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Breast cancer cells treated with proton beam: Immunological features and gene signatures

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Summary. — The breast cancer (BC) disease is characterized by a wide heterogeneity at both clinical and molecular level, showing distinct subtypes with different clinical outcomes. Thus, the choice of the therapeutic plan, such as the type of radiotherapy (RT) need to take into account this complexity. Indeed, the proton therapy (PT) shows a medical benefit compared to conventional X-ray RT, as regards the localized delivery of the radiation dose sparing health tissues, but few data regarding proton-induced molecular changes are currently available. The aim of this study was therefore to investigate the production of immunological molecules and gene expression profiles induced by proton irradiation on BC cell lines. Clonogenic survival assay, luminex assay and cDNA microarray gene expression analyses were performed both in the non-tumorigenic MCF10A cell line and in two tumorigenic MCF7 and MDA-MB-231 cell lines, following irradiation with 0.5, 2 and 9 Gy of clinical proton beams. We found that proton irradiation induced gene expression changes useful to define a cell line and dose-dependent gene signatures. The lack of molecular data in the literature can be filled by data here presented that could represent a useful tool to better understand the molecular mechanisms elicited by protons predicting the treatment outcome.

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1. – Introduction

The development of molecularly based signatures by gene expression profiling as biomarkers of disease is a promising strategy also to predict the response to the radiation treatment in the breast cancer (BC) disease [1, 2]. Specific assays, such as OncotypeDx, MammaPrint and Prosigna have been applied so far, modifying the conventional chemotherapy. The same goal can be reached for the evaluation of the response to the radiation treatment that is currently applied without taking account of the molecular characteristics and tumour heterogeneity [3, 4]. In this scenario, the application of the particle therapy requires the knowledge of radiation-induced biological mechanisms as well as of well-known physical beam features. Indeed, the accelerated proton beams showed potential advantages over conventional (photon-electron-based) radiation for BC, with a more localized delivery of the radiation dose on the tumor target, sparing heart and lungs surroundings [5-7]. However, these physical advantages can encourage studies evaluating proton-induced molecular changes, which are currently considered to be underexplored. In our previous studies we demonstrated that irradiation can influence the tumour progression control, since inflammatory factors related to the cell fate were specifically involved after ionizing radiation (IR) exposures [8, 9]. Therefore, the proton therapy (PT) effects need to be investigated at molecular level in order to identify biomarkers and gene signatures in response to the proton radiation treatment in BC.

In this study, the MCF10A mammary non-tumorigenic cell line, MCF7 and MDA-MB-231 BC cell lines were irradiated with protons beam at doses of 0.5, 2 and 9 Gy. The aim was to compare the cell loss of the reproductive capacity, radiation-induced gene expression profile (GEPs), cytokines, chemokines and growth factors produced by the three cell lines with different aggressive phenotypes. Our results highlighted the global molecular and immunological response of tumorigenic and non-tumorigenic BC cell lines to PT.

2. – Materials and methods

2˙1. *Proton irradiation set up.* – Proton irradiations were performed at INFN-LNS (Catania) using the clinical CATANA facility [10-12]. The protons were accelerated by a superconductive cyclotron, the beam was converted into a uniform clinical beam able to cover the entire target region passing through different passive elements, as previously reported [8]. Flasks were irradiated in the upright position facing the collimated beam exit by delivering separate shots: to cover entirely the flask surface, an *ad hoc* remotely controlled positioning system ensured that after each shot, the flask was moved so that the next shot would hit the adjacent area. Cell irradiations were conducted placing the cells in the middle of the Spread-Out Bragg Peak (SOBP, 1 cm width), to simulate a clinical condition, with dose values of 0.5, 2 and 9 Gy and a dose rate of 15 Gy/min.

2˙2. *Cell culture and clonogenic survival assays.* – The human non-tumorigenic breast epithelial MCF10A cell line and the human breast adenocarcinoma MCF7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC) and cultured according to the manufacturer's instructions (ATCC, Manassas, VA) as previously reported [9]. Cells were maintained in culture under standard growth conditions. Forty-eight hours before irradiation, cells were seeded in T25 tissue culture flasks and maintained at subconfluence. The surviving fraction (SF) was evaluated by a clonogenic assay performed as previously described [13, 14]. Untreated cells were used as a control to calculate the plating efficiency (PE). Cells were allowed to form colonies under

normal cell culture conditions for 10–12 days and then were fixed and stained for 30 min with methanol and 0.5% crystal violet (both from Sigma-Aldrich, St. Louis, MO, USA). Colonies with more than 50 cells were counted both manually under a phase-contrast microscope and with a software developed at the CNR-IBFM [15].

2˙3. *Cytokine, chemokine and growth factor analysis by Luminex assay.* – The assay was performed using the Luminex system according to the manufacturer's instructions (BioRad, Munchen, Germany) as previously described [9].

