



# Involvement of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1

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## ABSTRACT

Multidrug resistance-associated protein (MRP-1/ABCC1) transports a wide range of therapeutic agents and may play a critical role in the development of multidrug resistance (MDR) in tumor cells. However, the regulation of MRP-1 remains controversial. To explore whether miRNAs are involved in the regulation of MRP-1 expression and modulate the sensitivity of tumor cells to chemotherapeutic agents, we analyzed miRNA expression levels in VP-16-resistant MDR cell line, MCF-7/VP, in comparison with its parent cell line, MCF-7, using a miRNA microarray. MCF-7/VP overexpressed MRP-1 mRNA and protein not MDR-1 and BCRP. miR-326 was downregulated in MCF-7/VP compared to MCF-7. Additionally, miR-326 was downregulated in a panel of advanced breast cancer tissues and consistent reversely with expression levels of *MRP-1*. Furthermore, the elevated levels of miR-326 in the mimics-transfected VP-16-resistant cell line, MCF-7/VP, downregulated MRP-1 expression and sensitized these cells to VP-16 and doxorubicin. These findings demonstrate for the first time the involvement of miRNAs in multidrug resistance mediated by MRP-1 and suggest that miR-326 may be an efficient agent for preventing and reversing MDR in tumor cells.

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## 1. Introduction

Chemotherapy is the mainstream method of treatment against advanced breast cancer. However, cancerous cells frequently develop multidrug resistance (MDR) to chemotherapy agents [1]. MDR is the ability of tumor cells to resist several unrelated drugs after exposure to a single chemotherapy drug [2] and is the leading cause of cancer-related death. MDR has frequently been associated with elevated expression levels of one or more ATP binding cassette (ABC) transporters. There are three well-known transporters [3]: ABCB1 (MDR-1/P-gp) [4], multidrug resistance-associated protein ABCC1 (MRP-1) [1], and ABCG2 (BCRP) [5].

**Abbreviations:** miRNA, microRNA; MRP-1, multidrug resistance-associated protein 1; MDR, multidrug resistance; MDR-1, multidrug resistance 1; BCRP, breast cancer resistance protein; siRNA, small interfering RNA; RT-PCR, reverse transcription polymerase chain reaction.

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Despite some promising results were observed in hematological tumors, treatment involving solid tumors with MDR has been rather disappointing. It appears that other unknown MDR mechanisms co-exist, and the regulation of these transporters remains controversial. These factors suggest that the most practical and long-term solution for overcoming the vast majority of breast cancers today is not likely to be achieved by creating new drugs. Instead, an increased focus on the dominant causes of breast cancer drug resistance is warranted, especially in regard to the exploration of novel MDR mechanisms. A recently discovered class of small, functional, non-coding RNAs, named microRNAs (miRNAs), has been identified in animals and plants. miRNAs have been shown to function as regulatory molecules by inhibiting protein translation and to play an important role in development, differentiation, cell proliferation, and apoptosis [6]. A few studies suggest that downregulation of miRNAs may play a critical role in cancer progression [7,8]. More recently, miR-451 and miR-27 have been demonstrated to be involved in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin mediated by MDR-1 [9,10]. miR-328 was shown to regulate negatively expression of BCRP [11]. In the present study, we show

for the first time that miRNA is involved in multidrug resistance mediated by MRP-1.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

Wild-type MCF-7 (MCF-7/WT), VP-16-resistant MCF-7 (MCF-7/VP), and mitoxantrone-resistant MCF-7 (MCF-7/MX100) human breast cancer cell lines (a gift of Susan E. Bates from NCI/NIH, Frederick, MD) were grown in DMEM containing 10% FBS, 100 U/ml of penicillin sodium, and 100 µg/ml of streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. MCF-7/VP was selected in increasing concentrations of etoposide (VP-16) [12] and MCF-7/MX100 was selected with mitoxantrone in a stepwise manner [13].

### 2.2. Tissue samples

We obtained 35 archived normal breast and breast tumor samples from the Avon Tissue Bank for Translational Genomics Research at Grady Memorial Hospital in Atlanta, GA. These included 10 normal breast tissues (N), 5 early breast cancer tissues (stage I) without metastasis (P1), and 10 advanced primary breast cancer tissues (stages III and IV) with lymph node metastasis (P2).

### 2.3. miRNA microarray hybridization

For miRNA microarray analysis, total RNA was extracted from 70 to 85% confluence of MCF-7 and MCF-7/VP cells with Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. 10 µg total RNA was directly labeled with biotin and hybridized to a commercial microarray, GenoExplorer microRNA human array (GenoSensor Corporation, Tempe, AZ) according to manufacturer's instruction. A streptavidin-Alexa dye was used to stain the hybridized targets, and the fluorescent signals were then scanned using a GenePix 4000b Biochip Reader (Molecular Devices, Union City, CA) and analyzed using the Digital Genome System Suite (Molecularware, Irvine, CA). The average of three mean fluorescence signal intensities for each miRNA probe was normalized to that for tRNA. miRNAs detected as two-fold greater than background were considered to be expressed. Duplicates of each miRNA were printed on microarray and these experiments were repeated one time.

