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MicroRNAs in breast cancer progression and DNA damage response

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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-graduação em Fisiopatologia Clínica e Experimental, da Universidade do Estado do Rio de Janeiro. Realizada em cotutela com a Universidade Paris-Sud XI.

Orientadora: Prof.ª Dra. Cláudia Vitória de Moura Gallo

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RESUMO

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Os tumores de mama são caracterizados pela sua alta heterogeneidade. O câncer de mama é uma doença complexa, que possui o seu desenvolvimento fortemente influenciado por fatores ambientais, combinada a uma progressiva acumulação de mutações genéticas e desregulação epigenética de vias críticas. Alterações nos padrões de expressão gênica podem ser resultado de uma desregulação no controle de eventos epigenéticos, assim como, na regulação pós-transcricional pelo mecanismo de RNA de interferência endógeno via microRNA (miRNA). Estes eventos são capazes de levar à iniciação, à promoção e à manutenção da carcinogênese, como também ter implicações no desenvolvimento da resistência à terapia Os miRNAs formam uma classe de RNAs não codificantes, que durante os últimos anos surgiram como um dos principais reguladores da expressão gênica, através da sua capacidade de regular negativamente a atividade de RNAs mensageiros (RNAms) portadores de uma seqüencia parcialmente complementar. A importância da regulação mediada por miRNAs foi observada pela capacidade destas moléculas em regular uma vasta gama de processos biológicos incluindo a proliferação celular, diferenciação e a apoptose. Para avaliar a expressão de miRNAs durante a progressão tumoral, utilizamos como modelo experimental a série 21T que compreende 5 linhagens celulares originárias da mesma paciente diagnosticada com um tumor primário de mama do tipo ErbB2 e uma posterior metástase pulmonar. Essa série é composta pela linhagem obtida a partir do tecido normal 16N, pelas linhagens correspondentes ao carcinoma primário 21PT e 21NT e pelas linhagens obtidas um ano após o diagnóstico inicial, a partir da efusão pleural no sítio metastatico 21MT1 e 21MT2. O miRNAoma da série 21T revelou uma redução significativa nos níveis de miR-205 e nos níveis da proteina e-caderina e um enriquecimento do fator pró-metastático ZEB-1 nas células 21MT. Considerando a importância dos miRNAs na regulação da apoptose, e que a irradiação em diferentes espectros é comumente usada em procedimentos de diagnóstico como mamografia e na radioterapia, avaliamos a expressão de miRNAs após irradiação de alta e baixa energia e do tratamento doxorrubicina. Para os ensaios foram utilizados as linhagens não tumorais MCF-10A e HB-2 e as linhagens de carcinoma da mama MCF-7 e T-47D. Observou-se que raios-X de baixa energia são capazes de promover quebras na molécula do DNA e apoptose assim como, alterar sensivelmente miRNAs envolvidos nessas vias como o let-7a, miR-34a e miR-29b. No que diz respeito à resposta a danos genotóxicos, uma regulação positiva sobre a expressão de miR-29b, o qual em condições normais é regulado negativamente foi observada uma regulação positiva sobre miR-29b expressão após todos os tratamentos em células tumorais. Nossos resultados indicam que miR-29b é um possível biomarcador de estresse genotóxico e que miR-205 pode participar no potencial metastático das células 21T.

Palavras chave: Câncer de mama. MicroRNA. Série 21T. MiR-205. MiR-29b.

ABSTRACT

Breast tumors are characterized by their high heterogeneity. It is a complex disease, which has its development strongly influenced by environmental factors, combined with a progressive accumulation of genetic mutations and epigenetic dysregulation of critical pathways. Changes in gene expression patterns may be a result of a deregulation in epigenetic events as well as in post-transcriptional regulation driven by RNA interference endogenously represented by microRNA (miRNA) these mechanisms are capable to promote the initiation, maintenance and progression of carcinogenesis; they are also implicated on the development of therapy resistance. miRNAs form a class of non-coding RNAs which have emerged in recent years as one of the major regulators of gene expression through its capacity to silence messenger RNAs (mRNAs) containing a partially complementary sequence. The importance of regulation mediated by miRNAs was observed on their ability to regulate a wide range of biological processes including cell proliferation, differentiation and apoptosis.To gain insights into the mechanisms involved in breast cancer initiation and progression conducted a miRNA global expression on 21T series that are an in vitro model of breast cancer progression comprising cell lines derived from the same patient which include a normal epithelia (16N), primary in situ ductal carcinoma (21PT and 21NT) and cells derived from pleural effusion of lung metastasis (21MT-1 and 21MT-2). Considering the importance of miRNAs in the regulation of apoptosis, and that irradiation in different spectra is commonly used in diagnostic procedures as mammography and on radiotherapy, we evaluate the miRNA expression after cell low and high energy irradiation and doxorubicin treatment to determine whether miRNAs are useful biomarkers to detect cell response after DNA damage. The experiments were done on the non-tumoral cell lines MCF-10A and HB-2 and on the breast carcinoma derived cell lines MCF-7 and T-47D. We observed that of low energy X-rays is able to promote DNA strand breaks and apoptosis and to slightly change the expression of miRNAs involved on this pathway such as let-7a, miR-34a and miR-29b. Regarding DNA stress response pathways an upregulation on miR-29b expression, that in normal conditions is downregulated in tumor cell lines could be observed after all treatments. The microRNAome of 21T series revealed a significant downregulation of miR-205, an enrichment of the pro-metastatic factor ZEB-1, potential target for miR-205 and the consequent reduction of e-cadherin levels in 21MT cells checked by western blot. Our results indicate that miR-29b is biomarkers of genotoxic stress and that miR-205can participate on the metastatic potential of 21T cells.

Key words: Breast cancer. MicroRNA. 21T cells. MiR-205. MiR-29b

RESUME

Les tumeurs du sein sont caractérisées par leur grande hétérogénéité. C´ est une maladie complexe,qui a son développement fortement influencée par des facteurs environnementaux, combinés a une accumulation progressive de mutations et par la dérégulation épigénétiques des voies critiques. Changements dans les modes d'expression des gènes peut être le résultat de la déréglementation dans le contrôle des événements épigénétiques ainsi que dans régulation post-transcriptionnelle par le Fonds pour l'interférence ARN par l'intermédiaire microARN endogène (miRNA) susceptible de conduire à l'initiation, la promotion et le maintien de la cancérogenèse, ont également des implications pour le développement de la résistance au traitement Les miARN constituent une classe de ARN non-codants qui ont émergé ces dernières années comme l'un des principaux régulateurs de l'expression des gènes par sa capacité à réguler négativement l'activité des ARN messagers (ARNm) les patients atteints d'une séquence partielle complémentaire. L'importance de la médiation la réglementation par les microARN a été observé pour leur capacité à réguler un large éventail de processus biologiques incluant la prolifération cellulaire, la différenciation et l'apoptose. Pour évaluer l'expression des miRNAs au cours de la progression tumorale, nous avons utilisé comme modèle expérimental, la série comprenant 5 lignées de cellules 21T en provenance du même patient diagnostiqué avec ErbB2 du sein de type primaire de la tumeur et une métastase pulmonaire postérieure. Cette série se compose de la souche obtenue à partir du tissu 16N normale, les lignes correspondant aux 21pt carcinome primaire et 21NT et les lignes obtenu un an après le diagnostic initial d'un épanchement pleural à partir du site et métastatiques 21MT2 21MT1. Le miRNAoma série 21T a montré une réduction significative des niveaux de miR-205 et des niveaux de protéine E-cadhérine et un enrichissement des facteurs pro-métastatiques des cellules 1-ZEB 21MT. Considérant l'importance des miARN dans la régulation de l'apoptose, et que le rayonnement sur les différents spectres est couramment utilisé dans les procédures de diagnostic telles que la mammographie et de la radiothérapie, nous avons évalué l'expression des miARN après l'irradiation de traitement d'énergie haute et basse et de la doxorubicine. Pour les essais n'ont pas été utilisés lignes MCF-10A-2 et HB et la poitrine lignes carcinome MCF-7 et T-47D. Il a été noté que les rayons X de basse énergie sont capables de promouvoir des ruptures dans la molécule d'ADN et l'apoptose ainsi que le changement appréciable dans les miARN impliqués dans ces voies laissez-7a-miR-34a et miR 29b. Comme réponse à ce qui concerne les dommages génotoxiques a été observée sur une régulation à la hausse de miR-29b expression après tous les traitements sur les cellules tumorales, dans lequel dans des conditions normales, ce n'est régulés négativement miARN Nos résultats indiquent que miR-29b est un biomarqueur potentiel de stress génotoxique et miR-205 peut participer à le potentiel métastatique des cellules en 21T.

Mots clés : Cancer du sein. MicroARN. Serie 21T. MiR-205. MiR-29b

UTR- 3´Untranslated region

ADH- Atypical ductal hyperplasia

Ago- Argonaute proteins

cDNA- complementary DNA

Chip- Chromatin immunoprecipitation

CLL -chronic lymphocytic leukemia

CNPq- Conselho Nacional de Desenvolvimento Científico e Tecnológico

DCIS- Ductal carcinoma *in situ*

DDR- DNA damage response

DGCR8- DiGeorge syndrome critical region gene 8

DNA- Deoxyribonucleic acid

dsRNA- double-stranded RNA

EMT- epithelial to mesenchymal transition

ER- estrogen receptor

FAPERJ- Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro

FBS- fetal bovine serum

FC- fold change

h3k9me3- histone H3 trimethyl lys9

h4ac - acetylated histone H4

IDC- Invasive ductal carcinoma

iPS- induce pluripotent cells

miRNA- microRNAs

mRNA-messenger RNA

PR- Progesterone receptor

pri-miRNA- primary microRNA

qRT-PCR- quantitative reverse transcriptase polymerase chain reaction

RISC- RNA-induced silencing complex

RNA- ribonucleic acid

RNAi- RNA interference

SD- standard deviation

TLDA- Taqman low density assay

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I.Introduction

1. *Breast cancer –Progression and Molecular Classification*

Breast cancer is the leading cause of cancer deaths in women worldwide. This disease affects approximately one in ten women with cancer (Key *et al*, 2001). The number of new cases of breast cancer expected on 2012 in Brazil is 52,680, with an estimated risk of 52 cases per 100,000 women (Ministry of Health / INCa, 2011).

Breast cancer remains a major public health problem. The last worldwide breast cancer statistic report shows that mortality rate reaches 12.3 and 5.6/100,000 women in newly industrialized countries as Brazil and China *versus* 17.6 and 18.6/100,000 women in developed countries, such as France and UK (Globocan-IARC, 2008). Even if there is an increase in this cancer curability, when compared to previous years, metastatic breast cancer remains a lethal disease in the vast majority of cases. Therapeutic advances in the chemotherapeutic and targeted therapies fields induced an increase in survival rate. However physicians still have to deal constantly with non-responsive treatments and highly aggressive tumors.

Besides being a disease primarily influenced by environmental factors, breast cancer is a highly heterogeneous disease caused by a progressive accumulation of multiple genetic mutations combined with epigenetic deregulation of genes involved in critical pathways. As a result, breast cancer progression may be more or less aggressive and have different responses to therapy (Bertos and Park, 2011).

Patient management is an important challenge as it is difficult to predict which patients are at risk of cancer progression from an *in situ* disease to a metastatic one. Also, tumors exhibiting apparently the same histological type and grade can have quite distinct clinical courses, for example, in response to therapies or subsequently acquired resistance (Simpson and Lakhani, 2009). The molecular characterization of

these tumors is an important weapon that can help physicians to define the most appropriate therapy and prognosis of the disease (Olopade *et al*, 2008; Rakha and Ellis, 2009).

Currently, breast tumors are molecularly subdivided in four classes, based on microarray profiling: (a) luminal A, (b) luminal B, (c) HER2 (human epidermal growth factor receptor-2) over-expression and (d) basal, based on gene expression profiles, phenotype, prognosis and susceptibility to different treatments (Perou *et al*, 2000; Morris and Carey, 2007; Sempere *et al*, 2007). This classification has also been validated for tumor cell lines (Kao *et al*, 2009).

The luminal A (ER +, PR +, HER2-) usually responds well to treatment with antiestrogens and aromatase inhibitors, while the subtype luminal B (ER +, PR +, HER2 +) and HER2 (ER-, PR-, HER2 +) respond to treatment with trastuzumab (Herceptin). The basal subtype or triple negative (ER-, PR-, HER2-) is not responsive to any target therapy and, in this case, no specific treatment is used for this subtype, but the chemotherapy and radiotherapy (Sempere *et al*, 2007).

One of the most important measures of tumor behavior is the morphological classification and the rate of tumor cell proliferation, with high proliferation indices being significantly associated with aggressive tumor behavior (Simpson and Lakhani, 2009).

Ductal carcinoma *in situ* (DCIS) comprises a heterogeneous group of lesions that reflect the invasion of the mammary ducts by malignant cells, but without the basement membrane impairment (Polyak, 2010). During the transformation of normal cells into tumor, drastic molecular changes can be observed. However,

during DCIS to IDC evolution tumors essentially share the same epigenetic changes and the same gene expression profiles (Espina and Liotta, 2011).

One of the leading questions in translational breast cancer research is the ability to predict which patients diagnosed with atypical ductal hyperplasia (ADH) or DCIS are most at risk of progressing to invasive cancer.

Currently, considerably importance is being directed towards the lost of tight junctions in epithelial to mesenchymal transition (EMT), as the probable first step in the metastatic process (Martin and Jiang, 2009; Guttilla *et al*, 2012) (Figure 1).

In cancer, EMT is a multi-step and a reversible process allowing cell migration and later re-colonization. Broadly speaking, it is characterized by the loss of cell-cell junctions, cell matrix adhesion and reorganization of the cytoskeleton (Guttilla *et al* , 2012).

Tight junctions are the first barrier that cells must overcome to migrate, being related to the lost of epithelial cell polarity and the gain of mesenchymal characteristic such as cells with a spindle- shaped morphology and a higher degree of motility. Furthermore, one of the early hallmark of EMT is the lost of the epithelial cell adhesion molecule e-cadherin (Martin and Jiang, 2009; Guttilla *et al*, 2012).

Immunohistochemistry for e-cadherin is now used as an adjunct antibody for differentiating ductal from lobular pre-invasive and invasive lesions. This is particularly useful for lesions with undeterminated morphology and is clinically important because of the distinct management implications of ductal and lobular cancers (Simpson and Lakhani, 2009).

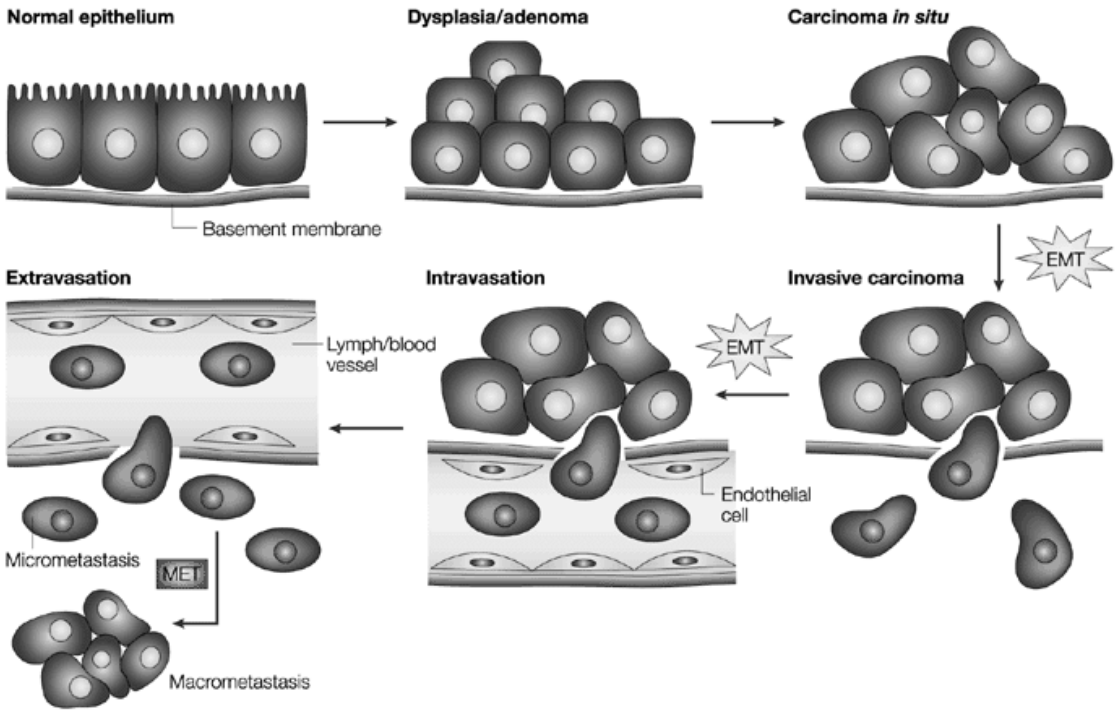

Figure 1: Cancer progression and EMT (Adapted from Thiery, 2002)

2. *Regulation of gene expression in cancer*

The amount of synthesized protein can be regulated by different mechanisms at different stages, such as promoter regulation, RNA splicing, epigenetic regulation, translation and post-translational modifications.

Epigenetic changes involve variations in gene expression that are not derived from alterations in the DNA sequence. Epigenetic mechanisms can regulate biological processes throughout life and be heritable, playing a crucial role, for example, in the genome reprogramming during embryogenesis, gametogenesis, and cellular differentiation and in X-chromosome inactivation in women (Delcuve *et al* 2009). Consequently, altered epigenetic signs are responsible for a range of diseases, including cancer (Delcuve *et al* 2009).

The epigenetic regulation mainly involves three elements that can act together: DNA methylation, histone modifications and chromatin remodeling. These processes modulate euchromatin (open chromatine with transcriptional activity) and heterochromatin (silenced gene expression) assembly. Changes in expression patterns due to a deregulation in the control of epigenetic events may lead to the initiation, promotion and maintenance of carcinogenesis (Lo and Sukumar, 2008, McKenna and Roberts, 2009).

Gene expression can also be regulated at transcriptional levels by interaction of an antisense RNA complementary to a messenger RNA. This phenomenon was named RNA interference (RNAi), described by Fire and colleagues (1998), when they observed that molecules of double-stranded RNA (dsRNA) could act as a potent gene silencing induced by pairing with complementary messenger RNAs and subsequent degradation (Bartel, 2004).

The discovery of an endogenous mechanism of RNA interference occurred in the early 1990s, through the identification of *lin-4* in the nematode *Caenorhabditis elegans*. This regulatory mechanism was, at first, considered as a feature of the biology of these animals. However, after its identification in many eukaryotic organisms, it was proved that these molecules represent an evolutionarily conserved mechanism capable of regulating the expression of thousands of genes (Wu and Belasco, 2008). Then, in 2001, the RNAi mechanism modulated by endogenous non-coding RNAs, known as microRNAs (miRNAs) was identified in mammals (Zhang and Farwell 2008).

3. MicroRNAs

The microRNAs are small (about 23 nucleotides long) non-coding RNA species that during the past five years have emerged as major elements of gene expression control, acting in post-transcriptional level by targeting a 3´UTR complementary region at mRNA sequence (Bartel, 2004).

Functional studies indicate that these small RNAs are involved in regulation of virtually all cellular processes and changes in their expression levels are closely related to the progression of various diseases, including cancer (Croce, 2009; Esteller, 2011). These results are not surprising when put into perspective with those obtained *in silico*, whose predictions estimate that up to 60% of all genes encoding proteins are potentially regulated by miRNAs (Nana-Sinkam and Croce, 2011).

