JBUON 2018; 23(3): 692-705
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

A miRNAs profile evolution of triple negative breast cancer cells in the presence of a possible adjuvant therapy and senescence inducer

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Summary

Purpose: *Breast cancer is a highly heterogeneous disease with an increasing number of cases resistant to chemotherapy. To increase the response to therapy, different adjuvant systems are tested, like the case of deuterium-depleted water (DDW).*

Methods: *For this study, we selected as in vitro model the triple-negative breast cancer cell line MDA-MB-231 and we performed a series of microscopy-based functional tests (apoptosis, autophagy assays, senescence detection) and microarray evaluation of the miRNA profile in order to evaluate changes induced by cisplatin and DDW treatment at cellular and molecular level.*

Results: *Cisplatin treatment led to increased mitochondrial activity and autophagy for cells kept in DDW, compared with those in standard conditions (SC). We also observed that cells treated with DDW medium promoted senescence in a higher level than SC. The exosomal miRNAs released in cell culture revealed an altered pattern in the case of cells maintained in DDW compared to SC.*

Conclusion: *DDW was proved to be non-toxic, and, when administered with cisplatin, to slightly increase the senescence of cancer cells, therefore, can be pondered as adjuvant therapeutic agent. However, future studies are needed to be done in order to further elucidate its mechanism of action.*

Key words: *adjuvant therapy, miRNAs profiling, senescence, triple negative breast cancer*

Introduction

According to the latest data, cancer has surpassed hearth diseases and represents the main cause of death worldwide [1]. One of the deadliest cancer types is breast cancer, and particularly triple-negative breast cancer, which is a highly heterogeneous disease and resistant to chemotherapy in many cases. This situation creates an urgent need of developing new strategies to defeat this old enemy, as it was presented by Workman et al. in 2002 [2].

Cisplatin is one of the most common chemotherapeutic agents used for breast cancer management. It has been related to the activation of apoptosis-associated mechanisms and oxidant effects, after preclinical, as well as clinical studies. Generally speaking, this drug is used in combina-

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Received: 19/11/2017; Accepted: 21/12/2017

tion with other chemotherapeutics or adjuvants in order to enhance its antitumor effects, especially by decreasing the chemoresistance of cancer cells [3], or by delaying tumor progression [4], but also to limit toxicity. The latest literature data presents DDW as a novel part of complementary therapy, used for cancer prevention and treatment [5].

Natural water contains approximately 150 ppm deuterium. A concentration of deuterium less than 140 ppm is considered deuterium-depleted. In Qlarivia bottled water, which we used for conducting the experiments presented in this article, the concentration of deuterium usually is between 20-30ppm [6,7]. A number of studies indicate that reduction in deuterium content might have a significant influence on multiple health parameters. Decreased concentration of deuterium in the organism is inducible after long-term consumption of DDW. DDW has been used as part of adjuvant therapy in some cancer types such as lung, prostate and breast cancer on a very limited number of patients, and the results showed improvement of response to therapy and increase of the patient survival [8-10].

Dietary supplementation with DDW is considered having no negative side effects. DDW can accompany the conventional treatment, or be administered after the chemotherapy is completed [11,12]. A retrospective study related to the survival in breast cancer patients who supplemented their treatment with DDW, revealed its potential application as adjuvant to conventional therapies [12]. Somlay et al. showed in their studies that the intake of DDW might have the capacity to affect cell proliferation and to modulate some cancer-related genes [10,13]. Another study revealed that the deuterium depletion is correlated with a reduction in the expression level of *C-myc, HRAS* and *p53* genes in carcinogen-treated mice [14]. This was confirmed in a publication related to the role of DDW as adjuvant therapy in four patients with brain metastases from lung cancer [8], but there is no publication on the impact DDW has on the transcriptomic profile, and nothing is known about the microRNA (miRNA) profile. MDA-MB-231 is a breast cancer cell line with high metastatic potential [15], characterized by the absence of HER and of the receptors for progesterone and estrogen; this cell line is regarded as the prototype for triple-negative breast cancer, the subtype with the worse response to therapy [16].

miRNAs are endogenous non-coding RNAs of 19–25 nucleotides in length [17] that are expelled from the cells during cell to cell communication. MiRNAs are negative regulators of the messenger RNA, and have been studied intensively over the last 15 years as prognostic and diagnostic markers, but also as therapeutic targets [18-21]. In order to gain a complete view over the beneficial effects of a therapy, it is necessary to understand its capacity to modulate the miRNAs found in the cytosol or released by the cell and thus possibly preventing drug resistance in cancer [19,21-23].

These short transcripts regulate physiological and pathological states, due to the fact that they are not only present in the cytosol, but also in the extracellular environment, including cell culture media. In our study, we focused on the quantification of the miRNA pattern released from the exosomal fraction, with direct impact on the cellular fate, by using the microarray technology, in correlation with basic functional tests performed for assessing the effects of DDW as a possible modulator of adjuvant therapy on MDA-MB-231 cells.

Methods

Cell culture

For the current experiment, the human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The first lot of the cells were maintained in standard growth conditions (SC), more precisely RPMI Medium 1640 (Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS) and the second experimental lot of the cells was grown in RPMI Medium 1640 (Sigma-Aldrich, Germany) prepared with DDW, as described by Mladin et al. in 2014 [7], and supplemented with 10% FBS. The culture medium was prepared in sterile conditions under a laminar flow hood and filtered through a 0.22 μm pore filter for the two experimental variants. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The cultured cells were at passage 20. Passages from 20 to 24 were used to perform the experiments. The therapeutic agent (cisplatin) used in our experiment was purchased from Actavis.

Apoptosis assay

Loss of mitochondrial transmembrane potential represents a key event in apoptosis, alongside several changes in nuclei morphology, depending on the apoptotic phases. For the microscopy evaluation of apoptosis, the Multi-Parameter Apoptosis Kit (Cayman Chemical, Michigan, USA) was used according to the manufacturer's protocol. After the staining protocol, the cells were analyzed at UV wavelength for Hoechst and at 560/595 nm for TMRE staining using an Olympus IX71 inverted microscope. Hoechst is a specific stain for nuclei morphology, while TMRE (tertamethylfhodamine ethyl ester) is a lipophilic rhodamine probe used to measure mitochondrial potential in living cells. MDA-MB-231 cell line apoptosis was evaluated at 24 hrs of exposure to cisplatin at IC_{50} value, which was determined at 24 hrs (35 μM in the case of SC and 76.58μM for the case of DDW).

Autophagy assay

Autophagy was assessed on the MDA-MB-231 cell line treated with similar cytotoxic concentrations as the ones used for the apoptosis test. The evaluation of autophagy was performed with the Autophagy/Cytotoxicity Dual Staining Kit (Cayman Chemical, Michigan, USA), according to the recommended protocol. Monodansylcadaverine (MDC) staining was used for the staining of autophagy vacuoles, and propidium iodide (PI) for the staining of DNA changes during cell death. After 24 hrs of cisplatin treatment, the cells were visualized with an Olympus IX71 inverted microscope.

Senescence detection

The detection of senescent cells was done using the Senescence Detection Kit (Abcam, San Francisco, USA), according to the manufacturer's instructions, for the cells kept in standard conditions versus those maintained in medium prepared using DDW. The kit detects β-galactosidase (SA-β-GAL), which is overexpressed in senescent cells and can be visualized under a microscope as small, dark dots. The cells expressing SA-β-GAL present a blue color staining. Cells were visualized using Olympus IX71 microscope.

