MicroRNA-34a Modulates Chemosensitivity of Breast Cancer Cells to Adriamycin by Targeting Notch1

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Background and Aims. MicroRNA-34a (miR-34a) as a tumor suppressor has been reported in many other studies. However, its role in modulating the sensitivity of breast cancer cells to adriamycin (ADR) remains unclear. The aim of this study is to evaluate the role of miR-34a in the sensitivity of breast cancer cells to ADR.

Methods. The role of miR-34a in breast cancer cells was detected using MTT assay, flow cytometry assay, real-time PCR and Western blot, etc. The association of miR-34a and Notch1 was analyzed by dual-luciferase reporter assay and Notch1-siRNA technology. Real-time PCR assay was performed to test the expression of miR-34a and Notch1 in 38 selective breast cancer tissue samples.

Results. Ectopic overexpression of miR-34a could sensitize MCF-7 breast cancer cells to ADR. MiR-34a mimic could inhibit the luciferase activity of the construct containing wild-type 3' UTR of Notch1 in MCF-7/ADR cells. Notch1-siRNA could partially reverse the effect of miR-34a inhibitor in inducing chemoresistance of MCF-7 cells to ADR. Further, there was an inverse association between Notch1 and miR-34a expression in breast cancer.

Conclusion. Dysregulation of miR-34a plays critical roles in the acquired ADR resistance of breast cancer, at least in part via targeting Notch1.

Keywords: MicroRNA-34a, Breast cancer, Adriamycin, Notch1, Chemosensitivity.

Introduction

Breast carcinoma is a leading cause of cancer mortality in women worldwide and poor response to chemotherapy remains to be a major clinical obstacle to the successful treatment of breast cancer (1). Adriamycin (ADR) is one of the most common chemotherapeutic drugs; however, as with many other chemotherapeutic drugs, resistance to ADR results in treatment failure in patients with breast cancer. Causes of anti-cancer drug resistance are believed to be complex and current studies have indicated that the acquisition of chemotherapy drug resistance may also be modulated via the changes in microRNA levels. In breast cancer, it was shown that increased miR-21 was associated with the chemoresistance of breast cancer MCF-7/ADR cells to ADR through direct interaction with PTEN (2). Kovalchuk et al. reported miR-451 expression was associated with the sensitivity of MCF-7/ADR to ADR by targeting MDR1 (3). In prostate cancer cells, it was suggested that upregulated miR-34a is associated with the sensitivity of tumor cells to camptothecin (4) and paclitaxel (5) cytotoxic agents. However, the role of miR-34a in breast cancer was not fully known. Current findings have shown that miR-34a acts as a tumor suppressor by targeting Bcl-2 (6), E2F3 (7), cyclinD1 (CCND1), c-Met (8) and Sirtuin 1 (9,10). Additionally, Fukuda et al. reported that miR-34a regulated Notch1 gene expression via binding to 3' UTR of Notch1.
in neuronal differentiation (11). Pang et al. demonstrated that forced expression of miR-34a caused a reduction of the invasion capacity of cervical cancer and trophoblast cell lines through blocking Notch1 and Jagged1 expression (12). Our study shows that miR-34a expression modulated breast cancer cells response to ADR by targeting Notch1 and Notch signaling pathway may play an important role in the acquisition of breast cancer cell resistance to chemotherapeutic agents.

Materials and Methods

Cell Culture

MCF-7 parental cells and resistant MCF-7/ADR cells obtained from the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (IBCB, Shanghai, China) were cultured as recommended by the supplier. The docetaxel-resistant MCF-7 cells (MCF-7/DOC) were established by a stepwise increase of docetaxel concentrations in the culture over 10 months to achieve statistically significant degrees of resistance relative to parental MCF-7 cell line. The initial and final concentrations of docetaxel were 2 nM and 80 nM in the culture medium.

Quantitative Real-time Polymerase Chain Reaction Assay

Total RNA was extracted from MCF-7, MCF-7/ADR and MCF-7/DOC cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. One µg of total RNA was reverse transcribed by using PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer’s instructions. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the ABI 7300 (Applied Biosystems). The miRNA sequence-specific reverse transcription—PCR (RT—PCR) primers for miR-34a were purchased from Ambion (Austin, TX). Endogenous control RNU6B was used for normalization. The primers used in the real-time PCR reaction for Notch-1, MDR1, and β-actin were also from Ambion. Cycling parameters were followed as protocol described. Each sample was analyzed in triplicate. Primer quality was analyzed by dissociation curve. Data were analyzed by comparing Ct values.

miRNA Target Gene Identification

For miRNA target gene identification, TargetScan (http://www.targetscan.org) and PicTar (http://pictar.mdc.berlin.de/) were used to identify target genes of identified miRNAs.

