

Upregulation of miR-21 expression is a valuable predictor of advanced clinicopathological features and poor prognosis in patients with renal cell carcinoma through the p53/p21-cyclin E2-Bax/caspase-3 signaling pathway

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Abstract. In the present study, we investigated whether the upregulation of miR-21 expression is a predictor of advanced clinicopathological features and poor prognosis in patients with renal cell carcinoma (RCC). It was found that the expression of miR-21 not only had a significant effect on the clinicopathological features and survival in patients with RCC, but also exerted an important influence on the occurrence rate of RCC. In addition, miR-21 expression was lower in the human kidney HRPTEpiC cell line than that noted in RCC A-498 cells. Meanwhile, the overexpression of miR-21 not only increased cell proliferation, inhibited apoptosis and reduced caspase-3 activity in the A-498 cells, but also suppressed p53, CDKN1A p21, cyclin E2 and Bax protein expression in the A-498 cells. In contrary, the downregulation of miR-21 expression promoted the protein expression of p53, CDKN1A p21 and cyclin E2, and decreased Bax protein expression and caspase-3 activity in the A-498 cells. Our data indicate a clear correlation between miR-21 expression and clinicopathological features and poor prognosis in patients with RCC through the p53/p21-cyclin E2-Bax/caspase-3 signaling pathway.

Introduction

As the most common renal parenchymal tumor, renal cell carcinoma (RCC) accounts for 2% of all adult malignant tumors (1). It is ranked third as the most common urinary tumor, following prostatic cancer and carcinoma of the urinary bladder. Its annual incidence rate and mortality rate are 271,000 and 116,000, respectively (2). In addition, the incidence rate shows a persistent increasing tendency. Due to differences in

diagnosis, the reported incidence rates of RCC in Europe and America are higher compared with those in Asia and Africa at present (3,4). In addition, the RCC morbidity of males is higher than that of females. Specifically, the incidence and mortality rates of males are 167,000 and 72,000, respectively, while those of female are 103,000 and 44,000, respectively (5).

Research in recent years has shown that the general biological characteristic of tumors is rampant growth. The main molecular mechanisms present extreme cell proliferation with too little apoptosis. Faithful cell replication is to be ensured and controlled by restriction points (6). The overexpression of cyclin D1 and E can promote the G/S limiting point of cells and cause excessive cell proliferation; moreover, cell cycle confusion is a key link of the genome (7). It may further increase the probability of carcinogenesis of labile cells, and thereby participate in tumorigenesis and development. At present, the relationship between tumors and the cell cycle is one of the main topics concerning tumorigenesis and development as well as gene therapy (8).

A large number of studies have demonstrated that p53 can transcribe the levels of numerous targeted genes and microRNAs (miRNAs) under induction of stress. Being able to damage DNA by stress, p53 mainly blocks the cell cycle and/or introduces cell apoptosis as a tumor-suppressor gene. It causes not only accumulation of DNA damage, but also cancer. For more than 50% of human types of cancers, p53 is mutated. Therefore, p53 in cells is recognized as a classic Knudson-type tumor inhibitor.

Actually, p21 can induce cells to convert to S and G2 phases (9). However, transient cell cycle arrest cannot eradicate tumors. In fact, low-dose mutant cell stress, or a low amount of cell mutation, may lead to monitoring point of cell cycle arrest and cytothesis (9). After the checking point of cytothesis, cells undergo cell cycle. That is to say, the cells maintain continuous growth and proliferation (9,10).

First detected in 1993, miRNAs are uncoded single small molecules containing 17-25 nucleotides (nts), which horizontally adjust genetic expression after transcription. Studies have shown that ~30% of human genes are regulated by miRNAs. They play a vital role in cellular differentiation, growth, proliferation, programmed cell death (apoptosis) and metabolism.

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Given this, they are also closely related to tumors. They can regulate the development and metastasis of tumors as oncogenes or cancer-suppressor genes. Among them, miRNA-21 (miR-21) has been given much attention, and has been reported in much research concerning tumors. Nevertheless, to the best of our knowledge, the underlying mechanisms of miR-21 in relation to advanced clinicopathological features and poor prognosis in patients with RCC have not been previously studied. On this basis, we explored whether upregulation of miR-21 expression predicates advanced clinicopathological features and poor prognosis in patients with RCC and the possible mechanism.

Materials and methods

Samples and cases. Sixty-eight patients (RCC and normal adjacent tissues) were collected from July 2014 to December 2014 at West China Hospital, Sichuan University. Blood and tissue samples from patients were collected and saved at -80°C.

