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# Effects of miR-222 on cisplatin resistance of renal cancer cell strains and related mechanisms

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Cisplatin is widely employed in combating renal cancers. However, a major challenge is the resistance of cisplatin by renal cancer cells. Increased miR-222 expression is known to promote this resistance and so ways of downregulating the expression of miR-222 is widely sought after as a means of combating the resistance. The study was performed to explore the effects of miR-222 on cisplatin resistance of renal cancer cell strains and related mechanisms. The expression of miR-222 and Dickkopf-3 (DKK3) was regulated to explore the effects of miR-222 and DKK3 on cisplatin chemotherapy in renal cancer cisplatin-resistant cell strains, and to figure out the regulatory relationship between miR-222 and DKK3. In renal cancer tissues and cell lines, miR-222 was highly expressed and DKK3 was lowly expressed. Renal cancer cisplatin-resistant cells had markedly higher miR-222 expression and lower DKK3 expression than normal renal cancer cells, suggesting a potential involvement of miR-222 and DKK3 in the cisplatin resistance in renal cancer cells. Down-regulated miR-222 expression led to stronger inhibition of the renal cancer cell growth by cisplatin and markedly higher DKK3 expression. Dual-luciferase reporter assay results showed that miR-222 had a targeted inhibition on DKK3. Co-transfection of miR-222 mimic and shDKK3, together with the up-regulation of miR-222 and DKK3 expressions, resulted in a higher sensitivity of renal cancer cells to cisplatin chemotherapy than the up-regulation of miR-222 expression alone. Down-regulated miR-222 expression can remove the inhibition of DKK3 expression and increase the sensitivity of renal cancer cells to cisplatin chemotherapy.

**Keywords**: Dickkopf-3, Kidney, microRNAs, PCR, Proliferation

Accounting for about 3% of all malignant tumors, renal cell carcinoma is quite common. In recent years, renal clear cell carcinoma has had a yearly stepwise increase in its incidence, with annual new cases of about 250000 patients and annual deaths of over 100000 people[1,2].

Despite the great progress in treatment strategies, the prognosis of patients using existing treatment methods is still disappointing. Chemotherapy resistance and metastasis are the main causes of deaths from renal cancer. Renal cancer generally shows low response to conventional chemotherapy, for example, patients with metastatic renal cell carcinoma (RCC) often develop resistance against anti-angiogenic therapy[3].

Cisplatin is the most widely used and most effective chemotherapeutic drug, which, unfortunately, results in a general resistance against cisplatin in cancer treatment[4]. MicroRNAs (miRNAs) are a group of non-coding RNAs, 19 to 24 nucleotides in length, forming a complex biological process regulatory network, enjoying an involvement in life activities such as tumorigenesis[5].

Recently, there is increasing evidence that miRNAs play a crucial role in chemical resistance, but the molecular mechanisms by which miRNAs regulate drug resistance in various cancers remain unclear[6,7]. miR-222, located in Xp11.3, is down-regulated in multiple tumors, including renal cancer[8,9]. MiR-222 is a promising predicting marker for the treatment of metastatic renal cell carcinoma with sunitinib, and it can affect the sensitivity of renal cancer cells to sunitinib treatment by a target binding to the vascular endothelial growth factor receptor. Gu *et al.*[10] discovered the involvement of miR-222 in tamoxifen resistance of breast cancer cells.

The above-listed studies believe miR-222 is closely related to tumor cell resistance, but little is reported on the relationship between miR-222 and cisplatin resistance in renal cancer cells. This study explored the effects of miR-222 on cisplatin resistance of renal

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cancer cell strains and related mechanisms, hoping to find out new therapeutic targets for clinical treatment of renal cancer.

## Materials and Methods

### Research subjects and source of patients

Nineteen samples of renal cancer tissues and 19 samples of corresponding tumor-adjacent tissues were collected from renal cancer patients (aged from 45 to 60 years) who were admitted to our hospital (Linyi People's Hospital, Linyi, Shandong, 276000, China) from March 2017 to May 2018. Inclusion criteria included patients with complete medical records and diagnosed with renal cancer by postoperative pathological diagnosis. Exclusion criteria included patients with metastatic renal cancer or previous presence of other tumors; pregnant or lactating women. This study was approved by the ethics committee of our hospital (Linyi People's Hospital, Linyi, Shandong, 276000, China) and awarded ethical committee number (LPH/PH/18/056). All patients and their families gave informed consent through the telephone or letter.

