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*Original Article*

# MiR-455-DNMT3A signaling is linked toacquisition of doxorubicinresistance in breast cancer

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**Abstract:** Breast cancer cells can develop resistance to chemotherapeutic agents. Epigenetic changes such as DNA methylation and histone modifications have been shown to play a critical role in acquisition of drug resistance bythese cells. microRNAs (miRNAs) are a class of short non-coding RNAs that are closely associated with cancer chemoresistance through regulating the genes involved in epigenetic modifications. In this study, we used a variety of biochemical techniques including cell culture, RT-PCR, Western blot, luciferase reporter assay, MTT and gene silencing to investigate the role of miR-455 in the resistance of human MCF-7 breast adenocarcinoma cells to doxorubicin (DOX). We demonstrated that DOX-resistant MCF-7 cells (MCF-7/DOX) show significant up-regulation of miR-455 compared to normal MCF-7 cells. The mechanistic link between miR-455 deregulation and DOXresistant phenotype in MCF-7/DOX ismediated by DNMT3A. Specifically, we showed that microRNA-455 down-regulates the expression of DNMT3A, thus possibly leading to global loss of DNA methylation in MCF-7/DOX cells. Furthermore, transfection of the MCF-7 cells with siRNAs targeting degradation of DNMT3 reduced their sensitivity to DOX. Our results support the idea that manipulating expression of miRNAs may have significant implications for therapeutic strategies aimedat overcoming thebreast cancer resistance.

**Keywords:** miR-455, DNMT3A, doxorubicin, resistance, breast cancer

## Introduction

Chemoresistance is one of the major obstacles for successful treatment of a variety of human cancers including breast cancer [1, 2]. While drug resistance to chemotherapeutic agents remain poorly understood, thepossible mechanisms include reduced intracellular concentrations of drug transporters and metabolic enzymes, impaired cellular responses that affect cell cycle, inducing of signaling pathways that promote malignant transformation and invasiveness of cell populations, alterations in the availability of drug targets, and dysregulation in DNA methylation and histone modifications [3].

Multiple genetic or epigenetic events are thought to be major players related to drug resistance [4]. Increasing body of evidence supports that acquired resistance of cancer cell to chemotherapeutic agents is closely associated with histone modifications and DNA methylation [5, 6]. Aberrant DNA methylation patterns are proven to be a prominent hallmark of cancer cell including breast cancer. Cancer cell survival and progression are dependent on the global loss of genomic methylation and changesin methylation levels at promoters of genes involved in cell signaling, proliferation, and apoptosis [7]. Three DNA methyltransferases (DNMTs) are involved in DNA methylation. DNMT1 is essential for the maintenance of established methylation status, while DNMT3A and 3B are responsible for *de novo* DNA methylation. DNMT3L possesses DNA methyltransferase motif, but lacks catalytic activity [8, 9]. DNA methylation usually inhibits local gene expression by recruiting epigenetic repressor complex [10, 11]. It is well known that the loss of function of certain tumor-suppressor genes results from hypermethylation in the promoter regions, and a number of studies have shown that a variety of genes are inactivated by DNA methylation in different cancer types. In addition, global hypomethylation thatcauses ge-

nomic instabilityalso contributes to cell transformation. Apart from DNA methylation alterations in promoter regions and repetitive DNA sequences, this phenomenon is also associated with the expression regulation of non-coding RNAs such as microRNAs (miRNAs) that may play role in tumor suppression and drug resistance in cancer [3].

Evidence for miRNA-mediated epigenetic changes in drug resistance of breast cancer has been recently brought to attention. Numerous studies have indicated the existence and importance of mechanisms of post-transcriptional regulation of gene function mediated by miRNAs. Aberrant levels of miRNAs have been reported in multiple human cancers including breast cancer [12, 13]. Currently, more than 1,200 miRNAs have been identified in mammals, which can regulate one-third of the protein-coding genes. These genes have been implicated in development, cell differentiation and proliferation, signaling transduction, metabolic pathways, and apoptosis. Dysregulation of miRNAs has been associated with almost every aspects of cancer biology such as tumor progression, invasion, metastasis, and acquisition of resistance to various chemotherapeutic agents [14].

