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MiR-18a upregulation decreases Dicer expression and confers paclitaxel resistance in triple negative breast cancer

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Abstract. – OBJECTIVE: MiR-18a is a miRNA that is aberrantly overexpressed in triple-negative breast cancer (TNBC). However, its biophysical function in TNBC is still not clear. In this study, we investigated the association among miR-18a dysregulation, Dicer dysregulation and paclitaxel (PTX) resistance in TNBC cells.

PATIENTS AND METHODS: 20 TNBC patients who received neoadjuvant chemotherapy before surgery were recruited. MiR-18a expression was quantified using QRT-PCR. The effects of miR-18a overexpression or knockdown on cell viability and apoptosis of PTX sensitive MDA-MB-231 cells and PTX resistant MDA-MB-231 cells after PTX treatment were studied. The influence of miR-18a overexpression on Dicer expression was measured by qRT-PCR and Western blot analysis.

RESULTS: Tissues from patients with stable disease (SD, n = 5) and progressive disease (PD, n = 2) to paclitaxel (PTX) containing neoadjuvant chemotherapy had significantly higher miR-18a expression than that from patients with partial response (PR, n = 13). MDA-MB-231/PTX cells had higher miR-18a expression than MDA-MB-231 cells. MiR-18a overexpression directly led to Dicer repression at mRNA and protein level. MiR-18a overexpression significantly increased PTX IC50 and reduced PTX induced cell apoptosis, while miR-18a suppression substantially decreased PTX IC50 and increased PTX induced cell apoptosis.

CONCLUSIONS: This study found that miR-18a is an important miRNA that suppresses Dicer expression and increases PTX resistance in TNBC cells.

Key Words:
miR-18a, Triple negative breast cancer, Paclitaxel, Dicer.

Introduction

Breast cancer is the one of most commonly female malignancies[1]. Although a large proportion of the breast cancer patients have tumors expressing the estrogen receptor (ER)[2,3], there is a small proportion (approximately 12-17%) of patients with breast tumors lacking the expression of ER, progesterone receptor, and HER-2 expression, which termed as Triple Negative Breast Cancer (TNBC)[4,5]. In fact, TNBC is the most aggressive type and is difficult to treat since it has no responses to endocrine therapy and HER2 targeted therapy[4]. Therefore, the treatment modalities for TNBC are still limited to surgery, chemotherapy and radiotherapy. Paclitaxel (PTX) is a widely used mitotic inhibitor-based chemotherapeutic reagent for TNBC[5]. It has been used in regimes of neoadjuvant chemotherapy before surgery and adjuvant chemotherapy after surgery[5]. However, the development of resistance to PTX is still common in the patients, which frequently results in the subsequent recurrence and metastasis[6]. Previous studies found that the mechanism of PTX resistance in the TNBC cells is quite complex. The known mechanisms include upregulation of P-glycoprotein, a drug-efflux pump, a mutation in drug target, change of cell cycle and the presence of cancer stem cells[7-9].

MiRNAs are a set of small noncoding RNAs, which exerts posttranscriptional suppression of gene expression by inducing mRNA degradation or translational repression[10]. Some recent studies suggest that dysregulated miRNAs are also an important mechanism of chemoresistance in TNBC cells[11-13]. Since a miRNA can exert regulative effects on multiple genes at the same time, they actually form a complex regulative network[14-16]. MiR-18a is a miRNA that is aberrantly overexpressed in TNBC[17]. However, the biophysical function of its dysregulation in TNBC is still not clear. Dicer is an enzyme playing an important role in miRNA processing[15]. In TNBC, previous studies[18,19] found that Dicer was significantly downregulated in cancerous tissues than in

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adjacent normal tissues. But the mechanism underlying its downregulation has not been fully revealed.

In this study, we investigated the association among miR-18a dysregulation, Dicer dysregulation and paclitaxel resistance in TNBC cells.

Patients and Methods

Patient Tissue Collection

This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. 20 patients with histopathologically confirmed stage IIA and IIB TNBC were recruited from the hospital. Written informed consent was obtained from the patients before the study. Before surgery, the patients received neoadjuvant chemotherapy based on epirubicin plus paclitaxel for three cycles. Two weeks after completion of the chemotherapy, their responses were assessed based on the Response Evaluation Criteria in Solid Tumors guidelines which defines four response categories, including complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Patients with CR and PR are considered as chemosensitive, while the patients with SD and PD are considered as chemoresistant. Cancerous and the corresponding paired normal adjacent tissues were obtained during surgery.

