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MicroRNA Profile in Response to Doxorubicin Treatment in Breast Cancer

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ABSTRACT

Chemotherapy treatment is the standard in triple negative breast cancers, a cancer subgroup which lacks a specific target. The mechanisms leading to the response, as well as any markers that allow the differentiation between responder and non-responder groups prior to treatment are unknown. In parallel, miRNAs can act as oncogenes or tumor suppressors and there is evidence of their involvement in promoting resistance to anticancer drugs. Therefore we hypothesized that changes in miRNA expression after doxorubicin treatment may also be relevant in treatment response. Objective: To study miRNAs that are differentially expressed in response to doxorubicin treatment. Methods: One luminal-A and two triple negative, breast cancer cell lines were exposed to doxorubicin. Microarray analysis was performed to identify the common and differentially modified miRNAs. Genes and pathways that are theoretically regulated by these miRNAs were analyzed. Results: Thirteen miRNAs common to all three lines were modified, in addition to 25 that were specific to triple negative cell lines, and 69 that changed only in the luminal-A cell line. This altered expression pattern seemed to be more strongly related to the breast cancer subgroup than to the treatment. The analysis of target genes revealed that cancer related pathways were the most affected by these miRNAs, moreover many of them had been previously related to chemotherapy resistance; thus suggesting follow-up studies. Additionally, through functional assays, we showed that miR-548c-3p is implicated in doxorubicin-treated MCF-7 cell viability, suggesting a role for this miRNA in resistance. J. Cell. Biochem. 116: 2061–2073, 2015.

KEY WORDS: BREAST CANCER; MIRNA; DOXORUBICIN

Triple negative breast cancer (TNBC) is characterized by the absence of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) expression [Yi et al., 2010]. It currently represents a minority (15%) of all breast cancers and but more frequently affects younger women, often those carrying BRCA mutations. However, despite its sparse prevalence, targeting TNBC is now one of the most important objectives in oncological research because of its highly aggressive and metastatic nature, and because it has a poorer prognosis [Carey 2011] than the other breast cancer subtypes [Andre and Zielinski 2012; Boyle 2012]. This is mainly due to the lack of known specific molecular therapeutic targets for it [Crown et al., 2012;Joensuu and Gligorov 2012], meaning that conventional chemotherapy is the main treatment used for these patients; anthracyclines, specifically doxorubicin, and taxane combinations (with cyclophosphamide and doxorubicin), are among the most commonly used for TNBC treatment [Liedtke et al., 2008 Oakman et al., 2010; Joensuu and Gligorov 2012]. Specifically, doxorubicin is an antracicline antibiotic that is considered to be the most effective agent in breast cancer treatment [Smith et al., 2006]. Nevertheless, resistance to this agent is frequent which represents a potential obstacle to successful treatment.

Women with TNBC treated with conventional chemotherapy respond differently to treatment [Rouzier et al., 2005; Carey et al.,

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2007; Liedtke et al., 2008; De Laurentiis et al., 2010]. Studies on neoadjuvant chemotherapy have shown that TNBC patients with pathological complete response (pCR) to treatment correlate with good clinical outcomes, with rates similar to non-TNBC patients. However, most TNBC patients (63%) have residual disease after treatment, showing a peak risk of relapse at around three years post-treatment, as well as an increased mortality rate [74%] [Carey et al., 2007; Dent et al., 2007; Cheang et al., 2008; Liedtke et al., 2008; Dent et al., 2009; Oakman et al., 2011; Andre and Zielinski 2012; Yu et al., 2013]. Although there are two TNBC patient groups: *chemotherapy-sensitive* and *chemoresistant*, the mechanism of this differing treatment response is unknown and so there is a clear need to resolve these differences and/or to identify specific targets as the basis for future therapies.

Data from gene profiling studies have been very useful in defining the characteristics of different cancers and have contributed to advancing both the treatment of breast cancer and predicting its outcome and therapeutic response. More recently, studies of epigenetic modifications, including miRNAs, have implicated these alterations in the initiation and progression of cancer [Peña-Chilet et al., 2014]. MiRNAs are molecules that can regulate gene expression at the post-transcriptional level by binding to 3′ UTR mRNA target sequences, and it has been postulated that each miRNA regulates up to 100 different mRNAs [Hummel et al., 2010]. MiRNAs play an important role in cellular processes including development, proliferation, differentiation, apoptosis and stress response, and several studies have shown that they are aberrantly expressed in different types of cancer. In fact, expression studies in normal breast tissue and tumors identified numerous miRNAs that are deregulated in breast cancer, thus allowing normal and malignant breast tissues to be distinguished [Iorio et al., 2009; Leivonen et al., 2014]. Therefore miRNAs may have a causal role in tumorigenesis by modulating the expression of known oncogenes or tumor suppressor genes [Garzon et al., 2006; Cowland et al., 2007; Cho 2007; Zhang et al., 2007; Hummel et al., 2010], and are also thought to be involved in differential treatment responses [Eroles et al., 2015; Tian et al., 2013]. Several microarray studies have demonstrated that drug-resistant and drug-sensitive cell lines have different miRNA expression profiles [Chen et al., 2010; Pogribny et al., 2010; Zhou et al., 2010) and so miRNAs can be considered not only as biomarkers but also as novel therapeutic anticancer targets.

Given the aggressive nature of TNBC, and the known role of miRNAs in cancer development and treatment resistance, in this study we focused on analyzing miRNA expression profiles in response to doxorubicin treatment in two TNBC cell lines (MDA-MB-231 and MDA-MB-468). We compared this profile to a non-TNBC luminal-A cell line (MCF-7), which is almost exclusively used for such studies, with the aim of better understanding the response and resistance mechanisms of TNBC.

METHODS

BREAST CANCER CELL LINES AND REAGENTS

MDA-MB-231, MDA-MB-468, MCF-10A and MCF-7 cell lines were obtained as kind gifts from Dr. Joan Albanell at the IDIBELL Institute, Vall D' Hebron Hospital, Barcelona. All cell lines were grown at 37°C in a humidified, 5% CO_2, 95% air incubator. MDA-MB-231 and MDA-MB-468 were grown in Dulbecco's Modified Eagle medium: nutrient mixture F-12 (DMEM/F12) with 2.5 mM L-Glutamine and 15 mM HEPES (Gibco), supplemented with 10% FBS and 1% penicillin-streptomycin. MCF-7 cells were grown in DMEM/F12 with 1 g/l D-Glucose, 4mM L-Glutamine, and 1mM Pyruvate (Gibco), supplemented with 10% FBS and 1% penicillin-streptomycin. MCF-10A cells were grown in DMEM/F12 with 0.1% insulin, 0.5 μg/ml hydrocortisone, and 16 ng/ml EGF, supplemented with 10% FBS and 1% penicillin-streptomycin. Doxorubicin (Ferrer Farma) was used at 5μM, according to previous reports [Tapia et al., 2007].

RNA ISOLATION

Total RNA, containing small RNAs, was isolated using a mirVana miRNA isolation kit (Ambion, Austin, Texas) according to the manufacturer's protocol. The concentration and purity of the RNA obtained was measured as the OD260/280 ratio using a GeneQuant pro spectrophotometer (GE, Healthcare). RNA integrity was determined by capillary electrophoresis using an RNA 6000 Nano Lab-on-a-Chip kit and a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Only RNA extracts with an integrity value of 8 or higher were used for further analysis. The time point selected for analysis was 24 h doxorubicin exposure.

SMALL NON-CODING RNA EXPRESSION-PROFILING

Small non-coding RNA expression-profiling was performed using a GeneChip miRNA 2.0 Array (Affymetrix, Santa Clara, CA). The array contains 15,664 mature miRNA sequences from the miRBase (v15), including encoded miRNAs from 131 organisms encompassing 2,334 snoRNAs and scaRNAs, and 2,202 probe sets unique to pre-miRNA hairpin sequences.

