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Tumorigenesis and Neoplastic Progression

Increased Expression of P-Glycoprotein and Doxorubicin Chemoresistance of Metastatic Breast Cancer Is Regulated by miR-298

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MicroRNAs (miRNAs) are short, noncoding RNA molecules that regulate the expression of a number of genes involved in cancer; therefore, they offer great diagnostic and therapeutic targets. We have developed doxorubicin-resistant and -sensitive metastatic human breast cancer cell lines (MDA-MB-231) to study the chemoresistant mechanisms regulated by miRNAs. We found that doxorubicin localized exclusively to the cytoplasm and was unable to reach the nuclei of resistant tumor cells because of the increased nuclear expression of MDR1/P-glycoprotein (P-gp). An miRNA array between doxorubicin-sensitive and -resistant breast cancer cells showed that reduced expression of miR-298 in doxorubicin-resistant human breast cancer cells was associated with increased expression of P-gp. In a transient transfection experiment, miR-298 directly bound to the MDR1 3′ untranslated region and regulated the expression of firefly luciferase reporter in a dose-dependent manner. Overexpression of miR-298 down-regulated P-gp expression, increasing nuclear accumulation of doxorubicin and cytotoxicity in doxorubicin-resistant breast cancer cells. Furthermore, down-regulation of miR-298 increased P-gp expression and induced doxorubicin resistance in sensitive breast cancer cells. In summary, these results suggest that miR-298 directly modulates P-gp expression and is associated with the chemoresistant mechanisms of metastatic human breast cancer. Therefore, miR-298 has diagnostic and therapeutic potential for predicting doxorubicin chemoresistance in human breast cancer. ***(Am J Pathol 2012, 180: 2490–2503; http://dx.doi.org/10.1016/j.ajpath.2012.02.024)***

A number of chemotherapy regimens have been used to treat metastatic breast cancer in humans. The success of treating breast cancer by chemotherapy is hampered by the development of multidrug resistance (MDR) of cancer cells.[1–3] MDR of cancer cell occurs because of the overexpression of one or more of the ATP binding cassette (ABC) transporters.[4,5] There are three well-characterized transporters, ABCB1 (MDR-1/P-gp), ABCC1 (MRP-1), and ABCG2 (BCRP), associated with the chemoresistance of breast cancer.[6–10] The P-glycoprotein (P-gp) overexpression in breast cancer cells has been found to be strongly associated with chemoresistant mechanisms of a variety of drugs.[11–13] P-gp is a 170-kDa transmembrane glycoprotein that acts as an energy-dependent efflux transporter that enhances drug efflux from the nucleus or prevents entry of drugs to the nucleus, thereby decreasing cytotoxicity of anticancer drugs.[12–14] A number of mechanisms have been proposed to explain the transcriptional activation of the P-gp gene (*ABCB1*) in cancer cells.[15–18] Studies indicate that increased resistance of chemotherapeutic agents is also associated with epigenetic alterations, including DNA methylation and histone modifications.[19–21] The precise cellular and molecular mechanisms underlying the increased *MDR1* gene expression is not clear.

New evidence indicates that changes in gene expression associated with cell proliferation, apoptosis, signaling, and

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chemotherapy response are regulated by altered expression of cellular microRNAs (miRNAs). miRNAs are small non–protein-coding RNAs that regulate gene expression through base pairing with target mRNAs, resulting in translation inhibition or mRNA cleavage.[22] miRNAs are produced through a series of steps that are initially generated in the nucleus where primary miRNAs are transcribed by RNA polymerase II. The primary transcripts are subsequently processed to shorter (70 to 85 nt) precursor (pre-) miRNA mediated by an RNase III enzyme called Drosha, and its cofactor DGCR8.[23–25] Subsequently, pre-miRNAs are exported to the cytoplasm by exportin 5 and then cleaved by Dicer, another RNase III enzyme, to produce a 22-nt double-stranded miRNA duplex.[26–30] The strand containing less stable hydrogen bonding at its 5′ end is the mature miRNA and is integrated into the RNA-induced silencing complex, whereas the other strand is degraded.[27]

To understand the role of miRNAs in the regulation of MDR of breast cancer cells, we developed doxorubicin-sensitive and -resistant metastatic human breast cancer cells (MDA-MB-231). We showed that high-level expression of P-gp leads to the impaired nuclear translocation of doxorubicin and the doxorubicin chemoresistance of MDA-MB-231. To study the role of miRNA involvement in the doxorubicin chemoresistance mechanism, we performed a miRNA array between the doxorubicin-sensitive and -resistant metastatic breast cancer cells. We found significant up-regulation and down-regulation of miRNAs in the doxorubicin-resistant human breast cancer cells compared with the sensitive cells. We have determined that miR-298 is down-regulated significantly in the doxorubicin-resistant MDA-MB-231 cells compared with the doxorubicin-sensitive MDA-MB-231 cells. Using the miRNA database, we found that human miR-298 targeted to the 3′ untranslated region (UTR) of the human P-gp mRNA. Because the role of miRNA-mediated development of resistance to the chemotherapeutic drug is largely unexplored, our study provides the evidence to suggest that the impaired processing of miR-298 because of low expression of Dicer enzyme is associated with an increased expression of P-gp and contributes to the doxorubicin resistance in breast cancer cells. This interaction may have an important functional consequence in the formation of cancer cell resistance to a variety of chemotherapeutic drugs used in the treatment of breast cancer.

Materials and Methods

Cell Culture and Reagents

The MDA-MB-231 and MCF-7 human breast cancer cell lines were obtained from ATCC (Manassas, VA). These two cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, nonessential amino acids, and 1% penicillin and streptomycin (Invitrogen, Grand Island, NY) at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Doxorubicin (Adriamycin) was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution of doxorubicin (1 mg/mL = 1.8 mmol/L) was prepared in distilled water. MDA-MB-231 cells were continuously cultured in growth medium in the presence of 0.18 μmol/L doxorubicin. After several passages, clones that grew in the presence of doxorubicin were selected as drug-resistant cancer (MDA-MB-231-R) cells. The MDA-MB-231-R cells had been cultured for >6 months in the growth medium supplemented with doxorubicin to assure that they were truly resistant to doxorubicin. With the use of a limiting dilution method, MDA-MB-231 cells showing increased doxorubicin-mediated cytotoxicity were isolated. Clones showing the highest cytotoxicity in the MTT assay were selected as doxorubicin-sensitive MDA-MB-231 (MDA-MB-231-S) cells. Doxorubicin-resistant MCF-7 cells (MCF-7-Dox) were obtained from the laboratory of Debasis Mondal (Tulane University School of Medicine, Tulane, LA) and were cultured in high-glucose DMEM supplemented with 10% FBS, sodium pyruvate, nonessential amino acid, 1% penicillin/streptomycin (Invitrogen), and 1 μmol/L insulin (Human Recombination, zinc solution; Invitrogen).

Cell Proliferation

Cell viability after doxorubicin treatment was measured by the MTT assay. The assay uses a tetrazolium compound (3-[4,5-dimethylthiazol-2-yl]−2,5-diphenyl tetrazolium bromide; Sigma-Aldrich, St. Louis, MO), which was reduced to formazan intracellularly by the mitochondrial dehydrogenase enzyme. The conversion of tetrazolium into purple formazan by metabolically active cells indicates the extent of cell viability. The viability of tumor cells was measured by the quantification of formazan dye by the colorimetric method. The MDA-MB-231 or MCF-dox cells were seeded on 24-well plates at a density of 2×10^4 cells/well in DMEM with 10% FBS and allowed to adhere at 37°C overnight. After 24 hours, culture medium was replaced and treated with four different concentrations (0.09, 0.18, 0.36, and 0.72 μmol/L) of doxorubicin. After 48 hours of incubation, cells were stained with 100 μL (5 mg/mL of PBS) of MTT solution along with 900 μL of growth medium added in each well. Cells were incubated at 37°C for 3 hours. Then, the tumor cells were washed in PBS and were solubilized with 1 mL of MTT solubilization buffer (anhydrous isopropanol containing 10% Triton X-100, 0.1N HCl) for 5 minutes. Absorbance of converted dye was measured in a spectrophotometer (Beckman Du 530, Life Science UV/Vis spectrophotometer; Beckman Coulter, Inc, Schaumburg, IL) at 570 nm. The percentage of cell viability was determined by comparison with untreated cells as controls.

Flow Cytometry

MDA-MB-231-S and MDA-MB-231-R cells (1×10^5) were seeded into 100-mm plates in DMEM with 10% FBS at 37°C overnight. On the next day, the medium was aspirated and replaced with 10 mL of fresh media containing 0.18 μmol/L doxorubicin. After 24 hours of treatment, cells were washed with PBS and trypsinized with 0.05% Trypsin-EDTA (Invitrogen). The cells were fixed in 70% ethanol in PBS and incubated for 2 hours on ice. The fixed cells were subsequently washed

twice with PBS and centrifuged at 2000 rpm for 5 minutes. The cells were resuspended in a commercially available propidium iodide staining solution (BD Pharmingen, San Diego, CA). Cells were incubated in the dark for 30 minutes at room temperature. The cell cycle analysis was performed with the use of 2×10^4 cells by a flow cytometer (BD LSR II; BD Biosciences, San Jose, CA). The percentage of tumor cells present in the G_1, S, and G_2 phases of the cell cycle was analyzed with the computer software (Modfit LT 3.0; Verity Software House, Topsham, ME).