Quantitative real-time PCR. Total RNA was extracted from blood, tissue samples or cells using an RNeasy Mini kit from Qiagen (Valencia, CA, USA). cDNA was prepared from total RNA (1 µg) using a reverse transcription system (Promega, Madison, WI, USA). 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to execute and analyze the gene expression of miR-21. Thermal cycling conditions consisted of 95°C for 40 sec, 40 cycles of 95°C for 30 sec and 60°C for 30 sec according to the TaqMan Fast Universal PCR protocol.

Cell lines and cell culture. Human kidney cell line HRPTEpiC and RCC cell line A-498 were cultured with RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 50 mg/ml penicillin and 50 mg/ml streptomycin (Invitrogen) in an incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C using a microplate reader (ELX800; GE, USA).

Cell transfection. A-498 cells were transiently transfected with miR-21, anti-miR-21 or anti-miR negative control using Lipofectamine 2000 (Invitrogen). The cells were pelleted after 24 h of transfection for cell proliferation, apoptosis, RNA and protein extraction.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A-498 cells transfected with miRNAs were seeded on 96-well plates, and collected and centrifuged at 700 x g for 1 min. Every well was administered 20 µl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and incubated for 4 h in an incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Dimethyl sulfoxide (DMSO) (150 µl) was added to incubate for 10 min at room temperature while being shaken. For assessment of cell proliferation the optical density was read at 570 nm using a microplate reader (ELX800).

Apoptosis assay. A-498 cells transfected with miRNAs were seeded on 6-well plates, and collected and centrifuged at 700 x g for 1 min. A-498 cells were stained with 5 µl

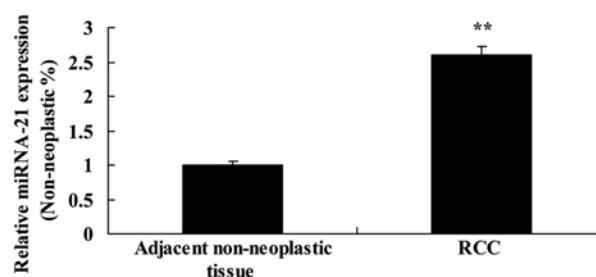


Figure 1. miR-21 expression in adjacent non-neoplastic and RCC tissues. Adjacent non-neoplastic tissue, adjacent non-neoplastic tissue group; RCC, renal cell carcinoma group. **p<0.01 vs. the adjacent non-neoplastic tissue group.

Annexin V-Alexa and propidium iodide (PI) for 15 min at room temperature in the dark. The apoptosis rate of the A-498 cells was analyzed by FACScan flow cytometry using CellQuest 3.3 analysis software (Becton-Dickinson, San Jose, CA, USA).

Caspase-3 activity. A-498 cells transfected with miRNAs were seeded on 6-well plates, and collected and centrifuged at 700 x g for 1 min. A-498 cells were washed once in phosphate-buffered saline (PBS) and lysed in RIPA lysis and extraction buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The protein concentration was determined using a BCA protein assay kit (Beyotime, Jiangsu, China). Equal sample volumes were incubated with 1 M DTT and the labeled caspase-3 substrate DEVD-p-nitroanilide (DEVD-pNA) for 4 h at 37°C. Caspase-3 activity was quantified by measuring the absorbance at 405 nm using a microplate reader (ELX800).

Western blot analysis. A-498 cells transfected with miRNAs were seeded on 6-well plates, and collected and centrifuged at 700 x g for 1 min. A-498 cells were washed once in PBS and lysed in RIPA lysis and extraction buffer. The protein concentration was determined using a BCA protein assay kit. Equal sample volumes were loaded onto 8-10% polyacrylamide-SDS gel and were then transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was incubated with blocking buffer and then incubated overnight at 4°C with anti-p53, anti-CDKN1A p21, anti-cyclin E2, anti-Bax and anti-β-actin (1:4,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with the corresponding secondary antibodies.

Statistical analysis. To analyze baseline characteristics, the continuous variables are presented as mean ± SD. Student's t-test was used to compare the different groups. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-21 expression and adjacent non-neoplastic and RCC tissues. To evaluate the expression of miR-21 expression in adjacent non-neoplastic and RCC tissues, miR-21 expression was detected by real-time PCR. As shown in Fig. 1, the expression of miR-21 in RCC tissues was much higher than that noted in the adjacent non-neoplastic tissues.

