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Original Article

miR-504 regulates chemosensitivity in triple negative breast cancer (TNBC) cells via targeting ABCB8

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Abstract: Emerging evidence suggest that miR-504 is implicated in carcinogenesis in several cancer types. However, its role in regulating chemo-sensitivity in TNBC has not been investigated. In this study, we investigated the role of miR-504 in regulating chemosensitivity in TNBC cells. We found that overexpression of miR-504 sensitized MDA-MB-231 and HCC38 cells to docetaxel whereas inhibition of miR-504 conferred resistance in these cells. ABCB8 was the direct target of miR-186 which was required for the regulatory role of miR-504 in chemosensitivity. In addition, miR-504 was down-regulated in TNBC patients who were chemoresistant. Taken together, our study demonstrates that miR-504 regulates chemoresistance of TNBC cells by modulating ABCB8 expression. miR-504 may represent a new therapeutic target for the improvement of clinical outcome in TNBC.

Keywords: miR-504, ABCB8, TNBC, chemosensitivity

Introduction

Triple-negative breast cancer (TNBC) is a type of invasive carcinoma of breast cancers that lack the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 or HER2 gene amplification. Due to the lack of expression of those receptors, chemotherapy has been the primary treatment option for the intervention of TNBC. However, chemoresistance has remained as a major obstacle in the therapeutic efficacy in TNBC patients.

MicroRNAs (miRNAs) are a class of endogenous, short (19-22 nucleotides), non-coding RNAs that regulate mRNA degradation or translational suppression by directly bind to the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs) [1]. Beyond its important roles in diverse biological processes, including cell apoptosis, cell proliferation, stress response, metabolism, cell differentiation, etc. [2], accumulating evidence suggests that the deregulation or dysfunction of miRNAs contributes to human cancer initiation and progression [3]. Depending on the functions of its target genes, miRNAs may function as either oncogenes or tumor suppressors.

miR-504 is an emerging miRNA that has been shown to play a role in tumorigenesis in several cancer types [4-6]. It was originally identified as a potential on comir through targeting tumor suppressor TP53 [4, 5]. A subsequent study revealed that TFF1 activates p53 through down-regulation of miR-504 in gastric cancer [7]. More recently, Cui reported that miR-504 can function as a tumor suppressor through its regulation of FOXP1 expression in glioma [8].

The ability to pump toxic drugs out of the cells is thought to be the most commonly observed mechanism of drug resistance which is mediated by the ATP-binding cassette (ABC) transporters. Of all the human ABC transporters, ABCB1, ABCC1 and ABCG2 are the best studied ones. However, they do not contribute to che-

mo-resistance in every cancer type. Therefore, it is necessary to investigate the functions of other transporter members in various cancers.

In this study, we investigated the role of miR-504 in regulating chemosensitivity in TNBC cells. We also evaluated the expression level of miR-504 in TNBC patient samples.

Materials and methods

Cell culture

Human TNBC cell lines MDA-MB-231 and HCC-38 were purchased from ATCC and were cultured in basal medium supplemented with 10% serum at 37°C and 5% CO_2.

Patient samples

TNBC tissue samples and matched non-tumor adjacent tissues were obtained from patients who underwent surgical resection at the Affiliated Zhongda Hospital of Southeast University, Nanjing, Jiangsu, PR China, between May 2012 and July 2015. All tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. Patients with disease progression or recurrence 6 months or less after completing neoadjuvant chemotherapy (NAC) were defined as being non-responders, while those without recurrence or recurrence more than 6 months after completing NAC were defined as responders. The study was approved by the Ethical Committee of the Affiliated Zhongda Hospital of Southeast University and informed consent was obtained from all patients.

Drug toxicity assay

Cells were seeded at a density of 5×10^3 cells/well in 96-well microtiter plates. Docetaxel was then added onto cells 24 hours afterwards and cells were cultured for an additional 72 h. Cell viability was assessed using CellTiter-Glo® reagent (Promega). Each value was normalized to cells treated with DMSO and the IC50 values are determined using Graphpad Prism software.