Western Blot Analysis

Briefly, the cells were cultured in a 6-well plate and lysed with 200 μL of RIPA lysis buffer. Total protein in the lysate was quantified by Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA). Approximately 10 μg of protein from each sample was mixed in 4× SDS-loading buffer. Proteins were separated by NuPAGE 12% gel and then transferred onto a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The membrane was blocked with 5% fat-free milk powder in 50 mmol/L Tris-buffered saline (TBS) pH 7.6 with 0.1% Tween-20 (TBS-Tw20) at room temperature for 1 hour. The membrane was washed three times and incubated overnight at 4°C with either the mouse monoclonal antibody to P-gp at 1:500 dilution (C219; AbCam, Inc., Cambridge, MA) or the rabbit monoclonal anti-β-actin clone (Cell Signaling Technologies, Danvers, MA) at 1:1000 dilution in TBS-Tw20 containing 5% fat-free milk powder. After this step, the membrane was washed three times with TBS-Tw20 and reacted for 1 hour with the secondary antibody, anti-mouse or anti-rabbit IgG (Cell Signaling Technologies) that was conjugated with HRP at a dilution of 1:2000. The bound antibodies were detected with the use of ECL Plus Western Blotting Detection system (GE Healthcare), and the chemiluminiscent signals were detected with the use of high-performance chemiluminescence film (GE Healthcare).

Immunocytochemistry

The MDA-MB-231-S and MDA-MB-231-R cells were cultured overnight on the chamber slides. The next day, slides were fixed with chilled acetone and treated with 1 mL of blocking reagent (Background Sniper; Biocare Medical, Concord, CA) for 10 minutes at room temperature. The slides were then incubated with a primary mouse monoclonal antibody C219 at 1:250 dilution (AbCam, Inc.) at room temperature for 1 hour, followed by a secondary reagent MACH 4 mouse probe (Biocare Medical) for 10 minutes, then a tertiary reagent Mach 4 HRP polymer (Biocare Medical) for 20 minutes. At the final step, the slides were counterstained with hematoxylin and bluing.

miRNA Array

miRNA was extracted from MDA-MB-231-S and MDA-MB-231-R cells with the use of the Purelink miRNA Isolation Kit (Invitrogen) according to the manufacturer's instructions. To compare the profiles of miRNA expression in these samples, we used the miRCURY LNA miRNA Array, 6th Generation - Human, Mouse & Rat (Exiqon Inc., Woburn, MA). This array platform allows a simultaneous screening of the expression of all known human, mouse, and rat miRNA molecules known to date. Samples extracted from the control (S) cells were labeled with Cy3, and those from the experimental (R) cells were labeled with cyanine 5, using the mercury LNA miRNA labeling kit (Exiqon Inc.). Cyanine-labeled samples were then mixed together in equivalent concentrations and cohybridized to the array overnight at 55°C in a rotary chamber. Slides were scanned on a dual confocal Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) with the use of GenePix version 6.2 software, and raw data were extracted. A stringent set of criteria was applied to remove background and highly variable data from consideration. The remaining data were $\log_2$ transformed and normalized, using Locally Weighted Scatter-plot Smoothing in Spotfire S^+, to remove intensity-specific bias. miRNAs were considered to be differentially expressed if they exhibited a twofold perturbation in expression magnitude.

Northern Blot Analysis

Expression miRNA in the doxorubicin-sensitive and -resistant breast cancer cells was examined by Northern blot analysis with the use of a miRNA Northern Blot Assay Kit (Signosis BioSignal Capture, Sunnyvale, CA). Briefly, total RNA was extracted from MDA-MB-231-S and MDA-MB-231-R cells with the use of the Purelink total RNA purification system (Invitrogen). Cellular RNA (5 μg) mixed with 3 μL of RNA loading buffer was heated at 70°C for 5 minutes and chilled on ice. RNA samples in a 10-μL volume were loaded onto a 15% urea-polyacrylamide gel at 60 V in 0.5× TBE buffer until bromophenol blue dye reached approximately 3 cm away from the bottom of the gel. RNAs were transferred to a nylon membrane (Signosis BioSignal Capture) with the use of XCell SureLock Electrophoresis (Invitrogen), immobilized with Stratagene UV cross-linker, and dried at 42°C for 15 minutes. The membrane was transferred into a hybridization tube, soaked with distilled H_2O, and incubated with 4 mL of prewarmed (42°C) hybridization buffer then rotated for 30 minutes at 42°C. The membrane was replaced with 4 mL of fresh hybridization buffer along with 10 mL of biotin-labeled miRNA probe and rotated at 42°C overnight. The membrane was removed to an empty container and rinsed with 10 mL of 1× detection wash buffer and then blocked with 15 mL of blocking buffer for 30 minutes at room temperature with moderate shaking. Then the membrane was incubated with 1 mL of the 1× blocking buffer containing 15 mL of streptavidin-HRP conjugate for 45 minutes at room temperature. Then the blocking buffer was removed, and the membrane was washed three times at room temperature with 15 mL of 1× detection washing buffer for 10 minutes each wash. The membrane was incubated with 2 mL of Tris-Buffer pH 7.4 with 200 μL of substrate A and B at room temperature for 5 minutes. After this step, excess substrate was removed by gently applying pressure over the top sheet with the use of a paper towel. The membrane was ex-

posed with Amersham Hyperfilm ECL (GE Healthcare). Membranes were stripped and reprobed to assess other miRNAs.

Plasmid Constructs

The 3′ UTR of P-gp gene (*ABCB1*) corresponding to 4262 to 4872 nt (610 bp; Accession no. NM-000927) was cloned into pMirTarget vector by SgfI and MluI restriction sites called pMirTarget-MDR1-3′ UTR (SC208086; Origene Technology, Inc., Rockville, MD). Two different control plasmids with deletion of miR-298 and miR-1253 binding sites were prepared by overlapping PCR and cloned into pMirTarget vector with the use of the unique AscI (GGCGCGCC) and MluI (ACGCGT) restriction sites (Origene Technology, Inc.). The 571-bp DNA fragment with 25-nt deletion in the miR-298 binding site was amplified by overlapping PCR of Fragment 1 (F1) and Fragment 2 (F2). The F1 (411 bp) was PCR amplified with the use of the sense primer (P1), 5′-TCGGGCGCGCCACTCTGACTGTATGAGATGTT-3′, and antisense primer (P2), 5′-AAAGAAAACTTTTTTAAAATTGAGAGAAGATATA-3′, and wild-type MDR1/3′ UTR plasmid as a template. The F2 (160 bp) was amplified from MDR1/3′ UTR plasmid with the use of sense primer (P3), 5′-TATATCTTCTCTCAATTTTAAAAAAGTTTTCTTT-3′, and antisense primer (P4), 5′-GGCACGCGTGAATCAGCAGGATCAAGTCCAAGAAGAATG-3′. AscI site was added to the outer sense primer, and MluI site was added to the outer antisense primer. The PCR-amplified DNA was digested with AscI and MluI restriction endonucleases and cloned into the downstream of the firefly luciferase reporter gene in the AscI/MluI cloning sites of pMirTarget vector. The sequence of the recombinant clones was confirmed by DNA sequencing and was called pMirTarget-MDR1-3′ UTR with deletion of miR-298. The PCR amplification cycles were 94°C for 5 minutes, then 33 cycles at 94°C for 30 seconds, 55°C for 30 second, 72°C for 1 minute, followed by 72°C for 10 minutes. Likewise, the 572-bp DNA fragment with 24-nt deletion in the miR-1253 binding site was amplified by overlapping PCR of Fragment 3 (F3) and Fragment 4 (F4). Briefly, the first 381-bp F3 was amplified with the use of sense primer (P1) and antisense primer (P5), 5′-CTACAATATTCCAATTGGGATAAGATGACTCCAG-3′, using the wild-type MDR1/3′ UTR plasmid as a template. The second 191-bp F4 was amplified with the use of the sense primer (P6), 5′-CTGGAGTCATCTTGTCCCAATTGGAATATTGTAG-3′, and antisense primer (P4) with the use of the wild-type MDR1/3′ UTR plasmid as a template. AscI site was added to the outer sense primer (P1), and Mlu I site was added to the outer antisense primer (P4) so that the recombinant DNA fragment can be cloned into pMirTarget vector with the use of the unique restriction sites. The PCR amplification cycles are the same as above. The sequence of the recombinant clones was confirmed by DNA sequencing and is called pMirTarget-MDR1-3′ UTR with deletion of miR-1253.

Luciferase Reporter Assay for MDR1-3′ UTR

The MDA-MB-231-R or MCF-7-Dox cells were seeded in 24-well plates and were transfected with 25 ng of the firefly luciferase MDR1-3′ UTR-reporter vector (PS100062; Origene Technology, Inc.) along with the miR-298, miR-1253 mimic (Ambion Pre-miR miRNA Precursor; Ambion by Life Technologies, Carlsbad, CA), or inhibitor (Ambion Anti-miR miRNA Inhibitor; Ambion by Life Technologies) with the use of LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen). After transfection for 24 hours, cells were lysed with a 1× Passive Lysis Buffer, and the activity of luciferase was assayed with the firefly luciferase reporter assay system (Promega, Valencia, CA) according to the manufacturer's instructions. The values were normalized by total protein of cell lysate. The efficiency of lipofectamine transfection to breast cancer cells was determined by fluorescence microscopy with the use of Cy3-labeled RNA oligonucleotides.

Doxorubicin Nuclear Accumulation

Doxorubicin accumulation was assayed by a method described previously.[31] Briefly, MDA-MB-231-R cells or MDA-MB-231-S cells were transfected with the miRNAs mimics or miR-298 inhibitor with the use of lipofectamine. After 24 hours, transfected cells were incubated overnight with doxorubicin (0.72 μmol/L). Then cells were washed thrice with PBS, fixed with methanol, and observed under a fluorescence microscope (1 × 70; Olympus, Tokyo, Japan).