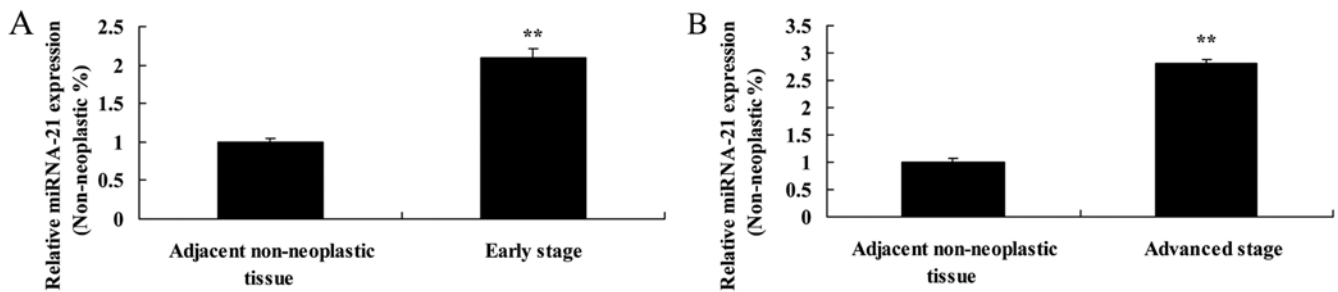


Figure 2. Expression of miR-21 and clinicopathological stage (A, early stage and B, advanced stage). Adjacent non-neoplastic tissue group; early stage, early stage renal cell carcinoma group; advanced stage, advanced stage renal cell carcinoma group. ** $p < 0.01$ vs. the adjacent non-neoplastic tissue group.

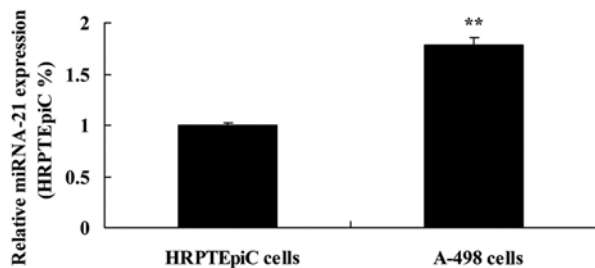


Figure 3. Expression of miR-21 in HRPTEpiC and A-498 cells. ** $p < 0.01$ vs. the HRPTEpiC cell group.

Expression of miR-21 and clinicopathological features. We investigated the correlation between the expression of miR-21 and the clinicopathological features of the patients with RCC (Table I). The expression of miR-21 in patients with early stage RCC or advanced stage RCC was higher than that detected in the adjacent non-neoplastic tissues, respectively (Fig. 2A and B).

Expression of miR-21 in HRPTEpiC and A-498 cells. We used HRPTEpiC and A-498 cells to detect the expression of miR-21. Similarly, the expression of miR-21 in A-498 cells was markedly higher than that in the HRPTEpiC cells (Fig. 3).

Effect of the overexpression of miR-21 on cell proliferation of A-498 cells. As shown in Fig. 4A, the miR-21 plasmid increased the expression of miR-21 in the A-498 cells, as compared to the negative control. Meanwhile, the miR-21 plasmid facilitated the cell proliferation of the A-498 cells, as compared to the proliferation noted in the cells transfected with the negative control (Fig. 4B).

Effect of the overexpression of miR-21 on apoptosis and caspase-3 activity in the A-498 cells. To determine whether overexpression of miR-21 affects apoptosis and caspase-3 activity of A-498 cells, flow cytometry and ELISA kit were used to analyze apoptosis and caspase-3 activity of the A-498 cells. Compared with the anti-miR-negative control, the apoptosis rate and caspase-3 activity were suppressed in the cells in the miR-21-overexpressing group (Fig. 5A and B).

Effect of the overexpression of miR-21 on the expression of p53. To determine the mechanism of miR-21 that affects A-498

Table I. Correlation of miR-21 expression and the clinicopathological features of the RCC cases.

Patient features	Low miR-21	High miR-21	P-value
Age (years)			0.761
<50	8	12	
≥50	25	23	
Tumor stage			0.021
Early (I-II)	11	7	
Advanced (III-IV)	19	31	
Tumor size (cm)			0.393
<5	14	16	
≥5	21	17	
AFP level (ng/ml)			0.112
≤20	14	11	
>20	20	21	
Venous infiltration			0.001
Absent	28	10	
Present	9	21	

RCC, renal cell carcinoma.

cells, p53 protein expression was investigated in the present study. Overexpression of miR-21 observably suppressed the protein expression of p53, as compared to the negative control (Fig. 6).

