal Health Department of the City of Rio de Janeiro and the National Committee for Ethics in Human Research (CONEP) – Brazilian Ministry of Health (Approval Number #CAAE46904715.2.0000.5279). Briefly, cells were detached from the internal wall of the vein through the addition of 0.1% collagenase type IV (Sigma-Aldrich) in PBS-glucose, and grown in M199 supplemented with 20% FBS, 2 mM L-glutamine, 2.5 fungizone, 500 U/mL penicillin (Sigma-Aldrich), 500 µg/mL streptomycin (Sigma-Aldrich) - in 75 cm^2 culture bottles previously coated with 1% sterile gelatin and kept at 37ºC and 5% CO_2, as described (Jaffe 1973). To obtain subcultures, the primary cultures were incubated for 5 min at 37ºC with Versène solution (0.5 mM EDTA in PBS) containing 0.025% trypsin. HUVEC cells were maintained in M199 supplemented with 20% SFB, L-glutamine and antibiotics.

Radiotherapy Irradiations

The cell lines were irradiated within a linear accelerator (Varian), at the Hospital Universitario Pedro Ernesto/UERJ. Brazil. To obtain the dose of 2 Gy, planning was performed in a clinical tomography (Varian/Eclipse) and then the cells were irradiated in the accelerator using a photon beam with energy of 6 MeV, the field of 12x10 cm^2, and dose rate equal to 400 cGy/min. Cells were irradiated at room temperature in flasks/culture plates placed between two 1 cm polymethyl methacrylate (PMMA) plates, resulting in approximately 4.5 cm thick. For the irradiation protocol, the following were established: (1) non-irradiated control; (2) single-dose equal to 2 Gy. For cell irradiation, in the clonogenic and apoptosis assays, the culture flask (25 cm^2) was placed inside a 4.5 cm thick polymethyl methacrylate (PMMA) breast phantom. For the immunostaining assay (nuclear γH2AX detection), cells were irradiated in a 24-well culture plate, for the analysis of EMT markers, individual cell invasion, analysis of metalloproteases, and transendothelial cell migration, cells were irradiated in a 6-well culture plate. The plate was placed between two 1 cm PMMA plates resulted in approximately 4.5 cm thick.

Detection of γH2AX foci by immunofluorescence

Cells were seeded ($3x10^4$ cells/well) in the wells of a 24-well plate containing round coverslips (∅ 13 mm) and subsequently irradiated. At four different times (0, 15, 30, and 60 min) after irradiation, the cells were fixed with absolute methanol for 5 min at room temperature. Cultures were then blocked with 1% bovine serum albumin (BSA) in PBS and incubated with anti-$γH2AX^{ser139}$ antibody (1:200, Merck-Millipore) in 50 mM Tris buffer containing 0.9% NaCl and 1% BSA (TBS/BSA) for 40 min. Three washes were performed with PBS. Cells were subsequently incubated with Alexa Fluor 555 goat anti-rabbit IgG (H + L) antibody (1:400, A-21428 Invitrogen) in TBS/BSA with 10% goat serum (G9023 Sigma) for 40 min. Coverslips were mounted on slides with ProLong Gold Antifade containing DAPI (P36935 Invitrogen). All slides were analyzed using the Evos-fl microscope, model M5000 (Thermo Scientific), equipped with fluorescence and triple-pass filter band. Two types of analyses were performed. In the first one, the number of γH2AX foci (immunofluorescence labeling) in 1000 cells was determined. The γH2AX label was quantified using

the image processing software Image J. In the second analysis, at 100x magnifications, 100 nuclei of the cultured epithelial cells bearing γH2AX foci were used for each experiment and the number of foci per cell was counted. A repair time of 15 min was chosen to count IR-induced DSBs. Based on the number of observed γH2AX foci in individual nuclei, a classification criterion was designed, in order to produce a scoring system: nucleus with less than 5 foci ($x < 5$), a nucleus with foci between 5 and 15 ($5 < x < 15$), and nucleus with more than 15 foci ($x > 15$).

Flow cytometry assay

The percentage of apoptosis was estimated from the number of cells positive for Annexin V-FITC and negative for propidium iodide (PI), using the Annexin V Apoptosis Detection Kit (BD Bio-sciences) [33], by flow cytometry on a BD Accuri C6 flow cytometer (5×10^3 events). MCF-10A, MCF-7, and MDA-MB-231 cells were grown in culture flasks (25 cm2). Before irradiation, the medium was removed, and the flasks were filled with PBS and placed inside a PMMA phantom. After irradiation, the apoptotic response in the cell lines was measured at 0, 24, 48, and 72 h.

Clonogenic assay

The cells ($3x10^4$ cells/flask) were irradiated in culture flasks (25 cm^2) filled with PBS (1:10) and placed inside a PMMA phantom. After irradiation, PBS was removed, and the cells were detached from the flask through the enzymatic action of 0.025% trypsin. Cells were then counted and seeded (2×10^2 cells/well) in 6-well plates, in their respective culture media. After 14 days at 37°C and 5% CO_2, the cells were fixed with 3.7% formaldehyde in PBS (1:10) and stained with 0.5% violet crystal. Colonies were counted on an inverted microscope (Zeiss Axi-overt) and only colonies with more than 50 cells were considered. The number of colonies formed in the respective control was used in the calculation of the plating efficiency, which was then used to calculate the survival fraction of the irradiated samples [13].

