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RESEARCH PAPER

Regulation of miR-19 to Breast Cancer Chemoresistance Through Targeting PTEN

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ABSTRACT

Purpose To explore whether miR-19 is involved in the regulation of multidrug resistance (MDR), one of the main causes of breast cancer mortality, and modulates sensitivity of tumor cells to chemotherapeutic agents.

Methods We analyzed miRNA expression levels in three MDR cell lines in comparison with their parent cell line, MCF-7, using a miRNA microarray. We investigated whether inhibitor of miR-19 sensitized MDR cells to chemotherapeutic agents *in vitro* and *in vivo*.

Results MiR-19 was overexpressed in all three MDR cell lines compared to their parental cell line, MCF-7. Expression levels of miR-19 in MDR cells were inversely consistent with those of PTEN. Inhibitor of miR-19a restored sensitivity of MDR cells to cytotoxic agents; administration of LNA-antimiR-19a, a chemo-modified miR-19a inhibitor, sensitized MDR cells to chemotherapeutic agents *in vivo*.

Conclusion Our findings demonstrate, for the first time, involvement of miR-19 in multidrug resistance through modulation of PTEN and suggest that miR-19 may be a potential target for preventing and reversing MDR in tumor cells.

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KEY WORDS breast cancer · microRNA · multidrug resistance · PTEN

ABBREVIATIONS

BCRP	breast cancer resistance protein
LNA	locked nucleic acid
MDR	multidrug resistance
MDR-1	multidrug resistance 1
miRNA	microRNA
MRP-1	multidrug resistance-associated protein 1
PTEN	phosphatase and tensin homolog
RT-PCR	reverse transcription polymerase chain reaction.

INTRODUCTION

The multidrug resistance (MDR) refers to the ability of tumor cells to resist several unrelated drugs after exposure to a single chemotherapy drug (1). Nearly all initially responsive breast tumors will eventually acquire an MDR phenotype (2). P-glycoprotein (MDR-1) (3), multidrug resistance associated protein (MRP-1) (4), and breast cancer resistance protein (BCRP) (2,5) have been considered as critical MDR-related factors. However, they are not strong as MDR predictive biomarkers and as targets for sensitizing MDR tumor cells to chemotherapeutic agents because one drug usually only induces overexpression of one transporter. More importantly, mechanisms of regulating the expression of these proteins remain controversial. The present lack of criteria to help individualized breast cancer treatment indicates a need for mechanistic understanding of miRNA in a MDR phenotype to develop prognostic and therapeutic tools for preventing and reversing MDR.

MicroRNAs (miRNA), a recently discovered class of small, functional, non-coding RNAs, have been shown to

function as regulatory molecules by inhibiting protein translation, and play an important role in development, differentiation, cell proliferation, and apoptosis (6–8). More recently, a few studies suggest that down-regulation of miRNAs may play a critical role in cancer progression (9–12). However, very little is currently known about the increasing MDR due to the overexpression of certain miRNAs. Recently, miR-451 and miR-27 have been shown to be involved in resistance of the MCF-7 breast cancer cells to the chemotherapeutic drug doxorubicin mediated by MDR-1 (13,14). Expression of BCRP was negatively regulated by miR-328 (15). Our recent studies found that the loss of miR-326 increased the resistance of tumor cells to VP-16 by modulating the expression of MRP-1 (16). In the present study, we show for the first time that miR-19 promotes MDR of breast cancer cells to chemotherapeutic agents through repressing PTEN, a critical tumor suppressor.

MATERIALS AND METHODS

Cell Lines and Cell Culture

MDR human breast cancer cell lines, MCF-7/TX200, MCF-7/VP-16, MCF-7/MX100 and wild-type MCF-7 (MCF7/WT) (a gift of Susan E. Bates from NCI/NIH, Bethesda, MD) were cultured 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_2. MCF-7/TX200, MCF-7/MX100, and MCF-7/VP were respectively selected in increasing concentrations of Taxol (paclitaxel), mitoxantrone, and VP-16 (etoposide) (17).

RT-PCR and Quantitative RT-PCR

Total RNA was extracted from MCF-7/WT and MDR cells using Trizol. Primer sequences of *MRP-1*, *MDR-1*, *BCRP*, *U6 snRNA* and *β-actin* were described in our recent publication (16,18). Primer sequences of *miR-19a* are as follows: *miR-19a* (GeneBank Accession number NR_029489), 5′-CCTCTGTTAGTTTTGCATAGTTGC-3′ and 5′-CAGGCCACCATCAGTTTTG-3′. Human *PTEN* (GeneBank Accession number NM_000314) primers are 5′-ACC AGGACCAGAGGAAACCT-3′ and 5′- GCTAGC CTCTGGATTTGACG-3′. Quantitative and regular RT-PCR were performed following our previous descriptions (19). U6 snRNA was used as an internal control for *miR-19* amplification and *β-actin* as an internal control for *PTEN*, *MDR-1*, *MRP-1*, and *BCRP*. Five hundred nanograms 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 thermal profile for cDNA PCR was 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 carried out in 20 μl of reaction volume with using a GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems). SYBR Green quantitative PCR 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 sample were measured using the $2^{-\Delta\Delta Ct}$ method (20). The results are presented as fold change of expression levels in the MDR cells relative to the parental MCF-7 cells or controls.