Plasmids and cell transfection

Transfection of miR-504 inhibitor, miR-504 mimic and its non-specific control (Invitrogen, USA) were performed according to the manual provided with the siPORTM NeoFXTM Transfection Agent (Ambion, USA). pLenti-C-Myc-DDK ABCB8 cDNA was obtained from Origene. cDNA transfections were performed with Lipofectamine LTX reagent (Invitrogen) according to manufacturer's protocol.

Real-time PCR

Total RNAs of cells and tissues were extracted with Qiagen miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and miRNA was reverse transcribed to cDNA. TaqMan miRNA assays (Applied Biosystems, Foster City, USA) with specific RT primers and probes were used to quantify the expression of mature miR-504. cDNA was generated from 500 ng of total RNA using PrimeScript™ RT Master Mix Perfect Real Time (TaKaRa, Dalian, China). U6 was used for miRNA template normalization and GAPDH was used for mRNA normalization. All samples were performed in triplicates.

Immunoblot analysis

Total cell lysates were prepared by harvesting cells in Laemmli S.D.S reducing buffer (50 mM Tris-HCl (pH 6.8), 2% S.D.S, and 10% glycerol), boiled and resolved on an 8% to 10% polyacrylamide gel, and transferred to polyvinylidinefluoride. After incubation with primary antibody overnight, the blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) at a dilution of 1:5000 and detected with SuperSignalWest Pico or Femto Chemiluminescent Substrate Kit (Thermo Scientific).

Luciferase assay

250 ng of pGL3 reporter vector containing the WT or mutant miR-504 binding site, 250 ng of the phRL-SV40 control vector (Promega), and 100 nM miRNA precursors or miRNA control (Ambion) were cotransfected into HEK293 cells in 24-well plates. Firefly luciferase activity was measured with a Dual Luciferase Assay Kit (Promega) 24 hours after transfection and normalized to the Renilla luciferase reference plasmid.

