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PRECLINICAL STUDY

MiR-181a enhances drug sensitivity in mitoxantone-resistant breast cancer cells by targeting breast cancer resistance protein (BCRP/ABCG2)

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Abstract Breast cancer resistance protein (BCRP)/ATP-binding cassette subfamily G member 2 (ABCG2) mediates multidrug resistance (MDR) in breast cancers. In this study, we aimed to investigate the role of microRNAs in regulation of BCRP expression and BCRP-mediated drug resistance in breast cancer cells. Microarray analysis was performed to determine the differential expression patterns of miRNAs that target BCRP between the MX-resistant breast cancer cell line MCF-7/MX and its parental MX-sensitive cell line MCF-7. MiR-181a was found to be the most significantly down-regulated miRNA in MCF-7/MX cells. Luciferase activity assay showed that miR-181a mimics inhibited BCRP expression by targeting the 3′ untranslated region (UTR) of the BCRP mRNA. Overexpression of miR-181a down-regulated BCRP expression, and sensitized MX-resistant MCF-7/MX cells to MX. In a nude mouse xenograft model, intratumoral injection of miR-181a mimics inhibited BCRP expression, and enhanced the antitumor activity of MX. In addition, miR-181a inhibitors up-regulated BCRP expression, and rendered MX-sensitive MCF-7 cells resistant to MX. These findings suggest that miR-181a regulates BCRP expression via binding to the 3′-UTR of BCRP mRNA. MiR-181a is critical for regulation of BCRP-mediated resistance to MX. MiR-181a may be a potential target for preventing and reversing drug resistance in breast cancer.

Keywords MicroRNA-181a · BCRP · Drug resistance · Breast cancer

Introduction

Breast cancer is one of the most common malignant diseases that seriously threaten the health of women [1]. Chemotherapy is often used as neoadjuvant and adjuvant therapy for breast cancer. The development of multidrug resistance (MDR) is a major hurdle for the effective treatment of breast cancer. It is known that overexpression of cell membrane-bound ATP-binding cassette (ABC) transporters that exclude a variety of chemotherapeutic drugs contributes to MDR in breast cancer. The ABC transporter subfamily B member 1 (ABCB1/MDR1/P-glycoprotein), subfamily C member 1 (ABCC1/MRP1), subfamily G member 2 (ABCG2/breast cancer resistance protein (BCRP)), and lung resistance protein (LRP) have been well known to play an important role in breast cancer chemoresistance [2].

The BCRP (*ABCG2*) gene, localized on chromosome 4q22.1, encodes a 655-amino acid protein [3]. BCRP recognizes and transports a variety of chemotherapeutic drugs, such as mitoxantrone (MX), out of cancer cells, thereby resulting in reduced drug concentration, and subsequent drug resistance [4, 5]. Accumulating evidence has shown that BCRP plays a critical role in the development of MDR in breast cancer

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[6–8]. Increased BCRP expression has been found in breast cancer cells that exhibit resistance to MX, topotecan and 7-ethyl-10-hydroxycamptothecin (SN-38) [6]. In addition, up-regulation of BCRP confers resistance to tamoxifen and BMS-536924 (an insulin-like growth factor receptor/insulin receptor inhibitor) in breast cancer cells [7, 8]. In addition, BCRP expression correlates with chemotherapeutic response to anthracycline in patients with breast cancer [9]. Furthermore, overexpression of BCRP in breast cancer stem cells contributes to its intrinsic resistance to chemotherapy [10]. However, although it is well known that BCRP contributes to MDR in breast cancer, the molecular mechanisms by which the expression of BCRP is regulated remain unclear.

MicroRNAs (miRNAs) are small, non-coding RNAs (~22 nucleotides in length), which post-transcriptionally regulate the expression of genes by targeting their mRNAs. miRNAs bind to the complementary target sites in the 3′-untranslated regions (3′-UTRs) of mRNAs, and directly induce mRNA degradation or translational inhibition. miRNAs have emerged as important gene regulators and key players in carcinogenesis by acting as oncogenes or tumor suppressors [11]. The 3′-UTR of the BCRP mRNA reported in GenBank (accession no. NM_004827), and in the UTR database (UTR database entry UTR: 3HSA117529) is about 2 kb in length, which is considerably longer than the average 770 bp observed for human mRNAs [12, 13], suggesting that the 3′-UTR of the BCRP mRNA may be a potential target for regulation of BCRP expression by miRNAs. To date, several miRNAs that target BCRP mRNA 3′-UTRs have been identified. For example, MiR-519c has been found to inhibit BCRP expression in the S1 colon cancer cell line, and this effect is lost in drug-resistant cells because of a shorter 3′-UTR of the BCRP mRNA in these cells [14, 15]. MiR-520h has been reported to inhibit the mRNA and protein expression of BCRP in PANC-1 pancreatic cancer cells [16]. MiR-328 is also involved in post-transcriptional regulation of BCRP in MDR cancer cells, such as MCF-7/MX cells [17]. Therefore, miRNAs play an important role in regulation of BCRP expression in cancer cells.

MiR-181a was initially identified as a tumor-suppressing miRNA in human primary glioblastomas [18, 19]. However, miR-181a has also been reported to function as a tumor oncogene in breast cancer [20], gastric cancer [21], and head and neck cancer [22]. The miR-181a expression is up-regulated in the breast cancer, gastric cancer, and head and neck cancer, but reduced in the glioma [18] and aggressive chronic lymphocytic leukemia [23]. In addition, aberrant expression of miR-181a contributes to different phenotypes of cancer cells, such as proliferation [24], apoptosis [25, 26] and drug resistance [27, 28]. It has been reported that serum miR-181a represents a potential novel biomarker for primary breast cancer as well as for early stage breast cancer diagnosis [29]. In addition, high expression of miR-181a is a predictive biomarker for breast cancer metastasis and patient survival [30, 31]. Furthermore, it has been reported that overexpression of miR-181a induces autophagy by targeting ATG5 in breast cancer cells [32]. However, the role of miR-181a in the drug resistance of breast cancer remains unclear.

In the present study, we performed miRNA microarray analysis to determine the differential expression patterns of miRNAs that target BCRP between the parental MCF-7 and its derivative BCRP-overexpressing MCF-7/MX cells. We found that miR-181a was the most significantly down-regulated miRNA in MX-resistant MCF-7/MX cells. Importantly, our study revealed that miR-181a, functioning as a BCRP suppressor, reversed BCRP-mediated drug resistance in vitro and in vivo. These findings suggest that miR-181a may be useful for reversing BCRP-mediated drug resistance in cancer chemotherapy.

Materials and methods

Cell culture

The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection. MCF-7/MX was kindly provided by Zhirong Zhan (Molecular Therapeutics Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA). Adherent cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA, USA) containing 10 % fetal bovine serum (HyClone, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere with 5 % CO_2 at 37 °C. To maintain the MDR phenotype of MCF-7/MX cells, MX was added to the culture media.

Antibodies and reagents

Primary antibodies against BCRP, multidrug resistance-associated protein (MRP), lung resistance protein (LRP), and P-glycoprotein (PGP) were from Abcam Inc. (Cambridge, MA, USA). MX and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St. Louis, MO, USA). MiR-181a mimics, mimic negative controls, inhibitors, inhibitor negative controls, cholesterol-conjugated miR-181a mimics (Chol-miR-181a), and negative controls (Chol-miR-NC) were from RiboBio (Guangzhou, China).

miRNA microarray analysis

Affymetrix® GeneChip miRNA array analysis was conducted to detect the expression pattern of miRNAs, using total RNAs (including small RNAs), which were provided

by Shanghai Biotechnology Corporation (Shanghai, China). Oligo-nucleotide hybridization was performed on the miRNA microarray chip (Affymetrix). Hybridization signals were detected using an Affymetrix® scanner. Scanner images were quantified by the software GCOS1.4. Hybridization data were analyzed with the miRNA QC tool using the default parameters. The genes with ≥2 fold changes between MCF-7 and MCF-7/MX cells were selected. For a straightforward comparison of differences between samples, the expression profiles of miRNAs were analyzed using Gene clustering 3.0 [33], and visualized with Java Tree View [34].