Analysis of EMT Markers by Western Blotting

The epithelial marker E-cadherin was analyzed, which indicates the loss of polarity and increased mobility, allowing cell migration and invasion, as well as the mesenchymal markers vimentin, fibronectin, and Snail, which indicate the acquisition of a mesenchymal phenotype [14]. The MCF-10A, MCF-7, and MDA-MB-231 cells ($3x10^4$ cells/well) in 6-well plates were irradiated and kept at 37ºC and 5% CO_2 for 72 h in the presence of their respective culture. Then, the cells were treated with 10 mM HEPES lysis buffer, pH 7.4 [150 mM NaCl, 1% Triton X-100, 20 mM NaF, protease inhibitor cocktail (p8340, Sigma-Aldrich/Merck) and phosphatase inhibitor cocktail (p5726, Sigma-Aldrich/Merck)]. The protein-enriched fractions were collected and centrifuged at 14000 x g for 10 min at 4°C. The total protein in the extracts was determined using the BCA method (Pierce® BCA Protein Assay kit). The proteins were electrophoresed on an SDS-PAGE gel and then transferred to a PVDF membrane (Immobilon, Merck-Millipore). After blocking for 4 h at room temperature with 1% bovine serum albumin (BSA, Sigma-Aldrich) in Tris-buffered saline (TBS) buffer [0.02 M Tris, pH 7.6, containing 150 mM NaCl], the membranes were treated overnight under agitation at 4ºC with the primary antibodies against the respective proteins of interest, diluted in TBS buffer with 0.15 Tween-20 (TBS-T) plus 1% BSA. The following primary antibodies

were used: anti-E-cadherin (1:1000, #3195, Cell Signaling), anti-fibronectin (1:5000, #A0245, Dako), anti-vimentin (1:1000, #550513, BD Pharmingen), anti-Snail (1:1000. #3879, Cell Signaling), and ant-tubulin (1:5000, #T5168, Sigma) as a control. The membrane was then incubated with respective rabbit or mouse secondary antibodies conjugated to HRP – (Cell Signaling). For the development of the bands, the membranes were treated with chemiluminescent HRP activity detection reagent (ECL Western Blotting Detection Reagents, Amersham), according to the manufacturer's instructions. The analysis of the bands was performed with the aid of the Adobe-Photoshop 6.0 program.

Analysis of Individual Cell Invasion

Individual cell invasion capacity was evaluated by counting cells that were able to invade a protein matrix (Matrigel™) and to cross a porous membrane towards a preferred condition (chemoattraction). Migration inserts fitting 24-well plates and bearing 8 µm pore size membranes (BD Falcon) were treated with 200 µg/mL Matrigel with reduced growth factor (#354230, Corning), diluted in Tris pH 8.0 buffer (0.01 M Tris and 0.7% NaCl), for 2 h at 37°C, for polymerization. MCF-10A, MCF-7, and MDA-MB-231 cells ($5x10^4$ cells/well) in 6-well plates were irradiated, and after 72 h, the cells were trypsinized and seeded ($3x10^4$ cells/well) on the polymerized matrix, in the presence of the respective culture media without supplements at 37ºC for 18 h, while the lower portion of the inserts received culture medium supplemented with 5% FBS (chemoattractant). The cells that invaded through the Matrigel layer reaching the other side of the membrane were fixed with 3.7% formaldehyde diluted in PBS for 10 min and stained with DAPI (Sigma-Aldrich). Twenty fields were selected at random and cells were counted using an EVOS M5000 PI-AMG fluorescence microscope.

Analysis of Metalloproteases by Zymography

To determine the effect of radiation in matrix degradation was evaluated the collagenolytic activity that is associated with the matrix metalloproteases MMP2 and MMP9. MCF-10A, MCF-7, and MDA-MB-231 cells ($5x10^4$ cells/well) in 6-well plates were irradiated and maintained for 72 h in the presence of an FBS-free culture medium. The media were then collected and centrifuged at 14000 x g and 4°C for 10 minutes. The amount of total protein was measured using the protein binding method to the Coomassie blue dye (Bradford, Sigma-Aldrich/Merck), following the manufacturer´s instructions. The zymography was performed in a 10% acrylamide fractionating gel, co-polymerized with 1% gelatin and 0.4% SDS. The samples (10 µg/well) were applied in a 3% acrylamide concentrating gel. After the run, the protein was renatured for 1 h in a solution containing 2.5% Triton X-100 while stirring. The gel was kept in a development buffer (50 mM Tris-HCl pH 7.8, containing 150 mM NaCl and 5 mM $CaCl_2$) for 72 h at room temperature and stained with 0.1% Coomassie Brilliant Blue R-250. The quantification of the bands was performed by densitometry with the aid of the Adobe Photoshop program.

Analysis of Transendothelial Cell Migration

This test mimics the invasion of tumor cells through blood endothelial cells. Endothelial cells (HUVEC) suspended in M199 medium supplemented with 20% FBS were seeded ($2x10^5$ cells) in 8.0 µm pore polycarbonate filters (BD Biosciences) previously coated with gelatin, inserted into 24-well plates, until

reaching confluence (48 h). Endothelial cell monolayers were treated with 10 ng/µl TNF-α in M199 supplemented with 0.1% BSA for 4 h. After this, the wells were washed with M199. Seventy-two hours after irradiation, MDA-MB-231 cells were marked with the viability fluorochrome PKH26 and seeded ($3x10^4$ cells/well) over the HUVEC monolayer. The lower compartment was filled with 800 µL RPMI medium supplemented with 5% FBS. After incubation for 16 h at 37°C and 5% CO_2, the transmigrated cells were fixed with 3.7% paraformaldehyde and stained with DAPI. Twenty fields were selected at random and cells were counted using an EVOS M5000 PI-AMG fluorescence microscope.

Statistical analysis

Expressed were data as mean ± standard deviation (SD) from at least three independent experiments. The difference between the experimental groups was assessed by the ANOVA test (analysis of variance), followed by Bartlett's post-test, through the GraphPad Prism 5.01 program (Intuitive Software for Science).

