

RESEARCH ARTICLE

Down-regulation of miRNA-452 is Associated with Adriamycin-resistance in Breast Cancer Cells

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Abstract

Adriamycin (ADR) is an important chemotherapeutic agent frequently used in treatment of breast cancer. However, resistance to ADR results in treatment failure in many patients. Recent studies have indicated that microRNAs (miRNAs) may play an important role in such drug-resistance. In the present study, microRNA-452 (miR-452) was found to be significantly down-regulated in adriamycin-resistant MCF-7 cells (MCF-7/ADR) compared with the parental MCF-7 cells by miRNA microarray and real-time quantitative PCR (RT-qPCR). MiR-452 mimics and inhibitors partially changed the adriamycin-resistance of breast cancer cells, as also confirmed by apoptosis assay. In exploring the potential mechanisms of miR-452 in the adriamycin-resistance of breast cancer cells, bioinformatics analysis, RT-qPCR and Western blotting showed that dysregulation of miR-452 played an important role in the acquired adriamycin-resistance of breast cancer, maybe at least in part via targeting insulin-like growth factor-1 receptor (IGF-1R).

Keywords: MicroRNA-452 - breast cancer - adriamycin - IGF-1R - chemosensitivity

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Introduction

Breast cancer is one of the most common malignant tumors in women worldwide (Siegel et al., 2013). Despite the use of multiple therapies including chemotherapy, the tumor does not actively respond to the therapies (Barrett 2010). Adriamycin (ADR) is an important chemotherapeutic agent frequently used in treatment of breast cancer. However, as with many other chemotherapeutic drugs, resistance to ADR results in treatment failure in patients with breast cancer. Causes of cancer-specific drug resistance are currently believed to be linked to the random drug-induced mutational events (genetic hypothesis) and the drug-induced nonmutational alterations of gene function (epigenetic hypothesis). Despite advances in the fields of resistance-associated molecular mechanisms, however, the underlying mechanisms of acquisition of resistance to chemotherapeutic agents are still poorly understood (Kovalchuk et al., 2008). Therefore, it should be acknowledged that other avenues must be explored.

Currently, extensive studies have indicated the existence and importance of another mechanism of nonmutational regulation of gene function mediated by means of short noncoding RNA (Kovalchuk et al., 2008). MicroRNAs (miRNAs) are a class of short (~22 nucleotide), single-stranded non-coding RNAs that usually bind their target mRNAs through imperfect base pairing in

the 3'-untranslated regions (3' UTRs) and impact protein expression by translational repression, mRNA degradation or the promotion of mRNA decay (Kutanzi et al., 2011). Widespread dysregulated expression of miRNA will make it another emerging hallmark of cancer, which provides not only biomarkers, but also novel therapeutic targets. Recently, the role of miRNAs in regulating drug-resistance is reported, but the studies evaluating their molecular mechanisms in drug-resistant are still rare (Tian et al., 2013). Accordingly, it is quite necessary to understand the potential functions of miRNAs in the process of drug resistance in the hope of overcoming drug resistance through modulating miRNA regulation.

Based on miRNA microarray data in our previous work (Zhong et al., 2013), microRNA-452 (miR-452) was found to be significantly down-regulated in the adriamycin-resistant MCF-7 cells (MCF-7/ADR) compared with the parental MCF-7 cells. However, the role or function of miR-452 has yet to be explored. Whether the abnormal expression of miR-452 contributes to acquisition of resistance? Basing on the finding and question, we investigated the association of miR-452 expression with the sensitivity of breast cancer cells to ADR and analyzed its potential mechanism. Our results showed, for the first time, that miR-452 could mediate chemosensitivity to adriamycin in human breast cancer cells, maybe at least partially by targeting insulin-like growth factor-1 receptor (IGF-1R).

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Materials and Methods

Cell Culture

Human breast cancer cell line MCF-7 was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The resistant subline selected at 500 nM adriamycin (MCF-7/ADR) was successfully established from human breast cancer parental cell line MCF-7, by exposing MCF-7 to gradually increasing concentrations of ADR in vitro in our laboratory. Parental MCF-7 cultured synchronously in the absence of drug was used as a control (called MCF-7). The IC₅₀ (inhibitory concentration to produce 50% cell death) values of ADR were 403.56 and 1.23 μ M for MCF-7/ADR and MCF-7 cells, respectively. The drug-resistant derivative cell lines were cultured in drug free medium for two weeks before subsequent experiments to avoid the influence of drug. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (HyClone) supplemented with 10% foetal bovine serum, 100U/ml penicillin, and 100 μ g/ml streptomycin at 37°C and 5% CO₂ in a humidified atmosphere.

