

Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin

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Abstract

Many chemotherapy regimens are successfully used to treat breast cancer; however, often breast cancer cells develop drug resistance that usually leads to a relapse and worsening of prognosis. We have shown recently that epigenetic changes such as DNA methylation and histone modifications play an important role in breast cancer cell resistance to chemotherapeutic agents. Another mechanism of gene expression control is mediated via the function of small regulatory RNA, particularly microRNA (miRNA); its role in cancer cell drug resistance still remains unexplored. In the present study, we investigated the role of miRNA in the resistance of human MCF-7 breast adenocarcinoma cells to doxorubicin (DOX). Here, we for the first time show that DOX-resistant MCF-7 cells (MCF-7/DOX) exhibit a considerable dysregulation of the miRNAome profile and altered expression of miRNA processing enzymes Dicer and Argonaute 2. The mechanistic link of miRNAome deregulation and the multidrug-resistant phenotype of MCF-7/DOX cells was evidenced by a remarkable correlation between specific miRNA expression and corresponding changes in protein levels of their targets, specifically those ones that have a documented role in cancer drug resistance. Furthermore,

we show that microRNA-451 regulates the expression of multidrug resistance 1 gene. More importantly, transfection of the MCF-7/DOX-resistant cells with microRNA-451 resulted in the increased sensitivity of cells to DOX, indicating that correction of altered expression of miRNA may have significant implications for therapeutic strategies aiming to overcome cancer cell resistance. [Mol Cancer Ther 2008;7(7):2152–9]

Introduction

Resistance of cancer cells to chemotherapy continues to be a major clinical obstacle to the successful treatment of cancer, including breast cancer (1–3). Causes of cancer-specific drug resistance are currently believed to be linked to the random drug-induced mutational events (genetic hypothesis), to the drug-induced nonmutational alterations of gene function (epigenetic hypothesis), and, recently, to the drug-induced karyotypic changes (karyotypic hypothesis; refs. 4–8). The absence of convincing evidence that genetic changes have a role in acquired clinical resistance following anticancer therapy undermines the genetic hypothesis (5). In contrast, conclusive data show that increased resistance of cancer cells to chemotherapeutic agents is associated with epigenetic alterations that include changes in DNA methylation and histone modifications (4, 5, 7). The karyotypic hypothesis (8) is closely related to the epigenetic one in view of the well-known fact that epigenetic changes are a necessary prerequisite to karyotypic changes (9). In this regard, karyotypic changes may be considered as a consequence of the epigenetic alterations progression and may serve as indirect evidence of the importance of epigenetic dysregulation in the acquisition of cancer drug resistance.

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 (10–12). Aberrant levels of microRNA (miRNA) have been reported in a variety of human cancers (13, 14), including breast cancer (15, 16). They have been shown to have both diagnostic and prognostic significance and to constitute a novel target for cancer treatment (17, 18). Considering the critical role of miRNA in cancer, we hypothesized that the acquisition of drug resistance by cancer cells may also be modulated via the changes in miRNA levels. A recent study by Climent et al. (19) suggests that the increased sensitivity of breast cancer patients to anthracycline-based chemotherapy may be related to the deletion of chromosome 11q, a region containing *miR-125b* gene. This finding was the first evidence to indicate a possible link between miRNA dysregulation and cancer drug resistance; however, the role of miRNA in the acquisition of drug resistance by cancer cells still remains elusive.

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Our present study for the first time shows that breast cancer cells resistant to doxorubicin (DOX) exhibit a pronounced deregulation of miRNA expression and the altered expression of miRNA processing enzymes. Moreover, we show that microRNA-451 (miR-451) regulates the expression of the multidrug resistance 1 (*mdr1*) gene, a crucial factor in drug resistance, and this interaction may have an important functional consequence in the formation of cancer cell resistance to chemotherapeutic agents.

Materials and Methods

Cell Lines and Cell Culture

The human breast adenocarcinoma MCF-7 cell line and MCF-7/DOX were cultured using Iscove's modified Dulbecco medium (Sigma) containing 10% newborn calf serum (HyClone) and 40 $\mu\text{g}/\text{mL}$ gentamicin at 37°C in a 5% CO₂ atmosphere. The MCF-7/DOX drug-resistant variant of the MCF-7 cell line was established by stepwise selection after prolonged (>6 months) treatment of MCF-7 cells to increasing concentrations of DOX at a range of 0.5 to 25 $\mu\text{mol}/\text{L}$ in the medium (20). After 6 months of culturing in the presence of DOX, the IC₅₀ (inhibitory concentration to produce 50% cell death) values were 24 and 1 $\mu\text{mol}/\text{L}$ DOX for the MCF-7/DOX and parental MCF-7 cells, respectively. Cells were seeded at a density of 0.5×10^6 viable cells per 100-mm plate, and the medium was changed every other day for 6 days. Trypsinized cells were washed in PBS and immediately frozen at -80°C for subsequent analyses. The experiments were independently reproduced twice, and each cell line was tested in triplicate.