Effect of the overexpression of miR-21 on the expression of CDKN1A p21. To ascertain whether overexpression of miR-21 is involved in the expression of CDKN1A p21 in A-498 cells, CDKN1A p21 protein expression was detected using western blot analysis. The protein expression of CDKN1A p21 was observably inhibited following the overexpression of miR-21, as compared to cells transfected with the negative control (Fig. 7).

Effect of the overexpression of miR-21 on the expression of cyclin E2. To ascertain how the overexpression of miR-21 affects the expression of cyclin E2 in A-498 cells, western

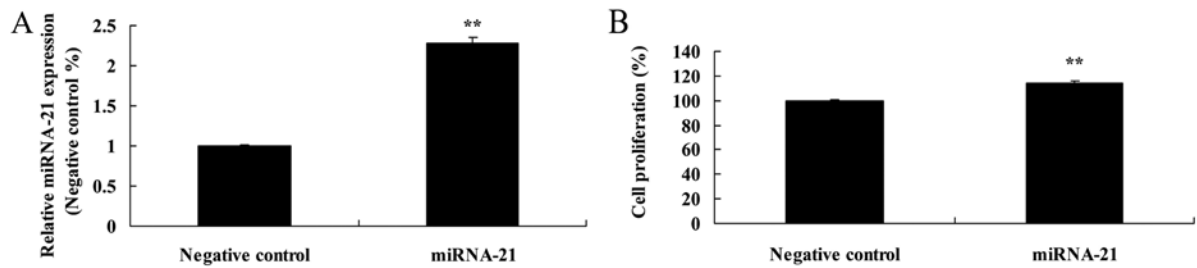


Figure 4. Effect of the overexpression of miR-21 on the cell proliferation of A-498 cells. Effect of the upregulation of miR-21 on the expression of miR-21 (A) and cell proliferation (B) in A-498 cells. **p<0.01 vs. the HRPTEpiC cell group.

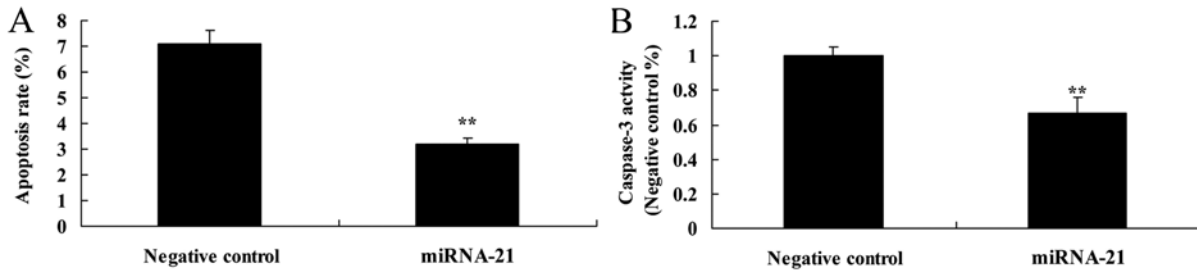


Figure 5. Effect of the overexpression of miR-21 on apoptosis and caspase-3 activity in the A-498 cells. Effect of the overexpression of miR-21 on the apoptosis rate (A) and caspase-3 activity (B) in A-498 cells. **p<0.01 vs. the negative control group.

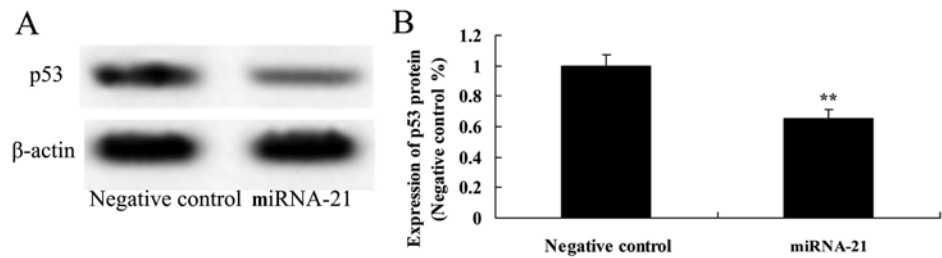


Figure 6. Effect of the overexpression of miR-21 on the expression of p53. Effect of the overexpression of miR-21 on the expression of p53 protein using western blot analysis (A) and statistical analyses of p53 protein expression (B). **p<0.01 vs. the negative control group.

