

MiR-18a upregulation enhances autophagy in triple negative cancer cells via inhibiting mTOR signaling pathway

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Abstract. – OBJECTIVE: We investigated the involvement of miR-18a upregulation in autophagy regulation and paclitaxel (PTX) resistance in triple negative breast cancer (TNBC) cells.

MATERIALS AND METHODS: PTX resistant MDA-MMB-231/PTX cells were generated using an intermittent, stepwise method. MiR-18a expression was assessed using qRT-PCR. The level of autophagy was assessed by Western blot analysis of LC3B expression and observation of LC3-GFP puncta formation under a fluorescence microscope. The effect of miR-18a mediated autophagy on PTX sensitivity was assessed by measuring IC₅₀ and PTX induced cell apoptosis.

RESULTS: MDA-MB-231/PTX cells had both higher miR-18a expression and basal autophagy than MDA-MB-231 cells. Enforced miR-18a overexpression directly led to increased autophagy in MDA-MB-231 cells, the effect of which was similar to that of rapamycin, a mTOR signaling inhibitor. Following Western blot analysis showed that miR-18a overexpression decreased the expression of p-mTOR and p-p70S6. Therefore, we infer that miR-18a increases autophagy level in MDA-MB-231 cells via inhibiting mTOR signaling pathway. Both drug sensitivity assay and flow cytometry analysis confirmed that the effect of miR-18a on increasing IC₅₀ and decreasing PTX induced apoptosis in MDA-MB-231 cells could largely be abrogated by treatment with bafilomycin A1 (Baf. A1).

CONCLUSIONS: MiR-18a upregulation results in enhanced autophagy via inhibiting mTOR signaling pathway in TNBC cells, which is a mechanism contributing to paclitaxel resistance.

Key Words:

miR-18a, Triple negative breast cancer, Autophagy, Paclitaxel, mTOR.

Introduction

Triple Negative Breast Cancer (TNBC) refers to the breast cancer lacking the expression of ER, progesterone receptor and HER-2 expression^{1,2}. Although the ratio of TNBC among all types of breast cancer is small, it is the most aggressive type and is difficult to treat since it has no responses to endocrine therapy and HER2 targeted therapy. Currently, paclitaxel (PTX) based chemotherapy is still an important treatment modality for this group of patients. Although the patients' initial responses to the chemotherapy is high, the development of PTX resistance is common and still a major hurdle is limiting its effectiveness³. Therefore, it is crucial to understand the mechanisms underlying PTX resistance in TNBC cancer cells. Previous investigations⁴⁻⁶ identified multiple mechanisms are involved in PTX resistance in TNBC, such as the presence of cancer stem cells, alteration of drug metabolism, mutation in drug target and change of cell cycle.

Autophagy is an evolutionarily conserved cellular process that basically consists of degradation and recycling of defective organelles and proteins to maintain cellular homeostasis⁷. Some recent re-

searches⁸⁻¹⁰ suggest that upregulated autophagy is also an important mechanism of chemoresistance in TNBC cells. Autophagy can increase epirubicin resistance in MDA cells by blocking the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB)-mediated pro-apoptotic signals¹¹. Enhanced autophagy also confers a cytoprotective function under paclitaxel treatment stress in MDA-MB-231 cells⁸. Inhibition of autophagy is considered as a strategy to improve chemotherapy efficiency in TNBC¹⁰. However, how autophagy is dysregulated in TNBC is still not fully understood. Therefore, it is quite necessary to further explore the underlying mechanisms. Previous studies found that multiple miRNAs participate in autophagy regulation in breast cancer, such as miR-25¹², miR-101¹³ and miR-214¹⁴. MiR-18a is a miRNA that is aberrantly overexpressed in TNBC¹⁵. However, whether its dysregulation in TNBC is related to autophagy is still not clear. In this work, we investigated the involvement of miR-18a upregulation in autophagy regulation and paclitaxel resistance in TNBC cells.

Materials and Methods

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

The human TNBC cell MDA-MB-231 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were grown in Roswell Park Memorial Institute 1640 (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) and were cultured according to the method introduced in one previous study¹⁶. All cells were cultured in an incubator with humidified atmosphere and 5% CO₂ at 37°C.