Immunocytochemistry and Immunofluorescence

Expression of P-glycoprotein (P-gp), a product of the *mdr1* gene, in the MCF-7 and MCF-7/DOX cells was detected by immunocytochemistry as described by Chekhun et al. (20) and by immunofluorescence. Cells were cultured on glass coverslips for 24 h and fixed in PBS containing 0.4% paraformaldehyde. The fixed cells were then rinsed with PBS and incubated with the primary mouse anti-human P-gp monoclonal (clone C494) antibodies (DAKO) diluted 1:100 at room temperature for 60 min. Horseradish peroxidase-coupled secondary antibodies and DAKO EnVision System were used for visualization. For immunofluorescence, the fixed and permeabilized cells were incubated with the primary anti-P-gp antibodies (1:100; Abcam). After washing, the cells were incubated with Alexa Fluor secondary antibodies and counterstained with 4',6-diamidino-2-phenylindole.

miRNA Microarray Expression Analysis

Total RNA was extracted from MCF-7 and MCF-7/DOX cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The miRNA microarray analysis was done by LC Sciences. Total RNA (10 μg) was size fractionated (<200 nucleotides) by using a *mirVana* kit (Ambion) and labeled with Cy3 and Cy5 fluorescent dyes. Dye switching was done to eliminate the dye bias. Pairs of labeled samples were hybridized to dual-channel microarrays. Microarray assays were done on a $\mu\text{ParaFlo}$ micro-

fluidics chip with each of the detection probes containing a nucleotide sequence of coding segment complementary to a specific miRNA sequence and a long nonnucleotide molecule spacer that extended the detection probe away from the substrate.

A miRNA detection signal threshold was defined as twice the maximum background signal. The maximum signal level of background probes was 180. Normalization was done using a cyclic LOWESS (locally weighted regression) method to remove the system-related variations (21). Data adjustments included data filtering, log₂ transformation, and gene centering and normalization. The *t* test analysis was conducted between MCF-7 and MCF-7/DOX samples, and miRNA with *P* values < 0.05 were selected for cluster analysis. The clustering analysis was done using a hierarchical method and average linkage and Euclidean distance metrics (22).

Quantitative Real-time PCR Analysis for miRNA Expression

The quantitative real-time PCR (qRT-PCR) was done by using SuperTaq Polymerase (Ambion) and a *mirVana* qRT-PCR miRNA Detection Kit (Ambion) following the manufacturer's instructions. Reactions contained *mirVana* qRT-PCR Primer Sets (Ambion) specific for human miR-127, miR-200a, miR-200c, miR-34a, miR-15a, miR-16, miR27b, let-7, miR-21, miR-28, miR-106a, miR-206, and miR-345. Human 5S rRNA served as a positive control. qRT-PCR was done on a SmartCycler (Cepheid). The level of each miRNA expression was measured using the $2^{-\Delta\Delta\text{Ct}}$ method (23). The results are presented as fold change of each miRNA in the MCF-7/DOX cells relative to the parental MCF-7 cells.

Western Immunoblotting

Total cellular extracts were prepared by homogenization of 3×10^6 to 5×10^6 cells in 500 μL lysis buffer [50 mmol/L Tris-HCl (pH 7.4); 1% NP-40; 0.25% sodium deoxycholate; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride; 1 $\mu\text{g}/\text{mL}$ each of aprotinin, leupeptin, and pepstatin; 1 mmol/L Na₃VO₄; and 1 mmol/L NaF], sonication, and incubation at 4°C for 30 min followed by centrifugation at $10,000 \times g$ at 4°C for 20 min. Small aliquots (10 μL) of extracts were reserved for protein determination using protein assay reagents from Bio-Rad. Equal amounts of proteins (20 μg) were separated by SDS-PAGE in slab gels of 8% or 12% polyacrylamide, made in duplicate, and transferred to polyvinylidene difluoride membranes (GE Healthcare Biosciences). The membranes were incubated with antibodies against Dicer (1:500; Santa Cruz Biotechnology), AGO2 (1:500; Santa Cruz Biotechnology), RB1 (1:750; Labvision Neomarkers), PTEN (1:1,000; Cell Signaling Technology), K-RAS (1:500; Santa Cruz Biotechnology), CYP1B1 (1:500; Santa Cruz Biotechnology), ER α (1:500; Cell Signaling Technology), P-gp (1:200; Abcam), BCL6 (1:200; Santa Cruz Biotechnology), BRCA1 (1:200; Santa Cruz Biotechnology), and NOTCH1 (1:250; Abgent). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and an ECL

Plus immunoblotting detection system (GE Healthcare Biosciences). Chemiluminescence was detected by GE ECL Hyperfilm (GE Healthcare Biosciences). The unaltered polyvinylidene difluoride membranes were stained with Coomassie blue (Bio-Rad), and the intensity of the M_r 50,000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software.

Luciferase Reporter Assay for Targeting *mdr1*-3'-Untranslated Region

For the luciferase reporter experiments, a 3'-untranslated region (UTR) segment of *mdr1* gene corresponding to a region of 610 bp (4,262-4,872 nucleotides of the total transcript) for *mdr1* (accession no. NM_000927) was amplified by PCR from human genomic DNA using primers that included a *Xba*I and *Eco*RI tails on the 5' and 3' strands, respectively. PCR products were restricted with both *Xba*I and *Eco*RI restriction endonucleases and then gel purified. The amplified 3'-UTR of *mdr1* contains a *Xba*I restriction site; therefore, *mdr1*-3'-UTR was ligated into the pGL3-control vectors (Promega) by using the *Xba*I site located immediately downstream of the stop codon of luciferase.

