Original Article
The role of miR-222 and miR-298 in breast cancer drug resistance

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Abstract: Docetaxel is widely applied in the clinic for the treatment of breast cancer. However, drug resistance might appear for the long-term usage. It was considered that breast cancer drug resistance was related to the aberrant expressions of some miRNAs. This study explored the role of miR-222 and miR-298 in the development of docetaxel resistance in the treatment of breast cancer. A total of 45 cases of breast cancer patients with drug resistance were enrolled in this study. RT-PCR and in situ molecular hybridization were used to determine the expression of miR-222 and miR-298 in breast cancer tissues. Mimic and inhibitor transfections were performed on breast cancer cell line MCF-7 to manipulate the expressions of miR-222 and miR-298. MTT assay was adopted to evaluate MCF-7 cell drug resistance. Western blot was performed to analyze the expression of PTEN and MDR1. Our results showed that miR-222 expression was significantly increased in breast cancer tissue with drug resistance, while miR-298 was obviously downregulated (P<0.05). Drug susceptibility test showed that miR-222 overexpression or miR-298 inhibition markedly enhanced drug resistance. In addition, miR-222 overexpression reduced PTEN level, whereas miR-298 inhibition elevated MDR1 expression. In conclusion, miR-222 was upregulated while miR-298 was declined in drug resistant breast cancer cells and they may regulate the development of drug resistance through targeting PTEN and MDR1.

Keywords: Breast cancer, miR-222, miR-298, PTEN, MDR1

Introduction

The incidence of breast cancer occupies the first among female malignant tumors. There are about 230,000 females who are diagnosed as breast cancer in America, accounting for 29% of all female malignant tumors [1]. At present, breast cancer is mainly treated by surgery, radiotherapy, chemotherapy, and endocrine treatment methods, of which chemotherapy is still an important approach [2]. Various commonly used chemotherapy drugs may induce drug resistance, including docetaxel and adriamycin, etc, thereby significantly reduces the curative effect of breast cancer treatment and increase the burden of patients [3, 4]. Thus, further in depth study on the mechanism of breast cancer drug resistance is of great significance to improve the effect of breast cancer treatment as well as the life quality of patients.

MiR-222 and miR-298 are small non-coding RNA molecules belonging to the miRNA family [5, 6]. It is reported that miRNAs can affect vari-

ous cell behaviors [6]. MiRNA expression profile analysis revealed that miRNAs play an important role in the regulation of malignant tumor cell behaviors [7]. Recently, it has been shown that miR-222 and miR-298 are associated with drug resistance in a large variety of malignant tumors, such as colorectal cancer, breast cancer, and liver cancer [8-10]. This study intends to investigate the role and related mechanism of miR-222 and miR-298 in the development of drug resistance in breast cancer cells.

Materials and methods

Subject selection

A total of 45 cases of primary breast cancer patients with a mean age of 46.3 ± 8.2 years in Tangshan People's Hospital from December 2012 to December 2015 were selected. All the enrolled patients were examined by breast ultrasound and X-ray mammography, and diagnosed by tissue pathology and cell pathology. All patients displayed adriamycin and docet-

Table 1. Primers for RT-PCR

Name	Sequence
miR-222-F	5'-AGCTACATCTGGCTACTG-3'
miR-222-R	5'-GAGACCCAGTAGCCAGAT-3'
miR-298-F	5'-CAGAAGCAGGGAGGTTC-3'
miR-298-R	5'-CTGAGCAAAGCAGCAGGC-3'

axel resistance after chemotherapy. Tissue samples were obtained from surgery before and after drug resistance and stored in liquid nitrogen for miRNA detection and paraffin embedding for histopathologic analysis as well as for in situ molecular hybridization. This study was approved by the ethics committee and obtained informed consents from all patients.

RT-PCR

RT-PCR was performed to measure the expression of miR-222 and miR-298 in breast cancer and hyperplasia of mammary glands. The primers used were designed according to their sequences (GeneBank: AJ550426 and NR_030580) (**Table 1**). Total RNA was extracted from tissues using RNAprep pure Tissue Kit (QIAGEN). RT-PCR test was applied using mirVanat qRT-PCR miRNA detection kit (Ambion). U6 was used as an internal reference and the results were analyzed by $2^{-\Delta\Delta Ct}$ method [11].