Results

Impaired Nuclear Translocation of Doxorubicin in MDA-MB-231-R Cells Occurs Because of the Increased Expression of P-gp

The antitumor effect of doxorubicin on the MDA-MB-231-R and MDA-MB-231-S breast cancer cells was investigated by MTT assay. A significant difference in the doxorubicin-mediated cytotoxicity between the drug-resistant (MDA-MB-231-R) and drug-sensitive (MDA-MB-231-S) breast cancer cell line was observed. The doxorubicin at a concentration of 0.36 μmol/L leads to a 20% growth arrest in the MDA-MB-231-S cells, whereas only 2% to 4% growth arrest was seen with the use of the MDA-MB-231-R cells (Figure 1A). The cellular cytotoxicity of doxorubicin (0.36 μmol/L) with the use of the two cell lines was also measured by MTT assay for 24, 48, and 72 hours, which shows a time-dependent increase in cytotoxicity (Figure 1B). The ability of doxorubicin to induce cell cycle arrest and apoptosis between the MDA-MB-231-R and MDA-MB-231-S cell lines was measured by flow cytometry (Figure 1C). These results indicate that doxorubicin treatment leads to G_2/M growth arrest in the majority of cells (89.59%) and that only 10% of cells stay in the S phase of cell cycle in MDA-MB-231-S, whereas only 16% G_2/M growth arrest was seen in MDA-MB-231-R.

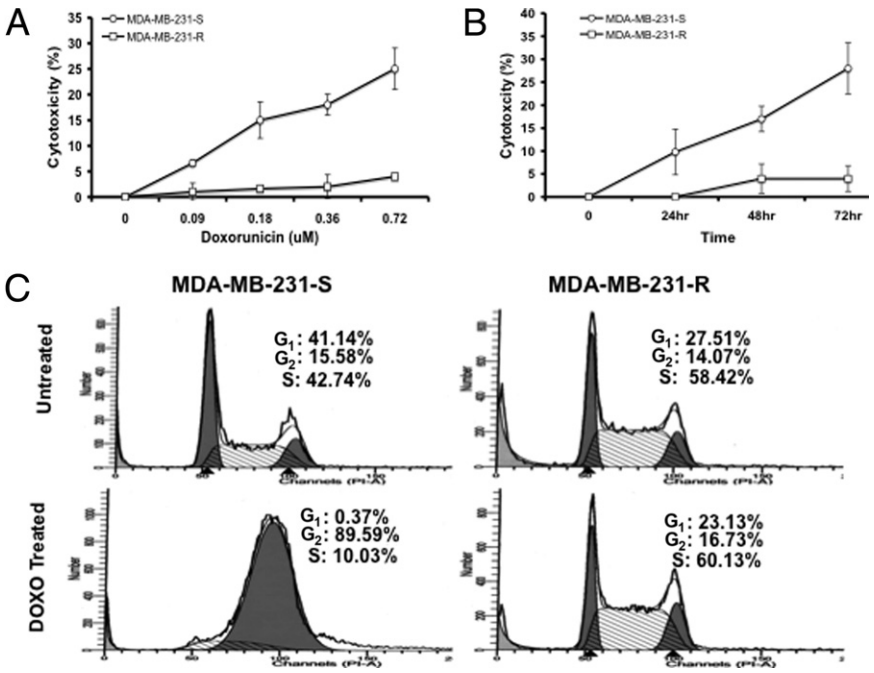

Figure 1. Characterization of the MDA-MB-231-R and MDA-MB-231-S cell lines. **A:** MTT assay showing the cytotoxicity of the two cell lines on dose-dependent treatment of doxorubicin (0.09 to 0.72 μmol/L). Cells were treated with different concentrations of doxorubicin, and after 48 hours of treatment doxorubicin-mediated cytotoxicity between the cells was determined by MTT assay. **B:** MTT assay showing cytotoxicity of MDA-MB-231-S and MDA-MB-231-R cell lines at 0, 24, 48, and 72 hours after doxorubicin treatment (0.36 μmol/L). **C:** Cell cycle analysis of MDA-MB-231-S and MDA-MB-231-R cells after doxorubicin (DOXO) treatment. Cells were cultured in 100-mm tissue culture dishes and treated with doxorubicin (0.18 μmol/L) for 48 hours. Cells were harvested by trypsin-EDTA, treated with propidium iodide, and analyzed by flow cytometry. The **left panels** show the cell cycle profile of doxorubicin-sensitive (MDA-MB-231-S) cells in the absence and the presence of the doxorubicin. The **right panels** show cell cycle profile of doxorubicin-resistant (MDA-MB-231-R) cells in the absence and the presence of doxorubicin. The percentage of cells in G_1, G_2, and S phases of cell cycle is listed for each analysis. Data are expressed as mean ± SD (**A** and **B**).

With the use of the TUNEL assay in the 4T1 breast cancer model, we have shown that doxorubicin treatment induced apoptotic cell death because of DNA break in most of the drug-sensitive breast cancer cells,[31] whereas in the resistant breast cancer cell line no TUNEL-positive cells were present. To understand the mechanism of doxorubicin resistance, the cellular uptake of doxorubicin between the MDA-MB-231-S and MDA-MB-231-R cells was compared by flow cytometry. MDA-MB-231-S and MDA-MB-231-R cells were treated with doxorubicin (0.18 μmol/L). After 24 hours, the intracellular doxorubicin fluorescence was measured by flow cytometry (Figure 2A). The results indicate that a shift in the fluorescence peak because of intracellular doxorubicin between the MDA-MB-231-S and MDA-MB-231-R cells indicates that doxorubicin is able to cross the cell membrane. The fluorescence microscopic results indicate that most of the

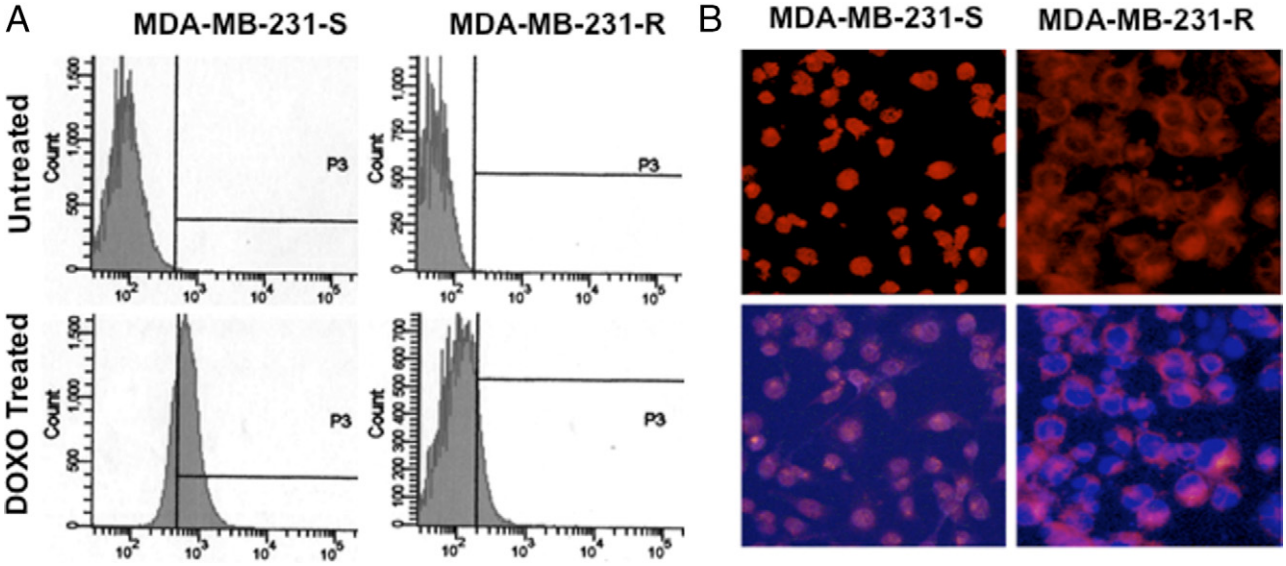

Figure 2. Impaired uptake and nuclear translocation of doxorubicin in the MDA-MB-231-R cells. MDA-MB-231-S and MDA-MB-231-R cells cultured in a 100-mm plate were treated with doxorubicin (0.18 μmol/L) for 24 hours. Cells were washed and analyzed for cellular uptake and fluorescence microscopy. **A:** Flow cytometric analyses of MDA-MB-231-S and MDA-MB-231-R cells show the intracellular uptake of doxorubicin (DOXO). There is a shift in the fluorescence peak in the doxorubicin-treated cells compared with the untreated cells. **B:** Fluorescence microscopy images show the MDA-MB-231-S and MDA-MB-231-R cells at 24 hours after doxorubicin treatment. The **left panels** show that doxorubicin localized in the nucleus of MDA-MB-231-S cells. The **right panels** show that most of doxorubicin fluorescence was observed in the cytoplasm of MDA-MB-231-R cells. The **upper panels** show the red fluorescence of doxorubicin. The **lower panels** are the composite image with Hoechst nuclear staining (original magnification, ×20).