Total RNA extraction from exosomal released medium

The total exosomal RNA was obtained by precipitating 4 ml of cell culture medium and using the commercial kit from Life Technology (Total Exosome Isolation Reagent for cell culture media, cat no. 4478359) according to the producer's recommendations. The exosome pellet was resuspended in the lysis solution provided by the kit, which is specially designed for low amounts of genetic material, and the RNA was extracted (Plasma/Serum Circulating and Exosomal RNA Purification Kit-Slurry Format, Norgene cat.no. 42800). The concentration of nucleic acids was assessed by using the ND-2000 spectrophotometer. Exosomal RNAs were extracted both from normal condition medium cultured cells and also from DDW prepared medium cultured cells.

Microarray evaluation

The labeling of total RNA samples was done by using the Complete Labeling and Hyb Kit (Agilent Santa Clara, USA, cat. no. 5190-0456) with 100 ng RNA from the samples, followed by purification with MicroBio-Spin 6 Columns (Bio-Rad, California, USA, cat. no. 732-6221). The final step of the assay was the hybridization on Human miRNA Microarray Kit Release 21.0 format 8x60K (code G4870C/AMADID: 070156) slides. The arrays were scanned with the Agilent Microarray Scanner G2565BA to acquire fluorescent images of each array, and analyzed with the use of Feature Extraction version 11.0 software.

Data analysis and identification of differentially expressed exosomal miRNAs released in cell culture media

The samples from cells kept in DDW and SC were grouped based on their duplicate status, and the data were used for differential expression analysis of miRNAs using the GeneSpring GX software, version 12.6.1. For the data analysis, we used the "Filter on volcano plot" module, with a fold change threshold of 1.5 and a p value ≤0.05. The classification of the biological processes affected by the altered miRNA pattern in the case of DDW was performed by using the NCBI Database (www.ncbi.nlm.nih.gov) and Ingenuity pathway analysis (IPA).

Breast cancer TCGA analysis

The results of the applied treatment and the effects on the expression of microRNAs were further investigated by using the data collected from The Cancer Genome Atlas (TCGA). TCGA data was downloaded from the University of California Santa Cruz (UCSC) website, and differential analysis on microRNA expression on patients with breast cancer was conducted. The miRNA expression data for breast cancer patients consists of 1090 tumor tissues and 104 normal tissues from TCGA data portal. Differential expression analysis was performed using the GeneSpring GX software (Agilent Technologies, Santa Clara, California, USA), setting a p value <0.05 and a fold change ≥1.5, applying the moderated *t test* with FDR (False Discovery Rate) correction for false positives. The list comprised the most up- and down-regulated microRNAs, generated after the comparison between tumor and normal tissue, and was used to test its similarity with the data generated in the microarray analysis.

Results

DDW showed a weak synergistic effect on mitochondrial activity in the case of cisplatin treatment of MDA-MB-231 cells

Evaluation of DDW supplementation in cisplatin-treated cells was assessed by using the fluorescence imaging of the apoptotic processes in MDA-MB-231 cells after 24 hours of treatment. No significant difference on cell viability could be observed between cell maintained in SC or DDW in terms of mitochondrial membrane potential activity and nuclei integrity (Figure 1). We assume that these changes at nuclear and mitochondrial level were possibly triggered by a drop in the mitochondrial membrane potential appearing 24 hrs after treatment with cisplatin in SC or cell culture media prepared with DDW, with a more pronounced condensation of the nuclei in cells cultured in DDW compared to cells grown in SC.

DDW showed a weak synergistic effect on autophagy activation in the case of cisplatin treatment of MDA-MB-231 cells

MDC/PI double stained MDA-MB-231 cisplatin-treated cells showed an increase in autophagy. In Figure 2, a difference can be observed between cells cultured in SC and DDW. The DDW caused an increase in the number of autophagy and ne-

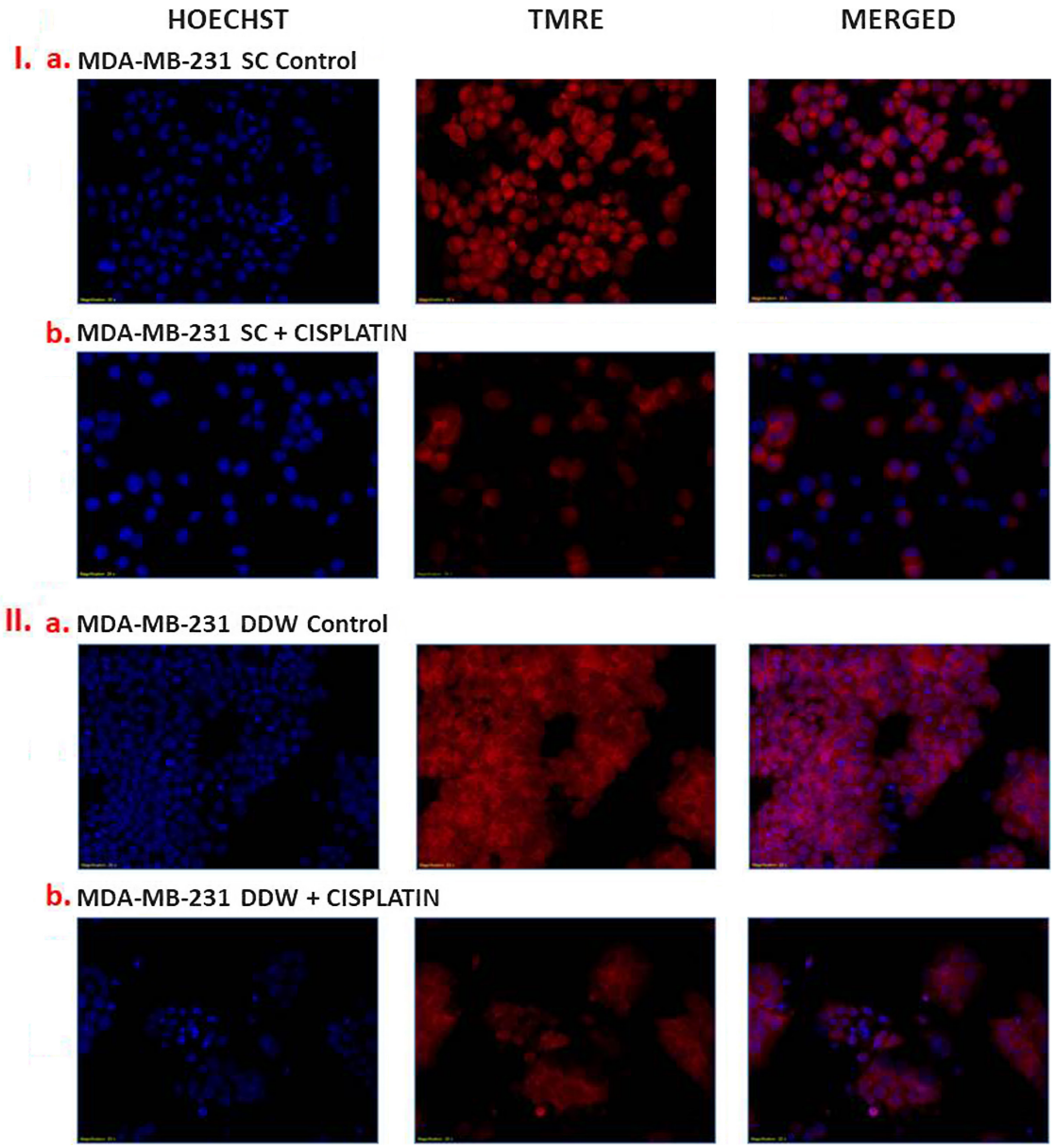


Figure 1. Evaluation of apoptosis by TMRE and Hoechst staining. **Ia/IIa:** MDA-MB-231 cells cultured in RPMI-1640 with SC and DDW for 24h do not show differences in nuclei morphology and mitochondrial membrane activity. **Ib/IIb:** MDA-MB-231 cells cultured in RPMI-1640 in SC and DDW, and treated for 24h with cisplatin show a decreased mitochondrial membrane activity in both cases and highest nuclei disintegration could be observed in cells maintained in DDW and treated with cisplatin.

crotic cells when compared to the cells cultured in SC. These results suggest that cisplatin therapy supplemented with DDW induced autophagy and cytotoxicity on MDA-MB-231 cells.