3.1.miRNA transcription, maturation and target recognition

The microRNAs synthesis requires some steps. They are transcribed mostly by RNA polymerase II, from independent genes, by miRNA clusters or represent introns of protein-coding genes and, in this case, their expression profiles are significantly correlated (Radfar *et al*, 2011). The primary transcript (pri-miRNA), is a capped and polyadenylated molecule that contain one or more long and imperfect pairing hairpin structures. This structure is recognized and cleaved by a multiprotein complex called microprocessor composed by Drosha, a RNaseIII enzyme, a RNA binding protein DGCR8 protein (DiGeorge syndrome critical region gene 8) and multiple RNA-associated proteins, including the DEAD box RNA helicases p68 and p72 (Suzuki and Miyazono, 2011; Starega-Roslan *et al*, 2011). p68 and p72 are required for the maturation of only a few number of miRNAs, especially those that

tend to be up-regulated under DNA-damaging conditions. Drosha cleaves the pri-miRNA hairpin at a distance of approximately 11 bp from the single-stranded RNA–dsRNA junction, which is recognized by DGCR8 and releases a precursor of about 60 nucleotides, named pre-miRNA (Suzuki and Miyazono, 2010).

At this step, the tumor suppressor protein p53 can, after stimulation, accumulate and interact by the central DNA- binding domain region with the microprocessor during certain miRNA species maturation. This interaction between p53 and Drosha complex enhances pri-miRNA processing of several miRNAs species both *in vitro* and *in vivo* under DNA-damage-inducing conditions (Suzuki *et al*, 2009)

After reach pre-miRNA conformation, the molecule is then actively exported into the cytoplasm by Exportin-5. In the cytoplasm, the final maturation step will take place, catalyzed by Dicer, another RNAse III enzyme, which converts the pre-miRNA to a mature miRNA. Dicer recognizes the 3´ends generated by Drosha and cleaves the pre-miRNA. The result is a miRNA duplex of 21 nucleotides on average with two 3' ends outgoing (2-nt 3' overhang) (Suzuki and Miyazono, 2010).

After Dicer processing, the mature single stranded miRNA is incorporated to the effector ribonucleoprotein complex called RISC (RNA-induced silencing complex). The RISC assembly occurs by the small RNA duplex active incorporation into Argonaute proteins (Ago). The RNA duplex is unwound by Ago protein and the most thermodynamically stable strand will be selected then reach and inhibit mRNAs expression by sequence complementarity (Kwak and Tomari, 2012).

The mature miRNA forming the RISC complex can silence mRNAs by base-pair complementarity. They were at first assigned to target sequences in the 3′-UTR of target mRNAs, only requiring a continuous base-pairing of miRNA nucleotides 2 to 8, known as the seed sequence (Pasquinelli, 2012).

Later, recognition sites located outside 3´UTR have been observed by computational tools and validated by functional approaches (Forman et al, 2008; Tay et al, 2008; Lee et al, 2009).

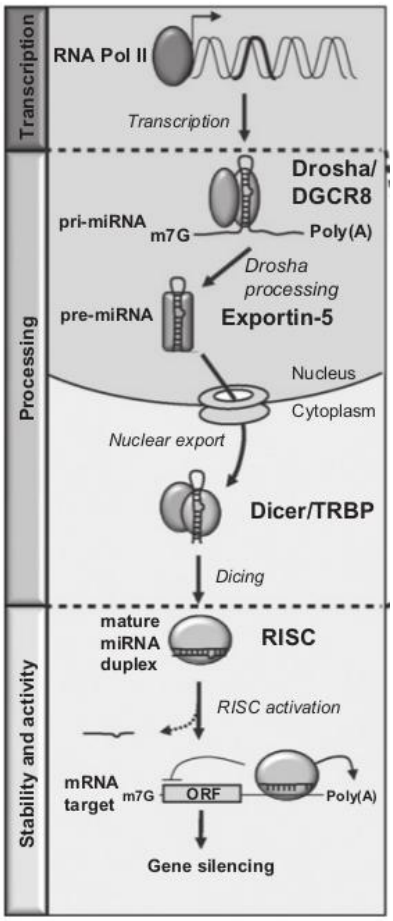

Figure 2: miRNA biogenesis (Adapted from Mockenhaupt et al., 2011)

A focus on miRNAs presented within 3´UTR region was supported by the proximity with the G-cap/poly-A tail interface, important for miRNA mediated silencing. It was also remarked that miRNAs tend to be more effective when located at the end of 3´UTR rather than the middle (Forman et al, 2009).

However, highly conserved miRNA complementary sequences were found in coding regions that were proved to be miRNA target sites. Furthermore, RNAm containing miRNA target sites on both 3´and 5´UTR presented greater alteration on protein levels, when subject to miRNA regulation, suggesting an interaction between 3´and 5´UTR on gene silencing (Lee *et al*, 2009).

After miRNA:mRNA assembly into RISC complex, protein synthesis will be inhibited (Krol *et al*, 2010; Pasquinelli, 2012) (Figure 2).

In plants, miRNAs generally base pair to mRNAs with nearly perfect complementarity and trigger endonucleolytic mRNA cleavage.

However, in most cases, metazoan miRNAs pair imperfectly with their targets and the mechanisms by which miRNAs regulate gene expression identified to date consist of silencing by regulating the mRNA stability, inducing degradation or by repressing translation (Figure 4) (for review Fillipowicz *et al*, 2008).

miRNA repression is often associated with RNAm target destabilization, which leads to a posterior degradation and reduction of target transcript levels.

The main mechanism identified to date, by which miRNA induce RNAm degradation, in animals, is by recruiting some RNA binding proteins (mRBP). The mRBP complex promotes mRNA deadenylation and decapping and consequent degradation. The degradation or at least its final steps seems to occur at P-bodies,

structures that are enriched with mRNA catabolizing enzymes and translational repressors (Fillipowicz et al, 2008; Hieronymus and Silver, 2004).

As part of the translational initial steps, the mRNA 5´cap is recognized by the eukaryotic translation initiation factor (eIF) that drives the assembly of the ribosome initiation complex, and facilitates the recruitment of the 40S ribosomal subunit.

Some evidences suggest that factors bound at the 3′ UTR exert their inhibitory effect on translational initiation by recruiting proteins that either interfere with the interaction between elf subunit eIF4E and the subunit eIF4G that acts as a scaffold on ribosome subunit 40S connection or they can bind directly to the 5´cap preventing 40S initiation complex assembly.

miRNAs can also, by targeting one of the two terminal mRNA structures prevent the synergy between the 5′ cap and 3′ poly(A) tail, by this way, mRNA cannot take a circular form and 40S initiation complex assembly will be compromised.

Another mechanism by which miRNAs can block translation is by preventing 60S subunit joining by RISC interaction with eIF6, a 60Sribosome-associated factor that prevents the assembly of a translationally competent (80S) ribosome

miRNAs coding sequences are located all over the genome with the particularity that miRNA encoding genes are commonly located at fragile chromosomal sites or regions undergoing changes in copy numbers, such as duplications or deletions, or other types of rearrangements in cancer (Esquela-Kerscher and Slack, 2006).

miRNAs can be transcribed from genes located far from their target, forming independent transcriptional units (Bartel, 2004). This organization provides an independent mechanism of expression, making miRNAs subject to the same

mechanisms that control gene expression of mRNA coding genes (Calin and Croce, 2006). They can also have their genes organized as miRNA clusters and, by this way, several miRNAs are activated by the same promoter and can also be subject to the same regulation. These miRNAs are usually functionally related, for example, the c-myc induced cluster miR-17-92 that comprises 15 miRNAs sharing oncogenic functions and are often overexpressed in some types of leukemias (Bartel, 2004; Mi *et al* , 2010).

3.2. Regulation of miRNA during cell differentiation and tumorigenesis

As a result of the widespread range of processes that miRNAs are able to influence, this includes cell cycle, growth, apoptosis, differentiation and stress response it is not surprising that miRNA deregulation is a hallmark for several pathological conditions, including cancer.

miRNA profiles can distinguish not only between normal and cancerous tissue and identify tissues of origin, but they can also discriminate different subtypes of a particular cancer or even specific oncogenic abnormalities. miRNAs, for example, are differentially expressed between basal and luminal breast cancer subtypes and can specifically classify estrogen receptor, progesterone receptor and HER2/neu receptor status (Lowery *et al*, 2009; Bockmeyer *et al* 2011) .

Even more importantly, several groups in recent years have reported how miRNA profiling can predict disease outcome or response to therapy (Andorfer *et al* , 2011). Taking into account that a unique miRNA can target several mRNAs and interfere in different pathways, a slightly alteration in miRNA signature can have a big impact in tumor development. This is the case of lung cancer, where miR-155

overexpression and let-7a downregulation are able to predict poor disease outcome. The significance of microRNAs as prognostic biomarkers is also supported by other reports (Raponi *et al*, 2009; Andorfer *et al*, 2011; Lu *et al*, 2012).

miRNA expression deregulation can be a result of increased or decreased transcription due to an altered transcription factor activity. miRNA can indeed be either positively or negatively regulated by transcription factor with oncosuppressive, as p53 activating miR-34a or miR-205 (Piovan *et al*, 2012) or oncogenic functions, as myc, activating miR-17-92 cluster and repressing let-7 and miR-29 family members, or zeb-1, directly repressing the transcription of members of the miR-200 family, which are in turn able to directly target *ZEB-1* and *ZEB-2*.

Plasticity is an important feature of the normal breast gland, representing the capacity of a given cell change dramatically over the course of gland development (for example, virgin ducts *versus* pregnancy, or lactation *versus* involution), and in response to hormones and growth factors.

Transitions between epithelial and mesenchymal states have crucial roles in embryonic development. Emerging data suggest a role for these processes in regulating cellular plasticity in normal adult tissues and in tumors. In cancer tissues, EMT helps on distinct cellular subpopulations generation, contributing to intratumoural heterogeneity. Some of these subpopulations may exhibit more differentiated features, whereas others have an undifferentiated phenotype, characteristic of stem cells.

Induction of the EMT program in the immortalized human mammary epithelial cell MCF-10A results in the acquisition of mesenchymal traits, but in addition the expression of stem-cell markers, and an increased ability to form mammospheres, a

property associated with mammary epithelial stem cells (Mani *et al*, 2008; Radisky and LaBarge, 2008).

miRNA expression patterns in embryonic stem cells often share a resemblance to those observed in tumor cells, especially in the most undifferentiated and aggressive subtypes. This evidence is not surprising, if we consider that miRNAs can control EMT process, differentiation and pluripotency. Usually, a global reduction of miRNA abundance appears as a particular characteristic of both cells that can play a causal role in the transformed phenotype, once miRNAs coordinate the expression of entire sets of genes, shaping the mammalian transcriptome (Kumar *et al*, 2007; Lu *et al*, 2005; Ozen *et al*, 2008).

An increasing body of evidence has linked some miRNAs to EMT process and cancer stem cells phenotype. In breast cancer, the gene network controlling the maintenance of the epithelial phenotype appears to be mainly controlled by miRNAs, as attested by multiple miRNAs inhibiting EMT, as miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-205 and miR-125a (Gregory *et al*, 2008; Burk *et al*,2008; Cowden-Dahl *et al*, 2009, Martello *et al*, 2010). Also, high levels of miR-103/107 attenuate Dicer expression, which empowers invasive and metastatic properties without major impact on primary tumor growth (Martello *et al*, 2010).

3.3.miRNAs in DNA damage response

The human genome is constantly threatened by potentially deleterious genotoxic events generated endogenously, resulting from cellular metabolism, generating reactive oxygen species or by exogenous genotoxic agents, such as radiation and

chemical mutagens. This results in a variety of nucleotide modifications and DNA strand breaks. Cellular DNA damage response (DDR) comprises a response system that induces cell cycle arrest to allow sufficient time to repair the incurred damage, activates the DNA repair pathways, or, in the case of irreparable damage, induces apoptosis (Lord and Ashworth, 2012). DDR is a potent barrier against carcinogenesis and defects within this response are observed in many, if not all, human tumors.

Significant evidence has suggested that miRNAs play a crucial role in regulating DDR, being able to modulate DNA repair, apoptosis and cell cycle related proteins, including p53.

Some examples of miRNAs involved in DDR and their validated targets comprise miRNAs that are able to target DNA repair genes, as *BRCA1* by miR-182 (Moskwa *et al*, 2011) and *H2AX* by miR-24 (Lal *et al*, 2009); cell cycle control genes as *p21* by miR-17–92 cluster (Ivanovska *et al*, 2008; Inomata *et al*, 2009) and *p27* by miR-221/ miR-222 (le Sage *et al*, 2007; Fornari *et al*, 2008) and apoptosis (related miRNAs that are summarized on figure 3; Lima *et al*, 2011).

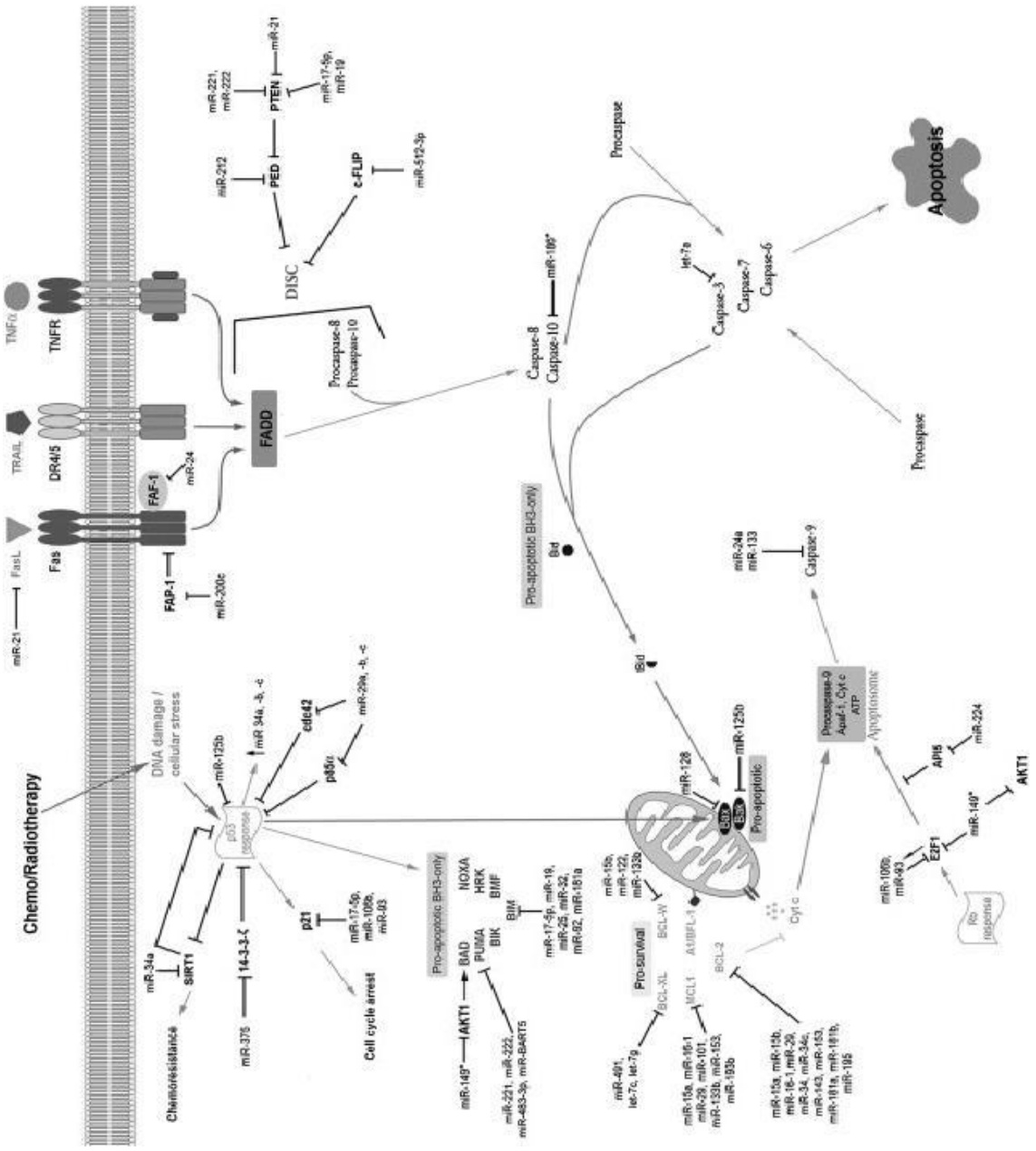

Figure 3: Apoptosis related miRNAs (Lima *et al*, 2010)

3.4.miRNAs and p53

The mechanism of action of the tumor suppressor p53, also known as the guardian of the genome, occurs mostly via transcription-dependent activities in the nucleus, by which p53 regulates transcription of genes involved in cell cycle, DNA repair, apoptosis, signaling, transcription, and metabolism (Olivier *et al*, 2009).

The promoter regions of miRNAs genes are highly similar to those of protein-coding genes. The presence of CpG islands, TATA box sequences, initiation elements and certain histone modifications indicate that the promoters of miRNA genes are controlled by transcription factors, enhancers, silencing elements and chromatin modifications, which is similar to protein-coding genes (Krol et al, 2010).

As a transcription factor, p53 can directly regulate some miRNAs. The miR-34 family was the first group of miRNAs identified as being subject to p53 stimulation (Hermeking, 2007; He *et al*, 2007).

miR-34 family is composed by miR-34a, miR-34b and miR-34c. They are encoded by two different genes: miR-34a is encoded by a gene located in chromosome 1 and miR34b/c share a common primary transcript located in chromosome 11. Both promoters have p53 response elements and these miRNAs are up-regulated after p53 activation, being part of genotoxic stress response. miR-34a is expressed at higher levels and miR34b/c are mainly expressed in lung tissues (Hermeking, 2010). Due to their high similarity, they share most of the targets, that include genes involved in cell cycle arrest and apoptosis as *BCL-2*, *Cyclin D1/E2*, cyclin dependent kinases 4 and 6 (CDK4/6), *c-MYC*, *MET* and *SIRT*, which is also a negative regulator of p53 (Hermeking, 2010). Thus, miR-34 can positively regulate p53 by repressing *SIRT*, which desacetylates p53, reducing its activity (Yamakuchi and Lowerstein, 2009).

So far, others miRNA that are directly activated by p53 have been identified as the cluster miR-143/145, miR-107, miR-192 and miR-215 (Feng, 2011) and all of them share tumor suppressor activities. High levels of miR-143/145 can result in apoptosis (Ostenfeld *et al*, 2010). miR-145 targets the proto-oncogene c-myc, reducing its expression (Sachdeva *et al*, 2009; Chen *et al*, 2010) and together with miR-143 and miR-145 it directly regulates *MDM2* expression, generating a positive feedback loop and increasing p53 response (Zhang *et al*, 2012). miR-107 is described as an inducer of cell cycle arrest in non-small cancer cell lines (Takahashi *et al*, 2009) and as a regulator of hypoxia inducible factor-1β (*HIF-1β*). Thereby it is able to decrease hypoxia signaling which stimulates tumor cell production of angiogenic factors, such as *VEGF-A*, basic FGF (*bFGF*) and placental growth factor (*PlGF*) (Yamakuchi *et al*, 2010). miR-192 and miR-215 are highly homologous and share a common seed region. A global gene expression analysis after their overexpression in cell lines, reveals that they induce virtually the same transcriptional profile. miR-192/215 can inhibit cell proliferation, probably through a coordinated action between these two miRNAs that are simultaneously stimulated by p53 (Georges *et al*, 2008). It was shown that acting together, miR-192/215 induce p21 accumulation (Braun *et al*, 2008) and retinoblastoma 1 (*Rb1*) was identified as being a miR-192 target (Feng *et al*, 2011).

Although p53 is certainly a transcriptional activator, this protein is also capable of down-regulating the expression of certain genes, including miRNAs. This is the case of let-7a and let-7b. Both are rapidly down-regulated after cell exposure to low and high doses of radiation and others genotoxic agents in a p53 dependent mechanism, as this down-regulation is not observed in p53 null cells (Saleh *et al*, 2011). These genes are clustered and have the same transcript origin moreover, it was shown that

p53 binds to let-7a/b gene enhancer and directly represses their expression (Saleh *et al*, 2011).