In situ hybridization detection

Antisense locked nucleic acid modified miR-222 and miR-298 oligonucleotide probes were used for analysis of in situ molecular hybridization (Boster) using paraffin tissue section. The sequences used were as follows: miR-222, 5'-AGCTACATC-TGGCTACTGGGTCTC-3'; miR-298, 5'-TGCTGCTTT-GCTCAGGAGTG-3'. Specially, paraffin section was routinely dewaxed and blocked. After washed by 0.5 M PBS, the section was treated by pre-hybrid liquid at 65°C for 4 h followed by addition of probe and incubated at 65°C for 15 h. Then, the section was washed by SSC solution to block nonspecific binding site. After treated with rabbit anti digoxin antibody at 37°C for 0.5 h, the section was developed and sealed for observation.

Results judgment: Violet particles in the cells indicate positive expression of miR-222 and miR-298 [12].

MiR-222 and miR-298 transfection

Mimic and inhibitor were adapted to upregulate or downregulate miR-222 and miR-298 expression in breast cancer cells. The mimic and inhibitor were purchased from Genepharma and transfected using INTERFERinTM transfection kit (Polyplus transfection). Breast cancer cell line MCF-7 was purchased from the cell bank of Chinese academy of sciences. The cells were maintained in DMEM supplemented with 10% FBS, 5000 U penicillin, and 5000 g/ml streptomycin (HyClone) at 37°C and 5% CO_2. The cells were seeded into 96-well plate for 24 h before transfection according to the manufacturer's instructions.

Drug resistance assay

MCF-7 cells were seeded into 96-well plates and incubated overnight at 37°C. The cells were incubated with different concentrations of docetaxel for 48 h at 37°C. After addition of MTT to each well for 4 h at 37°C, 150 μl DMSO was added into each well. Absorbance of each well at 550 nm (A550) was read using a spectrophotometer. The concentration of each drug producing 50% inhibition of growth (IC50) was estimated from the relative survival curves [13].

Western blot

Proteins were extracted from MCF-7 cells in logarithmic phase, separated by SDS-PAGE and transferred to PVDF membrane. After blocked by 5% skim milk, the membrane was incubated with anti-PTEN, and MDR1 antibodies. β-actin was selected as an internal reference. The membrane was detected by chemiluminiscence and the image was analyzed by using Image J.

Statistical analysis

All the data analysis was performed on SPSS 20.0 software. The data were represented as mean ± standard deviation (SD). T test was applied for data comparison. P<0.05 was considered as statistical significance.

Results

RT-PCR

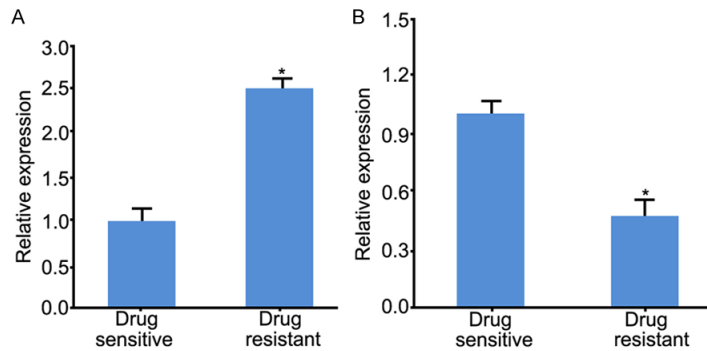RT-PCR was applied to test miR-222 and miR-298 expression in breast cancer tissues be-