The HEK293 cells were transfected with the firefly luciferase UTR-report vector, control *Renilla* luciferase pRL-TK vector (Promega), and precursor miR-451 for the *mdr1*-3'-UTR construct using LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen). Twenty-four hours after transfection, cells were lysed with a 1× passive lysis buffer and the activity of both *Renilla* and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Cell Survival Analysis

The MDR-7/DOX cells were seeded in six-well plates at density of 1×10^5 /mL and transfected with scrambled RNA oligonucleotide (control) or 100 nmol/L miR-451 (Ambion) in three independent replicates using a siPORT NeoFX transfection agent (Ambion) in accordance with the manufacturer's protocol. Seventy-two hours after transfection, cells were reseeded in 96-well plates in the presence of miR-451 at density of 1×10^4 per well and treated with DOX at a range of concentration of 2.5 to 200 μ mol/L in the medium for 72 h. Cell survival was analyzed by using the CellTiter-Blue Cell Viability Assay (Promega).

Results

Expression of miRNA in MCF-7 and MCF/DOX Breast Cancer Cells

MiRNA microarrays were used to analyze the miRNA expression profiles in the human breast adenocarcinoma MCF-7 cell line and its drug-resistant MCF-7/DOX variant. The cluster analysis revealed that the MCF-7 breast cancer cells with acquired resistance to DOX were characterized by significant changes in miRNA expression. We identified 137 miRNA genes (63 up-regulated and 75 down-regulated) that were differentially expressed ($P < 0.05$) in the MCF-7/DOX cells compared with the parental MCF-7 cells

(Supplementary Table S1).⁴ Furthermore, 84 of these miRNA genes were differentially expressed at a level of $P < 0.01$ (Fig. 1A). The results obtained by miRNA microarray analysis were independently confirmed by the qRT-PCR. We analyzed the status of differentially expressed *miR-127*, *miR-200a*, *miR-200c*, *miR-34a*, *miR-15a*, *miR-16*, *miR-27b*, *let-7*, *miR-21*, *miR-28*, *miR-106a*, *miR-206*, and *miR-345* genes in MCF-7 and MCF-7/DOX cells. The qRT-PCR confirmed the data obtained by microarray analysis (Fig. 1B).

Expression of Dicer and Argonaute 2 Proteins in MCF-7 and MCF/DOX Breast Cancer Cells

Having revealed the profound alterations in the miRNA profile in the MCF-7/DOX drug-resistant cells, we decided to analyze the protein levels of the main miRNA processing enzyme, Dicer. Several studies pointed toward the putative role of Dicer in tumorigenesis (16, 24, 25); however, its involvement in cancer drug resistance has not been addressed yet. Figure 1C shows a very strong down-regulation of Dicer levels in the MCF-7/DOX cells. Additionally, we have detected a pronounced down-regulation of the Argonaute 2 protein, a member of Argonaute protein family that has an important role in RNA silencing (26) in the MCF-7/DOX cells compared with MCF-7 cells. The significantly decreased levels of the Dicer and Argonaute 2 proteins may, in part, explain the profoundly dysregulated miRNAome profile in the MCF-7/DOX cells.

Association between miRNA Expression and Levels of miRNA Target Proteins

To establish the significance of miRNA expression dysregulation with respect to the acquired cancer cell drug resistance, we determined the protein levels of the experimentally confirmed targets of these differentially expressed miRNA. We found a strong negative correlation between expression of particular miRNA and levels of confirmed target proteins associated with cancer cell drug-resistant phenotype. For instance, Western blot analysis of the MCF-7/DOX cells showed the increased levels of antiapoptotic proteins BCL6, NOTCH1, and K-RAS and up-regulation of CYP1B1 protein leading to the increased metabolism of DOX. The up-regulation of these proteins was associated with down-regulation of the corresponding miRNA: *miR-127*, *miR-34a*, *miR-27b*, and *let-7* (27–31), respectively (Fig. 1D). In contrast, up-regulation of the *miR-206*, *miR-106a*, *miR-21*, and *miR-214* miRNA (Fig. 1A and B; Supplementary Table S1) in MCF-7/DOX cells was associated with the decreased level of their targets ER α , RB1, and PTEN proteins (32–36), respectively, resulting in estrogen insensitivity and increased survival of MCF-7/DOX resistant cells. Additionally, MCF-7/DOX cells were characterized by aberrant expression of several miRNA, such as *miR-10*, *miR-21*, *miR-155*, and *miR-200c*, associated with increased cell invasion and metastasis (36–38).