Cell Culture and Generation of PTX Resistant MDA-MB-231 Cells

The human TNBC cell lines, including MDA-MB-231 and MDA-MB-468, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All of the cancer cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units of penicillin/ml and 100 µg of streptomycin/ml. Immortalized human breast epithelial cell line MCF-10A were obtained from American Type Culture Collection (ATCC). MCF10A cells were grown in Ham's F12:DMEM (50:50) medium supplemented with 2 mM L-glutamine, 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mg/ml cholera toxin (CT) (Sigma-Aldrich), 10 mg/ml insulin (Sigma-Aldrich), 500 ng/ml hydrocortisone (Sigma-Aldrich) and 5% horse serum (Atlanta Biologicals, Norcross, GA, USA). All cells were cultured in an incubator with humidified atmosphere and 5% CO_2 at 37°C.

To generate the PTX-resistant MDA-MB-231 variant, an intermittent, stepwise method was used as described in one previous study[20]. Briefly, the parent MDA-MB-231 cells were firstly treated with the initially determined IC30 of PTX for 3-4 days. Then, the cells were cultured with fresh medium without drug for 3-4 days before drug treatment again. This process lasted for 4-6 weeks until the cells could bear IC60 treatment of PTX. Then, the cells were harvest and serially dilution was performed to get single clones. After expansion cultures, the cells that successfully grew under IC60 were termed as MDA-MB-231/PTX and were used for following studies. The culture medium for MDA-MB-231/PTX cells was additionally added 5 nM PTX to maintain the resistance.

Cell Treatment

MiR-18a mimics, antogamiR-18a (anti-miR-18) and the scramble negative controls were all purchased from Ribobio (Shanghai, China). MDA-MB-231 and MDA-MB-231/PTX cells were transfected with 100 nM miR-18a or 100 nM anti-miR-18a using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

QRT-PCR Analysis

Total RNAs in the cell or tissue samples were extracted using the TRIzol reagent (Invitrogen) according to manufacturer's instructions. The first strand cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). MiRNAs specific cDNA was synthesized using the stem-loop primers and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). To quantify miR-18a expression, qRT-PCR analysis was performed using TaqMan MicroRNA Assay Kit (Applied Biosystems), with U6 snRNA used as the endogenous control. To detect Dicer mRNA expression, the qRT-PCR was performed using the following primers, forwards: 5'-GGTGTTCTTGGTCAACTCTGC-3'; reverse: 5'-TTTCTCGATAGGGGTGGTCTA-3'. All PCR reactions were performed using an ABI Prism 7500 (Applied Biosystems). The results were calculated using the $2^{-\Delta\Delta CT}$ methods.

CCK-8 Assay of Drug Sensitivity

MDA-MB-231 cells with indicating treatments were seeded in a 96-well plate at a density of 3000 cells/well for 24 hours and then replaced by 200 µl full growth medium with varying concen-

trations of PTX (0, 0.5, 1, 5, 10, 15 and 20 or 0, 1, 5, 10, 20, 40 and 80 nmol/L) for 48 hours. Then, cell viability was measured using WST-8 assay using Cell Counting Kit-8 (CCK-8, Dojindo, Gaithersburg, MD, USA) according to manufacturer's instruction. In brief, 10 µl of CCK-8 solution was added to the medium and then incubated at 37°C for 2 h. Cell viability was reflected by the absorbance at 450 nm determined by a 96-well spectrophotometry. IC50 value was determined by creating dose-response curves.

Flow Cytometric Analysis of Cell Apoptosis

Cell apoptosis was detected by using Annexin V-FITC Apoptosis Detection Kit (ab14085, Abcam, Cambridge, UK) according to the manufacturer's instruction using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA).

Western Blot Analysis

Cell samples were lysed using a lysis buffer (Beyotime, Shanghai, China). Then, the protein concentration was quantified using a BCA protein assay kit (Beyotime). Then, a conventional western blot analysis was performed following a method described in one previous study[21]. Primary antibodies used included anti-Dicer (ab82539, 1:50, Abcam) anti-PARP (ab194586, 1:2000, Abcam), anti-cleaved PARP (#9546, 1:2000: Cell Signaling, Danvers, MA, USA), anti-active caspase-3 (1:500, ab32042, Abcam) and anti-β-actin (1:2000, ab8227, Abcam). The second HRP conjugated secondary antibodies were purchased from Abcam. The blot signals were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA). The relative gray scale intensity was quantified using ImageJ software.