Besides the well-known p53 functions in regulating cell cycle, apoptosis and senescence, studies show that the tumor suppressor p53 is also a barrier to cell dedifferentiation (Menendez *et al*, 2010; Jain *et al*, 2012). miRNA expression profile in a set of pluripotent cells from different origins present two distinct categories of pluripotent cells that differ on the state of p53 network, markedly on the over- expression of the p53- regulatory miR-92 and miR-141 (Neveu *et al*, 2010).

p53 can regulate EMT properties through a direct binding and activation of miR-200c and miR-183 promoters (Chang *et al*, 2011). During this process, epithelial cells acquire features of mesenchymal cells, such as loss of E-cadherin expression, a known epithelial cell marker. miR-200c and miR-205 were shown to regulate EMT by inhibiting *ZEB1/2*, transcriptional repressors of e-cadherin (Gregory *et al*, 2008). The miR192/215 both activated by p53 have also been reported to repress *ZEB-2*, helping in maintaining epithelial phenotype (Wang *et al*, 2010).

The current methodology used to induce pluripotent cells (iPS) from somatic differentiated is achieved by the ectopically introduction of four transcription factors: Sox2, Oct3/4, Klf4 and c-Myc. Moreover, studies show that the lack of p53 activity promotes a more efficient reprogramming and increases cell proliferation (Takahashi and Yamanaka, 2006; Yamanaka and Blau, 2010; Jain *et al*, 2012). The pluripotency factors Oct4, Sox2 and Klf4 are a miR-145 direct target, which in turn is directly activated by p53 (Xu *et al*, 2009). Another way by which p53 regulates pluripotency in the generation of Ips is through activation of let-7. This miRNA

participates from the regulatory loop involving *LIN28* and still has *E2Z2* as its target, and its low expression signature related to stem cells.

The control of gene expression exerced by microRNAs has recently been described and is emerging as an important factor in modulating the physiological response to cellular stress. Some of these microRNAs are regulated by p53, which is a key protein in maintaining the integrity of the genome in response to DNA damage, and is also an important barrier against undifferentiation.

II.Objectives

- Assess miRNA global expression during tumorigenesis and tumor progression, using as a model a series of cell lines, obtained from the same patient that mimics a HER2+ breast cancer progression.

- Investigate the role of miRNAs in tumor progression during the transition carcinoma *in situ* to metastasis in 21T series.

- Evaluate the expression of miRNAs during tumorigenesis in the established breast cancer cell lines MCF-7 and T-47D, when compared to the non-tumorigenic MCF- 10A.

- Evaluate the role of miRNAs involved in apoptosis after low energy X-rays irradiation in the cell lines MCF-7 and in the non-tumorigenic HB-2.

- Evaluate the miRNA global expression after different genotoxic treatments in the cell lines MCF-7 and T-47D.

III.Methodology

Cell lines

To analyze miRNA alterations during breast tumor progression we used as model the 21T series, composed by 5 immortalized cell lines, isolated from the same patient diagnosed with infiltrating and intraductal carcinoma, in different stages of the disease. In order to evaluate differences in miRNA expression after genotoxic treatments we used the well established cell lines MCF-10A, MCF-7 and T-47D. Major cell line features are listed in Table III-1.

Table III-1: Characteristics of the cell lines used in this study (Cancer Cell line project; Band *et al*, 1990; Liu *et al* 1994)

Cell line	**ER**[1]	**PR**[2]	***Her2***[3]	**Type**	**Source**	**Described Mutations**
MCF-10[a]		-		Non-tumoral	fibrocystic disease	-
HB-2		-		Non-tumoral	Normal breast tissue	
MCF-7	(+)	(+)	(-)	Adenocarcinoma	Derived from metastatic site: pleural effusion	CDKN2A- p.M1*157del
T-47D	(+)	(+)	(-)	IDC	Derived from metastatic site: pleural effusion	PIK3CA- p.E545K PIK3CA- p.H1047R *TP53*- p.L194F
H16N2		-		Non-tumoral	Normal breast tissue	-
21PT	(-)	(-)	(+)	DCIS	Breast tissue	*TP53*- c.96_97ins1
21NT	(-)	(-)	(+)	DCIS	Breast tissue	*TP53*- c.96_97ins1
21MT1	(-)	(-)	(+)	IDC	Derived from metastatic site: pleural effusion	*TP53*- c.96_97ins1
21MT2	(-)	(-)	(+)	IDC	Derived from metastatic site: pleural effusion	*TP53*- c.96_97ins1

[1]- Estrogen receptor status, [2]- Progesterone receptor status, [3]- *Her2 overexpression*

Tissue samples

Paraffin embedded formalin fixed biopsies from 10 breast cancer cases diagnosed as infiltrating ductal carcinoma, 1 mucinous sample and 6 biopsies from normal breast epithelia was used for total RNA extraction and posterior miRNA expression analysis.

Samples characteristics are described on table III-2. Tissue samples were obtained at Department of Pathology, from Fernandes Figueira Institute (IFF-FIOCRUZ), in Rio de Janeiro, with the local ethical committee approval.

Table III-2: Paraffin embedded formalin fixed breast samples

Tumor	Histological classification	EG[1]	Tumor size	Lymph node metastasis
T2	IDC[2]	1	2,0X1,2 cm	?
T19	Mucinous	1	?	?
T39	IDC[2]	1	0,6X0,5X0,4 cm	?
T15	IDC[2]	2	1,5X1,2X1,0 cm	?
T1	IDC[2]	2	2,7X1,7 cm	1 dos 16 linfonodes
T18	IDC[2]	3	2,0X2,0X2,0 cm	5 dos 18 linfonodes
T40	IDC[2]	3	1,7X1,5 cm	5 dos 11 linfonodes
T72	IDC[2]	3	3,0X3,0X3,0 cm	absent
T36	IDC[2]	3	0,7 cm	?
T26	IDC[2]	3	?	?
T21	IDC[2]	3	0,9X0,7 cm	absent
N16/1	Normal tissue	-	-	-
N16/2	Normal tissue	-	-	-
N21	Normal tissue	-	-	-
N26	Normal tissue	-	-	-
N5651.08.6	Normal tissue	-	-	-
N929.06.7	Normal tissue	-	-	-

[1] Elston Grade; [2]IDC- Intraductal carcinoma

Gene expression assays

miRNA expression levels was accessed from total RNA samples by both Microarray (Agilent Human miRNA v16.0 Microarray format 8x60K) and quantitative RT-PCR. Gene expression from 96 selected genes, involved in DDR and tumorigenesis was also analyzed by quantitative RT-PCR, using a TDLA (Taqman low density array) plate and by SYBR green technology for some selected genes. Microarray and TDLA reagents were obtained by the funds of "*Projet taxe d´aprendisage*" 2010/2011 and the experiments were done in collaboration with the IGR´s genomic platform, France.

Western blot

HER-2, Zeb-1 and e-cadherin protein levels were analyzed by western blot on 21T series, using the respective antibodies: HER-2/Neu (F11) (SC7301, 1:500, Santa Cruz Biotechnology), ZEB-1 (H-102, 1:200, Santa Cruz Biotechnology) and e-cadherin (610182, 1:2000, BD Biosciences). β-actine was used as load control (MAB1501, 1:2000, Millipore).

Cell treatments

Cell lines were submitted to a low energy X-ray irradiation (30kVs-50mA) at 5Gy dose performed on a mammography simulator located at the Radiobiology Laboratory at Universidade do Estado do Rio de Janeiro, Brazil. High energy Cesium irradiation at 0,5Gy dose was performed at PR2- Institut Gustave Roussy, France. Cells were also treated with doxorubicin at 1μM for 24h. Fresh doxorubicin samples were kindly provided by Dr Dante Pagnoncelli.

Genotoxicity testing

DNA single and double strand breaks were quantified respectively by Comet assay (Single cell electrophoresis assay) and *micronuclei* observation. Apoptosis occurrence was monitored by evaluation of nuclei morphology after HOECHST 33258 staining.

Chip assay

To access chromatin conformation on miR-29b promoter region, total chromatin was conjugated with the following antibodies: anti-H4Ac monoclonal antibody (catalog no 39243, Active Motif) euchromatin marker and anti-H3K9me3 (catalog no 05-1242, Millipore) heterochromatin marker.

Detailed description of all techniques can be found on the following section IV (Results) as part of the manuscripts.

IV.Results

The results achieved in this work were segmented in three manuscripts. The first one intitled “miR-205 is involved in metastatic potential of 21T series: a breast cancer progression model” describes a potential role of miR-205 on EMT transition, and consequently metastasis in 21T cells.

The second part of the work was dedicated to analyse the miRNAs expression after DNA damage.

The manuscript “Expression of apoptosis- associated microRNA after low-energy X-ray irradiation of breast cell lines”, already submitted to Mutation Research- Fundamental and Molecular Mechanisms of Mutagenesis, shows the capacity of low-energy X-rays to promote DNA strand breaks and to induce apoptosis in the non-tumoral cell line HB-2 and in breast cancer derived cell line MCF-7. The levels of the miRNAs let-7, miR-34a and miR-21, which have as targets genes involved in DDR, were measured and correated with DNA damage observed 4 and 24h after irradiation.

The manuscript “miR-29b downregulation in breast cancer cell lines is reverted by both low and high energy radiation” shows that miR-29b is downregulated in breast tumor cell lines that can be due to an epigenetic remodelation at miR-29b promoter region. However, after genotoxic damage, promoted by both low and high energy irradiation as well as doxorubicin treatment, this miRNA turns to be up-regulated. miR-29b is described to be a pro- apoptotic miRNA and can be a candidate as a biomarker of genotoxic stress.

This thesis is part of collaboration between Université Paris-Sud XI and Universidade do Estado do Rio de Janeiro and was developed under Dr Claudia Vitória de Moura Gallo direction at Laboratório de Biologia Molecular de Tumores, Brazil and under Dr Yegor Vassetzky direction at UMR8126- Interactions moléculaires et cancer, France.

During this period I also have the opportunity to participate as co-author of two papers: “The Kruppel-like factor 15 as a molecular link between myogenic factors and chromosome 4q transcriptional enhancer implicated in fascioscapulohumeral dystrophy” and “Defective Regulation of miRNA Target Genes in Myoblasts from Patients with Fascioscapulohumeral dystrophy”. Both of them were developed at Yegor Vassetzky´s lab. These papers are part of a work that aims to characterize genetic changes involved in fascioscapulohumeral dystrophy.

My contribution in these papers was to perform the techniques of plasmid cloning, luciferase assays and to quantify microRNA expression by qPCR.

Manuscript 1: miR-205 is involved in metastatic potential of 21T series: a breast cancer progression model

miR-205 is involved in metastatic potential of 21T series, a breast cancer progression model

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Running Title: Metastatic role of miR-205 in 21T cells

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Keywords: MicroRNA; miR-205; 21Tseries, EMT

Abstract

MicroRNA is a class of non-coding RNAs, which regulate gene expression at the post-transcriptional level. Many miRNAs have been implicated in several human cancers, as these regulatory molecules play important roles in key cellular processes, including cell proliferation, differentiation and response to DNA damage. To gain insights into the mechanisms involved in breast cancer initiation and progression we conducted a miRNA global expression on 21T series. These cell lines represent an *in vitro* model of breast cancer progression comprising immortalized cell lines derived from the same patient which include a normal epithelia (16N), primary *in situ* ductal cancinoma (21PT and 21NT) and cells derived from pleural effusion of lung metastasis (21MT-1 and 21MT-2). To confirm microarray results, the expression of the most significantly altered miRNAs were checked by qPCR. Analysis of 21T series revealed a significant downregulation of miR-205 together with an enrichment of its predicted target, the pro-metastatic factor *ZEB-1* and the consequent reduction of e-cadherin levels in 21MT cells. These molecular alterations, in special the inhibition of miR-205 in cancer cells, can participate on regulation of epithelial to mesenchymal transition and increase the metastatic potential on breast cancer.

1. Introduction

Breast cancer is the most frequent carcinoma in women and is also a highly heterogeneous disease. Diagnostic and treatment decision are mainly based on classical biological variables, including morphology, tumor grade, presence of lymph-node metastasis, molecular markers, such as estrogen and progesterone receptor (ER, PR) and human epidermal growth factor receptor 2 status (HER-2) (Reis-Filho and Pusztai, 2011). By histological classification, it is possible to identify ductal and lobular subtypes as those of highest incidence (Bombonati and Sgroi, 2011). The invasive ductal carcinoma (IDC) presents a high morphological and clinical variability and its prognostic is usually related with tumor grade (Bombonati and Sgroi, 2011, Bertos and Park, 2011). Transcriptional profiling studies have also contributed on our understanding of cancer heterogeneity and progression. The most accepted classification based on gene expression was proposed by Perou and colleagues (2000) and divides breast tumors into four intrinsic subtypes Luminal A and B, Her2 enriched and basal-like (Perou, 2000, Prat and Perou, 2011). The molecular classification revealed that breast tumors can be clustered according to their transcriptional profile, also taking into account some classical biomarkers, including ER, PR and HER-2. Furthermore, these subtypes have different incidence survival and response to treatments, being the basal and HER-2 enriched the ones with worse prognosis (Prat and Perou, 2011, Reis-Filho and Pusztai, 2011). Cancer progression according to the model of clonal evolution consists on a multistem process, progressing from normal epithelia to a hyperplasia to atypical ductal hyperplasia then to ductal carcinoma *in situ* (DCIS), culminating as an invasive ductal carcinoma (IDC) (Schnitt, 2010). The advances of the screening techniques since the 1980s made possible the diagnosis of breast carcinoma in earlier stages. Expression profiling analysis has shown that there are very few differentially

expressed genes in DCIS and IDC of similar grade (Espina and Liotta, 2011). These studies have demonstrated that there is no clear "*in situ*" and "invasive" gene expression signature. Also, the morphological analysis of these cells usually looks similar, if not identical, and the receptor status of coexistent DCIS and IDC is often the same. The knowledge on DCIS to IDC transition is still incomplete and there are a lot of remaining questions concerning breast cancer progression. It is not clear if the enrichment of a very few specific genes in IDC is enough to promote cell migration or if other factors, such as the microenvironment, breast stem cells enrichment or epigenetic changes, including microRNA (miRNA) regulation are acting together on DCIS progression to IDC.

miRNAs are small (about 23 nucleotides long) non-coding RNA species that during the past five years have emerged as major elements of gene expression control, acting in post-transcriptional level by targeting its complementary mRNA sequence. These small gene expression regulators are often deregulated in breast cancer and can have both tumor suppressor and oncogenic activity, being able to modulate nearly all relevant stages of cancer progression, including cell proliferation, apoptosis, cell migration, angiogenesis and stem cell maintenance. In breast tumors, special emphasis is given on miR-200 family, miR-21 and miR-205 that are able to regulate cell proliferation and invasion (Gregory *et al*, 2008; Wu *et al*, 2009). The miR-200 family and miR-205 can modulate epithelial to mesenchymal transition (EMT), mainly by e-cadherin regulation via *ZEB-1* inhibition (Gregory *et al*, 2008). Also, miR-205 can be negatively regulated by HER-2, possibly contributing for the worse prognostic associated to HER-2 enriched subtype (Adachi, 2011). Based on miRNA profiling data, breast cancers without vascular invasion show high miR-205 expression, compared to the more vascularized ones. It was also demonstrated that miR-205 is able to suppress lung metastasis in

animal models (Wu *et al*, 2009). When normally expressed, miR-205 can reduce its targets levels, such as *ZEB1* and *ZEB2* and, consequently, result in the expression of *E-cadherin,* keeping cell polarity and cell-cell junction integrity (Greene, 2010). To date miR-205 validated targets includes *E2F1, HER3, VEGF-A* and *PTEN* (Wu *et al*, 2009; Iorio *et al*, 2009, Dar *et al*, 2011; Qu *et al*, 2012). We hypothesized that miR-205 is expressed at higher levels in cancer subtypes with more epithelial characteristics, having a better prognosis and is lower in those that are more invasive with a poorer prognosis. To identify the role of miRNAs on cancer progression, we used as experimental model the 21T series, which is an *in vitro* model for breast cancer progression comprising cell lines derived from the same patient which include a normal epithelia (16N), primary ductal carcinoma (21PT and 21NT) and cells derived from the metastatic pleural effusion (21MT-1 and 21MT-2). Cells were obtained from different tissue samples from a woman initially diagnosed with infiltrating and intraductal carcinoma of nuclear grade 3 with 3 positive lymph nodes. The tumor receptor profile was ER-, PR- HER2+ (Band *et al* 1989; Band *et al* 1990). We also observed miR-205 expression on the well established cell lines MCF-10A (breast normal epithelia), MCF-7, obtained from a lung metastasis originated from a breast adenocarcinoma, T-47D, also obtained from a pleural effusion from a metastatic site originated from a breast invasive ductal carcinoma and from breast tissue samples. This study aims to evaluate changes in microRNA expression in different stages of epithelium to IDC progression and to accumulate evidence on the role of miR-205 in cell migration and metastasis.

2. Materials and Methods

2.1.Cell Lines and Culture

The cell lines from 21T series (21PT, 21NT, 21MT1 and 21MT2) were obtained from Dr. Pierre Hainaut (IARC-Lyon, France) and 16N from Dr. Vimla Band (University of

Nebraska Medical Center, USA). The cell lines MCF-10A, MCF-7 and T-47D were obtained from Dr. David Cappellen and Dr. Nancy Hynes (Friedrich Miescher Institute for BioMedical Research, Novartis Research Foundation, Basel, Switzerland). MCF-10A and cell lines from 21T series were maintained in DMEM-F12 media (Gibco) supplemented with 10% FBS, EGF (20ng/mL), insulin (10mg/mL) and hydrocortisone (0.5mg/mL) (Sigma-Aldrich) at 37^{o}C and 5% CO_2. Breast cancer cells MCF-7 and T-47D were maintained in DMEM media (Gibco) supplemented with 10% FBS, at 37^{o}C and 5% CO_2. All cell cultures were routinely checked for mycoplasma contamination.

2.2.Tissue samples

Paraffin embedded formalin fixed biopsies from 10 breast cancer cases diagnosed as infiltrating ductal carcinoma, 1 mucinous sample and 6 biopsies from normal breast epithelia were obtained at The Department of Pathology, from Fernandes Figueira Institute (IFF-FIOCRUZ), in Rio de Janeiro. The patient's data were obtained from the hospital records. Ethical approval for this study was obtained by local research Ethics Committees.

2.3.RNA extraction

The extraction of total RNA from cell cultures was performed with Trizol reagent (Life Technologies), following the manufacture´s protocols. The amount and quality of RNA samples were assessed on the Agilent 2100 Bioanalyzer with RNA 6000 Nano Reagents and Supplies (Agilent). Total RNA was isolated from formalin fixed paraffin embedded samples using High Pure RNA Paraffin Kit (Roche Applied Science), according to the manufacturer's protocol.

2.4.miRNA Microarray

Total RNA samples (100 ng) were dephosphorilated and labeled with the fluorophore Cyanine 3- pCp at the 3'end of the RNA molecule. After labeling, samples were purified with Micro Bio-spin 6 columns (Bio-Rad) and hybridized on array slides at 55^{o}C, 20 rpm for 20h. The label and hybridization reactions were performed using miRNA Complete Labeling and Hyb Kit (Agilent), following manufacturer's instructions. The microarray slides hybridized to the Agilent Human miRNA V16.0 Microarray format 8x60K(Agilent Technologies) were scanned on Agilent's High-Resolution C Scanner.

2.5.Microarray data analysis

To perform the microarray analysis, data were extracted from scanned images using Feature Extraction software (v 10.5.1.1 Agilent) with default settings. Data from all hybridizations was analyzed with LIMMA (Smyth, 2004), an R package from the Bioconductor project. First, a quantile inter array normalization was performed on the raw gMedianSignal values. Then the median of all probes for a given miRNA was computed to summarize the data. To asses differentially expressed genes, we start by fitting a linear model to the data. Then we used an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. The top-ranked genes were selected with the following criteria: an absolute fold-change > 2 and an adjusted p-value (FDR) < 0.05.