In this study, we tested the role of miR-455 in acquisition of drug resistance of breast cancer to doxorubicin (DOX). miR-455 has been previously found to be significantly up-regulated in DOX-resistance breast cancer cell lines [12]. Our results suggest that breast cancer acquires DOXresistance by down-regulating DNMT3A activity, thus likely leading to a global loss of DNA methylation in the breast cancer cells. The results further suggest that miR-455 is a potential target for reversing the chemoresistance in breast cancer.

## Materials and methods

### *Cell lines and cell culture*

Human breast adenocarcinoma MCF-7 cells were purchased from ATCC (VA, USA). The cells were cultured in DMEM/F-12 containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% $CO_2$. The MCF-7/DOX drug-resistant variant of the MCF-7 cell line was established as previously detailed [12]. Cells were seeded at a density of 0.5 × $10^6$ cells per 100-mm plate. Trypsinized cells were washed in PBS and immediately frozen at -80°C for subsequent analyses.

### *Antibodies and reagents*

Antibodies to DNMT3A and GAPDH were purchased from Santa Cruz Biotech (TX, USA). SYBR Green PCR Master Mix was obtained from QIAGEN (USA). PCR primers, miRNA mimics and inhibitors were synthesized by IDT (USA).

### *Cell transfection*

Hsa-miR-455 mimics and inhibitors were synthesized by IDT DNA (Iowa, USA). After being maintained in culture for 24 h, each well was transfected with the complex of miR-455 mimics or inhibitors-Lipofectamine 2000 (3 μl; Invitrogen, USA) in serum free media, prepared following the manufacturer's instructions with a final concentration of 50 μM. At 48 h after exposure, the cells were prepared for further analysis.

### *Real-time quantitative polymerase chain reaction assay (RT-qPCR)*

Cells at log phase were collected after reaching 90% confluence. Total RNAswere extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions. RNA was quantified by UV absorbance at 260 and 280 nm (260/280 nm) using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Reverse transcription was conducted with the use of Super Script First-Strand kit (ThermoFisher Scientific) according to the manufacturer's recommendations. RT-qPCR was performed using StepOne PCR system (ThermoFisher Scientific) with SYBR Green PCR Master Mix (Roche, USA). The cycle threshold (CT) values for each gene were normalized to internal control, GAPDH, and the relative expression levels were counted by the ΔΔCT method.

### *Cell survival analysis*

Cells were seeded in a 96-well plate at a density of 8 × $10^3$/well with 100 μl of complete medium per well and cultured for 24 h. Then, they were treated with serial dilutions of DOX at a range of 0.5 and 25 μM for 48 h. For the drug cytotoxic assays, the cells were supplemented

