Identification of miR-195-3p as an oncogene in RCC

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Abstract. There is increasing evidence that the deregulation of microRNAs (miRNAs; miRs) contributes to tumorigenesis. Previous studies have shown that miR-195 is downregulated in various types of cancer. The present study aimed to investigate the function and expression levels of miR-125b. Results of qPCR revealed that miR-195-3p, the mature sequence of miR-195, was upregulated in renal cell carcinoma (RCC) tissues and cell lines (786-O, 769P and ACHN). This indicated that the function and role of miR-195-3p may differ in different types of tumor. To assess the function of miR-195-3p in RCC cell lines, cell proliferation was examined using MTT and CCK-8 assays, mobility was assessed using a cell scratch assay, Transwell migration assay and invasion assay, and apoptosis was examined using flow cytometry. These assessments were also performed in cells with upregulated or downregulated miR-195-3p via transfection with synthesized miR-195-3p mimic or inhibitor. The results revealed that the overexpression of miR-195-3p promoted 786-O and ACHN RCC cell proliferation, migration and invasion, and inhibited cell apoptosis, whereas the downregulation of miR-195-3p suppressed cell proliferation, migration and invasion, and induced cell apoptosis. These results indicated that miR-195-3p was associated with the tumorigenesis of RCC, with further investigations to focus on the pathway and use of miR-195-3p as a clinical biomarker for RCC.

Introduction

Renal cell carcinoma (RCC), a common malignant tumor originating from renal tubular epithelial cells, is the most common type of renal cancer and the third most common type of urological cancer, accounting for 2-3% of all adult malignancies according to a survey conducted in 2013 in the USA (1,2). Worldwide, RCC accounts for ~2% of cancer-associated mortality (2,3), and clear cell carcinoma is the most common subtype of RCC, which accounts for ~80% (4). Early diagnosis and treatment for this type of RCC is difficult as it lacks characteristic symptoms, and is resistant to radiotherapy and chemotherapy (5). Therefore, it is essential to investigate the mechanism of RCC to identify a biomarker for early diagnosis and targeted therapy.

The roles of deregulated microRNAs (miRNAs; miRs) in tumorigenesis have attracted increasing attention. miRNAs are a class of short, single-stranded non-coding RNAs with a length of ~22 nucleotides (6,7). miRNAs can exert effects by imperfect binding with the 3’ untranslated region of mRNA and cause translational repression or mRNA cleavage (8,9). The function of miRNAs as oncogenes or tumor suppressors depends on the target gene they regulate. Previous studies have demonstrated that miRNAs are associated with various cellular processes, including proliferation, apoptosis, differentiation and stress response (6,10). miR-195, located on chromosome 17p13.1, has been shown to be downregulated and function as a tumor suppressor in different types of tumor, including bladder cancer (11,12), osteosarcoma (13) and cervical cancer (14). However, previous miRNA microarray chip analysis of RCC showed that miR-195-3p, the mature sequence of miR-195 also termed miR-195, was upregulated (15), which revealed that the role of miR-195-3p in RCC may be different, compared with other tumors. Therefore the present study performed reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis to detect the expression level of miR-195 in RCC tissues and cell lines, and the investigated the role of miR-195-3p in RCC tumorigenesis by performing cell proliferation, mobility and apoptotic assays.

Materials and methods

Tissue samples. In the present study, 31 paired tissues were collected from Peking University Shenzhen Hospital (Shenzhen, China) from December 2012 to December 2014.
Each pair of tissues included RCC tissue and adjacent normal tissue, which was a 2 cm distance from the visible RCC lesion. The collection and use of tissue samples were reviewed and approved by the Ethics Committees of Peking University Shenzhen Hospital, and written informed consent was obtained from all patients. The tissues were immersed in RNA later (Qiagen GmbH, Hilden, Germany) for 30 min on dissection and then stored at -80°C for further use. These tissues were reviewed and classified using hematoxylin and eosin staining. The clinical and pathological characteristics of the patients are presented in Table I.

**Cell lines.** The cell lines used in the present study comprised 293T human embryonic kidney cells (the Type Culture Collection of the Chinese Academy of Medical Sciences, Shanghai, China), and 786-O, ACHN and 769P RCC cell lines (the American Type Culture Collection, Manassas, VA, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% antibiotics (100 µl/ml penicillin and 100 mg/ml streptomycin sulfates) and 1% glutamine in the humidified incubator containing 5% CO₂ at the temperature of 37°C.