2.6. Quantitative RT-PCR

For miRNAs quantification, cDNA synthesis was carried out with total RNA, using NCode VILO miRNA cDNA Synthesis Kit (Life Technologies). For mRNAs quantification, cDNA synthesis was done with total RNA, using High Capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time PCR was performed using SYBR PCR Master Mix (Life Technologies). The primers sequences employed in qRT-

PCR reactions were: e-cadherin (F) 5´ CCCACCACGTACAAGGGTC 3´; e-cadherin (R) 5´ CTGGGGTATTGGGGGCATC 3´; b-actin (F) 5´ CATCGAGCACGGCATCGT 3´; b-actin (R) 5´ GCCTGGATAGCAACGTACAT 3´, miR-205 (F) 5´ CTTCATTCCACCGGAGTCTG 3´ with the universal reverse primer provided on miRNA cDNA synthesis kit. All experiments were performed in triplicate, using β- actin as a reference gene. Expression results were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001) and statistical analysis was done by Student's *t*-test, considering statistically significant p-value ≤ 0.05.

2.7. Western blot

Whole cell lysates were prepared using RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 1mM protease inhibitor cocktail). The preparation was separated using 10% SDS-polyacrylamide gel, transferred to Hybond-C extra nitrocellulose membrane (Amersham Biosciences) and incubated with primary antibodies against HER-2/Neu (F11) (SC7301,1:500, Santa Cruz Biotechnology), ZEB-1 (H-102, 1:200, Santa Cruz Biotechnology), E-cadherin (610182, 1:2000, BD Biosciences) and actin (MAB1501, 1:2000, Millipore) and Goat Anti-Mouse IgG Peroxidase Conjugated secondary anti-mouse (DC02L, 1:10000, Millipore) or Goat Anti-Rabbit IgG Peroxidase Conjugated (DC03L, 1:2000, Millipore) antibodies. Finally, the results were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

3. Results

3.1. miR-205 is potentially involved into metastatic transition in 21T cells

Intending to identify miRNAs species involved in 21T transition from primary breast tumor to a metastatic phenotype, we evaluated the microarray data of the differentially expressed miRNAs in metastatic 21T cell lines (21MT1 and 21MT2), compared to primary carcinoma 21T cells (21PT and 21NT) (Table 1). Only one miRNA, the miR-205 was strongly down-regulated (more than 2 fold) in 21MT1 and 21MT2 cells. The decreased expression of miR-205 is in agreement with the microarray data of other invasive cell lines (MCF-7 and T-47D), in comparison with the non-tumoral breast epithelial cell line MCF-10A (Table 2).

3.2.miR- 205 downregulation is associated to a high proliferative and invasive phenotype

The miR-205 reduced levels in 21MT cells were confirmed by qRT-PCR (Figure 1). We also verified miR-205 expression by qRT-PCR in metastatic derived cell lines MCF-7 and T-47D (Figure 2) and confirmed that this miRNA is also down-regulated in these cell lines. To check whether low levels of miR-205 correlate to a more aggressive phenotype *in vivo*, we quantified miR-205 expression by qRT-PCR in 11 paraffin conserved breast tumor and 6 non-tumoral epithelia samples. Table 3 shows the obtained results from the average expression of all tumor samples relative to the non-tumoral sample. Paraffin tumor samples were histological classified and clustered according to Elston grade. This classification takes into account tissue organization, nuclear size and emphasizes the tumor mitotic rate, being a good parameter to evaluate the tumor cells capacity to proliferate and invade other tissues (Elston and Ellis, 1991). Interestingly, we observed an inverse correlation between miR-205 and tumor grade,

with an important down-regulation detected in grade 3 invasive ductal carcinoma samples (Table 3).

3.3.Low miR-205 levels is associated to ZEB-1 up-regulation and e-cadherin down-regulation in HER-2 overexpressing 21T breast cancer cell lines

One of the hallmarks of metastasis is the epithelial to mesenchymal transition that allows cancer cells colonization in distant tissues. The main features of this process involve loss of epithelial markers, including E-cadherin, and the consequent loss of epithelial polarity and impairment of cell-cell junctions, allowing cell detachment. E-cadherin is encoded by the CDHI gene and some proteins have been identified as its transcriptional repressor, including ZEB1 and ZEB2. Both are encoded by mRNAs that are miR-205 targets (Gregory et al, 2008). All these observations lead us to verify the described repressive effect of endogenous miR-205 on ZEB-1 protein expression. Hence, we tested by western blot, the ZEB-1 protein levels different stages of tumor progression using the 21T series. Inhibition of miR-205 is correlated with elevated levels of ZEB-1 and with transcriptional repression of e-cadherin, quantified both at protein and mRNA levels (Figure 3 and Table 4).

3.4.Differential expression of miRNAs during breast cancer progression

In order to detect miRNA expression changes during breast cancer progression, we analysed the miRNA microarray obtained from each tumoral cell line (21PT, 21NT, 21MT1 and 21MT2) relative to the non-tumorigenic one (16N) (Suplementary data 1)

To better identify the altered miRNA during different stages of breast cancer progression, we compared the group formed by primary cancer cells (21PT and 21NT) and the group of metastatic cells (21MT1 and 21MT2) with the matched “normal” 16N cell line.

Tables 5 and 6 show the miRNA species, which presented significantly altered expression, over two-fold. Our results revealed that, among less studied miRNAs, some of the detected species, such as mir-29, miR-21, mir-210 and miR-205 have a established role in cancer development. Table 7 summarizes some of the detected miRNAs already described in litterature and their targets. As expected, most of the altered miRNAs species in 21T series tumor progression have their targets linked to proliferation, cell cycle control and metastasis pathways. Our results reveal a shift on miRNA´s signature during tumorigenesis and that only miR-205 expression is significantly altered in 21T cells during tumor evolution to metastasis.

4. Discussion

During the last 10 years, the knowledge about miRNA functions has enormously increased. Through their ability to repress gene expression by binding on an imperfect way to an mRNA 3´UTR region, they are able to control a myriad of different targets, becoming important regulators of different cellular processes, including tumorigenesis. It has been observed that cancers of different origins share similar miRNA signatures and these tumor-specific differences in the miRNAome can be a useful classifier and a prognostic tool.

The present work is dedicated to determine the differentially expressed miRNAs in 21T cells, a series of cell lines derived from the same patient, representative of breast cancer progression (Band et al, 1990; Qiao et al, 2007; Souter et al, 2010). Taking advantage of the possible comparison between the cancer cell lines 21PT, 21NT, 21MT1 and 21MT2 *versus* non-cancerous 16N or 21MT1 and 21MT2 *versus* non-metastatic 21PT and 21NT, our major goal was to highlight miRNAs with potential participation in the

breast cancer progression, taking advantage of this model that minimize genetic background interference.

The lungs and bones are frequent sites of breast cancer metastasis and metastases to these sites differ in terms of their evolution, gene expression, treatment, morbidity and mortality (Minn et al, 2005).

The metastatic related miR-205 has been considered one of the most important repressor of the epithelial to mesenchymal transition (EMT) (Wright et al, 2010) and is frequently down-regulated in malignant tumors (Gregory et al, 2008; Wu et al, 2008).

EMT concerns a molecular reprogramming and phenotypic changes involved in the conversion of polarized immotile epithelial cells to motile mesenchymal cells. This process represents the first barrier that cancer cells have to overcome to become metastatic (Gregory *et al*, 2008). EMT describes a series of reversible events involving some key signaling pathways that converge on a small number of transcription factors. Among them, *ZEB1* and *ZEB2* that are able to repress e-cadherin.

Our results are in agreement with the literature that identifies *ZEB1* as a miR-205 target, showing a significant reduction in miR-205 levels in metastatic 21T cells followed by an increase of zeb-1 protein levels and a consequent reduction in e-cadherin expression. E-cadherin is a central component of calcium dependent tight junctions and maintenance of cytoskeletal organization. Loss of *E-cadherin* expression in tumors is often associated with a more aggressive phenotype (Gregory et al 2008).

It was also demonstrated that miR-205 can target *E2F1* and that its expression is directly induced by the tumor suppressor p53 (Piovan et al, 2012). miR-205 seems to promote a barrier against cell proliferation, as its ectopic expression in breast cell lines significantly reduces cellular proliferation, clonogenic survival and anchorage

independent growth of MCF-7 and invasion and metastasis potential of MDA-MB-231 (Wu et al, 2009).

Comparison of miR-205 predicted target genes by target scan and transcriptome data of altered mRNA in 21NT-21MT1 transition (Souter et al, 2010) reveals a new miR-205 possible target, the *TBX3*, a critical transcription factor for mammary gland development that is also regulated by *Wnt* signaling pathway and is strong upregulated following induction of EMT program in squamous carcinoma cells, reinforcing the role of miR-205 in cancer cell migration (Humtsoe et al, 2012; Cho et al, 2012).

Analysis of microarray obtained data put on evidence several down and up-regulated miRNAs during cancer formation. In a survey of the recent bibliography, we could observe that part of the altered detected miRNAs has not yet a described function for example, miR-4299 and miR-3162. Together, this data show evidence that miRNAs plays an important role in tumorigenesis and during tumor maintenance and evolution, with a special focus on miR-205 driven EMT and metastasis in breast cancer cells.

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Table 1. Differentially expressed miRNA species in 21PT/21NT *versus* 21MT1/21MT2 cells ($p \leq 0.05$). Fold change cut-off was set at 2.0.

Name	Fold change	p-value

hsa-miR-205	-5.42729	3.62E-03

Table 2. miR-205 expression in MCF-10A, MCF-7 and T-47D cell lines detected by miRNA microarray.

hsa-miR-205	Fold Change	p-value
MCF-7	-91.25	1.05E-09
T-47D	-51.81	3.63E-09

Table 3: miR-205 levels in paraffin tissues

miR-205	Fold Change	p-value
EG1	1.740	0.313
EG2	-6.399	0.858
EG3	-8.834	1.34E-06

EG, Elston Grade.

Table 4. E-cadherin expression in 21T cell lines detected by qRT-PCR

21Tcells	
e-cadherin	**FC ± SD**
16N	1 ±0.07
21PT	4.09 ±0.06
21NT	1.143 ±0.07
21MT1	1.723 ±0.04

21MT2	-2.309 ±0.03
***In situ* x Metastatic**	
21PT, 21NT	1 ±0.8
21MT1, 21MT2	-2.405 ±0.7

Table 5. Differentially expressed miRNA species in 21PT/21NT *versus* 16N cell lines ($p \leq 0.05$). Fold change cut-off was set at 2.0.

Name	Fold change	p-value
hsa-miR-4299	-7.11502	2.94E-04
hsa-miR-3934	-5.44323	3.73E-02
hsa-miR-3162-5p	-2.2186	4.05E-02
hsa-miR-99b	2.200703	2.79E-02
hsa-miR-21	2.215094	1.56E-02
hsa-let-7e	2.242305	9.55E-03
hsa-miR-137	2.270638	1.01E-03
hsa-miR-29[a]	2.336743	9.34E-04
hsa-miR-30[a]	2.37167	3.28E-02
hsa-miR-200[a]	2.625427	2.65E-02
hsa-miR-10[a]	2.849836	4.71E-05
hsa-miR-93	2.976382	1.37E-04
hsa-miR-183	3.20894	6.42E-05
hsa-miR-135b	3.288252	2.43E-03
hsa-miR-148[a]	3.369391	5.27E-05
hsa-miR-29c	4.923525	2.79E-02
hsa-miR-106b	4.929391	4.71E-05

hsa-miR-96	5.813736	1.48E-03

Table 6. Differentially expressed miRNA species in 21MT1/21MT2 *versus* 16N cell lines ($p \leq 0.05$). Fold change cut-off was set at 2.0.

Name	Fold change	p-value
hsa-miR-4299	-10.1616	1.06E-04
hsa-miR-205	-7.87877	4.96E-04
hsa-miR-125b	-2.64046	4.09E-02
hsa-miR-130[a]	-2.53346	3.29E-03
hsa-miR-320d	-2.23462	1.06E-04
hsa-miR-3162-5p	-2.10762	4.54E-02
hsa-miR-181d	-2.09534	2.63E-03
hsa-miR-155	-2.0548	5.35E-05
hsa-let-7e	2.000388	1.66E-02
hsa-miR-137	2.060418	2.15E-03
hsa-miR-10[a]	2.230652	1.51E-04
hsa-miR-99b	2.607462	9.11E-03
hsa-miR-93	2.772225	1.63E-04
hsa-miR-183	2.834453	1.20E-04
hsa-miR-30[a]	2.901151	9.78E-03
hsa-miR-148[a]	2.908579	1.06E-04
hsa-miR-29[a]	3.00206	1.61E-04
hsa-miR-200[a]	3.376496	6.46E-03
hsa-miR-29c	3.890496	4.54E-02
hsa-miR-135b	3.924775	1.12E-03

hsa-miR-106b	4.409554	7.88E-05
hsa-miR-96	5.128198	2.15E-03

Table 7: Summary of the cellular processes the 21T altered miRNAs have been implicated in and their target genes identified to date.

miRNA	**Related pathway**	**Targets**	**Refs**
Up-regulated			
miR-29	Apoptosis, Cell adhesion	MCL1, PTEN, DNA methyl-transferases , Integrin B1	Fabbri et al, 2007; Mott et al, 2007; Xiong et al, 2010; Liu et al, 2010; Meunier, et al, 2012.
miR-93	Tumor growth, Angiogenesis	PTEN, Integrin-b8, VEGF, FUS1	Du et al, 2009; Long et al, 2010; Fang, et al, 2011; Fu et al, 2012
miR-96	Cell proliferation	Foxo1, Foxo3a	Lin *et al* 2010; Chen et al, 2012; Wang et al, 2012
miR-106b	Cell cycle arrest, EMT	CCND1, PTEN, CDKN1a, MAPK9, Smad7, p21	Ivanovska et al, 2008; Kan et al, 2009; Trompeter et al, 2011; Cai et al, 2011; Smith et al, 2012
miR-135b	Cell proliferation	Foxo1	Matsuyama et al, 2011;
miR-183	Cell migration, Apoptosis	EGR1, AKT1, AKT2, PDCD4	Sarver, et al, 2010; Li et al, 2010; Weeraratne et al, 2012
miR-200a	EMT/ Metastasis	Sec23a, ZEB1/2	Gregory et al, 2008 and Korpal et al, 2011
miR-21	Cell proliferation, Apoptosis, EMT, Metastasis	PTEN, PDCD4,TM1, p12, Spry2, TIAM1, RhoB, Cdc25A, MARCKS	Qian, 2009; Li et al, 2009; Wang et al, 2009; Cottonham et al, 2010; Zheng et al, 2011; Kwak et al, 2011; Liu et al, 2011; Zhang et al, 2012

miR-10a	EMT/ Metastasis	HOXA1	Ohuchida et al, 2012
Down-regulated			
miR-205	EMT/Metastasis	ZEB1/2, PTEN, E2F1, HER3	Gregory et al, 2008; Iorio et al, 2009; Dar et al, 2011; Qu et al, 2012
miR-210	Hipoxia, Cell proliferation and differentiation	FGFRL1, MNT, AcvR1b	Zhang et al, 2009; Mizuno et al, 2009; Tsuchiya et al, 2011
miR-181d	Apoptosis, cell cycle, and proliferation	K-Ras, Bcl-2	Zhu et al, 2010; Wang et al, 2012
miR-320	Angiogenesis, cell invasion	ETS2, CD71	Schaar et al, 2009; Bronisz et al, 2011
miR-130a	Cell migration	MET, ATG2B, DICER1, GAX, HOXA5	Chen and Gorski, 2008; Acunzo, et al, 2012; Kovaleva et al, 2012
miR-125b	Cell invasion	ERBB2, ARID3B	Shang, et al, 2012; Akhavantabasi et al, 2012

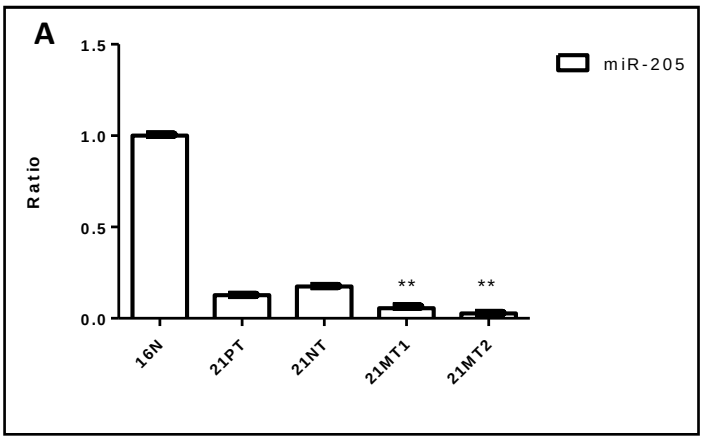

Figure 1: Quantitative RT-PCR of miRNA miR-205 on 21Tcell lines.

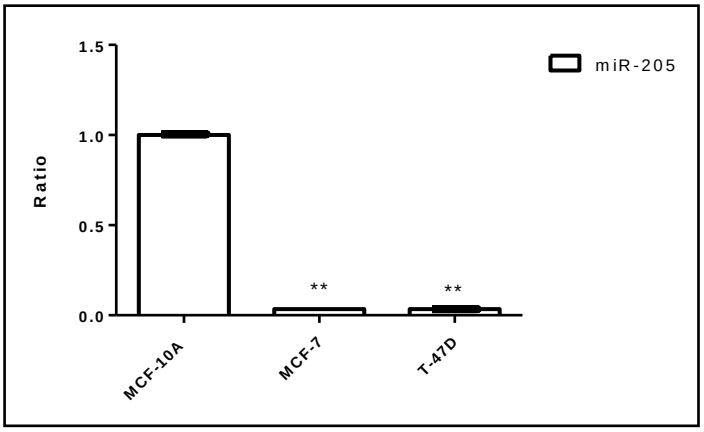

Figure 2: Quantitative RT-PCR of miRNA miR-205 on MCF-10A, MCF-7and T-47D cell lines.

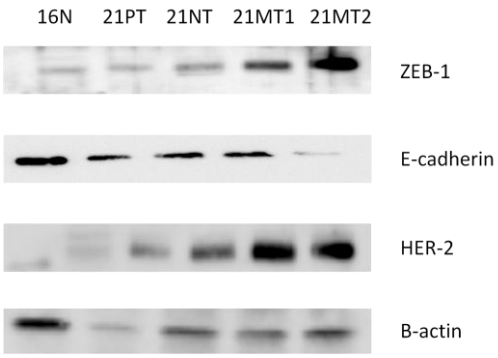

Figure 3: Western blot revealing increased levels of ZEB1 and a decrease in e-cadherin protein levels in accordance with tumor progression in ERBB2 positive 21T cells. ZEB1 is up-regulated while e-cadherin is down-regulated during cancer progression on 21T cells.

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Manuscript 2: Expression of apoptosis-associated microRNAs after low-energy X-ray irradiation of breast cell lines

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Expression of apoptosis-associated microRNAs after low-energy X-ray irradiation of breast cell lines

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Running Title: MicroRNA expression in X-ray irradiated breast cells.

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Abstract

Ionizing radiation provokes DNA damage in humans submitted to radiotherapy and medical imaging procedures, such as mammography. MicroRNAs, small non-coding RNAs which modulate important cellular processes, like apoptosis in response to DNA damage, may serve as potential biomarkers of genotoxic stress, as well as, therapeutic targets. We have studied the expression of the apoptosis-associated microRNAs let-7a, miR-34a and miR-21 in immortalized non-tumoral HB-2 breast epithelial cells and MCF-7 breast cancer cell line after 5Gy/30 kV X-ray irradiation. DNA lesions, as well as, apoptotic nuclei have been observed in both cell lines at 4 and 24 h after irradiation. We have detected upregulation of let-7a and miR-34a at 4 h after irradiation. Let-7a and miR-34a levels returned to the control levels at 24 h in MCF-7 cells. No significant changes in miRNA levels were observed in HB2. miR-21 expression was not significantly altered in both cell lines. We propose that miR-34a and let-7a up-regulation is associated to genotoxic stress during low energy X-ray irradiation, probably taking part in early cell responses to DNA injury in MCF-7 cells.

