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Inhibiting microRNA-449 Attenuates Cisplatin-Induced Injury in NRK-52E Cells Possibly via Regulating the SIRT1/P53/BAX Pathway

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Background: Acute kidney injury (AKI) is quite common in the patients who frequently use the anticancer drug cisplatin. microRNAs (miRNAs) are powerful tools in modulating the expression of key factors in disease progression, but little is known about roles of miRNAs in AKI. This study explored the expression and function of miR-449 in cisplatin-induced AKI.

Material/Methods: Rat renal proximal tubular cell line NRK-52E was used for cisplatin treatment and miR-449 sponge transfection. MTT assay and flow cytometry were performed to detect cell viability and apoptosis in different cell groups. Protein expression of sirtuin 1 (SIRT1), acetylated p53, and BCL-associated X protein (BAX) was detected to deduce the possible regulatory mechanism of miR-449.

Results: Results showed that cisplatin treatment in NRK-52E cells significantly up-regulated miR-449 levels ($P<0.05$), inhibited cell viability ($P<0.05$), accelerated cell apoptosis ($P<0.05$), and changed SIRT1, acetylated p53, and BAX protein levels ($P<0.01$). However, inhibiting miR-449 by its sponge transfection in cisplatin-treated cells significantly promoted cell viability ($P<0.05$), suppressed cell apoptosis ($P<0.05$), elevated SIRT1 expression ($P<0.01$), and inhibited acetylated p53 and BAX protein levels ($P<0.001$).

Conclusions: These results indicate that inhibiting miR-449 allows the attenuation of cisplatin-induced injury in NRK-52E cells, suggesting that miR-449 is a potential target for treating AKI. miR-449 regulates the SIRT1/p53/BAX pathway, which may be its possible mechanism in modulating cell apoptosis of cisplatin-induced AKI. Further verification and a thorough understanding are necessary for targeting miR-449 in AKI treatment.

MeSH Keywords: **Acute Kidney Injury • Apoptosis • Cisplatin • MicroRNAs • Sirtuin 1**

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Background

Acute kidney injury (AKI) is a series of clinical syndromes defined as increase in serum creatinine (SCr) by over 26.5 μM within 48 h, or increase in SCr to over 1.5 times baseline occurring within the prior 7 days, or urine volume less than 0.5 mL/kg/h for 6 h [1]. It is usually accompanied by oliguria, electrolyte imbalance, metabolic acidosis, azotemia, and other clinical manifestations. AKI can be divided into 3 categories according to the pathology: prerenal, intrinsic, and postrenal AKI. Molecular markers, such as cystatin C, neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, and interleukin-18, play important roles in the diagnosis of AKI [2–4]. Feasible treatment methods include the use of drugs like fenoldopam [5], and blood purification therapy such as peritoneal dialysis [6], intermittent renal replacement therapy [7], and continuous renal replacement therapy [8].

Cisplatin is a broad-spectrum anticancer drug widely used in the clinical treatment of diseases such as ovarian cancer, testicular cancer, lung cancer, esophagus cancer, and malignant lymphoma. It crosslinks with DNAs and thereby inhibits DNA replication and transcription [9]. Efforts have been made to improve the sensitivity of tumor cells to cisplatin treatment [10,11]. However, a difficult problem is the adverse effect of cisplatin in normal tissues, causing nephrotoxicity, myelosuppression, anaphylaxis, and neurotoxicity [12], severely limiting the clinical use of cisplatin. The impacts of cisplatin on the kidneys are mainly manifested in the apoptosis of renal tubular cells, because cisplatin induces endoplasmic reticulum stress, activates the apoptotic pathways (including the death receptor pathway and mitochondrial pathway), and leads to caspase-dependent or -independent apoptosis [13]. Inflammatory responses and reactive oxygen species induced by cisplatin also aggravate AKI [14,15].

microRNAs (miRNAs) are small endogenous RNA molecules that recognize specific target sequences, thus regulating mRNA stability and translation activity. Recent findings have reported the vital roles of miRNAs in modulating key factors in AKI. miR-34, miR-125b, and miR-127 are confirmed to play protective roles in the context of AKI, while miR-21, miR-29, and miR-192 are involved in the progression of renal fibrosis [16]. miR-21 is an especially promising biomarker for diagnosing and treating AKI because of its association with cell apoptosis, and inflammatory and fibrotic pathways [17]. From this point of view, research in miRNAs is of great value for a better understanding of and novel therapeutic strategies for AKI.

