**MicroRNA-645 promotes cell metastasis and proliferation of renal clear cell carcinoma by targeting GK5**

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**Abstract.** – OBJECTIVE: To dissect the functioning mode of miR-645 on renal clear cell carcinoma cell metastasis and growth, and provide therapeutic targets for renal clear cell carcinoma.

PATIENTS AND METHODS: Quantitative Real-time PCR (qRT-PCR) assay was employed to detect miR-645 expression level. Wound healing assay and transwell assay were performed to investigate metastasis capacity of renal clear cell carcinoma cells. Cell Counting Kit 8 (CCK8) assay was incorporated to assess cell proliferation capacity. Flow cytometry was used to identify cell apoptosis and cell cycle distribution. Protein levels were assessed by Western blotting assay. The target gene was predicted and verified by bioinformatics analysis and luciferase assay.

RESULTS: MiR-645 was upregulated in renal clear cell carcinoma tissues when compared with para-carcinoma tissues (n=32). Downregulated miR-645 could attenuate cell migration and invasion capacities, as well as inhibited cell proliferation capacity, promoted cell apoptosis and cell cycle arrest at G0/G1 phase. GK5 was chosen as the target gene of miR-645 by bioinformatics analysis and luciferase reporter assay. Moreover, silence of GK5 could rescue tumor suppression role of downregulated miR-645 on renal clear cell carcinoma metastasis.

CONCLUSIONS: Knockdown of miR-645 exerted tumor-suppressive effects on renal clear cell carcinoma metastasis and growth via targeting GK5 in vitro, which provided an innovative and candidate target for diagnose and treatment of renal clear cell carcinoma.

Key Words:
microRNAs, Metastasis, Proliferation, SOX9, Renal clear cell carcinoma.

**Introduction**

MicroRNA (miRNA) is a kind of non-coding small-molecule RNA with a length of 19-25 nucleotides. The primary product pri-miRNA is first transcribed from the coding gene, which is cleaved by Drosha into pre-miRNA with a length of about 80bp and then transported to the cytoplasm by Exportin-5 and processed by Dicer into double-stranded RNA with a length of about 22bp. Mature miRNAs combine with RNA-induced silencing complex (RISC) to form functional units, exerting effects. MiRNAs, through regulating the expressions of relevant target genes, are involved not only in the normal growth and development processes of organism, but also in the occurrence of disease, including the occurrence, development, metastasis and drug resistance of tumors. Therefore, it is helpful to elucidate the role of miRNA in the occurrence and development of tumor for deeply understanding the tumor and providing a theoretical basis for the treatment of tumor.

Renal cancer is a kind of common renal malignant tumor derived from renal tubular epithelial cells, accounting for 2-3% in all malignant tumors in adults and 85-90% in primary renal malignant tumors, 80-90% of which is the clear cell carcinoma. Besides, its incidence rate shows an increasing trend year by year. At present, the surgical treatment is still the preferred treatment method, because the therapeutic effects of chemotherapy, radiotherapy and hormone therapy are poor. It has been found that miRNAs play important roles in the occurrence and development of renal cancer, and
many kinds of miRNAs are abnormally expressed in renal cancer, affecting its growth, invasion and metastasis through regulating the downstream target genes. Looking for miRNA molecules associated with the renal clear cell carcinoma and elucidating the mechanism of action may enrich the etiology and molecular pathology of renal clear cell carcinoma. Moreover, the study on renal clear cell carcinoma-associated miRNAs can provide important theoretical support for the early diagnosis and prognosis evaluation of renal cancer, and development of new anti-cancer drugs.

In this study, the expression difference of miRNAs in renal clear cell carcinoma tissues and normal para-carcinoma tissues was mainly investigated; whether the expression of miR-645 in renal clear cell carcinoma tissues was correlated with the clinicopathological parameters of renal cancer, it was analyzed. Also, the role of miR-645 in the occurrence and development of renal cancer was explored, including the cell invasion, migration, proliferation, apoptosis and cycle, etc. Furthermore, the relevant target genes were predicted using the bioinformatics, and their regulatory effects and mechanisms involved in the pathogenesis of renal cancer were studied, so as to provide a theoretical basis for the in-depth exploration of pathogenesis and new treatment methods of renal clear cell carcinoma.