To generate the PTX-resistant MDA-MB-231 variant, an intermittent, stepwise method was used as described previously¹⁷. 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 supplemented with 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). Bafilomycin A1 (Baf. A1), an autophagy blocker and rapamycin, an inducer of autophagy were purchased from Sigma-Aldrich (St. Louis, MO, USA). To suppress autophagy, MDA-MB-231 cells, MDA-MB-231/PTX cells, MDA-MB-231 cells with miR-18a overexpression and MDA-MB-231/PTX cells with miR-18a knockdown were treated with 50 nM bafilomycin A1. To induce autophagy, MDA-MB-231 cells were treated with 50 nM rapamycin. MDA-MB-231 cells with miR-18a overexpression and MDA-MB-231/PTX cells with miR-18a knockdown were treated with 25 nM PTX with or without the presence of Baf. A1 (50 nM). Then, the cells were subjected to Western blot analysis and flow cytometric analysis.

Preparation of MDA-MB-231 Cells with Stable GFP-LC3 Expression

pSELECT-GFP-LC3 plasmid was purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). MDA-MB-231 cells were transfected with this plasmid were screened using 250 µg/ml Zeocin (Sigma-Aldrich, St. Louis, MO, USA). After three weeks' selection, clones with stable GFP-LC3 expression were obtained. GFP-LC3 puncta was observed using Olympus IX71 inverted microscope (Olympus, Tokyo, Japan).

qRT-PCR Analysis

Total RNAs in the cell samples were extracted using the TRIzol reagent (Invitrogen) according to manufacturer's instructions. 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, the qRT-PCR analysis was performed using TaqMan MicroRNA Assay Kit (Applied Biosystems), with U6 snRNA used as the endogenous control. All PCR reactions were performed using an ABI Prism 7500 (Applied Biosystems). The results were calculated using the 2^{-ΔΔCT} 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 μ l full growth medium with varying concentrations 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 μ l 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). A conventional Western blot analysis was performed following a method described in one previous study¹⁸. Primary antibodies used included anti-LC3B (ab51520, 1:3000,

Abcam), anti-mTOR (1:500, ab87540, Abcam), anti-phospho-mTOR (1:2000, ab109268, Abcam), anti-phospho-p70S6 (1:1000, #6198, Cell Signaling), anti-p70S6 (1:1000, #9202, Cell Signaling) 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 \pm standard deviation (SD) based at least three repeats of three independent studies. One-way 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 PTX Resistant TNBC Cells

To investigate the role of miR-18a in PTX resistance in TNBC cells, we firstly generated PTX resistant MDA-MB-231/PTX cells, which had about eight times higher PTX IC₅₀ (36.9 \pm 5.8 nM) (Figure 1A) and over three times higher miR-18a expression than the parent MDA-MB-231 cells (Figure 1B).

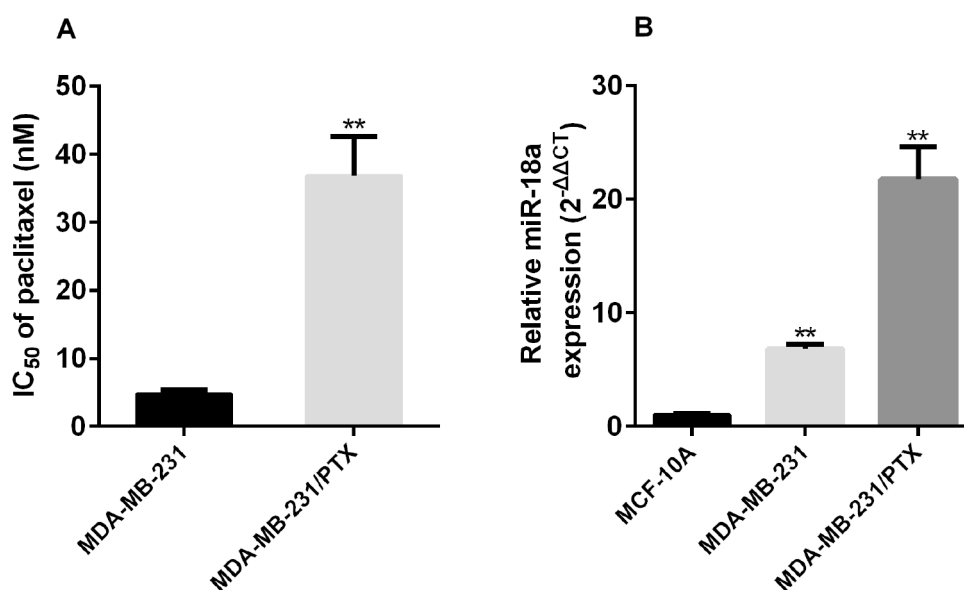


Figure 1. MiR-18a is significantly upregulated in PTX resistant TNBC cells. A. IC₅₀ value of the PTX sensitive MDA-MB-231 cells and the PTX resistant MDA-MB-231/PTX cells. B. QRT-PCR analysis and comparison of miR-18a expression in MCF-10A, MDA-MB-231 and MDA-MB-231/PTX cells. ***p*<0.01.