A recent study has indicated that miR-449 is almost absent in the kidneys [18], but its expression during AKI remains uncertain. This study investigated the expression pattern and roles of miR-449 in cisplatin-induced AKI. Rat renal proximal

tubular cell line NRK-52E was used to stimulate AKI *in vitro* by cisplatin treatment, and then miR-449 was inhibited by its sponge transfection. The changes in cell viability and apoptosis induced by cisplatin and miR-449 inhibition were detected by MTT and flow cytometry. This study also investigated the regulatory mechanism of miR-449 in AKI by detecting expression of its possible target sirtuin 1 (SIRT1) and downstream factors tumor protein p53 and BCL2-associated X protein (BAX). These results may provide an overview of miR-449 in cisplatin-induced AKI and offer a potential therapeutic target for treating this disease.

Material and Methods

Cell model

Rat renal proximal tubular cell line NRK-52E (Institute of Biochemistry and Cell Biology, Shanghai, China) was cultured in Dulbecco's modified Eagle medium/F12 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere with 5% CO₂ at 37°C. Cells were first divided into 2 groups: the cisplatin-induced renal injury model group and the control group. Cells of the cisplatin group were treated with 20 $\mu\text{g}/\text{mL}$ cisplatin (Abcam, Cambridge, UK) for 24 h before cell transfection.

Transfection

The cisplatin-treated cells were seeded in 6-well plates (1 \times 10⁶ per well) 1 day before transfection to 90% confluency. The cells were then divided into 3 groups: cisplatin (cisplatin-treated cells without transfection), cisplatin + blank vector (cisplatin-treated cells transfected with blank vector as a control), and cisplatin + miR-449 sponge (cisplatin-treated cells transfected with miR-449 sponge to inhibit miR-449). The cells were transfected with miR-449 sponge (2 $\mu\text{g}/\text{mL}$) or blank control synthesized by QuantoBio (Beijing, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The medium was changed at 6 h after transfection, and cells were collected for further analysis at 48 h after transfection.

Cell viability assay

Cells in the logarithmic phase were collected for cell viability assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method using MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, 100 μL of cell suspension (5 \times 10³ cells) was added to each well of a 96-well plate, and 10 μL MTT buffer was added for a 4-h incubation at 37°C, after which 100 μL of Formazan dissolving solution

was added to each well. The plate was incubated until formazan dissolved. Then the optical density (OD) at 570 nm was measured using a microplate reader (Bio-Rad, Hercules, CA). OD of cisplatin, cisplatin + blank control, or cisplatin + miR-449 sponge groups was compared to that of the control group.

Cell apoptosis assay

Cell apoptosis was detected by flow cytometry after treatment using the Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Briefly, cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in binding buffer. We added 100 μ L of cell suspension (1×10^5 cells) to each well of a 96-well plate, and 2 μ L of Annexin V-FITC was added for a 15-min incubation on ice in the dark. Before detection, 400 μ L of PBS and 1 μ L of propidium iodide (PI) were added. Flow cytometry (BD Biosciences) was used to detect the FITC-positive and PI-negative cells (early apoptotic), which were regarded as apoptotic cells in the analysis.