Western Blot Analyses

Immunoblot analysis was performed by using polyclonal antibody against PTEN (Cellsignaling, Danvers, MA), and monoclonal antibodies against MRP-1 (MAB4147, Chemicon/Millipore, Billerica, MA), MDR-1 (MAB4336, Chemicon), BCRP (Calbiochem, San Diego, CA), or β-actin (Sigma–Aldrich, St. Louis, MO). MDA-1, MRP-1, and BCRP monoclonal antibodies were used in TBST with 5% dried-milk at 1:1000 working concentration.

Human miRNA Microarray Analysis

Total RNA extracted from culturing MCF-7 and MDR cells was directly labeled with biotin and labeled RNA samples were hybridized to a commercial microarray (GenoSensor Corporation, Tempe, AZ, USA) according to manufacturer's instructions. The hybridized targets were stained with a streptavidin-Alexa dye. Duplicates of each miRNA were printed on microarray and these experiments were repeated once more. The microarrays were scanned in a GenePix 4000b Biochip Reader (Molecular Devices, Union City, CA, USA) and analyzed using the Digital Genome System Suite (Molecularware, Irvine, CA, USA). The average of three mean fluorescence signal intensities for each miRNA probe was normalized to that for 5sRNA. MiRNAs detected as two-fold greater than background were considered to be expressed.

Luciferase Reporter Assay

Two pGL3-PTEN-3′-UTR recombinants, which contained 3′-untranslated region (UTR) fragments of the *PTEN* gene with or without the putative binding site for miR-19, were constructed as reported (21). PCR was performed to amplify DNA fragments with and without the miR-19 target sequence using human DNA as the template. The primers used for *PTEN* 3′-UTR containing the putative binding site of miR-19 were 5′-ACTCTAGAGGCACCGCATATTAAAACGTA-3′ and 5′- ACTCTAGAATGCCATTTTTCCATTTCCA-

3′. The primers used for *PTEN* 3′-UTR excluding the putative binding site of miR-19 were 5′-ACTCTAGACA-TAACGATGGCTGTGGTTG-3′ and 5′-ACTCTA-GACCCCCACTTTAGTGCACAGT-3′. The product was cloned into the XbaI/XbaI site of the pGL3 control vector (Promega, Madison, WI) to generate the vector pGL3-PTEN/3′UTR. The ligated vector was amplified in *E. coli*. An antisense primer of *PTEN* and a sense primer of the vector (5′-AGGAGTTGTGTTTGTGGACG-3′) (21) were used to screen the clones. The resulting luciferase UTR-report vectors and increasing concentrations of miR-19a mimic were co-transfected into MCF-7/TX200 cells using LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, luciferase activity assays were performed using Steady-Glo Luciferase Assay System (Promega) following the manufacturer's instructions.

PTEN Plasmids, miR-19 Oligonucleotides, and Transfection

Plasmids pcDNA3.1/PTEN/wt and pcDNA3.1/PTEN/C124S (a gift of Donald L. Durden, UCSD, San Diego) (22), encoding respectively wild PTEN and mutant PTEN (Cys124Ser), were transfected into MDR cells to overexpress wild PTEN and mutant PTEN. AntimiR-19a (a miR-19a inhibitor) was transfected to MDR cells and miR-19a mimics to MCF-7 cells (Dharmacon, Chicago, IL) at a working concentration of 100 nM using LipofectAmine 2000 (Invitrogen) at 48 h following seeding cells. Control oligonucleotides were used as controls (Dharmacon). The transfected cells were used for the expression analysis of PTEN and MDR-related transporters, and for the sensitive analysis of chemotherapeutic agents.

Cell Survival Analysis

For drug sensitivity, twenty-four hours after transfection, cells were treated with cytotoxic agents at an increasing concentration for 48 h. Finally, cell viability was assessed using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) Kit (Promega, Madison, WI), following the manufacturer's instructions. Absorbance at 490 nm was used to determine IC_{50} of the sensitivity of cells to chemotherapeutic agents.

Animal Experiments

5×10^6 MCF-7/TX200 cells were injected laterally into the back of athymic female nude mice (nu/nu) (6–8 weeks of age) to generate subcutaneous breast cancer animal models. Drug treatment was initiated when mice bearing tumors reach a volume of about 150 mm^3. In total, there were 4 groups with different treatments for animal experiments. For the control group, only control oligonucleotides were injected at 10 mg/kg i.v. once daily for three consecutive days. Taxol alone was administered at 10 mg/kg once weekly for the second group. For the third group, , the locked nucleic acid (LNA)-antimiR-19a (Exiqon, Woburn, MA) was injected at 10 mg/kg i.v. once daily for three consecutive days and after two days post injection of LNA-antimiR-19a, Taxol was administered at 10 mg/kg i.v. once weekly. LNA-antimiR-19a alone was injected at 10 mg/kg i.v. once daily for three consecutive days for the fourth group. The measurement of tumor size started at the first Taxol treatment (day 0). Tumor volume was measured on two perpendicular axes using a Vernier caliper every other day and calculated using the following formula: $\text{tumor volume} = (\text{width})^2 \times \text{length}/2$. On day 14 post treatment, the mice were sacrificed and the tumors were removed and weighed. Data of tumor volume and weight were analyzed. In addition, the expression levels of *miR-19a*, *PTEN*, and *MDR-1* in the xenografts from control and LAN-antimiR-19a-treated groups were measured with qRT-PCR analysis.