MDA-MB-231/PTX Cells had Significantly Higher Basal Autophagy than MDA-MB-231 Cells

Previous studies suggested that elevated autophagy is an important mechanism of chemoresistance in TNBC cells^{8,9}. Therefore, we further investigated whether miR-18a is involved in the regulation of autophagy. We firstly investigated the basal level of autophagy in MDA-MB-231 and MDA-MB-231/PTX cells. Western blot analysis showed the relative LC3 II level was significantly higher in MDA-MB-231/PTX cells than in MDA-MB-231 cells (Figure 2A and B). The treatment with Baf. A1 led to increased LC3 II levels in both of the cells, but the increase in MDA-MB-231/PTX cells was higher, suggesting that MDA-MB-231/PTX cells had a higher basal autophagy level (Figure 2A and B). To verify further the autophagy levels, LC3-GFP puncta formation was observed using a fluorescence microscope. MDA-MB-231/PTX cells had more LC3 II puncta than MDA-MB-231 cells no matter with or without the treatment of Baf. A1 (Figure 2C and D). These results suggest that MDA-MB-231/PTX cells had significantly higher basal autophagy than MDA-MB-231 cells.

MiR-18a Mediated Autophagy Upregulation in MDA-MB-231 Cells

Then, MDA-MB-231 cells were transfected for miR-18a overexpression. Enforced miR-18a overexpression had a similar effect as rapamycin in stimulating LC3 II expression (Figure 3A and B) and LC3-GFP puncta formation in the cells (Figure 3C and D). These results suggest that miR-

18a upregulation confers increased autophagy to MDA-MB-231 cells. Since it exerts a similar effect as a mTOR inhibitor, we then detect whether it has regulative effects on the mTOR signaling pathway. By performing Western blot analysis, we found that miR-18a overexpression decreased the expression of p-mTOR and p-p70S6, which are mTOR substrates, while miR-18a knockdown showed opposite effects (Figure 3E). Based on these results, we inferred that miR-18a can increase autophagy level in MDA-MB-231 cells via inhibiting mTOR signaling pathway (Figure 3F).

MiR-18a Mediated Autophagy Upregulation is Involved in Development of PTX Resistance in MDA-MB-231 Cells

Since we verified the enhancing effect of miR-18a on autophagy, we further investigated whether this mechanism contributes to the development of PTX resistance. By performing drug sensitivity assay, we found that miR-18a overexpression increased PTX IC₅₀ in MDA-MB-231 cells (Figure 4A). However, treatment with Baf. A1, largely abrogated the effect of miR-18a (Figure 4A). In addition, anti-miR-18a had a similar effect as Baf. A1 in reducing PTX IC₅₀ in MDA-MB-231/PTX cells (Figure 4B). Following flow cytometric analysis also showed that treatment with Baf. A1 largely abrogated the effect of miR-18a on reducing PTX induced apoptosis in MDA-MB-231 cells (Figure 4C). Besides, anti-miR-18a had a similar effect as Baf. A1 in enhancing PTX induced apoptosis in MDA-MB-231/PTX cells (Figure 4D). These results suggest that miR-18a mediated autophagy upregulation is involved in the development of PTX resistance in MDA-MB-231 cells.

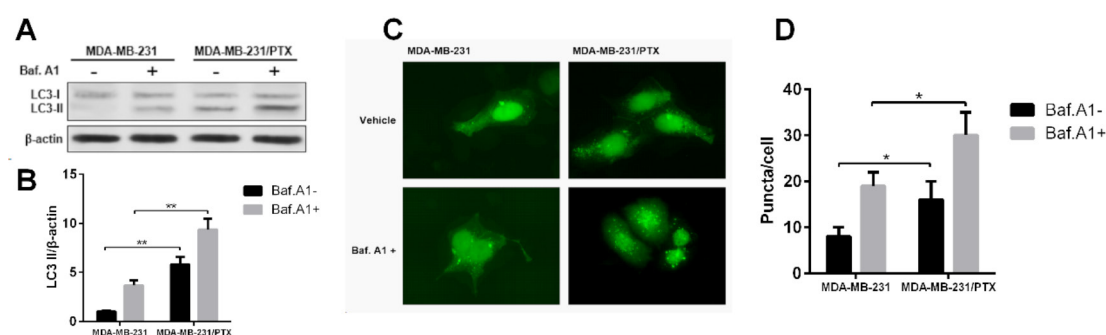


Figure 2. MDA-MB-231/PTX cells had significantly higher basal autophagy than MDA-MB-231 cells. **A-B**, Images of western blot analysis (A) and quantification (B) of relative LC3 I and LC3 II expression in MDA-MB-231 cells and MDA-MB-231/PTX cells 24 hours after treatment with vehicle (-) or 50 nM Baf. A1 (+). **C-D**, Representative images (A) and quantification (B) of LC3-GFP puncta accumulation in MDA-MB-231 cells and MDA-MB-231/PTX cells 24 hours after treatment with vehicle (-) or 50 nM Baf. A1 (+). ** $p < 0.01$.