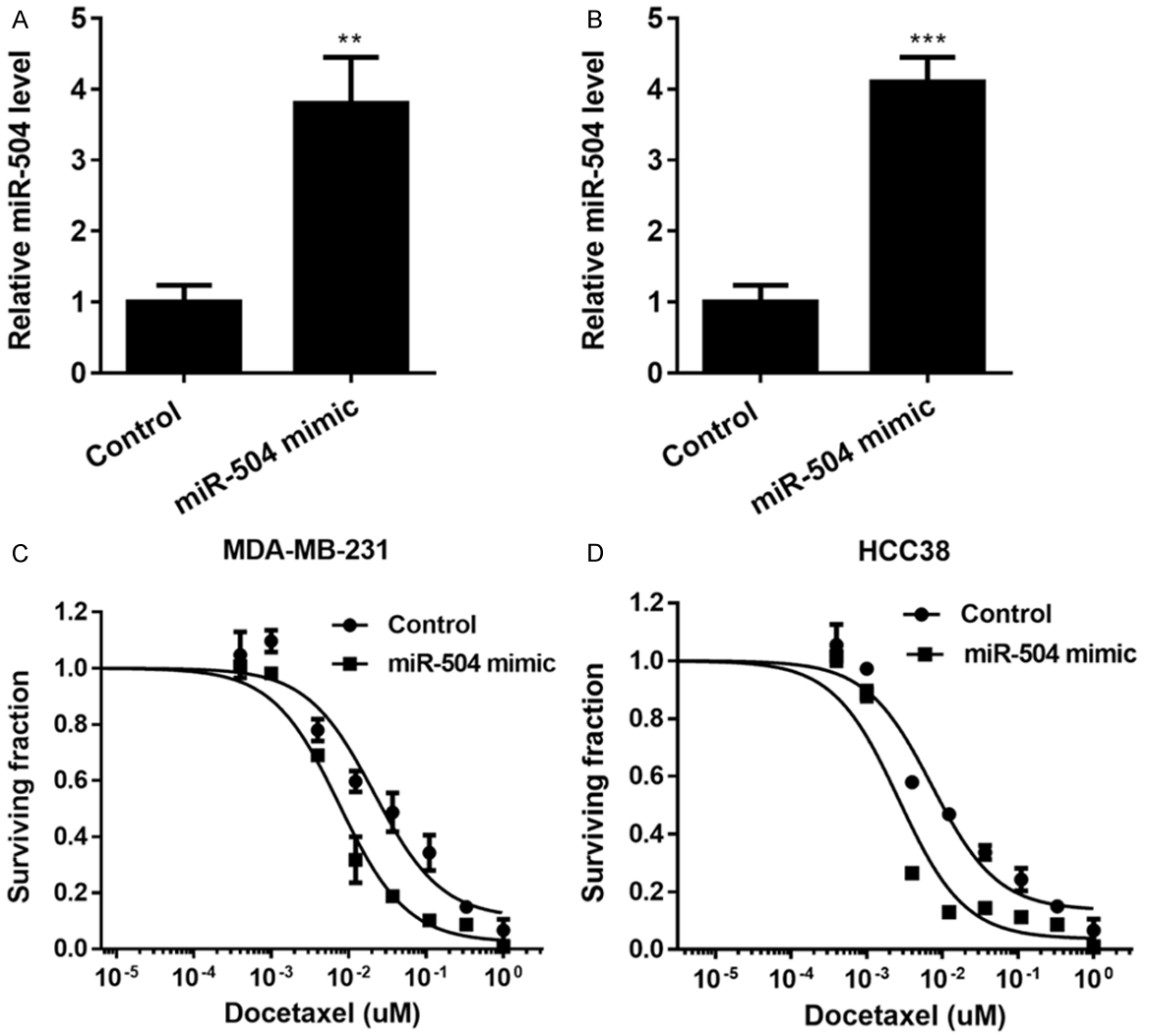

Figure 1. Overexpression of miR-504 sensitizes TNBC cells to docetaxel. (A, B) Relative level of miR-504 in MDA-MB-231 (A) or HCC38 (B) cells transfected with control miRNA or miR-504 mimic. Data represent mean ± SD. **, P<0.01; ***, P<0.001. (C, D) Dose response curves of MDA-MB-231 (C) or HCC38 (D) cells transfected with control miRNA or miR-504 mimic treated with docetaxel. Data represent the mean ± SD.

Statistical analysis

Quantitative data are presented as mean ± SD. Statistical significance was evaluated by the Student t test. Differences were considered to be significant when P<0.05.

Results

Overexpression of miR-504 sensitized TNBC cells to docetaxel

To examine the role of miR-504 in chemo-sensitivity in TNBC cells, we overexpressed miR-504 in MDA-MB-231 and HCC38 cells, followed by dose response curve study. Transfection of miR-504 mimic resulted in ~4 fold increase in the expression of miR-504 compared to the non-specific negative control (**Figure 1A**, **1B**). MDA-MB-231 and HCC38 overexpressing miR-504 were more sensitive to docetaxel (**Figure 1C**, **1D**).

Inhibition of miR-504 confers resistance to docetaxel in TNBC cells

We then transfected MDA-MB-231 and HCC-38 cells with miR-504 inhibitor to confirm the function of miR-504 in regulating chemoresistance. As shown in **Figure 2A**, **2B**, cells transfected with miR-504 inhibitor exhibited a significant decrease in miR-504 expression. miR-504 inhibitor transfected cells were more resistant to docetaxel compared to the control