### 2.4. RT-PCR and quantitative RT-PCR

To prepare total RNA from tissues, the tissues were cleaned twice with 100% followed by 95% of ethanol after 5-µM sections of each sample were deparaffined 2 times with xylene for 10 min. After washing 3 times for 3 min each with PBS, tissues were digested overnight in a 37 °C water bath with 100 µl digestion solution containing 2% SDS and 2 mg/ml of proteinase K. The digested tissue lysates were subjected to extraction of RNA with Trizol Reagent (Invitrogen). Total RNA was extracted from cultured MCF-7/WT, and MCF-7/VP cells with Trizol. Primer sequences of *MRP-1*, *MDR-1*, *BCRP*, *U6 snRNA* and *miR-326* are as follows: *MDR-1* (GeneBank accession number AF016535), 5'-GCTCCTGACTATGCCAAAGC-3' and 5'-TCTTCACTCCAGGCTCAGT-3'; *MRP-1* (GeneBank accession number NM\_004996), 5'-AGGTGGACCTGTTTCGTGAC-3' and 5'-ACCC-TGTGATCCACCAGAAG-3'; *BCRP* (GeneBank accession number AF098951), 5'-CACCTTATTGGCCTCAGGAA-3' and 5'-CCTGCTTG-GAAGGCTCTATG-3'; *miR-326* (GeneBank accession number MI0000808), 5'-CATCTGTCTGTTGGGCTGGA-3' and 5'-AGGAAGG-GCCAGAGGCG-3'; *U6 snRNA* (GeneBank accession number NM\_012321), 5'-CGGGTTTGTTCATTCT-3' and 5'-AGTCCAG-

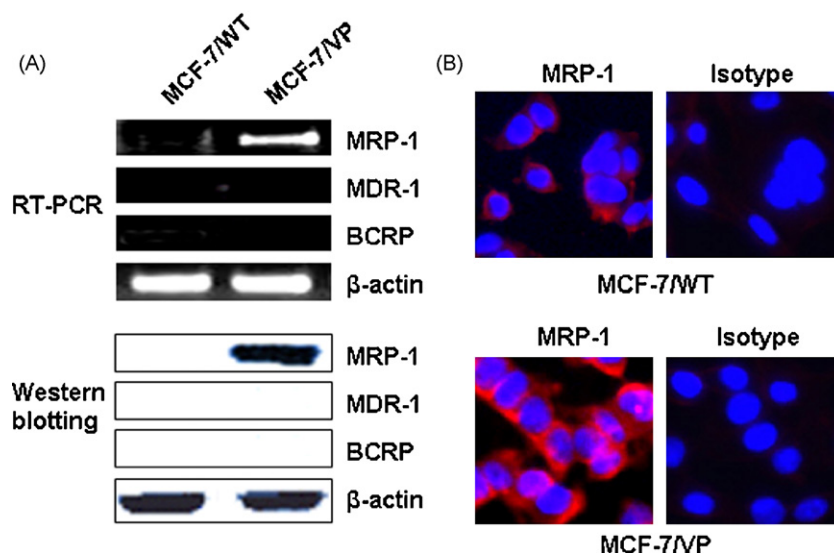
CATGAACAGCTT-3'. The primer sequence of  $\beta$ -actin was described in our previous publication [14]. Quantitative and regular RT-PCR was performed following our previous descriptions [15,16]. For regular and quantitative RT-PCR, 500 ng of total RNA were transcribed into cDNA in a 20 µl of reaction volume at 42 °C for 45 min with a GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA). The cycle conditions for cDNA PCR were 95 °C for 10 min followed by 40 cycles of 95 °C for 20 s, and 60 °C for 45 s. For regular RT-PCR, reactions were performed with using a GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems). For SYBR Green quantitative PCR amplifications, reaction was performed in a 20 µl reaction volume containing 10 µl of 2× SYBR Green PCR Master Mix (Applied Biosystems). The relative expression levels of each cell line or tissue samples of each group were measured using the  $2^{-\Delta\Delta Ct}$  method [17,18]. Briefly, the average  $\Delta Ct$  of each group was calculated by following formula:  $\Delta Ct = \text{average } MRP-1 \text{ or } miR-326 \text{ Ct} - \text{average of HK (housekeeping) gene } (\beta\text{-actin or U6 snRNA}) \text{ Ct}$ .  $\Delta\Delta Ct$  was calculated by  $\Delta\Delta Ct = \Delta Ct \text{ of control group} - \Delta Ct \text{ of the treated group}$ . The fold change for *MRP-1* or *miR-326* expression level was calculated using  $2^{-\Delta\Delta Ct}$ . The results are presented as fold change in the MCF-7/VP cells relative to the parental MCF-7 cells and tumor sample groups relative to the normal tissue group.