Microarray experiments were conducted according to the manufacturer's protocol. Briefly, 300 ng of total RNA was labelled with a FlashTag Biotin HSR RNA labeling kit from Genisphere. The labeling reaction was hybridized onto the miRNA array in an Affymetrix hybridization oven 640 at 48°C for 17 h. The arrays were stained using a Fluidics Station 450 with the fluidics script FS450_0003 (Affymetrix) and then scanned on a GeneChip Scanner 3,000 7G (Affymetrix, Santa Clara, CA), using the GeneChip Command Console® Software supplied by Affymetrix to perform gene expression analysis. MiRNA probe outliers were defined and further analyzed as per the manufacturer's instructions (Affymetrix), and quality control, as well as data summarization and normalization, was carried out using the web-based miRNA QC Tool (www.affymetrix.com).

MICROARRAY DATA ANALYSIS

Data (.CEL files) were analyzed and statistically filtered using Partek Genomic Suite v6.4 software (Partek Inc., ST Louise, MO), and input files were normalized with the multi-array average algorithm (RMA) for miRNAs. A one-way analysis of variance (ANOVA) was performed with the Partek Genomic Suite on all samples. Statistically significant small non-coding RNAs between the different groups and treatments studied were identified using an analysis of variance model with a probability value (*P*-value) of 0.05 or less.

The imported data were analyzed by principal components analysis (PCA) to determine the significant sources of variability in the data; PCA reduces the complexity of high-dimensional data and simplifies the task of identifying patterns and sources of variability in a large data set. The samples (eighteen biological replicates, each hybridized to a separate GeneChip) are represented by the spheres in the three-dimensional plot. The distance between any pair of points is related to the similarity between the two samples in high-dimensional space (in this case, each variable represents a one-dimensional space). Samples that are near each other in the plot have a large number of variables in common. Conversely, samples that are far apart in the plot are different in a large number of variables. Finally, the selected small non-coding RNAs were imported into Pathway Studio v8 software (Ariadne) to classify the biological processes represented by the miRNAs that were differentially expressed in treated and non-treated cells.

REAL TIME PCR VALIDATION

Validation was carried out in MDA-MB-231, MDA-MB-468, and MCF-7 cell lines. In addition, expression levels for the chosen miRNAs were checked in normal MCF-10A breast cells. Reverse transcription (RT) was performed with random hexamers using MultiScribe™ reverse transcriptase (Applied Biosystems). First-strand cDNA synthesis was performed at 42°C for 30 min; the reaction was stopped by heating the mixture at 95°C for 5 min, and was then stored at −20°C until further use. Previously-developed TaqMan primers specific for the housekeeping miRNA RNU43 and miRNAs of interest: miR-27a-5p, miR-27b-5p, miR-29b-1-5p, miR-424-3p (miR-424*), miR-1972, miR-23a-5p (miR-23a*), miR-449c-5p, miR-1207-5p, miR-1275, and miR-25-5p were purchased from Applied Biosystems and transcript levels were detected using the 9700HT Fast Real-time PCR System (Applied Biosystems). Each PCR reaction contained 5 ng of RT product, 10 μL TaqMan universal master mix II (Applied Biosystems), and 1 μL Taqman assay 20x in a final volume of 20 μL. The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All PCR reactions were cycled in the linear region of amplification; results were normalized according to RNU43 miRNA housekeeping control quantification. The threshold cycle (CT) was automatically determined, and then the relative miRNA expression was calculated as follows: relative amount $= 2^{-\Delta(\Delta CT)}$, where $\Delta CT =$ CT target-CT housekeeping control, and $\Delta\ (\Delta CT) = \Delta CT$ studied group-ΔCT baseline. MiRNA levels in non-treated cells were chosen as the baseline. All experiments were performed three times in triplicate. Data are represented as the mean plus or minus the standard deviation ($\pm$ *SD*). Comparison between two groups was performed with a two-tailed *t*-test. *P*-values of less than 0.05 were considered to be statistically significant.

ANALYSIS OF MIRNA–GENE INTERACTIONS

The miRBase database (http://www.mirbase.org/), a searchable resource containing published miRNA sequences and annotations, was used to rename all miRNAs with similar criteria. Each entry in the miRBase sequence database represents a predicted hairpin portion of an immature miRNA transcript (termed mir), and contains information on the location and sequence of the mature miRNA sequence (termed miR). The miRNA-gene interactions were computationally predicted and combined by calculating the Union of Targeted Genes (Gene Union) using the DIANA-microT-CDS web service [Vlachos et al., 2012]. The DIANA-miRPath v2.1 web service was used to identify pathways from KEGG (Kyoto Encyclopedia of Genes and Genomes) which were significantly targeted by the selected miRNAs. From the all of the targeted pathways sub-groups were selected by combining their statistical significance, number of targeted genes, and biological significance within the context of our study. The miRNA gene-interactions picture was built using the Heat map Builder® Version 1.1 software for each pathway.

FUNCTIONAL STUDIES: MIRNA TRANSFECTION AND CELL VIABILITY

In order to transfect the cell lines with either mimic or inhibitor miRNA, hsa-miR-548c-3p pre-miR miRNA precursor (#AM17100), or hsa-miR-548c-3p anti-miR miRNA inhibitor (#AM17000), were purchased from Ambion. CyTM3-labeled pre-miR (#AM17120; Ambion) was used as a negative control for transfection. The reaction was performed with the TransIT-X2 Dynamic Delivery System reagent (Mirus), following the manufacturer's instructions. The MDA-MD-231, MDA-MB-468, and MCF-7 cell lines were transfected in 96-well plates with different miRNA mimic/inhibitor concentrations, and then exposed to 5 μM of doxorubicin for different amounts of time. Cell viability was then measured using a MTT-based Cell Growth Determination Kit (#GDC1; Sigma); given that mitochondrial metabolic activity is often used as an indicator of cell viability, this assay is based on the reduction of tetrazolium salt by active mitochondria to produce insoluble formazan salt. MTT was added to each well under sterile conditions (final concentration was 10% of total volume) and the plates were incubated for 4 h at 37°C. Formazan crystals were dissolved in solubilization solution (1:1). The purple formazan crystals were formed from yellow MTT by succinate dehydrogenase in viable cells. Absorbance of the formazan product was measured at 570 nm with background correction at 690 nm using a microplate reader. Significant differences were assessed by using the t-student test.

COMPREHENSIVE SEARCH IN ARRAY DATA PUBLIC REPOSITORIES

We performed a comprehensive search in the currently available array data public repositories Gene Expression Omnibus and Array Express. The aim was to find miRNA array data from breast cancer patients coming from tumor biopsies obtained before and after doxorubicin treatment. To our knowledge, no studies following these criteria have been carried out to date. This is explained by the fact that breast cancer tumor re-biopsies are rare events in clinical practice which limits the feasibility of testing tumor miRNA transcriptomic changes in response to treatment in patient samples. As an alternative approach, we looked for coding RNA array data. The idea was to observe the indirect effect of the deregulated miRNA over target gene expression in post treatment tumor patient samples. We also failed to find public array data fitting our criteria.

However, we were able to validate miRNA differential expression between healthy breast tissues and breast tumor samples using the Gene Expression Omnibus dataset GSE45666 [Lee et al., 2013] which included array data from 101 breast tumors and 15 adjacent breast normal tissue samples. We downloaded raw data and used a custom

modification of AgiMicroRna R package to summarize probe level information into total gene signal using AFE algorithm. Normalization between arrays was carried out using quantile method. We analyzed the miRNAs selected for validation (miR-27a-5p, miR-27b-5p, miR-29b-1-5p, miR-424-3p, miR-1972, miR-23a-5p, miR-449c-5p, miR-1207-5p, miR-1275, and miR-25-5p) and a set of additional miRNAs (miR-125b, miR-1305, miR-200c, miR-222, miR-3163, and miR-548c-3p) chosen with the criteria of its implication in cancer pathways. Fifteen normal breast tissues, 36 TNBC and 30 luminal breast tumors were included in the analysis. The *t*-student test was used for statistical signification.

RESULTS

PRINCIPAL COMPONENTS ANALYSIS

In order to get an overview of the differences in the miRNA expression profiles in normal and doxorubicin treatment conditions, both between breast cancer cell lines and in each cell line separately, PCA was carried out on the small non-coding RNAs obtained in our study with a GeneChip miRNA 2.0 Array; each breast cancer cell line was represented (in triplicate) with colored dots (Fig. 1a) grouped onto colored ellipsoids (Fig. 1b, c and d). Similar miRNA expression profiles were observed for the MDA-MB-231 and MDA-MB-468 cell lines, as shown by the strong grouping of the dots from each condition and cell line. However, the MCF-7 cell line substantially differed from the others (Fig. 1a). In addition, strong differences were shown between normal conditions versus doxorubicin treatment in each separate breast cancer cell line (Fig. 1b, c and d).