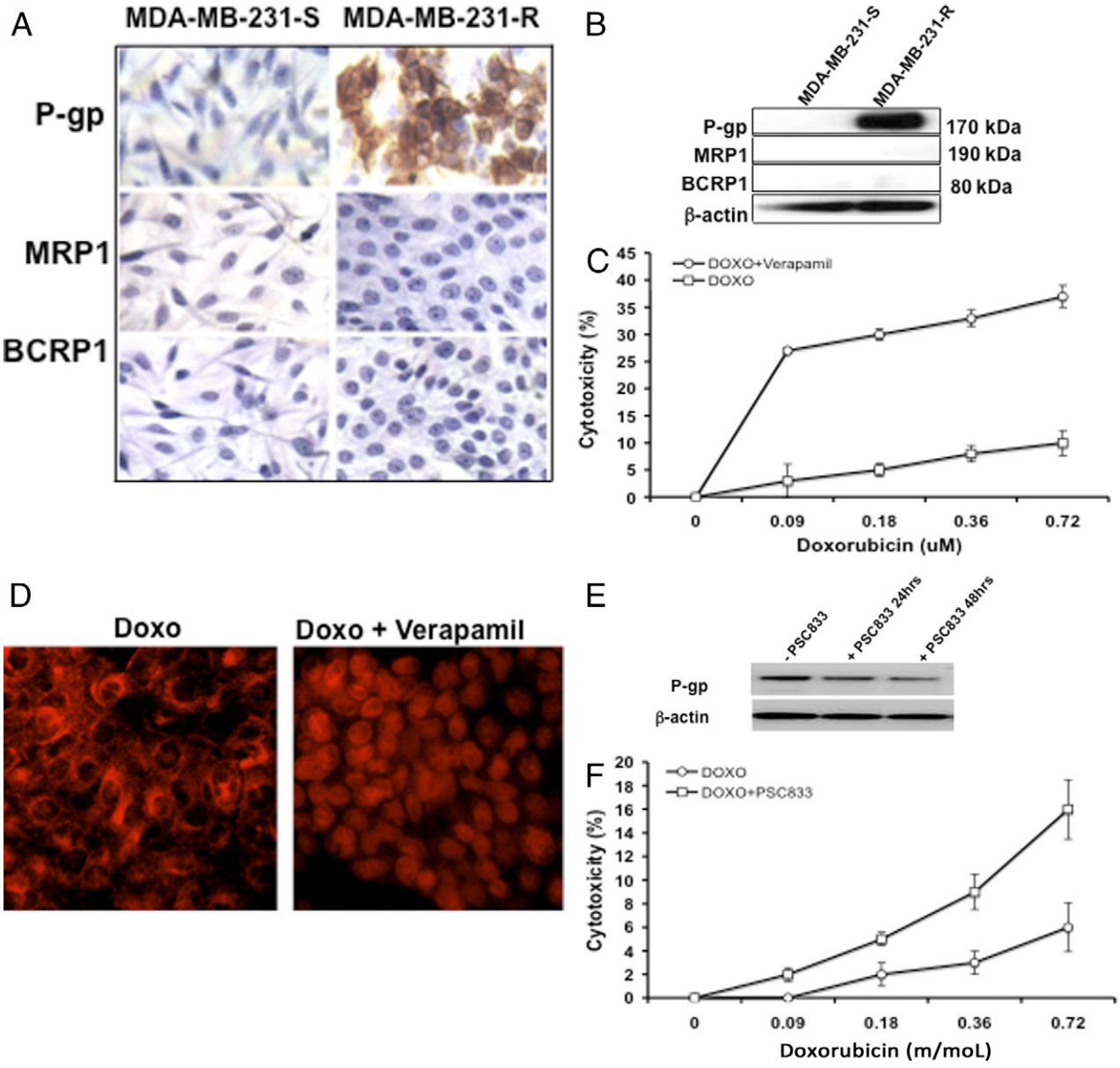

Figure 3. Increased expression of P-gp in doxorubicin-resistant breast cancer cell line and inhibition of its expression improved cellular cytotoxicity. **A:** Immunocytochemical staining shows the expression of three different drug efflux pumps (MDR1, MRP1, and BCRP) in MDA-MB-231-S and MDA-MB-231-R cells with the use of specific monoclonal antibodies against MDR1, MRP1, and BCRP. Strong cytoplasmic and nuclear stainings of P-gp expression were seen only in the MDA-MB-231-R cells. The expression of P-gp in MDA-MB-231-S cells was undetectable. The expression of MRP1 and BCRP1 in both cells was negative (original magnification, ×20). **B:** Western blot analysis confirms the expression of these proteins in MDA-MB-231-S and MDA-MB-231-R cells with the use of MDR1, MRP1, and BCRP antibodies. Antibody to β-tubulin was used as loading control. **C:** Verapamil pretreatment improves the cytotoxicity of doxorubicin. MDA-MB-231-R cells were pretreated with 20 μmol/L verapamil for 2 hours and then treated with an increasing concentration of doxorubicin for 48 hours, and cytotoxicity was measured by MTT assay. **D:** Verapamil pretreatment facilitates nuclear translocation of doxorubicin (Doxo). MDA-MB-231-R cells in culture were treated with 20 μmol/L verapamil for 2 hours and then treated with doxorubicin (0.72 μmol/L) for 24 hours. Nuclear translocation was examined under a fluorescence microscope. The **left panel** shows the nuclear translocation of doxorubicin in the MDA-MB-231-R cells without verapamil treatment. The **right panel** shows the nuclear translocation of doxorubicin (red) in the MDA-MB-231-R cells in the presence of verapamil. Original magnification, ×20. **E:** Treatment with the second-generation P-gp inhibitor (PSC833) at 10 μmol/L concentration reduces its expression in MDA-MB-231-R cells by Western blot analysis in a time-dependent manner (24 to 48 hours). **F:** MTT assay shows that pretreatment with PSC833 improves the doxorubicin cytotoxicity of MDA-MB-231-R cells. Data are expressed as mean ± SD (**C** and **F**).

doxorubicin efficiently localized in the nucleus of MDA-MB-231-S cells. In the MDA-MB-231-R cells, doxorubicin is unable to reach the nucleus and remained exclusively in the cytoplasm (Figure 2B). To examine whether expression of different drug efflux proteins could correlate with the impaired nuclear uptake of doxorubicin in MDA-MB-231-R cells, the expression of P-gp, MRP, and BCRP between the MDA-MB-231-S and MDA-MB-231-R cells was examined by immunocytochemical staining (Figure 3A). To support the immunocytochemistry results, the expression of P-gp, MRP1, and BCRP1 was also confirmed by Western blot analysis (Figure 3B). We observed a high-level nuclear and cytoplasmic expression of P-gp in MDA-MB-231-R

cells but lower expression in MDA-MB-231-S cells. Both cell lines showed negative expression of MRP and BCRP by immunocytochemical staining and Western blot analysis. To verify the role of increased expression of P-gp in MDA-MB-231-R cells, experiments were performed to examine whether inhibiting the expression of P-gp in the MDA-MB-231-R cells could increase the nuclear translocation of doxorubicin and cytotoxicity. We used verapamil, a first-generation MDR efflux pump inhibitor.[32] The MDA-MB-231-R cells in culture were pretreated with verapamil at a concentration of 20 μmol/L for 2 hours, the cells were then treated with doxorubicin at an increasing concentration (0.09, 0.18, 0.36, and 0.72 μmol/L), and MTT assay results indicate that pretreatment with verapamil improved doxorubicin toxicity of MDA-MB-231-R cells after 48 hours (Figure 3C). The improved cytotoxicity was due to increased nuclear translocation of doxorubicin seen under a fluorescence microscope (Figure 3D). We also used a second-generation P-gp inhibitor (PSC833) to examine whether it could improve doxorubicin-mediated cytotoxicity in MDA-MB-231-R cells. PSC833 is well known as a nonimmunosuppressant cyclosporine analog that functionally inhibits P-gp.[33] Western blot analysis showed that pretreatment of MDA-MB-231-R cells with PSC833 at a concentration of 10 μmol/L after 24 hours inhibited the expression of P-gp (Figure 3E). Treatment of MDA-MB-231-R cells with PSC833 followed by doxorubicin improved the cytotoxicity by MTT assay (Figure 3F). These results indicate that P-gp expression is partly responsible for impaired nuclear translocation of doxorubicin and cytotoxicity.

Down-Regulation of miR-298 in MDA-MB-231-R Cells

Because the miRNAs are an important modulator of cancer cell signaling and chemotherapy response, we generated an miRNA profile between MDA-MB-231-S and MDA-MB-231-R cells. We found a significant up-regulation and down-regulation of miRNAs in MDA-MB-231-R cells compared with MDA-MB-231-S cells. Among the list, we only show 10 miRNAs whose expression significantly changed between