Results

Detection of γH2AX foci by immunofluorescence

The Fig. 1 (a) shows that in the 60 min interval, the MCF-10A cell has a greater number of cells with γH2AX foci at 15 minutes after irradiation, while the MCF-7 cell has the greatest number of γH2AX foci at 30 minutes. Both breast cells showed an increase in γH2AX labeling followed by a further reduction over the analysis time. Or the contrary, the MDA-MB-231 cell showed an increasing increase in γH2AX focus in a time of 60 min. We also quantified the number of γH2AX foci per cell, after 15 min of irradiation as shown in Fig. 1 (b). Our results show that after irradiation with 2 Gy all cell lines have x >15. Showed that the biggest number of cells with x > 15 was found for the non-tumorigenic cell line MCF-10A, showing greater severity. Regarding the MDA-MB 231 cell line, the largest number of cells was classified as x > 15 group, regardless of exposure to radiation.

Flow cytometry assay

It was observed that the tumorigenic strains presented a similar apoptotic response profile. The cells, MCF-7 and MDA-MB-231, had greater apoptosis 24 h after irradiation. While the non-tumorigenic cell, MCF-10A had greater apoptosis within 48 h. However, 72 h after being irradiated, the three cell lines showed similar apoptosis (Fig. 1 (c).

Clonogenic assay

Increased proliferation is known to be a remarkable behavior of cancer cells [14]. We assessed the breast cell lines 14 days after irradiation for the ability to generate clonal colonies at low-density seeding (20 cells/cm^2). The results show significant differences, Fig. 1 (d). The studied breast cell lines had their proliferation capacity inhibited after 14 days of irradiation with a dose of 2 Gy. But lineage MDA-MB-231 had greater proliferation, while lineage MCF-7 had less proliferation.

Analysis of EMT Markers

In that evaluation we observed that dose 2 Gy was able to induce changes in the expression of EMT-related proteins, Figure 2 (a). The E-cadherin marker was up-regulated for an MCF-10A cell and down-regulated for an MCF-7 cell, Figure 2 (b). Vimentin marker was not detected in the MCF-10A and MCF-7 cells lines, Fig. 2 (c). The N-cadherin marker and the critical EMT regulator, Snail, were not expressed by any cell lineage.

Individual Cell Invasion Analysis

In this assay, it was possible to observe in Fig. 3 that the dose of 2 Gy induced a greater number of MDA-MB-231 cells in the invasion process. For MCF-10A and MCF-7 cell lines, no significant difference was observed.

Analysis of Metalloproteases by Zymography

There was no expression of collagenolytic activities compatible with MMP2 in irradiated cells in any of the cell lines, regardless of whether they were irradiated or not, Figure 4 (a). The reduction of collagenolytic activity compatible with MMP9 was observed in MCF-10A and MDA-MB-231 cells after irradiation compared to the respective non-irradiated control, Figure 4 (b) and (c). The MCF-7 cell, however, had increased collagenolytic activity compatible with MMP9 after exposure to dose 2 Gy, Figure 4 (d).

Analysis of Breast Cancer Cell Transendothelial Cell Migration

In the invasion assay, the MDA-MB-231 lineage had a greater number of cells invading, it is considered invasive and metastatic [15]. Therefore, the behavior of this cell was evaluated in transendothelial migration. Inhibition of the ability of MDA-MB-231 cells to migrate through the monolayer of endothelial cells was observed after exposure to dose 2 Gy compared to the non-irradiated control, Figure 5 (a). And when exposed to the pro-inflammatory cytokine TNF-α, there was also a reduction in the migratory capacity, Figure 5 (b).

Discussion

Recently, 2.3 million women were diagnosed with BC globally [16]. When the BC is identified early treatment can be highly effective. This treatment consists of a combination of surgical, radiation therapy, and medication (chemotherapy, hormonal therapy, and targeted biological therapy), prevent cancer growth and spread, thereby saving lives. Therefore, studies for advances in the treatment of this type of cancer have priority.

Radiotherapy is used to treat effectively various cancers in different stages, included advanced. However, has been shown that patients can acquisition of radioresistance during radiotherapy, which causes therapy to become ineffective [17]. Apart from this, damage to normal tissues is unavoidable during high-dose radiation treatments. For the radiotherapy treatment to be considered successful it is necessary that an increase in tumor cell death occurs while the adverse effects in the surroundings of healthy tissues are minimized. Thus, understanding the mechanisms that enhance radioresistance is important for advancing the development of new radiotherapeutic strategies [18].

High doses mainly cause damage to cellular DNA and proteins [19, 20], while doses of approximately 200 mGy can have anti-inflammatory events. However, it is not possible to know all inflammatory factors and their signaling pathways for all types of radiation doses administered. Distinguishing between effective doses with less damage to surrounding tissues in the field of radiotherapy is challenging. Our results, where different cell models received a single dose of 2 Gy, show that there was no change in the cellular microenvironment leading to changes in the mesenchymal epithelium.

The occurrence of DSB in the DNA molecule is considered the most critical damage and can lead to cell death, mutations, and genomic rearrangements that contribute to the development of cancer [21]. The activation of histone H2AX (γH2AX), important signaling damage in the DNA molecule, was investigated. [22], using the biomarker of histone H2AX phosphorylation on serine 139 (γH2AX), which is considered a sensitive and accurate marker of IR-induced DSBs [23]. The MCF-10A non-tumor cell had a greater number of γH2AX foci 15 min after irradiation, while in the MCF-7 tumor cell the greatest number of foci occurs at 30 min after irradiation. However, at 60 min the number of foci of the two strains is declining. In the MDA-MB-231 tumor cell, the number of foci increases over the time of experimental analysis. This suggests inefficient repair of that cell. It is also observed that the repair response in tumorigenic cells is slower when compared to the non-tumor cell MCF-10 A, which is successful in repair after 15 min. This data suggests that these cells have a repair mechanism sufficient to repair the damage to the DNA molecule [24]. Even so, it is not possible to say that all the damages were repaired efficiently, because no analysis of the repair mechanisms was carried out. This temporal reduction in the number of cells labeled for γH2AX may be associated with repair events of DNA breaks, and the permanence of others with the label may be associated with an incorrect repair. This is in agreement with the literature that suggests that tumor cells may have lower efficiency in repair mechanisms [25]. The persistence of γH2AX foci in DNA is associated with failures in the repair mechanism that can lead to genomic instability, cell death, and carcinogenesis [26, 27].