REGULATION OF SMALL NON-CODING RNAS IN DOXORUBICIN TREATMENT CONDITIONS

ANOVA (with a significance *P*-value of 0.05 or less) was performed to compare the normal condition versus doxorubicin treatment for the three breast cancer cell lines. According to these results seven groups (I to VII) were created, and a total of 218 miRNAs were found to have a significantly altered expression profile. Thirteen miRNAs were found to change in all three cell lines (group I), twenty-five changed only in the MDA-MB-231 and MDA-MB-468 cell lines (group II), sixty-nine were found only in the MCF-7 cell line (group III), a total of forty-six miRNAs changed significantly only in the MDA-MB-231 cell line (group IV), fifty-two changed only in the MDA-MB-468 cell line (group V), eight changed in the MDA-MB-231 and MCF-7 cell lines (group VI), and five changed in the MDA-MB-468 and MCF-7 cell lines (group VII). See Figure 2 and Supplementary Table 1. Given that our study focused on elucidating the global doxorubicin response and the differences in response between TNBC and Luminal breast cancer cells, groups I, II, and III were selected for further heat map analysis. This analysis again showed closer miRNA expression patterns between the MDA-MB-231 and MDA-MB-468 cell lines than the MCF-7 cell line, both in normal and doxorubicin treatment conditions (Figure 3).

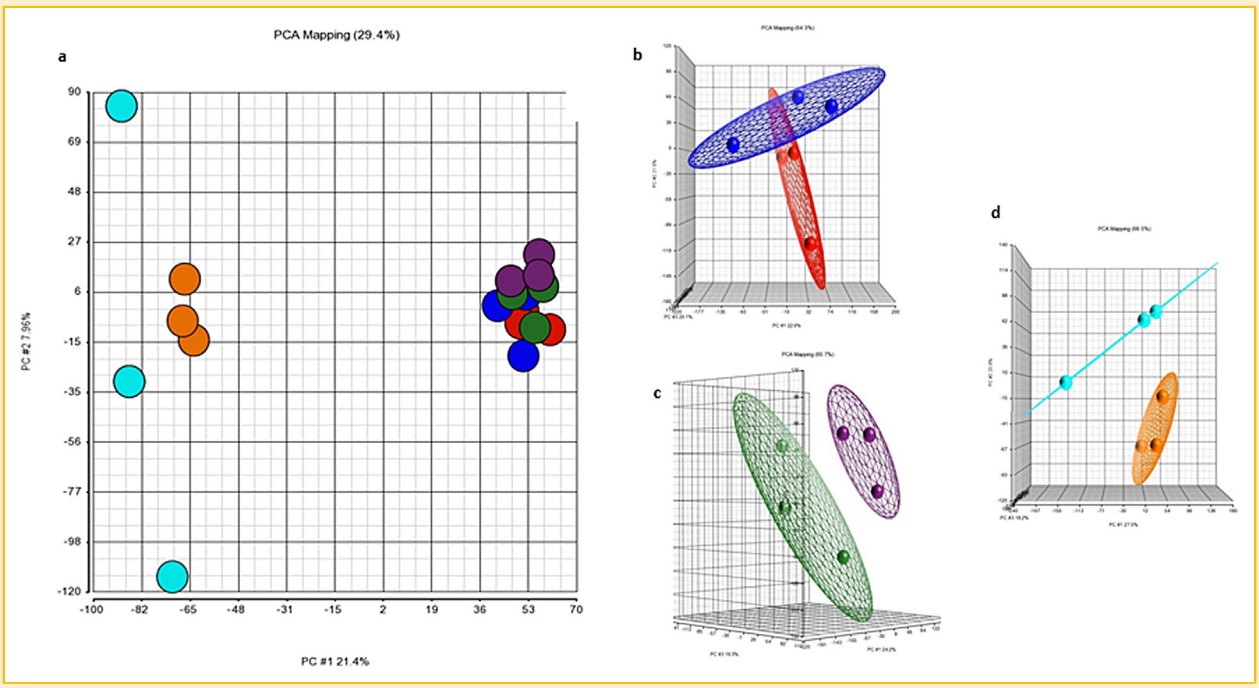

Fig. 1. Principal component analysis of the small non-coding RNA profiles in normal and doxorubicin-treated breast cancer cell lines. The small non-coding RNA expression profiles of the three breast cancer cell lines were analyzed by principal components analysis (PCA). The MDA-MB-231 cell line profile (a, b) is represented by red dots (control cells) and blue dots (treated cells). The MDA-MB-468 cell profile (a, c) is represented by green dots (control cells) and purple dots (treated cells). The MCF-7 cell profile (a, d) is represented by orange dots (control cells) and turquoise dots (treated cells). The colored ellipsoids show a different directionality in each cell line based on similarities and differences between normal conditions and doxorubicin-treatment cohorts. The axes correspond to principal components; PC1: x-axis, PC2: y-axis, and PC3: z-axis.

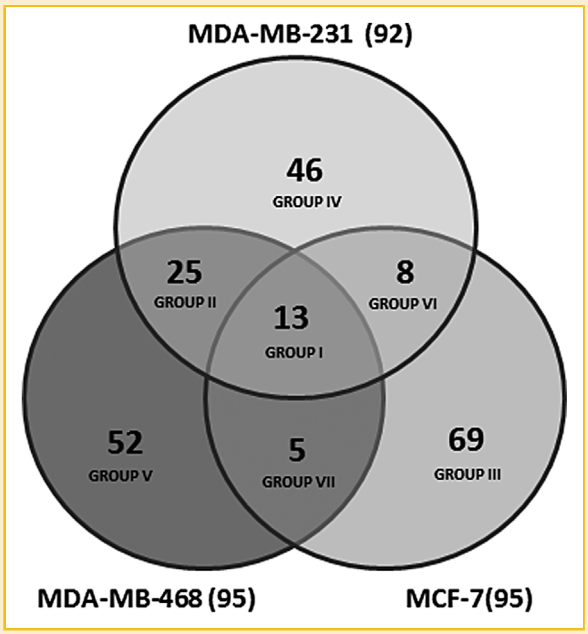

Fig. 2. Venn diagram showing the small non-coding RNAs regulated in the three classes of breast cancer cell lines when treated with doxorubicin. Venn diagram representing differentially expressed miRNAs observed in each cell line separately and comparisons between each of the three cell lines. A total of 218 miRNAs were found to have a significantly altered expression profile. These miRNAs were classified into different groups; 46 were found in MDA-MB-231 cells (group IV), 52 in MDA-MB-468 cells (group V), and 69 in MCF-7 cells (group III). Thirteen were commonly changed in all three lines (group I), 25 changed in MDA-MB-231 and MDA-MB-468 cells (group II), 8 changed in MDA-MB-231 and MCF-7 cells (group VI), and 5 changed in MDA-MB-468 and MCF-78 cells (group VII).

Finally, we focused on the expression values in terms of miRNA fold change between the control and treatment conditions in each groups. Real time PCR for several miRNAs was used to validate the expression changes observed in the microarrays. MiR-27a-5p, miR-27b-5p, miR-29b-1-5p, miR-424-3p, miR-1972, miR-23a-5p, miR-449c-5p, miR-1207-5p, miR-1275, and miR-25-5p were validated, confirming the downregulation of nine of them and the upregulation of miR-449c. The expression values obtained are shown in Supplementary Table 2.

In group I, representing miRNAs that changed in all three cell lines, we found that all of the miRNAs showed the same trend of increased or decreased expression in the three cell lines; however, not all of them were up-or downregulated by similar amounts. Several miRNAs, such as miR-27a-5p, miR-29b1-5p, miR-1972, and miR-449c-3p were more affected in TNBC cell lines than in the luminal-A cell line; others, such as miR-4298, and miR-1275, showed lower expression in MCF-7 cells than in TNBC cells, whereas miR-27b-5p showed a bigger fold change in MDA-MB-231 cells.