### 2.5. Construction of the vector and luciferase reporter assay

Two 3'-untranslated region (UTR) fragments of *MRP-1* gene (accession no. NM\_004996) with and without complementary sequence of *miR-326* were amplified by PCR using the primers that included a Bgl II restriction site on the 5' and 3' strands, respectively. The primers for the UTR segment containing complementary sites of *miR-326* were 5'-AATTAGATCTAAGAAAA-CAGGGAAGCAGCA-3' (sense) and 5'-AATTAGATCTGCTCTC-TGGGTTTGAAGTCG-3' (antisense). The primers for 3'-UTR segment of *MRP-1* without complementary sequence of *miR-326* were 5'-AATTAGATCTGCTGTGAAGACCCAGGAGAG-3' (sense) and 5'-AATTAGATCTACAGACTTCGGCTGGAGAGA-3' (antisense). PCR products were restricted with Bgl II restriction endonuclease (Biolabs, Ipswich, MA) and then gel purified with a kit (Qiagen, Valencia, CA). These two *MRP-1* 3'-UTR fragments were ligated into the pGL2-control vectors (Promega, Madison, WI) by using the Bgl II site. The resulting luciferase UTR-report vectors and increasing concentrations of *miR-326* mimic were co-transfected into MCF-7/VP cells using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). Twenty-four hours after transfection, luciferase activity assays were performed using Steady-Glo Luciferase Assay System (Promega) following the manufacturer's instructions.

### 2.6. Transfection of hsa-mir-326 miRIDIAN mimic and measurement of drug sensitivity

The hsa-mir-326 miRIDIAN mimic was purchased from Dharmacon (Lafayette, CO). The mimic or control oligonucleotide was transfected into drug-resistant MCF-7/VP cells and MCF-7/MX100 at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen). To determine the efficiency of the mimic, at 48 h post-transfection, the transfected cells were collected to measure the mRNA and protein levels of *MRP-1*. The sensitivity of the mimic-transfected cells to VP-16, doxorubicin and mitoxantrone (Sigma-Aldrich, St. Louis, MO), was determined by increasing concentrations of the tested drugs. Cell viability was assessed using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) Kit (Promega), following the manufacturer's instructions as previously described [14]. Absorbance at 490 nm was directly proportional to the number of living cells in culture.



**Fig. 1.** Expression of mRNA and protein of MDR-1, MRP-1, and BCRP in MCF-7/VP and MCF-7 cells. (A) Expression levels of mRNAs and protein of MRP-1, MDR-1, and BCRP in MCF-7/VP and its counterpart determined by RT-PCR and Western blotting analysis.  $\beta$ -actin was used as an internal loading control. (B) Immunofluorescence staining for MRP-1 in MCF-7/VP and MCF-7 cells. Red color represents MRP-1 and blue color is nuclear counter staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

## 2.7. Western blotting analyses

Proteins were resolved in an SDS/PAGE gel and subjected to immunoblot analysis using monoclonal antibodies against MRP-1 (MAB4147, Millipore, Billerica, MA), MDR-1 (MAB4336, Millipore), BCRP (EMD4Biosciences, Gibbstown, NJ), or  $\beta$ -actin (Sigma-Aldrich). All antibodies were used at 1  $\mu$ g/ml of working concentration in PBS with 5% dried-milk. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce Biotechnology Inc., Rockford, IL). Quantification of protein bands was performed using the ImageJ software.

## 2.8. Immunofluorescence staining

Expression of MRP-1 protein in the MCF-7/VP and MCF-7 cells was detected by immunofluorescence as described previously [14]. Cells were cultured on chamber slides for 24 h and fixed in PBS containing 3.8% paraformaldehyde. The fixed cells were incubated with the primary mouse anti-human MRP-1 monoclonal antibody (MAB4147, Millipore) diluted 1:50 at room temperature for 60 min. Rhodamine-coupled secondary antibody (R&D Systems, Minneapolis, MN) was used for MRP-1 visualization at 1:200 dilution and 1 mM Hoechst (Invitrogen) was used for counter-staining of nuclear at 1:500 at room temperature for 30 min.

## 2.9. Statistical analysis

Quantitative RT-PCR reaction was run in triplicate for each sample and repeated at least 2 times and the data were statistically analyzed with a Student's *t*-test. IC<sub>50</sub> data from the MTS proliferation assay were analyzed with Wilcoxon Matched Pairs Test.

## 3. Results

### 3.1. Expression of three MDR-related transporters in MCF-7/VP and MCF-7 cell lines

Prior to the analysis of miRNA expression, we analyzed expression of mRNAs and proteins of three MDR-related trans-