At 24, 48 and 72 h post-irradiation, irradiated conditioned media (ICM) were collected.

Untreated cells for each cell line were grown under the same experimental conditions and the conditioned medium (CM) was collected and used as a control to compare with treated samples. In addition, the blank controls, *i.e.*, the complete media without cells incubated under the same experimental conditions, were collected. The assay tests the following panel of 17 cytokines chemokines and growth factors: IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, IFN-c, TNF-a, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1b (MIP-1b), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony stimulating factor (G-CSF). An eight-point standard curve for every molecule was used and the reported data were normalised with respect to an untreated sample. Data were analyzed using the Bio-Plex Manager software (BioRad). All data are reported as means of duplicate measurements.

2˙4. *Gene expression profiling by cDNA microarray analysis.* – Whole-genome cDNA microarray expression analyses were conducted using the Agilent's Microarray Platform (Santa Clara, CA, USA) as previously described [13, 14]. MCF10A, MCF7 and MDA-MB-231 cells were collected 24 hr after irradiation and the pellet was stored immediately at −80 °C. The total RNA was extracted from the pellet using Trizol and the RNeasy mini kit according to the manufacturer's instructions (Invitrogen). Qualitative and quantitative analyses of isolated RNA, labeling with Cy5 dye and with Cy3 dye, hybridization onto Whole Human Genome 4×44 K microarray GeneChips containing all known genes and transcripts of an entire human genome, were conducted as previously described [13, 14]. Background correction of acquired images, normalization of the gene expression profiles (GEPs) and statistical data analysis were performed using the Feature Extraction and GeneSpring software (Agilent Technologies). Statistically significant differences were calculated by Students t-test and the significance level was set at $p < 0.05$. Differentially expressed genes were identified if they showed a fold change (FC) of at least 2 with a p-value < 0.05 compared to untreated MCF10A, MCF7 and MDA-MB-231 cells used as reference sample. The gene expression data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) [16] (`http://www.ncbi.nlm.nih.gov/geo`). Microarray data also are available in compliance with Minimum Information About a Microarray Experiment (MIAME) standards.

2˙5. *Pathway analyses of GEP lists.* – Pathway analyses were performed using the Reactome tool, in order to understand the biological meaning behind the lists of differentially expressed gene obtained by GEP analysis (`https://reactome.org/`). The most representative and significantly changed pathways and networks were selected and analyzed.

3. – Results

3˙1. *Cell survival..* – The clonogenic assay results revealed varying radiosensitivity according to the breast cell lines used. In particular, the SFs obtained following 0.5, 2 and 9 Gy are as follows: MCF10A cells: 0.78 (± 0.08); 0.44 (± 0.09); 0.050 (± 0.09); MCF7 cells: 0.67 (± 0.13); 0.38 (± 0.15); 0.0385 (± 0.0007); MDA-MB-231 cells: 0.81 (± 0.11); 0.53 (± 0.10); 0.040 (± 0.01), as shown in fig. 1.

3˙2. *Immunological molecules profiles secreted after radiation treatment*. – In this work we studied the cytokines, chemokines and growth factors profiles of non-tumorigenic mammary epithelial MCF10A cells and tumorigenic BC MCF7 and MDA-MB-231 cell lines, produced after proton irradiation with 0.5, 2 and 9 Gy doses, and assayed 24, 48 and 72 h after radiation exposures. The results of these assays are displayed in fig. 2. In the MCF10A cell line, a generalized cytokine down-regulation was observed 24 h post-treatment in each of the dose configuration tested, but a slight increase of cytokines secretion after 48 and 72 h post-irradiation was observed. In particular, all the molecules assayed showed an increased secretion 48 h post-treatment with 9 Gy, whereas a cytokine increase was found for all the three doses delivered at 72 h post-irradiation. These results suggest a time- and dose-dependent secretion of immunological molecules.

Regarding the two BC cell lines, we observed differences in the expression profiles of secreted molecules. The MCF7 cells showed a very low secretion of immunological factors in the ICM after radiation exposure, indeed only 8 out of the 15 immunological factors tested were detectable. These outcomes are in line with the literature data and with the results described by our group following electron radiation treatments in the MCF7 cells [9-17]. On the contrary, in the MDA-MB-231 cell line an earlier activation of almost all the immunological factors was observed in the ICM, effect that became more consistent especially 48 and 72 hours post-treatments. Therefore, MDA-MB-231 cells have revealed the strongest pro-inflammatory secretion profile compared to the other cell lines tested.

