Mir-1307 regulates cisplatin resistance by targeting Mdm4 in breast cancer expressing wild type P53

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Keywords
Breast cancer; cisplatin resistance; Mdm4; microRNA; miR-1307.

Abstract
Background: Many chemotherapy regimens are used to treat breast cancer; however, breast cancer cells often develop drug resistance that usually leads to relapse and poor prognosis. MicroRNAs (miRNAs) are short non-coding RNA molecules that post-transcriptionally regulate gene expression and play crucial roles in diverse biological processes, such as development, differentiation, apoptosis, and proliferation. We investigated the roles of miRNAs in the development of drug resistance in human breast cancer cells.

Methods: MiRNA expression was detected in human breast cancer cell lines MCF-7 and MDA-MB-468 via real time PCR; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, cell viability, colony formation, and luciferase reporter gene assays; Western blot; and immunohistochemistry.

Results: MiR-1307 was downregulated while MDM4 was upregulated in MCF-7/cisplatin (CDDP) and MDA-MB-468/CDDP cells compared with parental MCF-7 and MDA-MB-468 cells. In vitro drug sensitivity assay demonstrated that overexpression of miR-1307 sensitized MCF-7/CDDP cells to CDDP. Luciferase activity assay with a reporter containing sequences from the 3'-untranslated region of Mdm4 in MCF-7/CDDP cells suggested that Mdm4 was the direct target gene of miR-1307. Ectopic miR-1307 expression reduced the MDM4 protein level and sensitized MCF-7/CDDP cells to CDDP-induced apoptosis.

Conclusion: Our findings suggest, for the first time, that miR-1307 could play a role in the development of CDDP resistance in breast cancer, at least in part by modulating apoptosis by targeting Mdm4.

Introduction
Despite progress in methods for early diagnosis, breast cancer remains the most common cancer in women worldwide. Breast cancer is one of the most frequently diagnosed malignancies and the leading threat to women's health, currently comprising 25% of all cancer cases and 15% of all cancer-related deaths among women. Chemotherapy plays a pivotal role in the treatment of breast cancer; however, cancer cell resistance to chemotherapy continues to be a major clinical obstacle to successful treatment. Recently, efforts have focused on the characterization of biomarkers that can predict the response to neoadjuvant chemotherapy, with the aim of tailoring patient care programs, reducing chemotherapy-induced morbidity, and identifying novel targets to be used in the development of innovative and more efficient therapies for the treatment of breast carcinoma. MicroRNAs (miRNAs) are short single-strand RNAs (20–25 nucleotides) that regulate the expression of a wide variety of genes. miRNAs mediate regulation of gene expression at the post-transcriptional level by binding complementary sequences in the 3'-untranslated regions (UTRs) of target messenger RNAs (mRNAs). Various miRNAs have been reported to be involved in drug resistance, acting as potential oncogenes or tumor suppressors. These miRNAs function by repressing the expression of novel and known resistance-related genes and/or signaling pathways. For instance, overexpression of miR-451 sensitizes breast cancer cells to doxorubicin, and upregulation of miRNA-21 is associated with acquired trastuzumab...
resistance.\textsuperscript{10} Other research has reported that miR-1307 could promote cell chemoresistance by targeting ING5 expression in ovarian cancer. Thus, miRNAs play an important role in drug resistance in cancer.\textsuperscript{11}

Whether miR-1307 is involved in drug resistance in breast cancer remains unclear. In this study, we investigate the roles of miRNAs in the development of drug resistance in human breast cancer cells.

**Methods**

**Cell lines and cell culture**

Human breast cancer cell lines MCF-7 and MDA-MB-468 were cultured in RPMI-1640 medium with 10% fetal bovine serum, 100 IU/ml of penicillin, and 100 mg/ml of streptomycin, and maintained in a humidiﬁed atmosphere at 37°C with 5% CO\textsubscript{2}. A subpopulation of MCF-7 and MDA-MB-468 cells were cultured after four passages in complete RPMI-1640 medium supplemented with increased exposure to cisplatin (CDDP: 0.2 μM → 0.3 μM → 0.4 μM → 0.5 μM). When stable breast cells grew in RPMI-1640 medium containing 0.5 μM CDDP, the MCF-7 cells and MDA-MB-468 were intermittently attenuated to higher concentrations by incrementally increasing CDDP doses (0.5 μM → 1.0 μM → 2.0 μM). Thus, we established CDDP-resistant MCF-7/CDDP and MDA-MB-468/CDDP cell lines.