Statistical Analysis

Data were presented in the form of means ± standard deviation (SD) based at least three repeats of three times independent studies. Oneway ANOVA with Tukey's post-hoc test was performed to compare means of multiple group experiments. Comparison between groups was performed using the unpaired *t*-test. A two-sided *p*-value of < 0.05 was considered statistically significant.

Results

MiR-18a is Significantly Upregulated in TNBC and is Further Upregulated in Chemoresistant TNBC

Dysregulated miR-18a in TNBC was observed in one previous study[17]. However, its regulative effects in TNBC has not been fully revealed. In this study, we firstly measured miR-18a expression based on 20 cases of paired normal and TNBC tissues. QRT-PCR analysis confirmed that the TNBC tissues had significantly higher miR-18a expression than the normal tissues (Figure 1A). Based on the responses to the neoadjuvant chemotherapy, the 20 patients consist of 13PR, 5SD and 2PD. We found that the chemoresistant cases (SD and PD)

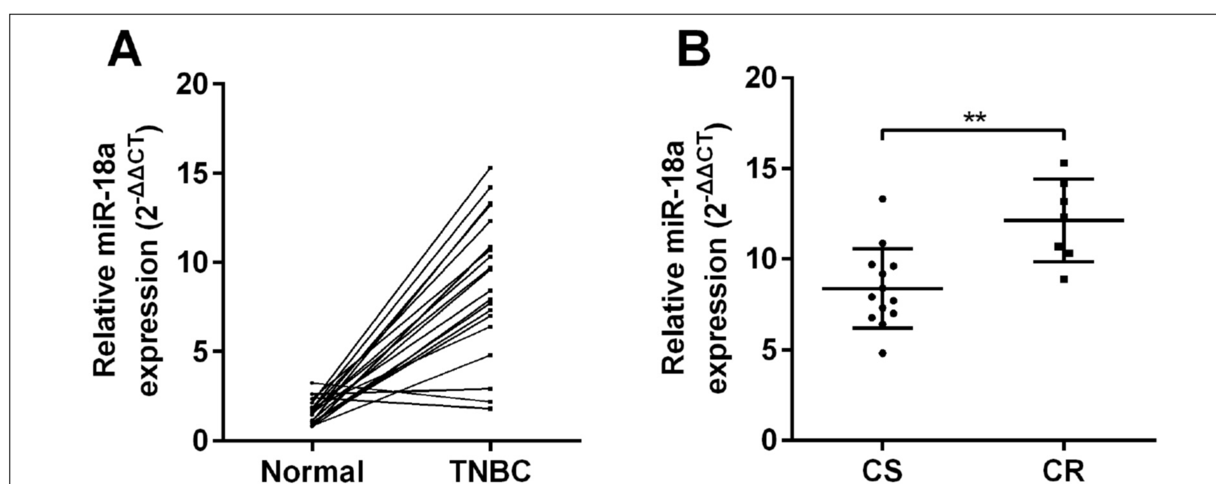

Figure 1. MiR-18a is significantly upregulated in TNBC and is further upregulated in chemoresistant TNBC. ***A,*** QRT-PCR analysis and comparison of miR-18a expression between TNBC cancerous tissues and paired adjacent normal tissues obtained from the 20 patients. ***B,*** Comparison of relative miR-18a expression in cancerous tissues between chemoresistant (CR) cases (5 SD and 2 PD) and chemosensitive (CS) cases (13 PD). $^{**}p < 0.01$.

had significantly even higher miR-18a expression than the chemosensitive cases (PD) (Figure 1B). Therefore, we decided to further investigate the association between miR-18a and chemoresistance of TNBC.