Transfection

MiR-452 mimics, miR-452 inhibitors and their negative control were synthesized by Genepharma (Shanghai, China). MiR-452 mimics and its negative control were used in MCF-7/ADR cells with low miR-452 expression, and miR-452 inhibitors and its negative control were used in MCF-7 cells with high miR-452 expression.

Cells were plated onto a six-well plate at a density of 3×10^5 cells/well. After 24h, cells were transfected with miR-452 mimics (10 nM), inhibitors (30 nM) or their negative control, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were collected for further analysis after 24h.

Real-time quantitative PCR (RT-qPCR)

Cells in logarithmic phase were collected when a confluence of 80-90% was reached. Total RNA was extracted from MCF-7 and MCF-7/ADR cells, transfected or untransfected, using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One μ g of total RNA including miRNA from each sample was reverse transcribed using the BU-Script RT Kit (Biouniquer Technology Co, Ltd, Nanjing, China) according to the manufacturer's protocol. Then, real time PCR was performed with a standard SYBR Green I quantitative PCR kit (Biouniquer Technology CO, LTD, Nanjing, China) according to the manufacturer's protocol. The sequence-specific RT primers for miR-452 and U6 snRNA were 5'-GTCGTATCCAGTGCCTGTCGTGGAGTCCGCAATTCGACTGGATACGACTCAGTTT-3' and 5'-GCGCGTGAGCAGGCTGGAGAAATTAACCACGCGCGGAACG-3', respectively. The PCR primers for miR-452 were 5'-GCCAACTGTTTGCAGAGG-3' and 5'-CAGTGCCTGTCGTGGAGT-3' and those for U6 snRNA were 5'-CGCAAGGATGACACG-3' and 5'-GAGCAGGCTGGAGAA-3'. All the primers were purchased from Springen Biotechnology CO., LTD, China. Primer quality was analyzed by dissociation

curves. The Ct values for each gene were normalized to endogenous control U6 snRNA, and the relative fold change values were calculated using the $\Delta\Delta$ Ct method. All reactions were run in triplicate and all experiments were carried three independent times.

Cell Viability Assay

MTT assay was performed to analyze the cell viability. Briefly, the cells, transfected or untransfected, were trypsinized and reseeded into 96-well culture plates at a density of 8×10^3 per well. After overnight incubation, cells were treated with ADR at different concentrations for 48 h. 20 μ l of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma, Germany) per well was added. The incubation was continued for 4h before the media was removed. 150 μ l dimethyl sulfoxide (DMSO) (Amresco, America) was added to each well and mixed to ensure cell lysis and dissolving of the formazan crystal. At last, the absorbance at 550 nm was measured using CliniBio128 (ASYS-Hitech, Austria). The IC₅₀ value of ADR was calculated using SPSS 16.0. Three independent experiments were performed.

Flow Cytometric Analysis

Apoptosis was analyzed by flow cytometry. Briefly, all the cells above, transfected or untransfected, were incubated with 100 μ M and 0.2 μ M ADR, respectively. After 48h, cells were harvested and washed twice with ice-cold PBS and incubated with Annexin-V-FITC and propidium iodide (PI) for 15 min in the dark at room temperature followed by using a FACScan flow cytometer (BD Biosciences) to analyze cellular apoptosis.

RT-qPCR Analysis for Target Gene Expression

Predicted target gene IGF-1R and internal control β -actin were detected using RT-qPCR as described above. The primers of reverse transcription for IGF-1R and β -actin were random primers. The sets of primers for IGF-1R were 5'-ATCCGACGGGGGAATAACA-3' and 5'-CCCACAGTTGCTGCAAGTTC-3', and those for β -actin were 5'-CACCTTCTACAATGAGCT-GCGTGTG-3' and 5'-ATAGCACAGCCTGGATAGCAACGTAC-3'. Endogenous control β -actin was used for normalization. Three independent experiments were performed.