qPCR

miRNAs of each cell group were isolated using RNAiso for Small RNA (TaKaRa, Dalian, China) according to the manufacturer's instructions. A specific primer for rno-miR-449a-5p (5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG ACC AGC TA-3') was used in the reverse transcription catalyzed by PrimeScript Reverse Transcriptase (TaKaRa). miR-449 expression level in these cell groups was detected by quantitative real-time PCR on platform LightCycler 480 (Roche, Basel, Switzerland) with each reaction system containing 20-ng templates and the specific primers for rno-miR-449a-5p (Fw: 5'-ACA CTC CAG CTG GGT GGC AGT GTA TTG TTA-3' and Rv: 5'-TGG TGT CGT GGA GTC G-3'). U6 (Fw: 5'-CTC GCT TCG GCA CA-3' and Rv: 5'-AAC GCT TCA CGA ATT TGC GT-3') was used as an internal control. Data were analyzed with $2^{-\Delta\Delta Ct}$ method.

Western blot

Protein samples of the 4 cell groups were extracted by lysis buffer (Beyotime). The protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with 20 μ g of protein loaded in each lane. The protein on gel was transferred to a polyvinylidene fluoride membrane and blocked in 5% skim milk for 2 h at room temperature, after which the membrane was incubated in primary antibodies for SIRT1, acetylated p53, or BAX (ab110304, ab61241, ab32503, Abcam) overnight at 4°C. GAPDH (ab181602) was used as an internal control. After washing 3 times in PBS, the membrane was incubated in horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were developed using the EasyBlot ECL Kit (Sangon Biotech, Shanghai, China),

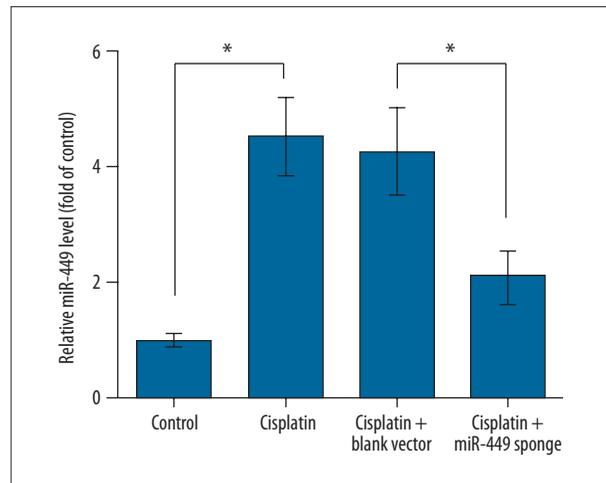


Figure 1. miR-449 is promoted by cisplatin treatment and successfully inhibited by its sponge in NRK-52E cells. qPCR shows that cisplatin treatment significantly elevates the expression of miR-449 compared to untreated cells, and that miR-449 is significantly down-regulated by its sponge transfection compared to the transfection control. qPCR detection is performed at 48 h after transfection (n=5). Control=NRK-52E cells without any treatment. Cisplatin=cells treated with cisplatin. Cisplatin + blank vector=cells treated with cisplatin and then transfected with blank vector as a transfection control. Cisplatin + miR-449 sponge=cells treated with cisplatin and then transfected with miR-449 sponge. * $P < 0.05$.

and the grey-scale value of tested proteins was analyzed using the ChemiDoc XRS System and compared to that of the GAPDH.

Statistical analysis

All the experiments were performed in triplicate. Results were indicated as the mean \pm standard deviation. Data were analyzed using the *t* test and one-way ANOVA in SPSS 20. Differences were considered significant at $P < 0.05$.