⁴ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

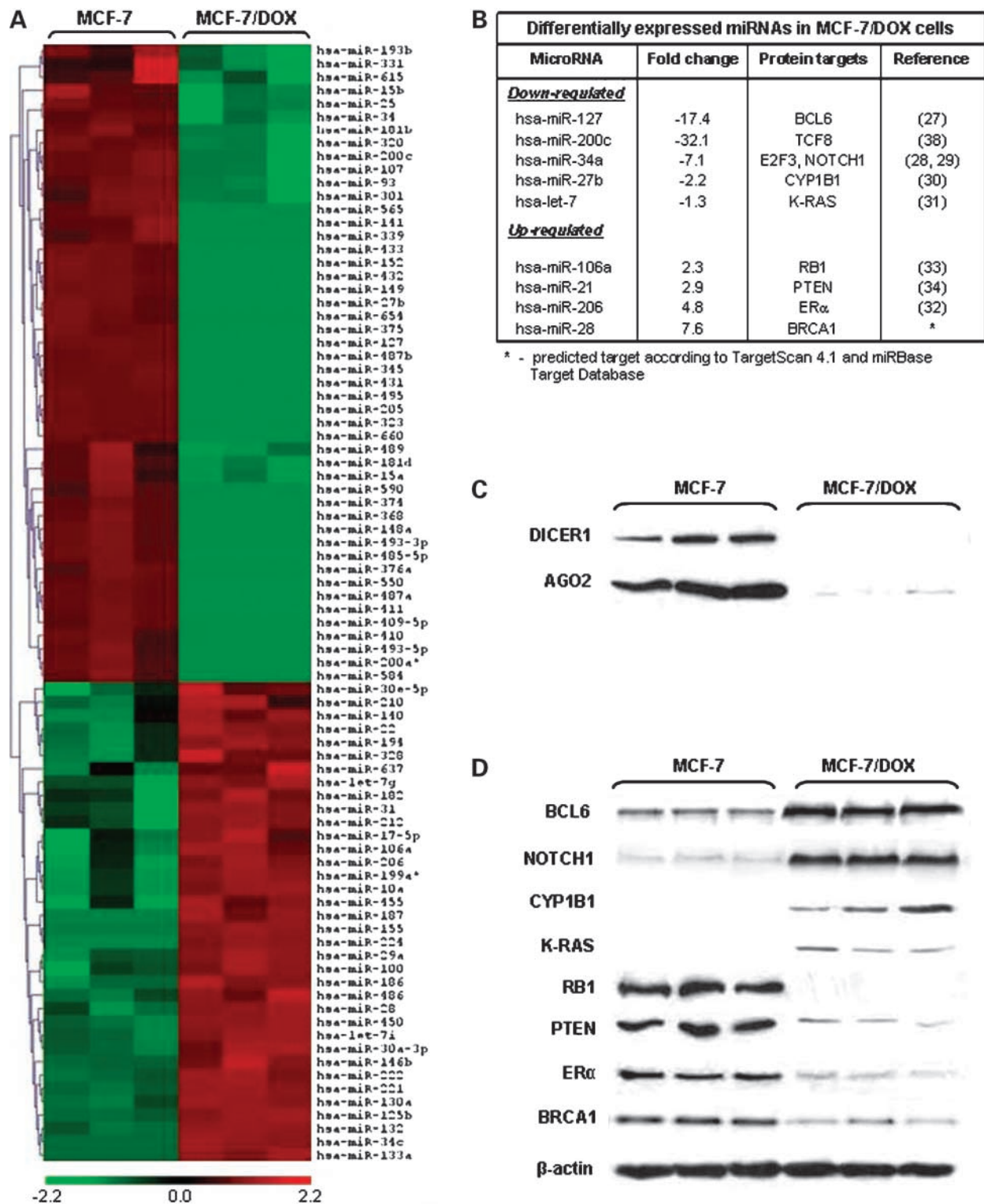


Figure 1. MicroRNA dysregulation in the MCF-7 human breast adenocarcinoma cells resistant to DOX. **A**, hierarchical clustering of the differentially expressed miRNA genes (as determined by ANOVA) in the MCF-7 and MCF-7/DOX cells. *Rows*, miRNA; *columns*, independent biological replicates. For each miRNA: *red*, high expression levels; *green*, low expression levels. Each miRNA listed is significantly differentially expressed ($P < 0.01$) between MCF-7 and MCF-7/DOX cells. **B**, qRT-PCR analysis of the differentially expressed miRNA in the MCF-7 and MCF-7/DOX cells. **C**, Western blot analysis of Dicer and Argonaute 2 expression in the MCF-7 and MCF-7/DOX cells. **D**, Western blot analysis of BCL6, NOTCH1, RB1, PTEN, K-RAS, CYP1B1, ER α , and BRCA1 proteins in the MCF-7 and MCF-7/DOX cells targeted by miR-127, miR-34a, miR-106a, miR-21, let-7, miR-27b, miR-206, and miR-28, respectively.

miR-451 Regulates Expression of *mdr1*

One of the major mechanisms involved in cancer cell resistance to chemotherapeutic agents is an increased energy-dependent efflux of drugs from cancer cells mediated by the ATP-binding cassette transporter P-gp, which is encoded by the *mdr1* gene (2, 39). Several studies have shown a critical role of this protein in the intrinsic or acquired drug resistance (2, 40). The importance of epigenetic mechanisms in the regulation of the *mdr1* gene has also been well documented (20, 41, 42). Specifically, the increased resistance of cancer cells, including breast cancer cells, to chemotherapy is associated with pronounced hypomethylation and altered histone modifications at the *mdr1* promoter region and up-regulation of the *mdr1* gene expression (20, 41, 42); however, the role of miRNA in the *mdr1* regulation has not been addressed. Figure 2 shows that the MCF-7/DOX cells exhibit very high levels of P-gp, a product of *mdr1* gene, compared with the parental MCF-7 cells as detected by immunocytochemistry and immunofluorescence.