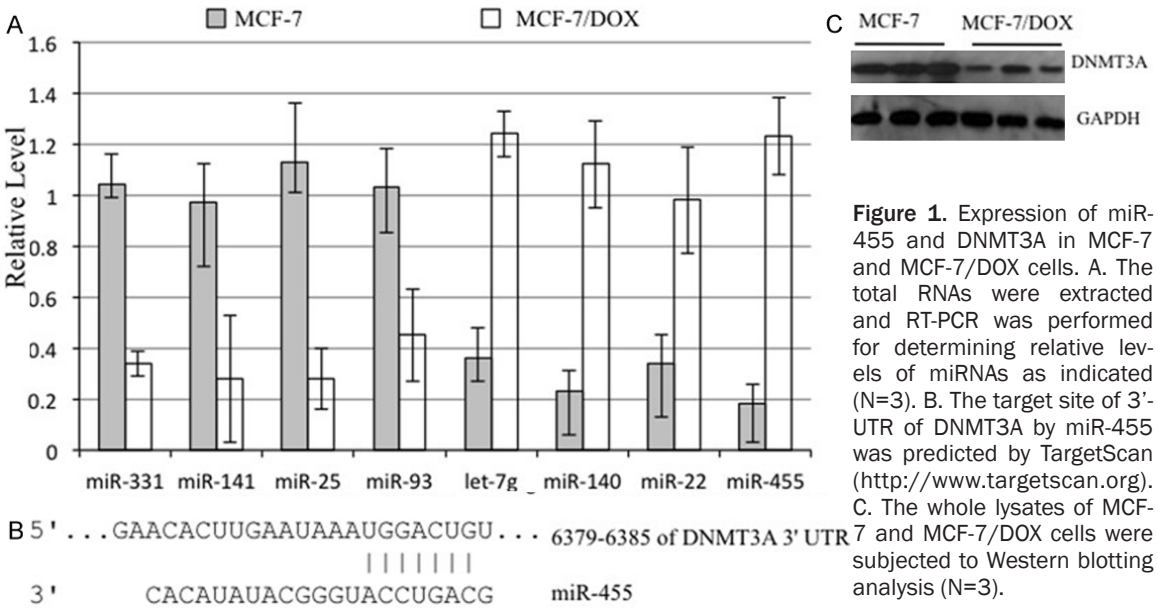


**Figure 1.** Expression of miR-455 and DNMT3A in MCF-7 and MCF-7/DOX cells. A. The total RNAs were extracted and RT-PCR was performed for determining relative levels of miRNAs as indicated (N=3). B. The target site of 3'-UTR of DNMT3A by miR-455 was predicted by TargetScan (http://www.targetscan.org). C. The whole lysates of MCF-7 and MCF-7/DOX cells were subjected to Western blotting analysis (N=3).

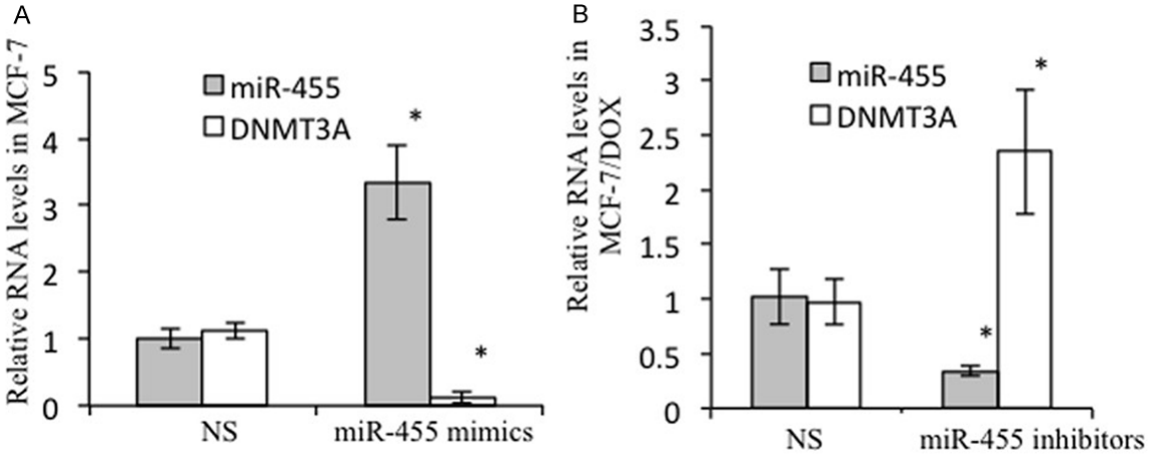


**Figure 2.** Effects of miR-455 inhibitors or mimics on mRNA levels of DNMT3A. A. MCF-7 cells were transfected with miR-455 mimics or non-specific controls (NS) for 48 h, and then total RNAs were extracted and subjected RT-qPCR for assessment of miR-455 and DNMT3A mRNA levels (N=3). B. MCF-7/DOX cells were transfected with miR-455 inhibitors or non-specific controls (NS) for 48 h, and then total RNAs were extracted and subjected RT-qPCR for assessment of miR-455 and DNMT3A mRNA levels (N=3). *P < 0.01.

with 20 μL ofMTT solution (5 mg/ml) (Sigma, USA) added into each well and then incubated for 4 h at 37°C. The absorbance at 490 nm was measured using a microplate reader (Victor3, Waltham, USA).