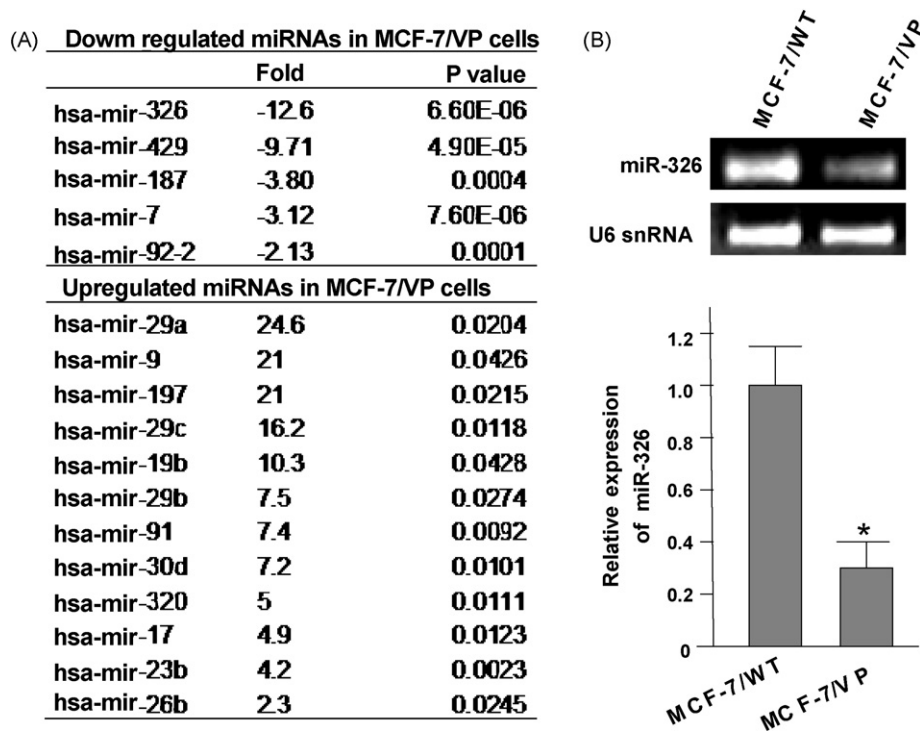
porters, MRP-1, MDR-1, and BCRP, in MCF-7/WT and VP-16-resistant MCF-7/VP cells with RT-PCR (Fig. 1A), Western blot analysis (Fig. 1A), and immunofluorescence staining (Fig. 1B). Results showed that MCF-7/VP, which was selected by VP-16, overexpressed MRP-1 mRNA and protein in comparison with MCF-7/WT. No expression of MDR-1 and BCRP was observed in both MCF-7/WT and MCF-7/VP (Fig. 1A).

### 3.2. Alteration of miRNA expression in MCF-7/VP cells

In order to determine whether the expression of certain miRNAs was altered in MDR tumor cells, we performed profile analysis of miRNA expression in MCF-7/VP cells compared with their parent cell line, MCF-7, using a microarray containing 463 human mature miRNA probes. Seventeen of miRNAs were differentially expressed in MCF-7/VP cells and their parent cells. The majority of these miRNAs exhibited increased expression levels, while miR-326, miR-429, miR-187, miR-7, and miR-92-2 showed decreased expression (Fig. 2A). These miRNAs may be specific modulators of MRP-1 and play a critical role in the development of MDR. To verify the findings obtained by miRNA microarray profiling, quantitative RT-PCR analysis for the down-regulated miRNAs was performed. Expression of miR-326, miR-429, miR-187, and miR-7 was downregulated significantly with quantitative RT-PCR while miR-92-2 was not (data not shown). As consistent with the microarray data, quantitative RT-PCR analysis demonstrated 3.3-fold decreased expression of miR-326 in MCF-7/VP cells in comparison with their parental cells (Fig. 2B), which is the largest downregulated expression among these four significantly downregulated miRNAs.

### 3.3. Negative correlation of miR-326 and MRP-1 in a selected panel of breast cancer tissues

We have observed the altered expression of certain miRNAs in MCF-7/VP cells compared with MCF-7/WT. These differentially expressed miRNAs may be specific modulators of MRP-1. To investigate further association of miRNAs to MRP-1, we measured expression levels of a panel of the downregulated miRNAs (miR-326, miR-187, miR-429, and miR-07) and MRP-1 mRNAs in 10 normal breast tissues (N), 5 early breast cancer tissues without



**Fig. 2.** Differential expression of miRNAs in MCF-7/VP cells and their parental cells. (A) Differentially expressed miRNAs in MCF-7/VP and its counterpart determined by microRNA microarray analysis. Data are shown as fold changes of miRNA levels (normalized median values) in MCF-7/VP relative to MCF-7. (B) Validation of expression levels of miR-326 with quantitative RT-PCR. Quantitative RT-PCR was performed in triplicate for each sample and assays were repeated once. The relative levels were normalized to U6 snRNA. The top panel is pictures of agarose gel electrophoresis of representatives of quantitative RT-PCR reactions. The lower panel shows fold changes of *miR-326* levels in MCF-7/VP cells in comparison to their parental cells, MCF-7. \* $P < 0.01$ .

metastasis (P1), and 10 advanced breast cancer tissues with metastasis (P2) with quantitative RT-PCR. Advanced breast cancer tissues expressed the highest levels of *MRP-1* and the lowest levels of *MRP-1* were observed in normal breast tissues (Fig. 3A and B). Conversely, the expression levels of *miR-326* in normal breast tissues were the highest, while advanced breast cancer tissues expressed the lowest levels of *miR-326* (Fig. 3A and B). Fig. 3C shows that expression levels of *miR-326* mRNA correspond negatively with *MRP-1* mRNA in tissues ( $R = -0.7178$ ,  $P < 0.01$ ). No significant difference between these three types of tissues was observed for expression of *miR-187*, *miR-429*, and *miR-07* respectively (data not shown). Therefore, we focused on *miR-326* for further experiments.