Luciferase activity assay

To construct pGL3-BCRP-3′-UTR, the full length 3′-UTR of the human BCRP mRNA was amplified by PCR and cloned into the vector pGL3-control (Promega). For luciferase reporter assays, 293T cells were transiently transfected with renilla-luciferase reporter plasmids (Promega) containing pGL3-BCRP-3′-UTR and miR-181a mimics, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, reporter gene activity was measured using the dual-luciferase assay-system (Promega). Renilla-luciferase activity was used to normalize for transfection efficiency.

Cell transfection

MCF-7 and MCF-7/MX cells (3×10^5) were seeded on 6-well plates, and cultured overnight. Cells were then transfected with 20 nM miR-181a mimics and inhibitors, or 20 nM control miRNAs using Lipofectamine 2000, according to the manufacturer's instruction. After 48 h, cells were used for Western blot and qRT-PCR.

Quantitative reverse transcription-PCR

To detect the relative level of MRP, PGP, LRP, and BCRP transcripts, quantitative reverse transcription-PCR (qRT-PCR) was performed. Briefly, Total RNA was isolated from MCF-7 or MCF-7/MX cells using trizol reagents, according the manufacture's protocol. A cDNA library was generated through reverse transcription using MMLV reverse transcriptases (Promega). The cDNA was used for the amplification of genes of MRP, PGP, LRP, and BCRP. β-actin was used as an endogenous control. The PCR primers were described by Liang Z et al. [35]. For mature miRNA quantification, cDNA was synthesized using specific sten-loop universal primers (60 ng) and a TaqMan microRNA reverse transcription kit. U6 small nuclear RNA was used as an internal control. The reaction condition was as follows: 30 °C for 10 min; 42 °C for 1 h; 85 °C for 5 min; 5 °C for 5 min; and 4 °C on hold for overnight. qRT-PCR primers for has-miR-181a were: 5′-GCTGGC AACATTCAACGCTGTC-3′ (forward) and 5′-GTGCAG GGTC CG AGGT-3′ (reverse). RT-PCR conditions were 95 °C for 2 min; 95 °C for 15 s and 60 °C for 30 s; then 40 cycles of 95 °C for 1 min; 55 °C for 30 s; 95 °C for 30 s. The fold change for each miRNA relative to the control was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

For Western blot analysis, MCF-7 and MCF-7/MX cells were homogenized on ice in lysis buffer. Proteins (30–50 μg) from each cell lysate was mixed 4:1 with 5× sample buffer (20 % glycerol, 4 % sodium dodecyl sulfate, 10 % β-mercaptoethanol, 0.05 % bromophenol blue, and 1.25 M Tris–HCl, pH 6.8; all from Sigma). Equal amount of proteins were loaded and were separated by electrophoresis in 10 % sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred onto PVDF membranes by electroblotting. Membranes were blocked with 5 % milk in Tris-buffered saline with 0.1 % Tween 20, and then incubated with an appropriate dilution of MRP, PGP, LRP, and BCRP antibodies (1:1,000–1:2,000) overnight at 4 °C. Blots were developed with horseradish peroxidase-conjugated anti-IgG antibodies (Santa Cruz Biotechnology, USA) and a chemiluminescent detecting system (Amersham, Freiburg, Germany).

MX accumulation assay

MX accumulation assay was performed as described previously [36] with modifications. Briefly, cells were exposed with 3 μM MX for 1 h at 37 °C in the darkness. The MX accumulation was stopped by addition of ice-cold phosphate-buffered saline (PBS). The intracellular MX level was determined by measuring MX fluorescence using a flow cytometer (Becton–Dickinson).

Cell viability assay

MTT assay was used to measure cells that survived drug exposure. Cells were seeded on 96-well plates at a density of 5×10^3 cells/well, and allowed to grow in the growth medium for 48 h. Cells After transfection with miR-181a mimic or inhibitor and control for 48 h, cells were treated with serial dilutions of MX for another 48 h. Cells were incubated with 5 mg/ml MTT for 4 h, and subsequently solubilized in DMSO (100 μl/well). The absorbance at 570 nm was then measured using an ELISA reader. Experiments were repeated at least three times.

Nude mouse xenograft model

BALB/c athymic nude mice (female, 4–6 weeks old and 16–20 g) were bred at pathogen-free conditions in the Animal Center of China Medical University. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of China Medical University, Shenyang. Two independent series of experiments were performed. To establish a human breast tumor xenograft model, 4×10^7 MCF-7/MX cells were suspended in 100 ml PBS and inoculated subcutaneously into the flank of nude mice. After 7 days, the transplanted nude mice were subjected to experiments. To investigate the effect of miR-181a on the expressions of ABC transporters, mice were randomly assigned into three groups: the Chol-miR-181a group ($n = 6$), the Chol-miR-NC group ($n = 6$), and the untreated control group ($n = 6$). To investigate the effect of miR-181a on the antitumor efficacy of MX, mice were randomly assigned into four groups: the untreated control group ($n = 6$), the Chol-miR-NC group ($n = 6$), the Chol-miR-NC+MX group ($n = 6$), and the Chol-miR-181a+MX group ($n = 6$). 1 nmol miRNA in 0.1 ml saline buffer was intratumorally injected into the tumor mass every 3 days for 2 weeks starting on day 7. MX (1.5 mg/kg) was administered intravenously twice a week for 3 weeks on day 7. Tumor growth was monitored by caliper measurement twice a week until termination of the experiment. Tumor volume (V) was determined by the length (L) and width (W) according to the following formula: $V = (L \times W^2) \times 0.5$. Mice were sacrificed, and tumors were removed and weighed 31 days after tumor transplantation. Tumor tissues were snap frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA and protein extraction.

Statistical analysis

Data were analyzed using the SPSS statistics 16.0 software package. Results are presented as mean ± standard deviation (SD).One-way ANOVA was used to compare differences among groups, followed by LSD post hoc tests. The P values <0.05 were considered statistically significant.

Results

MiR-181a is down-regulated in BCRP-overexpressing breast cancer cells

We first characterized the differential expression of MDR-related ABC transporters, including MRP, PGP, LRP, and BCRP, between the parental MCF-7 and its derivative MX-resistant MCF-7/MX breast cancer cells, using Western blot. As shown in Fig. 1a, compared to parental MCF-7 cells, the expression of BCRP, but not the MRP, PGP, and LRP, was up-regulated in MCF-7/MX cells. We next tested the cytotoxic effect of MX, a well-known BCRP substrate in MCF-7 and MCF-7/MX cells using MTT assay. MX dose-dependently inhibited the survival of MCF-7/MX and MCF-7 cells with the IC_{50} of 3.63 ± 0.34 μM and 0.52 ± 0.05 μM, respectively (Fig. 1b). MCF-7/MX cells exhibited 6.98-fold more resistance to MX than MCF-7 cells.

To identify novel miRNAs that target BCRP, we investigated the differential expression pattern of between MCF-7/MX and MCF-7 human breast cancer cells, using microarray. 33 miRNAs (17 up-regulated and 16 down-regulated) were found to be differentially expressed between MCF-7/MX and MCF-7 cells (Fig. 2a, b). MiR-181a was the most significantly down-regulated miRNA in MCF-7/MX cells (Fig. 2b). qRT-PCR was performed to confirm the result obtained by microarray analysis. qRT-PCR results showed that miR-181a was significantly down-regulated in MCF-7/MX cells compared to MCF-7 cells (Fig. 2c). These results suggest that miR-181a may be a BCRP suppressor in human breast cancer.