### Source of cells

Human renal epithelial cells 293F and human renal cancer cells A498, SN12C, A704, OS-RC-2, 769-P were purchased from BeNa Culture Collection (cell numbers: BNCC340697, BNCC338630, BNCC341858, BNCC342393, BNCC100729, BNCC101643). Normal cell culture medium was composed of 90% high glucose DMEM (Thermo Fisher Scientific Inc., item No. 11965084) + 10% fetal bovine serum (Thermo Fisher Scientific Inc., item No. 16250086). Renal cancer cell strain medium was composed of 90% RPMI-1640 medium (Thermo Fisher Scientific Inc., item No. 61870044) + 10% fetal bovine serum + penicillin at a concentration of 100 U/mL and streptomycin at a concentration of 100 μg/mL. Cells were incubated in a constant temperature incubator at 37°C under 5% $CO_2$. The cells were passaged for 2-3 generations before use.

### Construction of cell strains with drug resistance

Drug resistance of cells was induced by pulse therapy combined with continuous stepwise exposure to the drug. Logarithmically growing renal cancer cells were resuspended and routinely seeded in 96-well plates (3000 cells per well), and cultured in an incubator with 5% $CO_2$ at 37°C for 6-12 h. Then various concentrations of cisplatin (0.001, 0.01, 0.1, 1, and 10 μg/mL) were added to the culture plates, 5 parallel wells for each concentration. After 48 h of culture, MTT assay was used to determine the cell growth inhibition rate and the $IC_{50}$ value was calculated. Renal cancer cells were re-inoculated, and the cisplatin resistance of renal cancer cells was induced with an initial concentration of 10% of $IC_{50}$ value. The drug concentration was increased at the time of stable cell growth, finally the stable survive of cells was achieved at a cisplatin concentration of 0.5 μg/mL.

### Construction and transfection of the expression vector

miR-122-mimic, miR-122-inhibitor, miR negative control (miR-NC), and targeted Dickkopf-3 (DKK3) RNA (shDKK3) were constructed by Shanghai GenePharma Co., Ltd. The miR-NC group, miR-222 mimic group, miR-222 inhibitor group, and co-transfection group (miR-596 mimic + shDKK3) were set. The renal cancer resistant cell strains were digested by trypsin 24 h before the transfection. The transfection of expression vector was performed when the cells reached 80% confluence in strict accordance with the kit instructions. The incubator was cultured at 37°C with 5% $CO_2$ for 48 h, and the medium was changed every 6 h. Transfection results were detected by qRT-PCR and Western blot methods. The Lipofectamine™ 2000 Transfection Kit was purchased from Invitrogen, USA (item number No. 35050).

### qRT-PCR detection

This was performed as described by Crookenden *et al.* with little modifications[11]. The total RNA was extracted from collected cells using a TRIzol kit (Invitrogen, USA, 15590618). The purity, concentration, and integrity of total RNA were measured by ultraviolet spectrophotometry and agarose gel electrophoresis. The reverse transcription was conducted according to the cDNA synthesis kit manual. The amplification system of miR-222 was as follows: 1 μL of cDNA, 0.4 μL of forward primer, 0.4 μL of reverse primer, 10 μL of 2×TransTaq® Tip Green qPCR SuperMix , 0.4 μL of Passive Reference Dye (50×), and enough ddH2O to complement the system to 20 μL. PCR amplification conditions: 40 cycles of pre-denaturation at 94°C for 30 sec, denaturation at 94°C for 5 sec, annealing and extension at 60°C for 30 sec. Three replicate wells were set for each sample, and the experiment was carried out for 3 times. U6 was used as the internal reference of miRNA, GAPDH was the internal reference of mRNA. The data were analyzed using $2^{-\triangle\triangle ct}$. The qRT-PCR and reverse transcription kits

were purchased from TransGen Biotech, Beijing, China (item numbers: AQ201-01, AQ202-01). The primer sequences were shown in (Table 1).