Results

miR-449 level is elevated after cisplatin treatment in NRK-52E cells

The expression level of miR-449 was detected by qPCR in the 4 cell groups (Figure 1). Compared to the control group, cisplatin induced significant up-regulation of miR-449 expression in NRK-52E cells ($P < 0.05$), implying the possible relationship of miR-449 expression and the cisplatin-induced renal tubular cell injury, which inspired us to investigate the role of miR-449 by inhibiting its expression in renal cell injury. But before that, the inhibition of miR-449 by its sponge vector transfection

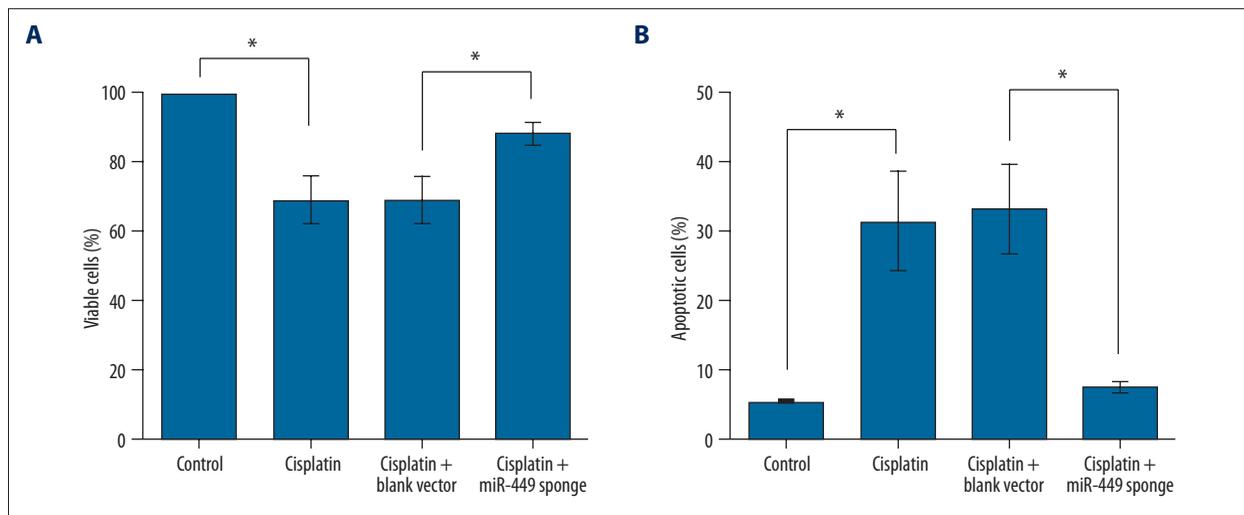


Figure 2. Inhibiting miR-449 promotes NRK-52E cell viability and suppresses cell apoptosis induced by cisplatin. MTT and flow cytometry were used to detect cell viability (A) and cell apoptosis (B), respectively, at 48 h after transfection (n=5). Cisplatin treatment inhibits cell viability and promotes cell apoptosis, which is abrogated by miR-449 sponge. * $P < 0.05$.

was verified to be effective, and results showed the miR-449 level after cisplatin treatment was significantly suppressed by its sponge compared to blank vector transfection ($P < 0.05$). Therefore, these transfected NRK-52E cells were used in the following experiments.

Inhibiting miR-449 promotes cell viability and suppresses cell apoptosis

The changes in NRK-52E cells by miR-449 were analyzed from cell viability and apoptosis. MTT results indicated that cisplatin significantly inhibited cell viability compared to the control ($P < 0.05$, Figure 2A), while miR-449 sponge transfection resulted in a recovery compared to the transfection control ($P < 0.05$). The percentage of FITC-positive and PI-negative apoptotic cells were increased after cisplatin treatment ($P < 0.05$, Figure 2B), and miR-449 sponge reduced the apoptotic cells compared to the transfection control ($P < 0.05$). These data suggest that cisplatin had cytotoxicity in NRK-52E cells, influencing both cell viability and cell apoptosis, and that inhibiting miR-449 promoted cell viability and suppressed cell apoptosis, which might protect NRK-52E cells from cisplatin-induced renal tubular cell injury.