MiR-18a Upregulation is Associated with Decreased Dicer and Increased PTX Resistance in TNBC Cells

By using *in-vitro* cell culture, we also found that the TNBC cell lines, including MDA-MB-231 and MDA-MB-468, had significantly higher miR-18a expression than the normal human breast epithelial cell line MCF-10A (Figure 2A). Previous studies[18,19] found that Dicer was significantly downregulated in TNBC tissues than in adjacent normal tissues, but the mechanism is largely unknown. Two recent studies[22,23] demonstrated that in bladder cancer cells and nasopharyngeal carcinoma, miR-18a can bind to the 3'UTR of Dicer and, thereby, suppress its expression. Therefore, we investigated whether this mechanism is involved in Dicer downregulation in TNBC. By performing qRT-PCR and western blot analysis, we confirmed that miR-18a overexpression directly led to Dicer repression at mRNA and protein level (Figure 2B). Then, by using an intermittent and stepwise method, we generate a PTX resistant MDA-MB-231 variant, which had approximately eight times higher PTX IC50 (36.9 ± 5.8 nM) to the parent cells (4.8 ± 0.68 nM) (Figure 2C). QRT-PCR analysis further confirmed that the MDA-MB-231/PTX cells had over three times higher miR-18a expression than MDA-MB-231 cells (Figure 2D).

