miR-137 restoration sensitizes multidrug-resistant MCF-7/ADM cells to anticancer agents by targeting YB-1

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Multidrug resistance (MDR) to chemotherapeutic agents is a major obstacle to successful treatment in breast cancer patients. The aims of this study were to investigate whether miR-137 was involved in the regulation of MDR, and to explore the mechanism of miR-137 on the sensitivity of MCF-7/ADM cells. miR-137 was downregulated in MCF-7/ADM cells, and its expression was found to inversely correlate with Y-box binding protein-1 (YB-1) and P-glycoprotein (P-gp) levels in breast cancer cells. Furthermore, YB-1 was confirmed as a target of miR-137 by luciferase reporter assay and western blot analysis. Moreover, elevated expression of miR-137 reduced the protein expression levels of YB-1 and P-gp, mimicking the effect of YB-1 knockdown in the sensitivity of MCF-7/ADM cells to anticancer agents, whereas restoration of YB-1 diminished this effect. In conclusion, our results demonstrated that miR-137 was involved in MDR in cancer through modulation of P-gp by targeting YB-1, suggesting that miR-137 might be a potential target for preventing and reversing MDR in tumor cells.

Keywords: miR-137; Y-box binding protein-1; multidrug resistance

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Introduction

Chemotherapy is the mainstream method of treatment against advanced breast cancer. However, cancerous cells frequently develop multidrug resistance (MDR) to chemotherapy agents [1]. MDR refers to the ability of tumor cells to resist several unrelated drugs after exposure to a single chemotherapy drug [2], and is the leading cause of cancer-related death. Nearly all initially responsive breast tumors will eventually acquire an MDR phenotype [1]. Although the etiology of MDR is multifactorial, one of the main causes is the overexpression of the membrane-associated 170-kDa glycoprotein (P-gp), encoded by the multidrug resistance gene-1 (MDR1). As other members of ABC membrane transporter family, P-gp acts as a drug efflux pump, lowers intracellular drug levels to sublethal concentrations, and helps cells escape from death [3]. It has been reported that notably natural product anticancer agents, including vinca alkaloids, anthracyclines (daunorubicin and adriamycin), and taxanes, are the substrates of P-gp. Many studies have been carried out on drug resistance reversal and three generations of drug resistance reversal agents to modulate P-gp-dependent MDR have been developed by decreasing the expression of the MDR1 mRNA and P-gp [4]. However, they do not have satisfactory effects because of the severe side effects or poor pharmacokinetics in vivo [5]. Therefore, increasing the drug sensitivity is a key step towards improving treatment for cancer patients.

Y-box binding protein-1 (YB-1) is a member of a family of DNA-binding proteins, characterized by a cold shock domain, a highly conserved nucleic acid recognition domain. It also interacts with a specific sequence motif, termed Y-box, which contains an inverted 5'-CCAAT-3' sequence [6]. Results from the literature suggested that overexpression as well as nuclear localization of YB-1 was predictive of drug resistance and tumor progression in breast, ovarian, lung, synovial, and prostate cancers [7–12]. Further studies confirmed that MDR1 expression was often upregulated due to promoter activation after drug treatment, and YB-1 acted as a major transcription factor for the activation of MDR1 promoter [12–14]. In a human breast cancer cell line, Bargou et al. [13] reported that the overexpression and nuclear localization of YB-1 was closely associated with MDR1 gene expression. We demonstrated previously that doxorubicin could increase expression of MDR1 through inducing YB-1 nuclear translocation, and enhancing DNA-binding activity of YB-1 [15].

miRNAs (miR) are short single-stranded RNAs consisting of 20–25 nucleotides. They are able to bind complementary
sequences in 3'-untranslated regions (3'UTR) of target genes to induce mRNA degradation, suppress translation, or both [16]. Zhu et al. [17] showed that miR-27a expression was increased in human ovarian MDR cancer cells compared with their respective parental cells. Furthermore, transfection of ovarian MDR cells with antagonists of miR-27a could reduce MDR1 mRNA level and decrease P-gp expression. Si et al. [18] found that suppression of miR-21 using antisense oligonucleotides sensitized MCF-7 cells to anticancer drug topotecan. Our previous results indicated that miR-181a sensitized a multidrug-resistant leukemia cell line K562/A02 to daunorubicin by targeting Bcl-2 [19], and miR-126 enhanced the sensitivity of non-small-cell lung cancer cells to anticancer agents by targeting vascular endothelial growth factor A [20]. These reports suggested a role of miRNAs in drug resistance. Further in-depth research is needed in order to fully understand this role and to find novel treatment strategies for cancer drug resistance.