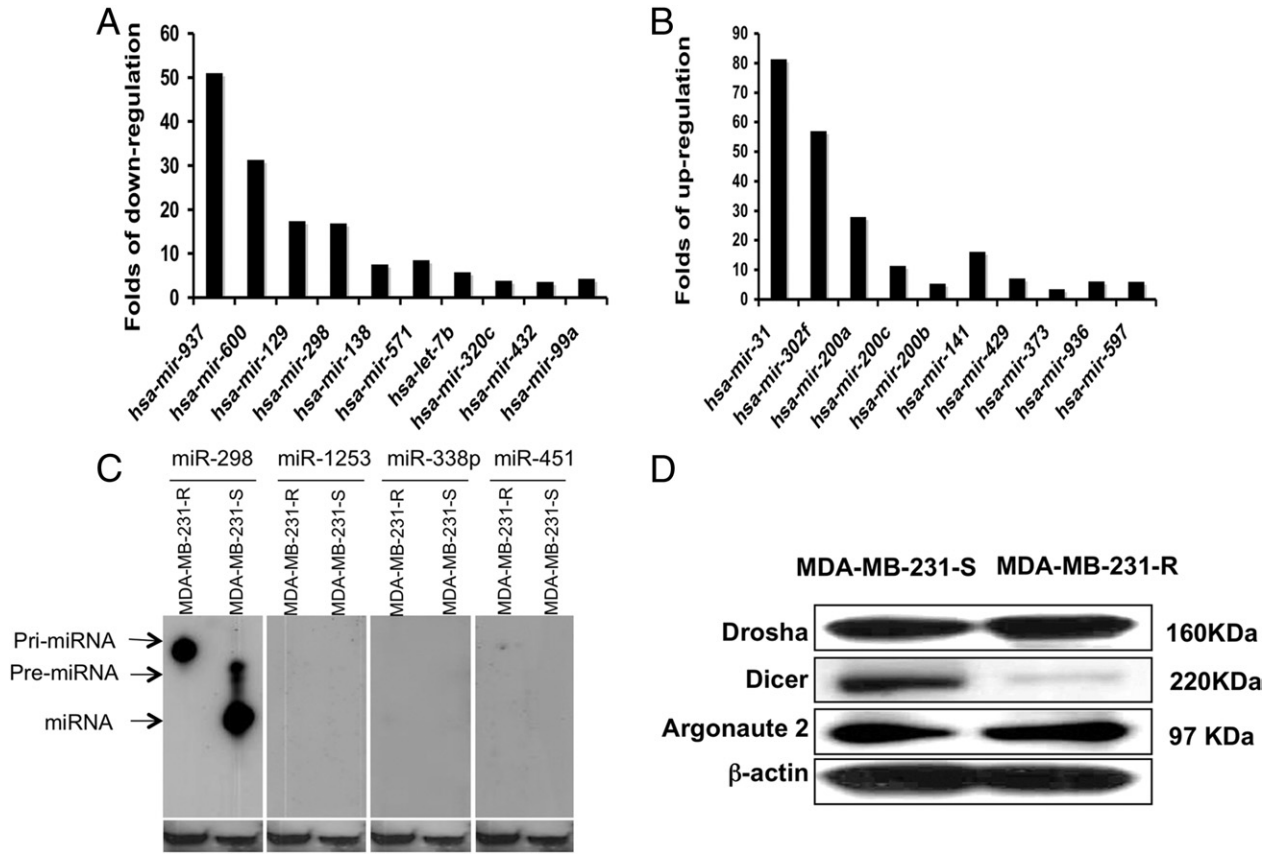

Figure 4. Differential expression of miRNAs between MDA-MB-231-S and MDA-MB-231-R cells by miRNA array analysis. Data are shown as fold changes of miRNA levels (normalized median values) in MDA-MB-231-S versus MDA-MB-231-R cells. The relative levels were normalized to U6snRNA. **A:** miRNAs that are down-regulated in the MDA-MB-231-R cell line relative to the MDA-MB-231-S cells. **B:** miRNAs that are up-regulated in the MDA-MB-231-R cell line relative to the MDA-MB-231-S. **C:** Detection of both pre-miRNA and mature miR-298 in MDA-MB-231-S and MDA-MB-231-R cells by Northern blot analyses. Five micrograms of total RNA isolated from MDA-MB-231-S and MDA-MB-231-R cells was processed for Northern blot analysis with the use of probes specific to miR-298, miR-1253, miR-338, miR-451, and miR-U6. Most of miR-298 in MDA-MB-231-R cells that were pre-miRNA remained in the unprocessed form (top band). Only mature miRNA-298 can be detected in MDA-MB-231-S cells. We could not detect other miRNAs in MDA-MB-231-S and MDA-MB-231-R cells by Northern blot analysis. The miR-U6 used as a control was detected in both the cells; because membranes were stripped and reprobed, the control is identical for each miRNA tested. The **arrows** indicate the different forms of miRNA. **D:** Western blot analysis for Drosha, Dicer, and Argonaute 2 expression in MDA-MB-231-S and MDA-MB-231-R cells. The expression of Dicer is low in MDA-MB-231-R cells compared with MDA-MB-231-S cells. β-actin was used as a loading control.

these two cell lines (Figure 4, A and B). The P-gp is the predicted target of miR-298 and showed down-regulated expression in MDA-MB-231-R cells. Northern blot analysis was used to show the difference of miR-298 expression between MDA-MB-231-S and MDA-MB-231-R cells. The results of this experiment indicate that mature miR-298 and its precursor are highly expressed in MDA-MB-231-S cells compared with MDA-MB-231-R cells (Figure 4C). We also found that the processing of miR-298 was blocked in MDA-MB-231-R cells because we could detect only the precursor miRNA but no mature miRNA. Web-based miRNA search (*http://www.microRNA.org*, 2008 release, last accessed October 1, 2009) as well as published papers indicate that miR-1253, miR-451, miR-338, and miR-27a regulate the expression of P-gp by binding to the 3′ UTR of the MDR1 gene.[34–36] We could not detect any of these miRNAs levels in MDA-MB-231-R cells by Northern blot analysis. To show the cause of profound alternations in the miRNA profile in the MDA-MB-231-R cells, we decided to analyze the levels of the miRNA processing enzymes, Drosha, Dicer, and Argonaute 2, by Western blot analysis. Drosha is the core nuclease that executes the initiation step of miRNA processing in the nucleus[27] and cleaves double-strand primary-miRNA (>1-kb nt) to produce a stem-loop precursor molecule (pre-miRNA), ~70 nt in length. Dicer, a member of the RNase III superfamily of bidentate nuclease, mediates the latter step. After the pre-miRNA is transported to the cytoplasm, the pre-miRNA undergoes its final processing step, which involves cleavage by Dicer.[28] The involvement of Dicer in MDA-MB-231-R cells was examined. Argonaute protein 2 was associated with small RNAs that guide miRNA degradation, translational repression, or a combination of both.[28] Western blot analysis showed a strong down-regulation of Dicer levels in MDA-MB-231-R cells, but no different expression of Drosha and Argonaute in MDA-MB-231-S and MDA-MB-231-R cells was observed (Figure 4D). The significantly decreased level of Dicer may explain the potential reason for the dysregulated expression of miRNAs in the MDA-MB-231-R cells.

MmiR-298 Targets the 3′ UTR of P-gp mRNA

For investigating the role of miRNAs in the P-gp regulation, we used a computational analysis of the 3′ UTR of MDR1 that found a putative binding site for miR-298, miR-1253.[34] Mature sequence of miR-298 (5′-AGCA-

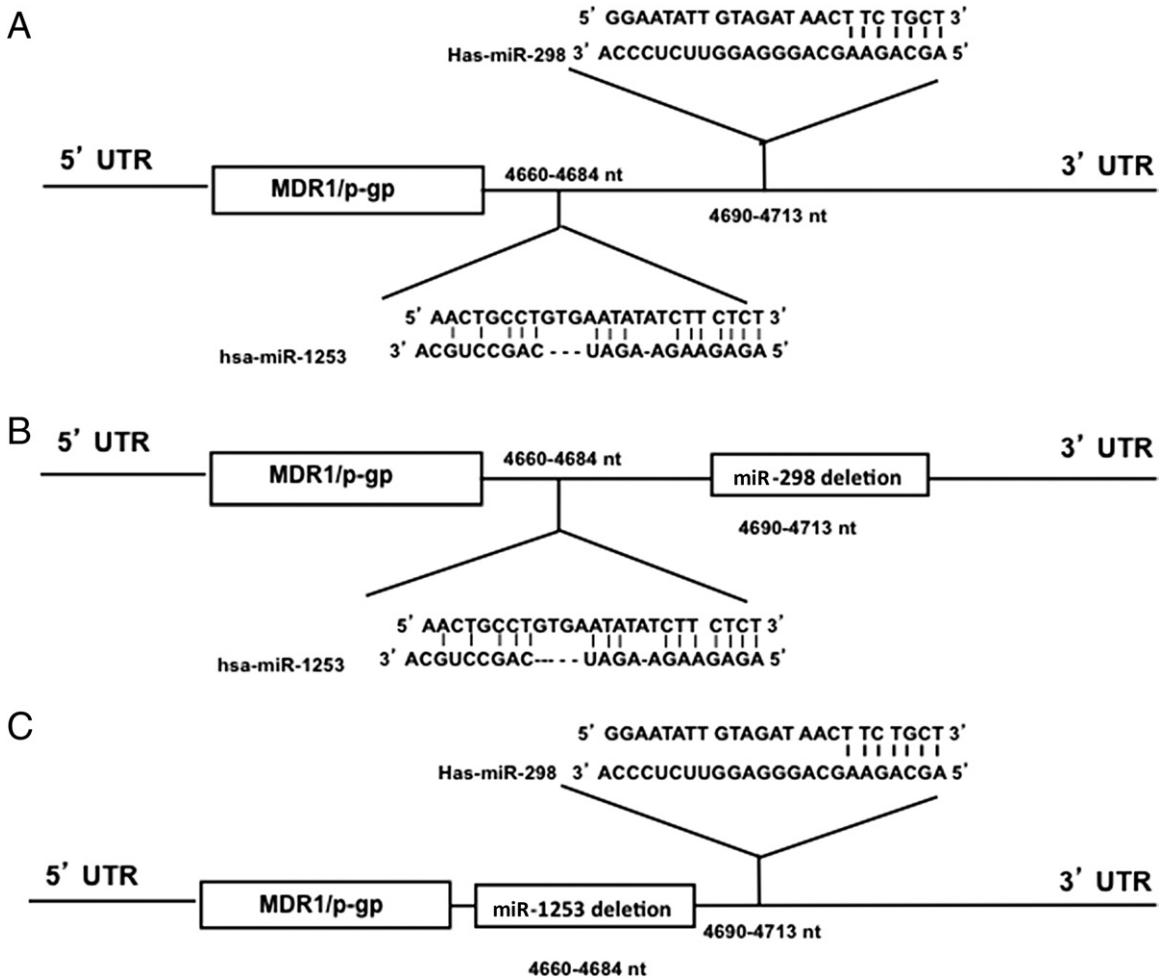

Figure 5. Schematic diagram showing the location and binding sites of miR-298, miR-1253 in the 3′ UTR region of p-gp mRNA of pmiRTarget-MDR1-3′ UTR-luciferase plasmid construct used in this study. **A:** Exact nucleotide (nt) location of miR-298 and miR-1253 binding sites in the 3′ UTR region of P-gp mRNA. **B:** Deletion of miR-298 binding site in 3′ UTR region of P-gp mRNA. **C:** Deletion of miR-1253 binding site in 3′ UTR region of P-gp mRNA.