Statistical Analysis

Real-time RT-PCR reaction was run in triplicate for each sample and repeated twice. The data were statistically analyzed with a Student's t-test. IC_{50} data from the MTS proliferation assay were analyzed with a Wilcoxon marched pairs test.

RESULTS

Elevated Expression Levels of miR-19 Correlated with Overexpression of Three MDR-Related Transporters in MDR Cells

Prior to the analysis of miRNA expression, we analyzed expression of mRNAs and the proteins of three MDR-related transporters, MRP-1, MDR-1, and BCRP, in three types of MDR cell lines and their parental cell line, MCF-7/WT, with RT-PCR (Fig. 1a) and Western blot analysis (Fig. 1b). Results showed that MCF-7/TX200, MCF-7/VP, and MCF-7/MX100, which were respectively selected by Taxol, VP-16, and mitoxantrone, overexpressed one of MDR-1, MRP-1, and BCRP in comparison with MCF-7/WT, while MCF-7 did not express any of these three transporters (Fig. 1).

In order to determine whether expression of certain miRNAs was altered in MDR tumor cells, we performed profile analysis of miRNA expression in three

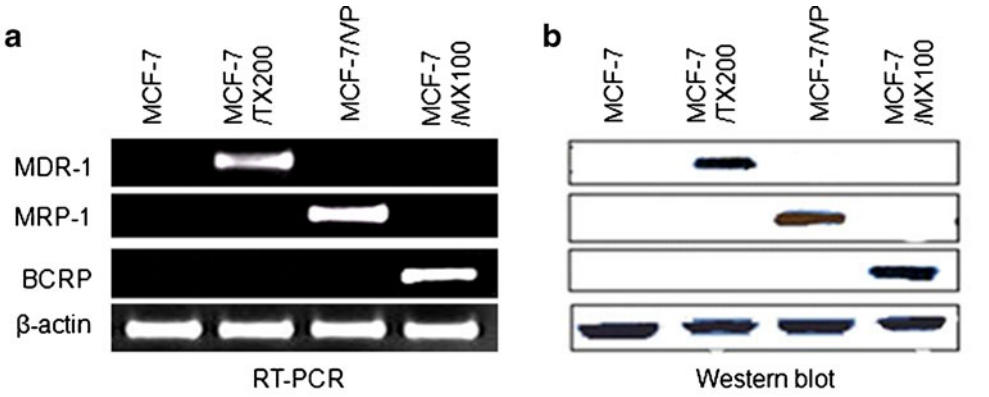

Fig. 1 Elevated levels of miR-19 are correlated with overexpression of MDR-1, MRP-1, and BCRP in MDR cell lines. Expression levels of mRNAs (**a**) and proteins (**b**) of MRP-1, MDR-1, and BCRP in three MDR cell lines and their counterparts determined by RT-PCR and Western blotting analysis. β-actin was used as an internal loading control. (**c**) Elevated expression of microRNA members in miR-17-92 cluster in MDR cell lines compared to their counterpart determined by microRNA microarray analysis.

c

Change fold of miR-19-92 cluster in MDR cell lines to MCF-7

	Normalized Median to 5S-rRNA					Fold to MCF-7		
	MCF-7	TX200	VP	MX100	*P* value	TX200	VP	MX100
hsa-mir-17 3p	0.7	1.5	3.4	3.7	0.0212	2.1	4.9	5.3
hsa-mir-17 5p	0.005	0.005	0.06	0.09	0.0364	1	12	18
hsa-mir-18	0.027	0.006	0.066	0.3	0.0491	-4.5	2.4	11
hsa-mir-19a	0.712	3.70	3.65	3.43	0.0067	5.2	5.3	4.8
hsa-mir-19b	0.060	1.08	1.14	0.62	0.0059	18	19	10.3
hsa-mir-20	0.056	0.066	0.46	1.21	0.0092	1.2	8.1	21.5
hsa-mir-92	0.58	0.57	2.1	2.4	0.0451	1	3.6	4.1

MDR cell lines compared with their parent cell line, MCF-7, using a microarray containing 463 human mature miRNA probes. Altered expression of the 115 miRNAs was observed in one or multiple MDR cell lines (data not shown). Similar miRNA expression profiles were shown in three MDR cell lines but they are different from that of MCF-7 (data not shown). Of note, the expression levels of all seven members in miR-17-92 cluster, a key oncogenic component, were significantly altered in all three MDR cell lines compared to their parental cell line (Fig. 1c). Particularly, high expression levels of *miR-19a* and *miR-19b* were observed in all three MDR cell lines compared to that in MCF-7 cells (Fig. 1c). To verify the findings obtained by miRNA microarray profiling, quantitative RT-PCR analysis was performed. Consistent with the microarray data, quantitative RT-PCR analysis confirmed significantly elevated expression levels of *miR-19* in all three MDR cell lines in comparison with their parental cells (data not shown). The miR-19 family contains *miR-19a* and *miR-19b*, which differ only by a single nucleotide at position 11, a region minimally important for target recognition. They share the same seed sequence, which is a binding sequence to targets. The seed sequence is a critical factor for microRNA function. Thus, we selected miR-19a as a representative of the miR-19 family for our further experiment analyses.