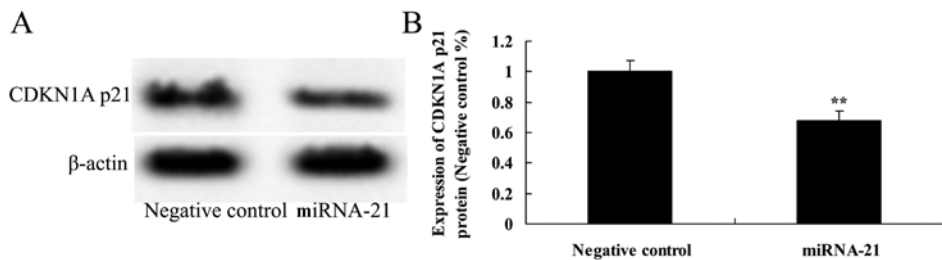


Figure 7. Effect of the overexpression of miR-21 on the expression of CDKN1A p21. Effect of the overexpression of miR-21 on the expression of CDKN1A p21 protein using western blot analysis (A) and statistical analyses of CDKN1A p21 protein expression (B). **p<0.01 vs. the negative control group.

blot analysis was used to reveal the protein expression of cyclin E2. We observed a significant inhibition of cyclin E2 protein expression following overexpression of miR-21 in the A-498 cells, as compared to the negative control. Thus, miR-21 affects the cell cycle of A-498 cells (Fig. 8).

Effect of the overexpression of miR-21 on the expression of Bax. To further explore how the overexpression of miR-21

affects the Bax protein expression in A-498 cells, western blot analysis was used to reveal Bax protein expression. As shown in Fig. 9, overexpression of miR-21 significantly suppressed the protein expression of Bax in the A-498 cells, as compared to the negative control.

Effect of the downregulation of miR-21 on cell proliferation and apoptosis in the A-498 cells. We further ascertained

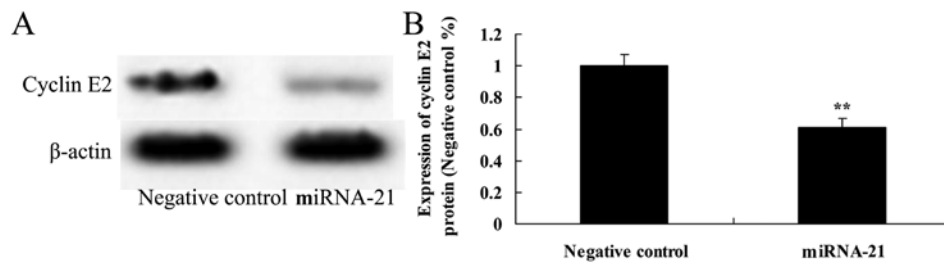


Figure 8. Effect of the overexpression of miR-21 on the expression of cyclin E2. Effect of the overexpression of miR-21 on the expression of CDKN1A p21 protein using western blot analysis (A) and statistical analyses of CDKN1A p21 protein expression (B). ** $p < 0.01$ vs. the negative control group.

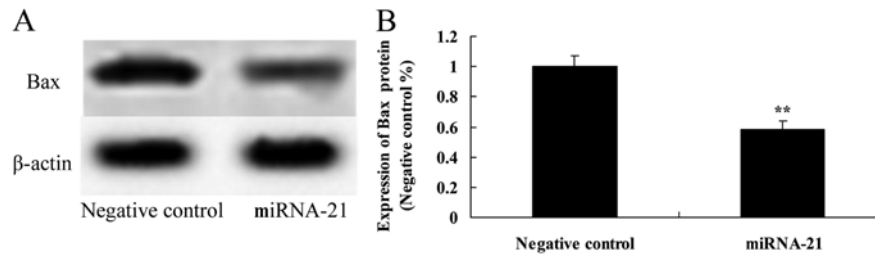


Figure 9. Effect of the overexpression of miR-21 on the expression of Bax. Effect of the overexpression of miR-21 on the expression of Bax protein expression using western blot analysis (A) and statistical analyses of Bax protein expression (B). ** $p < 0.01$ vs. the negative control group.