DDW slightly promoted senescence in MDA-MB-231 breast cancer cell line

We investigated whether a low concentration of deuterium can promote senescence in cancer cells, which would lead to a decline in the proliferation capacity of cancer cells. Senescent cells exhibited a high expression of β-galactosidase, which is absent in pre-senescent cells and immortalized cell lines. After the MDA-MB-231 cells, found in the 22nd passage, were cultured in a medium with reduced concentration of deuterium, evaluation of senescence showed the highest β-galactosidase expression. This result suggested that DDW can induce senescence in MDA-MB-231 cells (Figure 3).

Evaluation of the altered miRNA expression profiles based on microarray analysis

After analyzing miRNA microarray data, a list of differentially expressed miRNAs was generated in the case of MDA-MB-231 cells kept in DDW compared to those kept in SC conditions. For mi-

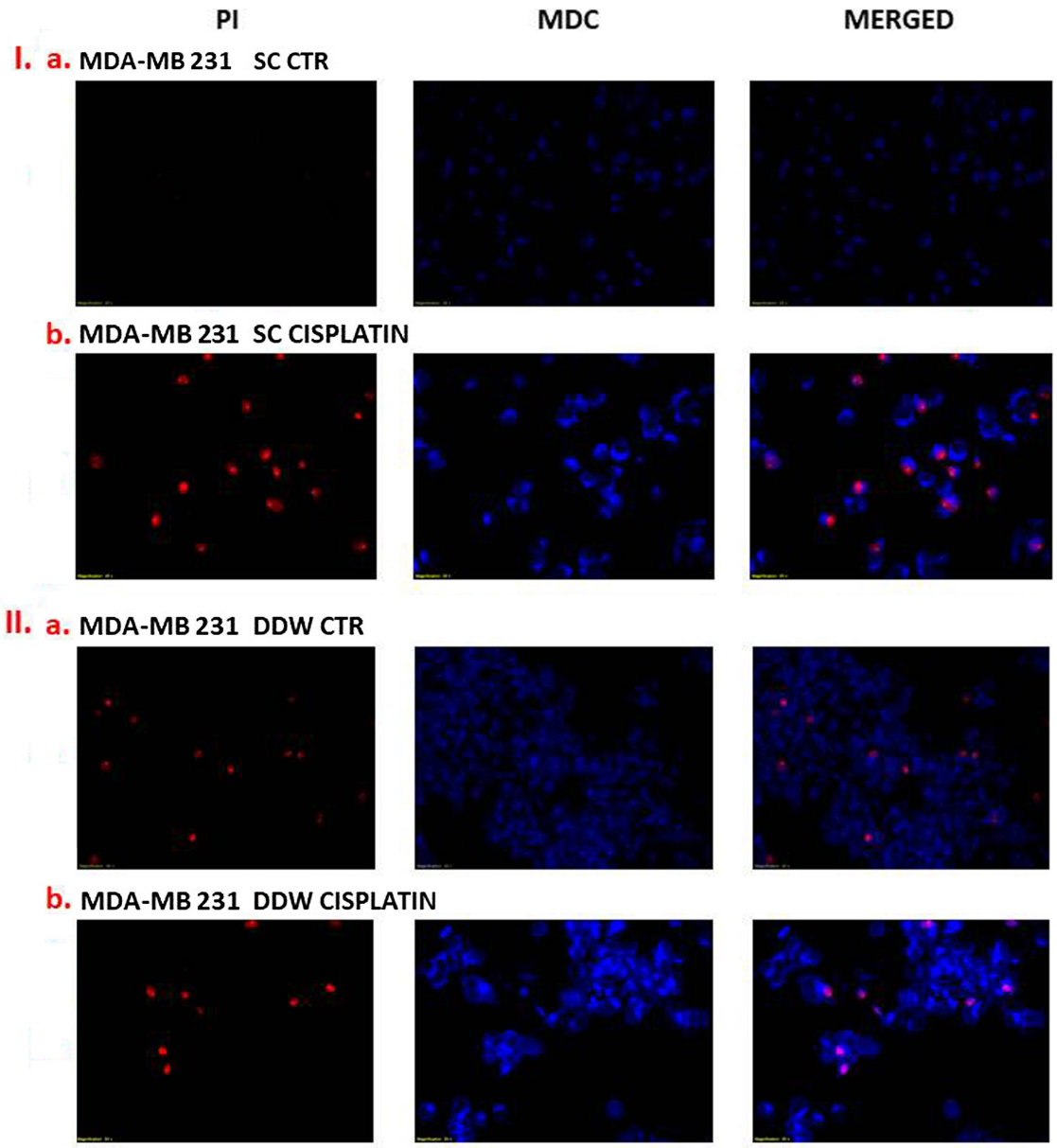


Figure 2. Autophagy/Cytotoxicity evaluation by MDC and PI staining. **Ia/IIb:** Highest autophagy can be observed in MDA-MB-231 cells cultured in medium with DDW than in cells cultured in medium with SC. Also a few number of necrotic cells (PI positive) was observed in cells cultured in medium with DDW. **Ib/IIb:** MDA-MB-231 cells cultured in RPMI-1640 with SC and DDW for 24h and treated with cisplatin show an increased autophagy, revealed by a strong MDC staining of the autophagy vacuoles, and PI positivity indicates a highest necrotic cell number in both cases.

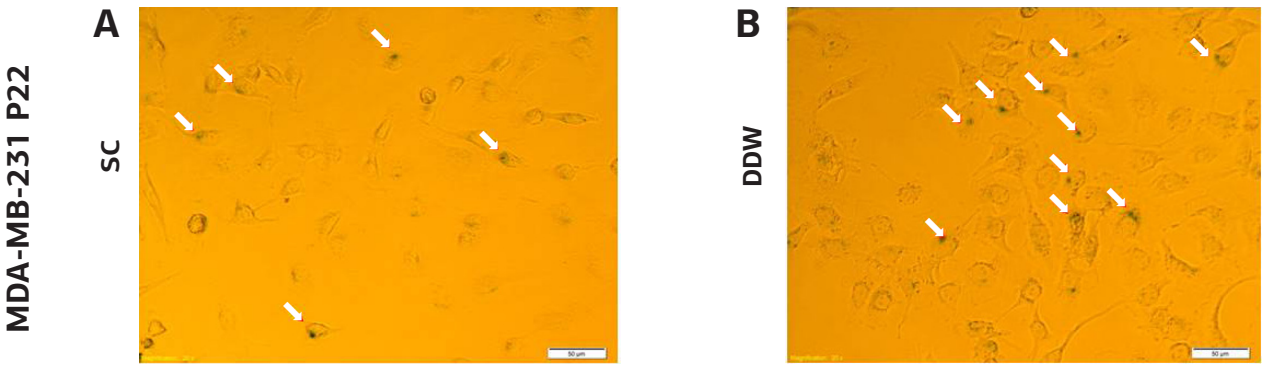


Figure 3. Senescent cells detection. **A:** MDA-MB-231 cells maintained in culture medium with SC present a lowest β-galactosidase after a 22 passages. **B:** MDA-MB-231 cells maintained in culture medium with DDW present a highest β-galactosidase expression. Positive cells for SA-β-gal activity (blue staining) are marked by arrows.