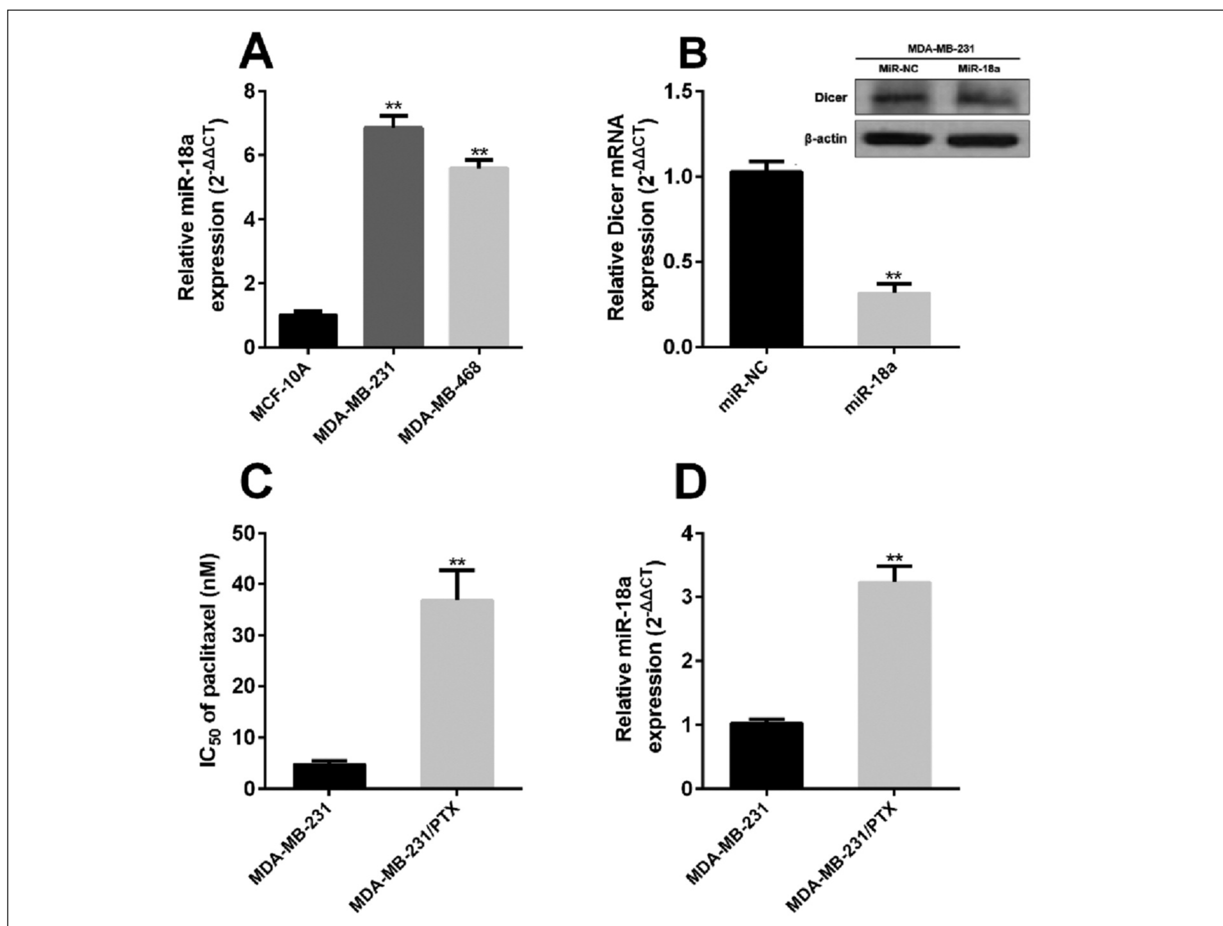

Figure 2. MiR-18a upregulation is associated with decreased Dicer and increased PTX resistance in TNBC cells. ***A,*** QRT-PCR analysis and comparison of miR-18a expression in MCF-10A, MDA-MB-231 and MDA-MB-468 cells. ***B,*** QRT-PCR and Western blot analysis of Dicer mRNA expression in MDA-MB-231 cells with miR-18a overexpression. ***C,*** IC_{50} value of the PTX sensitive MDA-MB-231 cells and the PTX resistant MDA-MB-231/PTX cells. ***D,*** QRT-PCR analysis of miR-18a expression in MDA-MB-231 cells and MDA-MB-231/PTX cells. $^{**}p < 0.01$.

MiR-18a Modulates Cell Viability After PTX Treatment

Since miR-18a is associated with PTX resistance, we further investigate whether there is a causative effect between miR-18a upregulation and PTX resistance. By performing CCK-8 assay, we found that miR-18a overexpression significantly increased the viability of MDA-MB-231 cells after PTX treatments (Figure 3A), while miR-18a suppression significantly decreased the viability of MDA-MB-231/PTX cells after PTX treatment (Figure 3B). By drawing dose-response curves, we observed that miR-18a overexpression significantly increased PTX IC50 of MDA-MB-231 cells (Figure 3C), while miR-18a knockdown significantly decreased PTX IC50 of MDA-MB-231/PTX cells (Figure 3D).

MiR-18a Modulates PTX-Induced Cell Apoptosis

Then, we performed flow cytometric analysis to investigate the effect of miR-18a on PTX induced apoptosis. The results showed that miR-18a overexpression substantially reduced the ratio of PTX-induced apoptotic MDA-MB-231 cells (Figure 4A-B), while miR-18a suppression substantially increased the ratio of PTX induced apoptotic MDA-MB-231/PTX cells (Figure 4A-B). Following Western blot analysis also showed that miR-18a overexpression substantially reduced PTX induced expression of cleaved-PRAP and cleaved-caspase-3 in MDA-MB-231 cells (Figure 4C-E), while miR-18a suppression significantly increased PTX induced expression of cleaved-PRAP and cleaved-caspase-3 in MDA-MB-231/PTX cells (Figure 4C-E). These results suggest that miR-18a upregulation confers increased PTX resistance to TNBC cells.

Discussion

One recent study found that miR-18a expression in TNBC was significantly higher than that in both normal breast tissues and luminal A tumors[17]. However, its regulative effects in cancer have not been clear understood. Another recent study found that miR-18a was significantly upregulated in breast cancer tissue samples and cancer cells[24]. MiR-18a can directly target 3'-UTR of Ataxia Telangiectasia Mutated (ATM) Kinase and downregulate its expression. In fact,