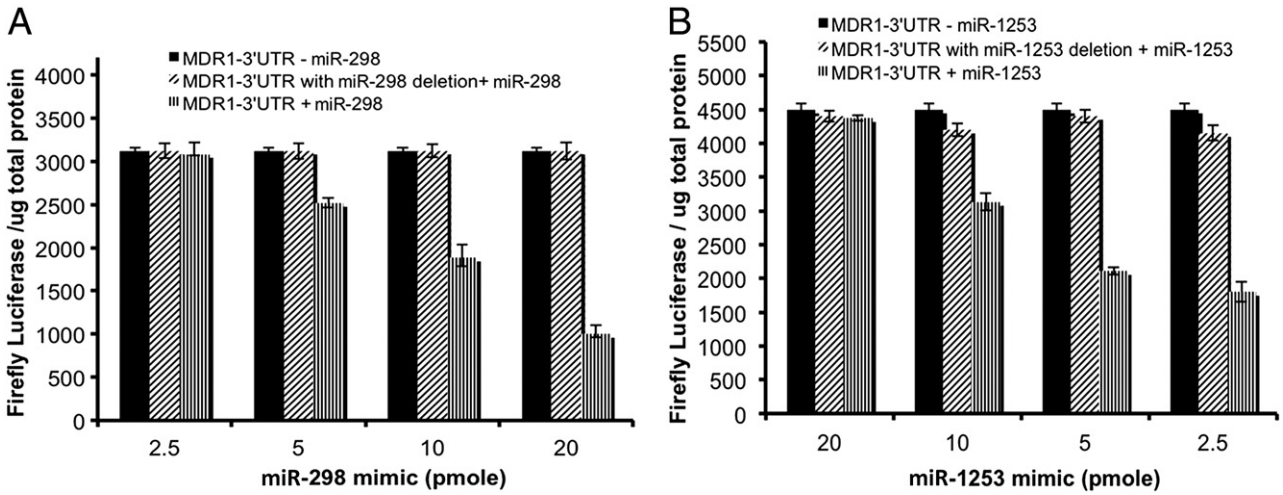

Figure 6. Effect of miRNA mimic transfection on the expression of MDR1-3′ UTR chimeric firefly luciferase in MDA-MB-231-R cells. **A:** miR-298 mimic inhibits the expression of chimeric firefly luciferase on pmiRTarget-MDR1-3′ UTR in a dose-dependent manner. The same miR-298 did not regulate the expression of firefly luciferase of pMirTarget-vector or pMirTarget-MDR1-3′ UTR with deletion of miR-298 binding site. **B:** miR-1253 mimic also inhibits the expression of pMirTarget-MDR1-3′ UTR dose-dependent manner but not the pMirTarget-vector and pMirTarget-MDR1-3′ UTR with deletion of miR-1253. Data are expressed as mean of triplicated values ± SD of the mean.

GAAGCAGGGAGGUUCUCCCA-3′) matched MDR1 mRNA at 4681 to 4705 nt, and miR-1253 (5′-AGAGAA-GAAGAUCAGCCUGCA-3′) matched at 4660 to 4681 nt (Figure 5A). To understand whether MDR1 is functionally targeted by miR-298 and miR-1253, 3′ UTR of MDR1 with miR-298 and miR-1253 complimentary site was cloned into pMirTarget, the clone vector for all 3′ UTRs for miRNA target validation. Two plasmid constructs with a deletion of each miRNA (miR-298 or miR-1253) sequences was prepared and used as a control in our assays (Figure 5, B and C). With the use of a transient transfection, we found that miR-298 mimic inhibited the firefly luciferase expression only on the wild-type pMirTarget-MDR1-3′ UTR construct with the miR-298 binding site in a concentration-dependent manner (Figure 6A). The luciferase expression of pMirTarget-MDR1-3′ UTR without the miR-298 binding sites did not change after mimic transfection and remained comparable with the untreated samples. Likewise, we observed that miR-1253 mimic showed a dose-dependent decrease in the luciferase expression of the pMirTarget-MDR1-3′ UTR construct by transient transfection. Both the miR-1253 deletion plasmid transfection and the untreated samples show similar readings, indicating that the miR-1253 did not inhibit luciferase expression (Figure 6B).

Overexpression of miR-298 Down-Regulates P-gp Expression and Nuclear Translocation of Doxorubicin and Increases Doxorubicin Cytotoxicity

Western blot analysis and immunocytochemical staining found that MDA-MB-231-R cells exhibit a high expression of P-gp, compared with MDA-MB-231-S cells. We used miRNA mimics to confirm that miR-298 alters the expression levels of P-gp in MDA-MB-231-R cells. MDA-MB-231-R cells were transfected with miR-298 and/or miR-1253 mimics by lipofectamine, and after 24 hours the level of P-gp expression in the presence and absence of miRNA mimic was examined by Western blot analysis. Results (Figure 7A) indicate that miR-298 and miR-1253 each treated either individually or in combination inhibited the expression of P-gp. We then examined whether inhibiting P-gp by miRNA mimics could also increase cytotoxicity. Cells were transfected overnight with miR-298 and/or miR-1253 into MDA-MB-231-R cells and the next day treated with doxorubicin, and cell viability was measured by MTT assay after 24 hours (Figure 7B). Because the impaired nuclear translocation was caused by the high-level expression of P-gp, we examined whether transfection of the miRNA mimics could improve the nuclear translocation of doxorubicin in MDA-MB-231-R cells under fluorescence microscopy (Figure 7C). The results clearly show that pretreatment with miR-298 and miR-1253 individually or in combination increased nuclear translocation of doxorubicin. The efficiency of miR transfection to breast cancer cells with the use of lipofectamine was determined to be 100% with Cy3-labeled RNA oligonucleotides (Figure 7D).

Down-Regulation of miR-298 in MDA-MB-231-S Cells Induced Expression of P-gp and Doxorubicin Chemoresistance

To assess the potential interaction between miR-298 and P-gp expression in chemoresistance, we performed reverse experiments to determine whether the doxorubicin chemoresistance can be restored by inducing the expression of P-gp in MDA-MB-231-S cells by down-regulating the expression of miR-298 by transient transfection with miR-298 inhibitor. MDA-MB231-S cells were trans-