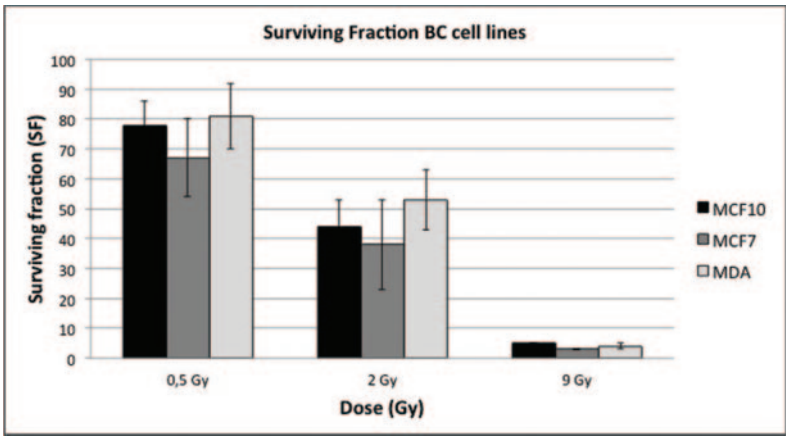

Fig. 1. – Surviving fractions of proton irradiation response in BC cell lines MCF10A, MCF7 and MDA-MB-231. Bar diagrams of the surviving fraction are in percentage. The data are mean ± SD of three independent experiments.

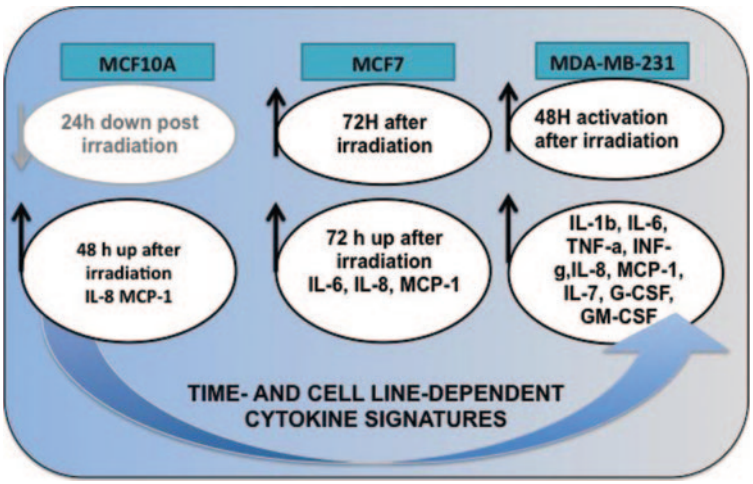

Fig. 2. – Overview of immunological proton irradiation response in BC cell lines MCF10A, MCF7 and MDA-MB-231.

3˙3. *Gene expression profiling (GEP) by cDNA microarray and pathway analysis*. – A two-color cDNA microarray-based gene expression analysis on the three breast cell lines treated with the same proton irradiation set-up reported above (*i.e.*, 0.5, 2 and 9 Gy of proton beam doses) was performed. GEPs of irradiated cells were compared to those of untreated cells, used as reference samples. A comparative differential gene expression analysis showed that multiple genes were significantly altered, at least 2-fold or greater, compared to untreated cells (fig. 3).

In order to select and group transcripts according to their involvement in specific biological pathways the Reactome tool was used [18]. For each cell line analyzed, deregulated

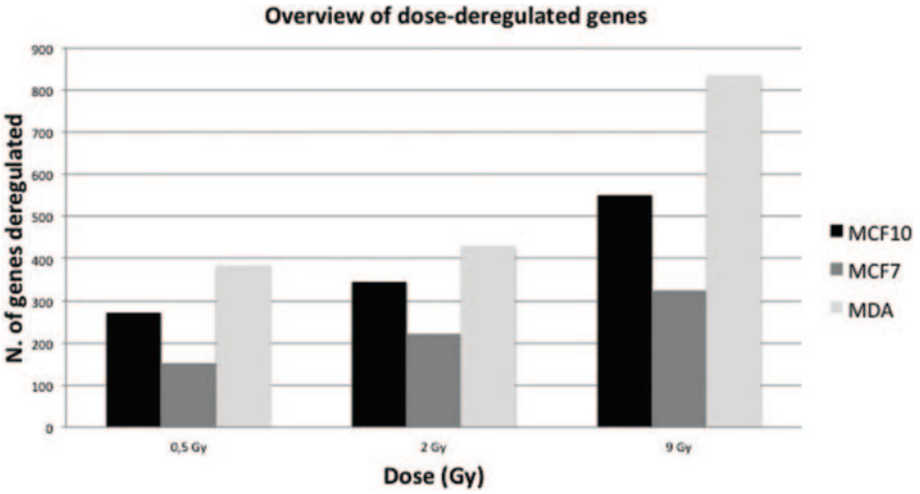

Fig. 3. – Number of deregulated genes in MCF10A, MCF7 and MDA-MB-231 cell lines proton irradiated with doses of 0.5, 2 and 9 Gy.