MiR-19 Directly Targets *PTEN* and Further Modulates Expression of Three MDR-Related Transporters

The *PTEN* gene possesses the putative miR-19 targeting sequence predicted by TargetScan (Fig. 2a). To validate whether miR-19 directly targets PTEN, a segment of PTEN 3′-UTR containing miR-19 binding sites was cloned into a luciferase reporter system. The plasmid lacking miR-binding sequence was used as a positive control of luciferase activity. The resulting reporter vector was transfected into the MCF-7/TX200 cells together with increasing concentrations of miR-19a mimics. Figure 2b shows that miR-19a inhibited luciferase activity from the construct with the PTEN 3′-UTR segment containing miR-19 binding sites in a dose-dependent manner. No luciferase activity change was observed when the cells were transfected with the plasmid with a PTEN 3′-UTR fragment lacking the miR-19a binding sequence (Fig. 2b). Furthermore, we examined whether miR-19a decreases the expression of PTEN. Three MDR cell lines were transfected with antimiR-19a or

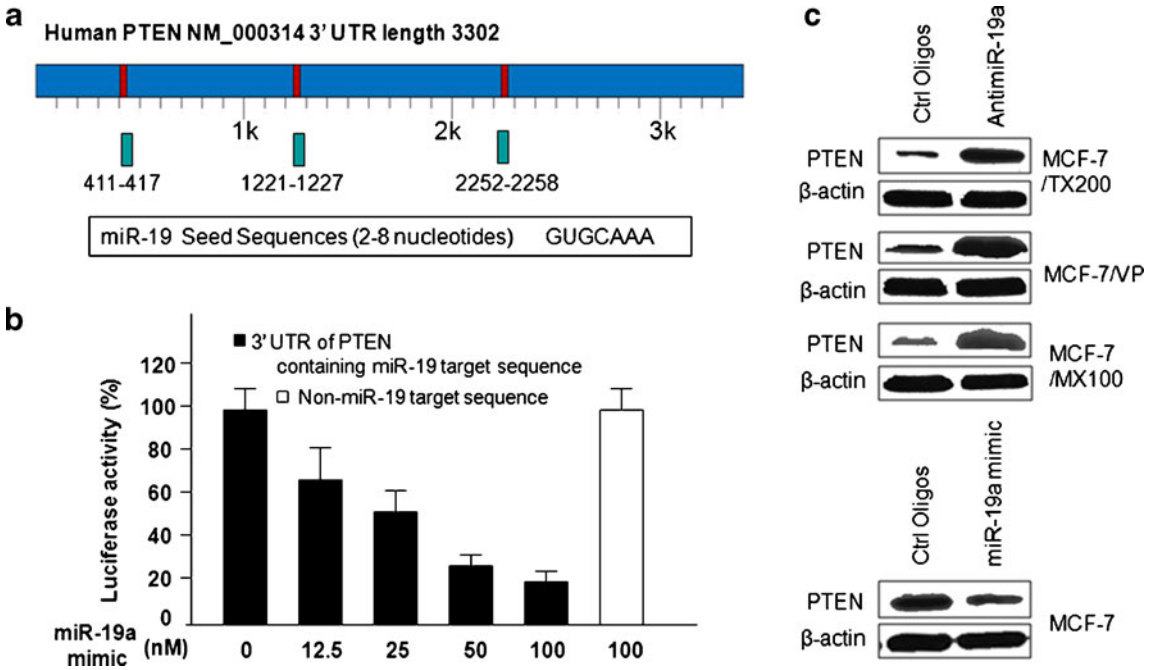

Fig. 2 PTEN is a predicted target of miR-19. (**a**) The putative miR-19a targeted sequence in the *PTEN* gene. TargetScan predicts three binding sites in *PTEN* 3′ UTR. (**b**) Luciferase reporter assays. The luciferase activity in MCF-7/TX200 transfected with the vector containing *PTEN* 3′ UTR fragment with binding sequence of miR-19a was inhibited by transfection of miR-19a mimic in a dose-dependent manner. (**c**) Expression levels of PTEN were restored in antimiR-19a-tansfected MDR cells (upper panel) and forced miR-19a expression reduced PTEN levels in MCF-7 cells (lower panel).

control oligonucleotides. PTEN protein expression levels were increased in MDR cells transfected with antimiR-19a compared to control oligonucleotides-transfected MDR cells (Fig. 2c). On the contrary, PTEN expression levels were downregulated in MCF-7 cells transfected with miR-19a mimics (Fig. 2c).

To address whether the overexpression of miR-19 contributes to breast cancer multidrug resistance, we down-regulated the expression levels of miR-19a to examine the effect of miR-19a on MDR-1, MRP-1, and BCRP mRNA and protein expression in MDR cells. MDR cells were transfected with antimiR-19a or control oligonucleotides. Expression levels of mRNA and protein of MDR-1, MRP-1, and BCRP were determined by quantitative RT-PCR and Western blotting 48 h after transfection. MDR-1, MRP-1, and BCRP mRNA (Fig. 3a) and protein (Fig. 3b) expression levels were significantly decreased in antimiR-19a-transfected MDR cells compared to control cells. On the other hand, miR-19a mimics were transfected into MCF-7 cells. Three MDR-related transporters were upregulated in the transfected cells compared to control cells (Fig. 3c).