**RNA extraction and RT-qPCR analysis.** Total RNA was extracted from the tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and purified using an RNeasy Maxi kit (Qiagen GmbH) according to the manufacturer’s protocol. The concentrations were measured on a NanoDrop2000/2000c spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, transcription was performed using the miScript Reverse Transcription kit (Qiagen GmbH), according to the manufacturer’s protocol. The transfected cells were plated in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% antibiotics (100 µl/ml penicillin and 100 mg/ml streptomycin sulfates) and 1% glutamine in the humidified incubator containing 5% CO₂ at the temperature of 37°C.

**Cell transfection.** Transfection of 786-O and ACHN cells with miR-195-3p mimic, inhibitor, negative control (NC) and inhibitor NC (GenePharma, Shanghai, China) was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), when cells reached 70-90% confluency. Cells were transfected for 4-6 h at 37°C. Alterations in the expression levels of miR-195-3p following transfection were determined by performing RT-qPCR analysis with the aforementioned thermocycling conditions. The primer sequences used are shown in Table II.

**Cell mobility assay.** A cell scratch assay and Transwell assay were performed to assess the mobility of the 786-O and ACHN cells. In the cell scratch assay, ~6x10⁵ cells were plated in each well of a 6-well plate. After 24 h, the cells were transfected with 200 pmol miR-195-3p mimic, inhibitor, NC or inhibitor NC. At 6 h post-transfection, a vertical horizontal line was scratched in the cell layer using a sterile 200 µl pipette tip. Images of the scratches at 0 and 24 h were captured using a digital camera system. The experiments were performed in triplicate and repeated at least three times. Transwell invasion and migration assays were performed to assess the migratory and invasive abilities of the 786-O and ACHN RCC cells. Transwell chamber inserts (BD Biosciences, Franklin Lakes, NJ, USA) with (to assess invasion) or without (to assess migration) Matrigel (BD Biosciences) were used in the assay, according to the manufacturer’s protocol. The transfected cells (1x10⁴) in 200 µl serum-free medium were seeded in the upper chamber of the insert. In the bottom of the inserts was medium containing 10% FBS. The cells were allowed to migrate for 36 h or invade for 48 h in the humidified incubator containing 5% CO₂ at the temperature of 37°C. The migratory or invasive cells on the bottom of the inserts were stained with crystal violet and counted using a microscope (Leica Microsystems GmbH, Wetzlar, Germany). The experiments were performed in triplicate and repeated at least three times.

**Cell proliferation assay.** MTT and CCK-8 assays were performed to assess cell proliferation ability. The cells (~3,000) were seeded in each well of 96-well plate and, 24 h later, were transfected with 5 pmol of miR-195-3p mimic, inhibitor, NC or

<table>
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AJCC, American Joint Committee on Cancer.
RT-qPCR analysis was performed to detect the expression levels of miR-195-3p in RCC tissues and cell lines. The ratios of expression of miR-195-3p in 31 paired RCC tissue samples are shown in Fig. 1A, which showed miR-195-3p was upregulated in 21 tissue samples. The mean relative expression of miR-195-3p in the RCC tissues was 3.88-fold higher, compared with the expression in adjacent normal tissues, as shown in Fig. 1B (P<0.05). The results demonstrated that the expression levels of miR-195-3p in 786-O, 769P and ACHN cells was 33.21-, 3.90- and 3.31-fold higher, compared with the expression in adjacent normal tissues, as shown in Fig. 1A, which showed miR-195-3p was upregulated in RCC tissues, compared with adjacent normal tissues, and miR-195-3p may have an oncogenic role in RCC.