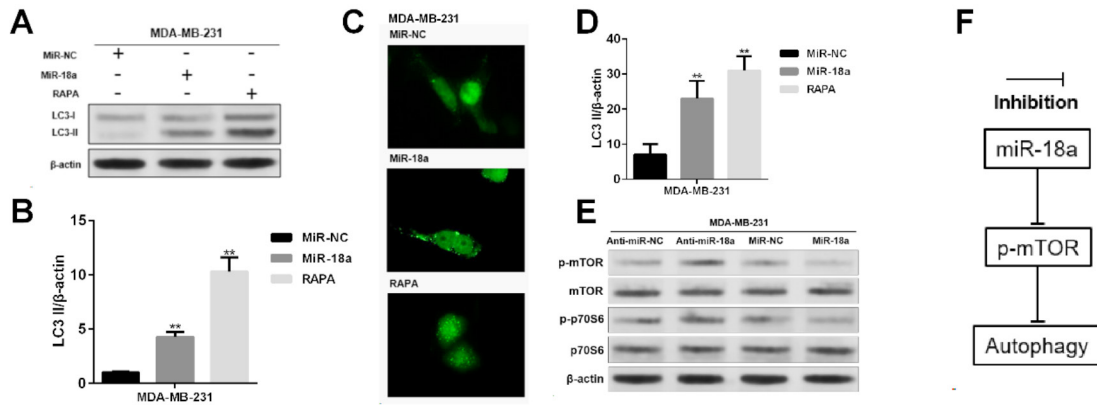


Figure 3. MiR-18a mediated autophagy upregulation in MDA-MB-231 cells. *A* and *B*. Images of Western blot analysis (*A*) and quantification (*B*) of relative LC3 I and LC3 II expression in MDA-MB-231 cells 24 hours after transfection of miR-18a or treatment with 50 nM rapamycin (RAPA). *C-D*, Representative images (*C*) and quantification (*D*) of LC3-GFP puncta accumulation in MDA-MB-231 cells 24 hours after transfection of miR-18a or treatment with 50 nM RAPA. *E*, Western blot analysis of p-mTOR, mTOR, p-p70S6 and p70S6 expression in MDA-MB-231 cells 48 hours after transfection of miR-18a mimics or anti-miR-18a. *F*, Schematic representation of the proposed model of the miR-18a-mTOR signaling pathway mediated autophagy upregulation in MDA-MB-231 cells. ** $p < 0.01$.

Discussion

Enhanced autophagy is utilized by some types of cancers to maintain intracellular homeostasis and promote survival under stressful environ-

ment¹⁹⁻²². Some recent studies⁸⁻¹¹ suggest upregulated autophagy is an important mechanism of chemoresistance in TNBC cells. Autophagy can increase epirubicin resistance in MDA cells by blocking the nuclear factor kappa-li-