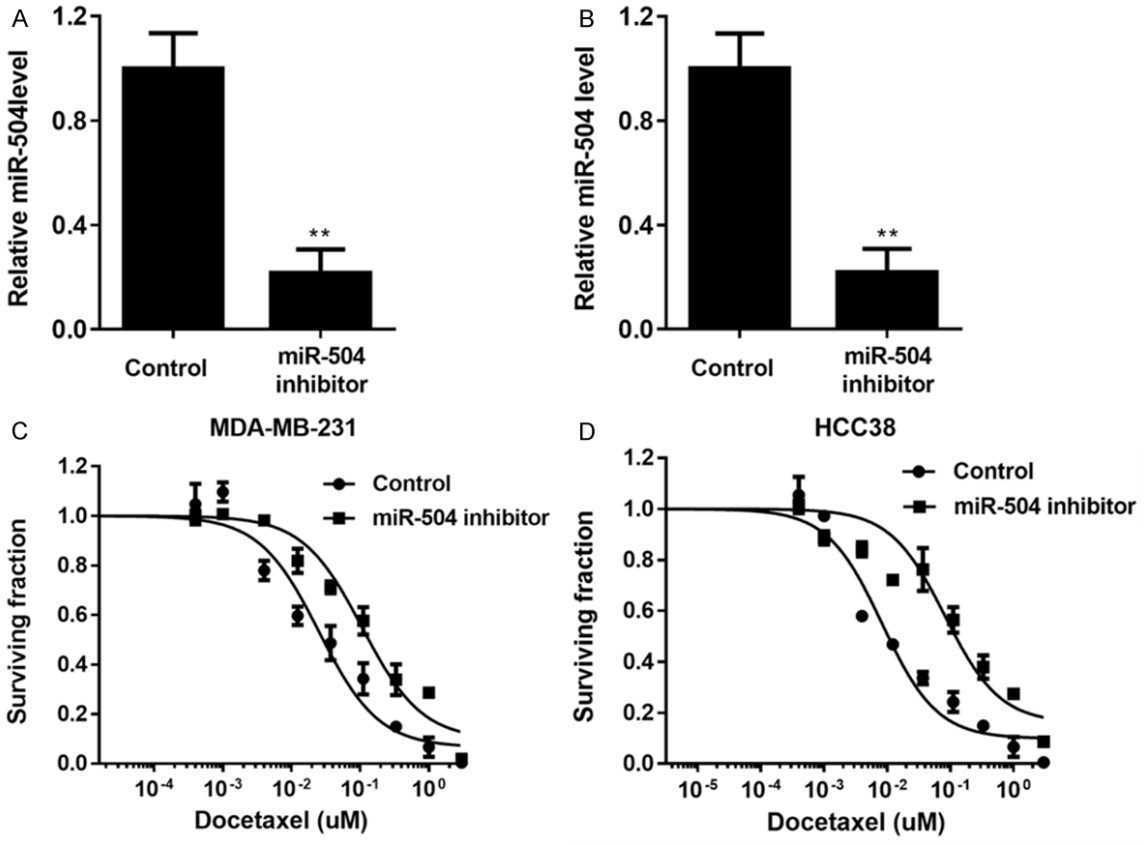

Figure 2. Inhibition of miR-504 confers resistance to docetaxel in TNBC cells. (A, B) Relative level of miR-504 in MDA-MB-231 (A) or HCC38 (B) cells transfected with control miRNA or miR-504 inhibitor. Data represent mean ± SD. **, P<0.01. (C, D) Dose response curves of MDA-MB-231 (C) or HCC38 (D) cells transfected with control miRNA or miR-504 inhibitor treated with docetaxel. Data represent the mean ± SD.

cells (**Figure 2C**, **2D**), suggesting that miR-504 plays a critical role in regulating chemoresistance in TNBC cells.

miR-504 directly targets the 3'UTR of ABCB8 and regulates its expression in TNBC cells

ABCB8 was identified as a putative target of miR-504 using miRNA target prediction program miR and a (www.microrna.org). To validate this prediction, we generated a construct containing the 3'-UTR of ABCB8 with the putative miR-504 binding site and a mutant form in which the putative binding site was mutated (**Figure 3A**). Co-transfection of miR-504 mimic with wild-type ABCB8 3'-UTR reporter construct significantly suppressed relative luciferase activity which was reversed by mutating the miR-504 binding site (**Figure 3B**). In addition, transfection with miR-504 mimic or inhibitor resulted in significant decrease or increase in the protein level of ABCB8 in MDA-MB-231 cells, respectively (**Figure 3C**, **3D**).

miR-504 regulates chemosensitvity of TNBC cells through ABCB8

To determine whether ABCB8 is required for the regulatory role of miR-504 in chemo-sensitivity, we ectopically expressed ABCB8 in MDA-MB-231 cells transfected with miR-504 mimic (**Figure 4A**). Overexpression of miR-504 sensitized A549 cells to docetaxel which was reversed by ectopic expression of ABCB8 (**Figure 4B**), suggesting that ABCB8 is directly involved in miR-504's function in chemoresistance.