Figure 1. Expression of miR-222 and miR-298 in drug sensitive or resistance tissues. A: miR-222 expression in breast cancer tissue. B: miR-298 expression in breast cancer tissue. *$P<0.05$, compared with drug sensitive group.
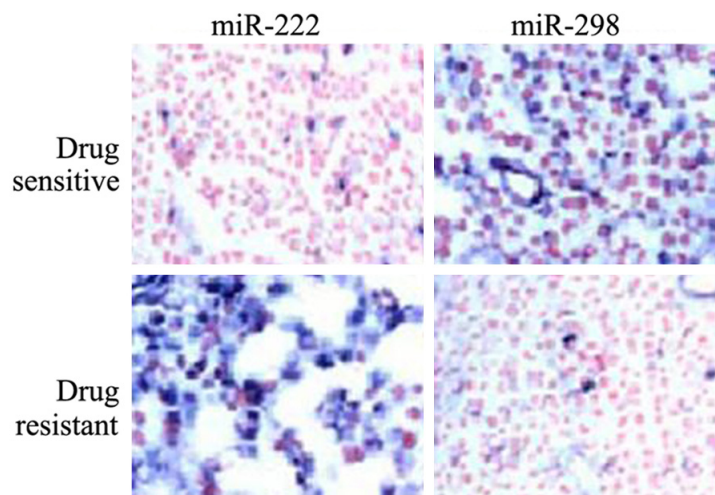

Figure 2. Analysis of the expression of miR-222 and miR-298 in drug sensitive or resistant tissues by in situ hybridization (×400).

fore and after drug resistance. As shown in **Figure 1**, miR-222 expression was significantly upregulated (around 2.5-fold change) ($P<0.05$), whereas miR-298 expression was reduced (around 2.1-fold change) in drug resistant group compared with drug sensitive group ($P<0.05$).

In situ hybridization detection

MiR-222 and miR-298 specific probes were used to detect miRNAs expression in breast cancer tissues by in situ molecular hybridization. Blue granules appeared in the cytoplasm when positive expression of miR-222 or miR-298 was observed. It was found that miR-222 expression in the drug resistant breast cancer tissues was obviously higher than that in the

drug sensitive group. On the contrary, miR-298 presented lower staining level in drug resistant group compared with drug sensitive group (**Figure 2**). It was demonstrated that miR-222 expression was upregulated while miR-298 was reduced in drug resistant breast cancer tissue, which was consistent with the results of RT-PCR.

miR-222 and miR-298 transfection

Mimic and inhibitor were adapted to manipulate the expression of miR-222 or miR-298 in MCF-7 cells. RT-PCR was applied to test miRNA relative expression. As shown in **Figure 3**, miR-222 expression was markedly increased, while miR-298 was obviously declined in MCF-7 cells after transfection ($P<0.05$), indicating successful manipulation of the expression of miRNA by mimic and inhibitor.

Drug resistance changes

MTT assay was performed to determine the drug resistance in MCF-7 cells after transfection. Different concentrations of docetaxel were applied to calculate IC50 value. As shown in **Figure 4**, IC50 of MCF-7 after miR-222 overexpression achieved 1.61 μM, which was obviously higher than that of 1.32 μM in negative control ($P<0.05$), while it was 1.27 μM in miR-222 inhibitor group. After miR-298 inhibition, IC50 for docetaxel in MCF-7 cells was 1.83 μM, which showed statistical difference to negative control which was 1.31 μM ($P<0.05$). It was only 1.25 μM in miR-298 mimic group. Thus, we speculated that miR-222 overexpression or miR-298 inhibition can increase MCF-7 cell proliferation in docetaxel, leading to drug resistance.