Furthermore, to verify if PTEN affects the expression of MDR-1, MRP-1, and BCRP, a PTEN vector was transfected to MDR cells to overexpress PTEN. Increased expression levels of these three transporters were observed in MDR cells with forced PTEN expression compared to controls (Fig. 3d).

Repression of miR-19a Restores Sensitivity of MDR Cells to Chemotherapeutic Agents *In Vitro*

To investigate whether repression of miR-19 results in the increased sensitivity of the MDR cells to chemotherapeutic agents, we transfected antimiR-19a to three MDR cell lines derived from MCF-7 and determined sensitivity of these antimiR-19a-transfected cells to chemotherapy. At 24 h post transfection, cells were treated with increasing concentrations of Taxol, VP-16 and mitoxantrone. At 48 h post cell treatment with drugs, an MTS cell proliferation assay was performed. Our data show that the IC_{50} of antimiR-19a-transfected MCF-7/TX200 cells to Taxol was 29.6 times lower compared with those of the control oligonucleotide-transfected corresponding cells (Fig. 4a). Additionally, Fig. 4b shows that transfection of MCF-7/VP with antimiR-19a resulted in 21-time decrease of resistance compared to that of control oligonucleotides-transfected cells. The transfection of MCF-7/MX100 with antimiR-19a increased 29.1 times in sensitivity of these cells to mitoxantrone (Fig. 4c). These results show clearly that suppression of miR-19 restores the sensitivity of MDR

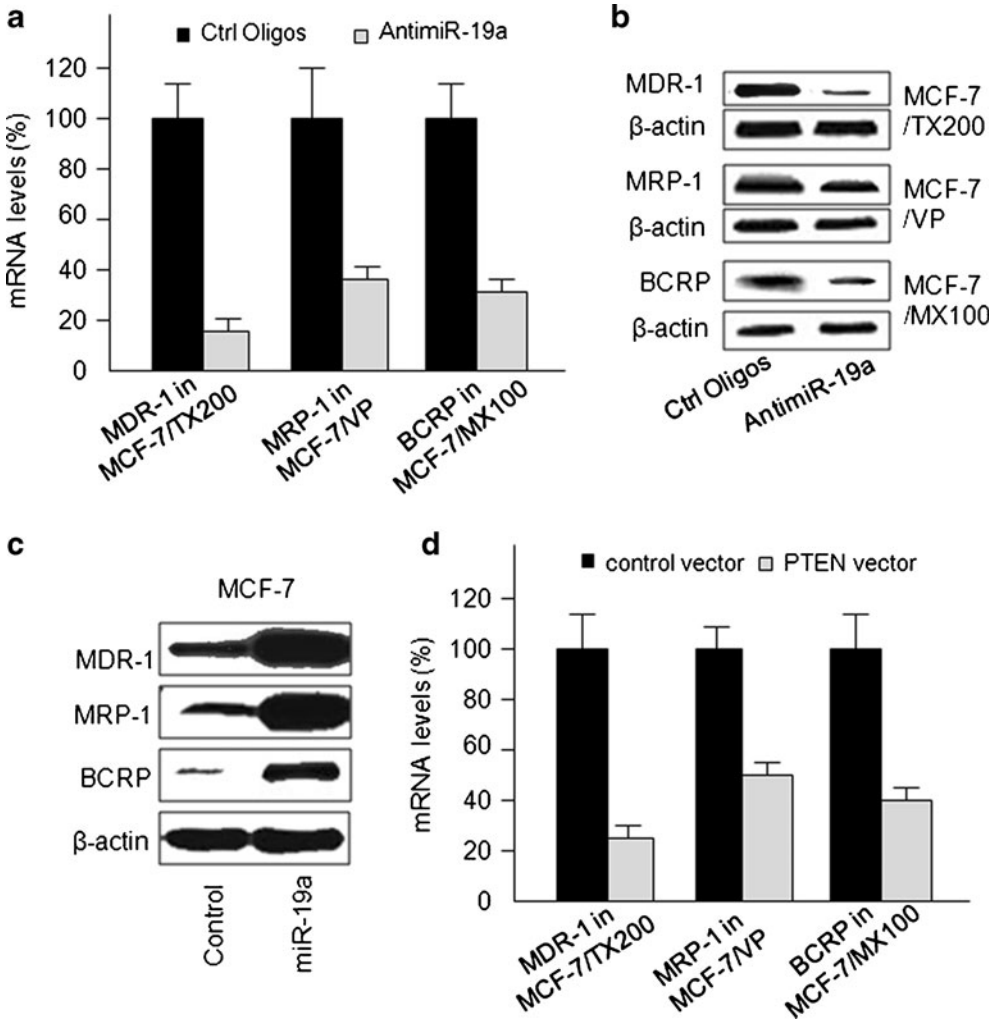

Fig. 3 MiR-19a modelates expression of MDR-1, MRP-1 and BCRP in MDR cells. (**a**) Expression levels of *MDR-1*, *MRP-1*, and *BCRP* mRNA in antimiR-19a-transfected MDR cells determined by real time RT-PCR were downregulated in comparison with the control cells. (**b**) Protein expression levels of MDR-1, MRP-1, and BCRP in antimiR-19a-transfected MDR cells were downregulated in comparison with control oligonucleotides-transfected MDR cells. (**c**) Overexpression of miR-19a upregulated MDR-1, MRP-1, and BCRP protein expression in MCF-7 cells. (**d**) Overexpression of PTEN in MDR cells reduced the expression of MDR transporters.

