Biomedicine & Pharmacotherapy 102 (2018) 286–294

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

MicroRNA-132 and microRNA-212 mediate doxorubicin resistance by down-regulating the PTEN-AKT/NF-κB signaling pathway in breast cancer

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ARTICLE INFO

Keywords:
Breast cancer
microRNA
miR-132
miR-212
PTEN

ABSTRACT

Breast cancer is a serious health problem worldwide. Acquisition of multi-drug resistance (MDR) during the treatment of breast cancer is still considered a major clinical obstacle. Despite the biological functions of miRNAs becoming increasingly apparent, the function of miRNAs in regulating drug resistance of breast cancer remains under investigation. Quantitative real-time PCR (qRT-PCR) was used to quantify the expression of miR-132/-212 (miR-132 and miR-212) in doxorubicin (DOX)-resistant and -sensitive breast cancer tumors and cells. The function of miR-132/-212 in drug resistance was investigated in vitro (MTT assay, TUNEL assay, fluorescence, immunohistochemistry, luciferase reporter assay, Western blotting). We found that miR-132/-212 were commonly overexpressed in DOX-resistant breast cancer tumors and cells. Silenced miR-132/-212 expression induced DOX accumulation in MCF-7/ADR cells, while overexpression of miR-132/-212 led to breast cancer resistance protein (BCRP)-based DOX efflux in MCF-7 cells. Further study showed that up-regulation of miR-132/-212 in MCF-7/ADR cells suppressed the expression of PTEN, a target gene of miR-132/-212, which activated AKT phosphorylation and the NF-κB pathway and led to increased BCRP expression. Down-regulation of miR-132/-212 sensitized MCF-7/ADR cells to DOX. Mechanistic investigations suggested that miR-132/-212 enhancement was a result of NF-κB-mediated transactivation of the pri-miR-132/-212 gene. Taken together, our findings are among the first to demonstrate a novel aspect of the miR-132/-212-PTEN-AKT/NF-κB-BCRP pathway in the generation of breast cancer resistance and provides a potential method to reverse drug resistance.

1. Introduction

To date, breast cancer is the most common cancer and the second leading cause of cancer death in women [1]. Chemotherapy is an effective treatment against breast cancer. However, its efficiency may be impeded by multi-drug resistance (MDR), a phenomenon that emerges during treatment [2]. The genetic basis of drug resistance is extraordinarily complex and involves multiple processes [3–6]. Currently, 30% of women with early-stage breast cancer have recurrent disease, and the phenomenon of MDR can occur in at least one-quarter of all cases [7]. Therefore, new therapeutic approaches that aim to resensitize the existing drugs to therapy and reverse drug resistance are of utmost importance.

MicroRNAs (miRNAs) are a class of small, non-coding cellular RNAs (19–25 bp) that post-transcriptionally regulate protein expression by inducing the degradation or translational repression of target mRNAs [8]. Approximately 50% of miRNA genes are located in tumor-associated genomic regions, suggesting that miRNAs play a significant role in cancer [9]. Computational prediction of miRNA target genes revealed that approximately one-third of all human protein-encoding genes may be regulated by miRNAs [9]. Recent evidence demonstrated that miRNAs may also take part in not only tumorigenesis and cancer

Abbreviations: MDR, multi-drug resistance; qRT-PCR, quantitative real-time PCR; BCRP, breast cancer resistance protein; PTEN, phosphatase and tensin homolog deleted on chromosome ten; DMEM, Dulbecco's Modified Eagle medium; RPMI 1640, Roswell Park Memorial Institute medium 1640; MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltet-razolium bromide; IHC, immunohistochemistry; TBP, TATA binding protein; P-gp, P-glycoprotein; ROC, receiver operating characteristic; AUC, area under the curve; NC, negative control

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https://doi.org/10.1016/j.biopha.2018.03.088
Received 2 September 2017; Received in revised form 14 March 2018; Accepted 14 March 2018

progression but also in the development of drug resistance [10]. Indeed, aberrant miRNA expression has been reported in a number of molecular pathways that are related to drug resistance. For example, miR-1268b could regulate chemosensitivity by targeting ERBB2-mediated PI3K-AKT pathway in breast cancer [11]. Additionally, miR-181c was shown to participate in the resistance of chronic myelocytic leukemia to DOX by targeting the ST8SIA4 protein [12]. Despite the biological function of miRNAs becoming increasingly apparent, the role of miRNAs in regulating drug resistance of breast cancer remains under investigation.

In the current study, we investigated, for the first time, the expression of miR-132/-212 and found that they are up-regulated in drug-resistant breast tumors and cell lines and modulated drug accumulation by suppressing the expression levels of phosphatase and tensin homolog deleted on chromosome ten (PTEN) in vitro. Our results also indicate that the expression of PTEN is significantly down-regulated in drug-resistant breast tumors and inversely correlated with miR-132 and miR-212 expression. We also found that the overexpression of miR-132 and miR-212, at least in part, is ascribed to transactivation by the NF-κB transcription factor and this interaction may have an important functional consequence in the acquisition of drug resistance.

