TRABAJO FIN DE MÁSTER

INFLUENCE OF IONIZING RADIATION IN miRNAS LEVELS OF BREAST CANCER STEM CELLS

Máster en Avances en Radiología Diagnóstica y Terapéutica y Medicina Física

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I. ABSTRACT

Objetive: Study the difference in expression of specific microRNAs in hypoxia and DNA damage response in breast cancer stem cells after receiving radiation doses versus a control that has not been radiated.

Methods: Breast adenocarcinoma cell line MDA-MB-231 was choosing for the study. CSCs of MDA-MB-231 isolation were performed by detecting activity of ALDH by flow cytometry, obtaining population positive (sorter+) and population negative (sorter-). Then cultivate these populations in *spheres culture medium* including general population (no sorter). After 72h cells were radiated by different doses and incubated 24h for later isolation RNA. To study the expression of two selected miRNAs was carried out a RT-qPCR.

Results: miRNAs examined were as follows: Hsa-mir 210 and Hsa-mir 24 together control GADPH standardized. When no sorter cells are radiated with 2 Gy the expression of mir 210 was 0,41 versus control when they are radiated with 6 Gy observed was 3,23. The expression of mir 210 in sorter+ was 91,40 in non-radiated cell, while when were radiated to 2 Gy and 6 Gy was 26,42 y 10,88 respectively. The expression of mir 24 in no sorter cells was 1,49 and 0,31 when were radiated to 2 Gy and 6 Gy decreases to 0,31 respectively. mir 24 expression in sorter+ non-radiated was 3,08, for 2Gy was 1,11 and for 6 Gy was 0,87.

Conclusions: Ionizing radiation affects the expression levels of miR 210 and miR 24 both in non-sorted population as in ALDH+ BCSCs. Ionizing radiation decreases miR 210 and miR 24 expressions in a dose-dependent manner in ALDH+ BCSCs.

II. INTRODUCTION

1. **Background**

Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). It is the most common cancer in women both in more and less developed regions with slightly more cases in less developed (883,000 cases) than in more developed (794,000) regions. Incidence rates vary nearly four-fold across the world regions, with rates ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 96 in Western Europe (GLOBOCAN).

Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) and while it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer. The range in mortality rates between world regions is less than that for incidence because of the more favourable survival of breast cancer in (high-incidence) developed regions, with rates ranging from 6 per 100,000 in Eastern Asia to 20 per 100,000 in Western Africa (GLOBOCAN).

In the province of Granada were reported, 19.634 cases corresponding to residents, in the period 2008-2011 first diagnosed with cancer during those years, representing an average of 4,908 new cases per year. In the period 2008-2011, the average annual incidence of breast cancer in Granada presented gross rates of 1 and 94 per 100,000 men and women, respectively (1 new case was diagnosed per 100,000 men and 94 new cases per 100,000 women) (Registro del cancer de Granada).

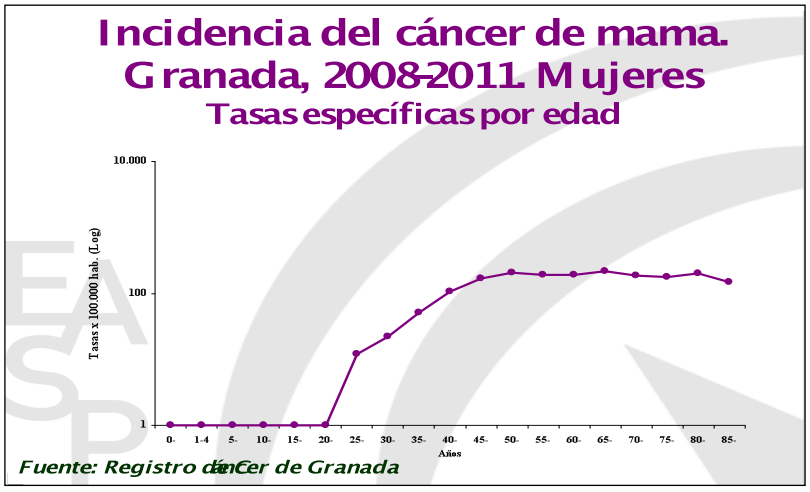


Figure 1: Breast cancer incidence 2008-2011, from Registro del cancer de Granada.

Cancer is a disease that primarily affects older adults. In both genders, the specific cancer incidence rates are higher with increasing age, following a different pattern in men and women and produced an increase from the 50. 36% of cases of breast cancer occur in women in the age group 65 years and approximately half of the cases occur in women aged 45-64 years (Table 1) (Registro del cancer de Granada).

Relative frequency

Years	00-14	15-44	45-64	65 & more
Women	0	16,0	47,9	36,1

Table 1. Breast cancer incidence in Granada, 2008-2011.

2. Cancer stem cell (CSCs)

The stochastic cancer model postulates that one or more tissue cells acquire a mutation and through an uncontrolled division process, are new genetic alterations are accumulated leading the selection of the fittest clones. According to this model, any cell of the tumor would be able to maintain and expand the tumor as well as to give rise to new tumors. Conversely, the hierarchical model of the cancer stem cell (CSC) implies the existence of a source cell in the tumor stem cell properties, able to proliferate and maintain indefinitely the growth due to its self-renewing ability. In this model, only the population of CSC has the ability to generate and maintain tumor, unlike other cells that form do not have that ability (Figure 2) (Eguiara et al., 2012).

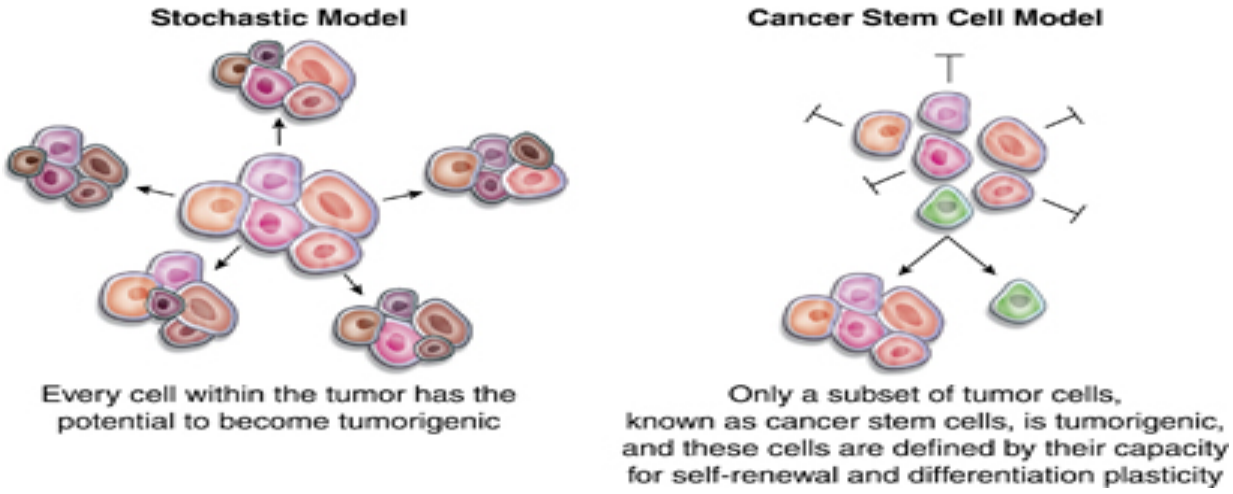


Figure 2: Stochastic model and Cancer Stem model picture from (Sasha D Girouard and George F Murphy, 2011)

Solid tumors similar to aberrantly developed organs and tissues are composed of many types of cells including neoplastic cells, supporting vascular cells, inflammatory cells, and fibroblasts. The majority of cells in bulk tumors have limited self-renewal ability and are non-tumorigenic. Only a small subpopulation of cancer cells is long-lived with the ability of extensive self-renew and tumor formation. This small population is called cancer stem cells (CSCs), or cancer initiating cells (CICs), or tumor stem cells (TSCs) (Han et al, 2013).