Western Blot

Western blotting was used to confirm the expression of protein. Briefly, according to the protocol from manufacturer, Total protein was extracted using RIPA lysis buffer (Biouniquer Technology CO, LTD, Nanjing, China). Equal amounts of total protein were applied to 10% polyacrylamide gradient gels. After electrophoresis, the samples were transferred to polyvinylidene fluoride (PVDF) membrane (Sigma, Germany). After blocking in 5% skim milk, IGF-1R proteins were detected with an anti-IGF-1R rabbit polyclonal antibody (1:6000; Abcam, America). Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Wuhan Boster Biological Technology Co., LTD., Wuhan, China) for 2 h at room temperature. The membranes were

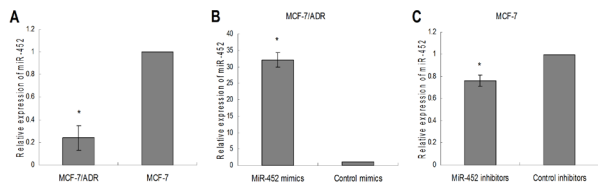


Figure 1. Relative Expression Level of miR-452 in the Breast Cancer Cells. (A) Relative expression of miR-452 in MCF-7/ADR cells and MCF-7 cells ($*P<0.05$). (B) Relative expression of miR-452 in MCF-7/ADR cells transfected with miR-452 mimics and its negative control ($*P<0.05$). (C) Relative expression of miR-452 in MCF-7 cells transfected with miRNA-452 inhibitors and its negative control ($*P<0.05$)

developed with an ECL Substrate (Biouniquer Technology CO, LTD, China). The levels of each protein were normalized to the level of β -actin (Sigma, Germany). The experiments were carried three independent times.

Statistical Analysis

SPSS 16.0 software was used for statistical analysis. Data were presented as mean \pm SD (standard deviation) of at least 3 independent experiments. Student's t-test was used for comparisons between 2 independent groups. All tests were two-tailed and $p<0.05$ ($*$) were considered to be statistically significant.

Results

MiR-452 was down-regulated in human MCF-7/ADR cells

According to the result of RT-qPCR analysis, miR-452 in MCF-7/ADR cells was down-regulated (0.24-fold \pm 0.11) in comparison with their parental MCF-7 cells ($P < 0.05$; Figure 1A), which is consistency with the previous microarray data.

MiR-452 modulated chemo-sensitivity of breast cancer cells to ADR

To study the function of miR-452 in MCF-7/ADR cells, miR-452 mimics were transfected into MCF-7/ADR cells. The results showed that the expression of miR-452 in the transfected MCF-7/ADR cells was up-regulated 32.05 folds compared to its NC ($P<0.05$; Figure 1B), and the IC₅₀ value of ADR ($319.91 \pm 12.23 \mu\text{M}$) was significantly lower than that in its NC ($408.94 \pm 8.24 \mu\text{M}$) or blank control ($404.65 \pm 11.34 \mu\text{M}$; $P<0.05$; Figure 2A).

Similarly, miR-452 inhibitors were transfected into sensitive cells (MCF-7). The results showed that the expression of miR-452 in the transfected MCF-7 cells was down-expressed 24% compared to its NC, $P<0.05$; Figure 1C), and the IC₅₀ value of ADR ($2.32 \pm 0.22 \mu\text{M}$) was significantly higher than that in its NC ($1.27 \pm 0.32 \mu\text{M}$) or blank control ($1.21 \pm 0.15 \mu\text{M}$; $P < 0.05$; Figure 2B). These findings suggested that up-regulation of miR-452 expression could decrease the drug-resistance of MCF-7/ADR cells to ADR, and down-regulation of miR-452 could increase drug-resistance of the MCF-7 cells to ADR in some degree.