Computational analysis of the 3'-UTR of *mdr1* revealed a putative binding site for a single miRNA, miR-451, mature sequence UUGAGUCAUUACCAUUGCCAAA, at 4,742 to 4,763 nucleotides (Fig. 3A). This prediction was further confirmed by the miRGen software (43). Therefore, we

determined the role of miR-451 in the regulation of *mdr1* expression. The cellular level of this miRNA in the MCF-7/DOX cells was below the detection limit of the microarray. To examine whether *mdr1* is indeed functionally targeted by miR-451, the segment of *mdr1*-3'-UTR containing the miR-451 complementary site was cloned into the 3'-UTR of a luciferase reporter system. The resulting reporter vector was transfected into the HEK293 cells together with transfection controls and miR-451, anti-miR-451, or miRNA that do not have binding sites within the 3'-UTR of *mdr1*.

Figure 3B shows that miR-451 inhibited the luciferase activity from the construct with the *mdr1*-3'-UTR segment in a concentration-dependent manner (Fig. 3B). There was no change in the luciferase reporter activity when the cells were cotransfected with the negative control (scrambled oligonucleotides) or unrelated miRNA such as miR-7, miR-127, or miR-345 (data not shown). No luciferase expression changes were observed when the cells were transfected with the plasmid lacking the *mdr1*-3'-UTR fragment (data not shown).

To further confirm that miR-451 indeed affects the protein levels of P-gp in the MCF7/DOX cells, these cells were transfected with either the miR-451 or anti-miR-451, and the level of P-gp was determined by Western blotting 48 h after transfection. Figure 3C shows

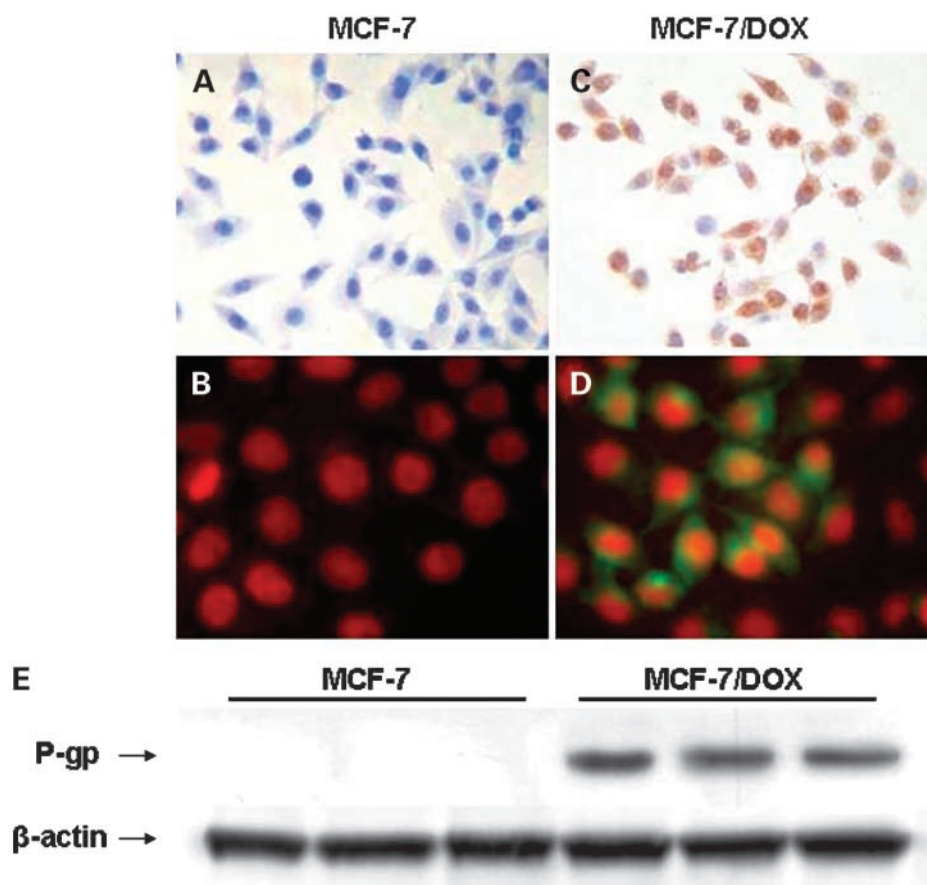
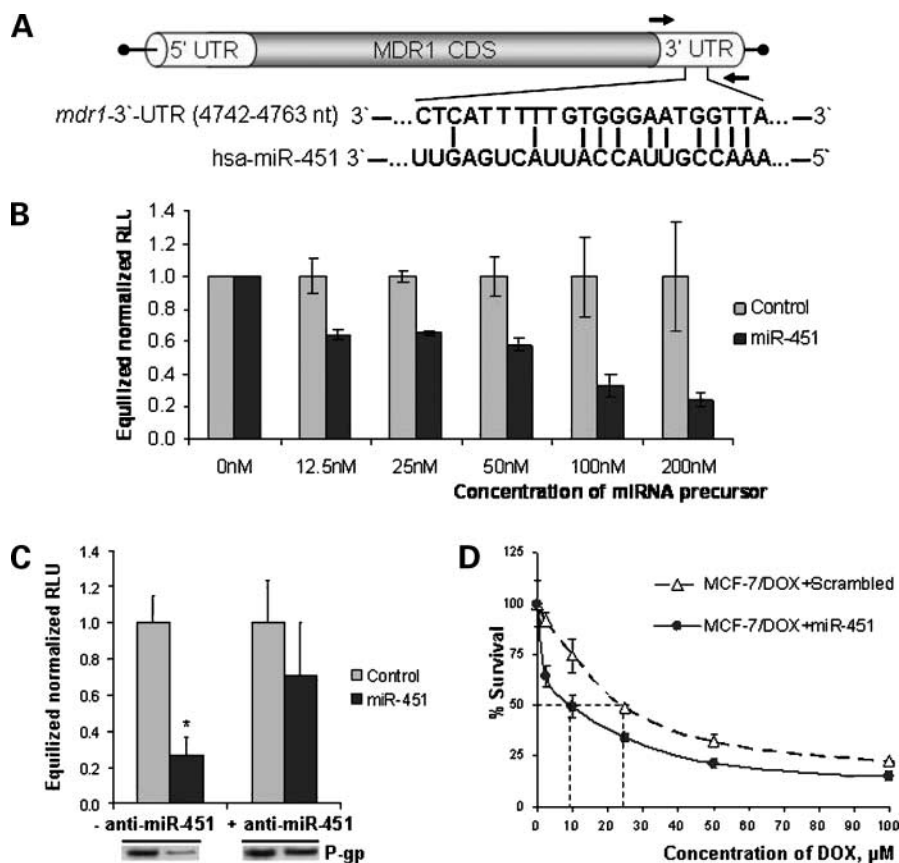