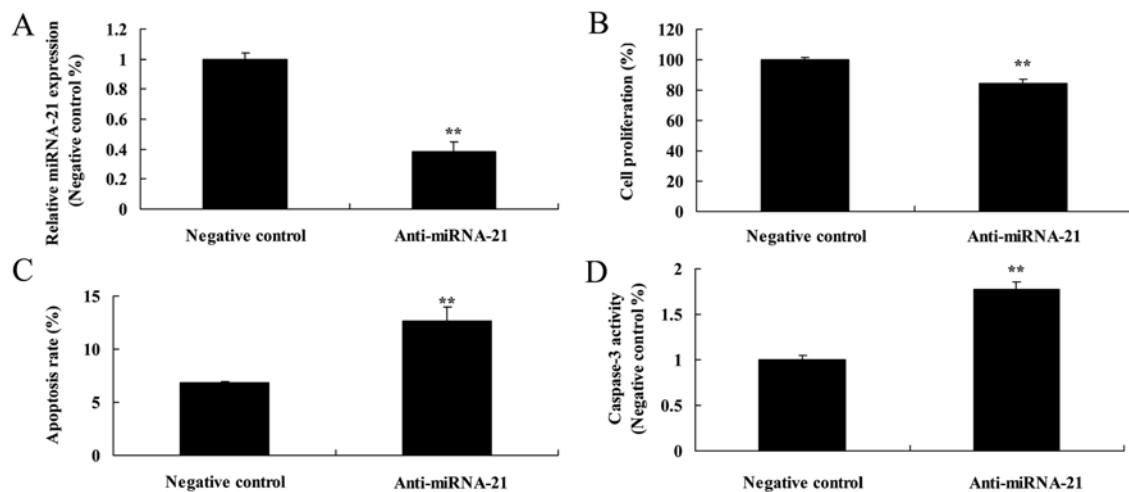


Figure 10. Effect of the downregulation of miR-21 on cell proliferation and apoptosis of A-498 cells. Effect of the downregulation of miR-21 on the expression of miR-21 (A), cell proliferation (B), apoptosis rate (C) and caspase-3 activity (D) in A-498 cells. ** $p < 0.01$ vs. the negative control group.

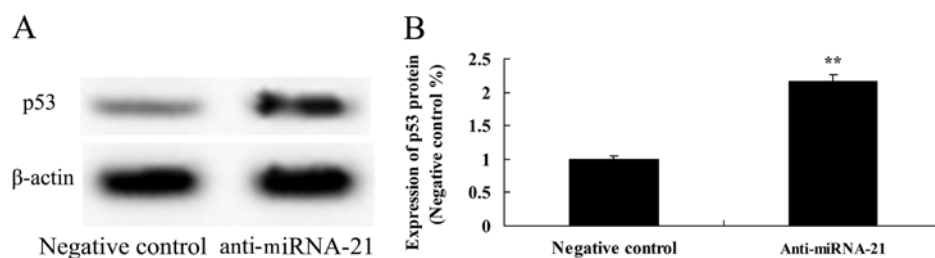


Figure 11. Effect of the downregulation of miR-21 on p53 protein expression in A-498 cells. Effect of the downregulation of miR-21 on the expression of p53 protein using western blot analysis (A) and statistical analyses of p53 protein expression (B). ** $p < 0.01$ vs. the negative control group.

how downregulation of miR-21 affects the cell proliferation and apoptosis of A-498 cells. miR-21 expression in the

anti-miR-21 expression group was lower than that of the negative control group (Fig. 10A). Meanwhile, downregulation

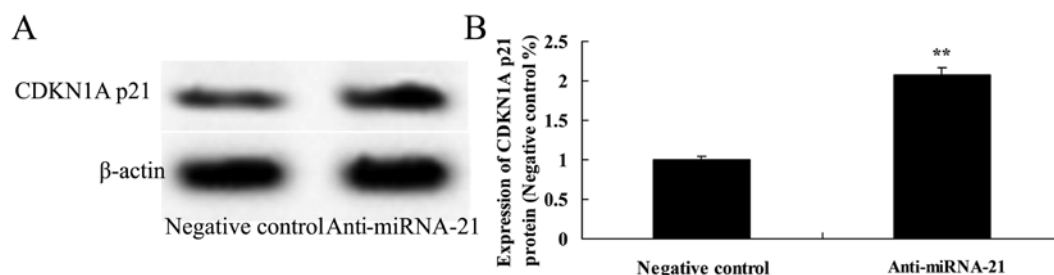


Figure 12. Effect of the downregulation of miR-21 on p21 expression in A-498 cells. Effect of the downregulation of miR-21 on the expression of p21 protein using western blot analysis (A) and statistical analyses of p21 protein expression (B). ** $p < 0.01$ vs. the negative control group.