miR-449 regulates SIRT1, p53 acetylation and BAX

This study tried to investigate the possible mechanism of miR-449 in NRK-52E cells. miR-449 was predicted to target *Sirt1* mRNA by online database microRNA.org-Targets and Expression (www.microrna.org), and some studies have indicated *Sirt1* mRNA to be a direct target for miR-449 [19], so the protein expression of SIRT1 and its downstream p53 and BAX was detected by Western blot analysis (Figure 3A). Results showed that, as predicted, SIRT1 was inhibited after cisplatin

treatment when miR-449 was up-regulated, and then it was promoted by miR-449 sponge transfection. Acetylated p53 and BAX showed the opposite changes: they were promoted by cisplatin treatment and then inhibited by miR-449 sponge. Repeated results of Western blot analysis indicated significant differences after cisplatin treatment or miR-449 sponge transfection ($P < 0.01$ or $P < 0.001$, Figure 3B). These data suggest that inhibiting miR-449 affects the expression of SIRT1, p53 acetylation and promote BAX expression, which might be a potential mechanism of miR-449 in cisplatin-induced renal tubular cell injury.

Discussion

miRNA research has become popular in AKI studies, but roles of miRNAs in cisplatin-induced AKI remain elusive. This study reveals the up-regulated miR-449 level in cisplatin-treated NRK-52E cells, and that inhibiting miR-449 reverses the pro-apoptotic effects of cisplatin on NRK-52E cells, and that miR-449 regulates SIRT1 and downstream p53 and BAX.

As a widely-used antitumor drug in chemotherapy, cisplatin is generally found to have adverse effects, especially its nephrotoxicity, which promotes apoptosis of renal tubular cells. Results of this study indicated that cisplatin could up-regulate miR-449 expression and affect NRK-52E cells, both inhibiting the viability and intensifying the apoptosis. These *in vitro* results are in line with the adverse effects of cisplatin found in clinical treatment. Moreover, findings of this study establish a possible relationship between miR-449 level and cisplatin-induced cell injury, which needs further verification.

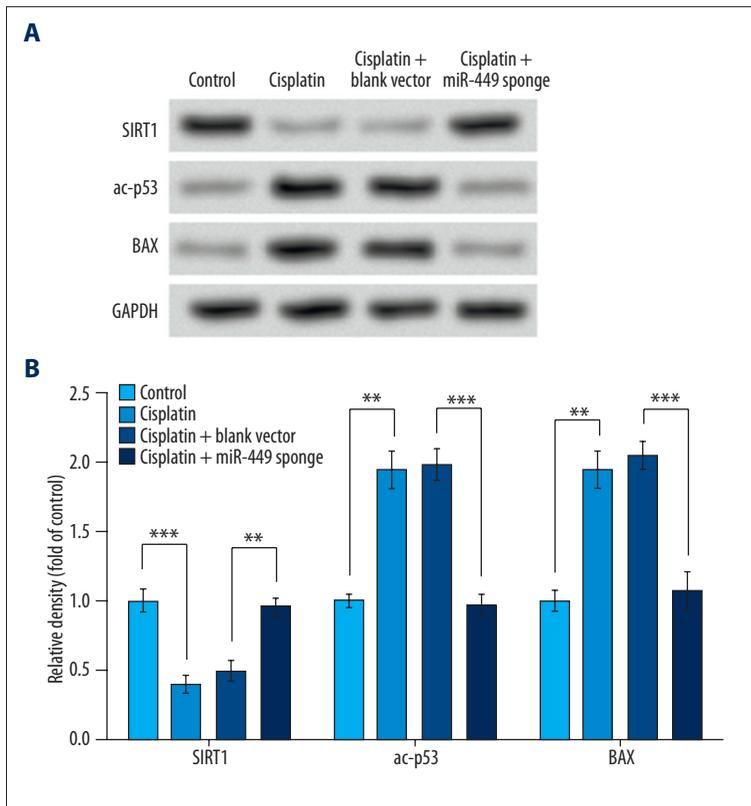


Figure 3. miR-449 regulates SIRT1, p53 acetylation, and BAX in cisplatin-treated NRK-52E cells. Cisplatin induces the down-regulation of SIRT1 and the up-regulation of acetylated p53 and BAX, while inhibiting miR-449 in the treated cells promotes SIRT1 expression and suppresses acetylated p53 and BAX levels. Western blot analysis was performed to detect protein levels, with GAPDH as an internal control (A), and a histogram is generated based on triplicate Western blot experiments (B). ** $P < 0.01$. *** $P < 0.001$. SIRT1, sirtuin 1. ac-p53=acetylated tumor protein p53. BAX=BCL2-associated X protein.