2. Methods

2.1. Breast cancer tumors

Tissue specimens treated with neoadjuvant chemotherapy were obtained with informed consent from patients at Nanjing Maternity and Child Health Care Hospital (Nanjing, China) and First Affiliated hospital of Nanjing Medical University (Nanjing, China). Tumor response to neoadjuvant chemotherapy was evaluated based on the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines [13]: partial response (PR) as greater than 30% tumor shrinkage; stable disease (SD) as a decrease of less than 30% or an increase of less than 20%; and progressive disease (PD) as an increase of at least 20% or the appearance of new lesions. For further study, patients with PR were regarded as clinical responders and drug sensitivity, while patients with SD or PD were regarded as non-responders and drug resistance [4,14]. Informed consent was obtained from the subjects. Tissue samples were stored frozen at −80 °C until analysis. Prior to RNA/protein extraction, tissue samples were thawed to room temperature and then rinsed thoroughly with deionized water. Fat tissue was removed and the remaining tissue was cut into small pieces and transferred to tubes.

2.2. Cell lines and cell culture

Human embryonic kidney cell line HEK293 (Chinese Academy of Sciences, Shanghai, China) were cultured using DMEM (Thermo Scientific Hyclone, MA, USA) containing 10% fetal bovine serum (Thermo Scientific Hyclone, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2. The drug sensitive MCF-7 was maintained in RPMI 1640 (Gibco, CA, USA) and MCF-7/ADR drug-resistant cell line in RPMI 1640 in the presence of 1 μg/ml DOX (Sigma-Aldrich, MO, USA) with the same conditions above.

2.3. Quantitative real-time PCR analysis for miRNA expression

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. cDNAs were synthesized from 1 μg of total RNA with RevertAidTM First-strand cDNA Synthesis Kit (Thermo Scientific, MA, USA) in a 25 μl volume containing 1 μg total RNA, 5 nM reverse transcription primer (Bulge-LoopTM miRNA primer from Ribobio, Guangzhou, China), 0.8 U/μl reverse transcriptase, 4 U/μl RNase Inhibitor and 0.2 mM dNTPs. Real time-PCR was carried out with the reagents of SYBR Green Master (ABI, CA, USA) in 20 μl reaction volume (10 μl SYBR Green Mix, 0.4 μM forward primers, 0.4 μM reverse primers and 2 μl cDNA template) by using 7500 real-time PCR system (Applied Biosystems, CA, USA). Data analysis was performed by correcting against the Ct level of U6.

2.4. Transfection of plasmids, siRNA, miRNA mimics and inhibitor

NF-κB p65 cDNA was cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). NF-κB p65 siRNA was purchased from Santa Cruz (CA, USA). All miRNA mimics and the hairpin inhibitor from Ribobio (Guangzhou, China) were respectively used for the overexpression and inhibition of miRNA activity in cells. Cells were grown to approximately 80% confluence in 6-well plates and transfected with 20 pM NF-κB p65 siRNA, 50 ng NF-κB p65 plasmid, 50 nM miRNA mimics or 100 nM miRNAusing Lipofectamine 2000 (Invitrogen, CA, Netherlands), as described by the manufacturer.

2.5. Target prediction

The miRNA 3′-UTR binding sites were predicted using miRcode (http://www.mircode.org/mircode/) [15], miRanda (http://www.microrna.org/ microrna/home.do) [16], and Diana-microT v3.0 (http://diana.cslab.ece.ntua.gr/microT/) [17].

2.6. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltet-razolium bromide (MTT) assay

Cells viability against DOX-induced apoptosis was determined by using an MTT assay in 96-well plates. Cells were transfected with miRNA mimics or inhibitor. ~5000 cells in 200 μL of medium were added to each well in 96-well culture plates and treated with various concentrations of DOX. After a post-drug treatment time (48 h), 0.5 mg/mL MTT (Sigma-Aldrich, MO, USA) was added. Then, the absorbance of every well was measured at 490 nm by the ELX800 (Biotek, VT, USA). All MTT assays were performed 3 times in triplicate and the IC_{50} was calculated using GraphPad PRISM 6.0.

2.7. TUNEL assay

Apoptotic cells were detected by DeadEndTM Fluorometric TUNEL System (Promega, Madison, USA). After the treatment of DOX, fixed and permeabilized cells were incubated in a buffer containing Equilibration Buffer, Nucleotide Mix and rTdT Enzyme, at 37 °C for 1 h. Then, the DAPI stain (10 μg/mL) for nucleus was added. Images were acquired at room temperature with a DMI3000B (Leica, Wetzlar, Germany). For quantification, the number of TUNEL positive cells was counted in at least five randomly selected high power fields with three independent samples.

2.8. 3′-UTR luciferase reporter assay

The wild type (WT) and mutant (MUT) PTEN 3′-UTR luciferase reporter vectors were constructed by subcloning the PTEN mRNA 3′-UTR and mutant 3′-UTR sequences into pGL3 Luciferase Reporter Vectors (Promega, Madison, WI, USA). HEK293 cells were transfected with 80 ng luciferase reporter plasmid and miR-132/-212 (a combination of miR-132, miR-212, miR-132 and miR-212) mimics (final concentration, 50 nM) using Lipofectamine 2000 (Invitrogen, CA, USA). After 24 h, luciferase activity was measured using the Dual-Luciferase Reporter System (Berthold Technologies, Stuttgart, Germany) according to the manufacturer's instructions.

2.9. Determination of DOX cellular accumulation using fluorescence microscopy and intracellular DOX assay

After transfected with miRNA mimics and inhibitor, cells were treated with DOX. The intracellular fluorescence of DOX was imaged by DMI3000B inverted fluorescence microscope. Then the cells were