#### 3.4. *miR-326* downregulated expression of *MRP-1*

The *MRP-1* gene possesses the putative *miR-326* targeting sequence predicted by TargetScan (Fig. 4A). To address whether the lowed expression of *miR-326* contributes to breast cancer MDR through modulating expression of *MRP-1* and increasing drug sensitivity of tumor cells, we employed a gene overexpression approach to examine the effect of *miR-326* on *MRP-1* mRNA and protein expression in MCF-7/VP cells. MCF-7/VP cells were transfected with *miR-326* mimics (Dharmacon) or control oligonucleotide. Expression levels of mRNA and protein of *MRP-1* were determined by quantitative RT-PCR and Western blotting 48 h after transfection. The expression levels of *MRP-1* mRNA (Fig. 4B) and protein (Fig. 4C) determined by real-time RT-PCR and Western blot were decreased in *miR-326* miRIDIAN mimic-transfected MCF-7/VP cells relative to control cells.

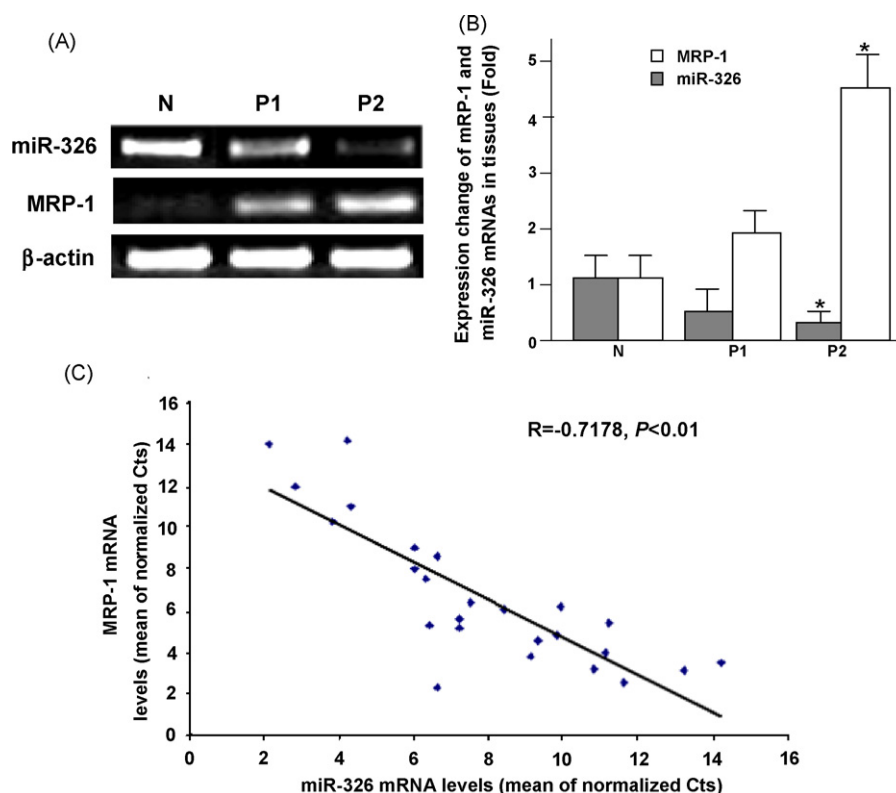
To examine whether *miR-326* directly targets *MRP-1*, a segment of *MRP-1* 3'-UTR containing *miR-326* binding sites was cloned into a luciferase reporter system. The plasmid lacking the *MRP-1* 3'-UTR

fragment was used as a positive control of luciferase activity. The resulting reporter vector was transfected into the MCF-7/VP cells together with increasing concentrations of *miR-326*. Fig. 4D shows that *miR-326* inhibited luciferase activity from the construct with the *MRP-1* 3'-UTR segment containing *miR-326* binding sites in a dose-dependent manner. No luciferase activity change was observed when the cells were transfected with the plasmid with a *MRP-1* 3'-UTR fragment without the *miR-326* binding sequence (Fig. 4D).

#### 3.5. Repression of *MRP-1* expression by *miR-326* increased sensitivity of MCF-7/VP MDR cells to VP-16 and doxorubicin

To address whether inhibition of *MRP-1* expression by *miR-326* results in the increased sensitivity of the MCF-7/VP cells, we transfected *miR-326* mimic to MCF-7/VP and determined sensitivity of these mimic-transfected cells to VP-16 and doxorubicin. A MTS cell proliferation assay was performed with increasing concentrations of V-16 and doxorubicin with 48-h treatment. The  $IC_{50}$  of resistant MCF-7/VP cells to VP-16 prior to transfection of *miR-326* was 15.3 times higher compared with their parental cells, MCF-7 (Fig. 5A). The  $IC_{50}$  of *miR-326*-transfected MCF-7/VP cells to VP-16 was 7.1 times lower than MCF-7/VP cells transfected with control oligonucleotide and only 2.1 times higher compared with their parental cells, MCF-7 (Fig. 5A). Additionally, Fig. 5B shows that transfection of MCF-7/VP with *miR-326* resulted in the decrease of resistance of MCF-7/VP cells to doxorubicin. The resistance of MCF-7/VP prior to transfection of *miR-326* to doxorubicin was 20 times compared with their parental cells, MCF-7 (Fig. 5B). After MCF-7/VP cells were transfected with *miR-326*, their  $IC_{50}$  to doxorubicin was 10 times lower compared with MCF-7/VP transfected with control oligonucleotide and 1.9 times higher compared with their parental MCF-7 cells (Fig. 5B). These results show clearly that inhibition of *MRP-1* by *miR-326* sensitizes MDR MCF-7/VP cells to VP-16 and