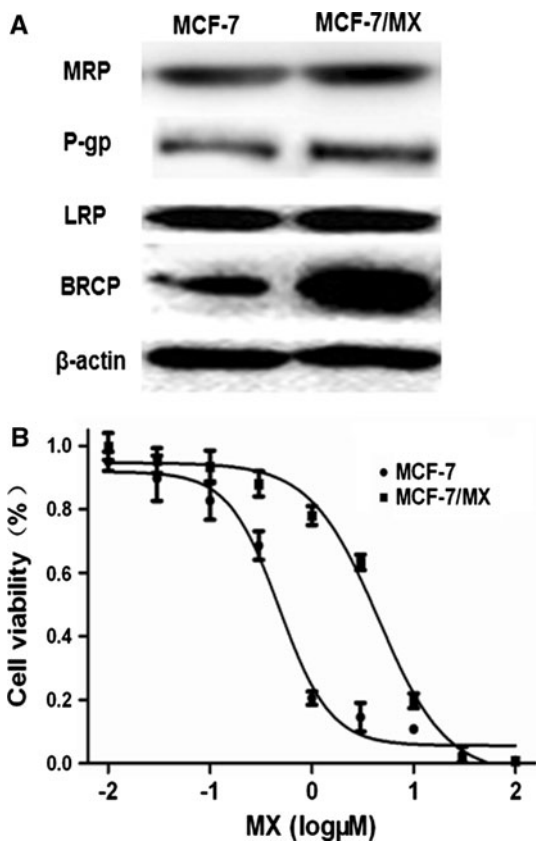


Fig. 1 BCRP is overexpressed in MCF-7/MX cell. **a** Western blotting analysis showing the protein expression of MRP, PGP, LRP, and BCRP in MCF-7 and MCF-7/MX cells. β-actin was used as an internal loading control. **b** MX sensitivity of MCF-7 and MCF-7/MX cells. Cells were treated with various concentrations of MX, and survived cells were measured by MTT assay

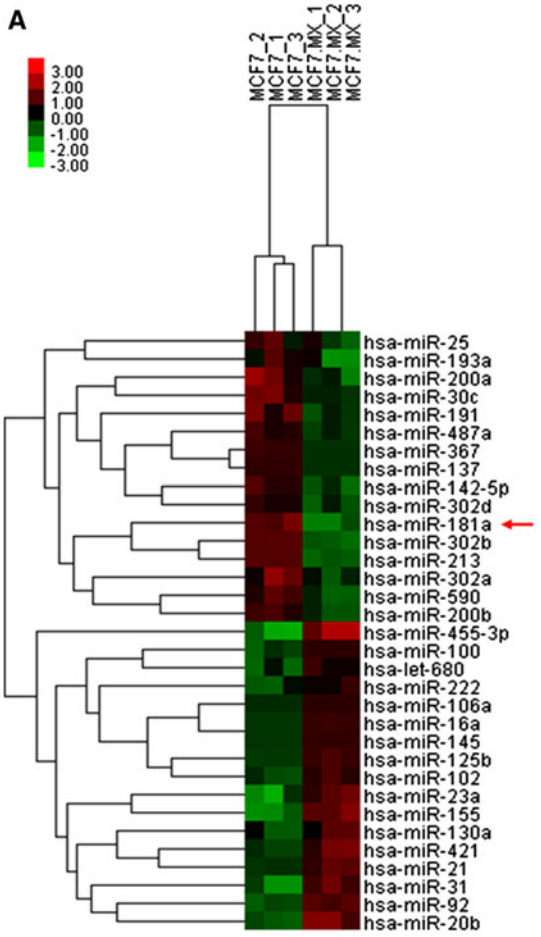
MiR-181a negatively regulates BCRP by targeting the BCRP mRNA-3′-UTR in MCF-7/MX cells

We performed searches for miR-181a targets using RNA-hybrid, and found that the 3′-UTR of the BCRP mRNA containing two putative miR-181a binding sites (Fig. 3a). To confirm that these sites were the biding sites of miR-181a, the 3′-UTRs of the BCRP mRNA were cloned downstream of a luciferase gene in a reporter plasmid, and the luciferase activity was measured in the presence or absence of miR-181a mimic. Compared with the negative control, miR-181a mimics significantly inhibited luciferase activity in MCF-7/MX cells (Fig. 3b), suggesting that the 3′-UTR of the BCRP mRNA is a target site of miR-181a.

To determine whether miR-181a suppresses endogenous BCRP expression, we transfected the mature miR-181a mimics into MCF-7/MX cells expressing high levels of BCRP. The expression level of miR-181a was confirmed by qRT-PCR, showing that MCF-7/MX cells transfected with miR-181a mimics expressed approximately 100-fold more miR-181a compared to those transfected with negative controls (Fig. 3c). The expression of BCRP, but not MRP, PGP, and LRP was significantly decreased in cells transfected with miR-181a mimics, compared to cells transfected with negative controls. (Fig. 3d–f), suggesting that miR-181a negatively regulated endogenous BCRP expression.

Ectopic miR-181a expression sensitizes MCF-7/MX cells to MX

We then investigated the effect of miR-181a on MX accumulation and cytotoxicity in BCRP-overexpressing MCF-7/MX cells. As shown in Fig. 4a, miR-181a mimics significantly increased intracellular MX accumulation in



B

Down-regulated		*Up-regulated*	
miRNA	Fold Change	miRNA	Fold Change
hsa-miR-590	3.37	hsa-miR-100	2.93
hsa-miR-302b	4.65	hsa-miR-23a	6.38
hsa-miR-367	2.41	hsa-miR-421	3.64
hsa-miR-302a	3.98	hsa-miR-130a	2.88
hsa-miR-200a	5.61	hsa-miR-125b	2.91
hsa-miR-25	2.47	hsa-miR-31	5.69
hsa-miR-193a	3.27	hsa-let-680	2.66
hsa-miR-142-5p	3.59	hsa-miR-21	2.98
hsa-miR-181a	5.77	hsa-miR-92	3.90
hsa-miR-191	3.45	hsa-miR-16a	2.48
hsa-miR-30c	3.19	hsa-miR-455-3p	11.86
hsa-miR-213	4.29	hsa-miR-222	2.43
hsa-miR-302d	2.61	hsa-miR-20b	5.21
hsa-miR-137	2.20	hsa-miR-102	2.98
hsa-miR-200b	2.85	hsa-miR-155	7.20
hsa-miR-487a	2.51	hsa-miR-106a	2.05
		hsa-miR-145	2.33

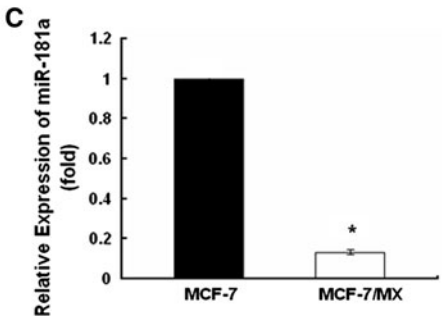


Fig. 2 Hierarchical clustering analysis of miRNAs in MCF-7 and MCF-7/MX cells. **a** Differential expression of 33 miRNAs between BCRP-overexpressing MCF-7/MX and its parental MCF-7 cells. The level of miRNA expression is color-coded. *Red* higher expression levels; *Green* lower expression levels; *black* no difference; *Rows* miRNA; *columns* sample. The miRNA tree is on the *left*, the sample tree is on *top*. **b** A list of significantly up-regulated and down-regulated miRNAs that exhibited more than twofold change in MCF-7/MX cells compared to MCF-7 cells. **c** qRT-PCR showing the miR-181a expression levels in MCF-7 and MCF-7/MX cells. Each sample was performed in triplicate, and the relative expression of miR-181a was normalized to endogenous control U6 snRNA. *Data* are shown as fold changes of miR-181a expression in MCF-7/MX cells relative to that in MCF-7 cells, which are set as 1. $*P < 0.05$