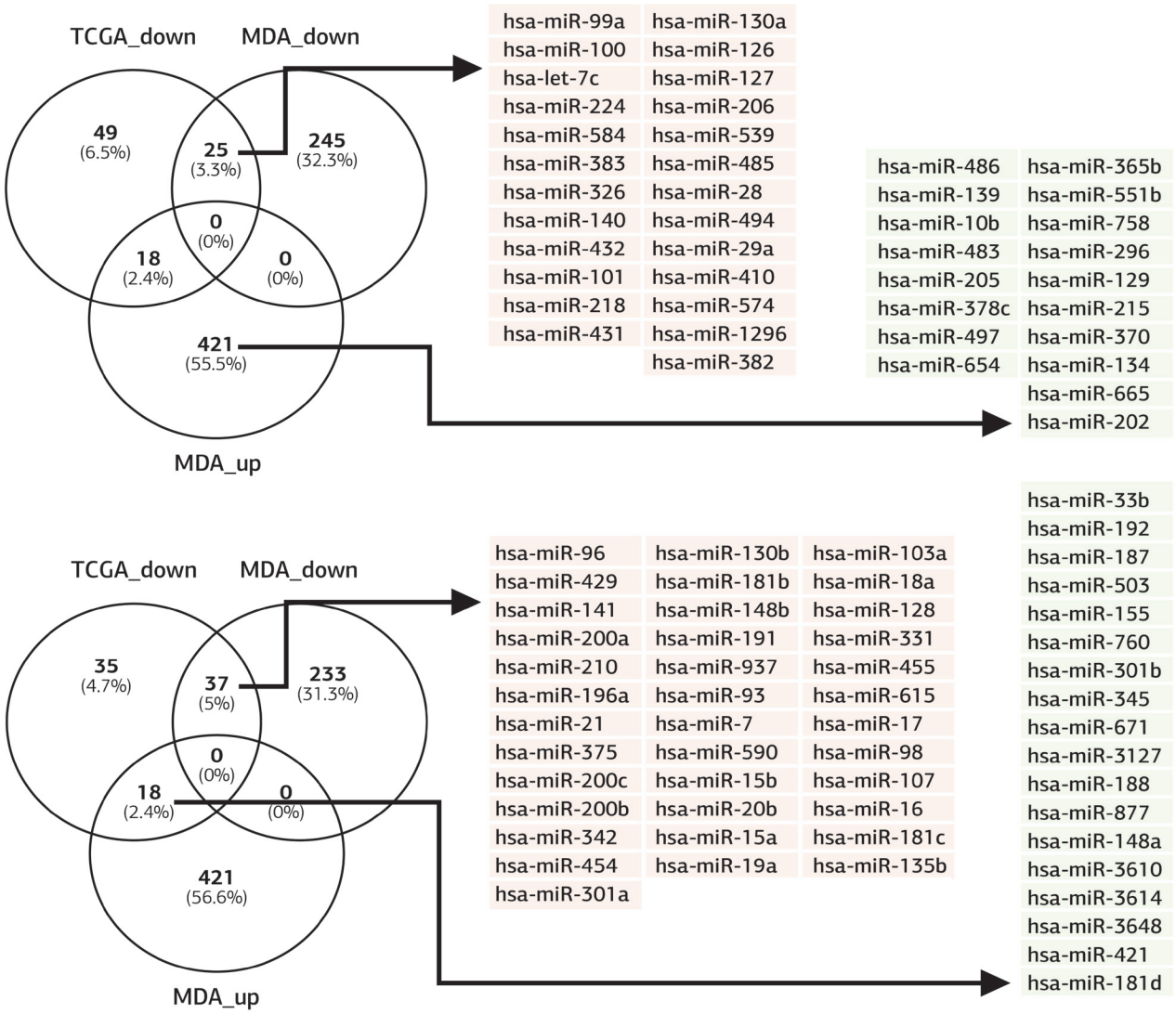


Figure 4. Venn diagram of overexpressed miRNAs from TGGA data overlapped with the cell culture data downregulated miRNAs from TGGA data overlapped with the cell culture data.

Table 1. The main disease and function targeted by the altered miRNAs for DDW maintained cells versus those maintained in SC

Name	*p value*	*Number of molecules*
Organism injury and abnormalities	4.92E-02 - 6.95E-59	215
Reproductive system diseases	1.76E-02 - 6.95E-59	133
Inflammatory diseases	3.69E-02 - 1.56E-53	97
Inflammatory responses	4.16E-02 - 1.56E-53	85
Renal and urological diseases	2.84E-02 - 1.56E-53	71

Table 2. The main molecular and cellular functions targeted by the altered miRNAs for DDW maintained cells versus those maintained in SC

Name	*p value*	*Number of molecules*
Cellular development	4.96E-02 - 1.81E-09	60
Cellular growth and proliferation	4.96E-02 - 1.81E-09	58
Cellular movement	3.76E-02 - 9.26E-07	39
Cell death and survival	4.27E-02 - 9.32E-05	35
Cell cycle	4.51E-02 - 1.10E-04	23

croarray analysis, a filter for corrected p value and fold change was set at p≤0.05 and a -1.5>fold change<+1.5 respectively.

When comparing the miRNA expression levels in the case of MDA-MB-231 cells maintained in DDW vs those maintained in SC, we identified a total number of 896 probes that belonged to transcripts that were differentially expressed and statistically significant. Out of these, 528 miRNAs were upregulated and 368 downregulated.

Construction of the extracellular miRNA-based networks by using IPA

Based on the altered miRNA profile, we assembled the IPA miRNA-based networks. We were able to generate four networks involved in key cellular and molecular processes that are highlighted between our two conditions (Tables 1 and 2). These altered transcripts caused dysregulation of numerous signaling pathways, with great implications for the response to therapy.

The altered miRNA pattern for cells maintained in DDW conditions versus those maintained in SC was introduced in IPA, in order to observe the interconnection with target genes to reveal the most relevant altered biological processes, as displayed in Figure S1 (A-C). Figure S1A presents the network where the *NFkB* is typical regulator of the inflammatory response, systemic injuries, and abnormalities; (B) is the second network, where *SMAD2/3, Akt* and *Rb* genes are highlighted and their involvement in systemic injuries and cancer is marked; the following network (C) is representative for cancer, systemic injuries and abnormalities, having as core gene the *Ago2*, interconnected with *TP63*, while the (D) network is representative for inflammatory disease or response, whose centerpieces are CCND1, SSB and TMER8B genes.