Expression of miR-504 in TNBC patients

We further investigated the expression level of miR-504 and ABCB8 in 5 pairs of cancerous tissues from TNBC patients as well as their matched normal tissues using qRT-PCR. As shown in **Figure 5A**, **5B**, the expression levels of miR-504 were significantly lower in tumor tissues and correspondingly the expression lev-

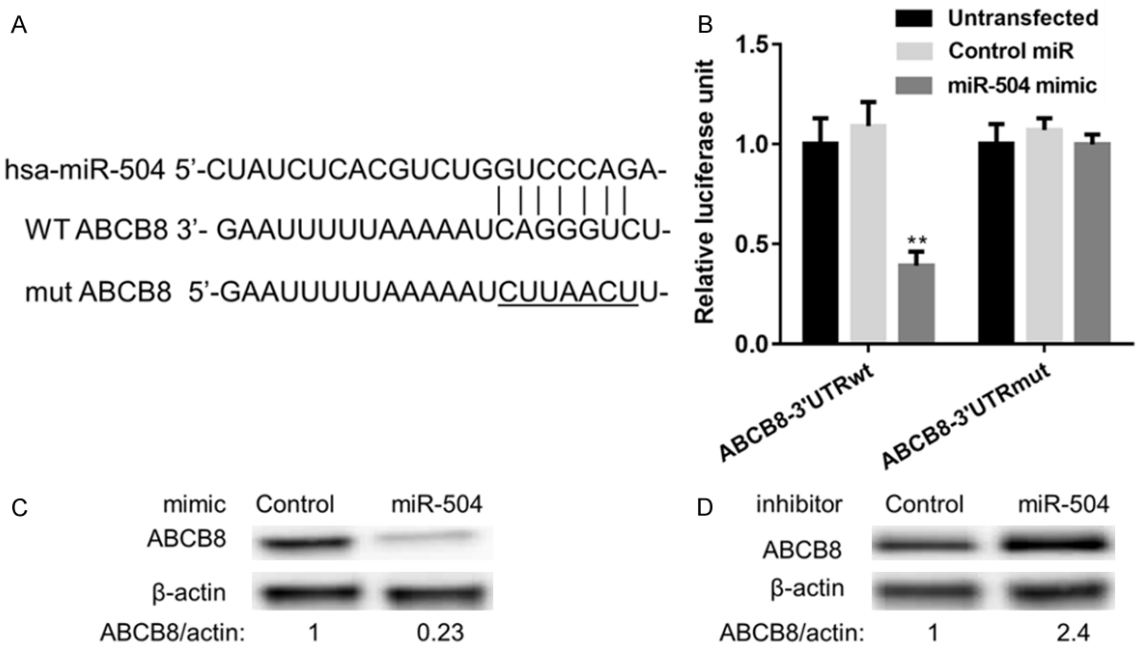

Figure 3. miR-504 directly targets the 3'UTR of ABCB8 and regulates its expression in TNBC cells. A. The wild type and mutant form of 3'-UTR of mammalian ABCB8 mRNA as well as putative miR-504 binding site. B. Luciferase reporter activity of HEK293 cells co-transfected with reporters containing the wild-type or mutant form of 3'-UTR of ABCB8 mRNA and control or miR-504 mimic. Data represent the mean ± SD, n=2. **, P<0.01. C. Western Blot analysis of ABCB8 protein level in MDA-MB-231 cells transfected with control or miR-504 mimic. β-actin was used as loading control. D. Western Blot analysis of ABCB8 protein level in MDA-MB-231 cells transfected with control or miR-504 inhibitor. β-actin was used as loading control.