Patients and Methods

Patients
A total of 32 cases of renal clear cell carcinoma tissue samples and corresponding para-carcinoma tissue samples were taken from patients receiving radical nephrectomy in Tongde Hospital of Zhejiang Province from February 2016 to January 2017, and the tissue samples were collected after the approval of Ethics Committee of Tongde Hospital of Zhejiang Province and the patients signed the informed consent.

Methods
The tumor samples were cut from the kidney within 10 min in vitro, while the para-carcinoma kidney was cut at more than 3 cm away from the distal tumor. All in vitro tissue samples were rinsed with RNase-free isotonic saline to remove the dirt or bloodstain, placed into the cryopreserved tube, immediately frozen into liquid nitrogen and stored in the ultra-low temperature refrigerator at -80°C for a long time used to extract RNA.

Cell Culture
The RCC cell lines 786-O, A498, Caki-1 and normal human renal cell line, HK-2, were purchased from Shanghai Model Cell Bank (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. All cells were cultured in an incubator at 37°C with 5% CO₂.

Plasmid and Transfection
For downregulation of miR-645 in RCCC cells, miR-645 inhibitor and corresponding negative control (inhibitor-NC) were obtained from the Ribobio (Guangzhou, China). Transfections were performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols.

For knockdown of GK5 for silencing, siRNA sequences that targeting for GK5 were designed as follows: siRNA-1: 5'-CCAATGCGGTTAATTTCCAGGAA-3'; siRNA-2: 5'-CCGTTCAATCTATCTTAGTGCT-3'; siRNA-3: 5'-CAATTAGTGACAGGGTTTCTTAA-3' and the siRNA-NC were purchased from Genechem (Shanghai, China).

RNA Extraction and qRT-PCR
Total RNA in tissues and RCCC cells were extracted via TRizol reagent (Invitrogen, Carlsbad, CA, USA). The relative expression level of miR-645 was determined using the mirVana™ qRT-PCR microRNA Detection kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocols. U6 was used for normalization. Then, we performed PCR reactions using the following primers: for miR-645, forward, 5'-AGA CAG TGG CAA TAC TGC UCA -3' and reverse, 5'-GGT CCG GTG CAG AGG T -3'; and for U6, forward, 5'-GCA CCT TAG GCT GAA CA-3' and reverse, 5'-AGC TTA TGC CGA GCT CTT GTG-3'.

PrimeScript RT reagent kit and SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used for qRT-PCR using the following primers: for GK5, forwards, 5'-TTTTGGGAAGGGGACCATTGA-3' and reverse, 5'-TTCGCCCATCATCTGTTTT-3'; and for GAPDH, forward, 5'-GAAGGTGAGGAACTCGAGG-3' and reverse, 5'-GAAGATGTTAGCATGGGATTTC-3'. qRT-PCR was carried out by using the ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Wound Healing Assay
Transfected cells were cultured in the 6-well plates marked by a horizontal line on the back.
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Cells were scratched by a pipette tip across the confluent cell layer. Next, cells were washed gently and continued to be cultured with the serum-free medium for 24-48 h. Wound closure was captured using a light microscope (DFC500, Solms Germany).

Transwell Assay

Cells were cultured in the upper invasion chamber (BD, Franklin Lakes, NJ, USA) coated with Matrigel. Serum-free medium was added into the upper chamber, whereas 10% FBS medium supplemented was then added into the lower. After 48 h, the cells cultured on the upside of the filter, which did not invade through the chamber, were removed. Then the chamber was suspended by 100% precoothing methanol, stained with 0.05% crystal violet and inspected with the microscope (Olympus, Tokyo, Japan). The values for the invasion cells were measured by counting five fields per membrane.

Cell Counting kit-8 Assay

Cell viability was assessed by cell counting kit-8 (CCK8) assay (Promega, Madison, WI, USA) after the transfection according to the manufacturer’s protocols. The transfected cells were grown in 96-well plates (2000 cells/well), and 10 μL CCK8 solution was added into 90 μL DMEM and incubated for 3 h. Then, the absorbance was measured at 490 nm.