The results obtained by immunofluorescence analysis showed differences between the sizes and quantity of γH2AX foci, being specific for each cell line. Both MDA-MB-231 and MCF-7 tumor lines exhibited fewer γH2AX foci when compared to the non-tumor cell MCF-10A. Furthermore, these quantification results suggest that the MCF-10A non-tumor cell is more radiosensitive while the MDA-MB-231 tumor cell is more radioresistant when irradiated with 2 Gy.

In this research, it was demonstrated that the evaluated strains had their ability to form colonies inhibited with the dose of 2 Gy. However, there are distinctions in the proliferative capacity of cells. Tumor cell lines had a higher rate of apoptosis 24 h after being irradiated, that is, IR was able to induce damage to DNA molecules, which could not be repaired by the cell's DNA system. However, the triple-negative adenocarcinoma cell, MDA-MB-231, when compared to the MCF-7 cell, has a higher survival fraction 14 days after receiving the 2 Gy dose, showing to be more aggressive in terms of proliferation. The non-tumor cell MCF-10A has a higher rate of apoptosis 48 h after irradiation. However, its clonogenic capacity suggests that the radiation dose used was not able to promote sufficient cellular alterations that lead a normal cell to undergo some type of malignant transformation. For, the transformation of non-tumor cells into neoplastic cells is a complex event that develops in multiple stages [14, 28, 29]. The ability to deal with DNA damage is highly dependent on the cell type, and this defines its radiosensitivity. Cell death is the loss of proliferative capacity, and survival is related to clonogenic capacity.

In addition to γH2AX labeling, apoptosis, and survival, this research aimed to evaluate characteristics directly related to the carcinogenesis process, such as EMT. Some studies demonstrate that EMT has been observed in breast cells after irradiation in radiotherapy [30, 31]. Cells with an epithelial phenotype acquire a mesenchymal phenotype. And this may be associated with disease recurrence and radioresistance. Therefore, we evaluated EMT markers when cells, MCF-10A, MCF-7, and MDA-MB-231, received a single dose of 2 Gy.

It was observed that the epithelial marker E-cadherin was expressed positively in the normal MCF-10A cell, and in tumor cells it was expressed negatively in the MCF-7 cell, showing that this cell lost adherence [30, 31, 32, 33], and had MMP9 degradation, which can facilitate the process of invasion and metastasis [31, 32]. However, this cell did not show changes in the mesenchymal markers N-cadherin, Vimentin, and Snail, while in the MDA-MB-231 cell this marker is absent. The absence of E-cadherin indicates a lack of adherence and increased motility, ability to migrate, invade and metastasize. However, after irradiation with a single dose of 2 Gy, there was no change in the mesenchymal markers N-cadherin, Vimentin, and Snail. This cell had its invasiveness doubled 72 h after irradiation, showing the absence of E-cadherin. Because it invaded and the ability to carry out metastasis is known, the transendothelial migration of this cell was evaluated. It can be said that 72 h after receiving a dose of 2 Gy, the MDA-MB-231 cell is induced to invade, but it cannot migrate through the endothelial cell monolayer. These data are in agreement with Young et al [34]. Young irradiated with 2.3 Gy the cell lines MCF-7 and MDA-MB-231 and observed that the marker vimentin did not show variation in expression. Furthermore, this does increase MDA-MB-231 invasion but did not increase invasion through the reconstituted basement membrane. It is important to note that these results were observed at the clinically used dose for standard fractionated radiotherapy treatment for CM.

Conclusion

These investigations suggest that a single dose of 2 Gy induces apoptosis and consequently an alteration in cell survival. However, a single dose of 2 Gy does not induce changes in the cellular

microenvironment that lead to changes in the mesenchymal epithelium.

A change in the regulation of the expression of the epithelial marker E-cadherin was observed in MCF-10A (up-regulation) and MCF-7 (down-regulation) cells, but no variation was observed in the mesenchymal markers in these cells. It can also be noted that there was no change in the phenotype of cells with respect to cell invasion. However, in the MCF-7 cell, there was an increase in collagenolytic activity compatible with MMP9, but this increase did not reflect the changes in the aforementioned phenomena.

Regarding the MDA-MB-231 cell, there was no change in the expression of the analyzed markers. However, there was an increase in cell invasion, showing that other pathways, in addition to the classic one associated with EMT, may be influencing this response. These data, taken together, show that the MDA cell was the one with the lowest radiosensitivity (smallest reduction in the survival fraction) and increased cellular invasion when exposed to a fractional dose of 2 Gy treatment. Suggesting a greater chance of recurrence and metastasis in patients with aggressive tumors, where tumor cells have survived radiotherapy treatment.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Each author to have made contributions to the conception or design of the work; acquisition, analysis, interpretation of data; have drafted the work or substantively revised it; or approved the submitted version.

Follow each author's contribution in this work:

- Leslie Pereira – orcid: 0000-0002-6055-836X

leslie@ird.gov.br; leslie.pereira@hotmail.com

LP is the lead author of this research. Set up all the experiments and interacted with the hospital's medical physicists to irradiate the cells. Analyzed and interpreted the results. Drafted and discussed the results to ensure the accuracy and integrity of any part of the work.

- Antonio Gilclêr F. Lima – orcid: 0000-0003-0106-923X

gilclerantonio@gmail.com

AL is the second author of this research. In the laboratory, monitored the performance of all experiments. Verified if all data, including reagents and code, comply with the transparency and reproducibility standards of both the field and journal. Discussed the results, substantively revised and approved the submitted version.

- Marcella T. Ferreira – orcid: 0000-0002-7719-0303

marcellatavaresferreira@gmail.com

MF is the third author of this research. Accompanied the performance of some experiments in the laboratory, being responsible for preparing the slides for microscope analysis. Verified if all data, including reagents and code. Discussed the results and approved the submitted version.