Validation of cell transfection efficiency. RT-qPCR analysis was performed to quantify the transfection efficiency of miR-195-3p mimics or inhibitors, compared with NC or inhibitor NC. The results indicated that the expression levels of miR-195-3p in the miR-195-3p mimic group were 424.58-fold higher (786-O) and 328.37-fold higher (ACHN), compared with the expression in 293T cells, respectively (Fig. 1C). The results suggested that miR-195-3p was upregulated in RCC tissues, compared with adjacent normal tissues, and miR-195-3p may have an oncogenic role in RCC.

miR-195-3p promotes cell proliferation. MTT and CCK-8 assays were performed to detect cell proliferation following transfection. The data are shown as the mean ± standard error of the mean. As shown in Fig. 2A, the results of the CCK-8 assay suggested that the overexpression of miR-195-3p promoted 786-O cell proliferation by 5.26, 14.61 (P<0.01) and 8.09% (P<0.001), and the downregulation of miR-195-3p (Fig. 2B) inhibited 786-O cell proliferation by 3.79, 10.99 (P<0.01) and 9.07% (P<0.001) at 24, 48 and 72 h post-transfection, respectively. In the ACHN cells, cell proliferation was promoted by 6.32 (P<0.05), 19.76 (P<0.01) and 11.89 (P<0.001) in the miR-195-3p mimic group. Cell proliferation in the miR-195-3p inhibitor group was inhibited by 5.25 (P<0.05), 6.32 (P<0.05) and 9.38% (P<0.01) at 24, 48 and 72 h post-transfection, compared with the NC or inhibitor NC groups, respectively (Fig. 2C and D).

The results of the MTT assay showed that the proliferation of 786-O cells (Fig. 2E and F) in the inhibitor group was

Table II. Sequences of transfectants and primers used in the present study.

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<tr>
<th>miR-195-3p mimic</th>
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<tr>
<td>NC</td>
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<td>miR-195-3p reverse primer</td>
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<td>miR-195-3p reverse primer</td>
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miR, microRNA; NC, negative control.
miR-195-3p decreases cell mobility. Cell scratch, Transwell migration and invasion assays were performed to investigate the effect of miR-195-3p on RCC (786-O and ACHN) proliferation reduced by 4.38, 9.83 (P<0.01), and 8.29% (P<0.01), and that in the mimic group was promoted by 3.65, 10.37 (P<0.05) and 25.34% (P<0.01), compared with the inhibitor NC or NC groups at 24, 48 and 72 h post-transfection. In ACHN cells, the results of the MTT assay showed that overexpression of miR-195-3p (Fig. 2G) promoted cell proliferation by 5.42, 10.88 (P<0.01) and 17.78% (P<0.01), whereas downregulation of miR-195-3p (Fig. 2H) inhibited 786-O cell proliferation by 5.69 (P<0.05), 9.21 (P<0.01) and 6.41% (P<0.01) at 24, 48 and 72 h post-transfection, respectively. The results of the proliferation assays showed that miR-195-3p promoted RCC cell proliferation.

miR-195-3p increases cell mobility. Cell scratch, Transwell migration and invasion assays were performed to investigate the effect of miR-195-3p on RCC (786-O and ACHN) proliferation reduced by 4.38, 9.83 (P<0.01), and 8.29% (P<0.01), and that in the mimic group was promoted by 3.65, 10.37 (P<0.05) and 25.34% (P<0.01), compared with the inhibitor NC or NC groups at 24, 48 and 72 h post-transfection. In ACHN cells, the results of the MTT assay showed that overexpression of miR-195-3p (Fig. 2G) promoted cell proliferation by 5.42, 10.88 (P<0.01) and 17.78% (P<0.01), whereas downregulation of miR-195-3p (Fig. 2H) inhibited 786-O cell proliferation by 5.69 (P<0.05), 9.21 (P<0.01) and 6.41% (P<0.01) at 24, 48 and 72 h post-transfection, respectively. The results of the proliferation assays showed that miR-195-3p promoted RCC cell proliferation.
cell mobility. The results of cell scratch assay are shown in Fig. 3. Overexpression of miR-195-3p by transfection with the miR-195-3p mimic promoted the 786-O cell migratory distance by 60.31% (P<0.001) and the ACHN distance by 34.23% (P<0.01) at 24 h post-transfection, compared with the NC cells. Downregulation of miR-195-3p by transfection with miR-195-3p inhibitor reduced cell migratory distance by 38.13% (P<0.001) in the 786-O cells and 40.01% (P<0.01) in the ACHN cells at 24 h post-transfection, compared with the cells transfected with inhibitor NC.