Our present study revealed that the elevated miR-137 expression could sensitize breast cancer cells to chemotherapeutic agents through modulating the expression of P-gp by targeting YB-1.

Materials and Methods

Cell lines and cell culture

The parental breast carcinoma cell line MCF-7, and its multidrug-resistant counterpart MCF-7/ADM, were obtained from Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). Cells were all cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (Gibco BRL, Grand Island, USA) in a humidified atmosphere containing 5% CO2 at 37°C. To maintain the MDR phenotype, ADM with a final concentration of 1 µg/ml was added to the culture media for MCF-7/ADM cells. Cells were cultured for 2 weeks in drug-free medium prior to their use in the experiments.

microRNA transfection assay

The miR-137 mimic and negative miRNA mimic control (5'-UUCUCCGAACGUGACACGU-3') were chemically synthesized by Shanghai GenePharma Company (Shanghai, China). MCF-7/ADM cells were plated in six-well plates (6 x 10^5 cells/well) and transfected with 100 nM of the miR-137 mimic, or negative miRNA mimic control using Lipofectamine 2000 (Invitrogen, Long Island, USA) according to the manufacturer’s protocol as previously described [20].

Real-time quantification of miRNAs by stem-loop reverse transcriptase polymerase chain reaction

Forty-eight hours after transfection, total RNA was extracted from the MCF-7 or MCF-7/ADM cells using Trizol (Invitrogen), and 10 ng RNA from each cell line was used for cDNA synthesis with the First strand cDNA synthesis kit (Fermentas, Glen Burnie, USA), which was determined using the SYBR green real-time PCR kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. The primers for miR-137 were: forward, 5'-GCAGCAAGAGTTCTGGTTG GC and reverse, 5'-TGAAACAGTGGC ACAA CAC. The relative amount of each miRNA was normalized to U6 snRNA. The relative expression levels of each sample were measured using the 2^(-ΔΔCt) method [21], where 

\[ \Delta \Delta C_t = (C_{\text{miRNA}} - C_{\text{U6 snRNA}})_{\text{transfected}} - (C_{\text{miRNA}} - C_{\text{U6 snRNA}})_{\text{control}}. \]

The results were presented as fold change of expression levels in the MDR cells relative to the parental MCF-7 cells.

In vitro drug sensitivity assay

The transfected cells were seeded into 96-well plates (2 x 10^3 cells/well), and then treated with serial dilutions of ADM, vincristine (VCR), or paclitaxel (Taxol). After being incubated for 48 h, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml; Sigma, St Louis, USA) was added into each well. After an additional 4 h at 37°C, the culture medium was removed and 200 µl of dimethyl sulfoxide was added into each well. The absorbance in each well was read at 490 nm by an automated microplate reader (Bio-Rad, Hercules, USA).

Intracellular ADM concentration analysis

The fluorescence intensity of intracellular ADM of transfected cells was determined by flow cytometry (FCM). Cells were seeded into six-well plates (1 x 10^6 cells/well) and cultured overnight at 37°C. After addition of ADM to the final concentration of 5 µg/ml, cells were cultured for another 1 h, then harvested for detection of ADM accumulation or, alternatively, and cultured in drug-free DMEM for another 1 h followed by being harvested for detection of ADM retention. Then cells were washed with phosphate-buffered saline, and the mean fluorescence intensity of intracellular ADM was detected by FCM.

Apoptosis assay

Surface exposure of phosphatidylserine in apoptotic cells was quantitatively detected using Annexin V/FITC and PI apoptosis detection kit (Becton Dickinson, Franklin Lakes, USA). Twenty-four hours after transfection, MCF-7/ADM cells were treated by ADM, with a final concentration of 0.1 µM. Forty-eight hours later, FCM was used to detect apoptosis of the MCF-7/ADM cells by determining the relative amount of Annexin V-FITC-positive, PI-negative cells as previously described [22].