*Western blot*

Cells were washed twice with PBS at room temperature and lysed with RIPA buffer (Sigma, USA) on ice for 5 min. Then, they were centrifuged at 14,000 g for 15 min at 4°C, and the supernatants were collected. Proteins were quantified using Nanodrop 2000 spectrophotometer (280 nM, Thermo Scientific, USA), mixed with LDS Sample Buffer (Life Technologies, USA) and boiled for 5 min. Equal amounts of proteins were separated by electrophoresis on 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA). Membranes were blocked in 5% non-fat milk power in 10 mM phosphate buffer (pH 7.2), 150 mM NaCl, and 0.1% Tween 20 (PBST) for 1 h, washed twice with PBST, and

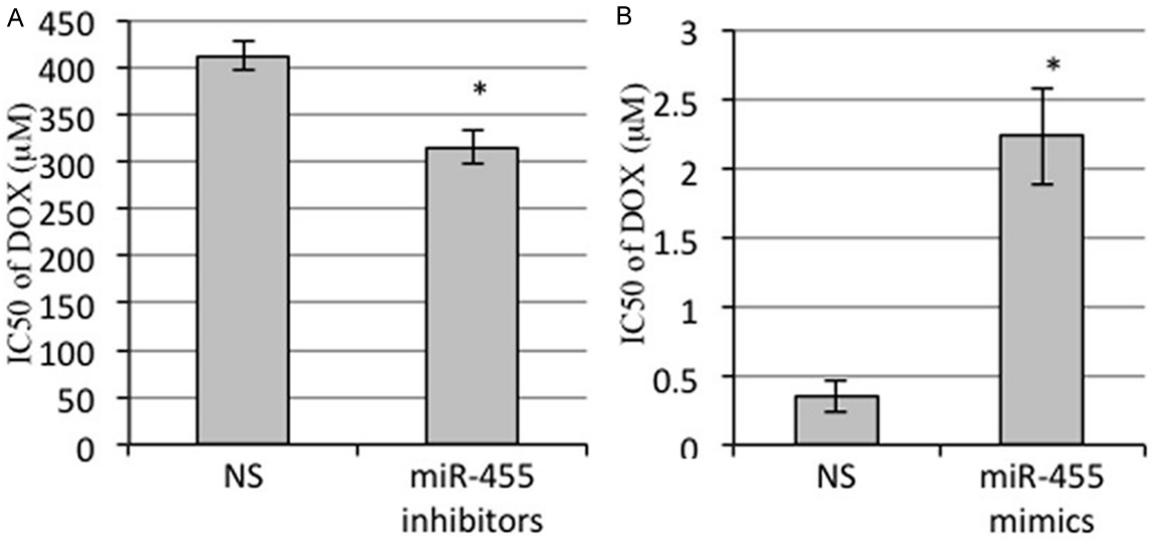


**Figure 3.** Effects of miR-455 inhibitors or mimics on the sensitivity of MCF-7 cell lines to DOX. A. The $IC_{50}$ values of DOX were measured using MTT assay after MCF-7/DOX cells were transfected with miR-455 inhibitors or non-specific controls (NS) for 48 h. B. The $IC_{50}$ values of DOX were measured using MTT assay after MCF-7 cells were transfected with miR-455 mimics or non-specific controls (NS) for 48 h. N=3. *P < 0.01.