**Western Blot analysis**

Western blot analysis was performed as described by Zhaowei *et al.* with slight modification[12]. The total protein was extracted by RIPA lysis method, and its concentration was determined by BCA method. Then the protein concentration was adjusted to 4 μg/μL and the protein was separated by 12% polyacrylamide gel electrophoresis. The initial voltage was 90 V, and then the voltage was increased to 120 V to move the sample to the appropriate position of the separation gel. After the accomplishment of electrophoresis, the membrane was transferred (100 V constant pressure for 100 min and 37°C for 60 min). The transferred membrane was then blocked in 5% skim milk and the immune reaction was carried out. The membrane was incubated with the primary antibody (1:1000) overnight at 4°C. On the next day, the membrane was washed for three times with PBS (5 min for each time) and then incubated with secondary antibody (1:1000) for 1 h at room temperature (25°C). Following that, color development and fixation were performed using ECL luminescence reagent. Statistical analysis was performed on the band after film scanning using Quantity One software. Protein relative expression level = gray value of band/gray value of internal reference. RIPA kit, BCA protein kit, ECL luminescence kit, and trypsin were purchased from Thermo Scientific™ (item number: 89901, 23250, 35055, 90058). The anti-rabbit DKK3 monoclonal antibody and goat anti-rabbit IgG secondary antibody was purchased from the Abcam (the United States, item number: ab38594, ab6721).

**Dual-luciferase reporter assay**

Dual luciferase reporter gene assay was performed as described by Ting *et al.* with adjustments[13]. Human embryonic kidney cell 293T was purchased from BeNa Culture Collection (cell number: BNCC100530). Logarithmically growing cells were harvested and transfected with pmirGLO-DKK3-3'UTR wild type (Wt), pmirGLO-DKK3-3'UTR mutant type (Mut), miR-222-mimics, and miR-NC. The fluorescence intensity was measured 48 h after the transfection using a dual-luciferase assay system (CytoFLE S flow cytometer, Beckman, the USA). The sequences were designed by Thermo Fisher Scientific (China) Co., Ltd.

Table 1 — Primer sequences

| | forward primer | reverse primer |
|---|---|---|
| miR-222 | 5′-GTTCGTGGGAG CTACATCTGGC-3′ | 5′-GCAGGGTCC GAGGTATTC-3′ |
| U6 | 5′-GCGCGTCGT GAAGCGTTC-3′ | 5′-GTGCAGG GTCCGAGGT-3′ |
| GAPDH | 5′-TGCACCACCA ACTGCTTAG-3′ | 5′-GATGCAGG GATGATGTTC-3′ |

**Cell proliferation assay *in vitro***

Cell proliferation assay using MTT was performed as described by Tazehkand *et al.*[14]. The cells were harvested 24 h after the transfection and seeded in 96-well plates at a density of $3*10^4$ cells/well, and incubated at 37°C for 48 h. Next, 20 μL of MTT solution (5 μg/mL) was added and the plates were continuously inoculated at 37°C for 4 h. Then, 150 μL of dimethyl sulfoxide was added to each well. Finally, the OD value of each group was measured using a microplate reader (Shanghai Flash Spectrum Biotechnology Co., Ltd.) at 490 nm. MTT assay kit was purchased from Beijing Fangcheng Jiahong Technology Co., Ltd.

**Statistical analysis**

Statistical analysis was performed sing SPSS19.0 (Asia Analytics Formerly SPSS China). The measurement data were expressed as Mean ± Standard deviation (Mean ± SD). The comparison between the two groups was performed by independent sample *t*-test, and the comparison between multiple groups was analyzed by one-way ANOVA and LSD post hoc test. Pearson correlation was used to analyze the correlation between miR-222 and DKK3 expression. A statistical difference was recognized when $P < 0.05$.

## Results

**Expression levels of miR-222 and DKK3 in renal cancer tissues**

Renal cancer tissues had notably higher miR-222 expression and notably lower DKK3 expression than adjacent normal tissues (both $P < 0.05$). Pearson analysis indicated a negative correlation between miR-222 and DKK3 expressions ($P < 0.05$). (Fig. 1)

**Expression levels of miR-222 and DKK3 in renal cancer cell strains**

miR-222 expression was significantly higher in renal cancer cells A498, SN12C, A704, OS-RC-2, and 769-P than in renal epithelial cells 293F ($P < 0.05$), while DKK3 was significantly lower than in 293F cells ($P < 0.05$) (Fig. 2).