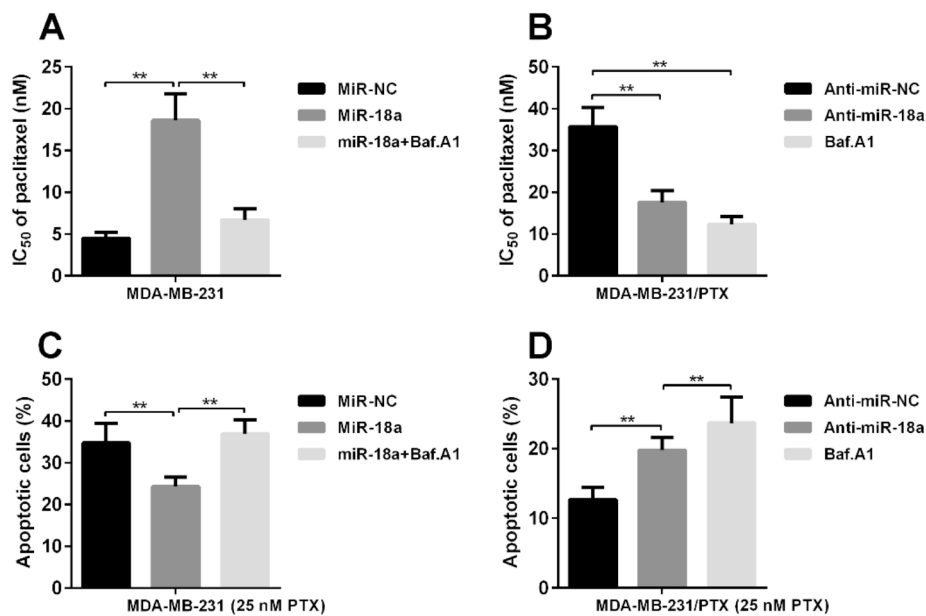


Figure 4. MiR-18a mediated autophagy upregulation is involved in development of PTX resistance in MDA-MB-231 cells. A-B. PTX IC₅₀ of MDA-MB-231 cells with miR-18a overexpression alone or in combination with 50 nM Baf. A1 treatment (*A*) and MDA-MB-231/PTX cells with miR-18a suppression or with 50 nM Baf. A1 treatment (*B*). C-D. Quantification of flow cytometric analysis of apoptotic MDA-MB-231 cells with miR-18a overexpression alone or in combination with 50 nM Baf. A1 treatment (*C*) and MDA-MB-231/PTX cells with miR-18a suppression or with 50 nM Baf. A1 treatment (*D*) 48 hours after 25 nM PTX treatment. ** $p < 0.01$.

ght-chain-enhancer of activated B cells (NF-kap-paB)-mediated pro-apoptotic signals¹¹. Enhanced autophagy also confers a cytoprotective function under paclitaxel treatment stress in MDA-MB-231 cells⁸. Another recent study¹⁰ demonstrated that LC3B, a protein involved in autophagosome formation, is a reliable marker of poor prognosis in TNBC. The inhibition of autophagy can enhance paclitaxel-induced cell death in the paclitaxel-resistant MDA-MB-231 cells⁸. Inhibiting autophagy via silencing ATG5 or ATG7 significantly reduced high-LC3B TNBC tumor growth *in vivo* and the use of autophagy inhibitor increased the effects of chemotherapeutic agents¹⁰. Therefore, for the TNBC with high expression of LC3B, autophagy inhibition is also considered as a strategy to improve chemotherapy efficiency¹⁰. However, how autophagy is dysregulated in TNBC is not quite clear.

In this study, we found that the MDA-MB-231/PTX cells had a higher level of basal autophagy and also higher expression of miR-18a than the parent MDA-MB-231 cells. Enforced miR-18a overexpression directly led to increased autophagy level in MDA-MB-231 cells, the effect of which was similar to that of rapamycin, a mTOR signaling inhibitor. The inhibitive effect of miR-18a on mTOR signaling was reported in some types of cancer and normal tissue development. For example, in HCT116 colon cancer cells, miR-18a can suppress mTORC1 activity, reflected by hypophosphorylation of p70S6²³. In gastric cancer, miR-18a overexpression reduced the phosphorylation of two mTOR substrates, S6K1 and 4E-BP1, indicating the inactivation of the mTOR pathway²⁴. Targeted disruption of miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a) can impair mouse spermatogenesis by activating mTOR signaling pathway²⁵. These findings suggest that miR-18a may exert a strong regulative effect on the mTOR signaling pathway.

Therefore, we further detect whether this inhibiting effect exists in TNBC cells. As hypothesized, miR-18a overexpression decreased the expression of p-mTOR and p-p70S6. Therefore, we infer that miR-18a increases autophagy level in MDA-MB-231 cells also via inhibiting mTOR signaling pathway. Since autophagy inhibition is a possible mechanism to sensitize TNBC cells to chemotherapy, we then observed whether miR-18a mediated PTX resistance can be abrogated by autophagy inhibition. Both drug sensitivity assay and flow cytometry analysis confirmed that Baf. A1 largely abrogated the effect of miR-18a on in-

creasing IC50 and decreasing PTX induced apoptosis in MDA-MB-231 cells. Baf. A1 also had similar, but stronger effect as anti-miR-18a in reducing PTX IC50 in MDA-MB-231/PTX cells and enhancing PTX induced apoptosis in the cells.

Conclusions

MiR-18a upregulation results in enhanced autophagy via inhibiting mTOR signaling pathway in TNBC cells, which is a mechanism contributing to paclitaxel resistance.

Conflicts of interest

The authors declare no conflicts of interest.

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