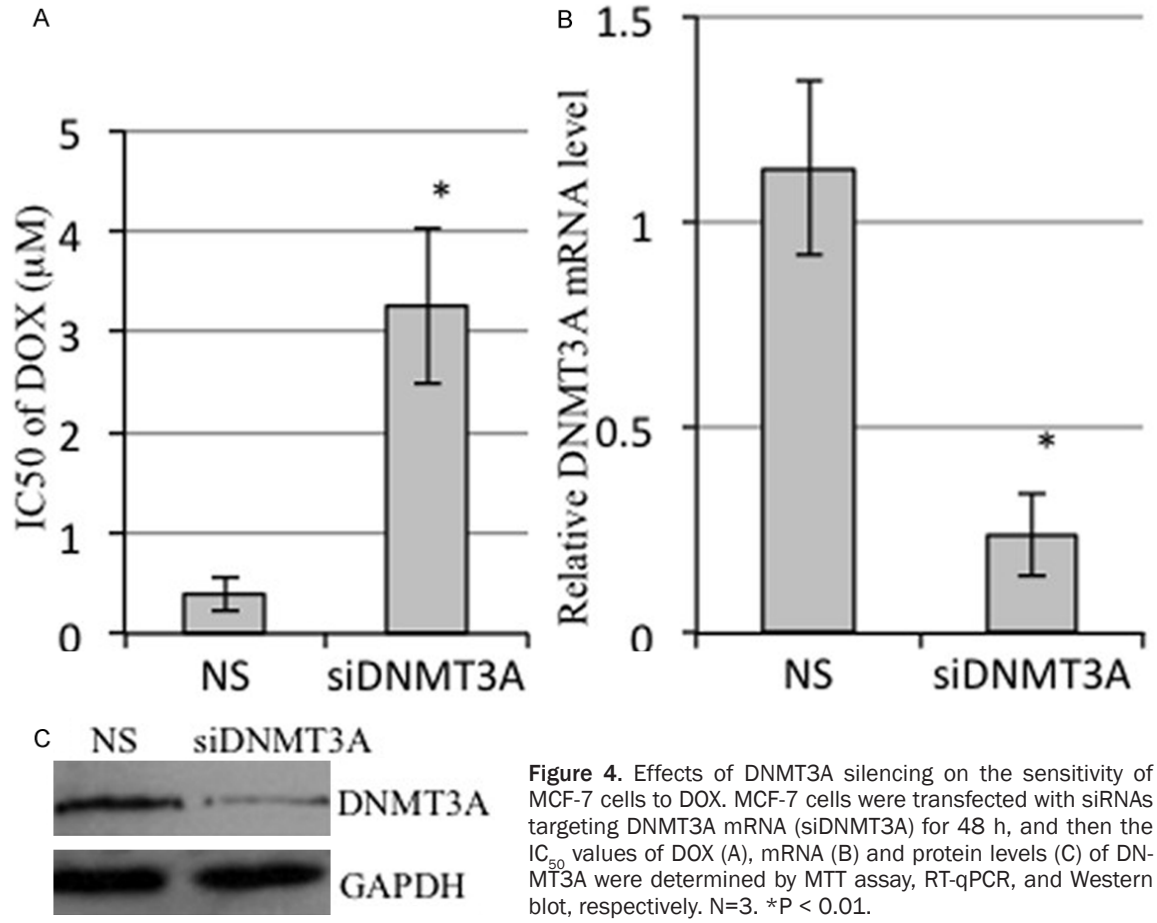


**Figure 4.** Effects of DNMT3A silencing on the sensitivity of MCF-7 cells to DOX. MCF-7 cells were transfected with siRNAs targeting DNMT3A mRNA (siDNMT3A) for 48 h, and then the $IC_{50}$ values of DOX (A), mRNA (B) and protein levels (C) of DNMT3A were determined by MTT assay, RT-qPCR, and Western blot, respectively. N=3. *P < 0.01.

incubated with appropriate antibodies in 1% nonfat milk powder-PBST at 4°C overnight. Blotswere washed 3 times with PBST, incubated with the appropriate horseradish peroxi-

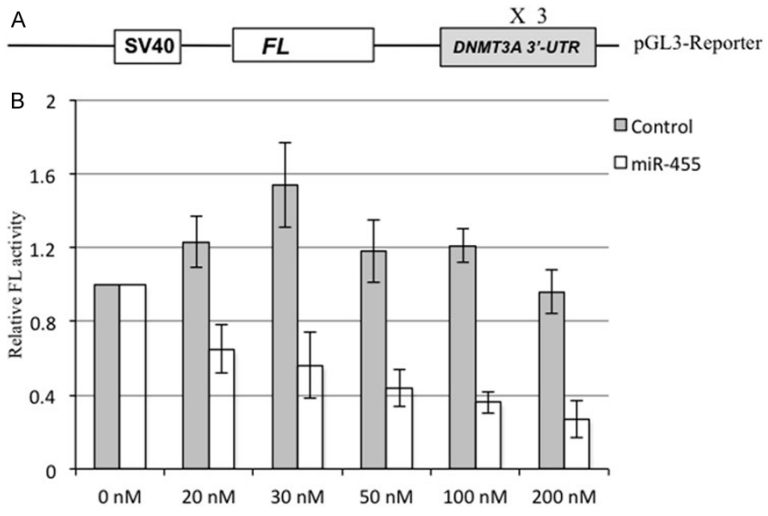


**Figure 5.** miR-455 directly targets 3'-UTR of DNMT3A mRNA. A. Schematic presentation of luciferase reporters. FL, firefly luciferase. B. HeLa cells were co-transfected with luciferase reporter and miR-455 mimics or non-specific control at serial concentrations as indicated for 48 h followed by dual-luciferase activity assay. Each experiment was repeated three times. *P < 0.01.