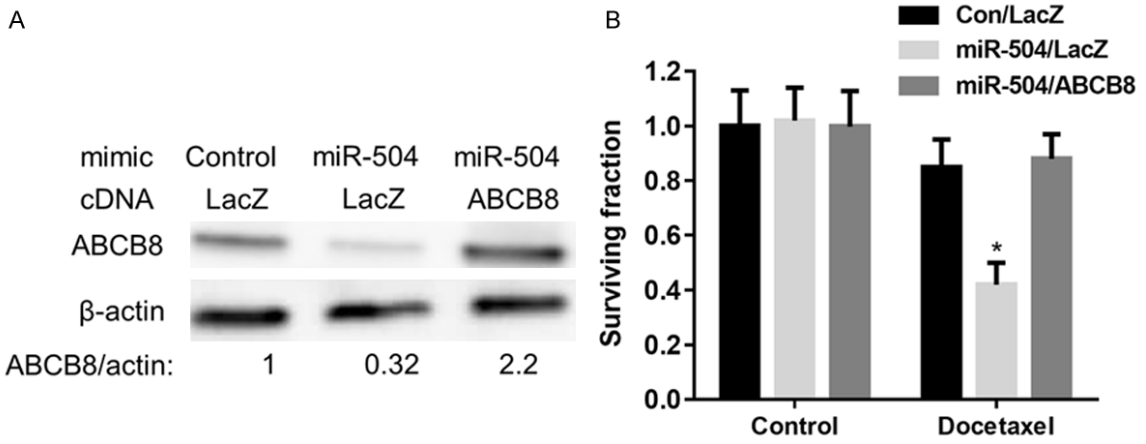

Figure 4. miR-504 regulates chemosensitvity of TNBC cells through ABCB8. A. Western Blot analysis of ABCB8 protein level in MDA-MB-231 cells co-transfected with miR-504 mimic and vector or ABCB8 cDNA. β-actin was used as loading control. B. Surviving fraction of MDA-MB-231 cells as described above with docetaxel (20 nM) treatments. Data represent the mean ± SD, n=2. *, P<0.05. Con: control.

els of ABCB8 were significantly higher in tumor tissues. In addition, expression levels of miR-504 were significantly lower in those patients who do not respond to NAC compared with the responder group (**Figure 5C**).

Discussion

Emerging evidence suggests that miR-504 is implicated in cancer progression. For example, it has been shown that miR-504 inhibits cell