Flow cytometry assay showed that the apoptotic rate of MCF-7/ADR cells transfected with miR-452 mimics were distinctly increased compared with that of the cells transfected with its NC or blank control ($18.51 \pm 2.15\%$ vs

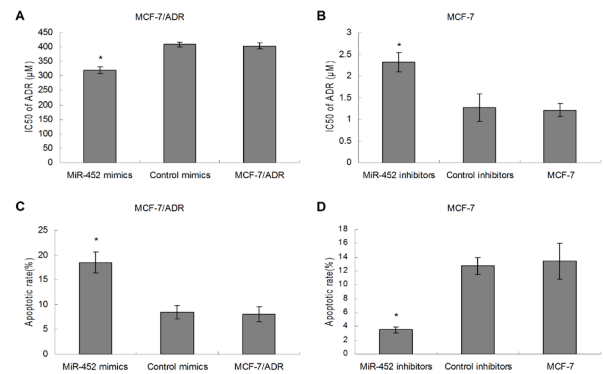


Figure 2. MiR-452 Modulated Chemo-sensitivity of Breast Cancer Cells to ADR. (A) After MCF-7/ADR cells were transfected with miR-452 mimics and its negative control for 24 h, the IC₅₀ value of DOC was determined by MTT assay ($*P<0.05$). (B) After MCF-7 cells were transfected with miR-452 inhibitors and its negative control for 24 h, the IC₅₀ value of DOC was determined by MTT assay ($*P<0.05$). (C) The apoptotic rate of MCF-7/ADR cells transfected with miR-452 mimics were distinctly increased compared with negative control and blank control ($*P<0.05$). (D) The apoptotic rate of MCF-7 cells transfected with miR-452 inhibitors were significantly decreased compared with its negative control and blank control ($*P<0.05$)

$8.46 \pm 1.39\%$ or $8.04 \pm 1.46\%$ Figure 2C, $P<0.05$). On the contrary, the apoptotic rate of MCF-7 cells transfected with miR-452 inhibitors were significantly decreased compared with that of the cells transfected with its NC or blank control ($3.51 \pm 0.43\%$ vs $12.74 \pm 1.19\%$ or $13.43 \pm 2.62\%$, Figure 2D, $P<0.05$). These results suggested that miR-452 could confer ADR resistance in breast cancer MCF-7 cells by inhibiting ADR-induced apoptosis.

MiR-452 regulated the expression of IGF-1R.

To explore the potential mechanisms of miR-452 in the drug-resistance of breast cancer cells to ADR, the prediction of miRNA target gene was performed by in silico analysis based on the computer-aided algorithms: TargetScan (<http://www.targetscan.org>), miRBase (<http://www.mirbase.org/>) and Miranda (<http://www.microrna.org>) in conjunction with KEGG pathway enrichment analysis for the predicted targets using web-based tool DAVID (<http://david.abcc.ncifcrf.gov/>). Satisfactory targets were predicted independently by at least two of the three applied tools. At last, IGF-1R was predicted as a candidate target of miR-452 (Figure 3A).

To identify the regulatory function of miR-452 in IGF-1R expression, the IGF-1R mRNA and protein expression were determined using RT-qPCR and Western blot. The results showed that both IGF-1R mRNA (Figure 3B, $P<0.05$) and protein (Figure 3E) expression were increased significantly in MCF-7/ADR cells compared to MCF-7 cells. After transfection, the relative level of IGF-1R mRNA (Figure 3C, $P<0.05$) and protein (Figure 3F) expression in MCF-7/ADR cells transfected miR-452 mimics were significantly decreased compared with control mimic. Reversely, IGF-1R mRNA (Figure 3D, $P<0.05$) and protein (Figure 3G) expression in MCF-7 cells transfected miR-452 inhibitors were significantly increased compared with control inhibitors.