**Quantitative real-time PCR analysis for microRNA (miRNA) expression**

Total RNA was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. For miRNA analysis, complementary DNA was synthesized using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen). miRNA real-time (RT)-PCR Primer Sets specific for human miR-141, miR-222, miR-24, miR-26a, miR-148b, miR-193a-3p, miR-21, miR-503, miR-302d, miR-611, and miR-486 were used and human U6 small nuclear RNA was used as an endogenous control. The primers were: forward 5′-CGCTAACACTGTCTAATAAGG-3′ and reverse 5′-CCTGACAGGTTCCGAGGT-3′; miR-222 forward 5′-AGGCGCAAGGTCCGAGGT-3′ and reverse 5′-GTGCAGGGTGAGGT-3′; miR-24 forward 5′-ACAGTGTGTCATACAGAGGAGT-3′ and reverse 5′-AACTGTGTGTCGGTGACTGC-3′; miR-611 forward 5′-CCGTAACATGTCTAATAAGG-3′ and reverse 5′-GTCGAAGGTTCCAGAAGT-3′; and miR-486 forward 5′-ATCCTGACTGCTGCC-3′ and reverse 5′-GTAATCGAGAAGGAG-3′.

Briefly, the miRNA in the total RNA samples was polyadenylated. The amount of miRNA was monitored using SYBR Green ER qPCR SuperMix Universal (TaKaRa, Dalian, China). Quantitative (q) PCR was performed under the following thermo cycler conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 20 seconds and 72°C for 20 seconds, and 78°C for 20 seconds (collecting ﬂuorescence). For qPCR of MDM4, the primers were: forward 5′-AGGTGCAGGAAAGTTAAATGTT-3′ and reverse 5′-CCATATGCTGCTCCGATGC-3′.

We analyzed the expression level of each miRNA using the 2\textsuperscript{ΔΔCT} method. The results are presented as fold changes of each miRNA in the MCF-7/CDDP and MDA-MB-468/CDDP cells relative to the parental MCF-7 and MDA-MB-468 cells.

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, cell viability, and colony formation assays**

MCF-7 and MDA-MB-468 cells were seeded in 96-well plates with 1 × 10\textsuperscript{4} cells per well in 100 μL of cell culture medium and incubated at 37°C for 24 hours. The cells were then transfected with miR-1307 antisense oligo and small interfering RNA-Mdm4 with control oligonucleotides. After 24 hours of incubation, the cells were then incubated with 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) at 37°C for four hours. The medium was then removed and the precipitated formazan was dissolved in 200 μL of dimethyl sulfoxide. After shaking the cells for 15 minutes, absorbance at 490 nm was measured on an enzyme immunoassay analyzer (Bio-Rad, Hercules, CA, USA). Meanwhile, colony formation assay was performed using 12-well plates with 150 cells per well in 1 mL of cell culture medium and incubated at 37°C. The medium was changed every two days. The experiment was performed in triplicate.

**Luciferase reporter gene assays**

Cells were transfected at 3 × 10\textsuperscript{4} cells per well into 24-well dishes. After 12 hours, pGL3-MDM 3′UTR wild-type/
mutant and miR-1307/anti-miR-1307 were transiently co-
transfected with the pRL-TK plasmid (Promega, Madison,
WI, USA) into cells. After 48 hours, cells were collected
and lysed in 1X passive lysis buffer. Luciferase activity was
determined using the Dual-Luciferase Reporter X Assay
System (Promega) according to the manufacturer’s
instructions.