dase-conjugated secondary antibodies at a 1:5000 dilution in 1% non-fat milk powder-PBST, and developed by Immuno-Star HRP substrate (Bio-Rad, USA).

*Dual luciferase assay*

3'-UTR segment *of DNMT3A* gene corresponding to the predicted target site was amplified by PCR from human genomic DNA using primers that included a XbaI and EcoRI tails on the 5' and 3' strands, respectively, as previously described [12]. PCR products were treated with both XbaI and EcoRI DNA restrictases, gel purified, and ligated into the pGL3 vector (Promega). Hela cells were transfected with the firefly luciferase UTR-report vector, control Renilla luciferase pRL-TK vector (Promega) 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 were assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

*Statistical analysis*

All experiments were performed at least three times and the data were presented as the means ± SD (standard deviation). Two-tailed unpaired Student's t-test was used to assess the statistical significance of difference between two independent groups. A *P* value < 0.05 was considered statistically significant.

## Results

*miR-455 and DNMT3A expression in human MCF-7 and MCF-7/DOX cells*

Using miRNA microarray assay and RT-PCR, a previous study has shown that miR-455 is regulated in MCF-7/DOX cells. Here we used RT-PCR to assess the levels of both miR-455 and DNMTs in MCF-7 and MCF-7/DOX cells. As shown in **Figure 1A**, the expression of miR-455 was significantly increased in MCF-7/DOX compared to MCF-7 cells, consistent with the previous study. Next, we used TargetScan to predict whether DNMT3A mRNA is targeted by miR-455, Data on **Figure 1B** show that DNMT3A is a highly putative target of miR-455. By using Western blot, we found that the protein level of DNMT3A is remarkably reduced in MCF-7/DOX (**Figure 1C**). These results suggest that up-regulated miR-455 and reduced activity of DNM3A may contribute to the DOX resistance in breast cancer.

*Changes in DNMT3A expression in response to treatments with miR-455 mimics or inhibitors*

To further validate whether miR-455 targets DNMT3A in breast cancer cell, we assessed the changes inexpression level of DNMT3A in response to transfection with miR-455 mimics in MCF-7 cellsor miR-455 inhibitors in MCF-7/DOX cells. As shown in **Figure 2**, the treatment with miR-455 led to significant decrease of DNMT3A expression.Conversely, the treatment with miR-455 inhibitors relieved the repression. These results further confirm that DNMT3A is targeted by miR-455 in breast cancer cells.

*miR-455 is involved in resistance of MCF-7 cells to DOX*

To investigate whether the levels of miR-455 have the effect on chemosensitivity of breast

cancer cells to DOX, MTT assay was performed to assess the $IC_{50}$ values of DOX both in MCF-7/DOX cells transfected with miR-455 inhibitors and in MCF-7 cells transfected with miR-455 mimics. Here we showed that the down-regulation of miR-455 attenuated the DOX resistance of MCF-7/DOX cells compared with non-specific controls (**Figure 3A**, $P < 0.01$). In contrast, the up-regulation of miR-455 increasedthe DOX resistance in MCF-7 cells (**Figure 3B**, $P < 0.01$). These results suggest that miR-455 is a potential target toreverse the DOX resistance in breast cancer cells.

*Inhibition of DNMT3A reduces sensitivity of breast cancer cells to DOX*

To further determine the effects of DNMT3A on drug resistance in breast cancer, MCF-7 cells were transfected with siRNA specifically targeting DNMT3A mRNA (siDNMT3A) for its degradation. The cells were exposed to DOX for 24 h, followed by cell survival analysis. As shown in **Figure 4**, transfection of siDNMT3Aled to reduced sensitivity of MCF-7 cells to DOX, as evident from the increased $IC_{50}$ values. These findings further suggest that miR-455-mediated drug resistance is dependent on the DNMT3A signaling.