**Construction of cell strains with drug resistance**

The growth inhibition rates of renal cancer cell strains A498 and OS-RC-2 exposed to different

concentrations of cisplatin are shown in (Fig. 3A & B). According to the linear regression equation, the $IC_{50}$ of A498 and OS-RC-2 were 0.1077 μg/mL and 0.0733 μg/mL, respectively. Cisplatin resistance was induced by continuous stepwise exposure to cisplatin combined with pulse therapy *in vitro*. The growth inhibition rates of cisplatin-resistant cells A498 (A498/DDP) and OS-RC-2 (OS-RC-2/DDP) exposed to different concentrations of cisplatin are shown in (Fig. 3C & D). The $IC_{50}$ of A498 and OS-RC-2 were 9.5170 μg/mL and 13.1129μg/mL, respectively, suggesting a success in constructing cisplatin-resistant cell strains.

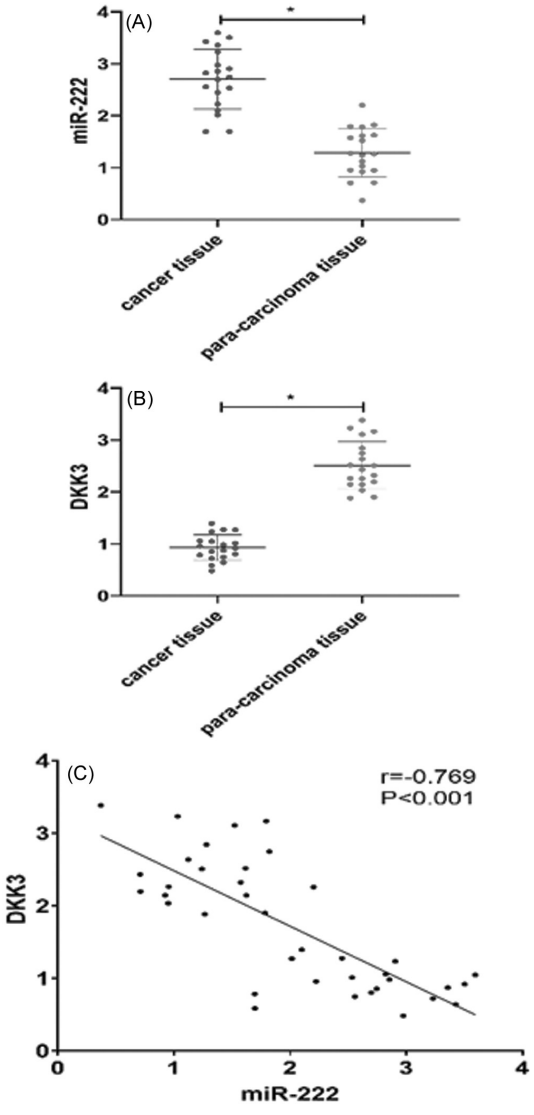


Fig. 1 — Expression levels of miR-222 and DKK3 in renal cancer tissues (A) Expression level of miR-222 in renal cancer tissues; (B) Expression level of DKK3 in renal cancer tissues; and (C) Correlation analysis between miR-222 and DKK3 expressions in renal cancer tissues

**Expression levels of miR-222 and DKK3 in renal cancer cell strains with drug-resistance**

A498/DDP and OS-RC-2/DDP cells had notably higher miR-222 level and lower DKK3 level than A498 and OS-RC-2 cells (both $P$ <0.05) (Fig. 4).

**Effects of down-regulated miR-222 on cisplatin chemotherapy in renal cancer cell strains**

After transfection with miR-222 inhibitor, the expression levels of miR-222 in A498/DDP and OS-RC-2/DDP cells in the miR-222 inhibitor group were significantly lower than those in the miR-NC group ($P$ <0.05). After 48 h of culture in cisplatin at a concentration of $IC_{50}$ for cisplatin-resistant cell strains, the growth inhibition rates of A498/DDP and OS-RC-2/DDP cells in the miR-222 inhibitor group were significantly higher than those in the miR-NC group ($P$ <0.05) (Fig. 5).

**Effects of down-regulated miR-222 on the DKK3 expression**

After transfection with miR-222 inhibitor, the expression levels of DKK3 in A498/DDP and OS-RC-2/DDP cells in the miR-222 inhibitor group were significantly higher than those in miR-NC group ($P$ <0.05). The dual-luciferase report assay revealed a targeted regulation on DKK3 by miR-222 ($P$ <0.05) (Fig. 6).