**Western blotting**
The proteins were resolved by sodium dodecyl sulfate-
polyacrylamide gel electrophoresis and then transferred
onto a nitrocellulose membrane. Antibodies against Mdm4
or β-actin were incubated with the membranes overnight
at 4°C. The membranes were washed extensively with 0.1%
tris-buffered saline plus Tween 20 and incubated with
horseradish peroxidase conjugated secondary antibodies.
Protein expression was assessed by enhanced chemilumi-
nescence and exposure to chemiluminescent film. Lab-
Works Image Acquisition and Analysis Software (UVP,
Upland, CA, USA) was used to quantify the band intensi-
ties. All antibodies were purchased from Abcam
(Cambridge, MA, USA).

**Immunohistochemistry**
Fresh human breast cancer specimens were obtained from
HangZhou Cancer Hospital. All tissue specimens were
fixed overnight in 10% paraformaldehyde and embedded
in paraffin. Samples were sectioned at 4 μM and stained
with hematoxylin and eosin or incubated with primary
antibodies. The primary antibodies were rabbit anti-Mdm4
(Abcam) and rabbit anti-BrdU (ZSGB-BIO, Beijing,
China). The tissues were counted manually using Image J
software (NIH, Bethesda, MD, USA).

**Statistical analysis**
Each experiment was repeated in triplicate. Numerical data
were presented as the mean ± standard deviation, and the
difference between means was analyzed by Student’s t-test.
Differences were considered significant when \( P < 0.01 \).

**Results**

**Expression of miRNA in cisplatin breast
cancer cells and cisplatin-sensitive breast
cancer cells**

In this experiment, two stable cell lines resistant to CDDP
were generated (MCF-7/CDDP and MDA-MB-468/
CDDP). The resistance index of the cells was measured by
MTT assay. The inhibitory concentration (IC\(_{50}\)) values of
CDDP to MCF-7 and MDA-MB-468 cells were 2.35 μM
and 1.82 μM, respectively. The IC\(_{50}\) values of CDDP to
MCF-7/CDDP and MDA-MB-468/CDDP cells increased
32.4 μM and 39.2 μM, respectively. The resistance
indexes of MCF-7/CDDP and MDA-MB-468/CDDP cells
were 13.8 and 22.0 times higher, respectively, than those of
the parental cells. Proliferation inhibition rates are shown
in Figure 1a,b.

MiRNA microarrays were used to analyze the miRNA
expression profiles in human breast cancer MCF-7 and
MDA-MB-468 cell lines and their cisplatin-resistant subpop-
ulations, MCF-7/CDDP and MDA-MB-468/CDDP cell lines.
We defined dysregulated miRNAs as those that displayed
more than threefold upregulation or downregulation. Results
showed that six miRNAs were significantly upregulated
and six were significantly downregulated in both the MCF-7/
CDDP and MDA-MB-468/CDDP cells. The upregulated
miRNAs were miR-141, miR-222, miR-24, miR-26a, miR-
148b, and miR-193a-3p, and the downregulated miRNAs
were miR-21, miR-503, miR-1307, miR-302d, miR-611, and
miR-486, as shown in Figure 1c. The microarray data were
also verified by the qRT-PCR assay, in which expression of
miR-222, miR-26a, miR-148b, miR-1307, miR-491-3p, miR-
21, and miR-486 was measured (Fig 1d). We combined the
expression of miRNA with their bio-functions and chose
miR-1307 as the target miRNA in our research.

**MiR-1307 enhances the inhibition rate of
cisplatin-induced cytotoxicity in MCF-7 and
MDA-MB-468 cells**

In MCF-7/CDDP and MDA-MB-468/CDDP cells com-
pared with the negative control, transfection of miR-1307
mimics markedly increased CDDP-induced cell cytotoxic-
ity (Fig 2a). By contrast, MCF-7 and MDA-MB-468 cells
transfected with miR-1307 inhibitor exhibited greatly
enhanced resistance to CDDP compared with the miRNA
inhibitor control transfected cells (Fig 2b). In colony-
formation assays, overexpression of miR-1307 suppressed
the formation of colonies and knockdown of miR-1307
promoted the formation of colonies (Fig 2c,d). These
results suggest that miR-1307 might regulate CDDP resis-
tance in these human breast cancer cell lines.