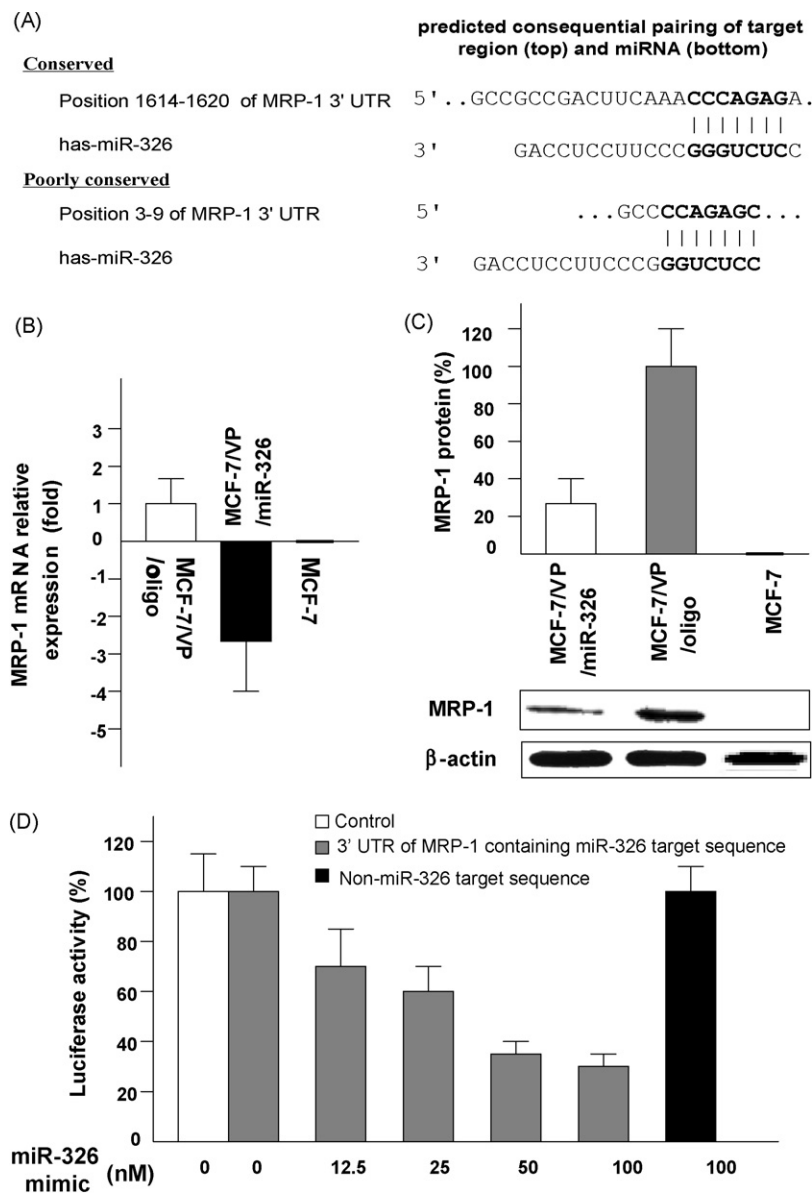
**Fig. 3.** Expression levels of *miR-326* in breast cancer tissues. (A) Expression levels of *miR-326* and *MRP-1* in representatives of early breast cancer tissues, advanced breast cancer tissues and normal breast tissues after quantitative RT-PCR. (B) Mean levels of *miR-326* with quantitative RT-PCR from P1, early breast cancer tissue without metastasis ( $N = 5$ ); and P2, advanced breast cancer tissue with metastasis ( $N = 10$ ), relative to that of control group, N, normal breast tissue ( $N = 10$ ).  $P$  value  $< 0.01$  compared to normal tissues. (C) The correlation between *miR-326* and *MRP-1* expression levels in breast cancer tissues ( $N = 15$ ) and normal breast tissues ( $N = 10$ ). Quantitative RT-PCR was performed in triplicate for each sample and assays were repeated once. The relative levels were normalized to *U6 snRNA* or  $\beta$ -actin. Each point in this scatter graph represents an individual sample, in which relative *miR-326* levels indicate on x-axis and *MRP-1* levels on y-axis. The x-axis shows normalized Ct values for *miR-326* determined by quantitative RT-PCR and the bigger value represents the lower expression levels of *miR-326*. The y-axis shows normalized Ct values for *MRP-1* determined by quantitative RT-PCR and the bigger value represents the lower expression levels of *MRP-1*. "Mean of normalized Ct values" is the subtraction of "mean of triple Ct values for *miR-326* (x-axis) or *MRP-1* (y-axis)" by "mean of triple Ct values for *U6 snRNA* or  $\beta$ -actin". The correlation coefficient,  $R = -0.7178, P < 0.01$ , indicates there is a strongly negative relationship between *miR-326* and *MRP-1*.

doxorubicin. VP-16 and doxorubicin have been shown to be substrates of MRP-1 [19]. To verify that the increased cell sensitivities to VP-16 or doxorubicin were mediated by MRP-1, we performed assays of sensitivity of the tested cells to another anti-cancer drug, mitoxantrone, a substrate of BCRP not MRP-1. The data showed that *miR-326* did not have significant effect on the sensitivity of the tested cells to mitoxantrone (Fig. 5C). Furthermore, to show a general mechanism, we performed the drug sensitivity analysis of a second cell line, MCF-7/MX100, selected by mitoxantrone and overexpressed BCRP not MRP-1, to VP-16 in the presence or absence of *miR-326* mimic. The results showed that *miR-326* did not have effect on the sensitivity of MCF-7/MX100 to VP-16 (Fig. 5D).