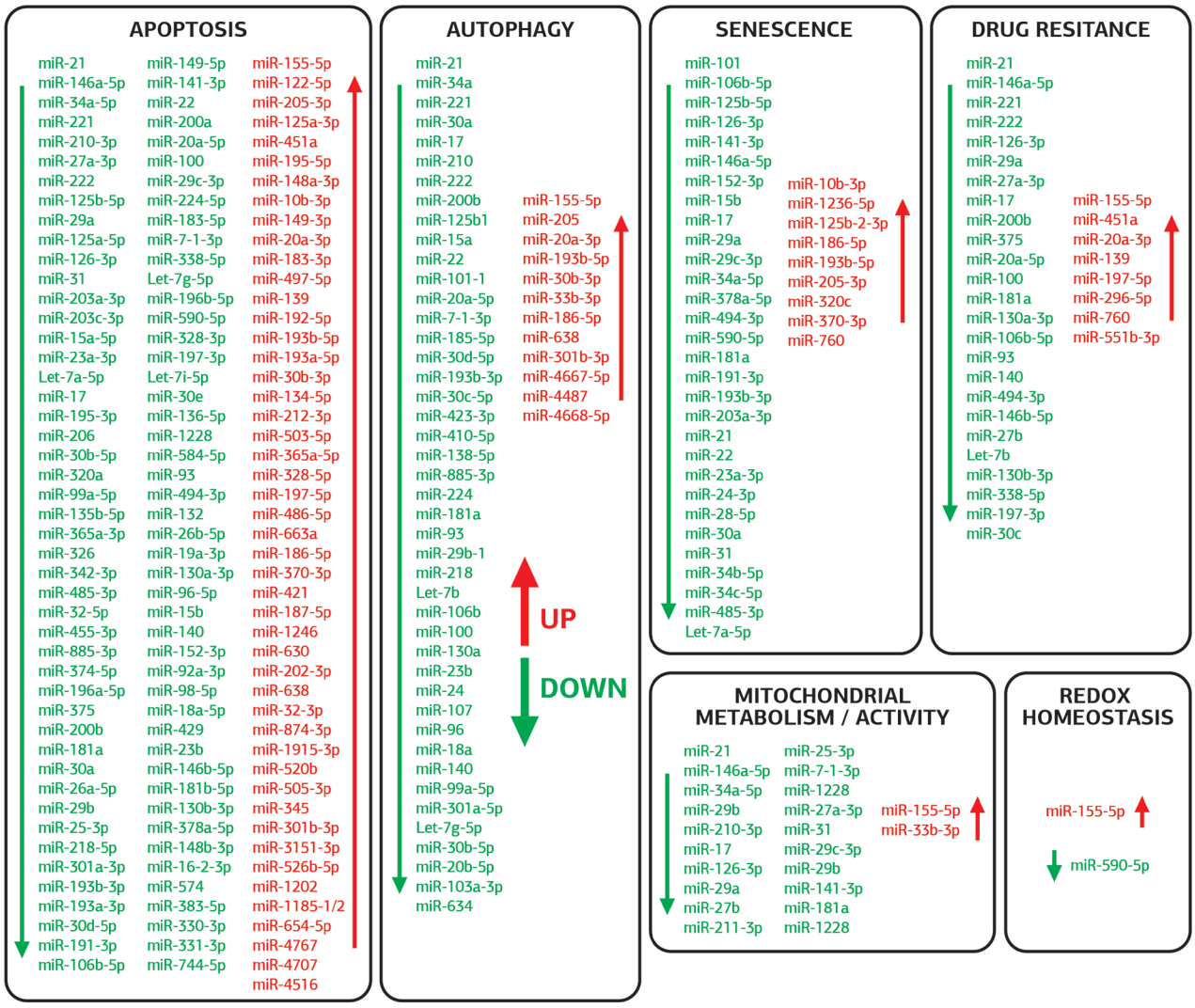


Figure 5. Classification of the altered biological processes, observing that most of the altered miRNAs in the case of cells cultured in medium prepared in DDW versus SC are related to apoptosis, autophagy, senescence, mitochondrial activity or those related to mitochondrial function, including redox homeostasis.

A summary of the most relevant biological processes affected by DDW, as was revealed by the microarray analysis, is shown in Figure 4, emphasizing the important role of miRNA-related key cellular processes.

Overlapping the altered miRNA pattern in MDA-MB-231 cells cultured in DDW medium with TGCA data

Differential expression analysis was also performed on the data generated by the one-color microarray assay performed on the MDA-MB-231 cell line maintained in SC, considered as control, and DDW.

Since the TCGA data matrices used for the differential expression analysis do not contain information regarding the -5p / -3p isoforms, the comparison between our results and the ones from the repository was challenging. As a result, an intermediate step was needed, in which the ambiguous isoforms of the microRNAs were eliminated, either because of having the same or opposite expression. In the end, 270 downregulated and 439 upregulated microRNAs from our microarray experiment were compared with the 92 downregulated and 90 upregulated microRNAs which were differentially expressed in the TCGA analysis. The results presenting the overlapping between the miRNA altered pattern in the case of DDW treatment and those obtained from TCGA data can be observed in Figure 5.

Discussion

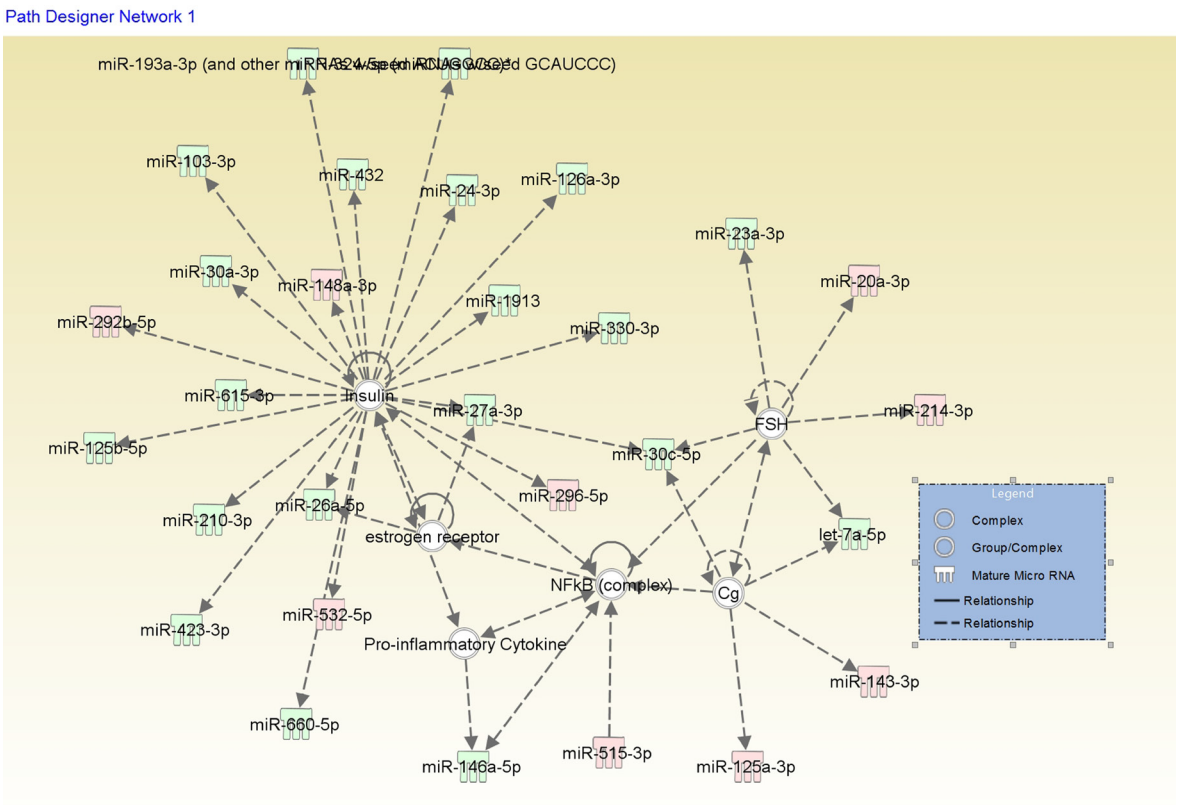
Due to its heterogeneity, most of the breast cancer related deaths occur because of therapeutic failure and resistance to standard therapies. Insuring a neoadjuvant approach through consumption of DDW determines a modification in miRNA profile that is also reflected on the pattern of gene and miRNA expression. In our situation, the exosomal released miRNAs are connected with cellular apoptosis, autophagy, mitochondrial activity and senescence (Figure 5), these being sustained by microscopy evaluation (Figures 1-3).