The first strong *in vivo* evidence in support of the CSC concept came from classical implantation studies in human leukaemia by Bonnet and Dick in 1997. They used fluorescence-activated cell sorting (FACS) to isolate a specific cell population from acute myeloid leukaemia (AML) patients that were able to initiate AML following implantation into non-obese diabetic mice with severe combined immunodeficiency (NOD/SCID). The leukaemia-initiating cells were defined by expression of the cell surface antigen CD34 and displayed self- renewal, differentiative and proliferative capacities similar to normal haematopoietic stem cells. The first evidence for the existence of CSCs in solid human tumours came from studies in breast cancer (Figure 3)(Ablett et al., 2012).

The CSCs have the characteristics of stem cells such as the unlimited self-renewal capacity, proliferation and differentiation to different cell lines features. Among the highlights of the CSCs that make fundamental in the development of tumors can be found tumorigenic, drug resistance, radiotherapy resistance, recurrence and metastasis.

While CSC theory appears to be attractive, it has its own pitfalls. A number of molecular pathways have been proposed to play a role in maintenance of CSC phenotype which is further complicated by the observation that none of the molecular markers of CSCs seems to be universally relevant. Most of the research is cancer specific, and the factors/pathways relevant in one cancer may or may not be relevant targets for therapy in other cancers (Ahmad et al., 2012).

Cancer type	CSC markers	% of CSC cells in tumor	Efficiency of tumor formation[a] (transplanted cells)
Lung	Sca-1^{+}CD45^{-}PECAM^{-}CD34^{+}	0.008–0.064	ND
	CD133+	0.3–22.0	ND
Colon	CD133	1.8–24.5	83% (500)
	EpCAM^{high}CD44^{+}	0.03–38.0 (mean 5.4)	75% (200)
	ALDH1	3.5±1.0	ND (25)
Breast	CD44^{+}EpCAM^{+}CD24^{-}Lineage^{-}	0.6–5.0	100% (1,000)
	CD44^{+}CD24^{-}ALDH1^{+}	0.1–1.2	100% (20)
	Thy-1^{+}CD24^{+}CD45^{-}	1.0–4.0	80% (50)
Prostate	CD44$^{+}\alpha_2\beta_1{}^{high}$CD133^{+}	0.1–0.3	ND
Brain	CD133	19–29	100% (100)
	CD15	5.6–70.5	100% (1,000)
	SP	0.15–1.2	ND
Melanoma	ABCB5	1.6–20.4	50% (100,000)
Pancreatic	CD44^{+}CD24^{+}EpCAM^{+}	0.2–0.8	50% (100)
	CD133	0.7–3.2	80% (500)
Liver	CD90^{+}CD45^{-}	0.7–6.2	50% (5000)
Head and neck	CD44	0.1–41.7	50% (5,000)
Skin	CD34	13.0–20.0	50% (1,000)
Ovarian	CD44^{+}CD117^{+}	0.1–0.2	100% (100)
	CD133^{+}	0.3–35.0 (median 8.9)	83% (500)
Bladder	CD44	3.1–36.3	50% (1,000–3,000)
Mesenchymal	SP	0.07–10.5[b]	55% (100)
Acute myeloid leukemia (AML)	CD34^{++}CD38^{-}	0,02–2,00[c]	100%[d] (100,000–500,000)
Acute lymphoblastic leukemia (ALL)	CD34^{+}CD10^{-}/CD34^{+}CD19^{-}	8±4/3±1[c]	100%[d] (50,000–200,000)

Figure 3: Markers used for the identification and isolation of cancer stem cells from different cancers (Ablett et al., 2012).

3. Breast cancer stem cell (BCSCs)

Al-Hajj and colleagues prospectively isolated a tumorigenic population of cells from primary human breast cancers using FACS based on the ESA^{+}/ CD44^{+}/CD24^{low}/lineage^{-} phenotype. (Ablett et al., 2012). The CD44+/CD24- phenotype has been used extensively to identify and isolate cancer cells with increased tumorigenicity. In addition to cell surface markers, other expresión based methods of CSC enrichment have been developed. Aldehyde dehydrogenase (ALDH) activity has been identified as a method of enriching for normal human breast stem and CSCs. Furthermore, by combining ALDH activity with CD44^{high}CD24^{-} expression, the CSC fraction was refined further compared to either method alone. Interestingly, the ALDH^{-}/CD44^{high}/CD24^{-} population was not enriched for CSCs demonstrating that the CD44^{high}CD24^{-} population retains significant heterogeneity (Figure 4) (Owens and Naylor, 2013).

Due to the intra and inter-tumor heterogeneity in cancer, it is possible that CSCs from different tumors have distinct expression profiles. Thus, isolating CSCs by function and detailing their expression profiles may prove extremely valuable where traditional markers fail.

	CSC isolation technique	Source	Type of treatment	Enrichment or preferential survival
Chemo-resistance	$ESA^{+}CD44^{+}/CD24^{low}$	Cell lines	5-Flurouracil or Paclitaxel	*In vitro* – FACS 5–30-fold ↑
	$CD44^{+}/CD24^{-}$	Cell lines	Doxorubicin-selected MCF7s	*In vitro* – FACS 30% ↑ and ↑ tumours *in vivo*
	$CD44^{+}/CD24^{-}$ mammosphere assay	Clinical samples	Neoadjuvant 5-flurouracil, epirubicin & cyclophosphamide	*In vivo* – FACS 9.5-fold ↑ *In vivo* MS ↑ 0.5% to 5.9%
	$CD44^{+}/CD24^{-}$ mammosphere assay	Clinical samples	Neoadjuvant docetaxel or doxorubicin & cyclophosphamide	*In vivo* – FACS ↑ 5% to 14% *In vitro* MS ↑ 5-fold
	$CD44^{+}/CD24^{-}$/mammosphere gene expression signature	Clinical samples	Neoadjuvant docetaxel	*In vivo* – ↑ gene signature following chemotherapy
Radio-resistance	Mammosphere assay	Cell lines	Radiation – single & fractionated	*In vitro* – clonogenic assay 2-fold ↑ in survival
	$CD44^{+}/CD24^{-/low}$	Cell lines	Radiation – fractionated	*In vitro* – FACS, up to 3-fold ↑
	Sca1+ BALB/c mice	Mouse	Radiation – single dose	*In vivo* – FACS 3-fold ↑
	$CD24^{+}Thy1^{+}Lin^{-}$ MMTV-Wnt1	Mouse	Radiation – fractionated	*In vivo* – FACS, 2-fold ↑
	$Lin^{-}CD29^{+}CD24^{+}$ P53-nullmouse	Mouse	Radiation – single dose	*In vitro* – clonogenic assay up to 10-fold ↑

Figure 4: Summary of preclinical, animal model and clinical evidence of breast cancer stem cell (CSC) resistance to chemotherapy and radiotherapy (Ablett, 2014).

4. Radioresistence

The CSC is thought to be directly responsible of the relapse in a tumor process after having received radiotherapy (RT). Radiation *in vivo* enriches the fraction of cells expressing CSC markers, which also have an enhanced self-renewal capacity and tumorigenicity compared to the tumor bulk. In addition, sorted CSC cells from different types of tumors survive such treatments in culture much better than unsorted or negative cells (Garvalov & Acker, 2010).

There are numerous studies on different tumor types, and CSC markers associated with them, supporting this hypothesis. It has been shown that CD133 positive cells, mostly associated brain cancer, are found in a greater proportion after receiving a fractionated radiation both *in vitro* and *in vivo* (Bao 2006). The population characterized as $CD44^{+}$/ $CD24^{-low}$ (breast cancer) is resistant to fractionated radiation treatment, keeping intact its capacity

for self-renew and being more aggressive and better able to reproduce the tumor and initiate metastasis (Phillips 2006, Lagadec 2010). Cells with high ALDH1 character a subpopulation with increased radioresistance, whose inhibition resulted in a sensitization to the same (Mihatsch 2011, Croker 2012).