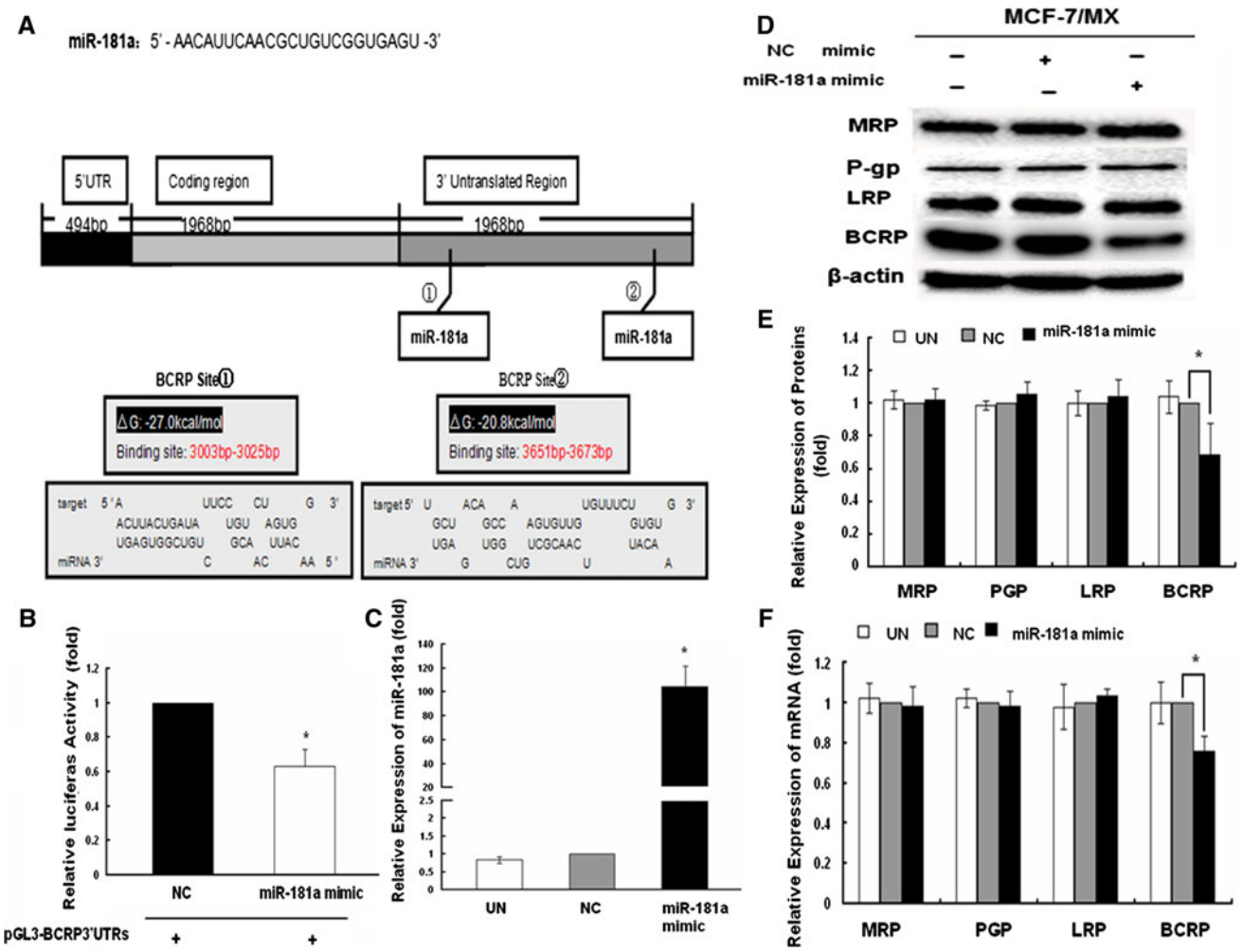


Fig. 3 MiR-181a targeted the BCRP mRNA-3′-UTR in MCF-7/MX. **a** The putative miR-181a targeted sequence in the BCRP mRNA. RNAhybrid predicts two binding sites in the BCRP 3′-UTR. **b** 293T cells were transfected with a BCRP 3′-UTR-luciferase reporter construct and miR-181a mimics or scrambled oligonucleotides as a negative controls (NC). Luciferase activity of cells treated with miR-181a is shown as a fraction of the control (*$P < 0.05$ vs. NC). **c** qRT-PCR showing the relative expression of miR-181a in untreated MCF-7/MX cells (UN), and MCF-7/MX cells treated with miR-181a mimics and negative controls (NC). **d** qRT-PCR showing the mRNA expression of MRP, PGP, LRP, and BCRP in untreated MCF-7/MX cells (UN), and MCF-7/MX cells treated with miR-181a mimics and negative controls (NC). **e** Representative Western blot showing the mRNA expression of MRP, PGP, LRP, and BCRP in untreated MCF-7/MX cells (UN), and MCF-7/MX cell treated with miR-181a mimics and the negative control (NC). **f** Densitometric analysis for the protein expression of MRP, PGP, LRP, and BCRP. $n = 3$.*$P < 0.05$ versus negative control (NC) or untransfected control (UN)

MCF-7/MX cells compared to negative controls ($P < 0.05$, Fig. 4a, b), suggesting that miR-181a inhibited BCRP-mediated transport of MX in MCF-7/MX cells. Furthermore, miR-181a mimics significantly reduced the IC_{50} of MX in MCF-7/MX cells compared to negative controls (1.57 ± 0.08 vs. 3.86 ± 0.36 μM, $P < 0.05$, Fig. 4c), suggesting that miR-181a sensitized MCF-7/MX cells to MX cytotoxicity. To verify that the increased sensitivity of MCF-7/MX cells to MX was mediated by BCRP, we tested the effect of miR-181a on the sensitivity of MCF-7/MX cells to another anti-cancer drug, etoposide, a substrate for MRP-1 but not for BCRP. There was no significant difference in the IC_{50} of MX between cells treated with miR-181a mimics and negative controls, suggesting that miR-181a did not sensitize MCF-7/MX cells to etoposide (Fig. 4d). These results suggest that miR-181a specifically inhibits BCRP-mediated drug efflux, resulting in increased sensitivity of MX-resistant MCF-7/MX cells to MX.

Intratumoral overexpression of miR-181a inhibits BCRP expression and reduces xenograft tumor growth in vivo

We then examined the effect of miR-181a on BCRP expression in nude mice bearing a human MCF-7/MX xenograft. Cholesterol-conjugated miR-181a mimics or