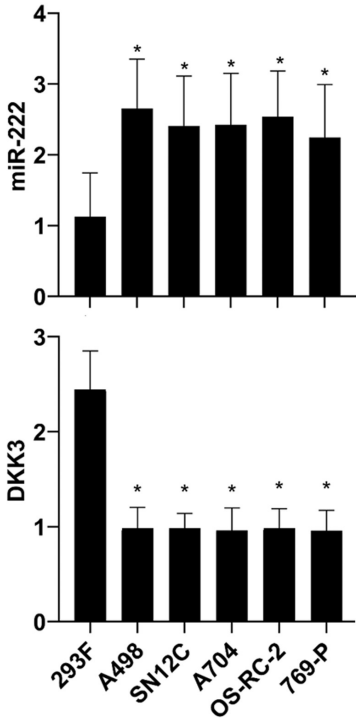


Fig. 2 — Expression levels of miR-222 and DKK3 in renal cancer cell strains (A) Expression level of miR-222 in renal cancer cell strains; and (B) Expression level of DKK3 in renal cancer strains

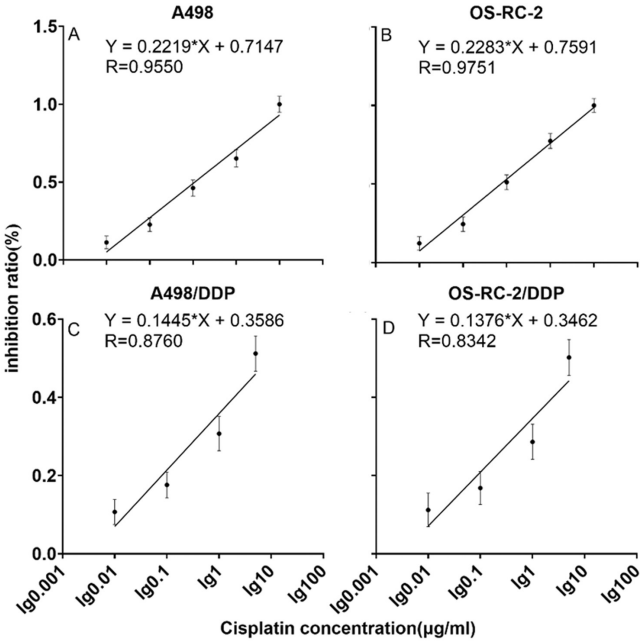


Fig. 3 — Construction of cell strains with drug resistance (A) Growth inhibition of renal cell strain A498 by different concentrations of cisplatin; (B) Growth inhibition of renal cell strain OS-RC-2 by different concentrations of cisplatin; (C) Growth inhibition of renal cell A498/DDP by different concentrations of cisplatin; and (D) Growth inhibition of renal cell OS-RC-2/DDP by different concentrations of cisplatin

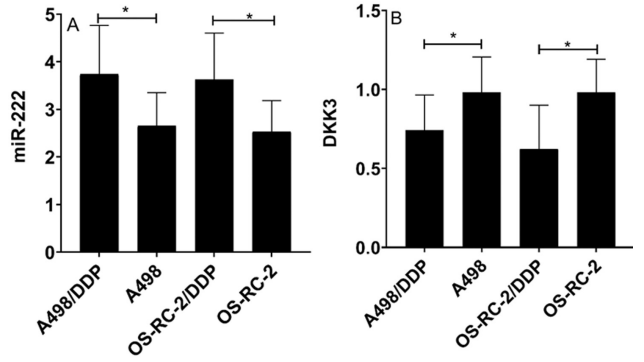


Fig. 4 — Expression levels of miR-222 and DKK3 in renal cancer cell strains with drug-resistance (A) miR-222 expression in A498/DDP and OS-RC-2/DDP cells; and (B) DKK3 expression in A498/DDP and OS-RC-2/DDP cells

**Rescue experiment**

After co-transfection with miR-222 mimic + shDKK3, the expression levels of DKK3 in A498/DDP and OS-RC-2/DDP cells in the co-transfection group were significantly higher than those in miR-222 mimic group ($P$ <0.05) while no significant difference was detect in the expression of miR-222 between the two groups ($P$ >0.05). After 48 h of culture in cisplatin at a concentration of $IC_{50}$ for cisplatin-resistant cell strains, the growth