A large panel of altered miRNAs was related to cell proliferation. There are studies focused on evaluating the antiproliferative effects of DDW [5,24]. In lung cancer cells, it was demonstrated to cause alterations of cell cycle and modifications of the cellular skeleton, by observing the cells through transmission electron microscopy [24]. DDW was an effective inhibitor of nasopharyngeal carcinoma, demonstrated through proliferation, colony formation, migration and invasion assays [25,26]. Researchers in the field demonstrated that tumor cells undergo inhibition of cell proliferation in the presence of DDW, while the opposite effect was obtained in normal cells. More precisely, the cell proliferation rate is much higher in DDW-treated splenocytes than in SC-cultured splenocytes [27]. Another study demonstrated that DDW increases immune defense and stimulates the proliferation of peripheral blood cells, hence having an important role in radioprotection [28]. On an animal model, *C. elegans*, it was demonstrated that DDW had antiaging effects, along with the capacity to protect against the toxic effects of manganese [29].

Mitochondria are key components of various cellular functions, including growth, division, energy metabolism and apoptosis, and they are directly related with cancer response to therapy [16]. Fluorescence microscopy allows the assembly of structural and functional data, and can be used for qualitative and/or quantitative fluorescent staining for monitoring the effects of different treatments scenarios [30]. In our study, we observed a slight activation of mitochondrial activity in the case of cells kept in DDW compared to those grown in SC. A similar study focused on the evaluation of the nuclei morphology and mitochondrial membrane (TMRE/Hoechst staining) revealed a low capacity of DDW to induce cell death through apoptosis. Another study showed that DDW has the capacity to induce apoptosis as measured by TUNEL assay and an increased percentage of DNA fragmentation [24]; in our data, we observed a slightly increased percentage of fragmented nuclei for the cisplatin-treated cells kept in DDW as opposed to the case of SC.

Autophagy is a biological process with an important role in cell homeostasis that implies a selective degradation of cellular components, such as damaged organelle and protein aggregates. Autophagy is induced under physiological stress conditions, such as hypoxia and nutrient deprivation, and by the lysosomal degradation of the intracellular components it ensures an increase in the energetic status of the cell in order to survive starvation [31]. Excessive autophagy induced by some therapeutic agents can have an apoptotic effect by accumulation of autophagosomes, leading to cell death [32], based on the fact that the mitochondria of the selected cell line displayed increased autophagic properties demonstrated in a previous study [16]. There are no studies focusing on the evaluation of autophagy after DDW application on cancer cells. In the current study, it was demonstrated that while cisplatin causes indeed induction of autophagy in the MDA-MB-231 cells,

A



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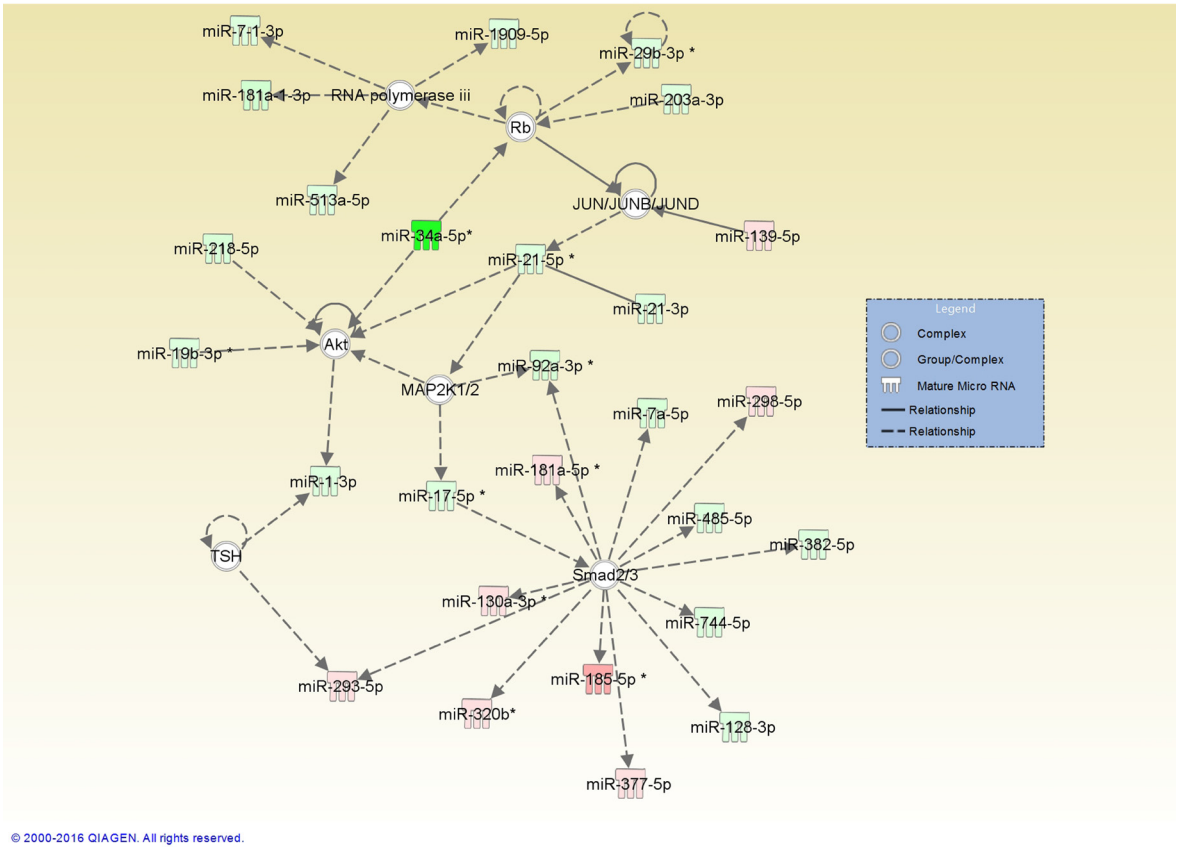