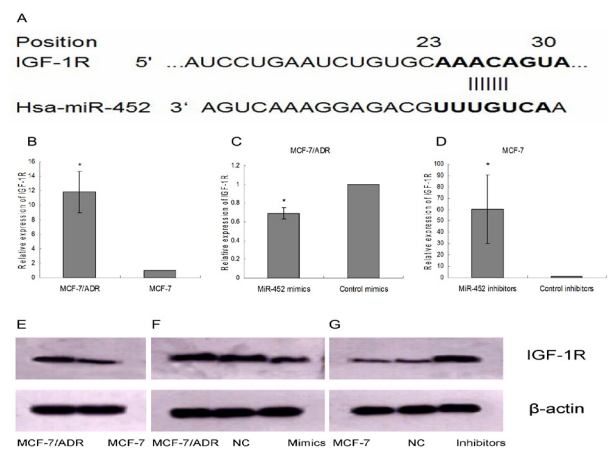


Figure 3. MiR-452 Regulated the Expression of IGF-1R. (A) Relative expression of IGF-1R-mRNA in MCF-7/ADR cells and MCF-7 cells ($*P < 0.05$). (B) Relative expression of IGF-1R-mRNA in MCF-7/ADR cells transfected with miR-452 mimics and its negative control ($*P < 0.05$). (C) Relative expression of IGF-1R-mRNA in MCF-7 cells transfected with miR-452 inhibitors and its negative control ($*P < 0.05$). (D) Expression of IGF-1R protein in MCF-7/ADR cells and MCF-7 cells. (E) Expression of IGF-1R protein in MCF-7/ADR cells transfected with miR-452 mimics and its negative control. (F) Expression of IGF-1R protein in MCF-7 cells transfected with miR-452 inhibitors and its negative control

Discussion

Breast cancer is a heterogeneous disease and its survival is dramatically improved through earlier detection and improvements in comprehensive treatment. Chemotherapy is the cornerstone of all the therapeutic strategies in breast cancer, however, drug resistance is a major obstacle in systemic treatment. Adriamycin is an important of chemotherapeutic agent commonly used in treatment of breast cancer, especially recurrent or metastatic patients. Adriamycin can intercalate directly DNA double helix and interferes with gene transcription to induce cancer cell apoptosis. Despite their widespread use, however, the clinical effectiveness of adriamycin is limited by the emergence of adriamycin-resistant cancer cells. Decades of research have not completed illuminated several unique or common mechanisms that could contribute to drug resistance in breast cancer. To better understand the biological mechanisms of chemo-resistance of breast cancer cells and search for the reversion opportunities, adriamycin-resistant breast cancer cells have been established in our laboratory after the selection by drug pressure and chosen as the subjects.

Currently, 2042 homo sapiens mature miRNAs have been identified (The miRBase Sequence Database-Release 19.0) that can potentially target up to one third of the protein-coding genes involved in development, cell differentiation, metabolic pathways, signal transduction, proliferation, and apoptosis (Anglicheau et al., 2010). Recently dysregulated miRNAs have been reported that associated with every aspect of breast tumor biology, including tumor progression (Li et al., 2013a), apoptosis (Anaya-Ruiz et al., 2013), different distribution in clinicopathological subtypes (Li et al., 2013b), and acquisition of resistance to various chemotherapeutic

agents. Causes of anti-cancer drug resistance are believed to be complex and current studies have indicated that the acquisition of chemotherapy drug resistance may also be modulated via the changes in miRNA levels. In breast cancer, it was shown that down-regulated miR-200c and miR-451 were associated with the sensitivity of MCF-7/ADR to ADR by targeting two members of ATP-binding cassette (ABC) transporters family *P*-glycoprotein (*P*-gp) and MDR-associated protein (MDR1) (Kovalchuk et al., 2008; Chen et al., 2012). Up-regulated miR-21, miR-222 and miR-29a were associated with the chemoresistance of breast cancer MCF-7/ADR cells to ADR through direct interaction with anti-apoptotic protein PTEN (Wang et al., 2011; Zhong et al., 2013). Down-regulated miR-34a and miR-200 modulates chemosensitivity of breast cancer cells to adriamycin by targeting Notch1 and E-cadherin, respectively, which regulated the formation of cancer stem cells (CSCs) and contributed to the acquisition of the epithelial-mesenchymal transition (EMT) phenotype (Tryndyak et al., 2010; Howe et al., 2011; Jurmeister et al., 2012; Li et al., 2012). Accordingly, it is quite necessary to understand the functions of miRNAs in the process of drug resistance in the hope of overcoming drug resistance through modulating miRNA regulation. In this study, we focused on miR-452, which was down-regulated miRNA in MCF-7/ADR cells according to the results of miRNA microarray and RT-qPCR. To further elucidate whether miR-452 expression could affect the sensitivity of breast cancer cells to ADR, MTT assay was performed to detect IC50 value of miR-452-transfected cells. Flow cytometry assays also indicated that miR-452 could significantly change apoptosis rate to ADR.