In group II (the TNBC group), once again, most miRNAs showed the same regulation trend, with the exception of miR-940 which was downregulated in MDA-MB-231 cells and upregulated in MDA-MB-468 cells, and miR-759 that was upregulated in MDA-MB-231 cells and downregulated in MDA-MB-468 cells. In addition, miR-21-3p, miR-1308, miR-222-5p, and miR-181a-3p were more affected by doxorubicin in MDA-MB-231 cells, while miR-92a-1-5p was more affected in MDA-MB-468 cells. The specific changes in groups III (MCF-7), IV (MDA-MB-231), V (MDA-MB-468), VI (MDA-MB-231, and MCF-7), and VII (MDA-MB-468 and MCF-7 cells) are shown in Supplementary Table 1.

Interestingly, a high percentage of star miRNAs (miRNAs*) were significantly changed after doxorubicin treatment. Specifically, 46.1% of the miRNAs in group I, 28% in group II, 15.9% in group III, 8.96% in group IV, and 13.46% in group V were miRNAs*.

EXPRESSION OF DOXORUBICIN-MODULATED MIRNAS IN NORMAL MCF-10A BREAST CELLS

In order to confirm that the changes in miRNA expression were specific to breast cancer tumor cells, we evaluated their expression in normal MCF-10A breast cells. The MCF-10A cell line was treated with 5 μM doxorubicin for 24 h and then the miRNAs were extracted. Expression of the selected miRNAs was then analyzed by quantitative PCR and compared to untreated cells. These results show that miR-1972, miR-1207-5p, and miR-1275 are elevated with treatment in this normal cell line, which is in contrast to observations in the breast cancer cell lines. However, miR-27b-5p is dowregulated with treatment. The rest of the miRNAs did not significantly change (Supplementary Table 2).

DIFFERENTIAL EXPRESSION OF DOXORUBICIN-MODULATED MIRNAS IN PRIMARY BREAST TUMOR VERSUS NORMAL BREAST TISSUE

We analyzed the data available in Gene Expression Omnibus dataset GSE45666, in order to know the status of expression of miRNAs in primary breast tumor (pre-treatment) and normal breast tissue. MiR-424* (miR-424-3p), miR-1207-5p, miR-125b were significantly upregulated in normal breast tissue versus breast tumor (P-values = 0. 0018, 0.0002 and $<2.2^{e-16}$ respectively). Oppositely, miR-200c was significantly downregulated in normal breast tissue versus breast tumor (P-value = 6.44^{e-7}). Analysis of the breast cancer subgroups revealed similar tendencies that analysis above for miR-1207-5p, miR-125b, and miR-200c (P-values = 0.0025, $<2.2^{e-16}$ and 0.0002 respectively) and a significant higher expression of miR-1305 in TNBC versus normal breast tissue (P-value = 0.0115). MiR-1207-5p, miR-125b, miR-1275, miR-23a* (miR-23a-5p), miR-424* showed a significant downregulation in the subgroup of luminal breast tumors versus normal breast tissue (P-values = 1.23^{e-5}, 4.11^{e-11}, 0.0022, 0.0015, and 1.75^{e-6} respectively). Again, MiR-200c was significantly lower in normal breast tissue than in luminal breast tumor (P-value = 1.06^{e-7}). No other miRNAs showed any significant changes between samples (Figure 4).

ANALYSIS OF GENE TARGETING BY DOXORUBICIN-MODIFIED MIRNAS

MiRPath V2.1 from Diana Tools was used to determine both the genes and the pathways (microT-CDS) which are theoretically regulated by the miRNAs altered by doxorubicin treatment in each subset. The miRNA regulation pathways found were based on 3′ UTR

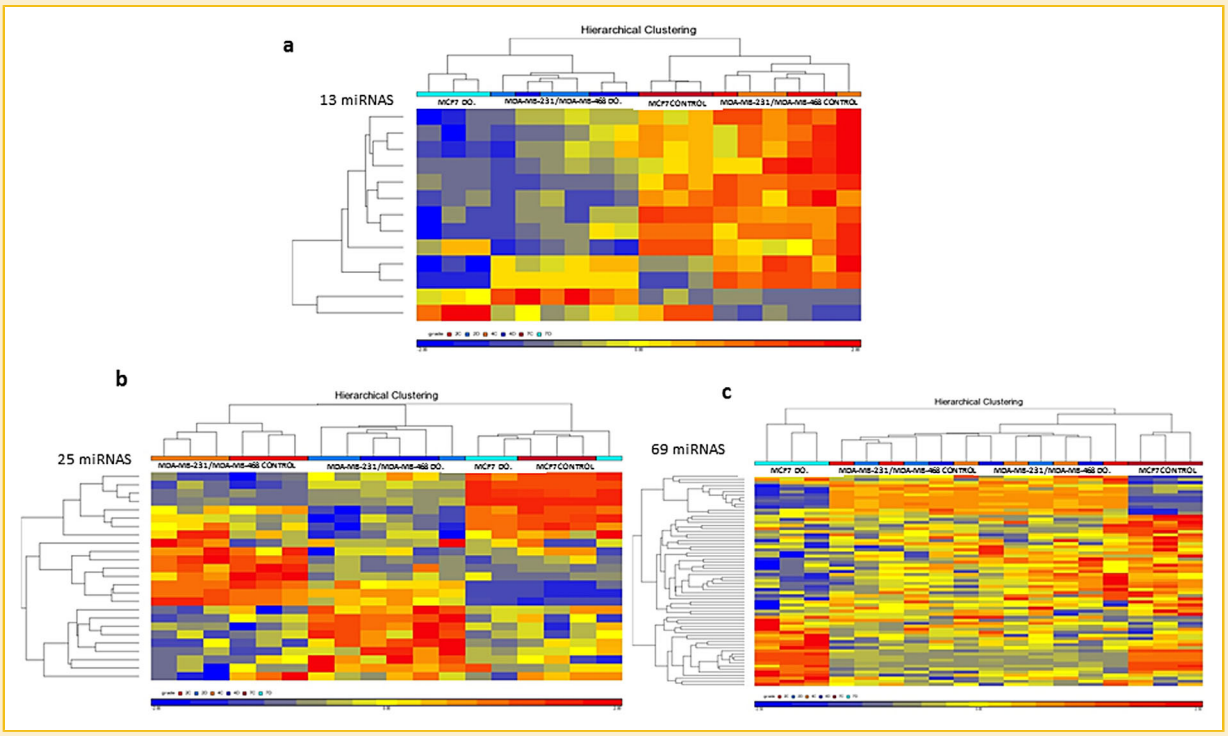

Fig. 3. Heat map of the expression profile changes between normal conditions versus doxorubicin treatment. Thirteen miRNAs were found to significantly change their expression in all three cell lines (3, a). Twenty-five miRNAs changed in MDA-MB-231 and MDA-MB-468 but not in MCF-7 cells (3, b). Sixty-nine miRNAs changed significantly in MCF-7 but not MDA-MB-231 and MDA-MB-468 cells (3, c). The expression scale is shown under each heat map. Blue boxes represent low-expression while red boxes represent high-expression. DO: doxorubicin treatment. Control: no treatment.

base alignment complementarity, and filtered according to a *P*-value threshold of 0.05 and a microT threshold of 0.8. Selection criteria were: statistical significance, number of genes targeted, number of miRNAs implicated in these pathways, and the biological meaning and impact in the context of our study. The main pathways (KEGG), including *PI3K-Akt* (hsa04151), *Wnt* (hsa04310), *cancer* (hsa05200), *focal adhesion* (hsa04510), *MAPK* (hsa04010), *ErbB* (hsa04012), and *adherens junctions* (hsa04520) were selected based on the criteria mentioned above and subjected to further analysis (Table I).