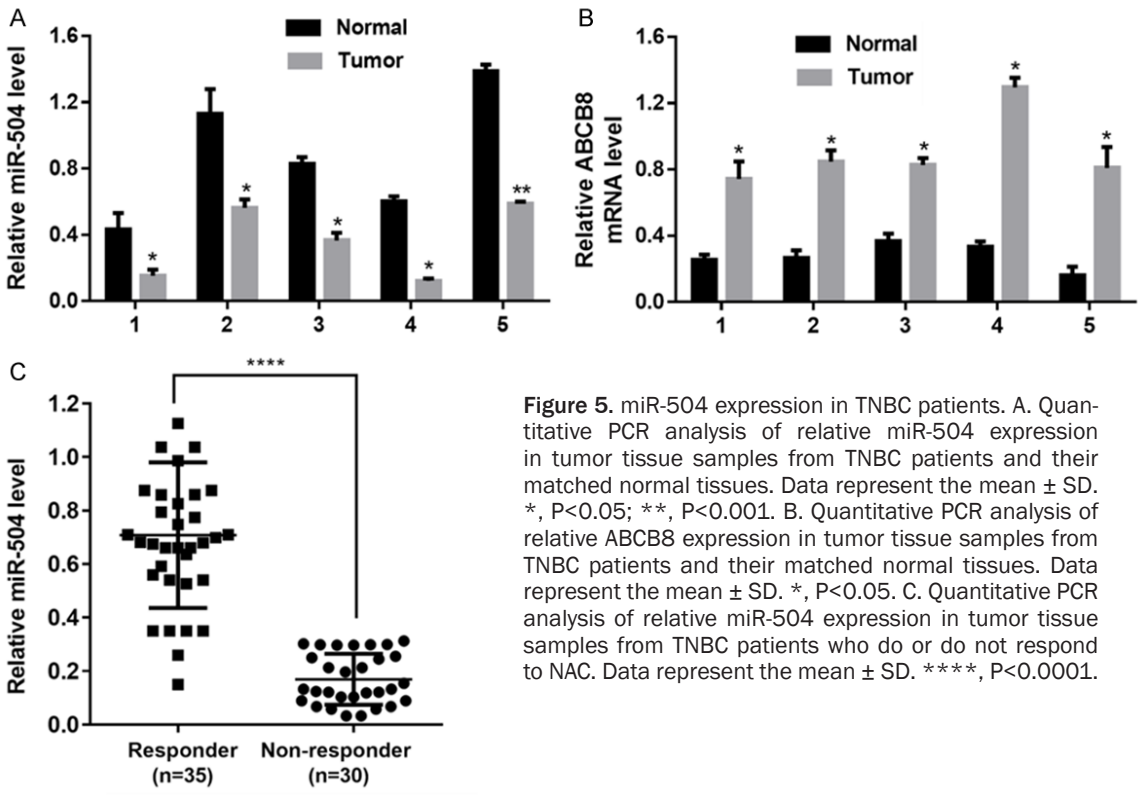

Figure 5. miR-504 expression in TNBC patients. A. Quantitative PCR analysis of relative miR-504 expression in tumor tissue samples from TNBC patients and their matched normal tissues. Data represent the mean ± SD. *, P<0.05; **, P<0.001. B. Quantitative PCR analysis of relative ABCB8 expression in tumor tissue samples from TNBC patients and their matched normal tissues. Data represent the mean ± SD. *, P<0.05. C. Quantitative PCR analysis of relative miR-504 expression in tumor tissue samples from TNBC patients who do or do not respond to NAC. Data represent the mean ± SD. ****, P<0.0001.

proliferation via targeting CDK6 in hypopharyngeal squamous cell carcinoma [9]. Connective tissue growth factor can mediate oral squamous cell carcinoma invasion by activating miR-504/FOXP1 signaling [6]. Additionally, Cui reported that miR-504 can function as a tumor suppressor through its regulation of FOXP1 expression in glioma [8].

Multi-drug resistance has been a major obstacle to the efficacy of chemotherapy in cancer treatment. A number of mechanisms have been proposed to be involved in cancer drug resistance, including genetic changes and variability and alteration of drug target, drug efflux, drug inactivation, increased repair of DNA damage, reduced apoptosis, altered metabolism of the drug, etc [10, 11]. More recent studies have indicated that both genetic changes including mutations, translocations, deletions and amplification of genes or promoter regions and epigenetic changes including aberrant DNA methylation, histone modifications, and non-coding RNA expression may also contribute to acquired drug resistance of cancer cells [12, 13]. We observed that overexpression of miR-504 sensitized TNBC cells to docetaxel whereas down-regulation of miR-504 conferred resistance (**Figures 1**, **2**), suggesting the implication of miR-504 in regulating chemo-sensitivity in TNBC cells.

ABCB8 encodes a multi-drug-resistance gene and is a member of ABC transporter family which has 12 putative drug transporters. ABCB8 is localized to the inner mitochondrial membrane and mitochondrial transporters are thought to be involved in the transport of heme, phospholipids, or peptides (mitochondrial ATP-binding cassette proteins). Its close family member, ABCB1, is well known to be one of the factors affecting the transport of chemotherapeutic drugs such as cisplatin and docetaxel; furthermore, it's often found to be upregulated in chemotherapy-resistant cancer cell lines [14]. This family of glycoproteins are known to contribute to the development of drug resistance by pumping drugs out of the cells, thus decreasing intracellular drug concentration [15, 16]. Recent evidence suggests that ABCB8 may also play an important role in chemoresistance. Gene expression analysis revealed that ABCB8 was significantly overexpressed in doxorubicin-resistant human T-lymphoblastoid leu-

kemic cells compared with the doxorubicin-sensitive parental cells [17]. It is found to be mutated in pre-malignant human colonic epithelial cells [18]. Mitochondria DNA is susceptible to doxorubicin induced damage with resultant cell death due to lack of protective histones [19]. However, direct evidence supporting the role of ABCB8 in regulating chemoresistance is lacking. In this study, we identified ABCB8 as the direct target of miR-504 as demonstrated by luciferase assay and Western blot analysis (**Figure 3**). Additionally, overexpression of ABCB8 reversed the chemo sensitizing effect of miR-504 mimic in MDA-MB-231 cells (**Figure 4**), demonstrating that miR-504 mediate chemoresistance via ABCB8 in TNBC cells. Moreover, miR-504 was significantly down-regulated in tumor tissues compared with matched normal tissues as well as TNBC patients who did not respond to NAC (**Figure 5**).

In summary, our study demonstrates that miR-504 regulates chemo-sensitivity via ABCB8 protein in TNBC cells. miR-504 is down-regulated in TNBC patients who do not respond to chemotherapy. Therefore, miR-504 may represent a novel therapeutic target for the improvement of clinical outcome of TNBC patients.

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Disclosure of conflict of interest

None.

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