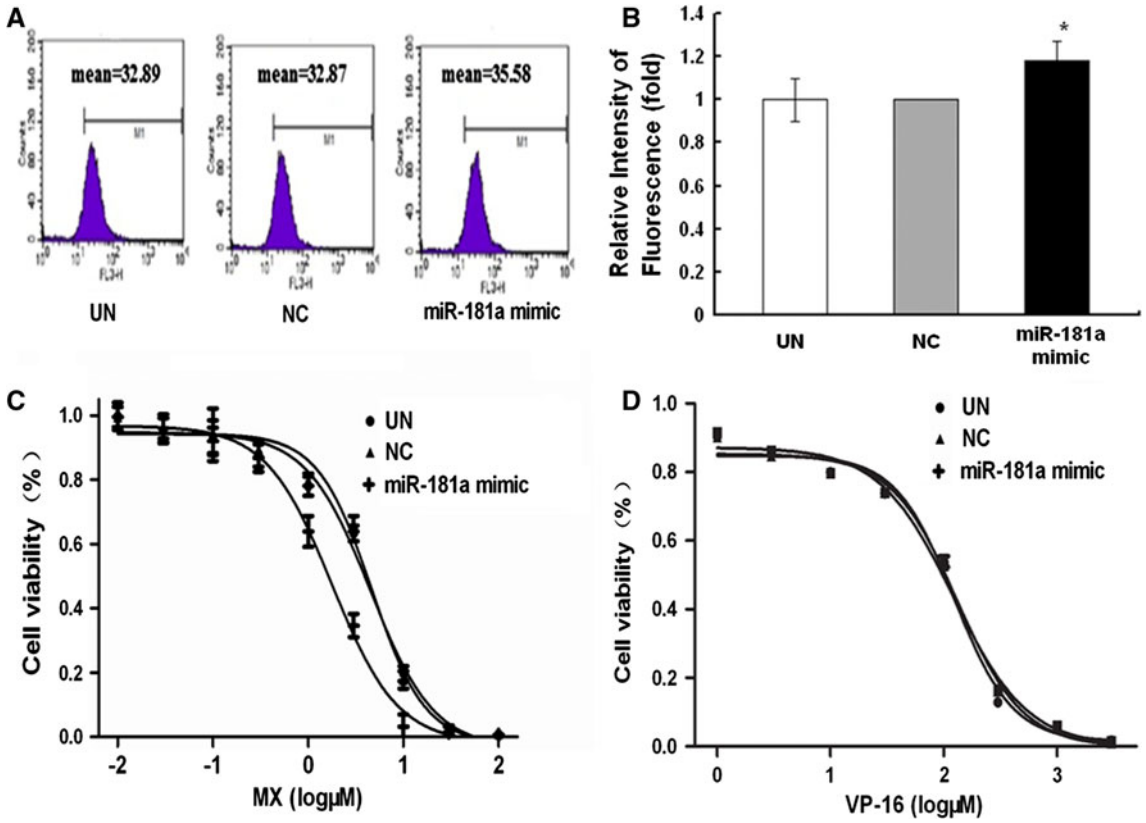


Fig. 4 MiR-181a increased the sensitivity of MCF-7/MX to MX. **a** Flow cytometry showing MX accumulation in untreated MCF-7/MX cells (UN), and MCF-7/MX cells treated with miR-181a mimics and negative controls (NC). **b** Quantitative analysis of the fold increase of fluorescence intensity in MCF-7/MX cells transfected with miR-181a mimics relative to that in cells transfected with the negative control. $^*P < 0.05$ **c**, **d** Sensitivity of MCF-7/MX cells to MX (**c**) and VP-16 (**d**) in untreated cells or cells transfected with miR-181a mimics and negative controls. Cells were treated with various concentrations of MX (**c**) and VP-16 (**d**) for1 h, and survived cells were measured by MTT assay. $n = 4$

negative controls were injected into the tumors. qRT-PCR analysis confirmed that miR-181a expression was significantly increased in the miR-181a group compared to the negative control group (Fig. 5a). We further found that the mRNA and protein expression of BCRP, but not MRP, PGP, and LRP, was significantly down-regulated in the miR-181 group (Fig. 5b–d), suggesting that ectopic expression of miR-181a specifically inhibited BCRP expression in vivo. We further examined the effect of miR-181a on the antitumor activity of MX in vivo. As shown in Fig. 6a, there were no significant differences in the tumor volume among groups within 17 days after transplantation of xenografts into mice. No significant differences in the tumor volume were observed between the untreated and Chol-miR-NC-treated groups from day 1 to day 31. MX treatment significantly inhibited tumor growth from day 20 to day 30. Intratumoral injection of Chol-miR-181a potentiated inhibition of tumor growth by MX. The tumor volume at sacrifice in the Chol-miR-181a plus MX group was much smaller than that in the MX alone group (Fig. 6b). We also measured tumor weight at sacrifice in each group (Fig. 6c). There was no significant difference in the average tumor weight between the Chol-miR-NC and untreated groups (0.41 ± 0.20 and 0.49 ± 0.17 g, respectively). MX treatment significantly reduced tumor weight compared to the untreated group or the Chol-miR-NC group ($P < 0.05$). The average tumor weight in the Chol-miR-181a+MX group (0.29 ± 0.09 g) was significantly less than that in the Chol-miR-NC+MX group (0.37 ± 0.13 g) ($P < 0.05$). These results further suggested that miR-181a enhanced the antitumor activity of MX via down-regulation of BCRP.

Down-regulation of miR-181a up-regulates BCRP and reduces the sensitivity of MCF-7 cells to MX

We then investigated whether inhibition of endogenous miR-181a in MX-sensitive MCF-7 cells resulted in an increase in the BCRP expression and a reduction in MX sensitivity, using miR-181a inhibitors. miR-181a expression was decreased about fivefolds by miR-181a inhibitors compared to control miRNAs (Fig. 7a). miR-181a inhibitors resulted

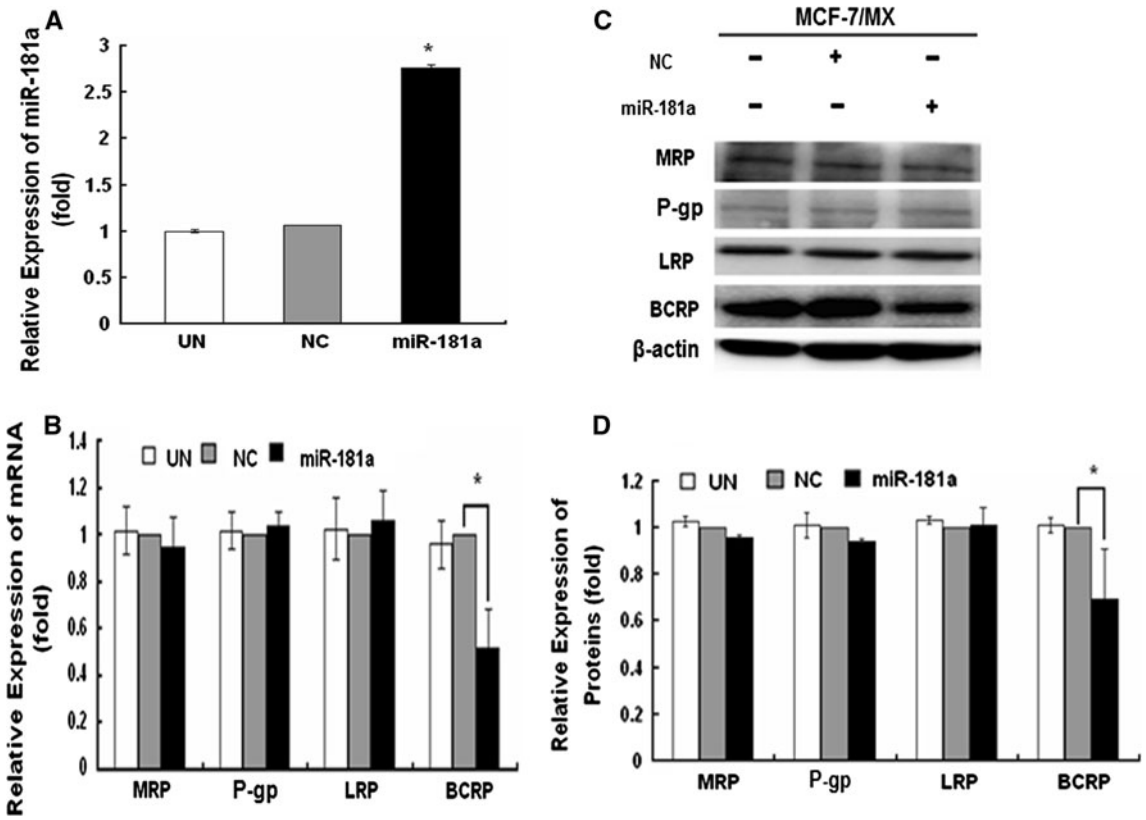


Fig. 5 BCRP mRNA and protein levels were inhibited by ectopic miR-181a in vivo. Mice were transplanted with untreated MCF-7/MX cells (UN), or MCF-7/MX cell treated with miR-181a mimics and negative controls (NC). **a** qRT-PCR results showing that the relative expression of miR-181a in tumor xenografts ($*P < 0.05$ vs. NC). **b** qRT-PCR results showing the mRNA expression of MRP, PGP, LRP, and BCRP. **c** Representative Western blot showing the protein expression of MRP, PGP, LRP, and BCRP. β-actin was used as an internal loading control. **d** Densitometric analysis of the protein expression of MRP, PGP, LRP, and BCRP. $*P < 0.05$ versus NC

in increased mRNA and protein expression of BCRP, but not MRP, PGP, and LRP, in MCF-7 cells (Fig. 7b–d). miR-181a inhibitors inhibited MX accumulation in MCF-7 cells (Fig. 8a, b), suggesting that inhibition of miR-181a facilitated BCRP-mediated transport of MX in MCF-7 cells. In addition, miR-181a inhibitors significantly increased the IC_{50} of MX in MCF-7 cells compared with negative controls (2.04 ± 0.08 vs. 0.56 ± 0.05 μM, Fig. 8c), suggesting that down-regulation of miR-181a in MX-sensitive cells made them resistant to MX. However, miR-181a inhibitors did not alter the sensitivity of MCF-7 cells to etoposide (Fig. 8d).