For each pathway, each gene theoretically targeted by one of our selected miRNAs was recorded and mapped onto a heat map (Supplementary Figure 1). The miRNAs with the highest number of targeted genes in each group per pathway were considered (Table II and Supplementary Figure 1). In group I, miR-1207-5p targeted a large number of genes; in group II, miR-200c-3p, miR-9-5p and miR-940 showed the highest number of gene interactions; in MCF-7 cells (group III) miR-548c-3p, and miR-1305 appeared to regulate a large number of genes per pathway; while miR-335-3p in MDA-MB-231 cells (group IV) and miR-3163 in MDA-MB-468 cells (group V) were found to regulate a higher percentage of the total number of genes targeted in each pathway. Bibliographic analysis of the significantly changed miRNAs in each group detected that 23% of the miRNAs had been previously related to chemotherapeutic resistance, and many of them have experimentally validated targets (Table III).

MIRNA-548C-3P EXPRESSION MODULATES SURVIVAL IN DOXORUBICIN-TREATED MCF-7 CELLS

Analysis of the genes and pathways regulated by the doxorubicin-treatment-modified miRNAs, specifically highlighted miRNA-548c-3p. Microarray results showed that this miRNA was significantly upregulated in MCF-7 cells after doxorubicin treatment (fold change = 1.37, $P = 0.045$), whereas there were no significant changes in this miRNA in the other two cell lines after similar treatment. MiRNA-548c-3p regulates a high percentage of theoretical target genes in cancer pathways (see Table II and Supplementary Figure 1). In order to evaluate the functional implication of miR-548c-3p in cell viability after doxorubicin treatment, we carried out transfection experiments to over-and underexpress this miRNA. The results show that miR-548c-3p overexpression significantly increased the viability of MCF-7 cells treated with doxorubicin at 24 h, 48 h and 72 h, but did not affect the other cell lines we tested (Fig.5). In addition, miR-548c-3p reduction not only reversed the resistance, but also increased sensitivity to the drug. This suggests that miR-548c-3p is implicated in doxorubicin treatment resistance in MCF-7 cells.

DISCUSSION

Deregulation of miRNA expression is common in all types of human cancer; some of these alterations correlate with the severity of the

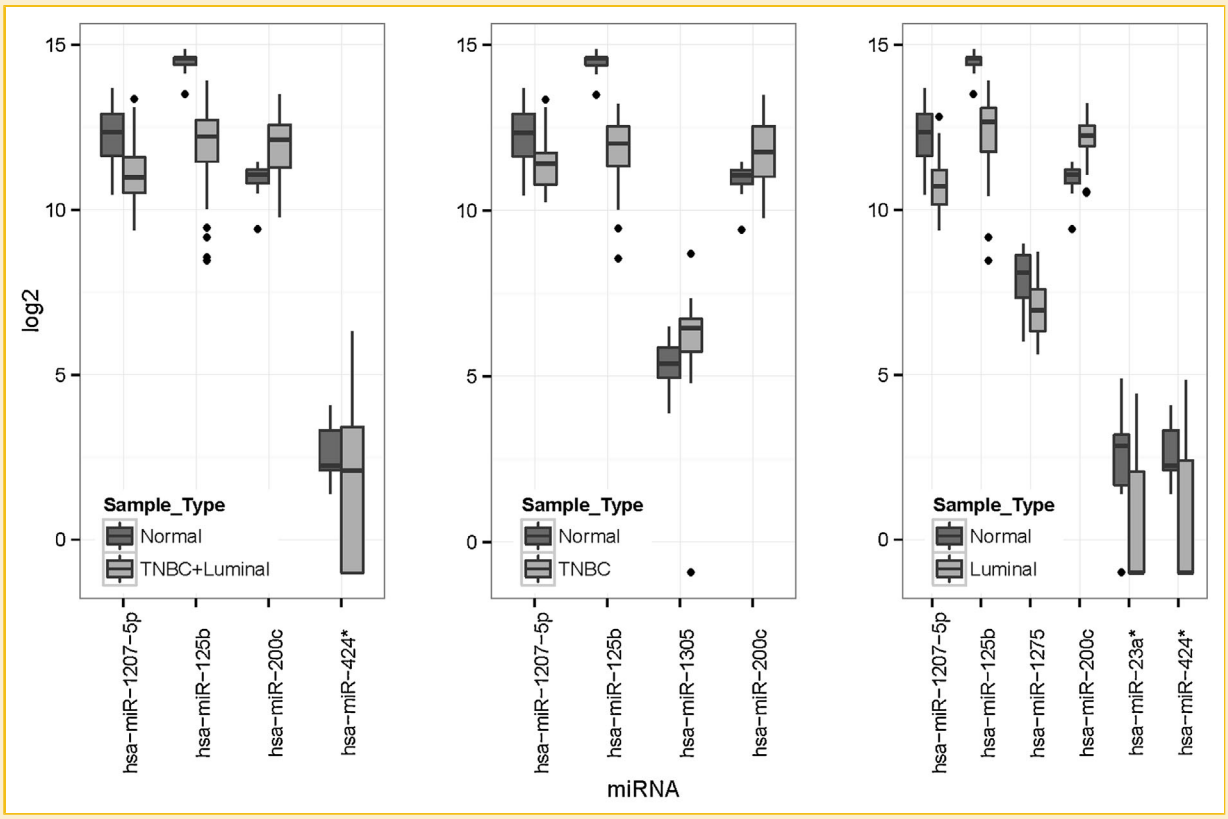

Fig. 4. Expression of miRNAs in primary breast cancer versus normal breast tissue. Figures from left to right show: expression of miRNAs in normal breast tissue (n = 15) versus tumor (triple negative and luminal breast cancer, n = 66); expression of miRNAs in normal breast tissue (n = 15) versus triple negative breast tumor (n = 36); expression of miRNAs in normal breast tissue (n = 15) versus luminal breast cancer (n = 30). MiRNAs with a significant difference in expression are shown.

TABLE I. MiRNA Targeted Pathways

	KEGG pathway	PI3K-Akt (hsa04151)	Wnt (hsa04310)	Cancer (hsa05200)	Focal adhesion (hsa04510)	MAPK (hsa04010)	ErbB (hsa04012)	Adherents junction (hsa04520)
GROUP I	p-value	7.43×10^{-6}	9.99×10^{-6}	2.82×10^{-3}	1.29×10^{-4}	4.42×10^{-6}	1.42×10^{-3}	1.47×10^{-4}
	#genes	60	33	52	37	50	16	17
	#miRNAs	12	10	10	9	8	7	7
GROUP II	p-value	$1.41 \times .10^{-16}$	1.51×10^{-11}	1.05×10^{-12}	1.52×10^{-22}	1.02×10^{-18}	1.84×10^{-30}	1.62×10^{-10}
	#genes	119	60	121	83	100	46	35
	#miRNAs	19	17	19	18	19	13	15
GROUP III	p-value	2.18×10^{-38}	5.83×10^{-26}	$6.08 \times .10^{-26}$	6.08×10^{-26}	2.69×10^{-39}	1.21×10^{-15}	1.25×10^{-5}
	#genes	161	78	162	97	129	47	38
	#miRNAs	28	27	31	29	31	26	24
GROUP IV	p-value	5.13×10^{-42}	1.91×10^{-21}	3.74×10^{-37}	4.49×10^{-26}	9.22×10^{-29}	6.04×10^{-16}	2.83×10^{-14}
	#genes	180	84	180	111	113	60	48
	#miRNAs	43	37	40	39	39	35	32
GROUP V	p-value	8.92×10^{-32}	2.92×10^{-18}	7.88×10^{-35}	9.39×10^{-21}	2.91×10^{-25}	5.36×10^{-11}	1.44×10^{-11}
	#genes	190	95	188	120	145	55	50
	#miRNAs	45	43	41	39	42	38	32
GROUP VI	p-value	–	–	–	–	2.61×10^{-2}	–	–
	#genes	–	–	–	–	7	–	–
	#miRNAs	–	–	–	–	3	–	–
GROUP VII	p-value	–	–	–	–	1.32×10^{-2}	–	1.77×10^{-6}
	#genes	–	–	–	–	30	–	16
	#miRNAs	–	–	–	–	4	–	5

Crosslink between number of altered miRNAs involved in each pathway and number of genes theoretically targeted inside of a given pathway. *P*-values <0.05 were considered to be significant.