Western Blots

Protein expression was examined by Western blotting, according to a standard procedure. The membranes were incubated with antibodies against human Notch1 (1:1,000; Cell Signaling Technology, Beverly, MA) overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membranes were developed with an ECL Substrate (Pierce Biotechnology, Rockford, IL). β-actin (1:20000; Abcam, Cambridge, MA) was used as an internal loading control. Three independent experiments were performed.

MiRNA-34a Transfection

MiR-34a mimics (5’-UGGCAGUGCUACUGGUU GU-3’), miR-34a inhibitor (5’-ACAACCAGCUAGACA CUGCCA-3’) and negative control (NC, 5’-CAGUACUU UUGUGUAGUACAA-3’) were synthesized by Genepharma (Shanghai, China). MiR-34a was overexpressed in MCF-7/ADR cells using miR-34a mimics. In MCF-7 sensitive cells, miR-34a was knocked down using an miR-34a inhibitor. Twenty four hours prior to transfection, cells were plated onto a six-well plate at a density of 2.5 × 10^5 cells/well. Then, cells were transfected with miR-34a mimics (10 nM) or inhibitor (30 nM) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were harvested for further analysis after 48 h. Three independent experiments were performed.

Cell Survival Analysis

MCF-7/ADR cells and MCF-7/sensitive cells were transfected with miR-34a mimics and inhibitor, respectively. Twenty four hour post-transfection, the cells were reseeded in 96-well plates and treated with ADR (Hisun Pharmaceutical Co., Zhejiang, China) at different concentrations for 48 h. MTT assay was performed to analyze the cell viability. Twenty µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide) (Sigma) per well was added and incubated at 37°C for 4 h. Then, 150 µL of dimethyl sulfoxide (DMSO) was applied to each well followed by measuring the absorbance at 550 nM on a microplate reader (CliniBio 128; ASYS Hitech, Eugendorf, Austria).

Apoptosis Assay

Cell apoptosis was determined using an Annexin-V-FITC apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ). Briefly, MCF-7/ADR cells and MCF-7/sensitive cells with transfection of miR-34a mimics and inhibitor were incubated with 30 µM adriamycin and 0.25 µM adriamycin for 24 h, respectively. Then 1.0 × 10^6 cells were washed twice with ice-cold PBS and incubated with Annexin-V-FITC and propidium iodide (PI) for 10 min in the dark at room temperature. A FACScan flow cytometer (BD Biosciences) was used to analyze cellular apoptosis.

Dual Luciferase Activity Assay

MCF-7/ADR cells were seeded into six-well plates and cotransfected with miR-34a mimics, control vector and pGL3
vector containing firefly luciferase reporter gene and the 3′ UTR of Notch1 gene (Promega, Madison, WI) using lipofectamine 2000 (Invitrogen). Forty eight hour after transfection, a luciferase assay kit (Promega) was used to assay luciferase activity according to the manufacturer’s protocol.

**Patients and Samples**

Thirty-eight patients with invasive primary breast cancer treated with neoadjuvant chemotherapy (NAC) at the Jiangsu Cancer Hospital, Affiliated Hospital of Nanjing Medical University, from 2008–2010 were enrolled in this study. The diagnosis of each case was confirmed independently by two pathologists based on WHO classification. The following criteria were met: a histological diagnosis of invasive ductal or lobular carcinoma with at least one measurable lesion; a clinical stage of IIA–IIB; patients were to receive 3–6 cycles of preoperative neoadjuvant chemotherapy with FAC or AC (adriamycin 40 mg/m² and CTX 0.6 g/m² combined with or without 5-Fu 0.5 g/m²) on day 1 with treatments repeated every 21 days. A modified radical mastectomy or conservative surgery was performed after NAC. Tissue samples were divided into responders (complete response or partial response) and non-responders (stable disease or progressive disease) according to the patient’s responses assessed by computed tomography (CT) tests and detection of serum tumor markers. Fresh tumor samples were immediately stored at 4°C overnight in RNAfixer and then preserved at −80°C. This study was carried out in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Jiangsu Cancer Hospital.