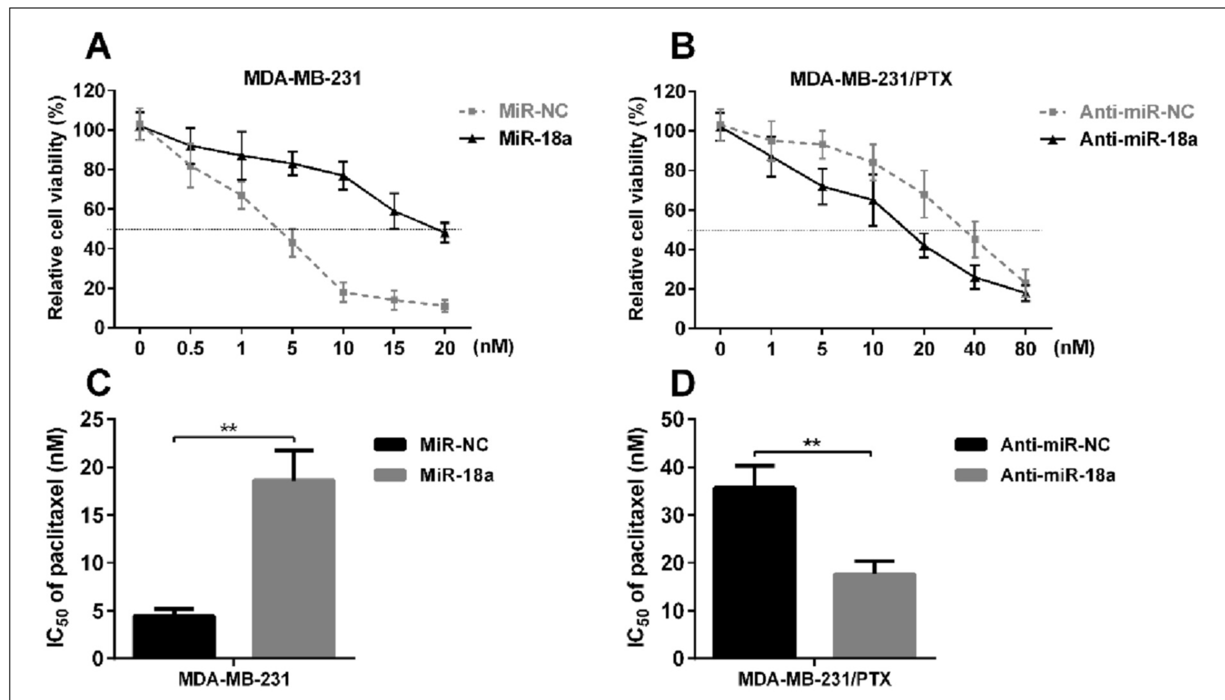

Figure 3. MiR-18a modulates cell viability after PTX treatment. A-B, CCK-8 assay of cell viability of MDA-MB-231 cells with or without miR-18a overexpression *(A)* and MDA-MB-231/PTX cells with or without miR-18a suppression *(B)* after treatment with varying concentrations of PTX for 48 hours. C-D, IC_{50} value of MDA-MB-231 cells with or without miR-18a overexpression *(C)* and MDA-MB-231/PTX cells with or without miR-18a knockdown *(D)*. $^{**}p < 0.01$.