The mechanisms by which CSCs may be resistant to RT can be framed into four groups: systems repair of DNA damage, redistribution of the cell cycle, cells tumor repopulation, and level of intratumor hypoxia (Figure 5).

i) The ability to repair DNA lesions when CSCs did not reach the threshold to be lethal. Among different control points altered in this process allowing survival are such activation of ataxia telangiectasia mutated (ATM) in $CD44^{+}/CD24^{-/low}$ cells (Yin 2011). Enrichment of polycomb group protein BMI1 in CD133 positive cells (Facchino 2010). The activation of the checkpoint kinases 1/2 which also leads to a survival of CD133 positive cells (Bao 2006). More evidence suggesting increased DNA damage repair capacity in CSCs came from the observation of γ-H2AX induction in BCSCs. γ-H2AX is the phosphorylated form of H2AX which is the gene encoding the histone H2A variant, H2AX. γ-H2AX is the sensitive surrogate of DNA double strain break, which can be quantified after radiation. It has been demonstrated by several groups that CSCs have lower γ-H2AX foci after radiation in human BCSCs. Wnt/β-catenin signaling pathway is a network of proteins essential in embryogenesis, stem cell maintenance and survival. More recently, this pathway has also been shown to be important in CSCs and their responses to DNA damages. One transcriptional target of β-catenin is survivin which can promote survival in response to apoptotic stimuli. Survivin has been linked to radiation resistance and it has been demonstrated that suppression of survivin with a small molecule inhibitor may sensitize the radiation effects and induce more apoptosis (Chumsri, 2013).

ii) Cell cycle phase distribution is important for sensibilisation to radiation. Cells in mitosis are more sensitive to radiation and those found in late S phase stronger, thus making a redistribution of the cell cycle resulting in a population in S phase. So if the radiation dose administered in the appropriate moment may act on the most sensitive stage making the treatment most effective (Pawlik 2005, Withers 1975).

iii) Hypoxia is a fundamental pathophysiological phenomenon strongly associated with the development and aggressiveness of various solid malignancies and also implicated in radioresistance (Brunner 2012). The oxygen is a key requirement for any biological process,

so that the concentration thereof is controlled with great precision. An imbalance that results in a hyperoxia induces formation of reactive oxygen species (ROS) that can go causing cell death, and hypoxia can trigger the activation of pro-apoptotic pathways and pro-angiogenic (Brunner 2012). In the case of the ROS a study based on $CD44^{+}$/ $CD24^{-/low}$ cells. They were grown in suspension, a higher level of ROS was found compared to monolayer culture. After receiving a radiation dose an increase thereof in monolayer culture was shown but not in the suspension, which suggests that a large removal control of ROS lead to cell death avoidance caused by radiation (Phillips 2006). Cellular responses to hypoxia are commonly regulated by the hypoxia inducible factor (HIF). The higher level of HIF in the tumor can be correlated with the level of oxygen and it has been shown to correlate with the radiation resistance (Keith, 2007). There are two isoforms, HIF1a and HIF2a with differential expression in CD133 glioma cells, while HIF2a significantly was present in the CD133 positive population HIF1a was detected in the complete pool of cells, but was stabilized under conditions of hypoxia, and HIF2a overexpression way further related with poor prognosis (Li, 2009).

As tumors develop, the requirement for oxygen increases, leading to regions of hypoxia. Hypoxia causes activation of HIFs, which enable to cells to adapt to the low-oxygen environment. Hypoxic culture conditions (1% O2) induced an increase in the $ALDH1^{+}$ proportion in breast cancer cell lines (Conley et al., 2012). Moreover, CSCs were enriched in hypoxic regions of tumor xenografts compared with normoxic regions (Conley et al., 2012).

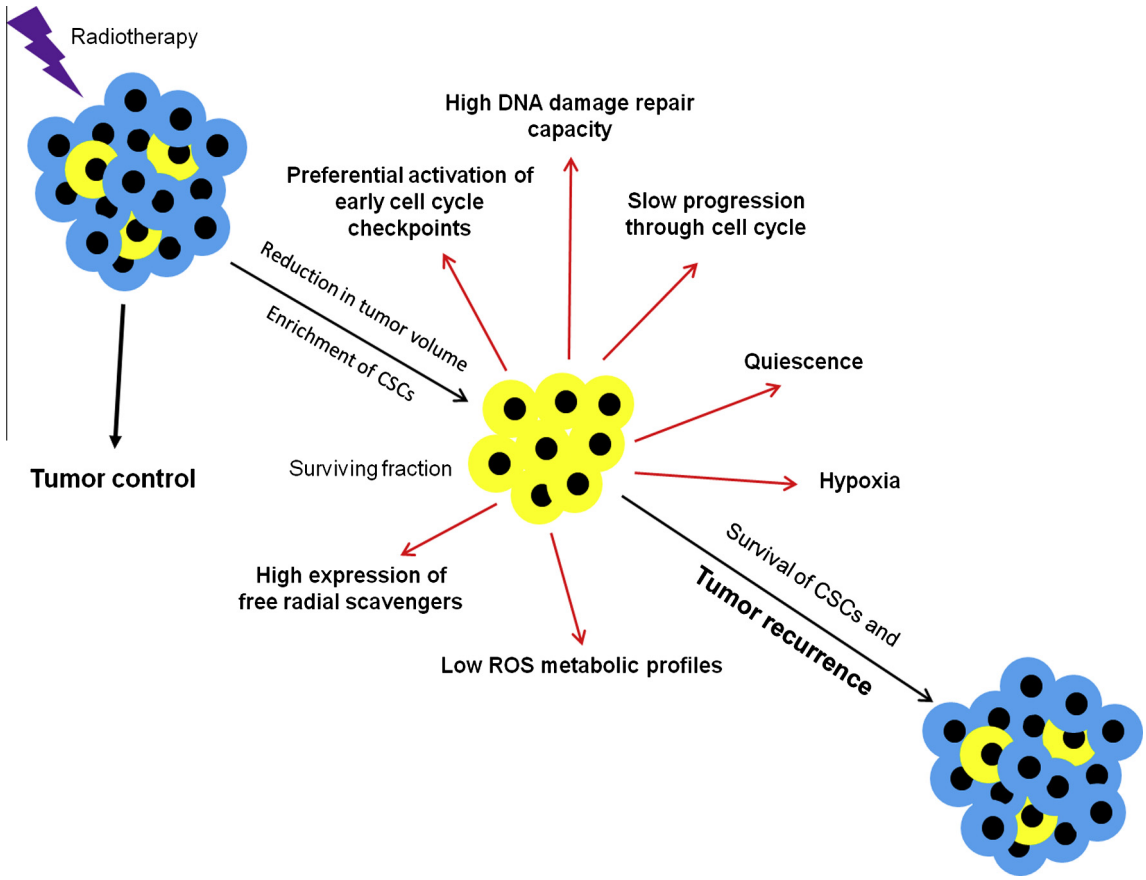


Figure 5: CSCs evade radiation-induced cell death through the activation of survival pathways (Marie-Egyptienne et al, 2013).

5. Metalloproteinases (MMPs)

MMPs are a family of zinc-dependent endopeptidases secreted as zymogens by tumour and stromal cells. They play a crucial role during tumour invasion and metastasis through their ability to degrade matrix proteins and are also involved in early steps of mammary carcinogenesis (Chabottaux & Noel, 2007). ECM remodelling is controlled by MMPs and endogenous tissue inhibitors of MMPs (TIMPs). Interactions between TIMPs and MMPs are responsible for the regulation of MMP activity, and the increased production or activity of MMPs has very often been linked to malignancy. Hence, the balance between MMPs and TIMPs plays a crucial role in cancer progression and metastasis. Several studies have reported increased levels of different MMPs in diverse cancer tissues including breast tumours (Artacho-Cordón, 2012).