1. Introduction

MicroRNAs (miRNAs) are a class of evolutionarily conserved small non-coding RNAs, which regulate gene expression, mainly at the post-transcriptional level. miRNAs exert their functions by binding to the 3'UTR of target mRNAs and subsequently directing them for translational inhibition and mRNA decay [1]. Many miRNAs have been implicated in several human cancers, as these regulatory molecules played important roles in several cellular processes, including DNA damage response [2]. Ionizing radiation such as X-rays, can damage cells by direct DNA breakage or through an indirect mechanism, which involves interaction with water molecules and creation of free radicals, which will also contribute to increase and prolong DNA damage eliciting cell responses to adapt or die [3,4]. Irradiated cells normally activate DNA repair system or apoptosis depending on cell type. Initial cell responses to genotoxic stress occur through molecular sensors, usually kinases that trigger important pathways. For example, the ATM-mediated DNA damage response activates the tumor suppressor protein p53, a transcription factor critical for genomic stability, regulating cell cycle progression and DNA repair, as well as apoptosis [5,6]. Apoptotic mechanisms are intricately connected with DNA damage and cancer. Increasing evidence associate three specific miRNAs, miR34a, let-7a and miR-21 with the control of cell damage response pathway [7]. miR-34a is a direct p53 target gene and its ectopic expression induces apoptosis, cell-cycle arrest in G1 or senescence [8]. *In vitro* experiments using *C elegans* and breast cancer cells as models showed that loss of function mutations in miR-34a gene generated an abnormal cellular survival response to radiation that was correlated with the increase of cell death after DNA damage [9]. Validated miR-34a targets include several genes involved in DNA damage response pathways as Bcl-2, Notch1, Cyclin D1, Cyclin E2, CDK4, MET and SIRT1 [10,11,12], suggesting that

miR-34a may serve as a marker of radiation injury and as a therapeutic target [9,13]. Let-7a is a member of a family that comprises 12 miRNAs. All of them are tumor suppressors and can be regulated in response to ionizing radiation. Among let-7a targets there are molecules involved in important cellular activities as proliferation (K-ras; c-myc; E2F2) and cell cycle control (Cdc25a; Cyclin D1). Let-7a is usually downregulated after ionizing radiation exposure, however its overexpression can increase radiosensitivity *in vivo* and in different tumor types, mainly by downregulation of K-Ras [14, 15]. Finally, miR-21, classified as an oncogenic miRNA, was described as a negative regulator of many suppressor genes related to proliferation, apoptosis and invasion such as PTEN, PDCD4, Tropomyosin-1 and Bcl-2 [16, 17, 18]. miR-21 is often deregulated in tumors and its overexpression is associated with a more proliferative and aggressive phenotype [19, 20]. *In vivo* and *in vitro* studies suggest a role for miR-21 in tumor initiation and progression and as a possible diagnostic and prognostic marker for human malignancies. In breast cancer, miR-21 knockdown cells can trigger apoptotic cell death accompanied by a decrease in cell proliferation suggesting a function as an anti-apoptotic factor [13, 21]. miR-21 is usually upregulated after irradiation and its inactivation can contribute to radiation induced apoptosis [13, 22, 23, 24]. Several miRNAs with aberrant expression are present in breast cancer, as well as in most other cancers. Microarray analysis shows a global change in miRNA expression in the presence of genotoxic agents, including irradiation [25].

Considering, thus, the importance of the above mentioned miRNAs in the regulation of apoptosis, and that low energy X-ray irradiation is commonly used in mammography exams, we have evaluated the level of expression of miR-34a, let-7a and miR-21, under low-energy X-ray cell irradiation, in the immortalized HB-2 non-tumoral breast cell line and the MCF-7 breast carcinoma derived cell line. The aim of the present work is to

determine whether the analyzed miRNAs are deregulated during irradiation of breast cells with low-energy X-rays. We have observed an upregulation of miR-34a and let-7a associated to DNA damage produced by low energy X-ray irradiation in MCF-7, but not in the control cells.

2. Materials and Methods

2.1. Cell culture

The human breast adenocarcinoma cell line MCF-7 and the non-tumorigenic epithelial breast cell line HB-2 were obtained from David Cappellen and Nancy Hynes (Friedrich Miescher Institute for BioMedical Research, Novartis Research Foundation, Basel, Switzerland). The MCF-7 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin, streptomycin. HB-2 cells were maintained in DMEN supplemented with 10% FBS, hydrocortisone 0.5µg/mL, insulin 5µg/mL and 1% penicillin, streptomycin. The culture medium and FBS were purchased from Invitrogen, Life Technologies (Carlsbad, CA, USA), all others supplements were from Sigma Aldrich (St Louis, MO, USA). Both cultures were routinely checked for mycoplasma contamination.

2.2. Irradiation of cells

Cells were submitted to a dose of 5 Gy (0.1 Gy/s) with x-ray (30 kV, 0.03 mm Mo filter) and seeded in logarithmic growth phase and just before irradiation the culture medium was replaced by PBS 1X (60mM NaCl; 0.2mM KCl; 0.1mM Na_2HPO_4; 0.1mM KH_2PO_4, pH 7.4) in a volume as much as necessary to fill the culture flask. These irradiation conditions were chosen in order to analyze biological effects caused by low

energy X-ray irradiation. After irradiation, PBS 1X was discarded and cells were incubated in culture medium at 37°C, 5% CO_2 for 4 or 24 h. A mock control done with cells under the same conditions but the irradiation was added in all experiments.

2.3. Comet assay (Single cell electrophoresis assay)

Comet assay was performed according to the method described elsewhere [26] with some modifications. Aliquots containing 2.5 x 10^5 cells were pelleted and ressuspended in 10μL of PBS 1X (60mM NaCl; 0.2mM KCl; 0.1mM Na_2HPO_4; 0.1mM KH_2PO_4, pH 7.4). Briefly, 120 μL of a 0.5% low melting point agarose kept at 37°C were added to these cell aliquots and then immediately distributed on glass slides previously covered with a thin layer of 1.5% normal melting point agarose. After upper agarose solidification, the slides were transferred to a lysis solution (2.5M NaCl; 10mM Tris; 100mM EDTA; 10% DMSO; 1% lauryl sarcosinate; 1% triton X-100; pH 10) at 4°C for 12 h and kept on electrophoresis alkaline buffer (0.3M NaOH; 1mM EDTA; pH 13) for 20 minutes before running electrophoresis at 25V/300mA for 20 minutes. After electrophoresis DNA was stained by silver according to Nadin *et al* (2001) and Brianezi *et al* (2009) [27, 28]. 100 cells were analyzed per slide and classified into four categories, according to the degree of DNA damage, where 1 corresponds to no damage and 4 to a highly damaged cell. All experiments were performed in triplicate and repeated at least two times.

2.4. Apoptosis and micronuclei estimation

For apoptosis and *micronuclei* estimation, HB-2 and MCF-7 cells were cultured as described before and, after 5Gy/30kV X-ray irradiation, they were incubated for 4 or 24 h, at 37°C, under 5% of CO_2. The cells were collected then and the evaluation of nuclei

morphology was performed by fluorescence microscopy, using HOECHST 33258 staining and scoring the number of apoptotic and micronucleated cells.

2.5. RNA extraction and quantitative RT-PCR

Total RNA samples were obtained using Trizol reagent (Life Technologies; Carlsbad, USA), according to the manufacturer´s protocol. Reverse transcriptase reaction and quantitative PCR were performed using miRVana qRT-PCR miRNA detection (Life Technologies; Carlsbad, USA) and NCode VILO miRNA cDNA Synthesis Kit (Life Technologies; Carlsbad, USA). Real-time PCR was done using SYBR green reagents (Life Technologies; Carlsbad, USA). Primer sequences used on qPCR reaction were: β-actin (F) 5´CATCGAGCACGGCATCGT 3´; β-actin (R) 5´GCCTGGATAGCAACGTACAT 3´; miR-34a (F) 5´GGCAGTGTCTTAGCTGGTTGT 3´; let-7a (F) 5´CCGCTGAGGTAGTAGGTTGTATAGTT 3´; and miR-21 (F) 5´GGCTAGCTTATCAGACTGATGTTGA 3´with the universal reverse primer provided on miRNA cDNA synthesis kit and the primers provided on miRVana qRT-PCR miRNA detection. All experiments were done in triplicate and repeated at least two times. Relative expression was analyzed using the ΔΔCt method [29,30].

2.6. Statistical analysis

All statistical analysis were performed on the irradiated samples of each type of cell line and its corresponding mock control using paired t-test with 95% confidence intervals.

3. Results

3.1. DNA lesions are observed in cultured breast cells after low energy X-ray irradiation

One of the main effects of radiation is the direct interaction with DNA molecules, causing their breakage. In order to detect DNA break, we performed comet assay and *micronuclei* analysis after low energy X-ray irradiation (5Gy /30 kV). The comet assay permits to detect DNA single-strand breaks, alkali-labile sites and double strand breaks associated with incomplete excision repair sites, while the *micronuclei* detects the occurrence of persistent double strand breaks [31]. An increase in all classes of analyzed lesions was observed on HB-2 and MCF-7 cell lines after irradiation. A significant increase in the amount and degree of damage was observed by the comet assay 24 h after irradiation of HB-2 cell line (Figure 1 A and B). With tumor cell line MCF-7, a significant increase in DNA damage level started from 4 h and lasted until 24 h after irradiation (Figure 1 C and D). We observed a similar result concerning the *micronuclei* formation. In HB-2 cells, a significant increase of *micronuclei* formation was seen 24 h after irradiation and in MCF-7, a progressive increase of *micronuclei* formation was observed from 4 h to 24 h after irradiation (Figure 2).

3.2. *HB-2 and MCF-7 cells undergo apoptosis after low energy X-ray irradiation*

One of the hallmarks of the terminal stages of apoptosis are genomic DNA fragmentation and chromatin condensation. Here, we have analyzed chromatin condensation, as well as other apoptotic features, such as cell shrinkage and membrane blebbing after 5Gy/30 kV cell irradiation by fluorescence microscopy. An increase in the percentage of apoptotic cells was observed at 4 and 24 h after irradiation, being statistically significant after 24 h incubation on both tested cell types (Figures 4 A and B). We also observed that HB-2 cells were more prone to undergo apoptosis than MCF-7, presenting higher percentages of apoptotic nuclei (Figures 4 A and B).

3.3. miR-34a and let-7a are up-regulated in cancer MCF-7 cells after low energy irradiation

We evaluated the expression of the apoptosis-associated miRNAs miR-34a, miR-21 and let-7a in HB-2 and MCF-7 cell lines 4 and 24 h after the irradiation with 5Gy/30 kV. These miRNAs were shown to be directly involved in cell stress response and apoptosis regulation. miR-34a and let-7a have also been considered as markers of radiation sensibility [13]. In HB-2 cells, only a slight increase in let-7a expression (2.3 fold relative to mock control) was observed at 4h after irradiation. At 24h, no significant differences in expression could be detected with any of the miRNAs (Figure 5). At the same time, an increase in expression of miR-34a and let-7a (6.8 and 3.0 fold relative to mock controls, respectively) was observed in MCF-7 cells at 4h after irradiation. The level of both miRNAs decreased at 24h (Figure 6). miR-21 did not show significant changes in expression in MCF-7 at 4 and 24h after irradiation. Hence, based on the obtained results, the expression of the analyzed miRNAs miR-34a and let-7a, is dependent on the time after irradiation and on cell type ("normal" or cancerous).

4. Discussion

The mechanisms of biological interaction promoted by low-energy radiations, such as medical X-rays are different from those of high-energy radiations, and these differences may affect the cellular response, including the type of damage. As low energy radiation is commonly used in diagnostic methods, for example in mammographic exams, it should be interesting to find out biological markers of cellular stress, including genotoxic damage. Recent studies demonstrate that the molecules known as miRNAs are important regulators of cell response to genotoxic stress [32]. The present study was conceived to determine if, among the miRNAs implicated in DNA damage response, the apoptosis-related miRNAs, let-7a, miR-34a and miR-21, are altered in consequence of genotoxic stress in breast cells submitted to low-energy X-rays. First we evaluated DNA damage through comet assay and by determination of *micronuclei* and apoptotic nuclei, after X-ray exposure at times 4 and 24h after irradiation. Following the confirmation of DNA alterations and apoptosis, we verified the miRNAs expression. The results suggest that low energy radiation is able to promote DNA damage and induce apoptosis at 4 and 24h. These observations are in agreement with previous publications that show that the low energy X-rays may be even more effective in causing mutational damage than high energy X-rays [33, 34]. Not only radiation doses have an impact on cellular responses, but radiation quality and cellular oxygen concentrations must also be taken into account, once they can substantially affect biological responses. Low-energy radiation is characterized by a low penetrance and consequently a higher indirect activity through reactive oxygen species generation and bystander effects. These effects persist for long periods, whereas DNA breaks induced by direct radiation are repaired relatively quickly [35,36]. X-rays can activate cellular responses such as G1 arrest, DNA repair and apoptosis. miRNAs are associated to the

above cited pathways, evoking a role of intermediate molecules on the regulation of DNA damage response [2, 32]. The results here presented, show that miR-34a and let-7a are up-regulated in MCF-7 cancer cells 4h after low energy X-ray irradiation, suggesting a relatively rapid activation of these miRNAs, which may be part of early events in the apoptotic response to radiation. In HB-2, the analyzed miRNAs did not present any significantly altered expression. Both cell lines, HB-2 and MCF-7, possess wild p53, a critical transcription factor able to regulate DNA damage response in part by miRNA activation [37]. miR-34a is a direct target of p53, which is up-regulated after genotoxic stress in breast cancer cells [9]. One of miR-34a validated target is SIRT1, a NAD-dependent deacetylase, which regulates apoptosis in response to oxidative and genotoxic stress [38, 39]. After miR-34a up-regulation there is an increase on p53 active form. This is due to a positive feedback involving the miR-34a transcriptional repression of SIRT1, which in turn inactivates p53 through deacetylation [40]. In MCF-7, a cancer cell line, which is usually more resistant to cell death by apoptosis, it is possible to observe, by comet assay, as well as by *micronuclei* formation, a greater accumulation of DNA damage. This high and persistent number of DNA breaks probably contributes to maintain the active p53 at high levels and, consequently, activate miR-34a, representing an early response to DNA injury that will culminate in cell cycle arrest or apoptosis. Therefore, our results reinforce an up-regulation of miR-34a, as a consequence of ionizing radiation activity followed by DNA damage and apoptosis [22, 38].We may suppose that this miRNA is a candidate to be a target in cancer therapy. Let-7a is another critical component on radiation response, once it targets the oncogenes k-ras and c-myc [41]. Ras signaling has been shown to increase the survival of tumor cells exposed to DNA-damaging agents. K-ras mutation is also related to poor prognosis in patients with non–small-cell lung cancer and pancreatic

cancer treated with radiotherapy or chemotherapy [14, 15]. A reduced expression of let-7a miRNA family members is associated with poor prognosis on lung cancer and, when exogenously, expressed let-7 can radio sensitizes lung cancer cells [42]. miR-21 up-regulation has been described in response to oxidative damage, including radiation [43, 44]. This response can be mediated by NFkB binding to miR-21 promoter region culminating on its direct activation [44]. The unchanged levels of miR-21 in our experiments may be linked to a lower sensitivity of this miRNA to low-energy radiation in these breast cell lines. Another possibility is that, as miR-21 may have its transcription directly activated by NFkB, a rapid-acting transcription factor [45], this can be part of an earlier response, not able to be detected after 4 and 24 hours time intervals. Women at high risk of breast and ovarian cancers are usually more susceptible to radiation-induced cancer because most of tumor suppressor genes implicated in breast cancer susceptibility are also implicated in the radio-induced DNA damage repair and signaling such as *TP53* and *ATM* [46, 47]. Unfortunately, this is the group of women most exposed to low energy radiation, since it is recommended an annual prophylactic screening by the age of 30-35, while in the rest of population, mammography is recommended at age 40 [48]. We believe that our observations may help to understand the effects of low energy X-ray irradiation in healthy women and breast cancer patients.

Conflicts of interest

"The authors declare that there are no conflicts of interest."

5. Acknowledgements

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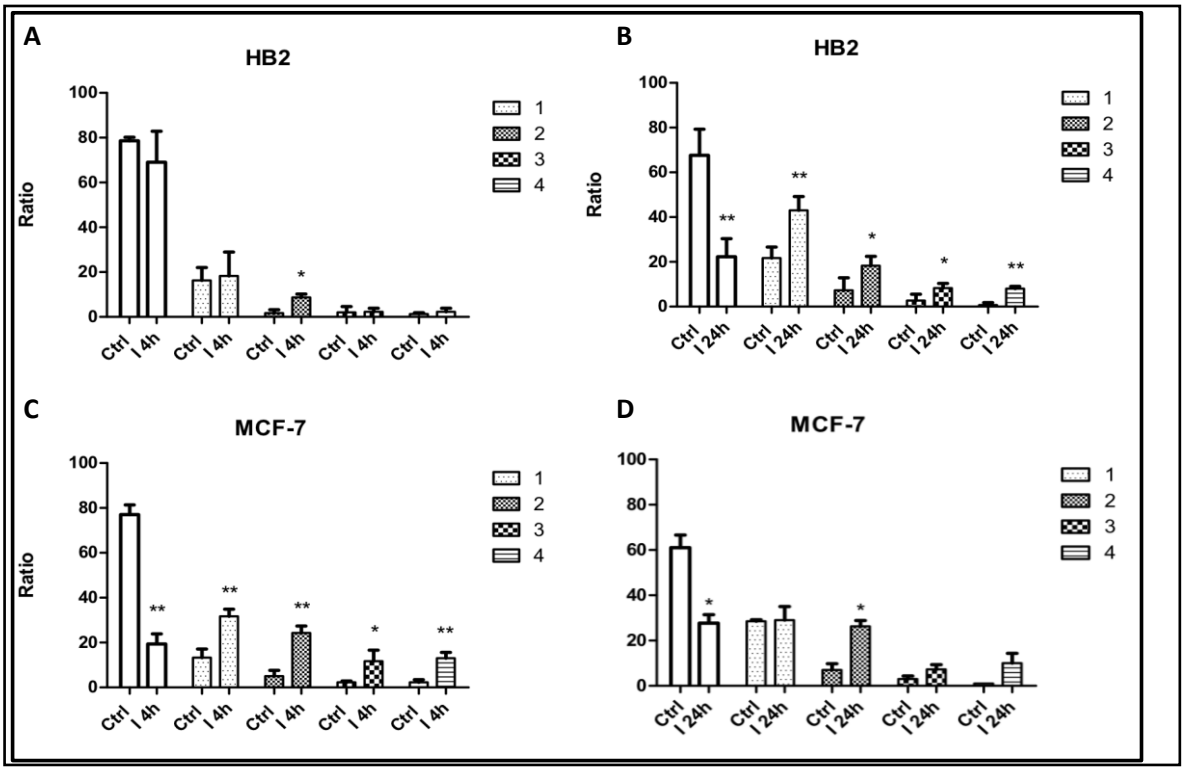


Figure 1: Percentage of DNA breaks observed by single cell electrophoresis assay after 5Gy/30kV X-ray irradiation of the mammary epithelial cell line HB-2 followed by 4 (A) and 24 (B) hours and MCF-7 followed by 4 (C) and 24 (D) hours of incubation at 37°C, 5%CO_2. Lesions were analyzed according to the length of comet tail and classified into four categories where (1) refers to absence of visible lesions and (4) a highly committed DNA, categories (2) and (3) are intermediates. Total analyzed cells per slide 100. * $P \leq 0.05$; ** $P \leq 0.01$.