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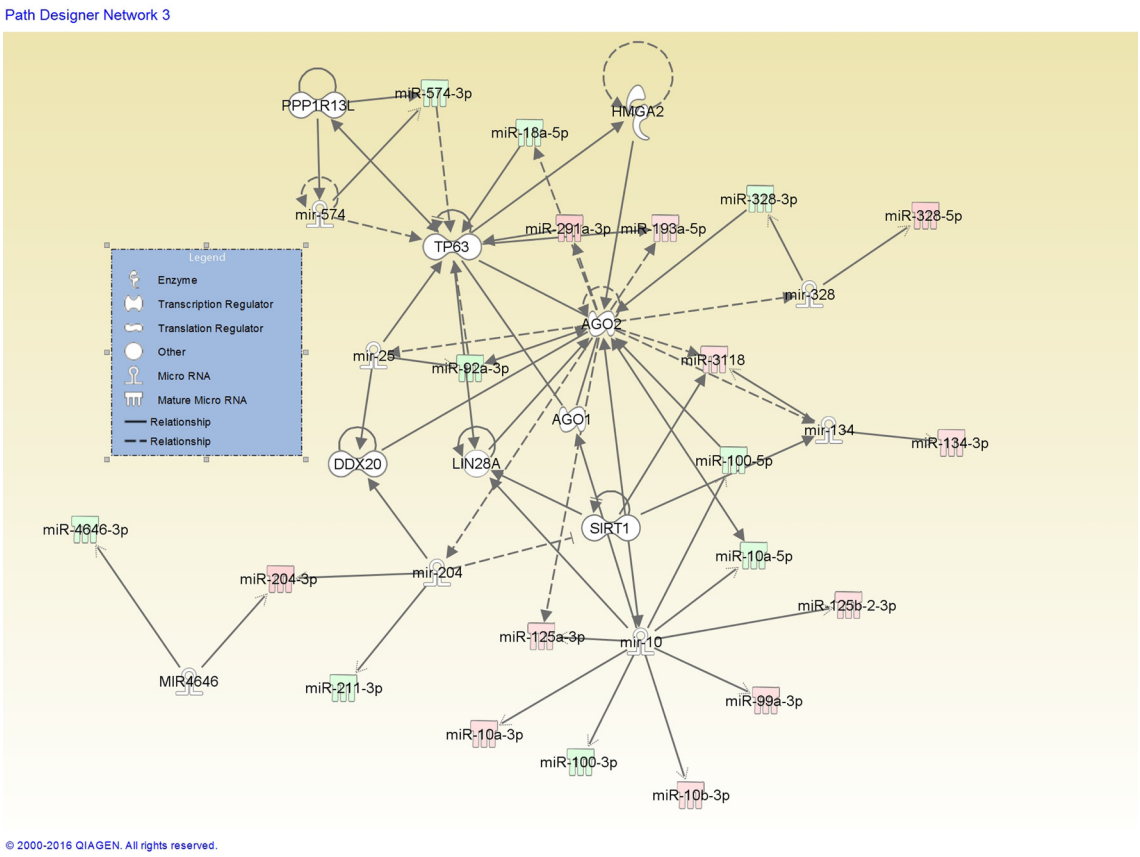

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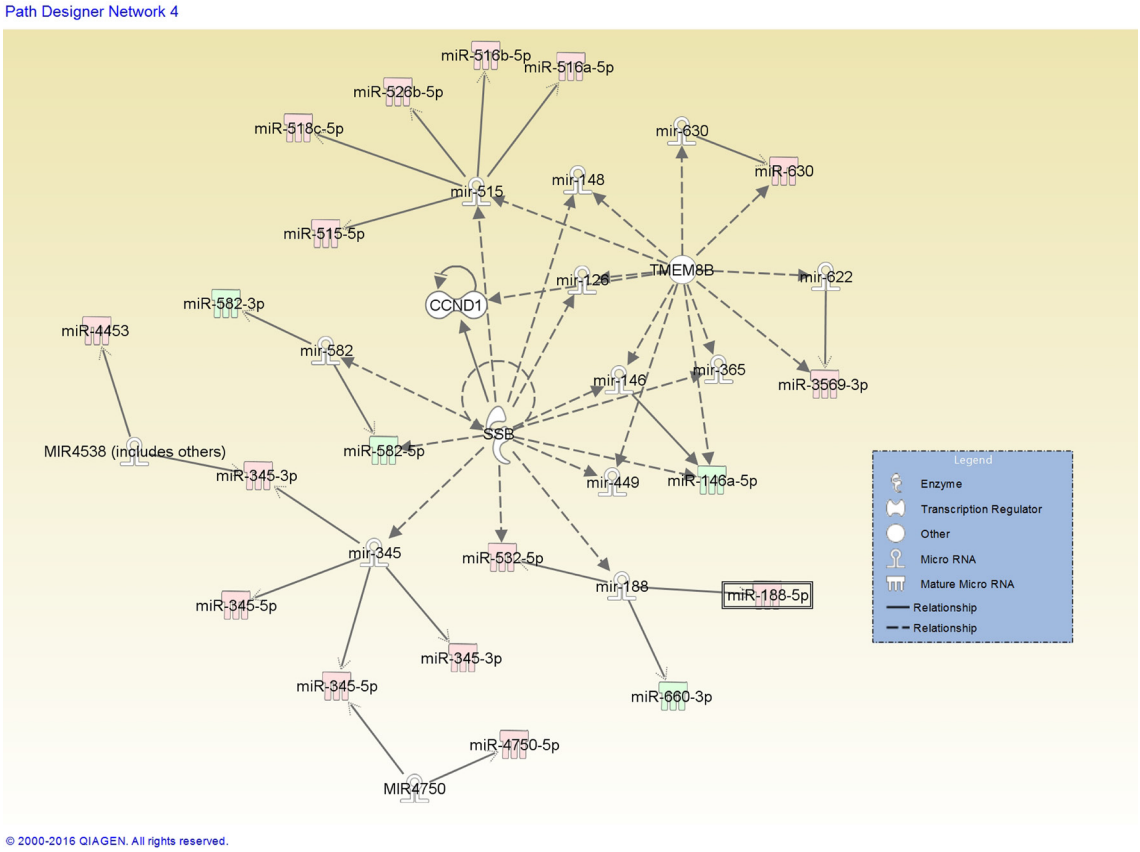

Figure S1. (A) The miRNA associated network generated using IPA altered by the DDW. The first network presents 29 transcripts, interacting with the NFkB signaling pathway, **(B)** while the second network includes 28 transcripts interacting with Akt, Jun, Mapk12, Rb and Smad2/3 pathways. **(C)** The third network includes 20 transcripts interacting with Ago, Tp63, Sirt1, Lin28A, and **(D)** the last or fourth network has 19 transcripts, which are interconnected with CCND1, SSB and TMEM8B.

the addition of DDW does not affect its capacity in any manner.

Senescence induces a growth arrest through an aging process associated with some pathologies, leading to loss of function of the cells [33]. Senescence also represents an antiproliferative mechanism for suppressing tumor growth and progression, and can be a potential anticancer target [33]. In our case, we cannot state for certain that inducing cellular senescence by DDW is friend or foe, as stated in the review paper written by Kahlem et al., related to the role of senescence [33].

The *in vitro* confirmation of the induction of a slightly higher senescence rate is done through microscopy evaluation of senescence and autophagy assays, sustained by data related to altered miRNA patterns (Figure 5).

The exosomal transfers of miRNAs have a relevant functionality and can affect cell fate via paracrine or autocrine-related mechanisms [34,35]. Our data reveals that the exosomal released miRNAs can modulate the response to DDW and thus can affect the cellular fate.

The altered exosomal miRNAs target the NFκB signaling pathway, which is recognized to be activated in breast cancer (Table 3). Therefore, this regulatory network (Figure S1A-D) seems to have an important role in breast cancer [36]. NFκB dysregulation is indispensable for the evaluation of the immune system modulation and for developing new therapeutic strategies [36], which can be done via microRNAs. Also, the regulation of NFκB signaling has an important role in the activation of the senescence-associated phenotype.

MiR-155 is upregulated in the TCGA patient cohort and was also overexpressed in the case of DDW-exposed cells. MiR-155 is considered in the literature as a key transcript that orchestrates inflammatory response and carcinogenesis, having the capacity to control an important number of cancer-related miRNAs [37]. This can be explained by the limited efficacy of DDW in the induction of autophagy, concluded after the functional assay done in the present study.

MiRNAs have lately arisen as essential regulators of cellular senescence [38]. One important axis related to cellular senescence is presented in Figure S2, where Akt and miR-21 and miR-34a are the key components [38] that are interconnected with Smad2/3 pathway and the TGFβ1 pathway that regulates EMT, a process necessary for cancer invasion [39].