#### 4. Discussion

More than 5300 human genes have been implicated as potential miRNA targets [20,21]. Previous studies suggest that down-regulation of miRNAs may play a role in cancer progression [22–24]. Despite strong evidence suggesting that miRNAs can be potential biomarkers for cancer diagnosis and personalized therapy, only a few of publications [9–11,25,26] are reported as to involvement of microRNAs in development of MDR in cancer. The role of microRNAs in development of cancer MDR remains largely unexplored. In this study, our findings demonstrating the involvement of *miR-326* in MDR mediated by MRP-1 provide new evidence about the roles of microRNAs determining MDR development of tumor cells.

Though MDR-1, MRP-1, and BCRP have been considered as critical MDR-related factors, they are not strong as predictive biomarkers for monitoring development of MDR and as targets for preventing and reversing MDR. Moreover, mechanisms of regulating the expression of these proteins remain controversial so far. microRNAs have been demonstrated as regulators of many function genes [20,21]. More recently, *miR-451* and *miR-27* have been demonstrated to regulate expression of MDR-1/P-gp and promote MDR development of tumor cells to cytotoxic drugs [9]. It was reported that *miR-328* was downregulated in MDR breast cancer cell line, MCF-7/MX100, and modulated expression of BCRP [11]. However, no report elucidates the involvement of microRNAs in MRP-1-mediated MDR. MDR-1, MRP-1, and BCRP are three critical transporters for development of MDR. In this present study, the expression profiles of miRNAs in MCF-7/VP MDR breast cancer cells, were demonstrated to be different from their parental MCF-7 cells. MCF-7/VP MDR cell line was selected from human breast cancer cell line MCF-7 in increasing concentrations of VP-16 [12]. Our data show that MCF-7/VP cells were overexpressed mRNA and protein of MRP-1 but not MDR-1 and BCRP compared to their parental MCF-7 cells. These results are consistent with previous reports [12,27]. The present study reveals that 17 miRNAs were differentially expressed in MCF-7/VP MDR cells and their parental MCF-7 cells. The altered expression of these miRNAs in MCF-7/VP cells not only provides important evidence for understanding mechanisms of MDR in cancer cells but also offers the chance to identify new biomarkers for diagnosis and targets for preventing and reversing MDR in tumor cells.