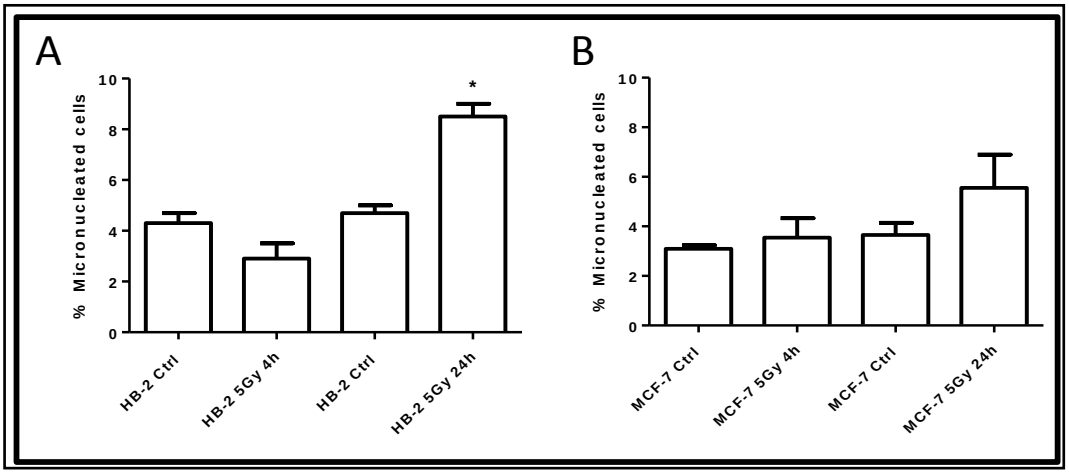


Figure 2: Percentage of *micronuclei* formation observed after Hoechst staining at 4 and 24 hours after 5Gy/30kV X-ray irradiation on mammary epithelial cell line HB-2 (A) and MCF-7 (B). * $P \leq 0.05$

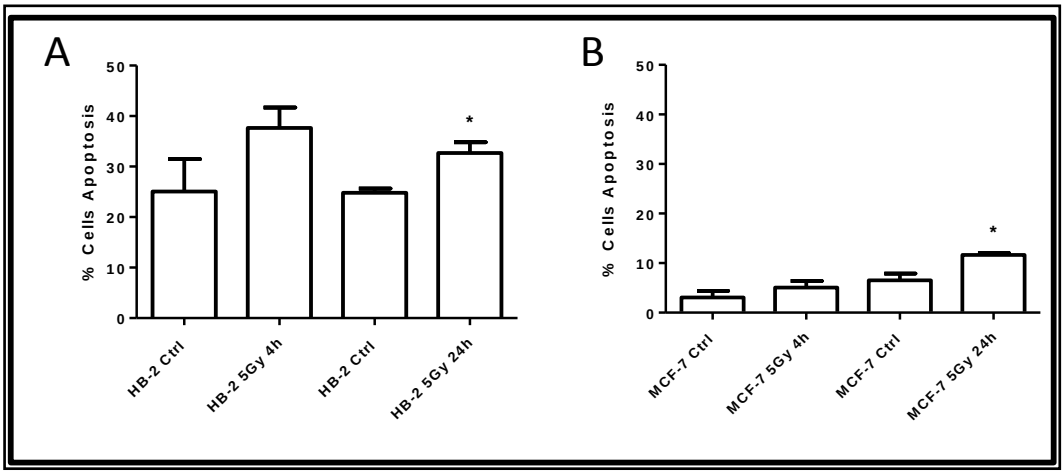
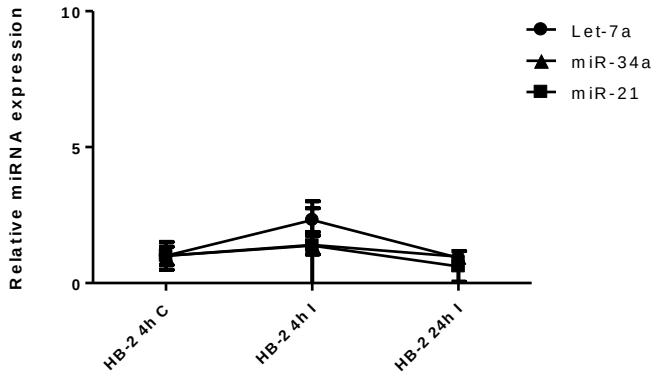
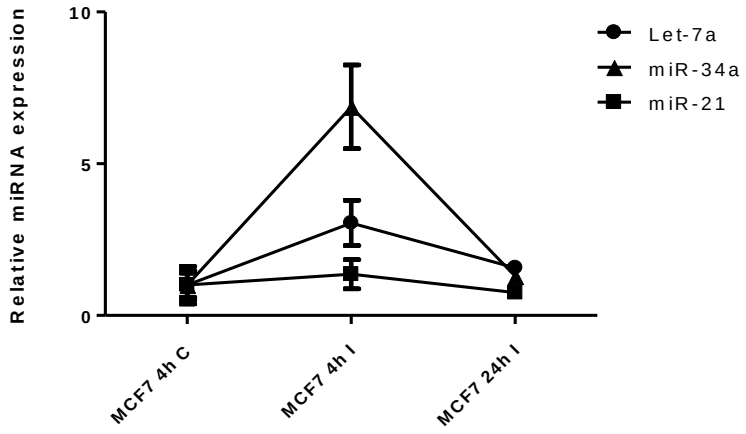


Figure 3: Percentage of apoptotic nuclei analyzed after Hoechst staining at 4 and 24 hours after 5Gy/30kV X-ray irradiation on the mammary epithelial cell line HB-2(A) and MCF-7 (B). * $P \leq 0.05$



HB-2					
miR-34a	**Ratio ± SD**	**Let-7a**	**Ratio ± SD**	**miR-21**	**Ratio ± SD**
Ctrl 4h	1.000 ± 0.337	Ctrl 4h	1.000 ± 0.101	Ctrl 4h	1.000 ± 0.509
Irr 5Gy 4h	1.402 ± 0.346	Irr 5Gy 4h	2.317 ± 0.434	Irr 5Gy 4h	1.381 ±0.627
Ctrl 24h	1.000 ±0.015	Ctrl 24h	1.000 ±0.297	Ctrl 24h	1.000 ±0.567
Irr 5Gy 24h	0.969 ± 0.135	Irr 5Gy 24h	0.932 ± 0.1435	Irr 5Gy 24h	0.610± 0.276

Figure 4: Relative expression of miRNAs let-7a, miR-34a and miR-21 detected by qRT-PCR at 4 and 24 hours after 5Gy/30kV X-ray irradiation of HB-2 cell line.



MCF-7					
miR-34a	**Ratio ± SD**	**Let-7a**	**Ratio ± SD**	**miR-21**	**Ratio ± SD**
Ctrl 4h	1.000 ± 0.398	Ctrl 4h	1.000 ± 0.621	Ctrl 4h	1.000 ± 0.488
Irr 5Gy 4h	6.874 ± 1.376	Irr 5Gy 4h	3.046 ± 0.745	Irr 5Gy 4h	1.360 ±0.481
Ctrl 24h	1.000 ± 0.083	Ctrl 24h	1.000 ±0.255	Ctrl 24h	1.000 ±0.103
Irr 5Gy 24h	1.295 ± 0.173	Irr 5Gy 24h	1.570 ± 0.044	Irr 5Gy 24h	0.755± 0.081

Figure 5: Relative expression of miRNAs let-7a, miR-34a and miR-21 detected by qRT-PCR at 4 and 24 hours after 5Gy/30kV X-ray irradiation of MCF-7 cell line.

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Manuscript 3: miR-29b downregulation in breast cancer cell lines is reverted by both low and high energy radiation

miR-29b downregulation in breast cancer cell lines is reverted by both low and high energy radiation

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Running Title: miR-29b expression is modulate by low and high energy radiation

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Abstract

MicroRNAs are small non-coding RNAs that regulate gene expression. Recent studies suggest roles of miRNAs in carcinogenesis. We showed by microarray and qPCR analysis that expression profiles of miRNAs are different in breast cancer vs. normal breast cell lines. Among the down-regulated miRNAs in breast cancer, we indentified the miRNA let-7 family and miR-29b. Considering the importance of miRNAs in the regulation of apoptosis and in the cell response to genotoxic stress, we evaluated the miRNA global expression after low and high energy cell irradiation and doxorubicin treatment, to determine whether miRNAs can modulate cell response after DNA damage. After the treatments, an overexpression of miR-29b could be observed in tumor cell lines, which correlates to a change in transcription of genes involved in apoptosis (BCL-2) and cell cycle control (CDC25 and E2F2). These results show that miR-29b can modulate important cellular processes in response to DNA damage and may be used as a biomarker of genotoxic stress.

1. Introduction

MicroRNAs (miRNAs) are a class of endogenous RNA interference system that acts on post-transcriptionally silencing of gene expression. They are evolutionary conserved molecules of approximately 23 nucleotides long able to bind, in an imperfect way, on 3´ untranslated region (3´UTR) of target mRNAs. In breast cancer some of miRNAs have a deregulated expression pattern, like miR-10b, miR-125b, and miR-145-21, and their expression is correlated with different molecular subtypes and clinical features such as HER2 overexpression and the presence of estrogen receptor (Iorio *et al*, 2005; McCafferty *et al*, 2009). In fact, in some cases, the expression profile of microRNAs can provide a more accurate prognosis than the mRNA expression analysis, which is the most common method for classification of breast cancer subtypes (Iorio *et al*, 2005; Lu *et al*, 2005; McCafferty *et al*, 2009). Recent studies describe the involvement of miRNAs in response to treatments as radiation and chemotherapy (Chaudhry *et al*, 2010; Hummel *et al*, 2010; Feng *et al*, 2011; Liu *et al*, 2011). Changes in their expression pattern are observed even at low dose irradiation, and tend to vary qualitatively and quantitatively according to the dose and time after the exposition (Maes *et al*, 2008). Radiotherapy is an important medical procedure in breast cancer management. Low energy X-rays are commonly used in diagnostic procedures, for example, mammography and high energy radiation, including gamma rays can be part of the treatment regime for breast cancer patients. Radiation effects include direct DNA damage causing single and double strand breaks and activation of pathways involved in DNA damage response (DDR), with the recruitment of DNA repair, cell cycle control and apoptosis proteins and indirect long term effects, known as bystander effects. In this context, miRNAs appears as new players on DDR cell responses. The first discovered

miRNAs were lin-4 and let-7, described in *C elegans*. They guide the nematode development through different larval stages, but only let-7 is conserved and present in humans. Let-7 family is composed by 11 miRNAs located in 8 different chromosomes (Boyerinas *et al*, 2010; Hertel *et al*, 2012). Let-7 family members possess very similar sequences, with consequent similar functions and redundant targets (Hertel *et al*, 2012). They are reported to be downregulated after genotoxic stress, especially after radiation exposure, probably due to a direct repression of let-7a and let-7b by p53. Let-7 accumulation is also a strong inducer of cell differentiation and let-7 is classified as a tumor suppressor by its ability of regulating tumor proliferation, differentiation and apoptosis by targeting, for example, RAS, Myc and caspase 3 (Tsang and Kwok, 2008; He *et al*, 2010; Oh *et al*, 2010). Other miRNAs also joint apoptosis pathways. In particular, miR-34 family, which is directly activated by p53 and enhances its effects by targeting SIRT1, leading to an increase of p53 deacetylated levels. In other words, these miRNAs increase the stability of p53 and, consequently, the expression of p21 and PUMA, targets of p53 and important apoptosis and cell cycle regulators. An apoptosis fine-tune regulation is also achieved by miR-29, a pro-apoptotic miRNA that targets Mcl-1, an anti-apoptotic Bcl-2 family member. miR-29b is also able to stimulate p53 expression by targeting p85alpha and CDC42 (Greene, 2010; Leung and Sharp, 2010; Dickey, 2011).

Some miRNAs are directly related to tumor progression and resistance to chemo and radiotherapy (Chaudhry *et al*, 2010), due to their capacity to control a vast range of pathways, including some cancer associated processes such as stem cell maintenance and apoptosis regulation.

In the present work, our purpose was to investigate miRNA expression changes in breast cancer cell lines compared to the non-tumoral cell line MCF-10A, in normal conditions and after different genotoxic treatments. Furthermore, we analyzed a possible correlation between the observed alterations and chromatin state changes.

2. Methodology

2.1.Cell Lines and Culture

The cell lines MCF-10A, MCF-7 and T-47D were obtained from Dr. David Cappellen and Dr. Nancy Hynes (Friedrich Miescher Institute for BioMedical Research, Novartis Research Foundation, Basel, Switzerland). MCF-10A was maintained in DMEM-F12 media (Gibco) supplemented with 10% FBS, EGF (20 ng/mL), insulin (10 mg/mL) and hydrocortisone (0.5 mg/mL) (Sigma-Aldrich) at 37^{o}C and 5% CO_2. Breast cancer cells MCF-7 and T-47D were maintained in DMEM media (Gibco) supplemented with 10% FBS, at 37^{o}C and 5% CO_2. All cell cultures were routinely checked for mycoplasma contamination.

2.2.*RNA extraction*

The extraction of total RNA from cell cultures was performed with Trizol reagent (Life Technologies), following the manufacture´s protocols. The amount and quality of RNA samples were assessed on the Agilent 2100 Bioanalyzer with RNA 6000 Nano Reagents and Supplies (Agilent).

2.3.miRNA Microarray

Total RNA samples (100 ng) were dephosphorilated and labeled with the fluorophore Cyanine 3- pCp at the 3'end of the RNA molecule. After labeling, samples were purified

with Micro Bio-spin 6 columns (Bio-Rad) and hybridized on array slides at 55^{o}C, 20 rpm for 20h. The label and hybridization reactions were performed using miRNA Complete Labeling and Hyb Kit (Agilent), following manufacturer's instructions. The microarray slides hybridized to the Agilent Human miRNA V16.0 Microarray format 8x60K(Agilent Technologies) were scanned on Agilent's High-Resolution C Scanner.

2.4.*Microarray data analysis*

To perform the microarray analysis, data were extracted from scanned images using Feature Extraction software (v 10.5.1.1 Agilent) with default settings. Data from all hybridizations was analyzed with LIMMA (Smyth, 2004), an R package from the Bioconductor project. First, a quantile inter array normalization was performed on the raw gMedianSignal values. Then the median of all probes for a given miRNA was computed to summarize the data. To asses differentially expressed genes, we start by fitting a linear model to the data. Then we used an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. The top-ranked genes were selected with the following criteria: an absolute fold-change > 2 and an adjusted p-value (FDR) < 0.05. Expression analysis comparisons were done between the cancer cells MCF-7; T-47D and the non-tumoral one MCF-10 and between the same cell line after treatments and their respective controls.

2.5.Quantitative RT-PCR

For miRNAs quantification, cDNA synthesis was carried out with total RNA, using NCode VILO miRNA cDNA Synthesis Kit (Life Technologies). For mRNAs quantification, cDNA synthesis was done with total RNA, using High Capacity cDNA Reverse Transcription Kit (Life Technologies). Real-time PCR was performed using SYBR PCR Master Mix (Life Technologies).

The primers sequences employed in qRT-PCR reactions were:

b-actin (F)	5′ CATCGAGCACGGCATCGT 3′
b-actin (R)	5′ GCCTGGATAGCAACGTACAT 3′
Let-7a	5′ CCGCTGAGGTAGTAGGTTGTATAGTT 3′
Let-7b	5′ CTGAGGTAGTAGGTTGTGTGGTT 3′
Let-7d	5′ CGAGAGGTAGTAGGTTGCATAGTT 3′
Let-7e	5′GCTGAGGTAGGAGGTTGTATAGTT 3′
Let-7g	5′ GCTGAGGTAGTAGTTTGTACAGTT 3′
Let-7i	5′ GGTGAGGTAGTAGTTTGTGCTGTT 3′
miR-29b	5′GCTGGTTTCATATGGTGGTTTAGA 3′

with the universal reverse primer provided on miRNA cDNA synthesis kit.

The expression of a set of 96 genes involved in DDR pathways were analyzed using Taqman array micro fluidic (Life Technologies) card according to the manufacturer's instructions. All tested genes are listed on (Supplementary data- Table 14). Experiments were performed in triplicate, using β- actin as reference gene. Expression results were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001) and statistical analysis was done by Student's *t*-test, considering statistically significant p-value ≤ 0.05.

2.6. Chip assay

Chromatin immunoprecipitation was performed using the SimpleChIP Enzymatic Chromatin IP kit- Agarose Beads (Cell Signaling) with minor modifications. 4 x 10^7 cells were cross-linked using 1% formaldehyde, chromatin was isolated and fragmented by sonication digestion with Micrococcal Nuclease, according to manufacturer's protocol. The antibody/chromatin complex was precipitated by protein G coupled with agarose beads, 20 μg of chromatin was used in the reaction performed using either 4 μg of Normal Rabbit IgG (#2729-Cell Signaling), anti-H4Ac monoclonal antibody (catalog no 39243, Active Motif), anti-H3K9me3 (catalog no 05-1242, Millipore). Following

immunoprecipitation, cross-linking was reversed, the proteins were removed by treatment with 0.5 μg/μL Proteinase K, and the DNA was purified by phenol/chlorophorm. Immunoprecipitated (IP) DNA was PCR-amplified using the following primers at 10 μM, miR-29 promoter (F) 5`ATGGAGCACTTGCTTGCTTT 3`; miR-29 promoter (R) 5`ATGCCTGGGACTCCCTTATT 3`, and control primers for heterochromatin (centromeric region of chromosome 16) and euchromatin (*B-actin* gene) regions. Quantitative PCR was performed using SyberGreen PCR Master Mix (Life Technologies). The IP samples were amplified using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA), and quantified normalizing by IgG with the Fold Enrichment method.

3. Results

3.1.Differential expression of apoptosis related miRNAs in cancer cell lines

Global miRNA expression was obtained by miRNA microarray on two established cancer cell lines MCF-7, derived from an adenocarcinoma ER+ and with wild type p53 and T-47D, obtained from a ductal carcinoma ER+/PR+, with mutated p53 when compared to the non-tumoral cell line MCF-10A (Supplementary data 1). The miRNA microarray analysis and further qPCR validation revealed a significant down regulation of the tumor suppressor miRNAs miR-29b and most of let-7 family members in both MCF-7 and T-47D cell lines (Table 1 and 2).

3.2.miR-29b downregulation in MCF-7 is consistent with the presence of heterochromatin markers in miR-29b promoter

As regulators of gene expression, miRNAs have been shown to be associated with many of the classic hallmarks of carcinogenesis, including cell proliferation, differentiation, and apoptosis control, and thus they can act both as oncogenes or tumor suppressor genes.

miR-29b downregulation has already been described in other cancer types, including cholangiocarcinoma, non-small cell lung cancer, nasopharyngeal cancer and chronic myeloid leukemia (Fabbri *et al*, 2007; Mott *et al*, 2007; Sengupta *et al*, 2008; Garzon *et al*, 2009).

In aggressive chronic lymphocytic leukemia (CLL), the down regulation of miR-29b is found to be selectively and is related to a worse prognostic. Shampath *et al* (2010), demonstrated that one of the mechanisms by which this miRNA is inactivated in cancer cell lines, in particular in CLL, is through chromatin remodeling at the miR-29b promoter region, located at 7q32.

In order to investigate whether the decreased levels of miR-29b in the analyzed breast cancer cell lines is due to the presence of heterochromatin, we observed histone modifications around miR-29b promoter by imunoprecipitating the chromatin of MCF-10A, MCF-7 and T47D cell lines. For this purpose, we used antibodies for anti-acetylated histone H4 (h4ac), representative of transcriptionally active euchromatin and histone H3 trimethyl lys9 (h3k9me3), which presence is associated with transcriptional repression.

Depletion in h4ac levels, accompanied by a slight increase in h3k9me3 levels was observed in MCF-7 cell line, when compared to the non- tumoral one (MCF-10A) (Figure 1), suggesting that the expression of miR-29b may also be subject to epigenetic control in certain breast tumors types.

3.3.miRNAs expression after genotoxic treatment reveals a shift in miR-29b expression in cancer cells

Given the importance of miRNAs in the regulation of DDR, in particular on p53 and apoptosis control, we investigated miRNA global expression in MCF-7 and T-47D cell lines after different genotoxic treatments. Cells were subject to chemical damage promoted by the DNA intercalating agent doxorubicin (1μM), low energy X-ray irradiation (5Gy/30Kv) and cesium 137 gamma-rays (0.5Gy). Gene expression was accessed by miRNA microarray 24h after treatment.