The effect of miR-222 overexpression on PTEN in breast cancer cells

Western blot was used to determine PTEN expression in breast cancer cells after miR-222
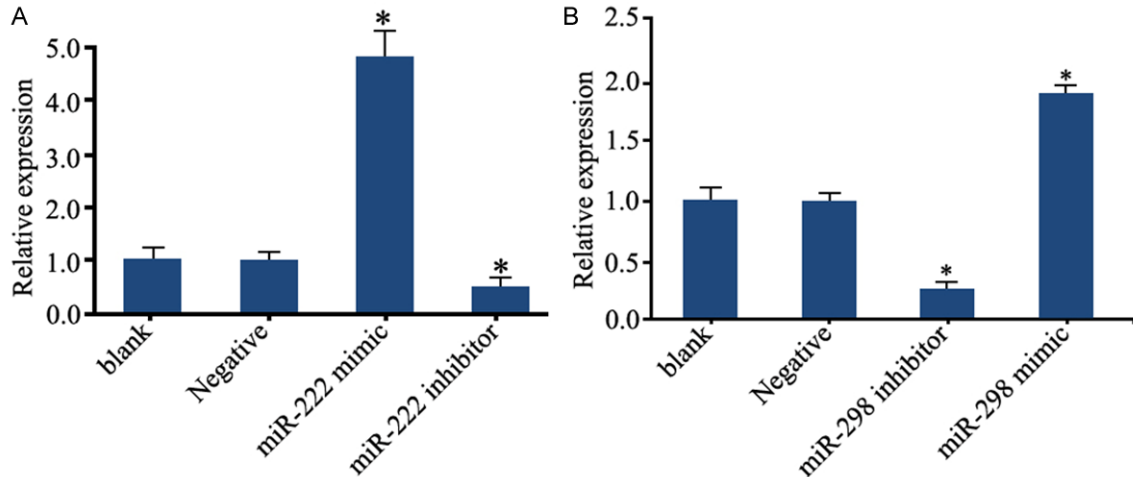
Figure 3. Expression of miR-222 and miR-298 after mimic or inhibitor transfection in MCF-7 cells. A: miR-222 expression in MCF-7. B: miR-298 expression in MCF-7. *P<0.05, compared with drug sensitive group.
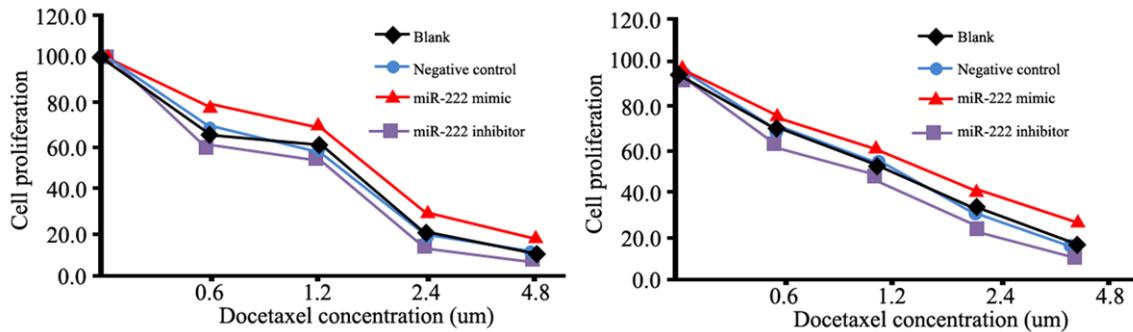

Figure 4. Cell proliferation after transfection.

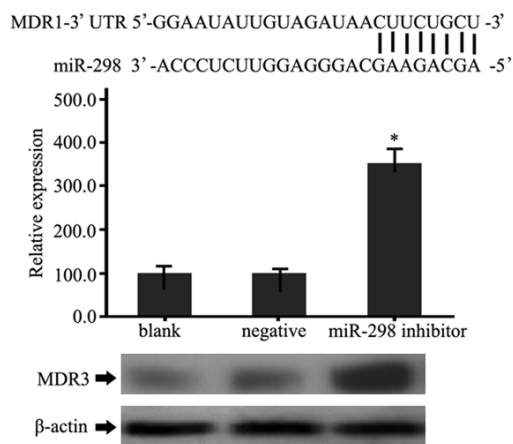
Figure 5. PTEN expression after miR-222 overexpression. *P<0.05, compared with drug sensitive group.

mimic transfection. As shown in **Figure 5**, miR-222 mimic transfection apparently reduced

PTEN protein level in MCF-7 cells compared with blank control or negative control (P<0.05).

The effect of miR-298 inhibition on MDR1 in breast cancer cells

Western blot was adopted to detect MDR1 expression in breast cancer cells after miR-298 expression was inhibited. It was demonstrated that MDR1 expression was obviously upregulated after miR-298 inhibitor transfection compared with control (P<0.05) (**Figure 6**).