**Statistical Analysis**

Statistical analysis was performed using SPSS 16.0 statistical package. Differences between groups were determined by Kruskal–Wallis test. Correlation between groups was analyzed using Spearman rank test. All tests were two-tailed; p < 0.05 was considered statistically significant.

**Results**

**Inverse Correlation Expression of miR-34a and Notch1 in MCF-7 and MCF/ADR Breast Cancer Cells**

The half maximal inhibitory concentration (IC₅₀) of ADR in MCF-7 and MCF-7/ADR cells was 0.52 ± 0.02 and 123.00 ± 9.90 µMol/L, respectively (p < 0.05; Figure 1A). MicroRNA microarray analysis identified miR-34a significantly downregulated in the MCF-7/ADR compared with its parental MCF-7 and MCF-7/DOC cell line. But there were no statistical differences in the level of miR-34a expression between MCF-7/DOC and MCF-7 cell lines (data not shown). The results obtained by miRNA microarray analysis were confirmed by the qRT-PCR (p < 0.05; Figure 1B). According to computational analysis, Notch1 was chosen for further validation by qRT-PCR and Western blot analysis to investigate its expression levels in breast cancer MCF-7 ADR-sensitive and MCF-7

![Figure 1](https://example.com/figure1.jpg)

**Figure 1.** Expression of miRNA34a and Notch1 in MCF-7 and MCF/ADR breast cancer cells. (A) IC₅₀ of ADR in MCF-7 and MCF-7/ADR cells was 0.52 ± 0.02 and 123.00 ± 9.90 µMol/L, respectively (p < 0.05). (B,C) Real-time RT-PCR assay was performed to detect the expression of mature miR-34a and Notch1 mRNA in MCF-7 and MCF-7/ADR cells, respectively. (D) Western blot assays were performed to detect the expression of Notch1 protein expression. All experiments were performed in triplicate.
ADR-resistant cells. In MCF-7 ADR-resistant cells, Notch1 (2.1-fold ± 0.2; *p* < 0.05) mRNA expression was increased compared to MCF-7 ADR-sensitive cells (*p* < 0.05; Figure 1C). Notch1 protein expression was significantly up-regulated in MCF-7 ADR-resistant cells compared with that in ADR-sensitive cells (Figure 1D).

**MiR-34a Regulates Expression of Notch1**

Notch1 was known to be targeted by miR-34a, which was significantly increased in the MCF-7/ADR cells. Whether downregulation of Notch1 induced by overexpression of miR-34a is involved in the resistance of MCF-7 to ADR remains unclear. In order to investigate whether miR-34a downregulated Notch1 expression, miR-34a mimic and inhibitor was successfully transfected into MCF-7/ADR and MCF-7 cell, respectively. At 48 h after transfection, expression of miR-34a was determined by quantitative RT-PCR assay. Compared with control MCF-7 cells, the relative level of miR-34a expression in miR-34a mimics-transfected MCF-7/ADR cells was significantly increased 14-fold (*p* < 0.05; Figure 2A). Compared with control MCF-7 cells, the relative level of miR-34a expression in miR-34a inhibitor-transfected MCF-7 cells was significantly decreased to 29% (*p* < 0.05; Figure 2B). Expression levels of mRNA of Notch1 were determined by quantitative RT-PCR 48 h after transfection. We show that the expression levels of Notch1 mRNA in MCF-7/ADR cells transfected with miR-34a mimic were significantly decreased compared with that in MCF-7/ADR cells transfected with control oligonucleotide (*p* < 0.05; Figure 2C). Reversely, the expression of Notch1 mRNA in MCF-7 cells transfected with miR-34a inhibitor were increased compared with that in control cells (*p* < 0.05; Figure 2D). Further, Western blot assays were performed to detect the expression of Notch1 protein. As shown in Figure 3A, the level of Notch1 protein expression in MCF-7/ADR/mimic cells was significantly decreased compared with that in MCF-7/ADR/control cells. On the contrary, the expression levels of Notch1 protein in MCF-7/inhibitor cells were increased compared with that in MCF-7/control cells (Figure 3B). These results suggested that Notch1 was transcriptionally regulated by miR-34a in MCF-7 cells.