**Mdm4 is the target gene of miR-1307 in
human breast cancer MCF-7 and MDA-MB-
468 cells**

To understand the possible mechanisms that underlie
miR-1307-mediated growth suppression, we performed a
bioinformatics study to identify gene targets of miR-1307
using the bioinformatics algorithms microRNA and Tar-
getScan (Whitehead Institute for Biological Research,
Both algorithms indicated that Mdm4 is a theoretical target of miR-137 and that complementary sequences exist between the 3' UTR of MDM4 and miR-1307 (Fig 3a).

To further demonstrate that miR-1307 can directly target MDM4 by binding to the 3’UTR, we constructed an enhanced green fluorescence protein (EGFP) reporter vector in which the predicted target regions of Mdm4 were...
inserted downstream of the EGFP coding region, with or without point mutations in the seed sequence. The constructs were then co-transfected with miR-1307 or control mimics for luciferase activity assays. As shown in Figure 3b, overexpression of miR-1307 significantly decreased the relative EGFP expression in two breast cancer cell types co-transfected with an Mdm4 wild-type 3‘UTR construct (P < 0.05 for MCF-7 cells and P < 0.01 for MDA-MB-468 cells). In contrast, no significant changes were observed in relative EGFP expression in the same cell types co-transfected with a mutated Mdm4 3‘UTR construct in anti-miR-1307 group compared to the control group (Fig 3b). These data indicate that Mdm4 is directly and negatively regulated by miR-1307 in MCF-7 and MDA-MB-468 cells.

We then determined whether expression of miR-1307 leads to the regulation of endogenous MDM4 expression. QRT-PCR showed that overexpression of miR-1307 reduced the levels of Mdm4 mRNA in MCF-7 and MDA-MB-468 cells (Fig 3c). Western blotting indicated that overexpression of miR-1307 also led to a substantial reduction of Mdm4 (Fig 3d), suggesting the negative regulation of Mdm4 by miR-1307. Therefore, Mdm4 is likely to be suppressed by miR-1307 through mRNA degradation and translational inhibition.

Repression of MDM4 in drug-resistant cells is essential for miR-1307-induced restoration of chemosensitivity

We developed a rescue experiment to verify whether the phenotypes observed after miR-1307 overexpression and knockdown were the result of Mdm4 regulation. MCF-7 and MDA-MB-468 cells were treated with 0.5 and 2 μM CDDP while Mdm4 was ectopically overexpressed. The addition of Mdm4 significantly reduced cell viability in both the 0.5 and 2 μM CDDP-treated miR-1307-overexpressing cells (Fig 4a). These inhibitory effects were...
ablaced by the supplementation of MDM4 in MCF-7 and MDA-MB-468 cells. Western blot analysis showed an increase of Mdm4 in MCF-7/CDDP and MDA-MB-468/CDDP cells compared with the parental MCF-7 and MDA-MB-468 cells, as shown in Figure 4b. These results suggest the Mdm4-dependent negative role of miR-1307 on CDDP resistance in breast cancer cell lines.

**Negative correlation between miR-1307 and Mdm4 expression in breast cancer tissues**

To confirm the relationship between miR-1307 and Mdm4 in clinical breast cancer, we analyzed miR-1307 and Mdm4 expression in 20 specimens of cultured breast tissues via RT-PCR. We observed an inverse correlation between miR-1307 and MDM4 expression levels (Fig 4a). We quantified MDM4 expression in both CDDP-sensitive and CDDP-resistant breast cancer tissues by immunohistochemistry (Fig 4b). The results showed higher expression of Mdm4 in the CDDP-resistant tumor tissues compared to CDDP-sensitive tumor tissues. These results demonstrate that miR-1307/MDM4 may play an important role in clinical breast cancer resistance to CDDP (Fig 5).