Discussion

At present, numerous studies have confirmed that there are many miRNA expression differences between breast cancer cells and normal cells, including miR-21, miR-10b, and miR-200 [14-16]. miRNA-mediated aberrant cell proliferation and differentiation has been
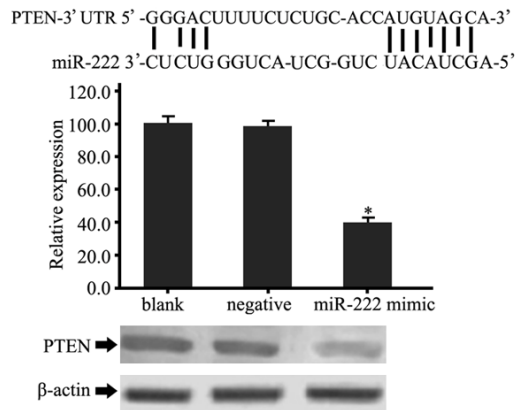
Figure 6. MDR1 expression after miR-298 inhibitor transfection. *P<0.05, compared with drug sensitive group.

observed in breast cancer cells. For example, miR-10b participates in the process of breast cancer cell invasion and metastasis [15]. MiR-21 also plays an important role in breast cancer cell proliferation [14]. It was also reported that some miRNAs expression was closely related to the drug resistance of breast cancer cells [8]. This study explored the role of miR-222 and miR-298 in the development of drug resistance in breast cancer cells. RT-PCR and in situ molecular hybridization detection revealed that miR-222 expression was upregulated, while miR-298 was declined in drug resistant breast cancer tissues compared with drug sensitive tissues.

It is still controversial on how miRNAs are differentially expressed in malignant tumor cells. Some scholars believe that cell growth factor signaling pathway, such as EGF and FGF activation can promote malignant tumor cell division, while this process is achieved by suppressing a part of miRNAs [17]. Some other scholars consider that miRNA expression changes in malignant tumor cells were caused by the irreversible modification of transcriptional regulatory sequences. It was found that methylation reagent 5'-mixed nitrogen-2'-deoxidization cytidine may upregulate miRNAs expression in glioma cells. MiRNA transcription regulatory sequences analysis has also confirmed this conclusion [18].

In order to further explore the impact of miR-222 and miR-298 on breast cancer drug resistance, we applied transfection method to over-

express miR-222 or inhibit miR-298 in drug sensitive breast cancer cell line MCF-7. Our results showed that MCF-7 proliferation was enhanced in docetaxel after transfection, suggesting increased drug resistance. Meanwhile, western blot analysis demonstrated that miR-222 upregulation declined PTEN expression, whereas miR-298 reduction elevated MDR1 level, indicating that miR-222 and miR-298 may regulate cell proliferation and drug resistance through mediating the expression of PTEN and MDR1.

The role of tumor suppressor gene PTEN exerts its function through PI3K/Akt signaling pathway. It can inhibit PI3K activation and maintain the activity of Akt, leading to the regulation of normal cell biological behaviors under physiological condition [19]. It was found that miR-222 can regulate cell proliferation and division by targeting PTEN [20]. Mardente et al. reported that miR-222 overexpression can enhance cell proliferation and invasion in thyroid cancer cells. It was further found that such process was completely impaired after inhibiting cell cycle regulatory protein PTEN [21]. Bao et al. found that docetaxel resistant breast cancer cell line MDA-MB-231 presented drug resistance through overexpressing MDR1/p-gp. MDR1 elevation may prevent the entry of docetaxel to nucleus, which is the mechanism of MDA-MB-231 cell line drug resistance [22]. Our study demonstrated that miR-298 can target MDR1 gene and inhibiting miR-298 expression can promote MDR1 expression, leading to the occurrence of docetaxel resistance.

Breast cancer is the leading malignant tumor in female that seriously affects the life quality of patients [1]. Docetaxel is a commonly used drug for the treatment of breast cancer in clinic. The presence of drug tolerance has a strong impact on the curative effect of breast cancer treatment. This study found that miR-222 expression was upregulated, while miR-298 was reduced in drug resistant breast cancer cell. Cell transfection, drug resistance assay, and Western Blot experiments determined a close relationship between miR-222 and miR-298 expression with breast cancer drug resistance, which can be used in the future for drug resistance judgment in clinic, thus providing better treatment strategies and

improving the curative effects. It can also provide certain theoretical basis for the application of RNA interference technology to reverse the drug resistance in the treatment of breast cancer in the future.

In conclusion, elevated miR-222 expression or reduced miR-298 expression may lead to docetaxel resistance in breast cancer cells which might be through mediating the expression of PTEN and MDR1.

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Disclosure of conflict of interest

None.

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