Cell Apoptosis Analysis

The cells were cultured in 6-well plates and treated with 0.25% trypsin and fixed in 70% ice-cold ethanol. These cells were double stained with 1 μL propidium iodide (PI, 50 μg/mL) and 5 μL Annexin V-FITC. The apoptosis rate was analyzed using a flow cytometer equipped with CellQuest Pro 5.1 software.

Cell Cycle Analysis

Transfected cells suspension were prepared and stained with propidium iodide using the BD Cycletest Plus DNA Reagent Kit (BD Biosciences, San Jose, CA, USA). The relative ratio of cells in G0/G1, S, or G2/M phase was analyzed by FACSCalibur flow cytometer.

Bioinformatics Analysis

TargetScan (http://www.targetscan.org/vert_71/) and miRecords (http://c1.accurascience.com/miRecords/index.php) were utilized to forecast the target genes. As shown in the database, GK5 was the candidate gene we chose. The result of bioinformatics software indicated that 3’-UTR of GK5 binds to miR-645. Then qRT-PCR was performed to detect whether GK5 was really inversely correlated to miR-645 expression in RCCC cells.

Luciferase Reporter Assay

The activity of luciferase was tested using the Dual-Luciferase reporter system (Promega, Madison, WI, USA). The GK5 3’-UTR region containing the wild type or mutant miR-645 binding site was amplified and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). Next, treated cells were co-transfected with the established vector and miR-645 mimics or scrambled using lipofectamine 2000. Then, the activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured as the fold-change to the basic pGL3 vector relatively.

Western Blot Analysis

A protein assay (Bio-Rad, Beijing, China) was conducted for measuring the total protein concentration. The target proteins were replaced to the polyvinylidene difluoride (PVDF) membrane, which was then blocked in 5% dry milk at 37°C for 1 hour after fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then membrane was immune-stained with antibodies (Cell Signaling Technology, CST, Danvers, MA, USA) overnight at 4°C: 1:1000 rabbit anti-GK5, and 1:5000 rabbit anti-GAPDH. Subsequently, 1:1000 goat anti-rabbit secondary antibody was used for cultivation. The bands were measured using ChemiDoc XRS imaging system and ImageJ software.

Statistical Analysis

Quantitative data was presented as mean ± SD. Data analysis software included SPSS.17.0 (SPSS Inc., Chicago, IL, USA). Chi-squared test was performed to evaluate the association between miR-645 levels and clinicopathological parameters. The method of 2-ΔΔCT was used to measure the relative expression of mRNA. Statistical significance was calculated by the log-rank test. Independent samples t-test was chosen as the method. Only p<0.05 was identified to be statistically significant.

Results

MiR-645 Expression was Elevated in RCCC Tissues and Cell Lines

Expression of miR-645 was detected in 32 pairs of RCCC tissues and para-carcinoma tissues.
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by qRT-PCR. The results indicated that miR-645 expression was remarkably increased in RCCC tissues compared with the matched para-carcinoma tissues on mRNA level (Figure 1A). This evidence implied that miR-645 might participate in RCCC tumorigenesis. In addition, the relationship between the expression level of miR-645 and the clinical parameters of patients was analyzed, and patients were grouped according to the median of miR-645 expression level. After the statistics with the mean value of tumor size as the critical value, it was found that miR-645 was correlated with the TNM staging of renal cancer tissues, tumor size and Fuhrman grades, but had no relationship with the gender, age, etc. (Table I).

Besides, we investigated expression of miR-645 in several BC cell lines and normal breast cell line with qRT-RCR. It showed that, compared with HK-2 cell line, all these RCCC cell lines expressed a relatively higher level of miR-645, in which A498 expressed the relatively highest (Figure 1B). To identify the mode of action of miR-645 in RCCC tumorigenesis in vitro, A498 cell line was transfected with miR-645 inhibitor and inhibitor-NC for knockdown of miR-645 (Figure 1C).

Knockdown of miR-645 Inhibited RCCC Cell Metastasis in vitro

We next evaluated the functional role of miR-645 in RCCC cell metastasis in vitro. As shown in wound-healing assay, downregulated miR-645 could suppress RCCC cell migration when compared with inhibitor-NC (Figure 2A). Meanwhile, influence of downregulated miR-645 on cell invasion measured by using transwell assay was the same as the former (Figure 2B). The results demonstrated that downregulated miR-645 could inhibit cell metastasis of RCCC.