**Discussion**

Cisplatin-based combination therapies are essential for the clinical treatment of breast cancer; however, various cancerous cells frequently develop multidrug resistance (MDR), which is characterized by tumor cells resisting several unrelated drugs after exposure to a single chemotherapy drug. MDR is observed in tumors with the highest instances of cancer-related mortality. The pathological process and molecular mechanism of drug resistance in breast cancer is complicated and is not yet fully understood. In our previous work, we generated two CDDP-resistant breast cancer cell lines, MCF-7/CDDP and MDA-MB-468/CDDP, and used a miRNA array and RT-PCR to screen the miRNAs in the CDDP-resistant cells that had different levels of expression compared with miRNAs in the non-drug-resistant cells. We found that miR-1307 was substantially downregulated in both human breast cancer cell line cells compared to the parental cell lines. MTT assay showed that transfection of miR-1307 antisense oligo markedly inhibited CDDP-induced cytotoxicity in parent...
MCF-7 and MDA-MB-468 cell lines and that the inhibitory effect was dose dependent. Furthermore, transfection of miR-1307 mimics substantially increased CDDP-induced cytotoxicity in MCF-7/CDDP and MDA-MB-468/CDDP cells.

To date, several miRNAs have been found to participate in drug resistance in cancer cells. miR-181b is known to play a role in the development of MDR in both gastric and lung cancer cell lines, by targeting BCL2.14 miR-508-5p plays a role in the regulation of MDR in gastric cancer.15 However, the mechanisms of miR-1307 involved in drug resistance remain largely unexplored in breast cancer. It has been reported that miR-1307 functions as a tumor suppressor by inhibiting AEG-1 in ovarian cancer,16 and can inhibit cell proliferation by targeting Cdc42 and Cdk6 in lung cancer cells.17

To explore the mechanisms underlying drug resistance in breast cancer by miR-1307, we identified potential target genes of miR-1307. Our bioinformatics analysis indicated that MDM4 was a potential target for miR-1307. MDM4 expression was increased in breast tumor tissue compared with adjacent normal tissue, suggesting a role in the pathological development of breast cancer.18 Mdm4 can negatively regulate p53 transcriptional activity by directly binding its binding domain, which is located in the N-terminal region, to the transactivation domain of p53.19 The tumor suppressor protein p53 is a transcriptional factor that controls multiple genes to regulate the cell cycle, apoptosis, DNA repair, and senescence.20–22

Our previous study explored the hypothesis that MDM4 is a direct target gene of miR-1307 in breast cancer through a series of experiments. First, luciferase activity assay showed that overexpression of miR-1307 significantly reduced the activity of a luciferase reporter containing the 3’UTR sequence of Mdm4. Second, we found that suppression of endogenous miR-1307 expression in MCF-7 cells effectively promoted Mdm4 expression at both mRNA and protein levels, whereas overexpression of miR-1307 moderately suppressed MDM4 expression. This suggests a potential inverse relationship between miR-1307 and MDM4 in breast cancer. Meanwhile, our functional study verified that transfection of miR-1307 inhibitor markedly inhibited CDDP-induced cytotoxicity in parent MCF-7 and MDA-MB-468 breast cancer cell lines. Furthermore, the inhibitory effect was dose dependent. These results show that miR-1307/Mdm4 is linked with chemotherapy resistance in breast cancer. The essential role of MDM4 in miR-1307-induced CDDP sensitivity was further confirmed by the finding that MDM4 knockdown could sensitize the treatment of CDDP in MCF/7MCF-7 and MDA-MB-468 cells in vitro.

Clinical studies have shown an inverse correlation between miR-1307 level and Mdm4 expression. Immunohistochemistry and Western blots indicated that the Mdm4 expression in resistant tissues was higher than in non-resistant tissues. Consistent with previous studies, this provides direct evidence that Mdm4 could be a critical inducer in the development of CDDP-resistance in breast cancer.

The present study revealed that high expression of miR-1307 could sensitize breast tumor cells to CDDP, by modulating Mdm4 expression. Our findings raise the possibility that miR-1307 mimics may have potential therapeutic value for the treatment of breast cancer. Strategies that promote miR-1307 expression in combination with other anti-cancer drugs might serve as a good therapeutic approach for the treatment of advanced breast cancer.

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Disclosure
No authors report any conflict of interest.

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