breast cells to corresponding chemotherapeutic agents. Additionally, after antimiR-19a was transfected into MDR cells, we did not observe statistically significant increase of sensitivity of MCF-7/TX200 cells to Mitoxantrone and VP-16, MCF-7/VP cells to Taxol and Mitoxantrone, and MCF-7/MX100 cells to Taxol and VP-16 (data not shown).

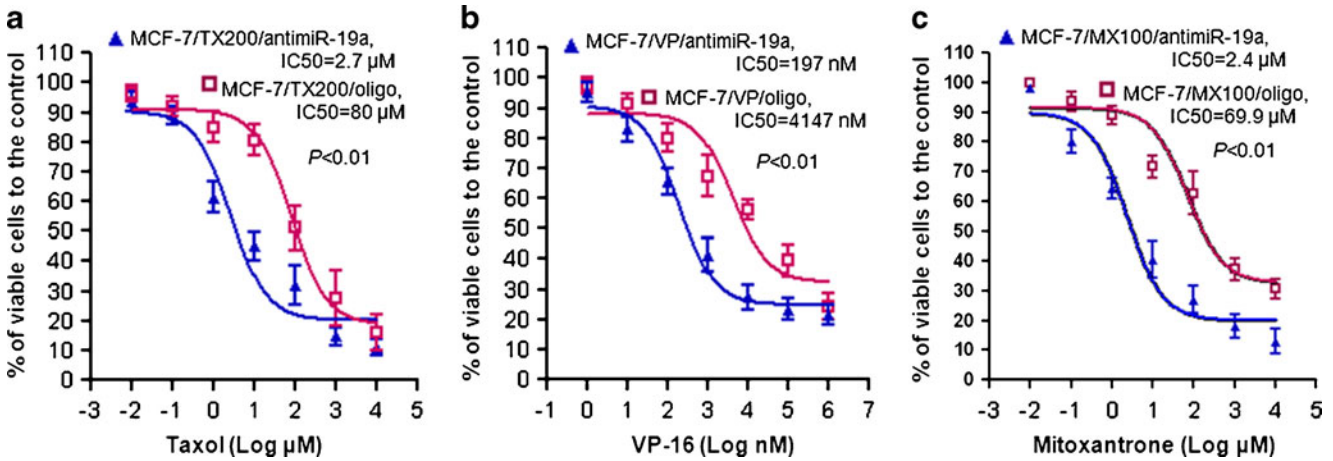

Fig. 4 Effect of miR-19a inhibitor on the sensitivity of MDR cells to Taxol, VP-16 and mitoxantrone. Drug sensitivity of miR-19a- or control oligonucleotides-transfected MCF-7/TX200, MCF-7/VP, and MCF-7/MX100 cell lines was tested by increasing concentrations of the tested drugs and measuring the surviving cells with the MTS cell proliferation assay at 48 h after the cells were treated. Each point is the average of triplicate determinations. AntimiR-19a restored significantly sensitivity of these cells to Taxol (**a**), VP-16 (**b**) and mitoxantrone (**c**).

LNA-antimiR-19a Increases Sensitivity of MDR Tumor Cells to Chemotherapeutic Agents *In Vivo*

Despite the efficiency of miR-19a inhibitor for restoring the sensitivity of MDR cells to chemotherapeutic agents *in vitro*, it is not clear whether suppression of miR-19 will sensitize MDR tumor cells to chemotherapeutic agents *in vivo*. Thus, we assessed the effects of LNA-antimiR-19a on sensitizing the tumors derived from MCF-7/TX200 MDR breast cancer cells to Taxol. Four groups of mice bearing xenografts derived from MCF-7/TX200 MDR cells were treated respectively with LNA-antimiR-19a alone, or LNA-antimiR-19a plus Taxol, or Taxol alone, or control oligonucleotides. The tumor volumes were evaluated at different times after injection (Fig. 5a). By day 14 when tumors were harvested, each tumor was weighed. Average weights for four groups are shown in Fig. 5b. As shown in Fig. 5, LNA-antimiR-19a significantly sensitized MDR MCF-7/TX200 xenografts to Taxol treatment compared to the Taxol alone group. At later time points, the volume of the tumors with LNA-antimiR-19a plus Taxol was significantly reduced compared to the Taxol alone on day 4, 6, 8, 10, 12, and 14 after the administration of Taxol (Fig. 5a) ($p<0.05$). In addition, we observed that LNA-antimiR-19a alone efficiently inhibited the growth of tumors compared to control oligonucleotides as Taxol alone (Fig. 5a). This may be explained because MCF-7/TX200 cells were resistant to Taxol and LNA-antimiR-19a alone may be efficient of inhibiting the growth of tumors. These results showed that LNA-antimiR-19a was effective for sensitizing MDR cells to chemotherapeutic agents.