Different types of MMPs have been studied seeing that they have different functions in the process of carcinogenesis like invasion and metastasis, tumor growth, inflammation, angiogenesis, apoptosis, epithelial mesenchymal transition and pre-metastatic niche formation (Figure 6) .(Artacho-Cordón et al, 2012).

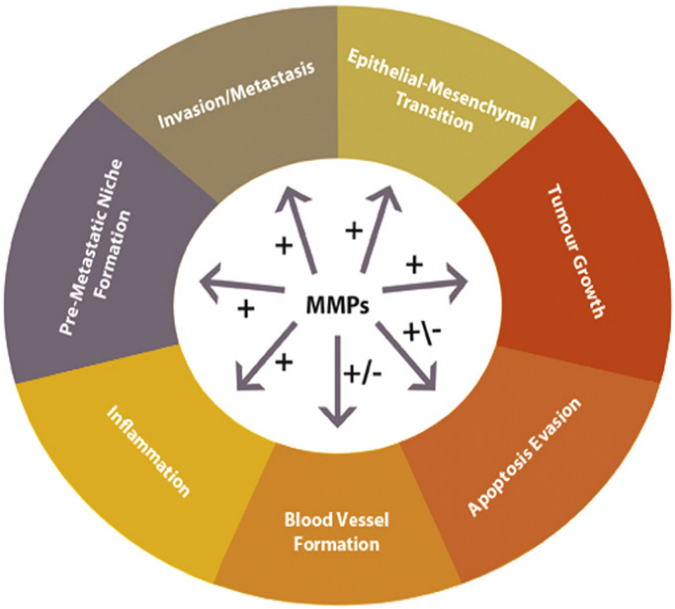


Figure 6. Role of matrix metalloproteinases in carcinogenesis. MMPs modulate tumour progression in a crucial manner (Artacho-Cordón et al, 2012).

On the other hand, MMP activity is related to the level of oxidative stress in cells, so that the free radicals generated during irradiation of a tissue can disrupt the normal regulation (Klaunig & Kamendulis, 2004). It has shown an increase in invasive capacity of breast tumor cells after irradiation of the basal membrane due to the increased expression of MMPs (Paquette et al., 2007). Actived MMPs by ionizing radiation (IR) may be involved in tumour growth, neovascularisation and dissemination, suggesting an increased risk of metastasis in survivor cells (Artacho-Cordón et al, 2012).

6. MicroRNAs

MicroRNA (miRNA) is a small endogenous non-coding RNA molecule that regulates gene expression in transcriptional and post-transcriptional specific sequences. In 1993, Victor

Ambros and colleagues discovered miRNAs studing the gene of the protein lin-14 in C. elegans development. Subsequent studies revealed that the 21 nucleotides transcript is complementary to the 3' untranslated region (3'UTR) of lin-14 and, most interestingly, negatively regulates the expression of lin-14. Initially, these findings were not appreciated by the scientific community, because it was believed to be a rare process occurring only in C. elegans. However, in 2000, another such 22 nucleotides non-coding RNA named as let-7, was identified in C. elegans . The discovery of two miRNAs, lin-4 and let-7, in Caenorhabditis elegans suggested that miRNAs are important regulators of embryonic development and stem cell functions in mammals (Lee et al., 1993).

Since then, thousands of miRNAs have been identified many of which have been shown to play important roles in a variety of biological processes, like development, differentiation, apoptosis, proliferation, and cell death. It is now clear, that miRNAs together with other non-coding RNAs (long non-coding RNAs, small nucleolar RNAs and ultraconserved regions) contribute to carcinogenesis. A miRNA deregulation is involved in initiation and progression of cancer. They modulate the expression of their target genes by either degrading their target mRNA or inhibiting their translation through pairing of miRNA sequences to complementary bases on the target mRNA. MiRNAs can function both as oncogenes and as tumor suppressors and are considered as emerging potential candidates for improved cancer diagnosis, prognosis and therapy (Schwarzenbacher, 2013).

Biogenesis of miRNAs is a complex process. miRNAs are transcribed for the most part by RNA polymerase II as long primary transcripts characterized by hairpin structures (pri-miRNA), and are processed in the nucleus by RNase III Drosha into 70–100 nucleotide long precursor miRNAs (pre-miRNAs) in combination with cofactors such as DGCR8, an evolutionarily conserved protein that interacts with prolinerich peptides through its WW domain (Lee et al., 2004).

Pre-miRNAs are then exported to the cytoplasm by the nuclear export factor Exportin 5 and the Ran-GTP cofactor, where they are cleaved by another RNase III type enzyme, Dicer, to generate a ~22 nt RNA duplex. One strand of the miRNA duplex is usually selected as a mature miRNA, and is assembled into an RNA induced silencing complex (RISC), while the other strand is degraded. The RISC complex interacts with the Argonuate proteins and they collectively act to silence target mRNAs. The mechanism of mRNA silencing is dependent on the degree of complementarity. In the case of completely aligned miRNA/mRNA pairs,

degradation occurs as a consequence of endonucleolytic cleavage resulting from the proteins bound to RISC. However, in the case of most animals, perfect complementarity rarely exists, and as such the target mRNA cannot be degraded by this mechanism. Consequently, these imperfect miRNA/mRNA pairs are either translationally repressed or silenced independent of the above-mentioned mechanism. The complementarity to the messenger RNA within positions 1–8 of the microRNA is the most crucial parameter for regulation, and binding sites on the mRNA are located in most instances on the 3' untranslated region (UTR) (Figure 7) (Abba et al, 2014).