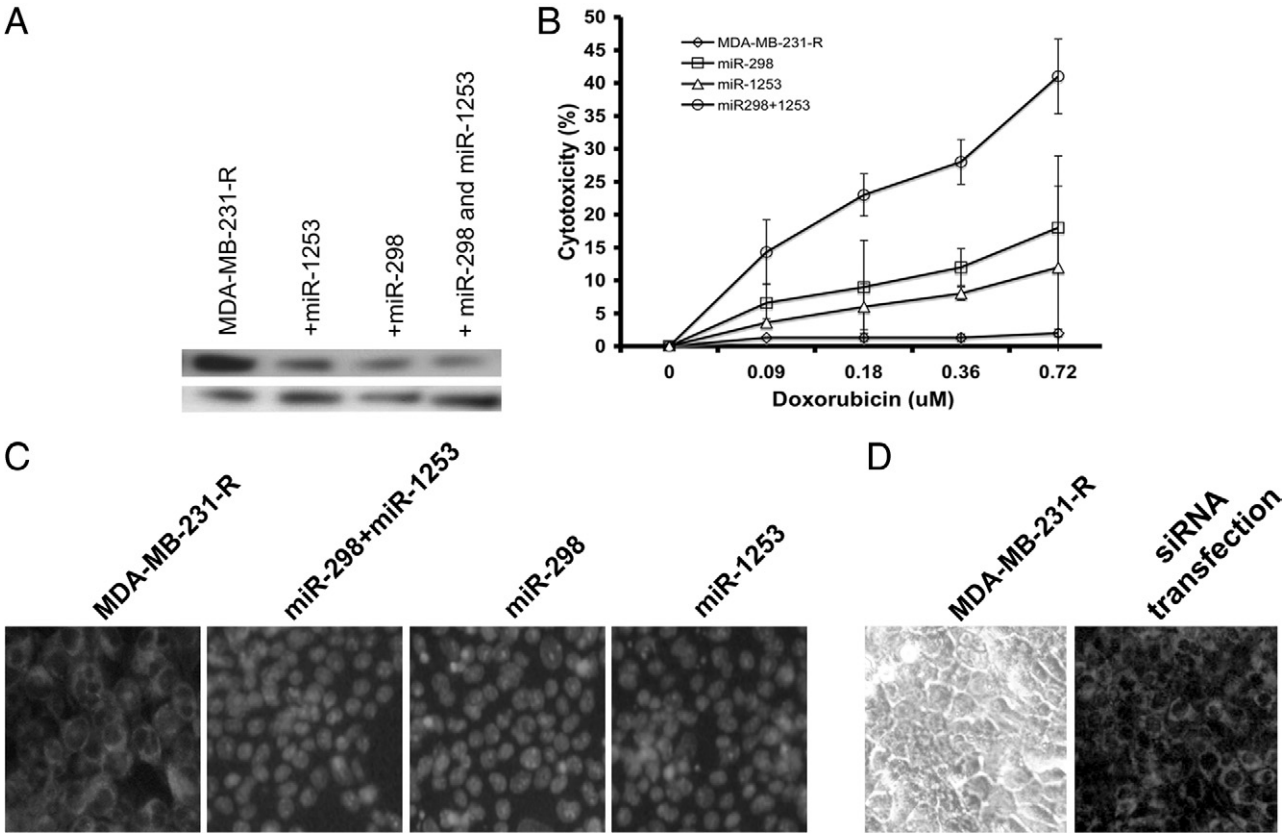

Figure 7. Overexpression of miR-298 inhibits P-gp expression, increased cytotoxicity, and nuclear translocation of doxorubicin in MDA-MB-231-R cells. **A:** MDA-MB-231-R cells were transfected individually with miR-298, miR-1253, or in combination with the use of lipofectamine. After 24 hours, P-gp expression in MDA-MB-231-R cells was examined by Western blot analysis. P-gp expression was inhibited in MDA-MB-231-R cells with miR-298 alone as well as in the combination with miR-1253. **B:** MTT assay shows the doxorubicin cytotoxicity in MDA-MB-231-R cells with or without miRNA transfection. MDA-MB-231-R cells were transfected with miR-298 and miR-1253 singly and in combination. After 24 hours, cells were treated with doxorubicin (0.09 to 0.72 μmol/L). MTT assay was performed after 24 hours. Doxorubicin cytotoxicity of MDA-MB-231-R cells was increased when transfected with miR-298 or miR-1253 individually or in combination. **C:** Fluorescence microscope images of doxorubicin nuclear translocation in MDA-MB-231-R cells without and with miRNA transfection. Original magnification, ×20. The miR-298, miR-1253, and the combination treatment improve the nuclear translocation of doxorubicin (fluorescence in nucleus). **D:** Efficiency of lipofectamine transfection in MDA-MB-231-Dox cells with the use of Cy3-labeled oligonucleotide. Data are expressed as mean ± SD (**B**). siRNA, small-interfering RNA (original magnification, ×20).

fected overnight with inhibitor. After 24 hours, the level of P-gp was examined by Western blot analysis and luciferase activity. We found that silencing miR-298 by selective inhibitor lead to a dose-dependent increase in the activity of MDR-3′ UTR luciferase activity in MDA-MB-231-S cells but not in the controls (Figure 8A). MTT assay was performed to determine whether down-regulation of miR-298 could alter the doxorubicin cytotoxicity of MDA-MB-231-S cells. MDA-MB-231-S cells were pretreated with miR-298 inhibitor by lipofectamine for 48 hours, and then cells were treated with an increasing concentration of doxorubicin for 72 hours. Cytotoxicity was measured by MTT assay (Figure 8B). The expression level of P-gp in MDA-MB-231-S cells with or without transfection with miR-298 inhibitor was examined by Western blot analysis (Figure 8C). MDA-MB-231-S cells transfected with miR-298 inhibitor showed impaired nuclear translocation of doxorubicin compared with the untreated cells (Figure 8D). These analyses concluded that transfection with miR-298 inhibitor in MDA-MB-231-S cells has made it partially resistant to doxorubicin because of increased P-gp expression.

Overexpression of miR-298 Down-Regulates P-gp Expression in MCF-7-Dox Cells

The role of miR-298 in regulating P-gp expression was verified with another human breast cancer cell line (MCF-7) resistant to doxorubicin. First, MTT assay was performed to confirm the doxorubicin cytotoxicity between doxorubicin-sensitive and doxorubicin-resistant MCF-7 breast cancer cells. MTT assay shows a significant difference in the doxorubicin-mediated cytotoxicity between the drug-resistant (MCF-7-Dox) and the parental MCF-7 breast cancer cell line (Figure 9A). The doxorubicin at a concentration of 2.88 μmol/L leads to a 40% growth arrest in the MCF-7 cells, whereas only 4% growth arrest was seen with the MCF-7-Dox cells. The expression of P-gp between the MCF-7 and MCF-7-Dox cell lines was analyzed by Western blot analysis (Figure 9B). We observed a high-level expression of P-gp in MCF-7-Dox cells but lower expression in MCF-7 cells. To confirm that the miR-298 mimic also regulates P-gp luciferase reporter, MCF-7-Dox cells were cotransfected with pMirTarget-MDR1-3′ UTR plasmid with or without miR-298 binding

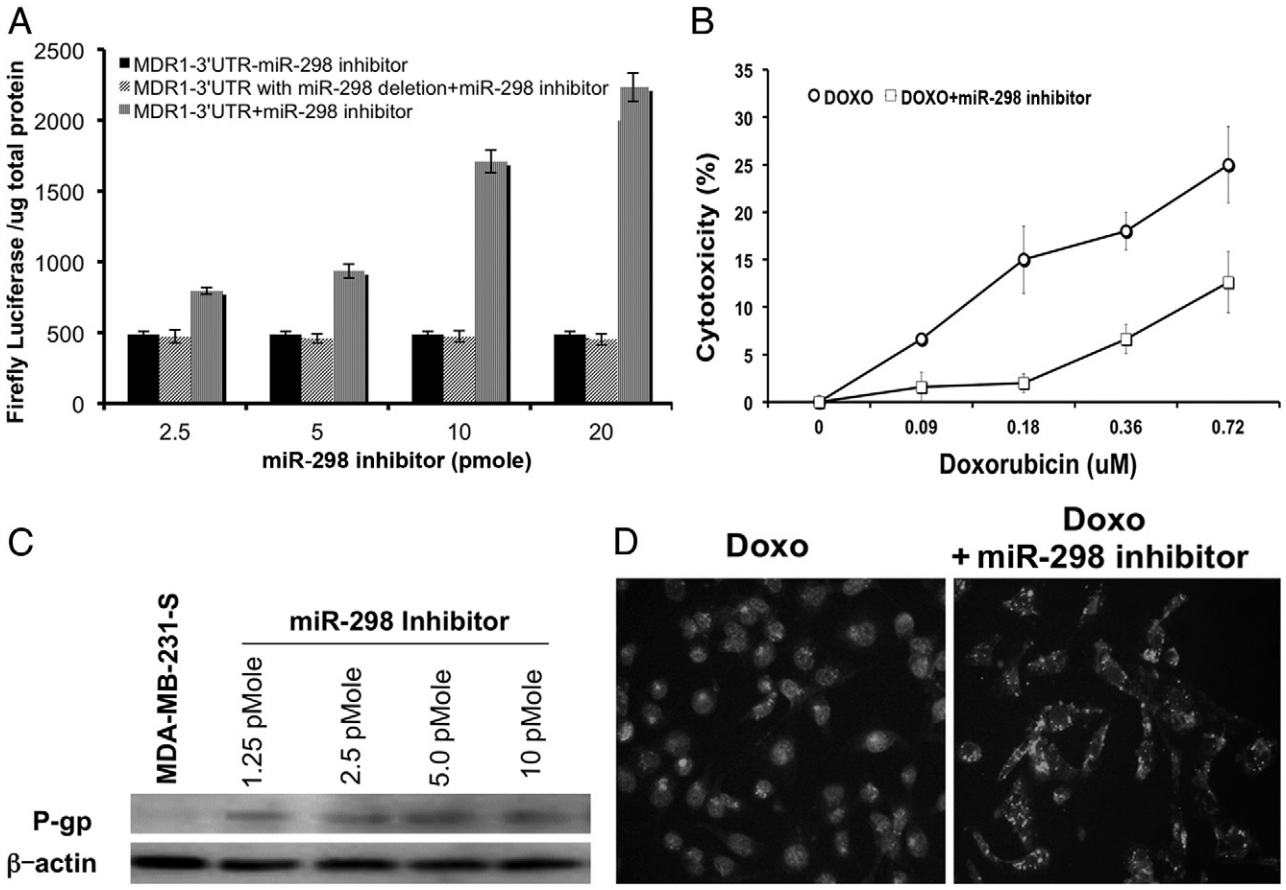

Figure 8. Inhibition of miR-298 in MDA-MB-231-S cells increased the expression of P-gp and induced doxorubicin resistance. **A:** Transient transfection of miR-298 inhibitor increased the expression of firefly luciferase gene containing the MDR-3′ UTR sequences in a dose-dependent manner (1.25 to 10 pmole). Data were normalized with total protein of the lysates. **B:** MTT assay shows the dose-dependent cytotoxicity of doxorubicin (DOXO) in MDA-MB-231-S cells with or without transfection with miR-298 inhibitor. β-Actin was used as a loading control. **C:** Inhibition of miR-298 expression in MDA-MB-231-S cells increased expression of P-gp on a dose-dependent manner as shown by Western blot analysis. **D:** Representative fluorescence microscopic pictures of the nuclear translocation of doxorubicin (0.72 μmol/L) in MDA-MB-231-S cells without and with miR-298 inhibitor (20 pmole) (original magnification, ×20). Data are expressed as mean ± SD (**A** and **B**).

sites and miR-298 mimic. After 48 hours, luciferase assay was performed with the protein lysate of transfected cells. We show that the miR-298 mimic inhibited the firefly luciferase expression only from pMirTarget-MDR1-3′ UTR construct in a concentration-dependent manner (Figure 9C). No change in the luciferase expression was observed with the use of pMirTarget-MDR1-3′ UTR with deletion of the miR-298 construct. The role of miR-1253 regulation of P-gp luciferase expression in MCF-7-Dox cells was examined in a transient transfection with miR-1253 mimic. We observed that miR-1253 mimic also inhibited pMirTarget-MDR-3′ UTR luciferase expression by a concentration-dependent manner to a lesser extent than miR-298. The luciferase expression of pMirTarget-MDR1-3′ UTR without deletion of miR-298 construct did not show inhibition and remained similar to the untreated cells (Figure 9D). The results of MCF-7-Dox experiments are identical to that with the MDA-MB-231-R cells.