TABLE II. The Number and Percentage of Theoretical Target Genes for the Mirnas Indicated, Grouped into Pathways

		Cancer pathways		Ad. junctions		Focal adhesion		ERBB		MAPK		PI3K		WNT	
Group	miRNA	genes	%	genes	%	genes	%	genes	%	genes	%	genes	%	n° genes	%
III	hsa-miR-548c-3p	113	42.80	38	59.38	65	39.63			74	38.34	92	56.10	47	36.15
V	hsa-miR-3163	85	32.20			49	29.88	24	33.33	57	29.53	79	48.17	54	41.54
IV	hsa-miR-335-3p	62	23.48	12	18.75	47	28.66	22	30.56	44	22.80	80	48.78	29	22.31
III	hsa-miR-1305	54	20.45	15	23.44	29	17.68	14	19.44	33	17.10	52	31.71	27	20.77
II	hsa-miR-9-5p	29	10.98	8	12.50	21	12.80	9	12.50	27	13.99	23	14.02	7	5.38
I	hsa-miR-1207-5p	16	6.06	7	10.94	15	9.15	4	5.56	21	10.88	19	11.59	11	8.46
II	hsa-miR-200c-3p	25	9.47	4	6.25	21	12.80	15	20.83	28	14.51	27	16.46	10	7.69
III	hsa-miR-27a-3p	32	12.12	5	7.81	27	16.46	19	26.39	30	15.54	41	25.00	17	13.08
III	hsa-miR-29a-3p	23	8.71	2	3.13	27	16.46	6	8.33	13	6.74	33	20.12	5	3.85
IV	hsa-miR-548o-3p	25	9.47	3	4.69	16	9.76	14	19.44	14	7.25	19	11.59	12	9.23
II	hsa-miR-940	28	10.61	12	18.75	18	10.98	10	13.89	20	10.36	23	14.02	13	10.00
III	hsa-miR-205-5p	21	7.95	11	17.19	10	6.10	7	9.72	11	5.70	16	9.76	14	10.77
VI	hsa-miR-548a-5p			10	15.63									17	13.08

The percentage refers the number of genes affected by the specific miRNA from the total number of genes analyzed in each pathway. The group number on the left indicates the origin of the miRNAs with significant expression changes after doxorubicin treatment.

disease because they regulate key transcripts involved in the initiation and progression of tumors. MiRNAs can act as true oncogenes or tumor suppressors to inhibit or exacerbate the expression of cancer related target genes, and to promote or suppress tumorigenesis [Zhang et al., 2007; Ng et al., 2009; López-Camarillo et al., 2013].

In our study we evaluated changes in miRNA expression after treatment with doxorubicin in one luminal-A cell line and two TNBC cell lines. Importantly, doxorubicin strongly affected/modulated miRNA expression in all three breast cancer cell lines. However, as we expected, this altered expression pattern seemed to be more closely related to the molecular subtype than to the treatment. PCA analysis and heat map clustering revealed that the changes between treatment conditions in MDA-MB-231 and MDA-MD-468 cells were closer than for MCF-7 cells. Another fact that supports this observation is the number of miRNAs with altered expression which were grouped in our study (shown in a Venn diagram in Fig. 2).

We analyzed the miRNAs with changes in expression in the two breast cancer subtypes, both separately and as a group, in order to highlight/propose different ways in which they might be able to respond to and/or resist doxorubicin treatment. Most of the miRNAs which were altered in response to doxorubicin treatment had not been widely described before, in part due to their star strand character, and so there was little or no evidence for their role in the main cancer-related pathways. Nonetheless, we reviewed the information available on validated targets in the literature using the Ariadne program (Supplementary Figure 2). The majority of miRNAs with validated targets were involved in processes related to cancer such as growth and proliferation, angiogenesis, invasion, metastasis, suppression of immune recognition, and cell survival pathways (Fig. 6); these data confirm that these pathways are regulated by miRNAs, as previously reported by others [Grammatikakis et al., 2013]. The drug absorption, distribution, metabolism and excretion (ADME) pathway also stood out because its regulation results in a distinct drug response [Harquail et al., 2012].

Reviewing these pathways identified that these miRNAs are principally involved in cancer and different neoplasms, although the relevance of this information is relative because most miRNA* target genes have not yet been validated. Accordingly, we used the DIANA miRPathv2.0 software to analyze the genes theoretically targeted by our miRNAs in order to find altered pathways that could explain modifications in response to doxorubicin treatment. The main potentially-targeted genes were classified into pathways related to cancer (described in the results section); we noticed that several miRNAs in each group seemed to regulate a high percentage of target genes within these pathways.

Group I (MDA-MB-231, MDA-MB-468, and MCF-7) contained 13 changed miRNAs that could be considered as "common response" miRNAs. First we found that all of these miRNAs showed the same trend of increased or decreased expression in all three cell lines, however not all of them changed by a similar amount (Supplementary Table 1). Indeed there were some miRNAs which were more greatly affected by doxorubicin in TNBC cells than in MCF-7 cells. For example miR-27a-5p, an miRNA that coordinates the regulation of the EGFR signaling axis by targeting EGFR, AKT1 and mTOR [Wu et al., 2013]. Increased miR-27a-5p expression leads to a profound cytotoxicity effect and decreased tumor growth in a murine model. Therefore, miR-27a-5p downregulation in MDA-MB-231 and MDA-MB-468 cells after doxorubicin treatment could be a mechanism of doxorubicin resistance. Conversely, miR-1275 was more strongly affected by doxorubicin in MCF-7 cells than in TNBC cell lines. This miRNA suppressed Claudin11 expression thus promoting tumor cell proliferation in human glioma stem cell-like cells [Katsushima et al., 2012], although no evidence for its action in breast cancer has been described so far. MiR-1207-5p expression was also significantly downregulated: its potential targets include adherence, MAPK, cell cycle progression and PI3K pathways (Table II).

Group II contained 25 miRNAs which were altered in both the MDA-MB-231 and MDA-MB-468 cell lines: we therefore considered this group to be the "TNBC-response fingerprint". Most miRNAs showed the same trend of regulation, with the exception of miR-940 that was downregulated in MDA-MB-231 and upregulated in MDA-MB-468. This miRNA regulates many cancer pathway genes (Table II), and it has been proposed that it has an anti-tumorigenic role in response to glyceollins in TNBC cells which is mediated by targeting RhoA, a prominent mediator of invasion and metastasis [Rhodes

TABLE III. MiRNAS Related to Chemotherapy Resistance in the Literature and Detected in Our Study