- Camila Salata – orcid: 0000-0001-7328-1200

camila.salata@cnen.gov.br

CS was a contributor in writing the manuscript and statistical analyses of this work.

- Samara C. Ferreira-Machado – orcid: 0000-0001-5306-1040

samararossatti@gmail.com

SM was a major contributor in writing the manuscript.

- Veronica Morandi – orcid: 0000-0002-5883-4847

veronica@uerj.br

VM coordinated the performance of all experiments in the laboratory. Greater contribution to the discussion of the biological effects of the entire work. This laboratory collaborated with physical spaces, materials, and reagents for this research. Revision all writing the manuscript.

- Luís A. G. Magalhães – orcid: 0000-0003-1677-197X

luisalexandregm@hotmail.com

LM it discussed the performance of all experiments in the field of physics. Greater contribution in the discussion of setup for cell irradiation. This laboratory collaborated with physical spaces, materials, and reagents for this research. Revision all writing the manuscript.

All authors read and approved the final manuscript.

Acknowledgements

We are grateful to the Medical Physicists Dr. Roberto Salomon and Msc. Aneli Silva for her dedication and availability, for always finding time to set up the setup and irradiation of the cells, at the Pedro Ernesto University Hospital, of the State University of Rio de Janeiro (HUPE/UERJ).

References

1. American Cancer Society. How Common Is Breast Cancer? Jan (2021) Available at: https://www.cancer.org/cancer/breast-cancer/about/how-common-is-breast-cancer.html
2. DeSantis CE, Bray F, Ferlay J, Lortet-Tieulent J, Anderson BO, Jemal A (2015) International Variation in Female Breast Cancer Incidence and Mortality Rates. Cancer Epidemiol Biomarkers Prev 24(10):1495–1506. http://www.ncbi.nlm.nih.gov/pubmed/26359465
3. Rindi G, Klimstra DS, Abedi-Ardekani B et al (2018) A common classification framework for neuroendocrine neoplasms: an International Agency for Research on Cancer (IARC) and World Health Organization (WHO) expert consensus proposal. Mod Pathol 31:1770–1786. https://doi.org/10.1038/s41379-018-0110-y
4. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35(4):495–516. doi:10.1080/01926230701320337
5. Galluzzi L, Vitale I, Aaronson S et al (2018) Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ 25:486–541. https://doi.org/10.1038/s41418-017-0012-4
6. Gray M, Turnbull AK, Ward C et al (2019) Development and characterisation of acquired radioresistant breast cancer cell lines. Radiat Oncol 14:64. https://doi.org/10.1186/s13014-019-1268-2
7. START Trialists' Group, Bentzen SM, Agrawal RK et al (2008) The UK Standardisation of Breast Radiotherapy (START) Trial A of radiotherapy hypofractionation for treatment of early breast cancer: a randomised trial. Lancet Oncol 9(4):331–341. doi:10.1016/S1470-2045(08)70077-9
8. Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Darby S, McGale P, Correa C, Taylor C, Arriagada R et al (2011) Effect of radiotherapy after breast-conserving surgery on 10-year recurrence

and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. Lancet 378:1707–1716

9. Ochieng J, Nangami GN, Ogunkua O et al (2015) The impact of low-dose carcinogens and environmental disruptors on tissue invasion and metastasis. Carcinogenesis 36:128–159. https://doi.org/10.1093/carcin/bgv034
10. Yang J, Antin P, Berx G et al (2020) Guidelines and definitions for research on epithelial–mesenchymal transition. Nat Rev Mol Cell Biol 21:341–352. https://doi.org/10.1038/s41580-020-0237-9
11. Luo M, Brooks M, Wicha MS (2015) Epithelial-mesenchymal plasticity of breast cancer stem cells: implications for metastasis and therapeutic resistance. Curr Pharm Des 21(10):1301–1310. doi:10.2174/1381612821666141211120604
12. Kaushik N, Kim MJ, Kim RK et al (2017) Low-dose radiation decreases tumor progression via the inhibition of the JAK1/STAT3 signaling axis in breast cancer cell lines. Sci Rep 7. https://doi.org/10.1038/srep43361
13. Franken N, Rodermond H, Stap J et al (2006) Clonogenic assay of cells in vitro. Nat Proto 7:2315–2319. https://doi.org/10.1038/nprot.2006.339
14. Hanahan D, Weinberg RA (2011) Hallmarks of Cancer: The Next Generation. Cell 144:646–674. https://doi.org/10.1016/j.cell.2011.02.013
15. The Physical Sciences - Oncology Centers Network (2013) A physical sciences network characterization of non-tumorigenic and metastatic cells. Sci Rep 3. https://doi.org/10.1038/srep01449
16. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/breast-cancer
17. Rajamanickam B, Jiawen D, Nei W, Richard Y, Kheng-Wei Y (2014) Biological response of cancer cells to radiation treatment. Frontiers in Molecular Biosciences. doi:10.3389/fmolb.2014.00024. 1;24
18. Galeaz Chiara T, Cristina (2021) Bisio Alessandra. Radiation Resistance: A Matter of Transcription Factors. Front Oncol 11:2055
19. Reisz JA, Bansal N, Qian J, Zhao W, Furdui CM (2014) Effects of ionizing radiation on biological molecules–mechanisms of damage and emerging methods of detection. Antioxid Redox Signal 21(2):260–292. doi:10.1089/ars.2013.5489
20. Lumniczky K, Impens N, Armengol G, Candéias S, Georgakilas AG, Hornhardt S, Martin OA, Rödel F, Schaue D (2021) Low dose ionizing radiation effects on the immune system. Environ Int 149:0160–4120. https://doi.org/10.1016/j.envint.2020.106212
21. Jeggo PA, Löbrich M (2006) Contribution of DNA repair and cell cycle checkpoint arrest to the maintenance of genomic stability. DNA Repair (Amst) 8:1192–1198. doi: 10.1016/j.dnarep.2006.05.011
22. Rothkamm K, Löbrich M (2003) Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. Proc Natl Acad Sci U S A 29;100((9):5057–5062. doi: 10.1073/pnas.0830918100

23. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 6;273((10):5858–5868. doi: 10.1074/jbc.273.10.5858
24. Dauer LT, Brooks AL, Hoel DG, Morgan WF, Stram D, Tran P (2010 Jul) Review and evaluation of updated research on the health effects associated with low-dose ionising radiation. Radiat Prot Dosimetry 140(2):103–136. doi: 10.1093/rpd/ncq141
25. Borrego-Soto G, Ortiz-López R, Rojas-Martínez A (2015) Ionizing radiation-induced DNA injury and damage detection in patients with breast cancer. Genetics and Molecular Biology 38:420–432. https://doi.org/10.1590/S1415-475738420150019
26. Chatterjee N, Walker GC (2017) Mechanisms of DNA damage, repair, and mutagenesis. Environ Mol Mutagen 58(5):235–263. doi:10.1002/em.22087
27. Huang RX, Zhou PK (2020) DNA damage response signaling pathways and targets for radiotherapy sensitization in cancer. Sig Transduct Target Ther 5:60. https://doi.org/10.1038/s41392-020-0150-x
28. Rosenwald I (2004) The role of translation in neoplastic transformation from a pathologist's point of view. Oncogene 23:3230–3247. https://doi.org/10.1038/sj.onc.1207552
29. Martin TA, Ye L, Sanders AJ et al Cancer Invasion and Metastasis: Molecular and Cellular Perspective. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013. https://www.ncbi.nlm.nih.gov/books/NBK164700/
30. Kim RK, Kaushik N, Suh Y et al (2016) Radiation driven epithelial-mesenchymal transition is mediated by Notch signaling in breast cancer. Oncotarget 7(33):53430–53442. doi:10.18632/oncotarget.10802
31. Wang Y, Zhou BP (2013) Epithelial-mesenchymal Transition—A Hallmark of Breast Cancer Metastasis. Cancer Hallm 1(1):38–49. doi:10.1166/ch.2013.1004
32. Repullés J, Anglada T, Soler D, Ramírez JC, Genescà A, Terradas M Radiation-Induced Malignant Transformation of Preneoplastic and Normal Breast Primary Epithelial Cells.Mol Cancer Res. 2019Apr; 17(4):937–948. doi: 10.1158/1541-7786.MCR-18-0938
33. Hollier BG, Evans K, Mani SA (2009 Mar) The epithelial-to-mesenchymal transition and cancer stem cells: a coalition against cancer therapies. J Mammary Gland Biol Neoplasia 14(1):29–43. doi: 10.1007/s10911-009-9110-3
34. Young AGH, Bennewith KL (2017) Ionizing Radiation Enhances Breast Tumor Cell Migration In Vitro. Radiat Res 188(4):381–391. doi: 10.1667/RR14738.1

Figures

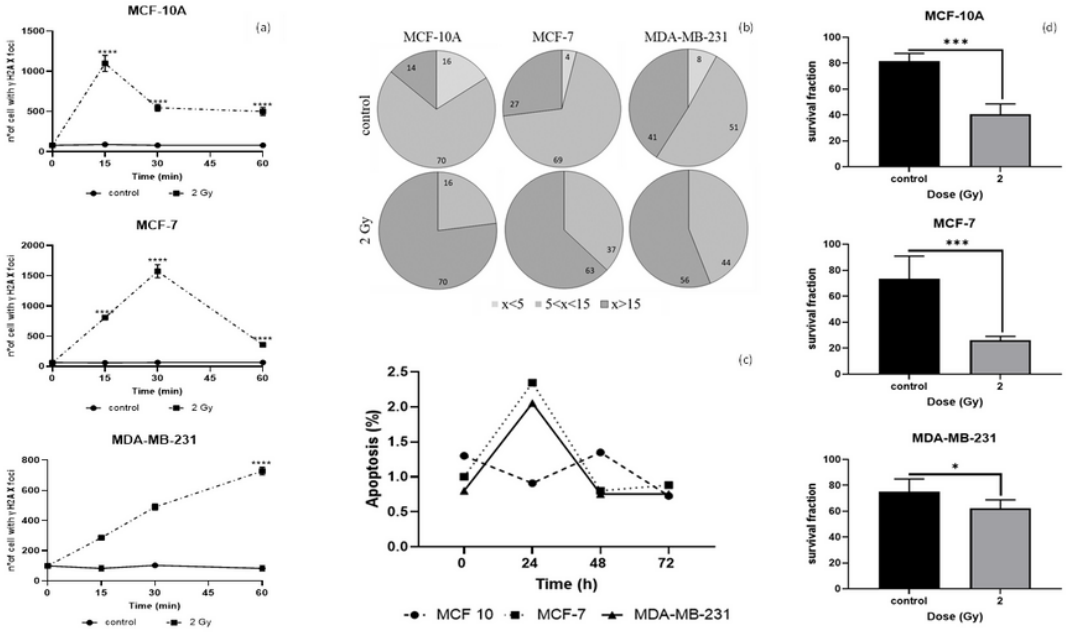

Figure 1

(a) Temporal analysis on the activation of histone γH2AX in breast cells irradiated with dose 2 Gy. The results represent the mean ± SD of at least three experiments, with significance of P<0.001 (****). (b) Analysis of the number of γH2AX foci per cell after 15 min irradiation with dose 2 Gy (x = foci). (c) Apoptosis in breast cells after irradiation with dose 2 Gy. Sample results were normalized to the control. The results represent the mean ± SD of at least three experiments and have a significance of 95%. (d) Clonogenic cell proliferation in breast cell lines irradiated with dose 2 Gy. (a) MCF-10A. (b) MCF-7. (c) MDA-MB-231. The results represent the mean ± SD of at least three experiments and have the significance of P<0.0001 (***), P <0.001 (*), as compared to the respective non-irradiated control.