Dual luciferase activity assay

The putative target sites of human YB-1 3'UTR segments for miR-137 were amplified using Pyrobest DNA polymerase (Fermentas) and then cloned into the XbaI site of pGL3 control (Promega, Madison, USA). Primers for YB-1 3'UTR region were: 5'-gtactcgagTGCCGGCTTACCCTCCTAC-3' and 5'-actgcggccgcCTTTATTAACAGGTGC TTGCAGT-3'. The mutated putative miR-137 binding site in the YB-1 3'UTR was generated by the Quick change site-directed mutagenesis kit (Stratagene, Cedar Creek, USA) according to the manufacturer's protocol. miR-137 site mutagenesis primers into YB-1 3'-UTR were: 5'-CATATCTGGTCAAGTTCTCGAGTTTAAGAA-3' and 5'-TTC TTAACTCGAAGCATTGACCAGATATG-3'. MCF-7/ADM cells were plated into 24-well plates (4 x 10^4 cells/well). Twenty-four hours later, cells were co-transfected with 800 ng luciferase vector, including the 3'UTR of YB-1, and miR-137 mimic or mimic control at a final concentration of 0, 50, 100, or 150 nM by Lipofectamine 2000. Forty-eight hours after transfection, luciferase activities were performed by the dual luciferase reporter assay system (Promega).

siRNA transfection assay

SignalSilence® YB-1 siRNA kit, the recombinant plasmid eukaryotic expression vector pcDNA3.1-YB-1 (YB-1) and the empty vector control (pcDNA3.1) was purchased from Cell Signaling Technology (Beverly, USA). Cells were plated at 6 x 10^5 cells/well in six-well plates, and YB-1-specific siRNA (100 nM), negative control siRNA (100 nM), pcDNA3.1-YB-1 (100 nM), or pcDNA3.1 (100 nM) were transfected into cells with Lipofectamine 2000 according to the manufacturer's protocol, respectively. The cells were prepared for next experiments 48 h after transfection. The transfection efficiency was evaluated by FCM by calculating the percentage of fluorescein-labeled cells.

Western blot analysis

Forty-eight hours after transfection, proteins from each group (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked and probed with antibodies against YB-1, P-gp (Santa Cruz Biotechnology, Santa Cruz, USA), and β-actin (Sigma) (each 1:5000). After being washed with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:3000) and visualized by super ECL detection reagent (Applygen, Beijing, China).

Statistical analysis

The results were calculated using SPSS version 12.0 software (SPSS, Chicago, USA). They were expressed as the mean ± standard deviation (SD) and compared using analysis of variance. Statistical significance was defined as P < 0.05. Results were representative of three independent experiments.

Results

Downregulation of miR-137 correlated with overexpression of YB-1 and P-gp in MCF-7/ADM cells

To establish functional association between YB-1 and miR-137, we verified differences of miR-137 expression in MCF-7/ADM cells and its parent cell line, MCF-7, using stem-loop reverse transcriptase polymerase chain reaction. Results showed that in MCF-7/ADM cells, miR-137 was markedly decreased compared with that in MCF-7 cells and could be significantly increased by miR-137 mimic transfection (P < 0.05) [Fig. 1(A)]. Meanwhile, higher amount of YB-1 and P-gp protein was detected in MCF-7/ADM cells [Fig. 1(B)]. It potentially indicated that the loss of miR-137 might be involved in the dys-expression of YB-1 and P-gp in breast cancer MDR.

Figure 1 Expression of miR-137, YB-1 and P-gp in breast cancer cells

(A) The relative expression of miR-137 in breast cancer cell lines by real-time PCR. Data were shown as fold changes of miR-137 levels in MCF-7/ADM cells relative to MCF-7 cells, or in mimic-transfected cells relative to control cells, respectively. (B) The expression of YB-1 and P-gp in MCF-7 and MCF-7/ADM cells detected by western blot. *P < 0.05 vs. control cells.
miR-137 interacted with YB-1 3'UTR

Using the algorithms for target gene prediction, including PicTar, TargetScan, and miRanda, YB-1 was identified as one of the potential targets of miR-137. To further confirm that YB-1 was the direct target of miR-137, a segment of the 3'UTR of YB-1, with or without point mutation sequence, was sub-cloned the downstream of the Firefly luciferase reporter, and then luciferase reporter vector together with the increasing amount of miR-137 mimic were transfected into MCF-7/ADM cells [Fig. 2(A,B)]. In MCF-7/ADM cells, a linear decrease of relative luciferase activity was observed when the YB-1 3'UTR was co-transfected with the miR-137 mimic (from 0 to 100 nM). Moreover, the decreased expression of YB-1 was also detected with the increasing levels of miR-137, suggesting that YB-1 is a direct target gene of miR-137 [Fig. 2(C)]. However, the luciferase activity and the expression of YB-1 remained relatively unchanged in cells treated with 100 or 150 nM mimic [Fig. 2(B,C)].