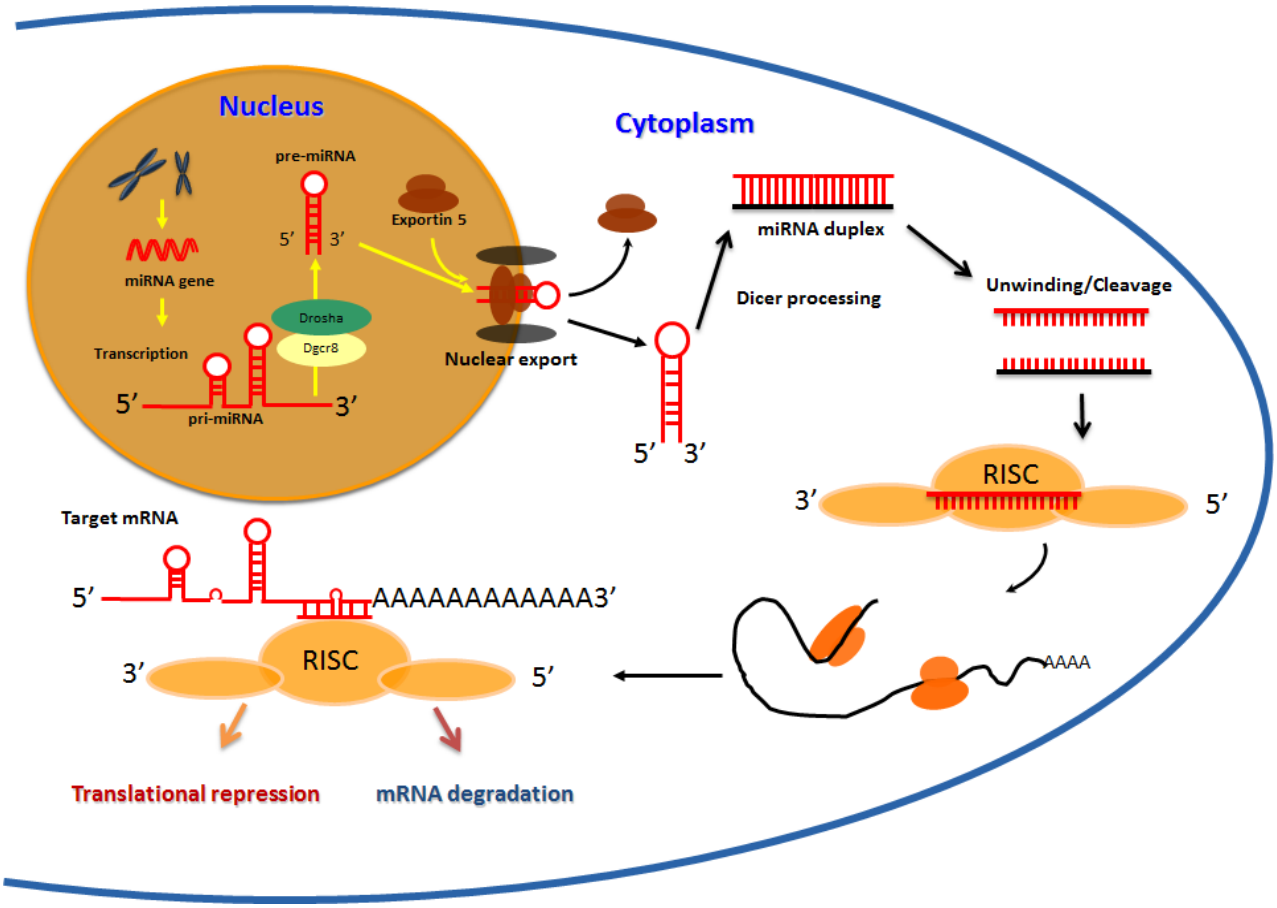


Figura 7: miRNAs biogenesis (Abba et al, 2014).

Radiotherapy, through ionizing radiations, aims to cure tumors determining damages by the production of free radicals at various levels in the neoplastic cell.

Cellular response to IR simultaneously activates a number of signalling pathways mediating the DNA damage response (DDR). Failure to repair radio-induced damages leads directly or indirectly to cell death, also changes ROS levels and hipoxia state. Complete recovery from these damages affects radiosensitivity, under physiological conditions that avoids the tumorgenesis, while in a clinical setting it determines tumor resistance to RT. miRNAs are deeply involved in the regulation of this processes. This issue promoted a high interest in the potential oncological applications of enhancing efficacy of RT through modification of tumor radiosensitivity. also useful to understand and manage treatment-related toxicity. Finally, miRNAs could be useful for monitoring and understanding professional and accidental exposures to IR (Cellini, 2014).

III. HYPOTHESIS AND OBJECTIVES

miRNAs are important in the regulation of metastasis, DNA damage and hipoxia after a radiation therapy. Moreover, BCSC subpopulations confer resistance to this physical agent because of: i) the highest repair rate of DNA damage, ii) the lower levels of ROS and iii) the increased expression of MMPs among others. So, BCSCs that are not eliminated by radiation would stimulate the recurrence, invasion and metastasis processes. Since radiation enriches the fraction of CSCs subpopulation, this physical agent may modulate specifics miRNAs of these cells.

The objective proposed in response to the previous working hypothesis is:

To study the difference in expression of specific miRNAs related with metastasis, hypoxia and DNA damage response in breast cancer stem cells after receiving radiation doses versus control non-radiated cells.

IV. MATERIALS AND METHODS

1. Cell culture:

We used the MDA-MB-231breast adenocarcinoma cell line (ATCC HTB-26 reference), derived from a woman of 51 years old and characterized as a triple negative cell line (ER^-/PR-/HER2-).

1.1 Culture conditions:

Cell culture was performed under sterile conditions in laminar flow closet (Micro-V, Telstar, Spain). Cells were grown in 75 or 25 cm^2 culture flasks in a CO^2 incubator (CO^2 Incubator Steri-Cult, Thermo Electron Corporation, Waltham, MA, USA) at 37°C and 90% humidity. When reaching 80-90% confluence, cells were detached from the surface using a trypsin-EDTA solution and subsequently washed in culture medium with FBS to inactivate the trypsin by centrifugation at 1500 rpm for 5 minutes. Finally they were replanted in new culture flasks.

Cells were growth in DMEM (Dulbecco's Modified Eagles Medium, Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, Lonza, Basel, Switzerland), inactivated at 56°C for 30 minutes and supplemented with 1% of a solution of penicillin / streptomycin (10,000 U / ml penicillin G and 10 mg / ml streptomycin, Sigma Chemical Co, St Louis, MO, USA).

1.2 Cell freezing method:

The maintenance of cell lines over long periods of time was achieved detaching cells from culture flasks by trypsin-EDTA, centrifuged at 1500 rpm for 5 min in medium supplemented with FBS and then the cell pellet was resuspended in freezing medium at 0.5 x 10^6 cells, and then introduced immediately in cryotubes. Then, cryotubes were storage in the freezer at -80°C for 24 h. Subsequently, for storage long time were transferred to liquid nitrogen at -196°C.

Freezing Medium: Fetal bovine serum (FBS) inactivated by wet heat at 56°C for 30 min and dimethyl sulfoxide (DMSO) at 5% (as recommended by the ATCC).

1.3 Cell thawing method:

Cell lines stored at -80°C were thawed in moist heat at 37°C and immediately resuspended in saline phosphate buffered saline (PBS) and centrifuged at 1500 rpm for 5 min to remove residual DMSO (two washes). The pellet was resuspended in culture medium to finally make the seeding of the cells in culture flasks of 75 cm^2.

1.4 Cell count:

For cell counting, after taking off and the cells centrifuged as previously described, the pellet was resuspended in culture medium. For crop density, a Neubauer chamber was used. Cells that appeared in each quadrant of the chamber were counted, the number obtained was divided by four and multiplied by 10,000 and, finally, it was applied the dilution factor used to taking the sample volume where the cells were resuspended.

2. Cancer stem cells isolation:

CSCs isolation was performed by detecting activity of the aldehyde dehydrogenase (ALDH) by flow cytometry in the breast cancer cell line MDA-MB-231. Therefore, the Aldefluor kit (StemCell Technologies, Vancouver, Canada) which is based in the quantification of the ALDH enzyme activity by metabolizing a substrate, BODIPY-aminoacetaldehyde (BAAA), resulting in a fluorescent product, the BODIPY-aminoacetate (BAA). The latter is accumulated in the interior of the cell, which allows the detection and subsequent separation, by flow cytometry. These ALDH + cells were separated using a flow cytometer FACS Aria III being selected cells had an increased fluorescence for FITC fluorochrome. The dietilbenzaldehide (DEAB) was used as negative control, since it is a potent inhibitor of ALDH activity

The cells were detached using trypsin-EDTA and harvested in cytometric tubes at a density 1.000.000 cells/mL. The cells were incubated at 37 ° C for 30 min, and centrifuged. The cell pellet was resuspended in 1 mL of Assay Buffer Aldefluor and maintained at 4 ° C until analysis.

For the cultivation of tumor stem cells spheres medium was used: DMEM/F12 (Sigma Chemical Co, St Louis, MO, USA), supplemented with 1X B27 (Gibco, Big Cavin, OK, USA), 1 μg / ml hydrocortisone, 4ng/ml heparin, 10 μg / ml insulin, 10 ng / ml EGF, 20 ng / ml of FGF, and 1% of a solution of penicillin / streptomycin (10,000 U / ml penicillin G and 10 mg / ml streptomycin, Sigma Chemical Co, St Louis, MO, USA).

3. Cell radiation:

The radiation of cells was done by the X-ray equipment Krautkramer-Foster, Smart Model 2006 for experimental animals and cell cultures. Cells sorted (sorter +, sorter - and general population) and maintained for 24 hours in sphere culture medium were radiated under a constant current of 4.5 mA and power of 200 kW at different doses and time (Table 2). Not irradiated cells were used as control.

DOSES	TIME
2 Gy	3' 21"
6 Gy	10' 09"

Table 2. Doses radiation and time

4. RNA isolation:

The total RNA was obtained one day after cells radiation. RNA extraction was performed using miRCURY^{TM} RNA Isolation Kit - Cell & Plant content (EXIQON seek find verify).

For this, we transfer cell suspension to an RNase-free tube and centrifuge at no more than 200 x g for 10 minutes to pellet cells. Carefully supernatant was decanted. Then 350 μL of Lysis Solution was added to the pellet and cells were lyses by vortexing for 15 seconds. We added 200 μL of 95 – 100% ethanol to the lysate and mixed by vortexing for 10 seconds.