As shown in Fig. 4, the results of the Transwell invasion assay showed that the invasive ability of 786-O cells was promoted by 52.83% (P<0.01) by upregulating miR-195-3p, and was suppressed by 51.69% (PP<0.01) by downregulating miR-195-3p (Fig. 4B). The migratory ability of 786-O cells was promoted by 80.79% by upregulating miR-195-3p, and suppressed by 45.44% (P<0.01) by downregulating miR-195-3p (Fig. 4C).

In ACHN cells, the Transwell invasion assay showed that the invasive ability of cells transfected with the miR-195-3p mimic was increased by 103.53% (P<0.01) and reduced by 27.85% (P<0.01) in cells transfected with the miR-195-3p inhibitor, compared with the NC or inhibitor NC group, respectively (Fig. 5B). As shown in Fig. 5C, the migratory ability of cells transfected with the miR-195-3p mimic was increased by 44.91% (P<0.01) and reduced by 41.35% (P<0.01) in cells transfected with miR-195-3p inhibitor, compared with cells transfected with NC or inhibitor NC. The results of the Transwell and wound scratch assays indicated that miR-195-3p promoted the mobility of RCC cells.

**Knockdown of miR-195-3p induces cell apoptosis.** Flow cytometry was performed to qualify the apoptotic rate of RCC cells following transfection. At 48 h post-transfection with the miR-195-3p mimic, miR-195-3p inhibitor, NC or inhibitor NC, cells were collected for measurement. As shown in Fig. 6A and B, the apoptotic rate of 786-O cells transfected with the miR-195-3p mimic was 10.45%, and was 22.27% in cells transfected with NC (P<0.01). The apoptotic rates of 786-O cells transfected with the miR-195-3p inhibitor or inhibitor NC were 33.58 and 20.50%, respectively (P<0.01). In ACHN cells transfected with the miR-195-3p inhibitor, the apoptotic rate was 18.91%, and was 8.73% in cells transfected with the inhibitor NC (P<0.01). However, no significant differences were found between cells transfected with the miR-19-3p 5 mimic and NC, with apoptotic rates of 4.83% in the mimic group and 6.62% in the NC group (Fig. 7A and B). These results revealed that the knockdown of miR-19-3p 5 induced RCC cell apoptosis.

**Discussion**

Tumorigenesis is involved with the activation of a series of oncogenes and inactivation of various tumor suppressors. The genes identified to be associated with RCC, comprising Von Hippel-Lindau, MET, folliculin, TSC1, TSC2, FH and SDH, are all possibly regulated by miRNAs, therefore, miRNAs are potential biomarkers for RCC for use as targeted therapy.

In the present study RT-qPCR analysis revealed that miR-195 was upregulated in RCC, whereas previous studies have shown that miR-195 is downregulated in the majority of types of cancer, including colorectal cancer (17), glioblastoma (18), bladder cancer (11,12), osteosarcoma (13), cervical cancer (14), gastric cancer (19), hepatocellular carcinoma (20), esophageal squamous cell carcinoma (10), breast cancer (21), non-small cell lung cancer (22) and prostate cancer (23). Therefore, the present study, to the best of our knowledge...
Figure 5. (A) miR-195-3p is involved in ACHN cell migration and invasion. (B) Upregulation of miR-195-3p promoted ACHN cell invasion, whereas downregulation inhibited invasion. (C) The migratory ability of ACHN cells was promoted by the upregulation of miR-195-3p and inhibited by its downregulation. **P<0.01. miRNA, microRNA; NC, negative control.

Figure 4. (A) miR‑195‑3p is involved in 786‑O cell migration and invasion. Magnification, x200. (B) Overexpression of miR‑195‑3p promoted 786‑O cell invasion ability, whereas downregulation of miR‑195‑3p inhibited invasion ability. (C) Migratory ability of 786‑O cells was promoted by the upregulation of miR‑195‑3p and inhibited by its downregulation. **P<0.01. miRNA, microRNA; NC, negative control.
was the first to report that miR-195 was upregulated in RCC. Subsequently, the function of miR-195 in RCC was examined, and the results revealed that the overexpression of miR-195 promoted RCC cell proliferation, migration and invasion, and reduced apoptosis, whereas the downregulation of miR-195 suppressed cell proliferation, migration and invasion induced apoptosis. With the exception of ACHN cells, the overexpression of miR-195 marginally reduced the
apoptotic rate of the ACHN cells with a characteristic low apoptotic rate.