*miR-455 directly targets DNMT3A*

We showed that miR-455 represses the luciferase activity from the construct with the DNMT3A-3'-UTR segment in a dose-dependent manner (**Figure 5**). There was no change in the luciferase reporter activity when the cells were co-transfected with the negative control. No changes in luciferase expression were observed when the cells were transfected with the plasmid lacking DNMT3A-3'-UTR fragment.

## Discussion

DOX is one of the commonly used chemotherapeutic agents in the treatment of breast cancer. However, the development of DOX resistance has become a prominent clinical obstacle in the treatment of this disease. A number of studies support the notion that miRNAs are critical players in tumor invasion, progression, and metastasis. miRNAs may function as either oncogenes or tumor suppressors, and some of them are associated with acquisition of drug resistance [15-17]. The present study showsthat expression of miR-455 is significantly associatedwith the resistance of MCF-7 cells to DOX. Specifically, sensitivity to DOX decreased in MCF-7 cells upon transfection with miR-455 mimics. In contrast, inhibition of miR-455 resulted in the increased sensitivity of breast cancer cells to chemotherapy.

This study shows that miR-455-associated drug resistance is mediated by reduced DNMT3A activities, thus supporting the idea that DNA hypomethylation is a major mechanism closely associated with acquisition of drug resistance by breast cancer cells [18, 19]. DNA hypomethylation ina variety of cancers promotes cell proliferation, invasion, metastasis, and drug resistance [20]. This effect was observed in many solid tumors such as cervical cancer, metastatic hepatocellular cancer, prostate tumors, as well as in hematologic malignancies such as B-cell chronic lymphocytic leukemia. In addition, a global hypomethylation observed in cancers, exemplified by breast, cervical, and brain cancers, demonstrates a progressive increase in line with the grade of malignancy [21, 22]. A mutation of *DNMT3B* has been found in patients with immunodeficiency and centromeric instability, which causes the instability of the chromatin [23, 24]. Moreover, hypomethylation likely contributes to oncogenesis by activation of oncogenes such as *cMYC* or *RAS* [18].

Our study adds a novel dimension to an already complex mechanism involved in the acquisition of drug (DOX) resistance in the course of chemotherapy of breast cancer [12, 13]. It has been previously reported that increased energy-dependent efflux of chemotherapeutic drugs is a major mechanism responsible for the resistance of cancer cells to DOX. Furthermore, Kovalchuk and co-workers reported that miR-451 regulates multiple drug resistance 1 gene (*MDR1*) expression [12]. By using luciferase reporter assay, they showed that miR-451 is a direct regulator of *MDR1*. Furthermore, they found that transfection of the MCF-7/DOX cells with miR-451 led to increasedsensitivity of resistant cells to DOX, suggesting that manipulatingthe expression of miRNA may have significant implications for therapeutic strategies aiming to overcome the cancer cell resistance. Recently, multiple studies supported the view that siRNAs or miRNAs can be used to quench

resistance of cancer cells to chemotherapeutic drugs. Our findings are consistent with these conclusions. A previous study using MCF-7/DOX cells showed modifications of epigenetic landscapes characterized by the loss of methylation of CpG islands, including hypomethylation of *MDR1* gene [25]. Our study is consistent with-the view that miR-455-mediated reduction of DNMT3A activity is responsible for the global loss of DNA methylationand, at least partially, for acquisition of DOX resistance by breast cancer cell.

## Conclusions

In summary, we showed that the development of DOX resistance in breast cancer is associated with the evident deregulation of miRNA expression, exemplified by miR-455. We have further shown that expression of miR-451 is inversely correlated with the DNMT3A expression in breast cancer drug-resistant cells. Moreover, the enforced increase of miR-455 levels in the MCF-7/DOX cells down-regulates the expression of DNMT3A and reducesthe sensitivity of resistant MCF-7 cancer cells to DOX. These results provide a valid base for the development of miRNA-based therapeutic strategies aiming to overcome the cancer cell resistance.

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## Disclosure of conflict of interest

None.

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