Moreover, we applied 400 μL of Wash Solution to the column and centrifuged for 1 minute at 14,000 x g. Finally we placed the column into a fresh 1.7 mL Elution tube and added 50 μL of

Elution Buffer to the column and centrifuged for 2 minutes at 200 x g, followed by 1 minute at 14,000 x g.
The purified RNA sample may be stored at –20°C for a few days. It is recommended that samples be placed at 80°C for long-term storage.

4.1 Quantitation of total RNA:

For quantification of RNA, we proceeded to the reading of the absorbance at 260 and 280 nm using a NanoDrop (NanoDrop ™ 2000/2000c Spectrophotometers, Thermo Scientific ™). The OD260/OD280 relationship allowed us to calculate the purity of nucleic acids, whereas an optimal range of values between 1.8 and 2. RNA concentration was calculated considering an OD unit at 260 nm corresponds to a concentration of 40 mg / ml nucleic acid.

5. Reverse transcription and qPCR to detect miRNAS

The miRCURY LNA™ Universal RT microRNA PCR system was the kit used to detect the expression of microRNA-specific, LNA™-based system designed for sensitive and accurate detection of microRNA by quantitative real-time PCR using SYBR® Green.

5.1 Reverse transcription:

For the reverse transcriptase reaction previously extracted RNA was used. For each reaction a volume of 20 µL in a nuclease free eppendorf was used. For each reaction 4µL of 5x Reaction buffer, 2 µL enzyme mix and 4.5 µL of nuclease-free water mix was added and 2.5 µL of RNA extracted. The reaction was mixed pipetting all the reagents.

The reverse transcriptase reaction was performed in a thermocycler (DOPPIO Thermal Cycler, VWR). The reaction tubes were heated at 42 ° C for 60 min and then the temperature rose to 95 ° C for 5 min. Finally samples were kept on ice for 5 min, and the cDNA was stored at -20 ° C.

5.2 Quantitative polymerase chain reaction qPCR:

Quantitative polymerase chain reaction in real time (qPCR) was carried out to validate the data obtained from cDNA and the primers of miRNAs selected. miRNAs selected were searched at the literature including as criterion hypoxia, metastasis and DDR. For this first experiment Hsa-mir-24 3p and Hsa-mir 210 3p were used together with an internal control GAPDH to normalize variations in the quality and quantity of the cDNA used.

For the analysis of the expression the "PCR Real-time PCR 7500 system"(Applied Biosystems, Inc.) system was used. The mix of 12.5 µL of SYBR Green PCR Master mix, 10 µL of diluted cDNA and 2.5 µL of primer was used with a volume total of 25 µL. Each reaction was carried out in triplicate.

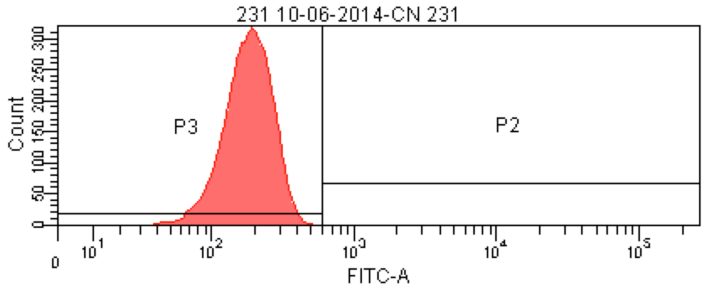
The process steps for qPCR was 95 ℃, 10 min to polymerase activation/denaturation, 40 amplification cycles at 95 ℃, 10 seconds and 60 ℃ 1 min. The Ct method was used to calculate the amplification factor as recommended by the supplier. The concentration of the miRNA of interest and the endogenous reference gene was determined by performing a calibration curve for each sample, from five serial dilutions of cDNA.

V. RESULTS

1. Flow cytometry based in the ALDH activity:

MDA-MB-231 cells were isolated by flow cytometry based in the ALDH activity.

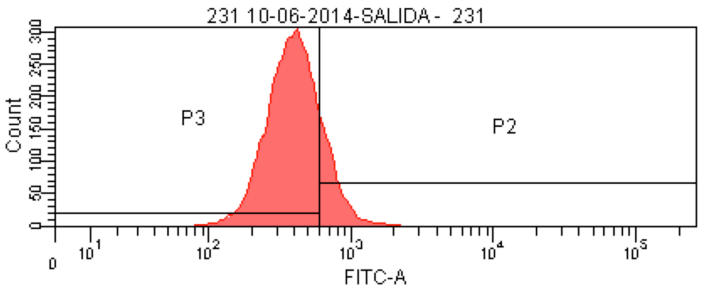
Inhibited ALDH activity



Tube: CN 231

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	7,278	72.8	72.8
P2	0	0.0	0.0
P3	7,278	100.0	72.8

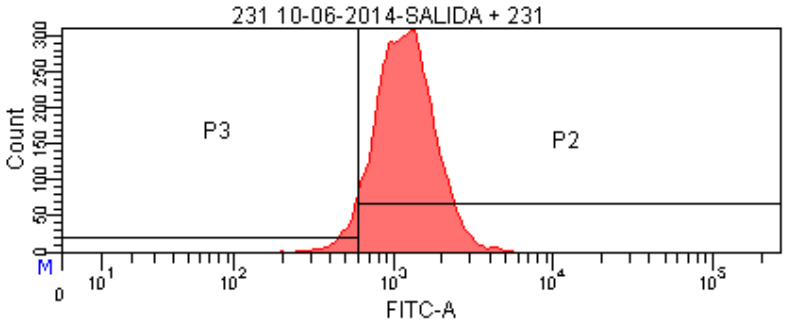
Negative ALDH activity



Tube: SALIDA - 231

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	7,768	77.7	77.7
P2	1,243	16.0	12.4
P3	6,457	83.1	64.6

Positive ALDH activity



Tube: SALIDA + 231

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	7,972	79.7	79.7
P2	7,563	94.9	75.6
P3	368	4.6	3.7

2. Enrichmented of MDA-MB-231 cell line after sorting with sphere culture medium

To determine expression of miRNAs in breast CSCs enriched subpopulation sorted cells were growth into low attachment plates with *sphere culture medium* for 72h before radiation.

As shown in Figure 8 sorted cells formed mammospheres at difference with control non-sorted cells and ALDH negative subpopulation

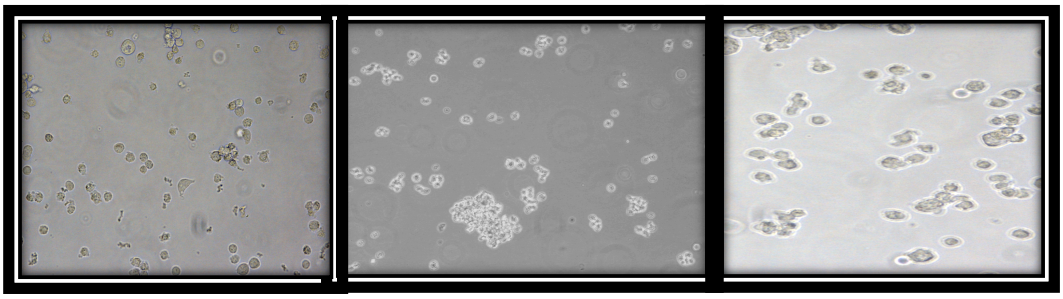


Figure 8: **a** Control non-sorted population in sphere culture medium (objective 20x), **b** ALDH + sorted cells in sphere culture medium (objetive 20x) and **c** ALDH - sorted cells in sphere culture medium (objetive 20x).