Discussion

It has been known that miRNAs can bind to target genes, and negatively regulate expressions and functions of genes. Identification of miRNAs that down-regulate the expression of MDR-related transporters such as BCRP is critical for reversing MDR. miRNA microarray is an effective method to screen miRNAs. In the present study, we performed miRNA microarray to identify the differential expression of miRNAs that target BCRP in the parental MCF-7, and its BCRP-overexpression MCF-7/MX cell lines. We identified 33 miRNAs, whose expression was significantly changed between the two cell lines, suggesting that these miRNAs may be related with drug sensitivity of breast cancer cells. miRNA microarray has been used to identify miRNAs that are differentially expressed between cisplatin-sensitive and -resistant breast cancer cells [37], and between tamoxifen-sensitive and -resistant breast cancer cells [38]. This study, for the first time, reported the differential expression of miRNAs between MX-sensitive and -resistant breast cancer cells.

It is well known that MX is the substrate of BCRP, and can be used to induce BCRP-overexpressing cell lines [6]. In consistence with previous studies, we found that the BCRP expression was significantly increased in MX-resistant MCF-7/MX cells compared to parental MCF-7 cells. In addition, MCF-7 cells specifically up-regulated BCRP, not other ABC transporters, such as MRP, PGP, and LRP, suggesting up-regulation of BCRP is a feature of MX-resistant cells. Furthermore, we also found that BCRP-

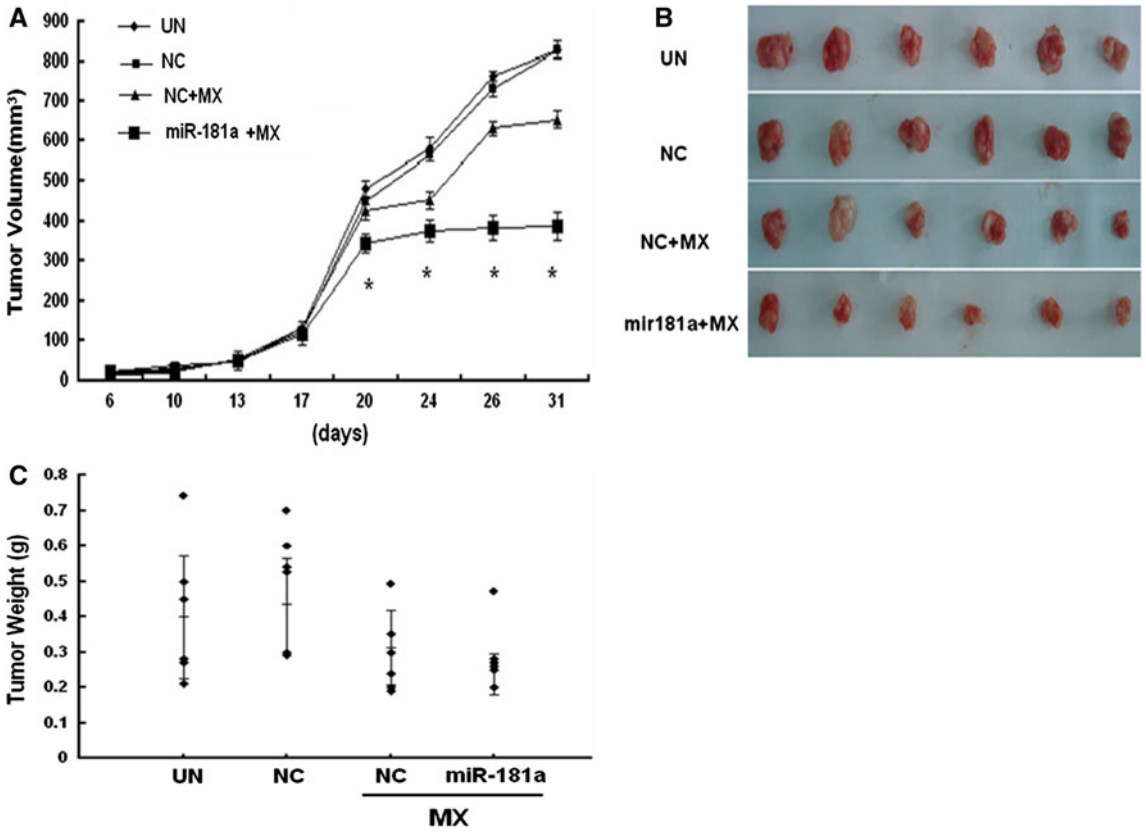


Fig. 6 MiR-181a potentiated the antitumor effect of MX in vivo. Mice were transplanted with untreated MCF-7/MX cells (UN), or MCF-7/MX cell treated with miR-181a mimics and negative controls (NC). MX was administered intravenously twice a week for 2 weeks 7 days after transplantation. **a** Tumor growth curves. Tumor volume was measured using the formula: $V = (L \times W^2) \times 0.5$. ($n = 6$ each). **b** Images showing the tumors removed 4 weeks after cell transplantation. **c** The mean tumor weight of each group. Data are presented as mean ± SD. *$P < 0.05$ vs NC + MX

overexpressing MCF-7/MX cells exhibited more resistance to MX than MCF-7 cells. The findings that MCF-7/MX and its parental MCF-7 cells have different BCRP expression and MX resistance indicate that these cells can be used for miRNA microarray to study the differential expression pattern of miRNAs that target BCRP.

In the present study, we performed miRNA microarray to identify miRNAs whose expression was down-regulated in MCF-7/MX versus MCF-7 cells. We found that miR-181a was the most down-regulated miRNA in BCRP-overexpressing MCF-7/MX cells, suggesting that miR-181a is involved in down-regulation of BCRP expression. We further used RNAhybrid to identify that the 3′-UTR of the BCRP mRNA contained miR-181a binding sites. The interaction of miRNA-181a with the 3′-UTR of the BCRP mRNA was confirmed by the luciferase assay, in which the luciferase activity of the reporter gene harboring 3′-UTR of BCRP mRNA was significantly inhibited by miR181a. Furthermore, we found that miR-181a mimics inhibited the expression of BCRP, but not MRP, PGP, and LRP, in MCF-7/MX cells, further suggesting that miR-181a specifically down-regulated BCRP. It has been reported that miR-181a can regulate many target genes, such as p27 [39], RAI1 [24], KLF6 [40], and K-ras [41]. The present study shows that BCRP is a novel target gene of miR-181a, suggesting that miR-181a contributes to drug resistance in breast cancer.