It was observed a significant change in expression patterns of miR-29b in MCF-7 and T-47D cell lines after irradiation, either in low and high energy, when compared to their respective non-treated controls (Table 3). miR-29b overexpression after irradiation was subsequently confirmed by qPCR (Table 3). The same effect was not observed after doxorubicin treatment.

The expression of other miRNAs involved in DDR, such as let-7 and miR-34 family did not revealed a consistent change in all the treatments. In brief, let-7 family members remain slightly downregulated and miR-34a, which is a p53 direct target, presented an increase only in MCF-7 cell line after all treatments (supplementary data)

3.4.Changes in mRNA expression after treatments comprises apoptosis and cell cycle related genes

DDR involves the recruitment of DNA-damage signaling pathways, the activation of cell cycle checkpoints and can ultimately result in apoptosis and the type of response varies within cell type and the kind of lesion.

As regulators of gene expression, miRNAs are able inhibit their target genes by imperfect complementarity to 3´UTR and each miRNA may thereby have several mRNAs as targets. So far, it is estimated that up to 30% of coding genes are subject to miRNA regulation (Brennecke *et al*, 2005). By this way, it is not surprisingly that even a change in a few number of miRNAs can cause significant changes in mRNAs expression.

We quantified the expression of a group of 96 mRNAs after doxorubicin (1μM), low energy X-ray irradiation (5Gy/30Kv) and cesium 137 gamma-rays (0.5Gy) treatments in the cell lines MCF-10A, MCF-7 and T-47D by qPCR, using the TLDA plate. The genes selected for this analysis have their function related to tumorigenesis, DDR and some of them have complementary sites on 3´UTR to miRNAs involved in apoptosis regulation. We observed a reduction in levels of genes involved in apoptosis, among them BCL-2, in cell cycle (CDC25 e E2F2) and RET protooncogene.

4. Discussion

The process of apoptosis consists in signaling cascades that culminate on cytochrome c release from the mitochondrial intermembrane space into the cytoplasm. The release of cytochrome c from mitochondria is a key event that triggers the rapid activation of caspases, the key cellular proteases that ultimately execute cell death.

In situations of cellular stress, apoptosis occurs mainly via p53 activation (Elmore, 2007; Lu and El-Deiry, 2009) and includes the activation of proapoptotic BH3-only proteins that are crucial for this process by either inhibiting the anti-apoptotic proteins Bcl-2, Mcl-1, and Bcl-xL, or directly activating proapoptotic Bax and Bak activation triggering, the caspase cascade and promoting cell death (Elmore, 2007).

Our group previously demonstrated that low energy radiation is able to promote DNA damage and induce apoptosis. These observations are also in agreement with previous publications that show that the low energy X-rays may be even more effective in causing mutational damage than high energy X-rays (Weidhaas, *et al*, 2007; Zhao *et al*, 2007). Some factors can influence cell response to radiation and not only radiation doses will determine the impact on cell survival, but radiation quality and cellular oxygen concentrations must also be taken into account, once they can substantially affect biological responses. Low-energy radiation is characterized by a low penetrance and, consequently, a higher indirect activity through reactive oxygen species generation and bystander effects. These effects persist for long periods, whereas high energy radiation tends to induce DNA breaks that are repaired relatively quickly. Both low and high energy radiation can activate cellular responses, such as G1 arrest, DNA repair and apoptosis. miRNAs are associated to the above cited pathways, evoking a role of intermediate molecules in the regulation of DNA damage response.

miR-29b is described to be a short-live miRNA in Hela cells (Zhang *et al*, 2011) and its expression can modulate p53 levels and induce apoptosis (Park *et al*, 2009). miR-29b can upregulate p53 by targeting p85α protein that is the regulatory subunit of phosphatidylinositol-3 kinase (PI3K) and has a central role in maintaining the balance of cellular survival and apoptosis and CDC42 protein is a member of the Rho family of GTPases, which regulate cell morphology, cell migration and cell-cycle progression. Both proteins negatively regulate p53 (Park *et al*, 2009).

Induction of miR-29b after genotoxic *stimuli* can induce p53 expression and contribute to apoptosis in a p53 dependent way once miR-29b is able to target Mcl-1.

Mcl-1 is an anti-apoptotic protein, member of BCL-2 family that binds to the BH3-only proteins Bim, Bid, Bik, Noxa, Puma and Bak, protecting cells against apoptosis (Mott *et al*, 2007). Either depletion of Mcl-1 or disruption of the interaction between Mcl-1 and Bak coincide with apoptosis following a cytotoxic stimulus (Mott *et al*, 2007).

The miRNA profile of tumor and normal cells reveals that several miRNAs have their expression altered in cancer cells and some of them seem to act in concert to promote and keep carcinogenesis. The down regulated let-7 family miRNAs suppress oncogenes, including Ras, Hmga2 and c-Myc, whereas miR-29b can have its expression repressed by c-Myc and targets, not only mcl-1 but also DNA methyltransferases 3A and 3B (DNMT3A, and DNMT3B) and can contribute to an aberrant DNA methylation (Mott *et al*, 2007; Garzon *et al*, 2009; Mott *et al*, 2010).

Our results demonstrated that miR-29b levels can be reestablished after genotoxic treatment in tumor cells, which draws attention to the role of miR-29b as a potential biomarker for treatment outcome in breast cancer.

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Table 1- miR-29b MCF-7 and T-47D *versus* the non-tumoral cell line MCF-10A

		Microarray		qRT-PCR	
		FC	p-value	FC	p-value
miR-29b	MCF-7	-8.314	9.48E-07	-63.4832	1.62E-03
	T-47D	-6.784	2.39E-06	-42.0751	3.31E-04

Table 2. MCF-7 and T-47D *versus* the non-tumoral cell line MCF-10A

		Microarray		qRT-PCR	
		FC	p-value	FC	p-value
let-7a	MCF-7	-1.158	4.63E-01	-3.080	7.22E-02
	T-47D	-1.046	8.18E-01	-4.474	5.48E-02
let-7b	MCF-7	-1.349	4.37E-02	-2.302	7.72E-03
	T-47D	-4.724	1.15E-06	-5.530	4.34E-03
let-7d	MCF-7	2.352	1.19E-04	2.512	5.12E-02
	T-47D	-8.533	5.28E-09	-2.850	3.59E-03
let-7e	MCF-7	1.444	1.29E-01	-2.159	8.87E-03
	T-47D	-2.137	4.98E-03	-1.729	6.78E-03
let-7g	MCF-7	1.005	9.77E-01	2.025	3.53E-01
	T-47D	-8.619	1.20E-06	-16.509	4.33E-04
let-7i	MCF-7	1.757	5.46E-04	1.600	1.55E-01
	T-47D	-13.921	1.61E-09	-7.423	6.48E-03

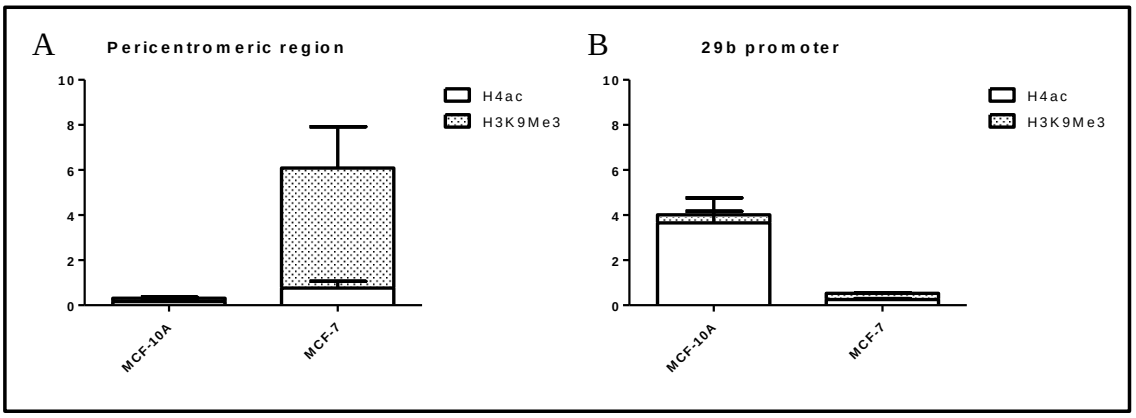

Figure 1: Presence of the chromatin markers H4ac and H3K9me3 at miR-29b promoter region, observed by Chip assay. (A) H3K9me3enrichment at the heterochromatin pericentromeric region (B) A depletion of of H4ac, euchromatin marker was observed in the breast tumor cell line MCF-7.

Table 3. miR-29b expression after genotoxic treatment

			Microarray		qRT-PCR	
			FC	p-value	FC	p-value
miR-29b	DOX	MCF-7	1.562718	0.026	-2.27994	0.036
		T-47D	-1.09176	0.631	-5.92114	0.088
	IRR 5Gy	MCF-7	2.241541	0.000	5.028934	0.118
		T-47D	2.099782	0.001	2.892018	0.414
	IRR 0.5Gy	MCF-7	1.92443	0.003	15.61775	0.045
		T-47D	1.171946	0.389	4.415022	0.037

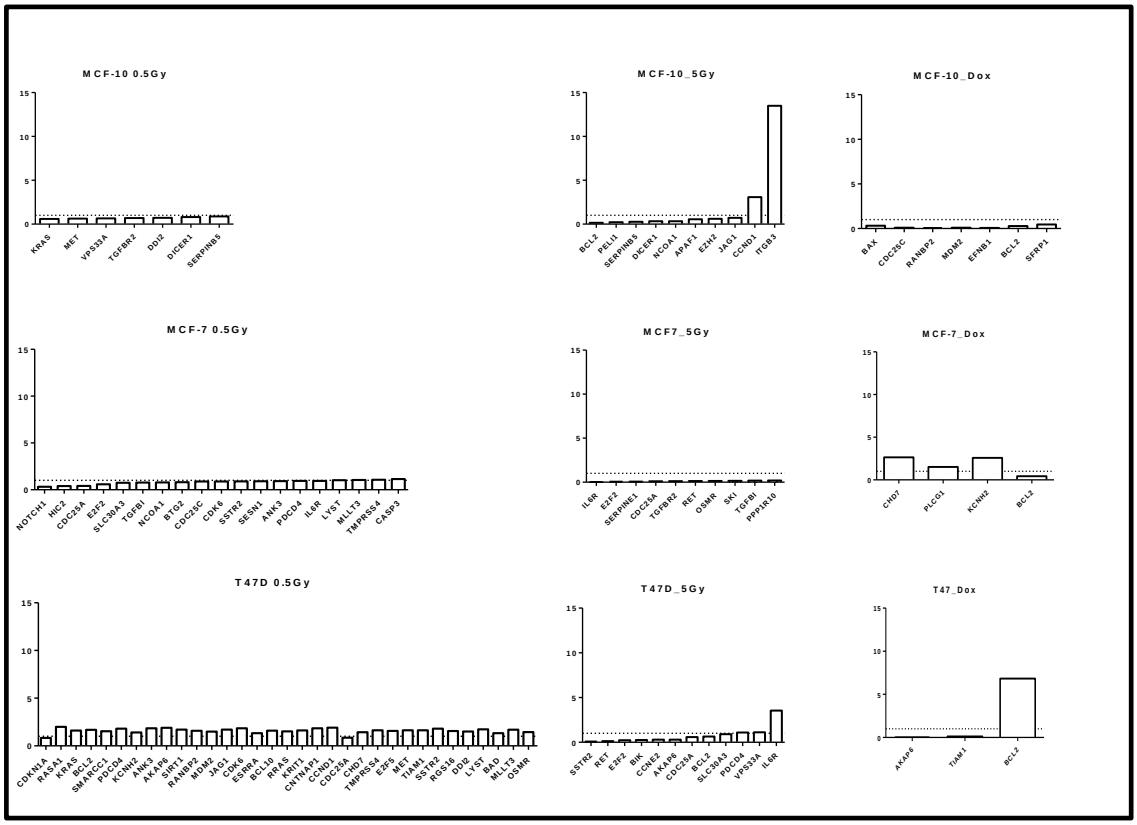

Figure 2. Carcinogenesis related mRNA after genotoxic treatment; Figure contains only the statistic significant data $p \leq 0.05$. Gene expression ratio was calculated according to DDCT method in comparison to the respective non-treated control.

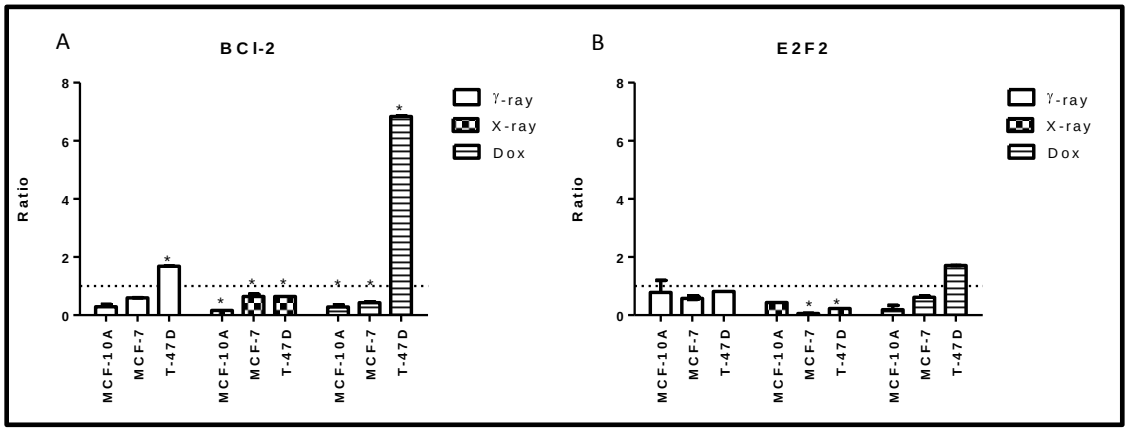

Figure 3: Gene expression levels of BCL-2 (A) and E2F2 (B) achieved by qPCR 24h after low and high energy irradiation and doxorubicin treatment

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Supplementary data

Manuscript 3: miR-29b downregulation on breast cancer cell line is reverted by both low and high energy radiation

Part 1: miRNA expression in breast cancer cell lines MCF-7 and T-47D versus the non-tumoral one MCF-10A

Table S1: Global miRNA expression profile of MCF-7cell line *versus* MCF-10A. Fold change cutoff 2.0.

Name	FC	Adj.P.Value
hsa-miR-205	-91.253	1.05E-09
hsa-miR-130a	-15.798	1.17E-12
hsa-miR-100	-13.608	6.20E-13
hsa-miR-29a	-8.668	4.88E-07
hsa-miR-29b	-8.314	9.48E-07
hsa-miR-221	-6.549	4.02E-13
hsa-miR-642b	-5.626	1.38E-08
hsa-miR-630	-4.523	5.50E-04
hsa-miR-125b	-4.516	5.49E-07
hsa-miR-22	-3.296	4.96E-06
hsa-miR-27a	-3.287	2.02E-07
hsa-miR-29c	-3.003	3.81E-05
hsa-miR-31	-2.617	2.02E-07
hsa-miR-18a	-2.542	3.81E-05
hsa-miR-31*	-2.528	5.87E-06
hsa-miR-24	-2.451	1.10E-05
hsa-miR-17	-2.397	2.38E-04
hsa-miR-19a	-2.362	1.11E-02
hsa-miR-205*	-2.275	6.77E-06
hsa-miR-19b	-2.275	4.19E-03
hsa-miR-29b-1*	-2.259	3.17E-08
hsa-miR-20b	-2.243	1.01E-04
hsa-miR-1290	-2.164	3.04E-05
hsa-miR-34b*	-2.158	6.12E-05
hsa-miR-365	2.083	9.16E-03
hsa-miR-99a	2.131	1.12E-03
hsa-miR-4284	2.189	2.12E-02
hsa-miR-193b	2.257	5.52E-03
hsa-miR-301a	2.271	2.74E-03
hsa-miR-425	2.291	7.68E-04

hsa-let-7d	2.352	1.19E-04
hsa-miR-324-5p	2.357	1.27E-04
hsa-miR-141	2.655	2.60E-02
hsa-miR-200c	2.951	1.12E-03
hsa-miR-7	3.055	3.25E-06
hsa-miR-375	3.235	4.56E-06
hsa-miR-93	3.363	5.37E-06
hsa-miR-342-3p	3.502	1.58E-05
hsa-miR-25	3.865	8.45E-06
hsa-miR-429	3.91	2.02E-07
hsa-miR-106b	4.676	1.41E-07
hsa-miR-196a	4.792	4.62E-06
hsa-miR-200a	5.049	5.57E-07
hsa-miR-203	9.252	2.02E-07
hsa-miR-200b	14.001	6.77E-09

(*) denotes the less predominant form when miRNA hairpin precursors give rise to two excised miRNAs, one from each arm.

Table 2: Global miRNA expression profile of T-47D cell line *versus* MCF-10A. Fold change cutoff 2.0.

Name	FC	Adj.P.Value
hsa-miR-205	-51.814	3.63E-09
hsa-miR-100	-14.134	5.09E-13
hsa-let-7i	-13.921	1.61E-09
hsa-miR-27a	-9.126	1.86E-10
hsa-let-7g	-8.619	1.20E-06
hsa-let-7d	-8.533	5.28E-09
hsa-miR-125b	-8.329	8.51E-09
hsa-miR-29a	-7.62	7.90E-07
hsa-miR-29b	-6.784	2.39E-06
hsa-miR-221	-6.609	3.77E-13
hsa-miR-642b	-5.474	8.51E-09
hsa-let-7b	-4.724	1.15E-06
hsa-miR-424	-3.884	6.60E-06
hsa-miR-23a	-3.655	8.35E-07
hsa-let-7c	-3.531	2.76E-06
hsa-miR-630	-3.302	3.76E-03
hsa-miR-31	-2.604	1.59E-07
hsa-miR-31*	-2.591	3.77E-06
hsa-miR-21	-2.545	4.55E-03
hsa-let-7f	-2.393	7.72E-03
hsa-miR-18a	-2.387	7.07E-05
hsa-miR-21*	-2.28	9.93E-06

hsa-miR-19a	-2.216	1.71E-02
hsa-miR-210	-2.204	4.75E-03
hsa-miR-29b-1*	-2.175	3.20E-08
hsa-miR-98	-2.154	6.98E-05
hsa-miR-197	2.045	7.76E-04
hsa-miR-148a	2.193	4.05E-03
hsa-miR-301a	2.26	2.71E-03
hsa-miR-193a-3p	2.271	7.50E-07
hsa-miR-16	2.275	1.44E-03
hsa-miR-148b	2.277	5.18E-04
hsa-miR-130a	2.285	1.52E-06
hsa-miR-375	2.333	1.07E-04
hsa-miR-4284	2.591	4.79E-03
hsa-miR-27b	2.757	4.63E-04
hsa-miR-365	3.017	2.61E-04
hsa-miR-107	3.255	4.01E-06
hsa-miR-324-5p	3.272	3.99E-06
hsa-miR-103a	3.402	1.41E-07
hsa-miR-203	3.84	2.82E-05
hsa-miR-15b	4.414	5.43E-04
hsa-miR-23b	4.592	4.01E-06
hsa-miR-200a	5.869	1.67E-07
hsa-miR-429	6.064	6.82E-09
hsa-miR-425	10.74	8.50E-09
hsa-miR-200b	18.46	1.61E-09
hsa-miR-196a	31.835	7.26E-10

(*) denotes the less predominant form when miRNA hairpin precursors give rise to two excised miRNAs, one from each arm.

Part 2: Gene expression after genotoxic treatments

Table S3: miR-34a expression accessed 24h after doxorubicin 1μM, 0.5Gy γ-rays and 5Gy/30Kv X-rays irradiation.

			Microarray		qRT-PCR	
			FC	p-value	FC	p-value
miR-34a	DOX 1μM	MCF10A	-1.7433	0.001	2.523404	0.224
		MCF-7	1.392693	0.020	1.528908	0.632
		T-47D	-1.04715	0.720	3.457116	0.284
	5Gy/30Kv X-rays	MCF10A	-1.8666	0.000	3.343046	0.279
		MCF-7	1.648072	0.001	2.591869	0.097
		T-47D	1.251641	0.097	-2.53447	0.287

0.5Gy γ-rays	MCF10A	1.012685	0.922	11.57334	0.406
	MCF-7	1.360008	0.029	-1.17243	0.855
	T-47D	1.154866	0.273	4.681938	0.249

Table S4: let-7 family expression accessed 24h after 5Gy/30Kv X-rays irradiation.