3. Expression levels of miRNAs:

miRNAs levels were measured in ALDH + CSCs (sorter positive) and non-sorted population (control cells)after 24 h of exposure to radiation. The ALDH negative CSC subpopulation was not measured in this first experiment; so only two populations were compared.

miRNAs examined were as follows: Hsa-mir 210 3p and Hsa-mir 24 3p together control GADPH. Results are shown in Table 3 and Figure 9 and 10. These data show that the expression of both miRNAs was different between control cells and ALDH+ CSCs with a significant higher expression in sorted cells in comparison with non-sorted. Surprisingly, Hsa-

mir 210 3p increased up to 91.3 folds and Hsa-mir 24 3p up to 3.1 in ALDH+ cells. After radiation, these levels decreased in the ALDH+ subpopulation in a dose-dependent manner.

However, in non-sorted subpopulations Hsa-mir 210 3p level after treatment with 2 Gy decreased and this level increased after 6Gy (Figure 9). Similarly, Hsa-mir 24 3p showed the same behaviour in ALDH+ cells after radiation with a decreased that was dose-dependent. In contrast, in non-sorted cells 2 Gy increased Hsa-mir 24 3p expression level and decreased after treatment with 6 Gy in comparison with control non-radiated cells (Figure 10).

In Table 3 the values obtained for miRNAs expression are shown.

		MDA-MB-231 NS				MDA-MB-231 S+		
Primers miRNAs	**Radiation Dose (Gy)**	**Mean**	**SEM**	**p-value**		**Mean**	**SEM**	**p-value**
Hsa-mir-210 3p	0	1	0,0670	0,0		91,27	27,68763	0,00084
	2	0,41	0,0256	0,00000		26,40	1,22998	0,00000
	6	3,23	0,0903	0,00000		10,88	0,58	0,00000
Has-mir-24 3p	0	1	0,0285	0,0		3,08	0,70112	0,00206
	2	1,49	0,0801	0,00002		1,11	0,11734	0,22211
	6	0,31	0,0048	0,00000		0,87	0,08	0,268897

Table 2. Expression levels miRNAs after radiation.

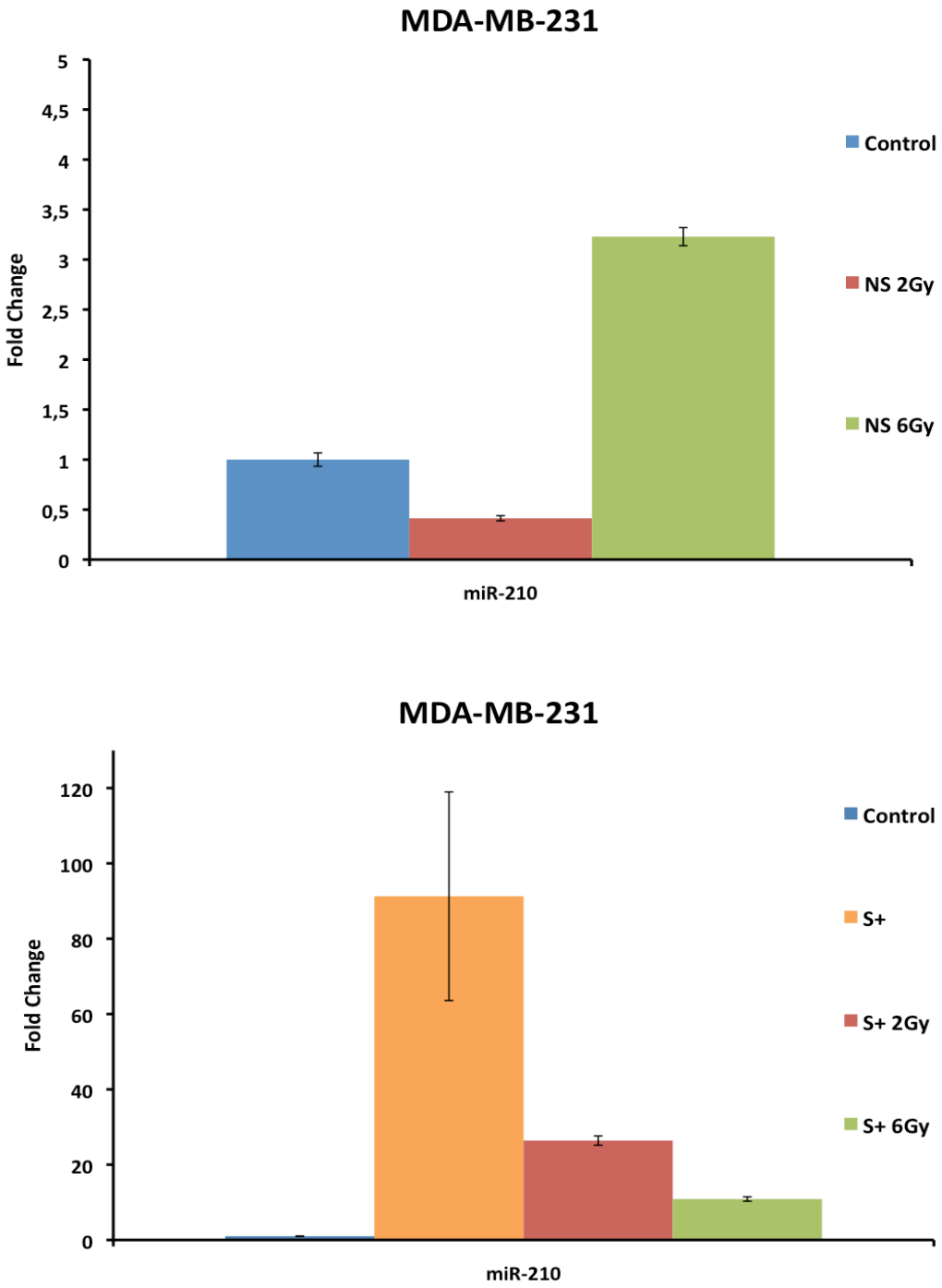
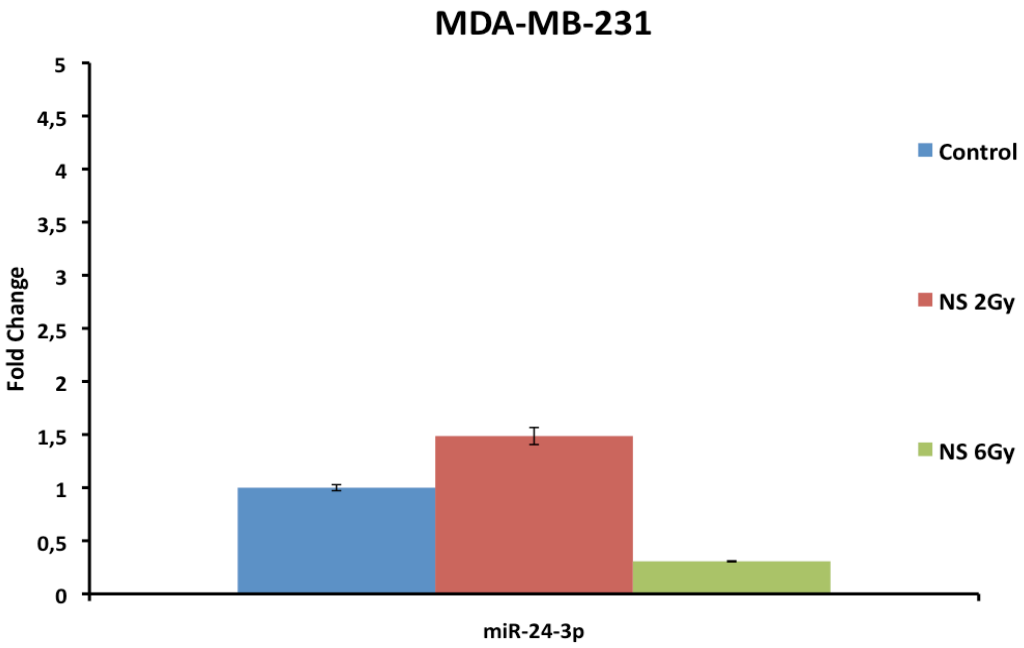


Figure 9: Relative miRNA 210 3p expression quantified by qRT-PCR after ionizing radiation in non-sorted MDA-MB 231 cells (general population) and ALDH+ cells compared with non-treated cells and normalized with *GAPDH* reporter gene.