**Effects of miR-34a on the Sensitivity of Breast Cancer Cells to ADR**

To test the association of miR-34a expression with the sensitivity of breast cancer cells to ADR, the IC$_{50}$ value of ADR in MCF-7/ADR cells transfected with miR-34a mimics or MCF-7 cells transfected miR-34a inhibitor were determined by MTT assay. The IC$_{50}$ value of ADR in MCF-7/ADR cells transfected with control and miR-34a mimics was 116.33 ± 16.80 and 39.28 ± 4.12 μMol/L, respectively (*p* < 0.05; Figure 4A), suggesting that upregulation of miR-34a expression could sensitize MCF-7/ADR cells to ADR. Additionally, the IC$_{50}$ value of ADR in MCF-7 cells transfected with control or miR-34a inhibitor was 0.29 ± 0.21 or 0.51 ± 0.03 μMol/L, respectively (*p* < 0.05; Figure 4B), suggesting that downregulation of miR-34a could inhibit the sensitivity of MCF-7 cells. From these

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Figure 2. MiR-34a regulates expression of Notch1. (A) Expression of miR-34a in MCF-7/ADR cells transfected with control or miR-34a mimic (*p* < 0.05). (B) Expression of miR-34a in MCF-7 cells transfected with control or miR-34a inhibitor (*p* < 0.05). (C-D) RT-PCR assays were performed to detect the expression of Notch1 mRNA in MCF-7/ADR and MCF-7 cells transfected with miR-34a mimic and inhibitor, respectively (*p* < 0.05). (D) RT-PCR and Western blot assays were performed to detect the expression of Notch1 mRNA and protein expression in MCF-7 cells transfected with control or miR-34a inhibitor, respectively (*p* < 0.05). All experiments were performed in triplicate.
experimental data, it was concluded that the level of miR-34a expression could significantly affect the sensitivity of breast cancer cells to ADR.

**Involvement of miR-34a in ADR-induced Apoptosis**

Cellular apoptosis were tested by Annexin V/propidium iodide staining with the Apoptosis Detection Kit (BD Pharmingen), the results revealed markedly more ADR-induced apoptotic cells in miR-34a mimic transfected MCF-7/ADR cells compared to negative controls ($p < 0.05$; Figure 5A). Furthermore, downregulation of endogenous miR-34a inhibited ADR-induced apoptosis in MCF-7 sensitive cells ($p < 0.05$; Figure 5B). These results showed that miR-34a confers ADR sensitive in MCF-7 cells by enhancing ADR-induced apoptosis.

**Notch1 Is a Target of miR-34a in MCF-7/ADR Cells**

According to the prediction of TargetScan software, a complementary site of miR-34a in the Notch1 3' UTR (position 180–186) and Notch1 was identified as a candidate target of miR-34a (Figure 6A). To identify the regulatory function of miR-34a in Notch1 expression, a dual luciferase reporter system analysis was performed. As shown in Figure 6B, 48 h post-transfection of the miR-34a mimic, luciferase activity significantly decreased by ($p < 0.05$; 65 ± 5%) in MCF-7/ADR-resistant cells that contained the 3' UTR of Notch1. In contrast, the luciferase activity of Notch1-3' UTR mutant reporter was unaffected ($p > 0.05$; 97.5 ± 10.6%). The result shows that miR-34a directly interacts with the 3' UTR of the Notch1 gene.

**Negative Correlation of miR-34a and Notch1 in Breast Cancer Patients with Adriamycin-based Neoadjuvant Chemotherapy**

We have observed the altered expression of miR-34a in MCF-7/ADR cells compared to MCF-7 cells. To investigate the further association of miR-34a with Notch1, we measured expression level of miR-34a and Notch1 mRNAs in 38 selected breast cancer tissues with neoadjuvant chemotherapy. Clinical responses to neoadjuvant chemotherapy according to RECIST criteria (13) were evaluated in 38 patients. Two patients (5.3%) had a complete response (CR), 19 patients (50%) had a partial response (PR), 14 patients (36.8%) had stable disease (SD), and three patients (7.9%) had progressive disease (PD). Patients with CR or PR were regarded as clinical responders and those with SD or PD were regarded as nonresponders. Thus, 21 patients were responders and 17 patients were nonresponders. It is known that ADR is one of the most important substrates of P-glycoprotein (P-gp), and we evaluated the expression of MDR1 mRNA in responder and nonresponder groups. Real-time RT-PCR analysis showed that MDR1 mRNA expression was upregulated in nonresponders ($n = 17$) as compared to responders ($n = 21$) (4.78 ± 0.87 vs. 9.67 ± 2.18, mean ± SD, $p < 0.05)$. To explore the potential implication of miR-34a for response to chemotherapy in breast cancer patients, miR-34a and Notch1 mRNA expression was evaluated by real-time RT-PCR in 38 breast tumors. MiR-34a expression is negatively correlated with Notch1 mRNA expression. As shown in Figure 7A, miR-34a was significantly downregulated in the nonresponders group ($n = 17$) compared with that in responders.