In cancer cells, BCRP transports its drug substrates, such as MX, doxorubicin, and topotecan, out of cells, leading to reduced intracellular substrate accumulation and subsequent resistance to these drugs. In the present study, we found that miR-181a mimics increased intracellular MX accumulation in MCF-7/MX cells, accompanied by increased sensitivity to MX, suggesting that miR-181a sensitized MX-resistant MCF-7/MX cells to MX. MiR-181a-induced increased sensitivity to MX in MCF-7 cells is specific for BCRP, as indicated by evidence that only the expression of BCRP, but not other MDR transporters such as MDR. PGP, and LRP was down-regulated. In addition, inhibition of miR-181a made MX-sensitive MCF-7 cells resistant to MX, accompanied by a decreased expression of BCRP, but not MDR, PGP, and LRP. Furthermore, miR-181a did not sensitize MCF-7/MX cells to etoposide, a substrate for MRP-1, but not for BCRP. These findings

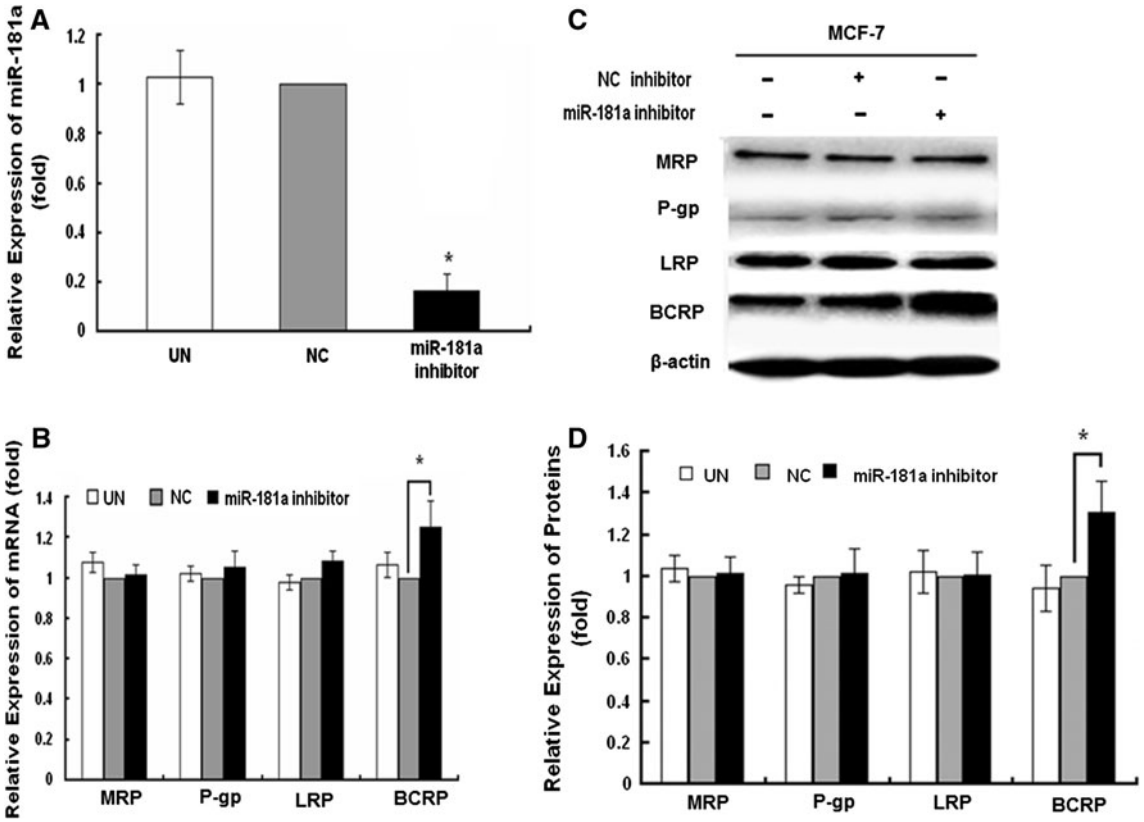


Fig. 7 Inhibition of miR-181a up-regulated BCRP expression in MCF-7 cell line. MCF-7 cells were transfected with miR-181a inhibitors and negative controls. **a** qRT-PCR showing the relative miR-181a expression in untreated MCF-7 cells (UN), and MCF-7/MX cells treated with miR-181a inhibitors and the negative control (NC). **b** qRT-PCR showing the mRNA expression of MRP, PGP, LRP, and BCRP in untreated MCF-7 cells (UN), and MCF-7 cells treated with miR-181a inhibitors and negative controls (NC).**c** Representative Western blot the mRNA expression of MRP, PGP, LRP, and BCRP in untreated MCF-7 cells (UN), and MCF-7 cells treated with miR-181a inhibitors and negative controls (NC). **d** Densitometric analysis for the protein expression of MRP, PGP, LRPs and BCRP. $n = 3$. $^*P < 0.05$ versus negative control (NC) or untransfected control (UN)

suggest that miR-181a can inhibit drug resistance mediated by BCRP, but possibly not by other MDR transporters. BCRP can transport many drugs such MX, doxorubicin, and topotecan [42]. In the present study, we used the MX-resistant cell line and identified that up-regulation of miR-181a inhibited BCRP-mediated resistance to MX. It has been reported that MCF-7/MX cells exhibit cross-resistance to MX doxorubicin, and topotecan [9, 42, 43]. Therefore, it is likely that up-regulation of miR-181a may inhibit resistance to many other drugs, such as doxorubicin and topotecan. However, whether the findings obtained using MX-resistant cells apply to other drug resistance remains to be determined.

Many other miRNAs, such as miR-520 h [16], -519c [14, 15], and -328 [17], have been found to inhibit the expression of BCRP by targeting the BCRP-3′-UTR. However, the roles of these micRNAs on the expression of BCRP have not been investigated in in vivo experiments. Intratumoral injection of miRNA mimics is a commonly used method to study the effect of miRNA in vivo. For example, Mercatelli et al. [44] reported that intratumoral injection of miR-221mimics every 4 days for a total of three injections per tumor enhanced tumor growth of prostate carcinoma xenografts in mice. Hou et al. [45] intratumorally injected cholesterol-conjugated miRNAs every 3 days for 2 weeks in mice inoculated with tumor tissues for 7 days. Similar to the report by Hou et al., at 7 days after transplantation of MCF-7/MX cells, we injected miR-181a into the tumor mass every 3 days for 2 weeks. We found that the miR-181a expression was significantly up-regulated in tumors after intratumoral injection of Chol-miR-181a, suggesting in vivo high expression of miR-181a was achieved. In addition, in consistence with the in vitro findings, we also found that BCRP was significantly up-regulated in tumor xenografts after intratumoral injection of miR-181a mimics. These in vivo findings supports the idea that miR-181a plays an important role in regulation of BCRP expression and drug sensitivity in breast cancer. We further investigated whether, miR-181a inhibition induced resistance in MX-