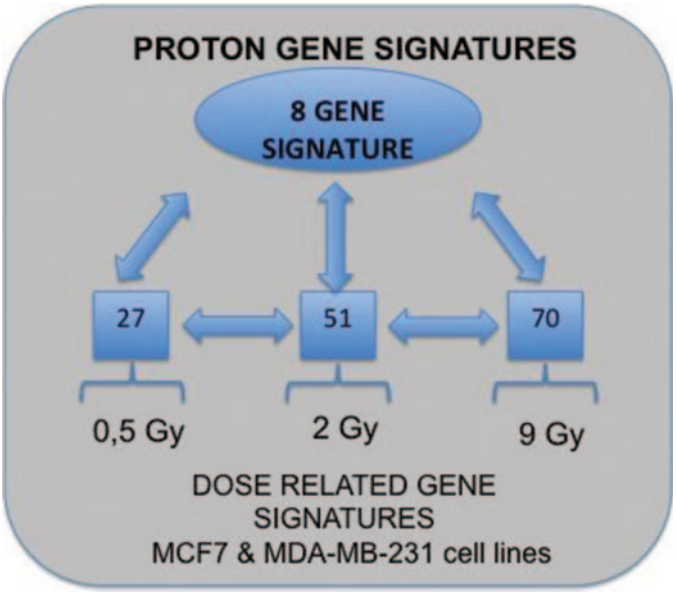

Fig. 4. – Specific 8-gene signature based on 27, 51 and 70 common dose-related genes in MCF7 and MDA-MB-231 cell lines exposed to 0.5, 2 and 9 Gy.

genes were unique and common between all the three dose configuration tested, defining gene expression profiles responsible for the activation of intracellular mechanisms and pathways able overall to respond to stress, such as those induced by IR. In particular, MCF10A cells revealed a gene response to irradiation with the activation of pathways related to inflammation, lipid metabolism and detoxification process. In MCF7 cells, a radiation-induced gene signature showed the involvement of general pathways not directly related to such stress response. The MDA-MB-231 cells showed the activation of pathways specifically induced in response to IR and related to several processes, such as gene transcription, apoptosis, inflammatory and anti-inflammatory response. In addition, we evaluated the differently expressed genes and pathways altered in the MCF7 and MDA-MB-231 BC cell lines, based on the delivered dose. Common and unique genes were deregulated in BC cells after proton irradiation using the same dose, able to define a proton dose-related gene signatures. Finally, a specific gene signature shared between BC cells exposed to the three doses of proton irradiation was identified, as shown in fig. 4.

3˙4. *Discussion.* – Nowadays, the PT showed potential advantages over photon-electron-based conventional RT for BC treatment, with a more localized delivery of the radiation dose on the tumor target, sparing heart and lungs surroundings [5-7]. However, studies evaluating proton-induced cell and molecular changes must be implemented, since this topic is considered almost completely unexplored, in order to the define molecularly based signatures to predict the response to the radiation treatment in BC.

The aim of this study was to identify gene signatures and immunological molecule profiles of breast cell lines treated with PT. Following the proton beam irradiation with doses of 0.5, 2 and 9 Gy on the non-tumorigenic MCF10A and tumorigenic MCF7 and MDA-MB-231 BC cell lines, a panel of cytokines, chemokines and growth factors was

analyzed by the Luminex assay. In addition, the whole genome gene expression profiling by cDNA microarray analysis was performed.

Time– and cell-line–dependent cytokine signatures were observed for all the three breast cell lines tested. In particular, among the three cell lines, MDA-MB-231 cells expressed immune response profiles in a time-dependent manner, with a strong activation of pro-inflammatory factors. The secretion IR-induced of cytokines, chemokines and growth factors can modulate the immune system towards survival/cell death balance and/or the senescence process in a highly specific way. These results are in line with those obtained by our group on the same breast cell lines following the electron beam irradiation [9].

We also analyzed GEPs induced by proton irradiation with the same doses, describing specific dose– and cell-type–dependent gene signatures. Each cell line revealed a specific alteration of a set of factors controlling several cellular processes, such as the cell death processes and inflammation. Through inflammation these cells can regulate intracellular mechanisms involved in stress radiation cell response. Among the GEPs, we selected 8 common deregulated genes (8-gene signature) in BC cells after 0.5, 2 and 9 Gy, which could represent specific biomarkers of the proton cell response.

In conclusion, our data confirm that the cell response to radiation is highly the heterogeneous resulting cell-line– and dose–dependent. The knowledge of radiation-induced molecular mechanisms is very useful for the future realization of personalized proton therapy protocols in combination with targeted therapies.

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