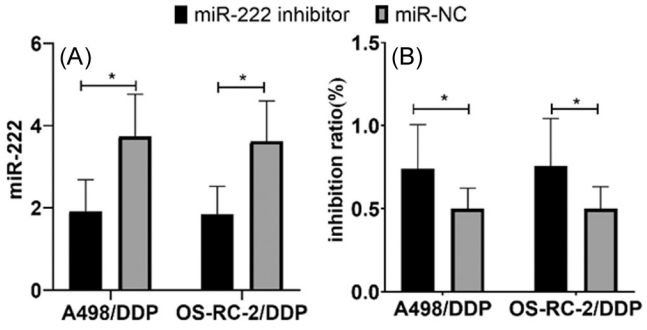


Fig. 5 — Effects of down-regulated miR-222 on cisplatin chemotherapy in renal can cell strains (A) Results of transfection with miR-222 inhibitor; and (B) Effects of down-regulated miR-222 on cisplatin chemotherapy in A498/DDP and OS-RC-2/DDP cells

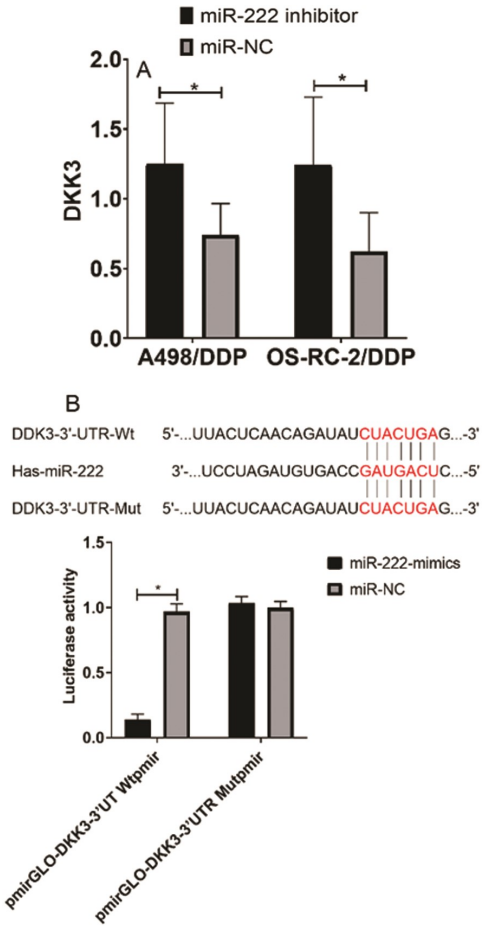


Fig. 6 — Certification analysis on the relationship between miR-222 and DKK3 (A) Effects of down-regulated miR-222 on the DKK3 expression; and (B) Dual-luciferase reporter assay

inhibition rates of A498/DDP and OS-RC-2/DDP cells in the miR-222 mimic group were significantly lower than that in the co-transfection group ($P$ <0.05) (Fig. 7).

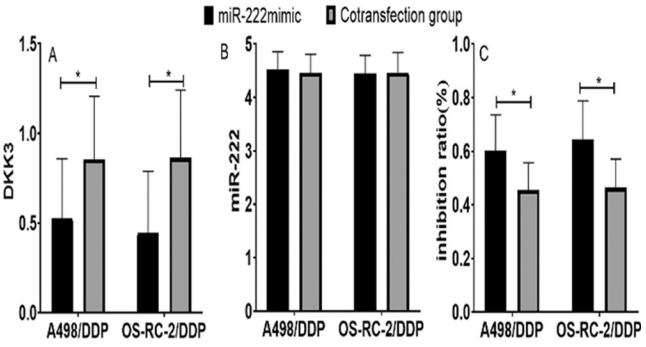


Fig. 7 — Rescue experiment (A) Effects of co-transfection on the DKK3 expression; (B) Effects of co-transfection on the miR-222 expression; and (C) Effects of co-transfection on cisplatin chemotherapy in A498/DDP and OS-RC-2/DDP cells

## Discussion

DKK3 is a soluble Wnt inhibitor that binds to low-density lipoprotein receptor-associated protein 5/6 and disrupts the stability of cellular plasma β-catenin to play a role in molecular therapy of tumors. Studies by Mohammadpour *et al.*[15] have reported the down-regulation of DKK3 in various tumors including renal cancer. In recent years, DKK3 has been found to be closely related to tumor cell resistance, but whether it has an influence on of renal cancer cell resistance is not sure. miR-222 has also been reported to be associated with a variety of tumor resistance. So far, it has not been figured out whether miR-222 is involved in tumor resistance by regulating DKKS. The present study revealed that down-regulated miR-222 expression can remove the inhibition of DKK3 expression and increase the sensitivity of renal cancer cells to cisplatin chemotherapy.