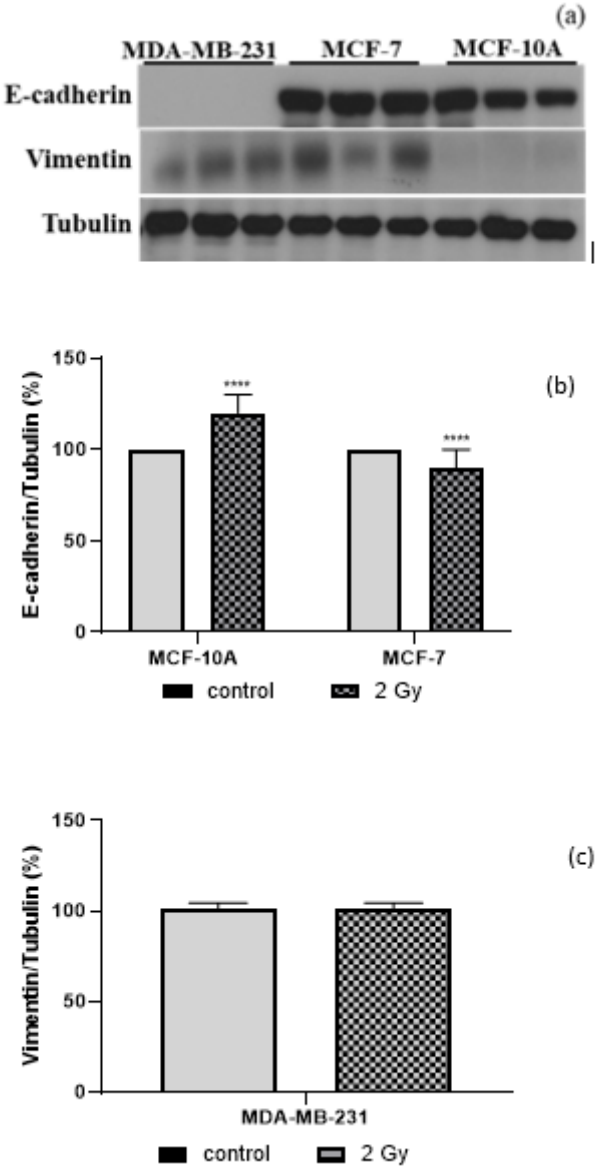

Figure 2

Evaluation of EMT markers, by Western blotting, of the non-tumor, MCF-10A, and tumor cells, MCF-7 and MDA-MB-231, at 72 h after irradiation with 2 Gy. (a) Representation of the bands for the E-cadherin, vimentin, and tubulin proteins. For each cell line, the first lane is the non-irradiated control cells, the second is from cells irradiated with 2 Gy. Densitometry was used to determine the relationship between the expression of (b) E-cadherin and tubulin, and (c) vimentin and tubulin. The results represent the mean + SD of at least three experiments and indicate significance of $P < 0.0001$ (****).

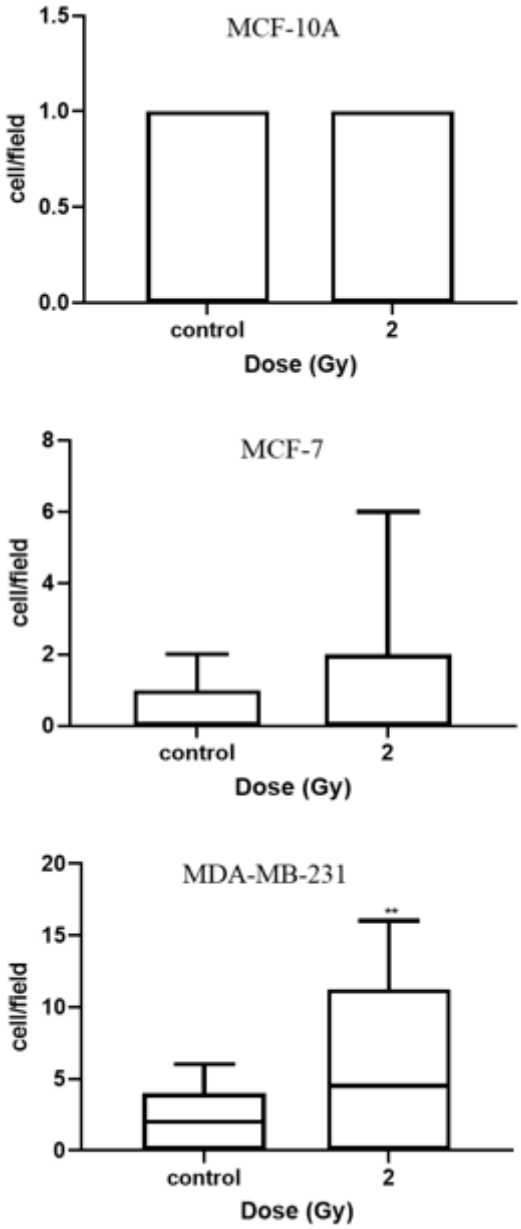

Figure 3

Individual cell invasion. The MCF-10A (a), MCF-7 (b), and MDA-MB-231 (c) breast epithelial cells were irradiated with 2 Gy, and after 72 h the invasion was evaluated using a Boyden chamber. The results represent the mean ± SD of at least three experiments indicate significance of P <0.0001 (**).