Figure 2. Expression of P-gp in the MCF-7 and MCF-7/DOX human breast adenocarcinoma cells as detected by immunocytochemistry (A and C), immunofluorescence (B and D), and immunoblotting (E).

Figure 3. miR-451 directly targets *mdr1*. **A**, complementary site for miR-451 in the 3'-UTR of *mdr1*. **B**, dose-dependent inhibition of *mdr1* expression in the luciferase assay after transfection of the HEK293 cells with miR-451 or a negative control. **C**, levels of *mdr1* mRNA and P-gp in the MCF-7/DOX cells transfected with miR-451 or a combination of miR-451 and anti-miR-451. **D**, transfection of the MCF-7/DOX cells with miR-451 increases their sensitivity to DOX treatment.



that transfection of MCF-7/DOX cells with miR-451 resulted in a decrease of P-gp levels, whereas the simultaneous transfection of miR-451 and anti-miR-451 abolished the inhibitory effect of miR-451 efficiently.

Inhibition of *mdr1* Expression Results in the Increased Sensitivity of the MCF-7/DOX Cells to DOX

The finding that the miR-451 targets *mdr1* suggested that down-regulation of expression of this miRNA contributes to the cancer drug resistance; therefore, the restoration of a miR-451 level in the resistant MCF-7/DOX cells may increase their sensitivity to DOX. To address this issue, we transfected MCF-7/DOX with miR-451 and determined the sensitivity of cells to DOX treatment. Figure 3D shows that transfection of MCF-7/DOX cells with miR-451 resulted in the increased sensitivity of the resistant MCF-7/DOX cells to DOX. The IC_{50} of MCF-7/DOX cells transfected with miR-451 was 2.5 times lower ($P < 0.05$) compared with the MCF-7/DOX cells transfected with scrambled oligonucleotide. In contrast, transfection of MCF-7 cells resistant to *cis*-dichlorodiammine platinum with miR-451 did not change sensitivity of the MCF-7/*cis*-dichlorodiammine platinum-resistant cells to *cis*-dichlorodiammine platinum treatment (data not shown).

Discussion

Recent findings have confirmed a critical role of miRNA as powerful diagnostic and prognostic indicators of human

breast cancer (15, 16, 37, 38, 44), resulting in the development of novel approaches to breast cancer management (44). Despite the well-established role of miRNA in cancer (14, 15) and the dedication of research and resources on the elucidation of the molecular mechanisms involved in the development of resistance cancer cells to chemotherapy, the role of miRNA in cancer drug resistance remains largely unexplored. In this report, we provide data indicating the importance of miRNA dysregulation in the acquisition of cancer cell resistance to chemotherapeutic drugs. This was evidenced by the pronounced alteration in expression of miRNA genes in the MCF-7/DOX-resistant cells compared with parental MCF-7 cells. The mechanistic connection of miRNAome dysregulation with the establishment of a multidrug-resistant phenotype in MCF-7/DOX cells was evidenced by the correlation between expression of specific miRNA and corresponding changes in the protein levels of their targets, specifically those targets that have a documented importance in the development of cancer cells drug resistance. At present, the cancer drug resistance is considered as a multifactorial phenomenon (4) involving several major mechanisms, such as decreased uptake of water-soluble drugs, increased repair of DNA damage, reduced apoptosis, altered metabolism of drugs, and increased energy-dependent efflux of chemotherapeutic drugs that diminish the ability of cytotoxic agents to kill cancer cell (1, 4). The pattern of miRNA expression in the