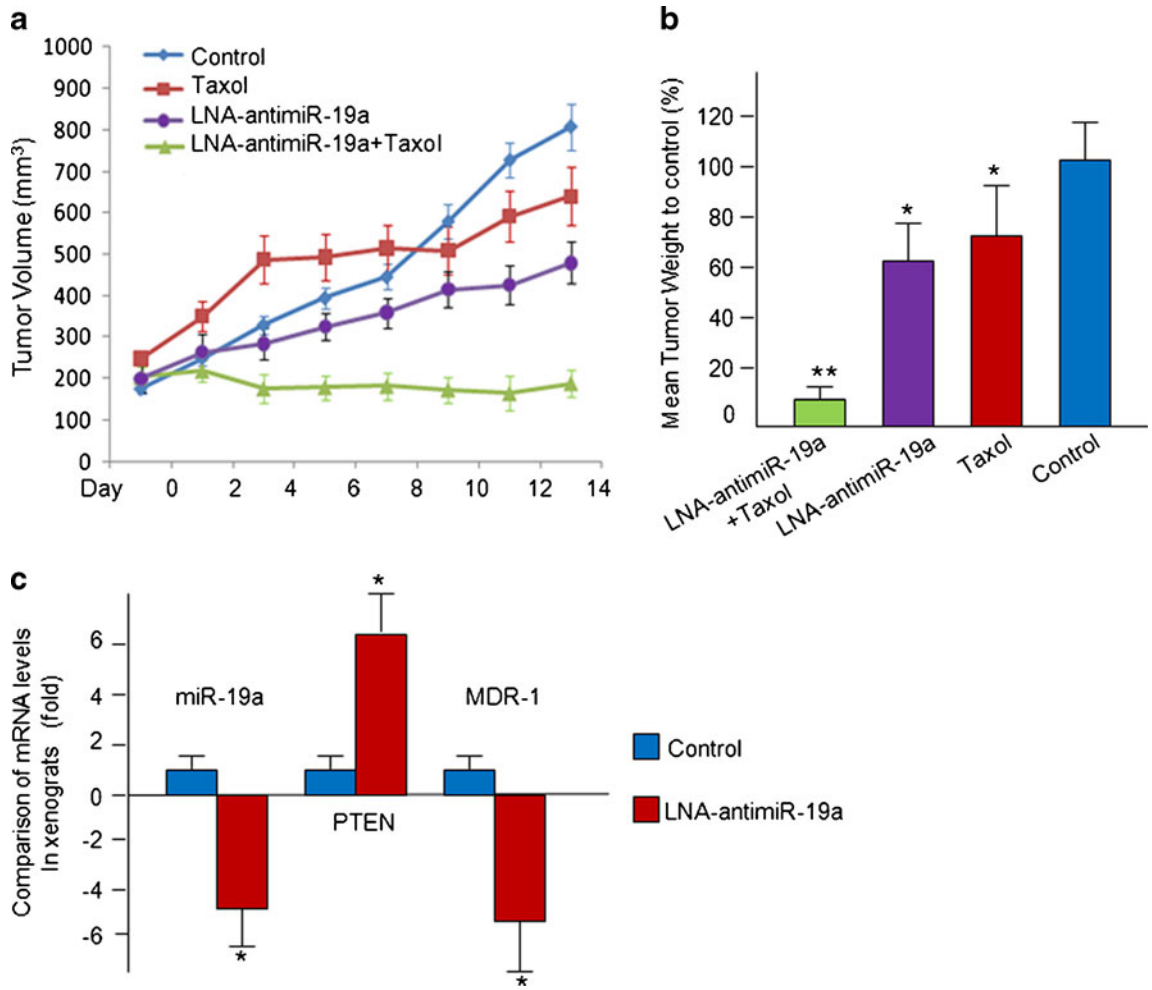

Fig. 5 LNA-antimiR-19a sensitizes MDR tumors to Taxol in nude mice. For the control group, only control oligonucleotides were injected at 10 mg/kg once daily i.v. for three consecutive days. Taxol was alone administered at 10 mg/kg once weekly In the Taxol Group. For the LNA-antimiR-19a+ Taxol group, LNA-antimiR-19a was injected at 10 mg/kg once daily i.v. for three consecutive days and after two days post last injection of LNA-antimiR-19a, Taxol was administered at 10 mg/kg once weekly. For the LNA-antimiR-19a group, LNA-antimiR-19a was alone injected at 10 mg/kg once daily i.v. for three consecutive days. Tumor volume was measured from the first Taxol treatment (0 day). (**a**) Mean tumor volume (mm^3) of each group at each time point Points. Bars mean standard errors. (**b**) Comparison of mean tumor weights from each group to control group. A significant decrease to the control is indicted with a star ($P<0.05$). (**c**) Comparison of mRNA expression levels of miR-19a, MDR-1, and PTEN in xenografts from the LNA-antimiR-19a-treated groups and control group.