miRNAs from the bibliography	miRNA doxorubicin-deregulated by groups in our study*	Validated Target	Mechanism	Drug	Cancer	Reference
hsa-let-7	III (let-7-5p) V (let-7d-3p)	ESR1, RAS, CASP3, HMGA2	Cellular response, EMT, hormone receptor status	Doxorubicin; Doxorubicin+verapamil; fulvestrant, cisplatin	breast	Kovalchuk et al. (2008); Chen et al. (2010); Salter et al.(2008); Xin et al.(2009)
hsa-miR-20	IV(miR-20a-3p)	VEGF, E2F1, CDKN1A, HIF-1α, RBL2, STAT3	Cellular response, EMT	Doxorubicin; taxol	breast	Kovalchuk et al. (2008); Zhao et al. (2008)
hsa-miR-21	II (miR-21-3p)	BCL2, CDC25, PTEN, RECK, TIMP3, TPM1, MSH6, MSH2, PDCD4,MASPIN	Cellular response EMT	Doxorubicin; taxol; doxorubicin+ verapamil; trastuzumab; cisplatin	breast, lung	Kovalchuk et al. (2008); Chen et al.(2010); Mei et al. (2010); Bourguignon et al. (2009);Yu et al. (2010)
hsa-miR-22	III (miR-22-3p)	ESR1, PTEN	Cellular response, hormone receptor status	Doxorubicin	breast	Kovalchuk et al. (2008)
hsa-miR-23	I (miR-23a-5p)	PTEN	Cellular response	VP-16	breast	Liang et al. (2010)
hsa-miR-27	I (miR-27a-5p) I (miR-27b-5p) III (miR-27a-3p)	CYPB1	Defense	Doxorubicin; taxol	breast	Kovalchuk et al. (2008);Zhou et al. (2010)
hsa-miR-29	I (miR-29b-1-5p) III (miR-29a-3p)	CDCD42, CDK6, ITGA11, ADAM12, ADEAMTS9	Cellular response EMT, epigenetic	Doxorubicin; taxol; cisplatin; VP-16	breast	Kovalchuk et al. (2008); Zhou et al. (2010); Liang et al. (2010); Pogribny et al. (2010)
hsa-miR-30	II (miR-30c-2-3p) V (miR-30b-3p)	CTGF	EMT	Docetaxel; taxol	breast	Zhou et al. (2010); Kastl etal. (2012)
hsa-miR-32	III (miR-32-3p)	PCAF	Hormone receptor status	Doxorubicin+verapamil	breast	Chen et al. (2010)
hsa-miR-34	IV (miR-34b-5p)	E2F3, MET, CDK4, CDK6, BCL2, BIRC3, CCDN1, CCNE	Cellular response, EMT	doxorubicin; doxorubicin+verapamil; docetaxel	breast	Kovalchuk et al (2008); Chen et al (2010); Kastl et al (2012)
hsa-miR-92	II (miR-92a-1-5p)				lung	Ranade et al (2010)
hsa-miR-93	III (miR-93-5p)	FUS1, E2F1	Cellular response	Doxorubicin	breast	Kovalchuk et al (2008)
hsa-miR-106	III (miR-106b-3p)	E2F1, BRMS1, RB, P21, AIB1, EP300	CelLular response, EMT, hormone receptor status	Doxorubicin; taxol	breast	Kovalchuk et al. (2008); Zhao et al.(2008); Zhou et al. (2014)
hsa-miR-107	IV (miR-107)	CDK6	Cellular response	Doxorubicin	breast	Kovalchuk et al. (2008)
hsa-miR-125	II (miR-125b-1-3p) III (miR-125b-5p)	CYP24, HER2/3, BAK1, P53, E2F3	Defense, cellular response	Doxorubicin; taxol; docetaxel; paclitaxel; 5-FU	breast	Kastl et al.(2012); Kovalchuk et al. (2008); Zhou et al.(2010); Wang et al.(2012)
hsa-miR-127	III (miR-127-3p)	BCL6	Cellular response	Doxorubicin; cisplatin	breast	Kovalchuk et al.(2008) Pogribny et al.(2010); Saito et al.(2006)
hsa-miR-132	III (miR-132-3p)	MMP9, MECP2	EMT, epigenetic	Doxorubicin; cisplatin	breast	Kovalchuk et al. (2008); Pogribny et al. (2010)
hsa-miR-140	V (miR-140-5p)	HDAC4	Epigenetic	Doxorubicin;taxol	breast	Kovalchuk et al.(2008); Zhao et al. (2008)
hsa-miR-145	III(miR-145-3p)	ESR1, IGFR, MUC1, SOX2, IRS1, RTNK	Cellular response, EMT	Doxorubicin+verapamil	breast	Chen et al.(2010)
hsa-miR-146	III (miR-146a-5p) IV (miR-146b-5p)	BRCA1	Cellular response	Doxorubicin+verapamil; taxol; cisplatin	breast	Pogribny et al. (2010); Chen et al. (2010); Zhou et al. (2010)
hsa-miR-152	II (miR-152)	DNMT1	Epigenetics	Doxorubicin	breast	Kovachuk et al. (2008)
hsa-miR-181	II (miR-181a-3p) IV (miR-181a-2-3p)	BCL2, TIMP3, HOXA11, PCAF, ATM, SIRT1, TGFβ, WNT, MAPK, VEGF	Cellular response, EMT, epigenetics	Doxorubicin; doxorubicin+verapamil; fuvestrant	breast	Kovachuk et al. (2008); Chen et al.(2010); Xin et al. (2009); Pogribny et al. (2010); Zhu et al.(2013)
hsa-miR-182	IV (miR-182-5p)	BRCA1	Cellular response	Doxorubicin	breast	Kovalchuk et al. (2008)
hsa-miR-183	II (miR-183-5p)	EZRIN			osteosarcoma	Zhu et al. (2012)
hsa-miR-192	IV (miR-192-5p)					
hsa-miR-194	II(miR-194-1)	RAC1, SOCS2, CDH2, MDM2, ANXA1, DNMT3A, MECP2	Cellular response,EMT, epigenetics	Doxorubicin; cisplatin	Breast	Kovachuk et al. (2008); Pogribny et al. (2010)
hsa-miR-195	III (miR-195-5p)	GLUT3			Bladder	Fei et al. (2012)
hsa-miR-200	II (miR-200c-3p) III(miR-200c-5p) V (miR-200b)	MSN, ZEB1, ZEB2, WASF3, CTNNB1, NTRK2	Cellular response; EMT	Doxorubicin; cisplatin; doxorubicin+verapamil; paclitaxel; vincristine	breast	Kovachuk et al. (2008); Chen et al.(2010); Pogribny et al. (2010); Cochrane et al. (2009)
hsa-miR-205	III (miR-205-5p)	E2F1, PTEN, HER3, VEGFA, ZEB1, ZEB2	Cellular response, EMT, hormone receptor status	Doxorubicin;cisplatin;lapatinib; gefitinib	breast	Kovalchuk et al.(2008); Pogribny et al. (2010); Iorio et al.(2009)
hsa-miR-212	V (miR-212)	MMP9, MECP2, PED	Cellular response, epigenetic , hormone receptor status	Doxorubicin;fulvestrant	breast	Kovalchuk et al. (2008); Xin et al. (2009)
hsa-miR-215	V (miR-215)	DHFR/TS		Methotrexate	colorectal	Song et al. (2010)
hsa-miR-222	II (miR-222-5p) III (miR-222-3p)	ESR1, FOXO3, P27, PTEN, P57, BMF, TRPS1, PUMA, DICER	Cellular response, EMT, hormone receptor status	Doxorubicin; cisplatin; doxorubicin+verapamil; fuvestrant; tamoxifen		Kovalchuk et al. (2008); Pogribny et al. (2010); Chen et al. (2010); Miller et al. (2008); Rao et al. (2011); Xin et al. (2009); Zhao et al. (2008)
hsa-miR-424	I (miR-424-3p)	N/A		Erlotinib; vandetinib	lung	Weiss et al. (2010)
hsa-miR-449	II (miR-449b-3p) II (miR-449b-5p) II (miR-449-3p) II (miR-449a)			tamoxifen	breast	Lau et al. (2010)

*Specific miRNA in each group.

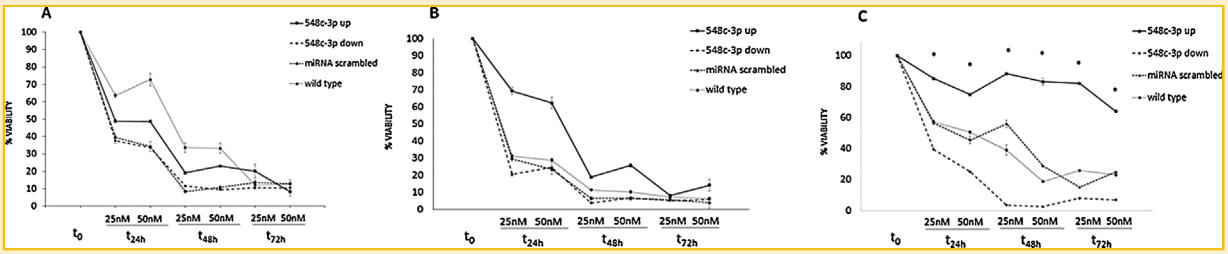

Fig. 5. Modulation of doxorubicin-treated cell viability by miR-548c-3p. MDA-MB-231 (A) MDA-MB-468. (B) and MCF7 (C) cell lines were transfected with miRNA mimic (548c-3p up) or inhibitor (548c-3p down) at different concentrations, and then treated with 5 μM doxorubicin for different amounts of time. MiRNA scramble was used as a negative transfection control. Results are represented as the average of three independent measurements, and statistical analysis of miR-548c-3p up versus miRNA scrambled is shown (*P-value < 0.05).

et al., 2012]. With regard to our results, upregulation of this miRNA in MDA-MB-468 cells could lead to lower invasiveness, while downregulation in MDA-MB-231 cells could reinforce their ability to invade and metastasize. We also focused on miRNAs in this group whose expression level was different between the two cell lines. For example, miR-92a-1-5p was more downregulated in MDA-MB-468 than in MDA-MB-231 cells; it belongs to the 17-92 "oncomiR" cluster known to promote tumorigenesis by antagonizing tumor-suppressing mechanisms, apoptosis, and oncogene-induced senescence through the activities of the different miRNAs it encodes [Hong et al., 2010].