miR-449 expression was significantly up-regulated in cisplatin-treated NRK-52E cells compared to the untreated cells, and its inhibition was confirmed to promote cell viability and inhibit cell apoptosis of cisplatin-treated NRK-52E cells, implying the pro-apoptotic role of miR-449 in cisplatin-induced AKI. Similar results have been found in gastric cancer, where miR-449 inhibits cell proliferation and is down-regulated [19], and in prostate cancer with miR-449-induced cell cycle arrest [20]. In primary human bronchial epithelial cells exposed to volatile cigarette smoke, both miR-449 and its transcription factor E2F1 levels are increased, which may allow the elimination of cells with damaged DNA [21]. Taken together, the functions of miR-449 in various cell types and diseases are relatively conserved: its pro-apoptotic role helps to prevent cancer progression in cancer cells and aggravates injury degree in AKI, as found in this study.

SIRT1 is a direct target for miR-449 based on existed studies [19] and online prediction, so this study did not perform verification using luciferase report assay. It is generally considered that SIRT1 plays crucial roles in cisplatin-induced AKI, with its overexpression protects renal proximal tubular cells [22]. Tumor protein p53 functions as an apoptosis facilitator in various diseases, including kidney injury and carcinoma [23,24], as well as cisplatin-induced AKI, with its down-regulation attenuating the injury [25,26]. SIRT1 deacetylates p53, thus regulating cell apoptosis during kidney injury [27,28]. BAX is a downstream factor of p53 that indicates cell apoptosis in numerous

studies [29]. Upon DNA damage, p53 induces BAX translocation to mitochondria and cytochrome C release is promoted accordingly [30,31], which is also one of the molecular mechanisms of renal cell apoptosis [32]. Thus, SIRT1/p53/BAX has an important role in signaling in the mitochondria apoptosis pathway.

Based on the above information that the mitochondria apoptosis pathway is one of the mechanisms in AKI, this study attempted to reveal the possible regulatory mechanism of miR-449 in AKI, and found that inhibiting miR-449 promoted SIRT1 expression and inhibited p53 acetylation and BAX expression, which were altered by cisplatin treatment in NRK-52E cells. It can be deduced that the cisplatin-induced miR-449 up-regulation inhibits SIRT1, which further elevates acetylated p53 and BAX levels, thus activating the p53/BAX signaling of the mitochondria apoptosis pathway. Therefore, miR-449 may be a promising target in AKI treatment. The regulation of the SIRT1/p53/BAX mitochondria apoptosis pathway may be a potential functioning mechanism of miR-449 in cell apoptosis of cisplatin-induced AKI. However, more verification is necessary to confirm the direct association between miR-449-regulated SIRT1/p53/BAX signaling and cisplatin-induced AKI. It is possible that miR-449 targets other factors, such as histone deacetylases [33], and affects brain development, spermatogenesis and multiciliogenesis [34–36]. Therefore, a comprehensive understanding of the miR-449-involved regulatory network is indispensable for its application to AKI clinical treatment.

Conclusions

Our study indicates that inhibiting miR-449 in cisplatin-treated NRK-52E cells attenuates the affected cell viability and promoted cell apoptosis caused by cisplatin, indicating miR-449 is a potential therapeutic target for treating AKI. In addition to the possible SIRT1/p53/BAX signaling regulated by miR-449,

more thorough information on the mechanism will be helpful for future research.

Conflict of interests

No conflict of interests exists.

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