MCF-7/DOX cells affecting multiple genes simultaneously provided support for this multifactorial polygenic drug resistance hypothesis. This was evidenced by (a) down-regulation of *miR-27b* and consequent up-regulation of CYP1B1 leading to the increased metabolism of DOX; (b) down-regulation of *miR-127* and *miR-34a* resulting in a reduced apoptotic program via inhibition of p53 network; (c) down-regulation of *miR-200c* resulting in up-regulation of TCF8 (38) and consequent decreased expression of E-cadherin inducing cell invasiveness and metastasis, (d) up-regulation of *miR-21* and *miR-214* (Supplementary Table S1) targeting PTEN (34, 35) leading to increased cell survival; (e) up-regulation of *miR-28* leading to loss of BRCA1 expression, which is associated with increased resistance to DOX treatment (45); and (f) up-regulation of *miR-206* leading to loss of the ER α -mediated signal transduction. Furthermore, our results of *miR-125* down-regulation in the MCF-7/DOX-resistant cells (Supplementary Table S1) support suggestion by Climent et al. (19) about the link between miRNA dysregulation and breast cancer drug resistance.

Because the increased energy-dependent efflux of chemotherapeutic drugs is a major mechanism associated with resistance of cancer cells to DOX, we have focused on a potential role of miRNA as regulators of the *mdr1* expression. The results of our reporter assay experiments that show the significant reduction of MDR1 expression clearly show that *miR-451* is a regulator of MDR1. More importantly, transfection of the MCF-7/DOX cells with *miR-451* resulted in the increased sensitivity of resistant cells to DOX. This finding indicates that correction of altered expression of miRNA may have significant implications for therapeutic strategies aiming to overcome cancer cell resistance. Indeed, recent reports on the positive results in the use of small interfering RNA (46, 47) or miRNA (48) to suppress resistance of cancer cells to chemotherapeutic drugs support this suggestion. In our previous study using MCF-7/DOX cells, we have found profound alterations of cellular epigenetic landscape (20), including hypomethylation of the *mdr1* gene. Those epigenetic abnormalities in MCF-7/DOX cells, specifically loss of cytosine methylation, may be partially related to the increased expression of *miR-22*, *miR-29a*, *miR-194*, and *miR-132* miRNA that target DNA methyltransferases 3A and 3B and methyl CpG binding protein 2 (49, 50).

In conclusion, we have shown that the development of multidrug resistance is associated with the pronounced deregulation of miRNA expression. Additionally, we have shown that expression of *miR-451* is inversely correlated with *mdr1* expression in breast cancer drug-resistant cells. Furthermore, the enforced increase of *miR-451* levels in the MCF-7/DOX cells down-regulates expression of *mdr1* and increases sensitivity of the MCF-7-resistant cancer cells to DOX. These results provide a strong rationale for the development of miRNA-based therapeutic strategies aiming to overcome cancer cell resistance. However, these alterations are not necessarily indicative of the causative

role of miRNA deregulation in the cancer drug resistance development and the ultimate goal of future studies is to address this question.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Lehner M. Chemotherapy resistance in breast cancer. *Anticancer Res* 1998;18:2225–6.
- Szakacs G, Paterson JK, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Disc* 2006;5:219–34.
- Cox MC, Dan TD, Swain SM. Emerging drugs to replace current leaders in first-line therapy for breast cancer. *Expert Opin Emerg Drugs* 2006;11:489–501.
- Roberti A, La Sala D, Cinti C. Multiple genetic and epigenetic interacting mechanisms contribute to clonally selection of drug-resistant tumors: current views and new therapeutic prospective. *J Cell Physiol* 2006;207:571–81.
- Glasspool RM, Teodoridis JM, Brown R. Epigenetics as a mechanism driving polygenic clinical drug resistance. *Br J Cancer* 2006;94:1087–92.
- Iwasa Y, Nowak MA, Michor F. Evolution of resistance during clonal expansion. *Genetics* 2006;172:2557–66.
- Fojo T. Multiple paths to a drug resistance phenotype: mutations, translocations, deletions and amplification of coding genes or promoter regions, epigenetic changes and microRNAs. *Drug Res Update* 2007;10:59–67.
- Duesberg P, Li R, Sachs R, Fabarius A, Upender MB, Hehlmann R. Cancer drug resistance: the central role of the karyotype. *Drug Res Update* 2007;10:51–8.
- Matarazzo MR, Boyle S, D'Esposito M, Bickmore WA. Chromosome territory reorganization in human disease with altered DNA methylation. *Proc Natl Acad Sci U S A* 2007;104:16546–51.
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
- Sevignani C, Calin GA, Siracusa LD, Croce CM. Mammalian microRNAs: a small world for fine-tuning gene expression. *Mamm Genome* 2006;17:189–202.
- Bushati N, Cohen SM. microRNAs functions. *Annu Rev Cell Dev Biol* 2007;23:175–205.
- Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834–8.
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–66.
- Iorio MV, Ferracin M, Liu C-G, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005;65:7065–70.
- Blenkiron C, Goldstein LD, Thorne NP, et al. MicroRNA expression profiling of human breast cancer identifies new markers of tumour subtype. *Genome Biol* 2007;8:R214.
- Tricoli JV, Jacobson JW. MicroRNA: potential for cancer detection, diagnosis, and prognosis. *Cancer Res* 2007;67:4553–5.
- Blenkiron C, Miska EA. miRNAs in cancer: approaches, aetiology, diagnostics and therapy. *Hum Mol Genet* 2007;16:R106–13.
- Climent J, Dimitrow P, Fridlyand J, et al. Deletion of chromosome 11q predicts response to anthracycline-based chemotherapy in early breast cancer. *Cancer Res* 2007;67:818–26.
- Chekun VF, Lukyanova NYu, Kovalchuk O, Tryndyak VP, Pogribny IP. Epigenetic profiling of multidrug-resistant human MCF-7 breast adenocarcinoma cells reveals novel hyper- and hypomethylated targets. *Mol Cancer Ther* 2007;6:1089–98.
- Bolstad BM, Irizarry RA, Astrandand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;19:185–93.