Knockdown of miR-645 Inhibited RCCC Cell Proliferation in vitro

As shown in CCK8 assay, the viability of RCCC cells was significantly inhibited after transfected with miR-645 inhibitor compared with inhibitor-NC, in a time-dependent manner (Figure 2C). To better illustrate how miR-645 inhibited cell proliferation, we analyzed whether cell apoptosis and cell cycle were also affected by miR-645. As shown in flow cytometric analysis, the apoptotic rate of RCCC cells transfected with miR-645 inhibitor increased remarkably compared with inhibitor-NC (Figure 2D). Moreover, the percentage of RCCC cells transfected with miR-645 inhibitor increased in G0/G1 phase while decreased in S phase obviously compared with inhibitor-NC (Figure 2E). These results indicated that miR-645 impaired RCCC cell proliferation capacity by promoting cell apoptosis and inducing cell cycle arrest at G0/G1 phase.

GK5 is Directly Targeted by miR-645

To better understand the mechanism about how miR-645 participated in these biological processes, we selected GK5 as the potential downstream of miR-645 via using TargetScan and miRecords database (Figure 3A). According to the consequence of prediction, miR-645 was transfected with GK5 3’UTR luciferase reporter gene into
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A498 cell. The result of dual-luciferase assay displayed a significant activity decrease in the WT group but no difference in mutant group (Figure 3B), indicating that GK5 was a target of miR-645.

Meanwhile, we further detected expression level of GK5 in transfected RCCC cells. The results indicated that GK5 was upregulated in A498 cells transfected with miR-645 inhibitor on mRNA level and protein level when compared with inhibitor-NC (Figure 3C&D). All these results indicated that GK5 was directly targeted by miR-645.

Silencing of GK5 Rescued Tumor Suppression of Downregulated miR-645

To further identify the interaction relationship of miR-645 and GK5, we firstly measured the expression of GK5 in RCCC tissues. The results indicated that GK5 was obviously downregulated in RCCC tissues compared with the para-carcinoma tissues on the mRNA level (Figure 4A), and the expression of GK5 was negatively correlated with the expression of miR-645 in RCCC tissues (Figure 4B).

Secondly, we explored whether GK5 is responsible for the functional effects of miR-645 in RCCC tumorigenesis. We found that siRNA-3 had the strongest ability to knock out GK5 (Figure 4C), thus we silenced GK5 expression by transfected with siRNA-3 in miR-645-decreased A498 cells (Figure 4D). GK5 silencing not only increased cell migration and invasion compared with miR-645 inhibitor (Figure 4E&F), but also increased the proliferation capacity (Figure 4G), attenuated cell apoptosis and cell cycle distribution at G0/G1 phase (Figure 4H&I). These results implied that miR-645 promoted RCCC tumorigenesis by repressing GK5 expression partially.

Discussion

At present, more than 1500 miRNAs have been found in the human genome, and it is estimated that about 30% protein-coding genes are regulated by miRNAs12. A large number of studies have shown that miRNAs may participate not only in...
a variety of normal biological processes of organism, but also the occurrence of diseases, and its abnormal expression plays an important role in occurrence and development of tumors\textsuperscript{13,14}. In recent years, the incidence rate of renal cancer has been increasing year by year, and it has become one of the top ten malignant tumors in China according to the latest statistics in 2012\textsuperscript{15,16}. The incidence rate of renal cancer is 4-5/0.1 million in males and 3-4/0.1 million in females. Besides, renal cancer is the second major tumor of urinary system only to the bladder tumor\textsuperscript{17}. The incidence rate of renal cancer increases by about 2.5% per year, so the rapid growth rate should attract enou-
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Figure 4. Silencing of GK5 rescued tumor suppression of downregulated miR-645. A, Analysis of GK5 expression level in RCCC tissues (T) and matched paracarcinoma tissues (N), n=32; B, Correlation between miR-645 and GK5 expression in BC tissues (n=32); C, Analysis of transfection efficiency in A498 cells transfected with siRNAs; D, Analysis of transfection efficiency in A498 cells transfected with miR-645inhibitor-NC, inhibitor and/or siRNA-GK5; E, Downregulated GK5 increased cell migration of miR-645-transfected cells; F, Downregulated GK5 increased cell invasion of miR-645-transfected cells; G, Downregulated GK5 promoted cell proliferation of miR-645-transfected cells; H, Downregulated GK5 attenuated cell apoptosis of miR-645-transfected cells; I, Downregulated GK5 attenuated cell cycle distribution of miR-645-transfected cells. Data are presented as the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