MiR-21 is a key transcript that regulates apoptosis, autophagy, mitochondrial activity and senes-

Table 3. Correlation of the altered expression level for some relevant miRNAs retrieved to have altered expression level, with literature data

miRNAs	*Expression level*		*Biological process*	*Literature target gene*	*Reference*
	TCGA*	DDW** vs SC***			
Let-7s	↓	↓	Apoptosis, autophagy, drug resistance	D1/Akt1/Wnt1	[9,10]
miR-141	↑	↓	Apoptosis	ZEB1, FAK and PI3K/AKT	[1,2]
miR-155	↑	↑	Apoptosis, autophagy, mitochondrial processes	STAT3, SOCS1, IL-6, NF-κB	[4]
miR-181a/b/c	↑	↓	Apoptosis, drug resistance, mitochondrial processes	Akt	[8]
miR-193a/b	↓	↑	Apoptosis, senescence	K-Ras and PLAU	[5]
miR-200b	↑	↓	Apoptosis, autophagy	ZEB1	[1,2]
miR-205	↓	↑	Apoptosis, autophagy, drug resistance, mitochondrial processes	ErbB3 and VEGF-A	[12]
miR-210	↑	↓	Apoptosis, autophagy, autophagy, mitochondrial processes	FBXO31, BDNF/TrkB	[6,11]
miR-21	↑	↓	Apoptosis, autophagy, drug resistance, mitochondrial processes	PTEN, Akt	[3,7]

*The Cancer Genome Atlas, **deuterium depleted water, ***standard condition

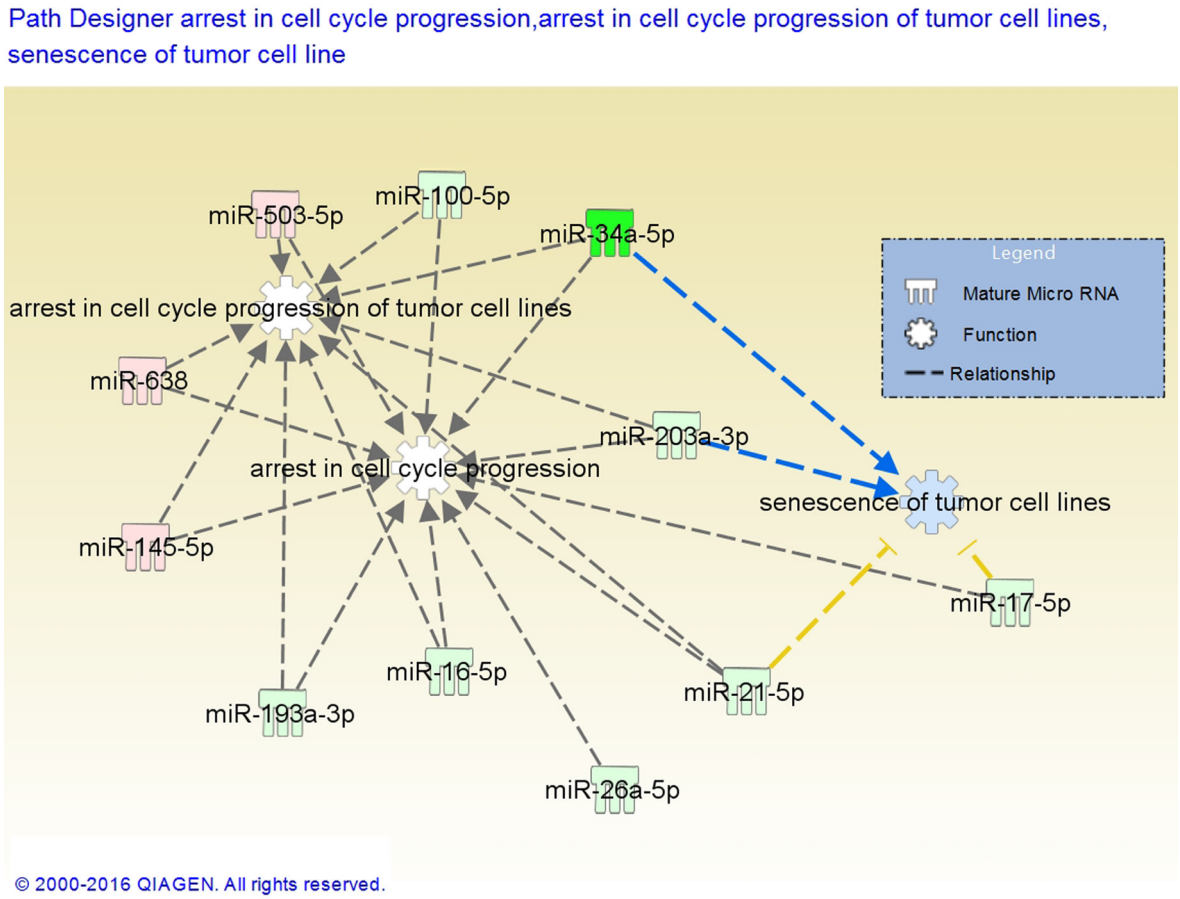

Figure S2. miRNAs network related to cell cycle and senescence.

cence. In the case of DDW, we had a low expression in exosomes vs SC. This has important relevance since this is a miRNA that was observed to have a higher expression level in plasma exosomes, when compared to healthy subjects [40]. MiR-21 and miR-210 overexpression is related with poor survival in breast cancer [41]. MiR-210 is a transcript that was demonstrated to be overexpressed in tumor tissue and tumor microenvironment [41]; in our experiment, we observed a downregulation of miR-210 after DDW exposure.

Other miRNAs which are overexpressed in breast cancer are miR-181a/b/c, which were proved to be related to cell proliferation via Akt hyperactivation [42]. MiR-205 is significantly downregulated in breast cancer, and restoring its expression level was related with reduced cell proliferation [43].

MiR-200 family members (miR-200b, miR-429, and miR-141) are involved in cell plasticity, regulating epithelial-to-mesenchymal transition (EMT) and stem-like characteristics. This miRNA family is regarded to be at the basis for tumor aggressiveness and drug resistance [44], and DDW appears to be able to downregulate these miRNAs.

The let-7 family members are involved in the resistance to therapy and are downregulated in the case of DDW exposure. This is in concordance with the literature data, suggesting let-7 members as therapeutic targets for cancer stem cells, and having an important role in sensitizing the response to radiotherapy [45,46], particularly for the TNBC subtype that has the worse survival rate [47,48].

Conclusion

DDW was proved to be non-toxic, and when administered with cisplatin to slightly increase the senescence of cancer cells, and therefore can be pondered as an adjuvant therapeutic agent. However, future studies are needed to be done in order to further elucidate its mechanism of action. DDW owns selective biological effects to inhibit cell proliferation in the presence of chemotherapeutics.

The exosomal released miRNA pattern is significantly altered by DDW, hence this may underline a new therapeutic approach to controlling the deleterious effects of tumor exosomes, and can have important application in the prevention or managing metastatic breast cancer.

Acknowledgements

This work is part of the research grant No. 128/2014; PN-II-PT-PCCA-2013-4-2166 "New strategies for improving life quality and survival in cancer patients: molecular and clinical studies of the tumour genome in deuterium-depleted water treatment augmentation-GenCanD". We want to thank to the SC Microsystem SRL for providing Qlarivia- deuterium depleted water as part of the GenCanD project.

Conflict of interests

The authors declare no conflict of interests.

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