Discussion

miRNAs are short noncoding RNAs that posttranscriptionally regulate expression of the target genes in a specific tissue and/or cells at certain stages of their development and/or cellular response to chemotherapy drugs. More studies are now providing evidence to indicate that they are important in regulating the expression of genes involved in chemoresistant mechanisms in cancer treatment.[37] To address the chemoresistant mechanisms of human breast cancer, we have developed chemoresistant human breast cancer (MDA-MB-231) cells. We used doxorubicin as a model drug that was marked as Adriamycin. We showed that the increased expression of P-gp in MDA-MB-231-R cells is associated with impaired nuclear translocation of doxorubicin. In our experimental model system, we show that doxorubicin was localized mostly in the cytoplasm in MDA-MB-231-R cells, whereas doxorubicin was efficiently localized to the nucleus in the MDA-MB-231-S cells.

We show that doxorubicin chemoresistance of breast cancer is due to deregulated expression of miRNA expression. This was supported by miRNA array data between MDA-MB-231-S and MDA-MB-231-R cells. The miRNA data indicated that numerous miRNAs are up-regulated and down-regulated in MDA-MB-231-R cells compared with MDA-MB-231-S cells. Because the in-

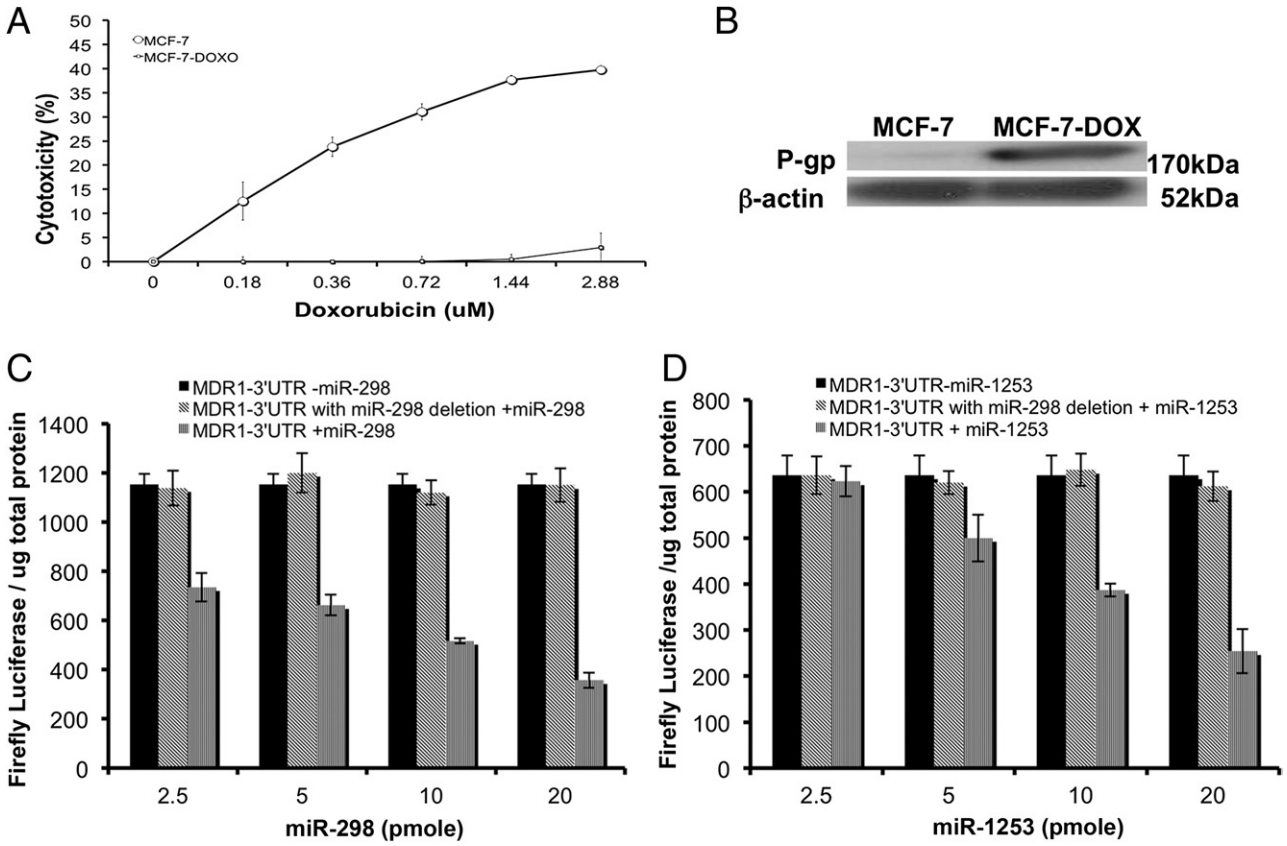

Figure 9. MCF-7-Dox cells show increased expression of P-gp, and miR-298 down-regulates expression of MDR1-3′ UTR chimeric firefly luciferase **A:** MTT assay showing the doxorubicin (0.18 to 2.88 μmol/L) cytotoxicity between MCF-7 and MCF-7-Dox cell lines at 48 hours. **B:** Western blot analyses show the expression of P-gp between MCF-7 and MCF-7-Dox cells. Antibody to β-tubulin was used as loading control. **C:** miR-298 mimic inhibits the expression of chimeric firefly luciferase pMirTarget-MDR1-3′ UTR in a dose-dependent manner. The same miR-298 did not regulate the expression of firefly luciferase pMirTarget-vector and pMirTarget-MDR1-3′ UTR with deletion of miR-298. **D:** miR-1253 mimic also inhibits the expression of pMirTarget-MDR1-3′ UTR dose-dependent manner but not on the pMirTarget-vector and pMirTarget-MDR1-3′ UTR with deletion of miR-1253. Data are expressed as mean of triplicated values ± SD of the mean (**C** and **D**).

creased drug efflux because of the high-level expression of P-gp is the main mechanism involved in the doxorubicin chemoresistance, we specifically found the down-regulated expression of miRNAs that targeted to the P-gp mRNA. We have identified miR-298 whose low-level expression is associated with increased P-gp expression. Our results clearly show that the miR-298 expression is altered in MDA-MB-231-R cells by Northern blot analysis. Because of the low-level expression of Dicer, most of the miR-298 remained in the unprocessed form. To confirm the role of miR-298 in regulating the expression of P-gp, we show that transfection of miR-298 mimic into MDA-MB-231-R cells down-regulated expression of P-gp by Western blot analysis as well as luciferase activity. We also found that transfection of miR-298 mimic into MDA-MB-231-R cells improved drug transport and nuclear translocation of doxorubicin and cytotoxicity by MTT assay. The nuclear translocation of doxorubicin in the miR-298-transfected cells is comparable with nontransfected cells. Results of these functional studies support the role of miR-298 in regulating the expression of P-gp. Taken together these findings suggest that correcting the expression of miRNAs overcame drug resistance and improved cytotoxicity to doxorubicin.

The role of miRNAs in mediating chemotherapy resistant mechanisms is relatively new. At present there are few laboratories, including ours, that study the modulation of P-gp by miRNAs.[35,36] On the basis of the published reports, there are four miRNAs claimed to be associated with P-gp-related chemoresistant mechanisms. Zhu et al[36] reported that increased expression of miR-27a and miR-451 contributes to the MDR and modulates the expression of P-glycoprotein in human ovarian cancer cell line. These investigators showed that transfection of drug-resistant cells with antagomirs of miR-27a or miR-451 decreased the expression, suggesting that these two miRNAs regulate expression of P-gp. In another study, Kovalchuck et al[35] found that low-level expression of miR-451 is associated with increased expression of P-gp and doxorubicin chemoresistance of the MCF-7 breast cancer cell line. There are also reports that other multidrug-resistant proteins (MRP-1 and BCRP/ABCG2) are also regulated by miRNAs.[38,39] In this study, we examined the role of miR-298, miR-338, miR-1253, and miR-451 in regulating the expression of P-gp in our MDA-MB-231-R cells. Among these four miRNAs tested, only miR-298 expression was confirmed by Northern blot analysis. We could not detect any other miRNAs reported

earlier by Northern Blot analysis. The microRNA-298 has showed promising results in regulating the expression of P-gp and increased uptake of doxorubicin and in inducing cytotoxicity. Our results also bring evidence to indicate that the altered expression of miRNA-processing machinery (Dicer) is significantly altered in the doxorubicin-resistant cell line, leading to improper processing of miR-298. Our results are also supported by a recent study indicating that altered expression of miRNA regulators, Drosha and/or Dicer, are associated with a specific population of triple-negative human breast cancer, such as the MDA-MB-231 cell line.[40] The exact mechanism underlying the effect of altered expression of miR-298 and P-gp need to be established in the future. In summary, the results of our study suggest that miR-298 is a potential marker for identifying chemoresistance of human breast cancer. We propose that targeted delivery of miR-298 should reduce the expression of P-gp and improve chemotherapy response of metastatic human breast cancer.

Acknowledgment

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