miR-137 downregulated P-gp through inhibition of YB-1

Based on our previous study [15], we suspected that the miR-137-dependent suppression of YB-1 might contribute to the downregulation of P-gp. To confirm this hypothesis, we evaluated P-gp level in the YB-1 siRNA or miR-137 mimic-transfected MCF-7/ADM cells. As shown in Fig. 2(D), the protein expression of YB-1 was significant decreased after YB-1 siRNA transfection, and the downregulation of P-gp was detected in YB-1 siRNA as well as mimic-transfected cells. To further confirm that YB-1 plays a role in the regulation of P-gp by miR-137, we evaluated YB-1 levels after the addition of pcDNA3.1-YB-1 to mimic transfected cells. As shown in Fig. 2(E),...
downregulation of P-gp by miR-137 was impaired by the YB-1 restoration.

Identification of potential gene targets of miR-137

Previous studies showed that cyclin-dependent kinase 6 (Cdk6) was a target of miR-137 in uveal melanoma cell lines [23]. Otherwise, in colorectal cancer cells, miR-137 targeted cell division cycle 42 (Cdc42), inducing cell cycle G1 arrest and inhibiting invasion [24]. To determine whether they were also affected by ectopic expression of miR-137 in breast cancer, western blot analysis was performed. Results showed remarkable downregulation in both selected genes after miR-137 mimic transfection [Fig. 2(F)].

Overexpression of miR-137 partially sensitized the MCF-7/ADM cells to chemotherapeutic agents

We further investigated the effects of miR-137-related transfection and YB-1 siRNA transfection in MCF-7/ADM cells. As shown in Table 1, after transfection with miR-137 mimic or YB-1 siRNA, the IC50 of MCF-7/ADM cells for P-gp-related drugs VCR, ADM, and taxol were significantly decreased compared with control RNA-transfected cells (P < 0.05). Moreover, YB-1 siRNA had a similar effect on cells with miR-137 overexpression. However, with the elevated YB-1 expression, the decreased IC50 of chemotherapeutic agents by transient expression of miR-137 were eliminated, suggesting that miR-137 confers MDR via regulating YB-1 in MCF-7/ADM cells.

ADM content in miR-137-related transfectants

Since MDR of cancer was mainly due to alterations of drug influx and efflux, we measured the intracellular accumulation and releasing of ADM by FCM in MCF-7/ADM cells. As shown in Fig. 3(A,B), increased accumulation of ADM and decreased releasing index of MCF-7/ADM + miR-137

<table>
<thead>
<tr>
<th>MCF-7/ADM+</th>
<th>IC50 (µg/ml)</th>
<th>VCR</th>
<th>Taxol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADM</td>
<td>52.12 ± 2.92</td>
<td>11.69 ± 0.91</td>
<td>6.03 ± 0.27</td>
</tr>
<tr>
<td>Mimic-NC</td>
<td>51.56 ± 4.12</td>
<td>12.09 ± 1.02</td>
<td>6.43 ± 0.32</td>
</tr>
<tr>
<td>Mimic</td>
<td>11.17 ± 1.01*</td>
<td>3.61 ± 0.05*</td>
<td>2.17 ± 0.01*</td>
</tr>
<tr>
<td>scramble siRNA</td>
<td>53.01 ± 5.04</td>
<td>11.96 ± 1.17</td>
<td>6.17 ± 0.41</td>
</tr>
<tr>
<td>YB-1 siRNA</td>
<td>9.87 ± 0.91*</td>
<td>3.88 ± 0.11*</td>
<td>1.87 ± 0.03*</td>
</tr>
<tr>
<td>pcDNA3.1</td>
<td>52.85 ± 6.02</td>
<td>12.46 ± 1.68</td>
<td>6.37 ± 0.52</td>
</tr>
<tr>
<td>YB-1</td>
<td>137.54 ± 10.34*</td>
<td>29.12 ± 3.21*</td>
<td>17.81 ± 2.04*</td>
</tr>
<tr>
<td>YB-1 + mimic-NC</td>
<td>130.12 ± 9.78</td>
<td>23.12 ± 1.32</td>
<td>15.97 ± 1.03</td>
</tr>
<tr>
<td>YB-1 + mimic</td>
<td>67.01 ± 8.11*</td>
<td>14.96 ± 2.07*</td>
<td>8.17 ± 1.01*</td>
</tr>
</tbody>
</table>

*P < 0.05.
cells were observed compared with that of control cells ($P < 0.05$).