22. Eisen MB, Spellman PT, Brown PO, Boltstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998;95:14863–8.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. *Methods* 2001;25:402–8.
24. Chiosea S, Jelezcova E, Chandran U, et al. Overexpression of Dicer in precursor lesions of lung adenocarcinoma. *Cancer Res* 2007;67:2345–50.
25. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet* 2007;39:673–7.
26. Schmitter D, Filkowski J, Sewer A, et al. Effects of Dicer and Argonaute down-regulation on mRNA levels in human HEK293 cells. *Nucleic Acids Res* 2006;34:4801–15.
27. Saito Y, Liang G, Egger G, et al. Specific activation of microRNA-127 with downregulation of proto-oncogene *BCL6* by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 2006;9:435–43.
28. Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* 2007;26:5017–22.
29. Fukuda Y, Kawasaki H, Taira K. Exploration of human miRNA target genes in neuronal differentiation. *Nucleic Acids Symp Ser* 2005;49:341–2.
30. Tsuchiya Y, Nakajima M, Takagi S, Taniya T, Yokoi T. MicroRNA regulates the expression of human cytochrome *P450 1B1*. *Cancer Res* 2006;66:9090–8.
31. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. *Cell* 2005;120:635–47.
32. Adams BD, Furneaux H, White BA. The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor- α (ER α) and represses ER α messenger RNA and protein expression in breast cancer cell lines. *Mol Endocrinol* 2007;21:1132–47.
33. Volinia S, Calin G, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A* 2006;103:2257–61.
34. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 2007;133:647–58.
35. Yang H, Kong W, He L, et al. MicroRNA expression profiling in human ovarian cancer: *miR-214* induces cell survival and cisplatin resistance by targeting PTEN. *Cancer Res* 2008;68:425–33.
36. Hurteau GJ, Carlson A, Spivack SD, Brock GJ. Overexpression of the microRNA has-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. *Cancer Res* 2007;67:7972–6.
37. Ma L, Teruya-Feldstein J, Weinberg RA. Tumor invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 2007;449:682–8.
38. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res* 2008;18:350–9.
39. Gottesman MM, Ling V. The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research. *FEBS Lett* 2006;580:998–1009.
40. Takara K, Sakaeda T, Okumura K. An update on overcoming MDR1-mediated multidrug resistance in cancer chemotherapy. *Curr Pharm Des* 2006;12:273–86.
41. Baker EK, El-Osta A. MDR1, chemotherapy and chromatin remodeling. *Cancer Biol Ther* 2004;3:819–24.
42. David GL, Yegnasubramanian S, Kumar A, et al. MDR1 promoter hypermethylation in MCF-7 human breast cancer cells. *Cancer Biol Ther* 2004;3:540–8.
43. Megraw M, Sethupathy P, Corda B, Hatzigeorgiou AG. miRGen: a database for the study of animal microRNA genomic organization and function. *Nucleic Acids Res* 2007;35:D149–55.
44. Lowery AJ, Miller N, McNeil RE, Kerin MJ. MicroRNAs as prognostic indicators and therapeutic targets: potential effect on breast cancer management. *Clin Cancer Res* 2008;14:360–5.
45. Tassone P, Tagliaferri P, Perricelli A, et al. BRCA1 expression modulates chemosensitivity of BRCA1-defective HCC1937 human breast cancer cells. *Br J Cancer* 2003;88:1285–91.
46. ILage H. MDR1/P-glycoprotein (ABCB1) as target for RNA interference-mediated reversal of multidrug resistance. *Curr Drug Targets* 2006;7:813–21.
47. Kaszubiak A, Holm PS, Lage H. Overcoming the classical multidrug resistance phenotype by adenoviral delivery of anti-MDR1 short hairpin RNAs and ribozymes. *Int J Oncol* 2007;31:419–30.
48. Weidhaas JB, Babar I, Nallur SM, Trang P, Roush S, Boehm M, Gillespie E, Slack FJ. MicroRNAs as potential agents to alter resistance to cytotoxic anticancer therapy. *Cancer Res* 67:11111–6.
49. Fabbri M, Garzon R, Cimmino A, et al. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3^o and 3B. *Proc Natl Acad Sci U S A* 2007;104:15805–10.
50. Klein ME, Liroy DT, Ma L, Impey S, Mandel G, Goodman RH. Homeostatic regulation of MeCP2 expression by a CREB-induced microRNA. *Nat Neurosci* 2007;10:1513–4.