In this study, miR-222 was highly expressed in renal cancer tissues and cell lines and DKK3 was lowly expressed in them, besides, renal cancer cisplatin-resistant cells had higher miR-222 expression and lower DKK3 expression than ordinary renal cancer cells, suggesting that miR-222 and DKK3 may be associated with cisplatin resistance in renal cancer cells. The down-regulation of miR-222 expression resulted in stronger inhibition of the renal cancer cell growth by cisplatin and markedly up-regulation of DKK3 expression. Dual-luciferase reporter assay results showed that miR-222 could enhance the fluorescence intensity of DKKS, indicating a targeted inhibition on DKK3 by miR-222.

The rescue experiment demonstrated that the up-regulation of miR-222 and DKK3 expressions combined with the co-transfection of miR-222 mimic and shDKK3 resulted in a higher sensitivity of renal cancer cells to cisplatin chemotherapy than the up-regulation of miR-222 expression alone did, indicating that DKK3 is a target for miR-222 to regulate cisplatin resistance in renal cancer cells.

Recently, some studies have unveiled the role of miR-222 in affecting tumor cell resistance and its mechanism. Yu *et al.*[16] reported that exosomes of adriamycin-resistant breast cancer cells can transfer miR-222 to transmit drug resistance. Shen *et al.*[17] stated that miR-222 can enhance the resistance of breast cancer cells to doxorubicin by regulating the PTEN/Akt/FOXO1 pathway. In addition to breast cancer, miR-222 can also modulate the sensitivity of multiple tumors such as multiple myeloma, glioma[18], bladder cancer[19] to chemotherapeutic drugs, but no studies have reported the effects of miR-222 on the resistance of renal cancer cells so far.

According to the above-listed studies, the induction of tumor resistance by miR-222 is very likely to be ubiquitous, which means miR-222's influence on the resistance of renal cancer cells is also possible. Fortunately, this study confirmed this possibility. Dkk 3 is a tumor suppressor that performs it functions by regulating Wnt signaling pathway. Also, miR-222 can target DKK2 to activate Wnt signaling pathway to promote glioma development. As a member of the DKK family, DKK2 has similar amino acid sequences to DKK3, but unlike DKK3, DKK2 is an activator of the Wnt signaling pathway[20]. Studies by Wang *et al.*[20] reported that miR-221 was discovered to target DKK2 expression and mediate the drug-resistance of esophageal adenocarcinoma. miR-221 and miR-222 have the same seed sequences, which means that DKK2 may be another target of miR-222 to affect tumor cell resistance. Such facts also indicate that miR-221, miR-222, DKK2, and DKK3 may all participate in tumor cell resistance by forming a complex regulatory network, but this requires further research and validation.

This study marks the first to uncover the involvement of miR-222 in cisplatin resistance in renal cancer cells through its targeted regulation of DKK3, not verified by other studies. So, we hope to see more similar studies in the future to provide validation for the results and conclusions of this study. This study is subject to certain shortcomings. For example, despite the large number of renal cancer resistant cell strains, the in-vitro cell experiment in this study failed to simulate the complex tumor microenvironment of the body, waiting to be verified by *in vivo* experiments. In

addition, the clinical value of miR-222 and DKK3 in the diagnosis and prognosis evaluation of renal cancer is rarely studied. We will include this part in our study purpose in future researches.

## Conclusion

In summary, miR-222 regulates the drug-resistance of renal cancer cells by targeting DKKS. Down-regulated miR-222 expression can remove the inhibition of DKK3 expression and increase the sensitivity of renal cancer cells to cisplatin chemotherapy, suggesting the possibility of miR-222 to work as a potential target for clinical treatment of renal cancer in the future.

## Conflict of interest

All authors declare no conflict of interest.

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