To check if anitimiR-19a targets PTEN and modulates the expression of MDR-1 *in vivo*, we measured the expression levels of miR-19a, PTEN, and MDR-1 in the tumors from the LNA-antimiR-miR-19-treated group and control group with RT-PCR analysis. MiR-19a expression levels were significantly downregulated in the tumor tissues from the LNA-antimiR-19a-treated group compared to control group (Fig. 5c). The upregulation of PTEN and downregulation of MDR-1 were observed in the treated group compared to control group (Fig. 5c).

DISCUSSION

Despite strong evidence suggesting that miRNAs can play a critical role in cancer (23) and have a documented importance in cancer chemoresistance, the role of miRNA in cancer multidrug resistance remains largely unexplored. In this report, our findings demonstrating the involvement of miR-19 in MDR via the modulation of PTEN provide new evidence in MDR development mechanisms of tumor cells.

The current study shows regulatory functions of miR-19 in breast cancer chemoresistance. MicroRNAs have been demonstrated to be regulators of many functional genes (24). 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 (7). These microRNAs are usually downregulated in tumor cells, which leads to overexpression of their targeted genes. Several microRNAs have been demonstrated to be involved in multidrug resistance as suppressors. miR-451, miR-27 and miR-328 have been documented to be downregulated in MDR breast cancer cell lines, to regulate the expression of MDR-1/P-gp (13) and BCRP (15) respectively, and to promote MDR development of tumor cells to cytotoxic drugs. Our recent study reported that miR-326 regulates negatively the expression of MRP-1 (16), an important MDR-related transporter. The present study finds that miR-19 family members are overexpressed in all three MDR cell lines of breast cancer compared with their parental cells and directly target *PTEN*, which suggests that these miRNAs may function as modulators to regulate breast cancer chemoresistance. Previous studies have shown that the activation of the PI3K/Akt signaling network plays a critical role in MDR and loss of PTEN induced activation of Akt (25,26). Furthermore, it has been observed that PI3K/pAKT induced MDR by increasing the expression of MDR-1 (27), MRP1 (3) and BCRP (28). In the present study, we found that miR-19a inhibitor decreased the expression of MDR-1, MRP-1, and BCRP. In addition, we observed that forced expression of PTEN in MDR cells reduced the expression of MDR-1, MRP-1, and BCRP. Thus, our findings suggest that miR-19 modulates breast cancer chemoresistance via miR-19/PTEN/PI3K/AKT/MDR-related transporter pathways.

In the present study, we find that LNA-antimiR-19 sensitized MCF-7/TX200 cells to Taxol and also was effective as Taxol in inhibiting tumor growth. Recent studies showed that certain microRNAs in cancer cells may function as oncogenes to inhibit expression of tumor suppressors (29). MiR-21 has been well documented as a critical oncogenic microRNA and plays an important role in tumor progression (30,31). MiR-19 has been shown to be a key oncogenic component of the miR-17-92 cluster and to confer tumorigenicity (32,33). Therefore, the possible mechanism for LNA-antimiR-19a having an effect as Taxol may be that LNA-antimiR-19 targets the PTEN/AKT signaling pathway not only to sensitize MDR tumor cells to chemotherapeutic agents but also to inhibit tumor growth. In addition, MCF-7/TX200 cells were resistant to Taxol, so it is less efficient to inhibit the growth of MCF-7/TX200 tumors.

Our studies suggest that directly targeting miR-19 would be a potential strategy of sensitizing cancer cells to chemotherapeutic agents and suppressing the development of multidrug resistance. Despite the development of newer chemotherapeutic agents and combination chemotherapy regimens, metastatic and advanced breast cancer is often resistant to chemotherapy. MicroRNAs may be a class of emerging targets for preventing chemoresistance. Small interfering RNA (siRNA) is likely not effective for targeting the pri-miRNA, as siRNAs work primarily in the cytoplasm, where the pri-miRNA substrate is inaccessible. Modified antisense oligonucleotides (ASOs) complementary to miRNA have been used by several groups to inhibit miRNA activity in cell culture (34,35). Modified oligonucleotides can provide increased resistance to endogenous nucleases, increased hybridization affinity to the target sequence, and reduced toxicity. One of the most promising modifications is a recent use of locked nucleic acid residues in a couple of independent studies (36–38). The phosphorothioate linkage in DNA was found to be an effective modification for antisense molecules, significantly improving resistance to nuclease degradation, promoting protein binding, and delaying plasma clearance (39,40). Our data have demonstrated that LNA-antimiR-19a sensitized xenografts derived from MDR cells to Taxol treatment in an animal model. These results suggest that LNA-antimiR-19a is a stable and efficient agent for sensitizing tumors to chemotherapeutic agents.

CONCLUSION

We have shown that elevated levels of miR-19 are correlated with overexpression of MDR-1, MRP-1, and BCRP in MDR breast cancer cells. Additionally, miR-19

inhibitor restored the expression of PTEN and decreased the expression of MDR-1, MRP-1, and BCRP, and also sensitized MDR breast cancer cells to chemotherapeutic agents *in vitro* and *in vivo*. We demonstrate, for the first time, that miR-19 is involved in breast cancer chemoresistance. These findings contribute further to the understanding of MDR regulation in breast cancer cells.

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