IGF-1R is a transmembrane protein that contains two extracellular α subunits with the ligand-binding site and two transmembrane β subunits with intracellular tyrosine kinase activity. It is activated by engagement with IGF1 and IGF2 and has an important physiological role in regulating cell growth, proliferation and anti-apoptosis. It is a strategy for the development of cancer drugs to inhibit the expression of IGF-1R or its signaling pathway. Apoptotic resistance is a critical factor in the development of drug resistance. IGF-1R activates three signaling pathways to block apoptosis. The first pathway activated by IGF-1R stimulates PI3-kinase and the AKT pathway to phosphorylate BAD and block apoptosis. A second pathway activated by IGF-1R involves ras mediated activation of the map kinase pathway to block apoptosis. A third pathway involves interaction of raf with mitochondria in response to IGF-1R activation. These results provide molecular evidence indicating that activation of IGF-1R signaling maybe related to chemo-resistance (Peruzzi et al., 1999). Furthermore, some studies have showed that it can enhance the adriamycin cytotoxicity of human breast cancer cells by inhibiting IGF-1R (Beech et al., 2001; Zeng et al., 2012).

In this study, the data suggested that miR-452 could modulate the sensitivity of breast cancer cells to ADR, maybe in part by regulating the expression of IGF-1R. First, according to the different algorithms, we chose three kinds of tools to predict target gene, respectively. To improve the accuracy, a preliminary screening was carried.

Satisfactory targets were predicted independently by at least two of the three applied tools. Afterwards, KEGG pathway enrichment analysis, consisting of genomic, network information and possible mechanisms important in the development of resistance to drugs, was performed. According to the final predicted results of bioinformatics analysis, it indicated that the miRNA-452 was likely to target IGF-1R to modulate the drug-resistance. Second, expression of IGF-1R was up-regulated in MCF-7/ADR cells compared with MCF-7 cells. More importantly, miR-452 mimics could down-regulate the expression of IGF-1R mRNA and protein in MCF-7/ADR cells, on the contrary, miR-452 inhibitors could up-regulate the expression of IGF-1R mRNA and protein in MCF-7 cells. According to the overall results of bioinformatics analysis, mRNA and protein expression, it showed that miRNA-452, maybe in part via targeting IGF-1R, involved in the ADR resistance formation of breast cancer cells in vitro.

The efficiency of the miR-452 was only moderate, but the protein level IGF-1R was significantly changed in MCF-7 cells transfected with miR-452 inhibitors. The reason maybe as following: First, other than 3'UTRs, miRNA can position on the 5'UTRs or coding regions of their target, also. Therefore, miR-452 maybe regulated the expression level of IGF-1R in different ways. Second, one miRNA can target multiple mRNAs and signaling pathways, thus, miR-452 maybe regulate the other target gene or signaling pathways to affect the expression of IGF-1R. Third, the organism itself and IGF-1R signaling pathways had complicated physiologic function and feedback mechanisms, which maybe regulate the expression of IGF-1R in some degree.

According to the results of miRNA microarray, there were many aberrant expressions of miRNAs including miR-452 in MCF-7/ADR cells. However, current study was solely focused on miR-452, this miRNA represented only one of the miRNAs differently expressed that were likely to be involved in adriamycin resistance. Thus, the role of a single miRNA was limited. Meanwhile, one miRNA can target multiple mRNAs, and one target can be repressed by multiple miRNAs (John et al., 2004), so role of miRNAs in cancer was complex and difficult to unravel at present. Also, the role of IGF-1R was complicated in cancer in cell growth and survival (Xiong et al., 2007). Importantly, the purpose of this study was to explore of the miRNA-452 in acquiring drug-resistance, the attempt to restore the physiological miRNA expression could provide a more successful and sustainable strategy than to target the function of a single protein specifically (Haenisch and Cascorbi 2012). It is worth encouraging that evidence of miRNA-mediated adriamycin resistance is accumulating. Before we use these differentially expressed miRNAs as novel therapeutic targets to overcome resistance, many preclinical studies remain to be done.

In conclusion, we demonstrate that miR-452 is involved in adriamycin-resistance in breast cancer cells in vitro, maybe at least in part via targeting IGF-1R. The result provides a supportive rationale for the development of miRNA-based therapeutic strategies aiming to overcome cancer cells drug-resistance. Further studies, including the function analyses of miR-452 in

vivo, including in clinical breast cancer tissues and animal models would be warranted.

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