Previous studies of miR-195 have focused on urological cancer, with the exception of renal tumors. Guo et al. (24) found that miR-195 suppressed prostate cancer cell proliferation and metastasis by targeting BCOX1. Another study of miR-195 in prostate cancer revealed that miR-195 suppresses prostate cancer cell migration and invasion through its direct target gene, Fra-1 (25). miR-195 was also found to inhibit prostate cancer cell metastasis and EMT by targeting FGFR2 (26). Therefore, in prostate cancer, miR-195 functions as a tumor suppressor partially by targeting BCOX1, Fra-1 and fibroblast growth factor 2 FGFR2. In bladder cancer it has been demonstrated that miR-195 induces G1-phase arrest by targeting CDK4 (27), and inhibits bladder cancer cell proliferation, at least partially, through the inhibition of Cdc42/STAT3 signaling (12). miR-195 has been indicated to be associated with the glycometabolism in bladder cancer by suppressing glucose uptake through regulating the expression of GLUT3 (11). In other tumors of the urological system miR-195 is predominantly a tumor suppressor and can affect cellular migration, invasion, metastasis, EMT and glycome-tabolism.

Various studies of miR-195 have revealed that miR-195 acts as a tumor suppressor in hepatocellular carcinoma (HCC) and colorectal cancer (CRC). miR-195 has been reported to regulate HCC cell apoptosis, proliferation, invasion and migration (9,19,28-30). It has also been reported that miR-195 is involved as a tumor suppressor by targeting LAST2 (27), SRC-3 (28), CBX4 (30), tumour necrosis factor-α/nuclear factor-κB (31) and PCMT1 (32). Wang et al. (20) found that miR-195 suppresses HCC angiogenesis and metastasis by inhibiting VEGF, VAV2 and CDC42 (20). Another study showed that the miR-497-195 cluster can regulate HCC cell proliferation and cell cycle by targeting CCNE1, CDC25A, CCN3, CDK4 and BTRC (33). All studies on miR-195 in HCC have indicated that miR-195 functions as a tumor suppressor. In CRC, miR-195 has been described as a tumor suppressor by regulating cell proliferation, migration, invasion and apoptosis (17,34,35). A study investigating miR-195 as a biomarker in CRC demonstrated that the downregulation of miR-195 was associated with poor prognosis and lymph node metastasis (6). miR-195 has also been described as a biomarker in cervical cancer, osteosarcoma, adenocortical cancer and breast cancer (21,36-40). Zhao et al. (38) found that miR-195 has a higher sensitivity for breast cancer detection, and the expression level of miR-195 has been found to significantly predict the survival rates of patients with HER2-positive breast cancer (36). Zhang et al. (37) reported that use of a serum miRNA panel, comprising miR-16-2*, miR-195, miR-2861 and miR-497, was able to distinguish cervical cancer from cervical intraepithelial neoplasia and healthy controls with high accuracy. Down-regulated miR-195 can predict a poor prognosis in patients with osteosarcoma or adenocortical cancer (39,40). Therefore, miR-195 is a potential biomarker for multiple types of cancers, and can be used for diagnosis, targeted therapy or predicting prognosis.

miR-195 has been reported to have the ability to regulate the sensitivity of cancer cells to chemotherapeutic drugs. Yang et al. (41) found that miR-195 sensitizes HCC cells to 5-FU by targeting BCL-w. In colon cancer, miR-195 has been shown to sensitize cells to doxorubicin by targeting BCL2L2 (42). In breast cancer, the overexpression of miR-195 sensitizes cells to adriamycin by inhibiting Raf-1, and enhances the radiosensitivity of cells by inhibiting BCL2 (43,44). Thus, miR-195 is a novel anticarcinogen in certain types of cancer that are particularly resistant to certain chemotherapeutics.

miR-195 has been associated with diseases other than cancer. In Alzheimer's disease miR-195 can negatively regulate BACE1, which offers potential therapy for Alzheimer's disease.

In conclusion, the present study is the first, to the best of our knowledge, to describe miR-195-3p as an oncogene in RCC by regulating RCC cell proliferation, mobility and apoptosis. Further investigations aim to focus on the pathway of miR-195-3p in RCC and the possibility of using as a biomarker for RCC.

Acknowledgements

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References