In contrast miR-21-3p, miR-222-5p, and miR-181a-3p were more downregulated in MDA-MB-231 than in MDA-MB-468 cells. MiR-21 has been described to play an important role in breast cancer resistance to chemotherapy [Mei et al., 2010]. In addition, miR-222 has been related to breast cancer cell drug-resistance by targeting PTEN [Zhong et al., 2013] and miR-181a-3p belongs to the 181a family of tumor suppressor miRNAs which has been associated with enhanced drug sensitivity in breast cancer cells by targeting the Breast cancer resistance protein [BCRP] [Jiao et al., 2013]. Fifty-six percent of the miRNAs which were significantly modified by doxorubicin in the MDA-MB-468 and MDA-MB-231 group had been previously related to chemotherapy resistance in breast cancer (Table III). For example, miR-200 upregulation has been described in several cancers and is implicated in promoting cell growth and epithelial-mesenchymal transition [Park et al., 2008]. Our results show its upregulation in doxorubicin-treated cells, suggesting that it has a role in resistance. Moreover, the FOG2 protein, which inhibits PI3K activity and suppresses cell growth, has been validated as one of its targets [Hyun et al., 2009].

In group III (MCF-7) miR-548c-3p and miR-1305 were the most strongly involved in the modification of cancer genes and pathways: both were upregulated in doxorubicin-treated MCF-7cells. Our results show that overexpression of miR-548c-3p increases the viability of doxorubicin-treated MCF-7 cells, while its down-regulation reduces cell viability. This suggests a specific role for miR-548c-3p in doxorubicin resistance in MCF-7 cells which was not observed in the other two cell lines assayed. A possible mechanism of action is a miR-548c-3p-mediated decrease in DNA damage after chemotherapy treatment that consequently increases drug resistance, thus determining its effectiveness [Srikantan et al., 2011].

MiR-335-3p was significantly upregulated in MDA-MB-231 cells (Group IV). Interestingly, its non-carrier strand, miR-335-5p suppresses metastasis and migration by targeting the progenitor cell transcription factor SOX4 and extracellular matrix component tenascin C, however the role of miR-335-3p remains unknown [Tavazoie et al., 2008]. In Group V the expression of miR-3163 was significantly decreased in MDA-MB-468 cells after doxorubicin treatment; interestingly its expression is also downregulated in colorectal cancer [Hamfjord et al., 2012] but there are no data available for breast cancer.

Additionally, evaluation of miRNA expression in the normal MCF-10A breast cell line, both with and without doxorubicin treatment, showed results in contrast to the observations in the breast cancer cell lines. It supports the idea that the changes observed in response to treatment are specific to tumor cells and do not occur in normal breast cells. In another step of validation/confirmation of our results, search carried out in available array data public repositories regarding to the analysis of miRNAs in primary human breast cancer samples (not-treated) versus normal human breast tissue revealed significant changes in several miRNAs. Comparing these results with those obtained in tumor cell lines, we noticed that some miRNAs (miR-424*, miR-1207-5p, miR1275, and miR-200c) showed coincidence of expression trend in both primary tumor and doxorubicin treated tumor cells (Supplementary Table 1 and Figure 4). These data are closer to resistance than to a favorable response to treatment, since it is supposed that doxorubicin treatment would reverse the expression of tumor associated miRNAs.

One of the most surprising findings of our study was the high percentage of star miRNAs that were significantly changed after doxorubicin treatment in all groups (data shown above). MiRNAs*, or "passenger" or "carrier strands", had been thought to be biologically inactive, however mature functional miRNAs* have recently been reported, and therefore miRNA/miRNA* ratios may dramatically vary at different developmental and/or disease stages [Lai et al., 2003; Kuchenbauer et al., 2011]. Several studies have described important roles for miRNAs* [Yang et al., 2011] in response to treatments such as metformin [Avci et al., 2013], berberine [Lo et al., 2013], and 5-fluorouracil [Shah et al., 2011] . This suggests that miRNA* expression is a generalized response to anti-cancer treatments, which might represent possible new (and so far unexplored) drug-response and/or resistance generation

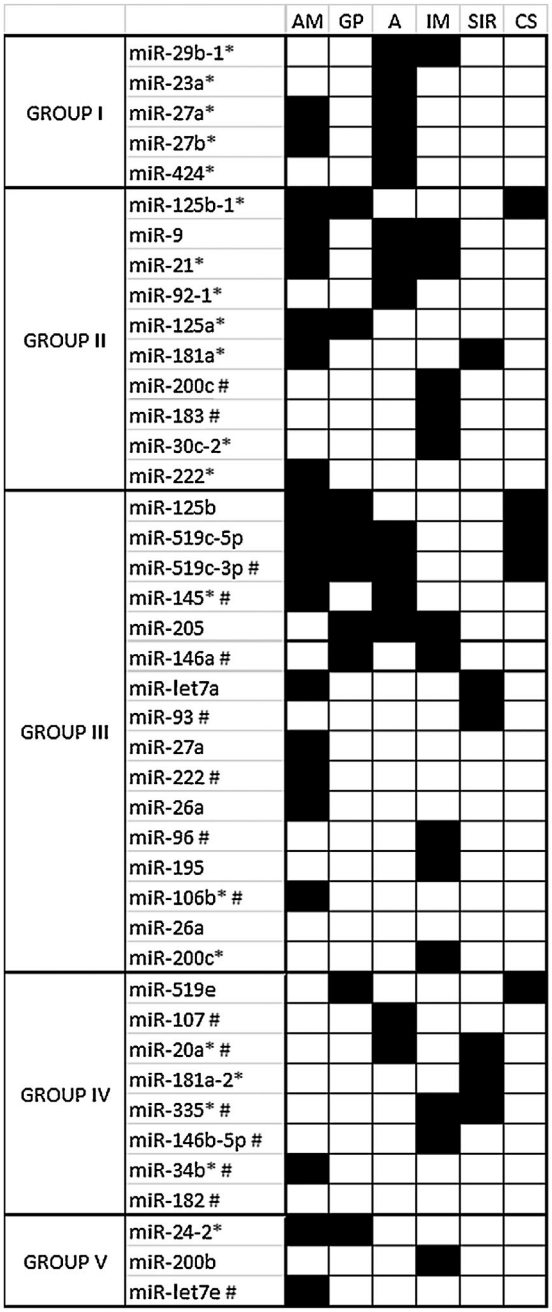

Fig. 6. Representation of five major clustered groups of analyzed miRNAs, and their relationship with several processes connected with cancer. AM: ADME (drug absorption, distribution, metabolisms and excretion), GP: Growth and proliferation, A: Angiogenesis, IM: invasion and metastasis, SIR: suppression of immune recognition, CS: cell survival. # upregulated miRNAs. Group I (MDA-MB-231, MDA-MB-468, and MCF-7); Group II (MDA-MB-231 and MDA-MB-468); Group III (MCF-7); Group IV (MDA-MB-231); Group V (MDA-MB-468).

mechanisms. Therefore it is important to examine the role of miRNAs* in greater depth in future studies.

In conclusion, our results suggest an important role for miRNAs in gene transcriptional regulation after doxorubicin treatment in breast cancer. A high number of the miRNAs that were differentially expressed after treatment were implicated in the modulation of genes and pathways relevant in cancer. Furthermore, an elevated percentage (23% of the total) of miRNAs with altered expression after doxorubicin treatment had already been related to chemotherapy resistance in the literature. This data supports the idea that one of the consequences of doxorubicin treatment is the generation of resistance to therapy, which is exemplified by the implication of miR-548c-3p in MCF-7 cell viability after doxorubicin treatment. Therefore, further investigation including functional and mechanistic studies must be undertaken in order to address the biological implications of these miRNAs, and to elucidate their involvement in cancer-related pathways and in treatment response and resistance

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