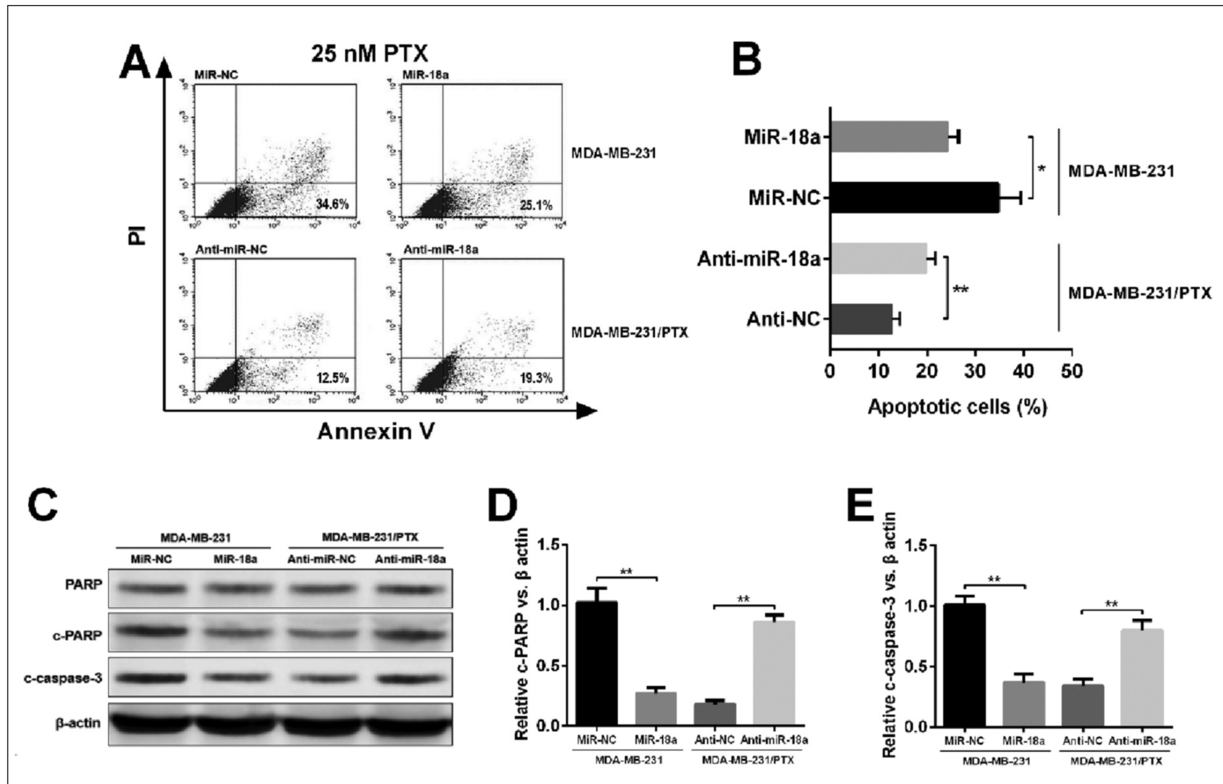

Figure 4. MiR-18a modulates PTX induced cell apoptosis. ***A-B,*** Representative images ***(A)*** and quantification ***(B)*** of flow cytometric analysis of apoptotic MDA-MB-231 cells and MDA-MB-231/PTX cells 48 hours after 25 nM PTX treatment using Annexin V and PI staining. ***C,*** Western blot analysis of PRAP, cleaved-PRAP and cleaved-caspase-3 expression in MDA-MB-231 cells and MDA-MB-231/PTX cells 48 hours after 25 nM PTX treatment. ***D-E,*** Quantification of relative cleaved-PRAP (c-PRAP) and cleaved-caspase-3 (c-caspase-3) expression showed in Figure C. $^*p < 0.05$, $^{**}p < 0.01$.

ATM kinase is an important transducer of the DNA damage signal[24]. Ectopic expression of miR-18a could significantly abrogate the IR-induced cell cycle arrest via downregulating ATM kinase[24]. In fact, PTX is a mitotic inhibitor, which induces mitotic arrest and triggers cell apoptosis[25]. Considering the interfering effect of miR-18a on cell cycle arrest, we then investigated whether it is related to PTX resistance in TNBC. By quantifying miR-18a level in the chemosensitive and chemoresistant cases, we observed that miR-18a upregulation was significantly associated with PTX resistance. By using *in-vitro* cell line models, we also found that miR-18a expression was significantly higher in PTX resistant MDA-MB-231/PTX cells than in MDA-MB-231 cells. To further investigate whether there is a causative effect between miR-18a upregulation and PTX resistance. We, then, performed CCK-8 assay, flow cytometric and Western blot analysis to investigate the effect of miR-18a overexpression or knockdown on PTX sensitivity. The results showed that miR-18a overexpression significantly increased PTX IC50 and reduced PTX induced cell apoptosis, while miR-18a suppression substantially decreased PTX IC50 and increased PTX induced cell apoptosis. Therefore, we infer that miR-18a upregulation directly confers increased PTX resistance to TNBC cells.

Dicer is an important enzyme that plays a critical role in the maturation of miRNAs[19]. In TNBC, Dicer expression is usually downregulated in cancerous tissues than in adjacent normal tissues[18,19]. Reduced expression of Dicer is correlated with high grade, shorter metastasis-free survival and certain TNBC subtypes[26,27]. However, how it is downregulated in TNBC is not clear. Two recent studies[22,23] demonstrated that in bladder cancer cells and nasopharyngeal carcinoma, miR-18a can bind to the 3'UTR of Dicer mRNA and thereby suppress its expression. Considering significantly upregulated miR-18a in TNBC, we further assessed whether miR-18a involves in

Dicer downregulation. The results showed that miR-18a overexpression directly led to Dicer repression at mRNA and protein level.

The effect of miR-18a in breast cancer is paradoxical. Although this study found that miR-18a overexpression confers paclitaxel resistance to TNBC cells, some other works also reported that miR-18a might act as a tumor suppressor in breast cancer. One research[28] found that miR-18a can inhibit hypoxia-inducible factor 1alpha activity and lung metastasis in basal breast cancers. MiR-18a, together with miR-124, miR-365, miR-34b* and miR-50 might also reverse the p27/myc/phospho-Rb protein signature in both breast and ovarian cancer[29]. Since a miRNA may target multiple genes simultaneously and exert diversified regulative effects, the exact regulation might be closely related to the specific tumor microenvironment. Therefore, more investigations are required to explore further the role of miR-18a in breast cancer.

Conclusions

We observed that miR-18a is an important miRNA that suppresses Dicer expression and increases PTX resistance in TNBC cells.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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