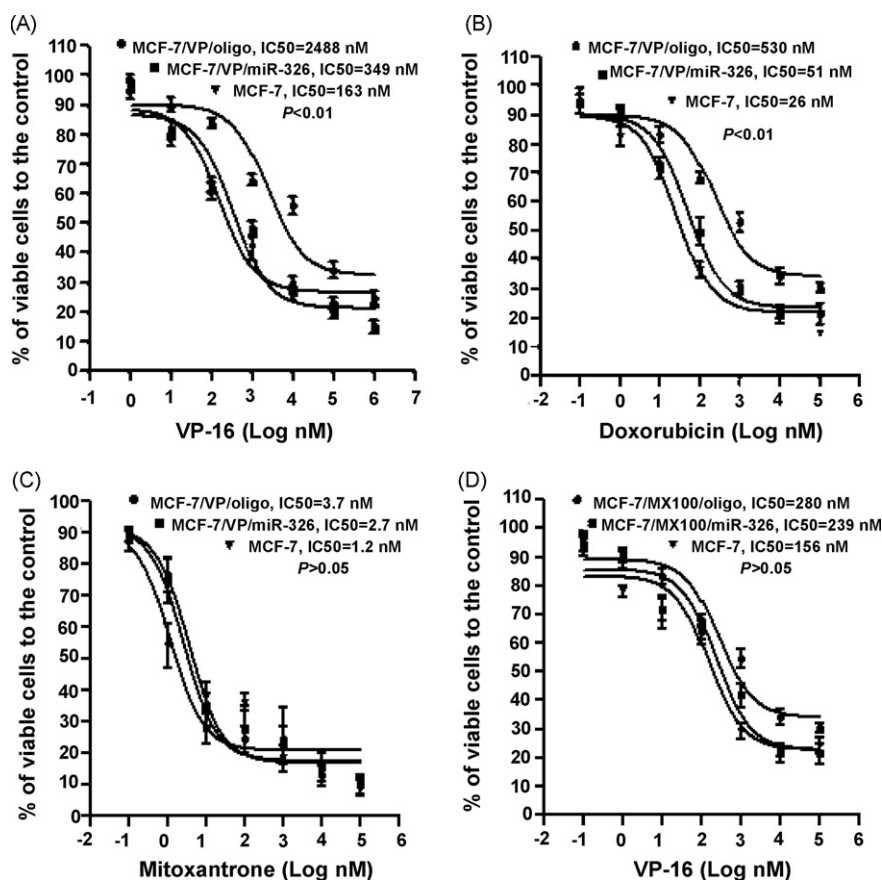


**Fig. 4.** Elevated levels of miR-326 inhibit expression of MRP-1 in MCF-7/VP cells. (A) The putative miR-326 targeted sequence in the *MRP-1* gene. TargetScan predicts two binding sites in *MRP-1* 3'-UTR. (B) Expression levels of *MRP-1* mRNA in the miR-326 mimic-transfected MCF-7/VP cells determined by real-time RT-PCR were downregulated in comparison with control cells ( $P < 0.01$ ). (C) Protein expression levels of MRP-1 in miR-326-transfected MCF-7/VP cells were downregulated in comparison with control oligonucleotide-transfected MCF-7/VP cells. Proteins were quantified with ImageJ software and relative expression levels show in the top panel. The low panel shows representatives of three experiments. (D) Luciferase reporter assays. The luciferase activity in MCF-7/VP transfected with the vector containing MRP-1 3'-UTR fragment with binding sequence of miR-326 was inhibited by transfection of miR-326 at a dose-dependent manner.

Some miRNAs in cancer cells may function as oncogenes to inhibit expression of tumor suppressors like BCL2 [25,28]. miR-21 has been shown to function as an oncogene and plays a critical role in tumorigenesis, invasion, and metastasis of breast cancer cells, in part through the downregulation of tumor suppressor genes [29]. More recently, miR-126 and miR-335 have been demonstrated to be lost in a majority of primary breast tumors from recurrent patients and identified as suppressor miRNAs of breast cancer metastasis [30], while miR-373, miR-520C, and miR-10b have been demonstrated to be metastasis-promoting miRNAs [31,32]. However, few investigations have addressed the relationship between miRNAs and MDR. The current study found that 12 of 17 miRNAs were overexpressed in MCF-7/VP MDR cells compared with their parental cells, suggesting that certain miRNAs of these 12 miRNAs may function as oncogenes to promote expression of MDR-related genes through targeting certain tumor suppressors. On the other

hand, it has been shown that certain miRNAs function as suppressors that can be incorporated into the RNA-induced silencing complex and downregulate target mRNAs through initiation of degradation [6,28]. The loss of these suppressor miRNAs may lead to overexpression of their targeted genes such as MDR-related genes in cancer cells. The current study found that miR-326, miR-429, miR-187, miR-7, and miR-92-2, were down-regulated in MCF-7/VP MDR cells compared with their parental cells, which suggests that these miRNAs may function as suppressors that block the expression of MRP-1.

Furthermore, the elevated levels of miR-326 in miR-326 mimic-transfected MCF-7/VP MDR cells not only downregulated expression of MRP-1 mRNA and protein, but also increased sensitivity of these cells to VP-16 and doxorubicin. Recently, miR-326 was identified as a suppressor of Hedgehog developmental signaling pathway to control the development of cerebellar neuronal



**Fig. 5.** Effect of increasing levels of miR-326 on the sensitivity of MCF-7/VP cells to VP-16 and doxorubicin. Drug resistance of miR-326-transfected MCF-7/VP, control oligonucleotide-transfected MCF-7/VP, and MCF-7 cells was tested by increasing concentrations of the tested drugs and measuring the surviving cells with the MTS cell proliferation assay at 48 h after the cells were treated. Each point is the average of triplicate determinations. Elevated levels of miR-326 in the mimic-transfected MCF-7/VP cells sensitized these cells to VP-16 (A) and doxorubicin (B) while miR-326 did not change the sensitivity of MCF-7/VP cells to another anti-cancer drug, mitoxantrone, a substrate of BCRP not MRP-1 (C). (D) The drug sensitivity analysis of a second cell line, MCF-7/MX100, selected by mitoxantrone and overexpressed BCRP not MRP-1, to VP-16 in the presence or absence of miR-326 mimic.

progenitor and tumor cells [33]. It was reported that miR-326 was downregulated in the adipogenic differentiation process of adipose-derived stem cells [34]. Here, we report for the first time miR-326 as a suppressor to modulate MRP-1-mediated MDR of tumor cells to chemotherapeutic agents. Our results demonstrated that the loss of miR-326 in MCF-7/VP MDR cell line contributed to the overexpression of MRP-1 and sensitized MCF-7/VP MDR cells to VP-16 and doxorubicin. Currently, a few investigations have successfully targeted oncogenes with artificial synthetic miRNAs [17,35,36]. Our results suggest that targeting MRP-1 by miR-326 may have significant implications for prevention and reversal of tumor cell MDR.

In conclusion, we have shown that expression of miR-326 is negatively correlated with MRP-1 expression in breast cancer cells and tissues. Furthermore, the elevated level of miR-326 by transfection of miR-326 mimic not only downregulates expression of MRP-1 and also sensitizes MCF-7/VP MDR cells to cytotoxic drugs. Our findings contribute further to the understanding of MDR regulation in cancer cells. Additionally, these findings may be beneficial for further research of predicting MDR in patients and designing personalized therapy for breast cancer patients.

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