![Figure 3](image-url)  
**Figure 3.** (A) Western blot assays were performed to detect the expression of Notch1 protein in MCF-7/ADR cells transected with mimic and negative control. (B) Expression of Notch1 protein in MCF-7/inhibitor and control cells.

![Figure 4](image-url)  
**Figure 4.** Effect of miR-34a expression on the sensitivity of MCF-7 and MCF-7/ADR cells to ADR. (A) After MCF-7/ADR cells were transfected with control or miR-34a mimic for 24 h, the IC50 value of ADR was determined by MTT assay ($p < 0.05$). (B) After MCF-7 cells were transfected with control or miR-34a inhibitor for 24 h, the IC50 value of ADR was determined by MTT assay ($p < 0.05$). All experiments were performed in triplicate.
the responders group \((n = 21)\) \((p < 0.05)\), with a positive correlation with ADR sensitivity \((\text{Spearman rank test } \rho = 0.94; p < 0.05)\). On the contrary, Notch1 mRNA expression was upregulated in the nonresponders group \((p < 0.05; \text{Figure 7B})\), with a negative correlation with ADR sensitivity \((\text{Spearman rank test } \rho = -0.85; p < 0.05)\). Linear regression analysis was used to analyze the inverse correlation between miR-34a and Notch1 mRNA expression \((\text{Spearman rank test } \rho = -0.89; p < 0.05; \text{Figure 7C})\). It suggests that downregulated miR-34a expression and upregulated Notch1 expression are associated with poor response to adriamycin-containing AC and FAC neo-adjuvant chemoregimens in breast cancer patients.

**Discussion**

miR-34a is a widely studied human miRNA, but it is still a controversial small non-coding RNA in mediating drug resistance. For example, Kastl et al. showed that increased
et al. showed that the upregulated expressions of Notch1 and Jagged1 by miR-34a are associated with the invasive ability of cervical carcinoma and choriocarcinoma cells (12). Notch signaling pathway is a highly conserved signaling pathway. There are four Notch receptors (Notch 1–4) and five Notch ligands (Delta-like [DLL]-1, -3 and -4 and Jagged1 and 2) in mammals. Notch signaling pathway was involved in differentiation, cell fate specification, adhesion and proliferation (15). Recently, emerging evidence shows that abnormal Notch signaling may contribute to cancer chemoresistance (16) and metastasis (17). More importantly, the studies have demonstrated that Notch signaling pathway regulates the formation of cancer stem cells (CSCs) and contributes to the acquisition of the epithelial–mesenchymal transition (EMT) phenotype, which are typically drug resistant and are believed to be the root cause of tumor recurrence (18). For example, Phillips et al. (19) reported that cancer stem CSCs can be identified by phenotypic markers and their fate is controlled by the Notch pathway in breast cancer. In addition, Notch signaling pathway has been reported to be involved in the acquisition of EMT in drug-resistant cancer cells. Moreover, downregulation of Notch signaling by siRNA approach led to partial reversal of the EMT phenotype, resulting in the mesenchymal-to-epithelial transition (MET). These results provide molecular evidence indicating that activation of Notch signaling is mechanistically related to chemoresistance phenotype. But the correlation between miR-34a and Notch1 in regulating tumor cells response to chemotherapeutic drug is still not fully known. In this study, expression of Notch1 was downregulated in MCF-7 cells compared with MCF-7/ADR cells. Additionally, miR-34a mimic could downregulate the expression of Notch1 mRNA and protein in MCF-7/ADR cells. Luciferase reporter gene assay shows that miR-34a modulates the expression of Notch1 by directly binding to the 3’ UTR of Notch1 in breast cancer MCF-7/ADR cells. These data suggested that miR-34a could modulate the sensitivity of breast cancer cells to ADR in part by downregulating Notch1 protein expression. However, the role of Notch signaling pathway in miR34a-mediated drug resistance needs further exploration.

In conclusion, we have shown that expression of miR-34a is inversely correlated with Notch1 expression in breast cancer drug-resistant cells. Furthermore, upregulated miR-34a in the MCF-7/ADR cells downregulates expression of Notch1 and increases sensitivity of MCF-7/ADR cells to ADR. These findings show that miR-34a could not only act as a predictor of tumor response to ADR-based chemotherapy, but also as a potential to develop new strategies in treating breast cancer.

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