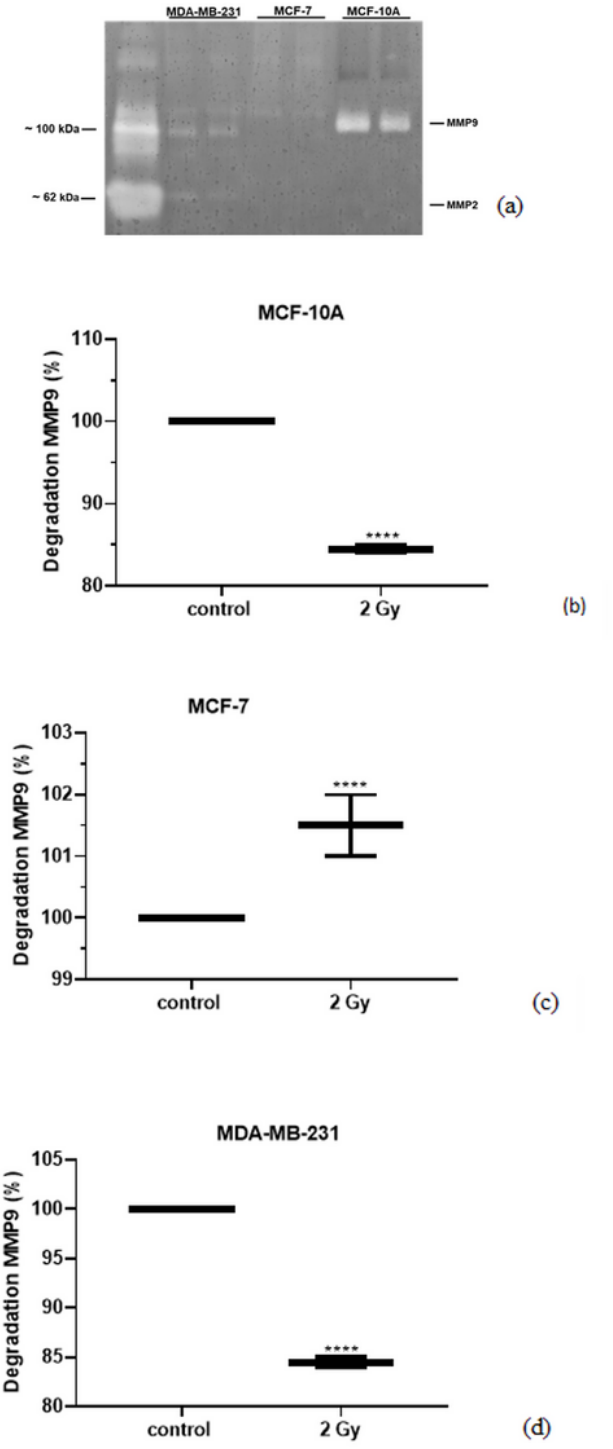

Figure 4

Evaluation of collagenolytic activities compatible with MMP2 and MMP9 metalloproteases. The breast epithelial cells, MCF-10A, MCF-7, and MDA-MB-231 were irradiated with 2 Gy and after 72 h were evaluated by zymography. (a) Representation of the bands for molecular weights compatible with the metalloproteases MMP2 and MMP9. An area of degradation of the control was considered to be 100% and the relative expression of MMP9 was determined by densitometry for (b) MCF-10A, (c) MCF-7, and (d)

MDA-MB-231. The results represent the mean + SD of at least three experiments indicate significance of P <0.0001 (****).

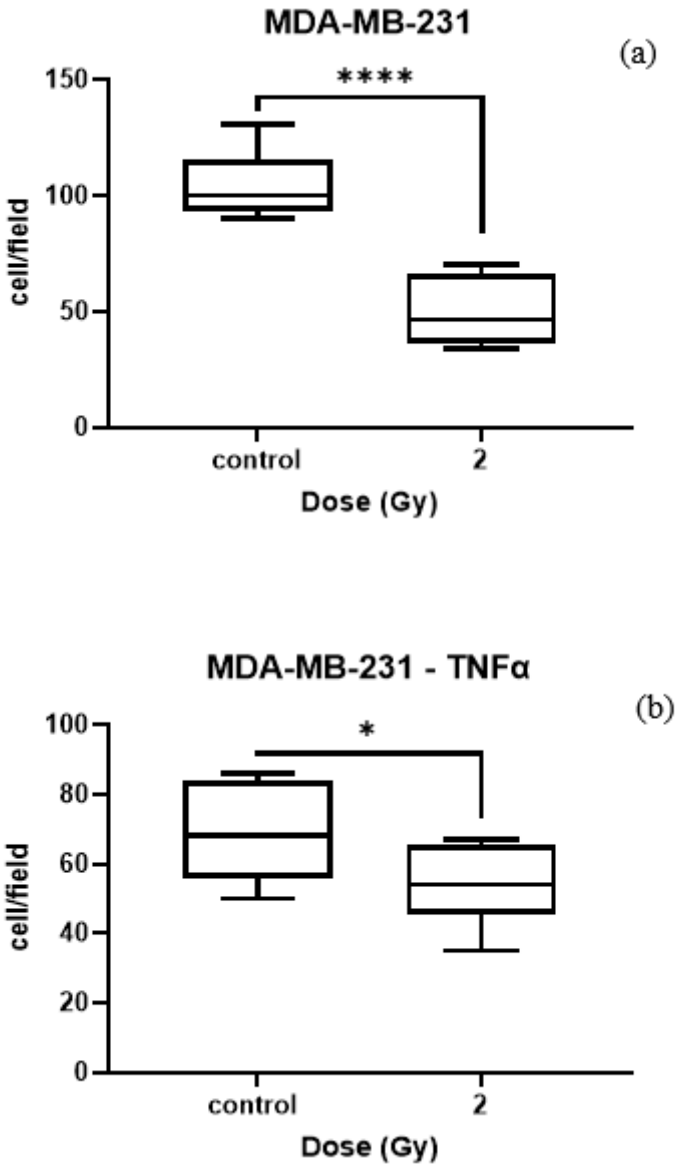

Figure 5

Transendothelial migration assay with the tumoral breast lineage MDA-MB-231 irradiated with dose 2 Gy. The results represent the mean + SD of at least three experiments indicate significance of P <0.05 (*) and P <0.0001 (****).