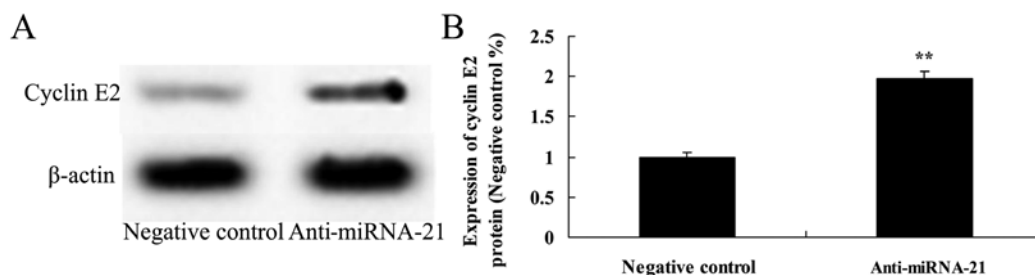


Figure 13. Effect of the downregulation of miR-21 on cyclin E2 expression in A-498 cells. Effect of the downregulation of miR-21 on the expression of cyclin E2 protein using western blot analysis (A) and statistical analyses of cyclin E2 protein expression (B). ** $p < 0.01$ vs. the negative control group.

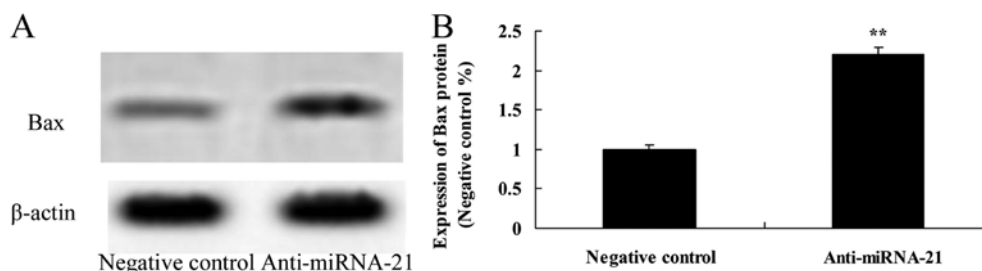


Figure 14. Effect of the downregulation of miR-21 on Bax expression of A-498 cells. Effect of the downregulation of miR-21 on the expression of Bax protein using western blot analysis (A) and statistical analyses of Bax protein expression (B). ** $p < 0.01$ vs. the negative control group.

of miR-21 markedly suppressed the cell proliferation of the A-498 cells, as compared to the negative control (Fig. 10B). However, downregulation of miR-21 also markedly induced the apoptosis and caspase-3 activity in the A-498 cells, as compared to the negative control (Fig. 10C and D).

Effect of the downregulation of miR-21 on p53 protein expression in the A-498 cells. To further observe whether downregulation of miR-21 is involved in the expression of p53 in A-498 cells, p53 protein expression was detected using western blot analysis. On the contrary, downregulation of miR-21 significantly activated the protein expression of p53, as compared to the negative control (Fig. 11).

Effect of the downregulation of miR-21 on CDKN1A p21 expression in the A-498 cells. To next ascertain whether downregulation of miR-21 affects CDKN1A p21 protein expression in the A-498 cells, CDKN1A p21 protein expression was also analyzed using western analysis. As shown in Fig. 12, down-

regulation of miR-21 significantly promoted CDKN1A p21 protein expression in the A-498 cells, as compared to the negative control.

Effect of the downregulation of miR-21 on cyclin E2 expressions in the A-498 cells. To further ascertain whether the downregulation of miR-21 affects cyclin E2 expression in the A-498 cells, we assessed cyclin E2 expressions using western blot experiments. Downregulation of miR-21 significantly increased cyclin E2 expressions in the A-498 cells, as compared to the negative control (Fig. 13).

Effect of the downregulation of miR-21 on Bax expression in the A-498 cells. We further ascertained how the downregulation of miR-21 affects Bax expression in the A-498 cells. Using western blot experiments we assessed Bax expressions. Downregulation of miR-21 significantly increased Bax expression in the A-498 cells, as compared to the negative control (Fig. 14).

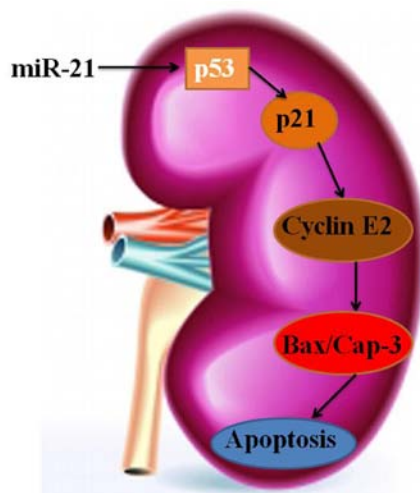


Figure 15. Upregulation of miR-21 expression predicates advanced clinicopathological features and poor prognosis through p53/p21-cyclin E2-Bax/caspase-3 signaling pathway in patients with renal cell carcinoma.