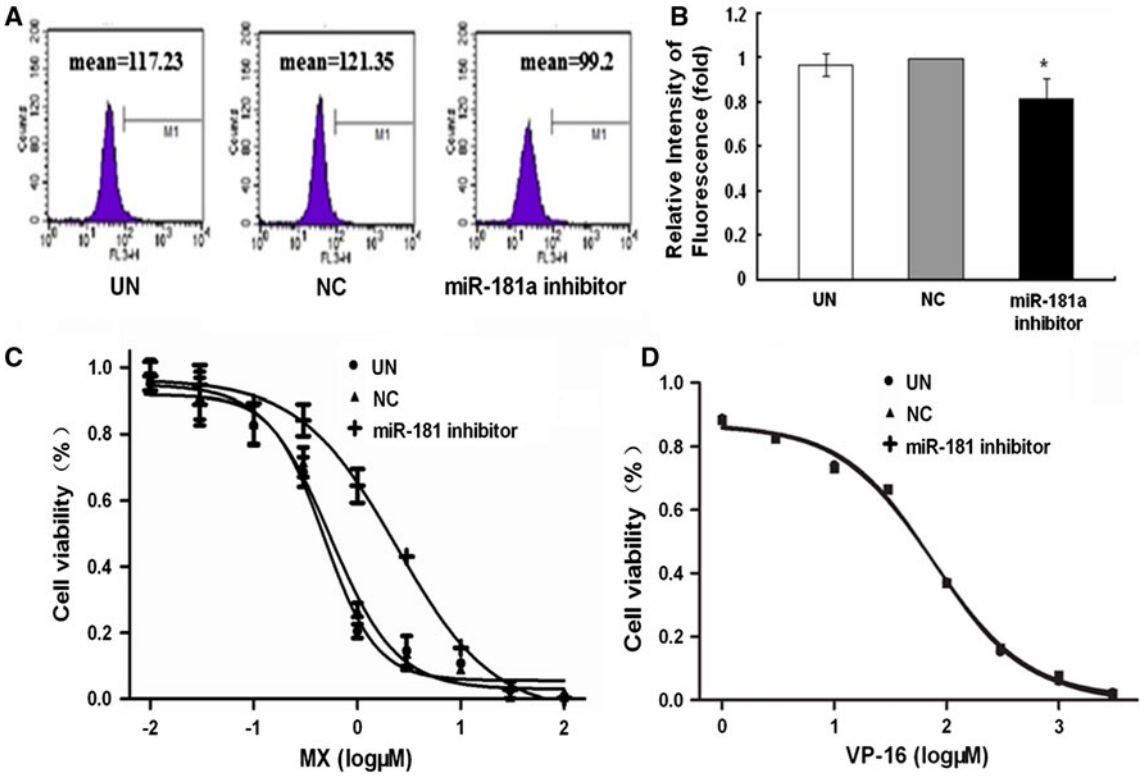


Fig. 8 Inhibition of miR-181a reduced MX sensitivity of MCF-7 cells. **a** Flow cytometry showing MX accumulation in untreated MCF-7 cells (UN), and MCF-7 cells treated with miR-181a inhibitors and negative controls (NC). **b** Quantitative analysis of the fold increase of fluorescence intensity in MCF-7 cells transfected with miR-181a inhibitors relative to that in cells transfected with the negative control. $^*P < 0.05$. **c**, **d** Sensitivity of MCF-7 cells to MX (**c**) and VP-16 (**d**) in untreated cells or cells transfected with miR-181a inhibitors and negative controls. Cells were treated with various concentrations of MX (**c**) and VP-16 (**d**) for1 h, and survived cells were measured by MTT assay

sensitive MCF-7 cells. We found that miR-181a inhibitors increased BCRP expression, and decreased MX sensitivity. MiR-181a inhibition did not alter the expression of PGP, MRP, and LRP, and the sensitivity to etoposide, further suggesting that miR-81a specifically regulated BCRP-mediated drug resistance in breast cancer cells.

MX, a topoisomerase inhibitor, is used in cancer treatment, but its application is greatly limited due to its easy induction of drug resistance. In the present study, we found that overexpression of miR-181a reduced the IC_{50} of MX in MCF-7/MX cells from 3.86 ± 0.36 to 1.57 ± 0.08 μM, which was still higher than the IC_{50} of MX (0.56 ± 0.05 μM) in MCF-7 cells. In addition, inhibition of miR-181a increased the IC_{50} of MX in MCF-7 cells from 0.56 ± 0.05 to 2.04 ± 0.08 μM, which was still lower than the IC_{50} of MX (3.86 ± 0.36 μM) in MCF-7/MX cells. These findings showed that overexpression of miR-181a partially reversed resistance to MX in MCF-7/MX cells, and inhibition of miR-181a partially made MCF-7 cells resistant to MX. These results suggest that mechanisms other than BCRP-mediated drug resistance may be involved in cancer cell resistance to MX. Multiple mechanisms have been reported to confer resistance to MX in cancer cells. Signaling pathways that are involved in DNA damage repair [46], cell apoptosis [47], and PI3K-Akt activation [48] have been shown to contribute to MX resistance in breast cancer cells.

Recent evidence has shown that miR-181a exhibits seemingly paradoxical roles in different cancers. For example, miR-181a is up-regulated in many cancers such as the breast cancer [20], gastric cancer [21], and head and neck cancer [22], and functions as a tumor oncogene. In contrast, in the glioma [18] and aggressive chronic lymphocytic leukemia [23], the expression of miR-181a is down-regulated, and miR-181a functions as a tumor suppressor. Furthermore, miR-181a has been found to regulate targets that include both tumor suppressors and oncogenes. It has been reported that miR-181a promotes growth of many cancers, such as gastric cancer and leukemia, via down-regulation of some tumor suppressors, such as KLF6 [40] and p27 [39]. In contrast, miR-181a has been found to inhibit tumor growth in the chronic myelogenous leukemia and oral squamous cell carcinoma via down-regulation of some oncogenes such as RalA [24] and K-ras [41]. The findings that aberrant miR-181a expression exhibits a tumor-specific manner suggest that signaling pathways that

induce miR-181a expression may be different in different tumors. For example, TGF-β has been found to up-regulate miR-181a expression in breast cancer [30], while Wnt/β-catenin induces miR-181a expression in hepatocellular carcinoma [49]. In addition, the expression of miR-181a may be also affected by other cellular conditions. For example, the miR-181a is particularly up-regulated in triple-negative breast cancers [30]. Given a large number of targets of miR-181a, the expression of its specific targets may be regulated by many cellular conditions. For example, TGF-β-mediated up-regulation of miR-181a in breast cancer results in deregulation of the pro-apoptotic molecule Bim, but not other targets such as Smad7, TIMP3, Bcl-2, and KLF-6 [30]. It seems that the expression of miR-181a and its targets are regulated by multiple complex signaling pathways in a cell-specific manner. The mechanisms underlying the selective activation of miR-181a targets in breast cancer remain unknown. Although up-regulation of miR-181a is associated with breast cancer metastasis and poor patient survival [30, 31], it remains to be determined, which signaling pathways are involved in miR-18a activation, what targets are expressed, and whether up-regulation of miR-181a alone can lead to breast cancer metastasis.

Similarly, several different targets are involved in miR-181a-mediated increase in drug sensitivity. miR-181a sensitized MDA-MB-231 breast cancer cells to the poly-ADP-ribose-polymerase1 inhibitor olaparib by targeting ataxia telangiectasis mutated (ATM) [50], and enhances drug sensitivity to fludarabine, daunorubicin and Ara-C in multidrug-resistant leukemia cells by targeting multiple anti-apoptosis genes [51]. In addition, miR-181a have also been found to cooperate with miR-30d and miR-199a-5p to increase the sensitivity of human prostate, colon, and bladder cancer cells to the histone deacetylase inhibitor trichostatin A (TSA) by targeting GRP78 [52]. In the present study, we found that miR-181a sensitized MCF-7/MX cells to MX. Although the signaling pathways that are involved in miR-181a-mediated increase in drug sensitivity remain unclear, it is likely that different signaling pathways may be responsible for different targets activated by miR-181a in distinct cancer cells. Down-regulation of miR-181a seems to be a common feature for these cancer cells to exhibit drug resistance. Overexpression of miR-181a may be a potential target for promoting drug sensitivity in cancer cells. However, since many miR-181a targets are oncogenes [24, 41], and overexpression of miR-181a has been reported to be associated with breast cancer metastasis [30, 31], there are concerns about use of miR-181a alone in the treatment of breast cancer. A combination use of miR-181a and inhibition of its targets involved in cancer metastasis may represent a therapeutic strategy for breast cancer treatment.

In summary, we found that miR-181a was down-regulated in MX-resistant MCF-7/MX cells. MiR-181a regulated BCRP expression via binding to the 3′-UTR of BCRP mRNA. Overexpression of miR-181a sensitized MX-resistant cells to MX, and down-regulation of miR-181a made MX-sensitive cells resistant to MX. Our findings suggest that miR-181a may be a potential target for reversing BCRP-mediated drug resistance in breast cancer.

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Conflict of interest The authors declare no conflict of interest.

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