**Effects of miR-137 on apoptosis**

As the blockade of apoptosis was another important mechanism of MDR, we investigated the capacity of MCF-7/ADM + miR-137 and MCF-7/ADM + YB-1 siRNA cells to undergo ADM-induced apoptosis. As shown in Fig. 3(C), miR-137 as well as YB-1 siRNA could promote ADM-induced apoptosis. The apoptotic rate of MCF-7/ADM + miR-137 and MCF-7/ADM + YB-1 siRNA cells was higher than that of control cells ($P < 0.05$).

**Discussion**

Despite the development of newer chemotherapeutic agents and combination chemotherapy regimens, metastatic and advanced breast cancer is often resistant to chemotherapy [1]. Although much remains to be learned, there is emerging evidence suggesting that in addition to protein-encoding genes, miRNAs have more than a cursory role in preventing cancer chemoresistance. Up to now, the biological roles of only a small fraction of identified miRNAs in chemoresistance have been elucidated. miR-27a and miR-451 expression were shown to be downregulated in MDR breast cancer cell lines relative to parental cells [27]. miR-328 was also documented to be downregulated in MDR breast cancer cell lines, and to regulate the expression of BCRP [28]. However, the role of miRNA in cancer MDR remains largely unexplored. In this report, our findings demonstrated the involvement of miR-137 in cancer MDR via the modulation of P-gp by targeting YB-1, providing new evidence in MDR development.

miR-137 is on chromosome 1p21.3 and lies across a large CpG island. Promoter methylation of miR-137 has been described in several solid tumors, including colorectal cancer [29], gastric cancer [30], uveal melanoma [23], oral cancer [31,32], glioblastoma multiforme [32], squamous cell carcinoma of the head and neck [33,34], and breast cancer [35]. It has also been shown to potentially act as a tumor suppressor microRNA in these tumors. However, the biological roles of miR-137 as well as its specific downstream mRNA targets in MDR breast cancer cells remains unknown.

Bargou et al. [13] reported that YB-1 expression in the nuclei of untreated primary breast cancer showed an almost perfect correlation between YB-1 and P-gp expression, indicated that YB-1 may be involved in modification of sensitivity in breast cancer to paclitaxel. Studies showed the Y-box, a putative AP-1 site that overlaps with a CAAT-like box, a binding motif for CCAAT-enhancer-binding protein β (C/EBPβ), and a novel head-to-tail site within the MDR1 promoter for the tumor suppressor protein p53. Thus, several corresponding transcription factors of these sites such as Sp1, NF-Y, YB-1, c-fos, c-jun, p53, and C/EBPβ have been implicated in the regulation of MDR1 [36]. Because microRNAs have been considered as the increasing important regulators of gene expression following transcription, we sought to determine whether the expression of YB-1 could be under the control of microRNAs, and then lead to the regulation of cancer MDR via the modulation of P-gp.

Zhao et al. [35] reported that miR-137 downregulated in breast cancer cells, and could impair the proliferative and migratory capacity of breast cancer cells by targeting estrogen-related receptor alpha. In our study, we found that in ADM-resistant breast cancer cell line MCF-7/ADM, compared with MCF-7, the expression level of miR-137 was dramatically decreased, and correlated with overexpression of YB-1 and P-gp. Then we found restoration of miR-137 or inhibition of YB-1 in MCF-7/ADM cells sensitized MCF-7/ADM cells to P-gp-related drugs VCR, ADM, and taxol, increased ADM accumulation and retention, decreased ADM releasing index, and displayed a higher proportion of apoptotic cells as well. The sequence alignment of human miR-137 indicated that YB-1 was one of the potential targets of miR-137. A further analysis of the regulatory mechanisms revealed that miR-137 interacted with YB-1 3′UTR, which subsequently resulted in the downregulation of P-gp.

In conclusion, here we examine the role of miR-137 in the modulation of MDR of breast cancer. The results suggested that up-regulation of miR-137 could reverse MDR phenotype of MCF-7/ADM cells at least in part through regulation of P-gp by targeting YB-1 and promotion of apoptosis.

**References**