Discussion

Originating from renal tubular epithelial cells (RTECs), renal cell carcinoma (RCC) is a common malignant tumor of the urinary system (11). As a common malignant renal tumor, it accounts for approximately 2-3% of all adult solid malignant tumors, and 85-90% of primary malignant neoplasms (12). RCC tissue typing mainly includes clear cell carcinoma, chromophobe cell tumor, collecting duct carcinoma and unclassified RCC. The morbidity of RCC in different countries or regions is diversified (13). Specifically, it is higher in developed countries than in developing countries. The morbidity and mortality rate of RCC in China have been presenting an escalating trend in recent years (14). We found that the expression of miR-21 in RCC tissues was much higher than that in adjacent non-neoplastic tissues. Meanwhile, the expression of miR-21 in RCC patient tissues at the early or advanced stage was higher than that in the adjacent non-neoplastic tissues.

Research has also shown that miR-21 plays an important role in a variety of malignant tumors (including tumors in solid organs and the blood system) (15,16). As the only miRNA overexpressed in almost all malignant solid tumors, it has been regarded as a proto-oncogene. Generally, it is upregulated in a variety of tumor tissues and cells, such as glioma, cervical, ovarian, bladder, prostatic, lung, breast, thyroid, esophageal and liver cancer, and bile duct carcinoma, pancreatic, colorectal and gastric cancer, B cell lymphoma, leukemia and multiple myelomas. Owing to its participation in cell differentiation, proliferation and apoptosis (15-18), it is closely related to genesis and development, as well as infiltration and metastasis (19). Moreover, the overexpression of miR-21 was found to increase cell proliferation, and inhibit apoptosis and caspase-3 activity in RCC A-498 cells.

Briefly, p53 can induce cell cycle progression and regulate G2/M transformation. It not only prevents cells from entering into the mitotic phase by inhibiting cdc2 (20), but also inhibits cyclin B1 and blocks the cells at the G2 phase (21). In addition, p53 enables the transcriptional activation of cell cycle protein

relying on kinase inhibitor p21 protein expression, thus, as to block the cell cycle at the G1 phase (21,22). Ma *et al* suggested that miR-21 may be beneficial in apoptosis-inducing cancer therapies for p53-deficient tumors (23). Observably, the overexpression of miR-21 affects the protein expression of p53 in A-498 cells.

It is noteworthy that p21 can play the role of an oncogene. The root cause is that p21 can be phosphorylated by Akt and is located in the cytoplasm (24). Nonetheless, the cytoplasmic localized p21 inhibits caspase-3, and thus restrains apoptosis. In addition, it can form a composite with procaspase-3 to resist Fas-mediated apoptosis (10). Consequently, the functional loss of p53 of transcription factor p21 may verify tumor inhibition and promote cancer activity of cytoplasmic localization in various situations. With two different functions of p21, p53 can generally activate the downstream of cell cycle inhibitors (25). In the present study, it was found that miR-21 regulated CDKN1A p21 protein expression in the A-498 cells. Haghpanah *et al* suggested that miR-21 enhances differentiation/apoptosis by increasing the expression of p21 in anaplastic thyroid cancer (26).

The increase in p21 protein expression may result in the blocking of the cell cycle at G1/S. However, there are reports concerning DNA damage induced by exogenous factors such as UV irradiation (27) in recent years. Generally, p21 is activated to upregulate and p53 is utilized to inhibit cyclin/CDK composite, so as to induce blocking of the cell cycle at the S phase (28). Based on a large number of studies, p53 can induce apoptosis. As a transcription factor, it is of vital importance to clarify the target genes related to the regulation of apoptosis (22). Additionally, wild-type p53 can combine with the bax promoter, and increase bax expression at the transcription level. Bax is a family member of Bcl-2 (28). It can form heterology of two polymers to inhibit the activity of the Bcl-2 family, which plays an important role in apoptosis and cancer (27). For instance, Bcl-2 is able to control cytochrome *c* to release from mitochondria, thus activating caspase-9, -9 and -3, eventually leading to apoptosis (29). The present study results showed that miR-21 affects cyclin E2 protein expression in the A-498 cells. Zaman *et al* reported that miR-21 can not only serve as a tumor marker in kidney cancer survival but also affects cyclin E2 protein expression (30).

In conclusion, our results demonstrated that miR-21 expression predicates advanced clinicopathological features and poor prognosis in patients with RCC through p53/p21-cyclin E2/caspase-3 (Fig. 15). Moreover, examining new targets and biological factors may facilitate to clarify the miR-21/p53/p21-cyclin E2/caspase-3 pathway in RCC.

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