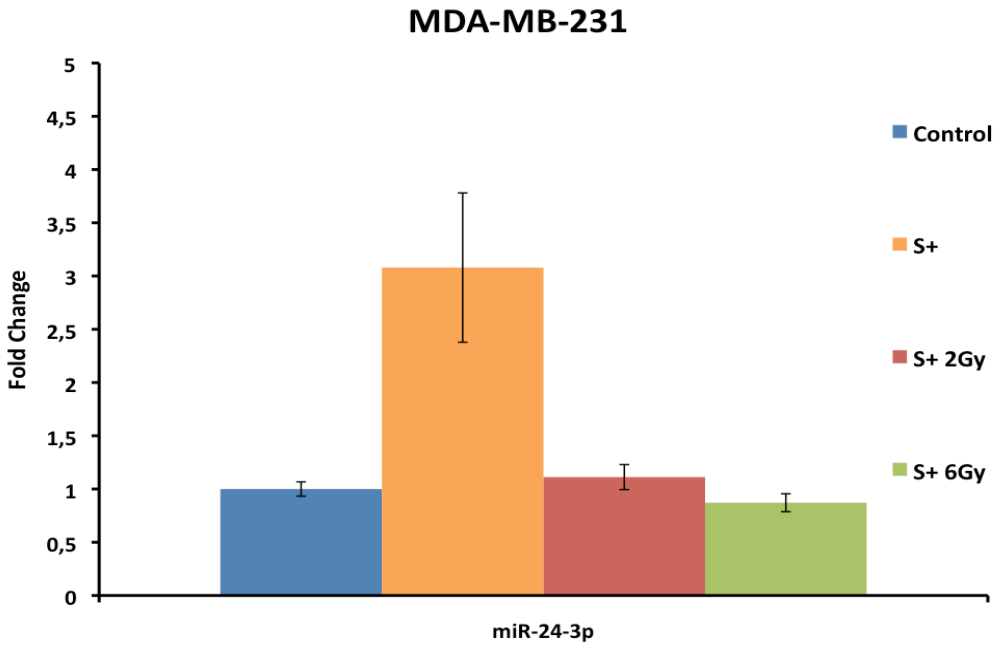


Figure 10: Relative miRNA 24 3p expression quantified by qRT-PCR after ionizing radiation in non-sorted MDA-MB 231 cells (general population) and ALDH+ cells compared with non-treated cells and control normalized with *GAPDH* reporter gene.

VI. DISCUSSION

The hypothesis proposed in this paper suggests that the response to treatment with ionizing radiation in breast cancer is relatedwith CSC subpopulation present in the tumor. Local recurrence and distant metastases, despite therapy, indicate that BCSCs are able to evade the effects of chemotherapy and radiotherapy and thereby repopulate the tumour following treatment (Ablett, 2014). Moreover, miRNAs have an important relevance in all the process of resistance at radiotherapy

Gene analysis studies have demonstrated aberrant miRNA expression in tumors compared to normal tissues and that miRNAs are deregulated in an array of solid cancers as well as haematological malignancies. The findings of the role of miRNAs in cancer is supported by the fact that about 50% of miRNA genes are located in cancer associated genomic regions, or in fragile sites, further strengthening the evidence that miRNAs do play a crucial role in cancer. As a result, human miRNAs are likely to be highly useful as biomarkers, especially for future cancer diagnostics, and are emerging as attractive targets for disease intervention (Abba et al, 2014).

In this paper CSCs were isolated by ALDH activity. Aldehyde dehydrogenase activity has been identified as a method of enriching for normal human breast stem and CSCs (Ginestier et al., 2007). In our experiments, we isolated and enriched MDA-MB-231 CSCs using this ALDH activity and culturing then in spheres medium. After three days of culturing in spheres medium, cells were radiated at different doses and miRNAs expression were measured 24 hours later.

We determined mir 210 3p expression since it has been identified as the most frequently deregulated miRNA in response to hypoxia. Hypoxia-inducible factors (HIF) are transcription factors that regulate various genes involved in response to hypoxia, including miR-210 (Jacobs et al., 2014). Some works have shown that hypoxia can also promote genetic instability by affecting the DNA repair capability of cancer cells, due to transcriptional downregulation of MLH1, MSH2, BRCA-1, and RAD51 observed in hypoxic cells. Forced expression of miR-210 was able to suppress levels of RAD52, a key factor in homology-dependent repair (Tessitore et al., 2013). Our results show that when non- sorted cells were radiated with 2 Gy, the expression of mir 210 3p decreases to 0,41 versus control although when they are radiated with 6 Gy we observed an increment up to 3.23 folds. This increases

can be explain by the effect that the ionizing radiation has in the enrichment of "stemness" in cancer cell. For example, previous studies have demonstrated that ionizing radiation can enrich CD133+ glioma CSCs *in vitro* and *in vivo*. Moreover, these authors showed that this enrichment effect preferentially activation of the DNA damage checkpoint in CD133+ glioma CSCs compared to CD133- non-stem glioma cells (Ghisolfi et al, 2012).

Surprisingly, the mir 210 3p expression increased up to 91.4 in ALDH+ cells but decreased in radiated cells up to 26.42 y 10.88 for 2 Gy and 6 Gy, respectively. We can interpret that when population is enriched in BCSCs the expression of mir 210 3p which is related with hypoxia and DDR is overexpressed. In fact, hypoxia may be a major component of the BCSCs niche, providing the BCSCs with cues for maintenance of an undifferentiated state. There is increasing evidence that hypoxia can affect expression of genes and pathways controlling stemness such as Oct4 and Notch, and that it can affect the epigenetic control of gene expression to promote an undifferentiated state (according to Marie-Egyptienne et al, 2013). In contrast, when we radiate cells this level decreases significantly in a dose-dependent radiation manner, which means that it is killing the CSC population, although there is a considerable bulk of BCSCs that survives to ionizing radiation.

miR 24 3p has been identified by miRNA arrays during post-mitotic differentiation of hematopoietic cell lines. Moreover, overexpression of miR-24 downregulates the histone variant H2AX, the initial sensor protein in the double strain break (DSB) response. miR-24-mediated suppression of H2AX renders hematopoietic cells hypersensitive to gamma-IR and genotoxic drugs, which might account for the reduced DNA repair capacity of terminally differentiated hematopoietic cells (Lal et al., 2009). Also, this miRNA has been relationed with ionizing radiation in a study overexpressing miR 24 3p leads to higher chromosomal breaks and sensitivity to ionizing radiation and other cytotoxic drugs in various cell lines (Metheetrairut and Slack, 2013). In our experiments, miR 24 3p expression in non sorted cells increased at 2 Gy up to 1.49 and decreases at 6 Gy up to 0.31 in comparison to the control non-radiated cells. The miR 24 3p overexpression in this population can be explain because of a low radiation dose could dowregulate H2AX and therefore, the DSB response. The downregulation observed after 6Gy treatment leads to the elimination of BCSCs in the non-sorted subpopulation.

The behaviour of miR 24 3p expression in ALDH+ cells was the same that miR 210 3p but the difference were not significant. In not radiated ALDH+ cells there was an increase of 3.08 folds, which decreased up to 1.11 for 2 Gy and up to 0.87 for 6 Gy , respectively.

VII. CONCLUSSIONS

1. Our results showed that miR 210 (hypoxia) and miR 24 (DDR) are overepressed in BCSCs.

2. The miR 210 expression levels were higher than those found for miR 24 in BCSCs.

3. Ionizing radiation affects the expression levels of miR 210 and miR 24 both in non-sorted population as in ALDH+ BCSCs.

4. Ionizing radiation decreases miR 210 and miR 24 expression in a dose-dependent manner in ALDH+ BCSCs.

4. Our results suggest that the determination of these miRs could be useful markers of response to radiotherapy in patients with BC.

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