Irr 5Gy/30Kv		Microarray	
		FC	P.Value
let-7ª	M10	-1.47409	0.066569
	MCF-7	-1.02228	0.911615
	T-47D	-1.1147	0.586328
let-7b	M10	-1.05362	0.70493
	MCF-7	-1.31055	0.065285
	T-47D	-0.96526	0.797441
let-7d	M10	-1.69257	0.000434
	MCF-7	-1.01611	0.891274
	T-47D	-0.88207	0.292924
let-7e	M10	-1.4129	0.151858
	MCF-7	-1.05066	0.831516
	T-47D	-1.0087	0.970235
let-7g	M10	-2.19485	0.000975
	MCF-7	-0.83282	0.349253
	T-47D	-0.86887	0.469105
let-7i	M10	-1.31733	0.0468
	MCF-7	-0.96833	0.802611
	T-47D	-0.92804	0.563872

Table S5: Global miRNA expression profile of MCF-10A cell line submitted to 1uM Doxorubicin treatment *versus* non- treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-141	-3.906	1.36e-03
hsa-miR-29b	-3.828	6.65e-05
hsa-miR-19a	-3.653	2.07e-04

hsa-miR-19b	-2.827	3.01e-04
hsa-miR-18a	-2.516	2.57e-05
hsa-miR-125a-5p	-2.283	8.64e-04
hsa-miR-15a	-2.141	1.21e-03
hsa-miR-200c	-2.109	1.18e-02
hsa-miR-29b-1	-2.097	9.80e-08
hsa-miR-106b	-2.008	2.25e-04
hsa-miR-20a	-2.006	5.97e-03
hsa-miR-642b	2.41	1.02e-05
hsa-miR-575	2.091	2.90e-05
hsa-miR-1268	2.135	2.25e-04
hsa-miR-1275	2.178	3.46e-05
hsa-miR-134	2.448	1.74e-08
hsa-miR-188-5p	2.461	1.13e-06
hsa-miR-4270	2.499	2.19e-05
hsa-miR-718	2.607	1.74e-08
hsa-miR-3656	3.025	1.81e-04
hsa-miR-1207-5p	3.065	5.76e-04
hsa-miR-1225-5p	3.113	1.08e-03
hsa-miR-1246	3.125	3.75e-03
hsa-miR-3667-5p	3.391	8.29e-08
hsa-miR-320c	3.807	6.65e-05
hsa-miR-3663-3p	3.825	6.35e-05
hsa-miR-1915	4.369	2.25e-04
hsa-miR-3665	4.703	2.37e-03
hsa-miR-4281	4.725	9.48e-04
hsa-miR-3679-5p	5.241	7.76e-07
hsa-miR-638	6.252	6.68e-06
hsa-miR-762	6.944	8.29e-08
hsa-miR-2861	7.706	1.41e-06
hsa-miR-630	19.852	4.58e-07

Table S6: Global miRNA expression profile of MCF10A cell line submitted to 0.5Gy Cs γ irradiation treatment *versus* the non-treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-29b	-4.111	2.76e-04
hsa-miR-642b	-2.275	1.47e-04
hsa-miR-100	-2.162	3.20e-06
hsa-miR-29a	-2.152	2.14e-02
hsa-miR-424	-2.074	9.08e-03
hsa-miR-663	2.001	2.33e-07
hsa-miR-200b	2.036	9.87e-03
hsa-miR-224	2.484	1.08e-03
hsa-miR-1181	2.488	2.33e-07
hsa-miR-1915	2.521	3.65e-02

hsa-miR-203	21.35	2.72e-08

Table S7: Global miRNA expression profile of MCF10A cell line submitted to 5Gy/30Kv X-ray irradiation treatment *versus* the non-treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-29b	-2.593	2.08e-02
hsa-miR-642b	-2.286	5.47e-04
hsa-miR-224	2.185	9.73e-03
hsa-miR-200b	2.637	3.05e-03

Table S8: Global miRNA expression profile of MCF-7 cell line submitted to 1uM Doxorubicin treatment *versus* the non-treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-1275	-2.181	6.19e-04
hsa-miR-125a-5p	-2.642	1.91e-03

Table S9: Global miRNA expression profile of MCF-7 cell line submitted to 0.5Gy Cs γ irradiation treatment *versus* the non-treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-203	-3.926	1.25e-04
hsa-miR-196a	-3.246	3.51e-04
hsa-miR-93	-2.214	9.37e-04
hsa-miR-320d	-2.121	1.38e-02
hsa-miR-135b	-2.011	7.04e-04
hsa-miR-4284	0.398	1.06e-02
hsa-miR-200b	0.495	6.17e-03
hsa-miR-148a	2.29	4.79e-03
hsa-miR-29c	3.1	1.25e-04
hsa-miR-21*	3.307	1.85e-06

(*) denotes the less predominant form when miRNA hairpin precursors give rise to two excised miRNAs, one from each arm.

Table S10: Global miRNA expression profile of MCF-7 cell line submitted to 5Gy/30Kv X-ray irradiation treatment *versus* the non-treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-29b	2.242	2.90E-02

Table S11: Global miRNA expression profile of T-47D cell line submitted to 1uM Doxorubicin treatment *versus* the non-treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-4284	-4.393	1.02e-04
hsa-miR-574-3p	-4.172	8.69e-07
hsa-miR-197	-3.949	1.13e-06
hsa-miR-125a-5p	-3.865	1.58e-05
hsa-miR-181d	-2.323	4.19e-04
hsa-miR-4281	4.17	3.03e-03
hsa-miR-483-5p	14.87	5.20e-13
hsa-miR-21	2.025	2.82e-02
hsa-miR-1290	2.042	7.05e-05
hsa-miR-718	2.086	2.50e-07
hsa-miR-134	2.113	1.25e-07
hsa-miR-3679-5p	2.182	1.54e-03
hsa-miR-4271	2.273	1.05e-03
hsa-miR-301a	2.458	1.12e-03
hsa-miR-188-5p	2.563	1.08e-06
hsa-miR-4270	2.577	2.75e-05
hsa-miR-1202	2.866	4.60e-03
hsa-miR-3665	2.927	4.18e-02
hsa-miR-1246	2.952	7.71e-03
hsa-miR-3663-3p	3.282	3.44e-04
hsa-miR-1225-5p	4.574	1.29e-04
hsa-miR-638	4.834	5.33e-05
hsa-miR-765	5.161	7.31e-12
hsa-miR-1915	5.225	1.29e-04
hsa-miR-1207-5p	5.459	2.02e-05
hsa-miR-2861	6.747	4.65e-06
hsa-miR-762	11.22	1.02e-08
hsa-miR-630	20.27	5.71e-07

Table S12: Global miRNA expression profile of T-47D cell line submitted to 0.5Gy Cs γ irradiation treatment *versus* the non-treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-363	-2.522	6.79e-08
hsa-miR-181d	-2.394	1.41e-03

hsa-miR-3656	-2.268	1.07e-02
hsa-miR-197	-2.135	2.01e-03
hsa-miR-3162-5p	-2.051	2.88e-02
hsa-miR-1268	-3.54	2.24e-05
hsa-miR-101	2.04	1.23e-03
hsa-miR-125b	3.23	3.85e-05
hsa-miR-30a	2.041	3.92e-03
hsa-miR-181a	2.234	2.39e-03
hsa-miR-26b	2.496	5.35e-03
hsa-let-7g	2.853	5.51e-03
hsa-let-7c	2.858	1.07e-04
hsa-miR-99a	3.775	2.85e-05

Table S13: Global miRNA expression profile of T-47D cell line submitted to 5Gy/30Kv X-ray irradiation treatment *versus* the non-treated control. Fold change cutoff 2.0.

Name	FC	p-value
hsa-miR-196a	-2.391	5.09e-03
hsa-miR-29b	2.1	2.67e-02
hsa-miR-301a	2.095	1.05e-02
hsa-miR-19a	2.171	3.38e-02
hsa-miR-1290	4.397	2.41e-07
hsa-miR-1246	7.889	1.94e-04

Table S14: List of selected genes and their respective assay number for gene expression analysis by Taqman array micro fluidic (Life Technologies)

ID Gene- Assay
ACTR1A-Hs00194913_m1
AKAP6-Hs00188681_m1
ANK3-Hs00241738_m1
APAF1-Hs00185508_m1
AXL-Hs00242357_m1
ACTB-Hs00357333_g1
BAD-Hs00188930_m1
BAX-Hs00180269_m1
BCL10-Hs00961847_m1
BCL2-Hs00608023_m1
18S-Hs99999901_s1
BIK-Hs00609635_m1
BTG2-Hs00198887_m1
CASP3-Hs00234385_m1
CCL1-Hs00171072_m1
CCND1-Hs00765553_m1
CCND2-Hs00277041_m1
CCNE2-Hs01051897_g1
CCR7-Hs99999080_m1

CDC25A-Hs00947994_m1
CDC25C-Hs00156407_m1
CDK4-Hs01565683_g1
CDK6-Hs00608037_m1
CDKN1A-Hs01121168_m1
CHD7-Hs00214990_m1
CNTN2-Hs00543989_m1
CNTNAP1-Hs00182533_m1
CXCL5-Hs00171085_m1
CYCS-Hs01588974_g1
DDI2-Hs00260555_m1
DICER1-Hs00229023_m1
DOCK3-Hs00389427_m1
E2F2-Hs00231667_m1
E2F3-Hs00605457_m1
E2F5-Hs00231092_m1
EFNB1-Hs00270004_m1
ESRRA-Hs00607062_gH
EZH2-Hs00544830_m1
F2RL2-Hs00765740_m1
FAS-Hs00163653_m1
FASLG-Hs00181225_m1
MDM2-Hs99999008_m1
HGF-Hs00300159_m1
HIC2-Hs00740546_s1
HMGA1-Hs00600784_g1
HNF1A-Hs00167041_m1
IKBKG-Hs00415849_m1
IL13-Hs01124272_g1
IL1B-Hs99999029_m1
IL6R-Hs00169842_m1
ITGB3-Hs00173978_m1
ITGB7-Hs00168469_m1
JAG1-Hs01070036_m1
KCNH2-Hs00918375_m1
KRAS-Hs00270666_m1
KRIT1-Hs00184988_m1
LHCGR-Hs00174885_m1
LYST-Hs00915897_m1
MET-Hs00179845_m1
MLLT3-Hs00180312_m1
MYC-Hs99999003_m1
NCOA1-Hs00186661_m1
NFKBIA-Hs00153283_m1
NOTCH1-Hs01062014_m1

OSMR-Hs00187782_m1
PDCD4-Hs00205438_m1
PEA15-Hs00269428_m1
PELI1-Hs00221035_m1
PIP5K1A-Hs00801004_s1
PLCG1-Hs00234046_m1
PPP1R10-Hs00160391_m1
RANBP2-Hs00397898_g1
RASA1-Hs00243115_m1
RECK-Hs01019179_m1
RET-Hs01120030_m1
RGS16-Hs00892674_m1
RRAS-Hs00196699_m1
SDHA-Hs00188166_m1
SERPINB5-Hs00985282_m1
SERPINE1-Hs00167155_m1
SESN1-Hs00902787_m1
SFRP1-Hs03928965_s1
SIRT1-Hs01009000_m1
SKI-Hs00161707_m1
SLC30A3-Hs00185728_m1
SMARCC1-Hs02559508_s1
SSTR2-Hs00265624_s1
TBP-Hs00920498_m1
TGFBI-Hs00932747_m1
TGFBR2-Hs00234253_m1
TIAM1-Hs00180075_m1
TMPRSS4-Hs00854071_mH
TNFRSF1B-Hs00153550_m1
TP53-Hs01034249_m1
VPS33A-Hs00224777_m1
WNT1-Hs00180529_m1

V.Discussion

Breast cancer is the malignant neoplasia with highest incidence of deaths among women. Despite major efforts to understand the main mechanisms involved in its development, there are still many gaps in understanding this disease, generating uncertainties, especially regarding the response to different therapies and recurrence.

Diagnosis and treatment decision are currently based on tissue morphology taking into account the grade of the tumor, the mitotic rate, the presence of metastatic lymph nodes, and the expression of molecular variables, like the presence of estrogen, progesterone and HER-2 receptor (Colombo *et al*, 2011; Eroles *et al*,2012).

During the last decade a high amount of transcriptome data was generated on breast tumors samples allowing a better understanding of the disease progression, an improvement in diagnosis and the development of new therapies. The high degree of heterogeneity between breast tumors is a challenge for physicians and the development of a more personalized treatment could play a crucial role on it (Eroles *et al*, 2012). To date some diagnostic and treatment predictors have been developed based on gene expression signatures to measure the probability of patient relapse and estimate the need of adjuvant chemotherapy (Espinosa *et al*, 2012). The most known are Mammaprint, a microarray based technology that analyses 70 genes and Oncotype DX a qPCR based method that checks the expression of 21 genes. Both assays are being tested under phase III prospective clinical trial (Espinosa *et al*, 2012). These approaches help to stratify tumors into molecular classes and in treatment decision. However, this first generation of breast cancer molecular diagnostic can still be improved, in order to increase their prognostic power and to be more incorporated into clinical practice (Colombo *et al*, 2011).

The class of molecules known as microRNAs is emerging as an important factor in controlling cell differentiation and tumorigenesis. They often present an altered expression in breast tumors, when compared to normal tissue and can be classified as proto-oncogenes and tumor suppressor genes.

These small regulators of gene expression are able to control virtually all stages of tumor progression. miRNA regulation can modulate cell processes, such as cell proliferation and migration (e.g. miR-200, miR-205 and miR-21), apoptosis (e.g. miR-34 and miR-29 family), angiogenesis (e.g. miR-126 and miR-92) and maintenance of the pool of stem cells (e.g. let-7 family) (Mott *et al*, 2007; Gregory *et al*, 2008; Bonauer *et al*, 2010; Hermeking 2010; Han *et al*, 2012) .

We observed, through a microarray analysis and quantitative PCR on the 21T cell line series that mimic HER-2 $^{+}$ breast cancer progression and on tissue samples that miR-205 levels are significantly reduced in metastatic tumors. This downregulation is associated to an invasive high proliferative profile and a worse poor prognosis.

The miR-205 downregulation occurs along with the reduction on levels of the transcription factor ZEB-1, a miR-205 target and consequently with the inhibition of the epithelial marker e-cadherin, transcriptionally regulated by ZEB-1(Gregory *et al*, 2008).

These results indicate a role of miR-205 on the maintenance of epithelial tissue polarity and then preventing the epithelia-mesenchyme transition, one of the earliest events that contribute for the development of metastases.

Recent data link miRNAs regulation to pathways activated by genotoxic stress, for example by the ability of miRNAs on regulating p53 response, DNA repair systems and apoptosis (Hummel *et al*, 2010).

According to miRNA target prediction softwares that takes into account the complementarity between miRNA sequence and mRNA 3'UTR, each miRNA has an average of hundred targets (TargetScan Human v 6.3). Considering that many miRNAs orchestrate a response to environmental *stimuli* and that this class of molecules is subject to the same genomic mechanisms of gene regulation observed in protein coding genes it is not surprisingly that subtle changes in their expression patterns can drive cell behavior. Alterations in this global mechanism of gene regulation are also commonly observed in cancer and other diseases (Lu *et al*, 2005).

Putting all this information together we can deduce that the expression profile of miRNAs may be a more sensitive prognostic marker to cytotoxic treatment than the mRNA profile.

Based on this idea, we first analyzed the expression of miRNAs involved in the regulation of apoptosis after submit the non-tumoral cell line HB-2 and the breast cancer cell line MCF-7 to low energy irradiation. The ability of soft rays in causing lesions was confirmed by the increase in the number of DNA strands breaks observed by Comet assay and *micronuclei* formation and the occurrence of apoptosis was checked by characteristic changes in cell morphology.

Concerning miRNAs expression analysis, we could observe a let-7a and miR-34a up-regulation 4 h after irradiation and the return to control levels 24h after X-ray exposure. These results suggest that miR-34a and let-7a up-regulation probably take part from early cell responses to DNA injury.

After submitting the tumor cell lines MCF-7 and T-47D to different genotoxic *stimuli* generated by doxorubicin and low and high-energy radiation, we observed 24 hours after all treatments an increase in miR-29b expression. The miR-29 family (miR-29a, miR-29b and miR-29c) have its expression triggered after chronic DNA damage and

during senescence (Ugalde *et al*, 2011). This upregulation can in part be a p53 dependent mechanism, as it was demonstrated before that doxorubicin treatment is able to activate all miR-29 promoters only in p53 wild type models (Ugalde *et al*, 2011). Our results confirm the activation 24h after doxorubicin treatment only in MCF-7 (p53 wt) cells and suggest that another mechanism is involved in miR-29b upregulation after irradiation, as both T-47D (mutated p53) and MCF-7 showed increased miR-29b levels 24h after treatment.

This miRNA, in turn is capable to stimulate p53 transcription by targeting p85α and CDC42 and, at protein level by repressing Ppm1d phosphatase and enhance p53 activity (Ugalde *et al*, 2011; Park *et al*, 2009). miR-29b is downregulated in some cancer types and, until now, it has been demonstrated it can induce apoptosis by silencing at mRNA level the Bcl-2 family protein Mcl-1, regulate collagens and cell adhesion related genes and revert aberrant methylation in lung cancer by targeting DNA methyltransferases A/B (Mott *et al*, 2007; Liu *et al*, 2010; Fabbri, 2007).

We observed that in normal conditions miR-29b is strongly downregulated in MCF-7 and T-47D, when compared to the non-tumoral cell line MCF-10A. The miR-29b downregulation in MCF-7 seems to be an epigenetic event, as an enrichment of the heterochromatin marker h3k9me3 in the promoter region of this miRNA could be observed.

These results suggest that the genotoxic stress caused primarily by radiation from both low and high energy can stimulate miR-29b expression and possibly increase apoptosis by the silencing of MCL-1 and p53 activation.

This work allows us to conclude that miRNAs are involved in regulating both tumor progression and cellular responses to environmental damage. We could classify miR-

205 as a possible EMT regulator and miR-29b as a potential marker of DNA damage. We believe that miRNAs may become an important class of biomarkers and potential therapeutic targets in breast cancer treatment.

VI.Conclusions

- A miRNA global expression panel of 21T, a HER2+ breast cancer progression, shows a strong downregulation of miR-205 on the DCIS to IDC transition.
- miR-205 reduced levels is correlated with elevated ZEB-1 protein levels and reduced e-cadherin levels corroborating the role of this miRNA in EMT regulation in breast cancer.
- The miRNA global expression analysis reveals a deregulation of several miRNAs, including let-7 and miR-29 family members on the tumoral cell lines MCF-7 and T-47 when compared to the non-tumoral one MCF-10A
- Low energy X-rays (30kv spectrum), is efficient on promoting DNA damage and on inducing miRNA expression. The irradiation responsive miRNAs varies according to the time after irradiation and cell line.
- miR-29b downregulation in breast cancer cells (MCF-7 and T-47D) is reverted after irradiation and doxorubicin treatment drawing attention to the possible role of miR-29b as a biomarker of genotoxic damage.

VII.Perspectives

As a result of this work we aim to investigate the role of miR-205 in cell proliferation and metastasis through the following technical approaches:

- Silencing of miR-205 by siRNA in 16N, 21PT and 21NT cell lines followed by 3D matrigel cell culture and anchorage independent cell growth and invasion assay.
- Overexpression of miR-205 after transfection with pre-miRNA 21MT1 and 21MT2 in the cell lines followed by 3D matrigel cell culture and anchorage independent cell growth and invasion assay.
- Confirm the role of ErbB2 as miR-205 transcriptional regulator, by Chromatin imunopreciptation (Chip assay)

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IX.Annexes