Radical nephrectomy is still the preferred treatment method of renal cancer, but the effects of radiotherapy, chemotherapy and other conventional treatment means on advanced renal cancer with the opportunity of surgical therapy lost are not satisfactory. Besides, immunotherapy has a certain curative effect on advanced renal cancer, but the effective rate is only about 15% and the survival benefit is limited\(^9\). In 2006, sunitinib was officially approved by Food and Drug Administration (FDA) as a kind of therapeutic drug of advanced renal cancer, opening a new chapter for the treatment of advanced renal cancer and marking the entry of renal cancer treatment into a new era of targeted therapy\(^9\). It is hoped that the research on miRNAs can provide
new targets for the targeted therapy of renal cancer in the future.

Many studies have revealed that more than 50% annotated miRNAs are located in the genomic regions or fragile sites associated with tumorigenesis and play a function similar to the anti-oncogene or proto-oncogene, which is closely related to the occurrence and development of a variety of tumors. For example, Ma et al\textsuperscript{20} found that miR-10b can inhibit the expression of HOXD10 and indirectly activate RHOC by direct targeting effect, significantly enhancing the invasion and metastasis capacities of breast cancer cells. Meng et al\textsuperscript{21} found that the miR-21 expression is significantly increased in human liver cancer tissues, and the proliferation, invasion, and metastasis of liver cancer cells can be promoted via down-regulating the expression of PTEN. Although more and more relationships between miRNAs and tumor have been elucidated, there are few studies on the miRNAs and renal cancer. This work aimed to study the relationship between miRNAs and the occurrence and development of renal cancer. We demonstrated that miR-645 was upregulated in RCCC tissues compared with para-carcinoma tissues, implying that miR-645 might play a potential and vital role in the development and progression of RCCC. Besides, downregulated miR-645 inhibited RCCC cell invasion and migration capacities, as well as attenuated cell proliferation, promoted cell apoptosis, and induced cell cycle arrest at G0/G1 phase. All these findings suggested that downregulated miR-645 exerted its suppressive effect on cell metastasis and proliferation of RCCC.

To further identify the underlying mechanism of how downregulated miR-645 suppressed RCCC cell tumorigenesis and metastasis, we predicted and selected glycerol kinase 5 (GK5) as the novel target gene of miR-645 by bioinformatics analysis. GK5 is located on chromosome 3q23, and it is a member of glycerol kinase, as well as a kind of phosphotransferase involved in the synthesis of triglycerides and glycerophospholipids\textsuperscript{22}. In addition, studies have found that GK5 is also closely related to the occurrence and development of tumors. Yu et al\textsuperscript{23} found that GK5 can regulate the malignant progression of gliomas via its interaction with miR-135b. However, the underlying upstream mechanism of GK5 in RCCC has not been well identified and reported yet. In our present study, we ini-

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The expression level of miR-645 was cut off by median expression level and * indicated p<0.05.
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potentially revealed that GK5 was directly targeted by miR-645, and GK5 expression was negatively correlated with miR-645 in RCCC tissues and cell lines. Moreover, silencing of GK5 could rescue tumor suppression role by downregulated miR-645 on RCCC cell metastasis and proliferation. The evidence indicated that miR-645 might be the upstream of GK5 involved in RCCC tumorigenesis and metastasis.

Conclusions

We demonstrated that downregulated miR-645 had tumor-suppressive effect on RCCC metastasis and proliferation via targeting GK5 in vitro. Our findings may help to elucidating molecular mechanisms underlying RCCC progression and provide miR-645 as an innovative and candidate target for diagnose and treatment of RCCC.

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Conflict of interest

The authors declare no conflicts of interest.

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