MicroRNA-195 inhibits proliferation and metastasis in renal cell carcinoma via regulating HMGA1

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Abstract: Growing evidence indicates that aberrant expression of microRNAs (miRNAs) contributes to tumorigenesis in various human malignancies. In this study we revealed that miR-195 acted as a tumor suppressor in renal cell carcinoma (RCC) through inhibition of HMGA1 expression. qRT-PCR was used to detect the miR-195 expression in RCC tissues and cell lines. RCC cell line Caki-1 and Caki-2 cells were used in this study. The luciferase report assay and rescue assay were performed to identify HMGA1 as the target gene of miR-195. Additionally, Kaplan-Meier method and log-rank test was used to explore the relationship between HMGA1 expression and RCC prognosis. We observed that miR-195 expression was significantly downregulated both in RCC tissues and in RCC cell lines. We observed that miR-195 overexpression inhibits the abilities of RCC cell proliferation, cell cycle progression and metastasis in vitro by targeting HMGA1 via epithelial to mesenchymal transition (EMT) pathway. In clinical specimens, HMGA1 was overexpressed in high-grade RCC when compared with its levels in normal tissues and low-grade RCC cancer, its expression levels were inversely correlated with overall survival. Our findings highlight an important role of miR-195 and HMGA1 in the molecular etiology of RCC, indicating that they can serve as potential biomarkers and therapy targets of RCC.

Keywords: miR-195, HMGA1, RCC, proliferation, metastasis

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults [1]. Men are more likely to be diagnosed with this type of cancer [1, 2]. Epidemiological studies have shown that various factors such as smoking, obesity, hypertension and use of non-steroidal anti-inflammatory drugs, may be involved in the development of renal cell carcinoma [3, 4]. Although the five-year survival rate of localized RCC patients is around 80%-95%, the five-year survival rate is around 5% to 15% when RCC metastasized to lymph nodes [5]. The symptoms of RCC is usually hidden as a result people with RCC often have advanced disease by the time it is discovered [6]. Therefore, identifying efficient targets for the early diagnosis of RCC for the therapy of RCC is needed.

MicroRNA (miRNA) is a single-stranded, small non-coding RNA molecule which functions in RNA silencing and post-transcriptional regulation of gene expression [7, 8]. Previous studies have reported that miRNA may be an important causal factor causing DNA repair deficiencies and associated with tumorigenesis [9, 10]. It is reported that down-regulation of miR-195 occurred very frequently in human hepatocellular carcinoma [11], clear cell renal cell carcinoma [12], gastric [13], pancreatic [14] and prostate cancer [15]. Here, we showed that down-regulation of miR-195 in RCC tissues and cell lines. Furthermore, we demonstrated the role of the miR-195/HMGA1 axis on epithelial to mesenchymal transition (EMT) cell cycle, invasion and proliferation of RCC cells. These findings suggest an important role of miR-195 in the molecular etiology of RCC and cell cycle.
Control, which may provide evidence for cancer therapy.

**Materials and methods**

**Tissue microarrays (TMAs)**

Tissue specimens were obtained from 133 renal cell carcinoma patients who underwent radical nephrectomy between 2008 and 2011 at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). All patients gave their written informed consent. The follow-up deadline was December 2017. The protocols used in the study were approved by the ethics committee of the hospital. Primary tumors from 133 patients were positioned on TMAs with 0.6 mm core specimens in triplicates.

**Immunohistochemistry (IHC) and TMA scoring**

Sections from TMA blocks were deparaffinized in xylene and rehydrated and heated in a steam pressure cooker in citrate buffer (pH 6.0). Then samples were blocked for 5 min and incubated overnight with primary antibodies against HMGA1 (Abcam, UK) (1:100) at 4°C overnight. The results of the IHC staining were determined independently by two pathologists without knowledge of the clinical data. Assessments were performed according to the following standards: the intensity was 0 (no immunoreactivity), 1 (weak), 2 (moderate) or 3 (strong), and the proportion of cells stained was scored as 0 (0%), 1 (0-30%), 2 (30-60%), or 3 (>60%). Expression of HMGA1 was graded as high expression (proportion plus intensity scores ≥4), or low expression (proportion plus intensity scores ≤3).

**Cell culture and transfection**

The human RCC cell lines (Caki-1 and Caki-2) and 293T were purchased from the Chinese Academy of Sciences (Shanghai, China). Caki-1 and Caki-2 cell lines were cultured in McCoy’s 5A (modified) Medium (Gibco, USA). 293T were cultured in DMEM (Gibco, USA). The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) in a humidified air atmosphere at 37°C and 5% CO₂.

RCC cells were transfected with miR-195 mimics, non-specific miRNA control and HMGA1 siRNA (GenePharma, China) by using Lipofectamin 2000 (Invitrogen, USA) according to the manufacturer’s protocol.

**Western blotting**

The total proteins of RCC cells were lysed in RIPA buffer (KeyGene biotech) supplemented with protease inhibitors. Protein were separated by 10% SDS/PAGE after boiling the samples for 10 min. Then the lysates were transfected onto PVDF membranes in transfer buffer. The PVDF membranes were blocked in 5% non-fat milk with Tris-buffered saline with Tween (TBST) for 2 h, then the PVDF membranes were treated overnight at 4°C with the following primary antibodies: E-cadherin (Cell Signaling Technology, USA), HMGA1 (Abcam, UK), N-cadherin (Cell Signaling Technology, USA), vimentin (Cell Signaling Technology, USA).

**Total RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)**

Using Trizol (Invitrogen, USA) for RNA extraction from RCC cell lines according to manufacturer's instruction. miRNA was reverse-transcribed into cDNA using MiR-X™ miRNA First-Strand Synthesis (Takara, JPN). Total RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara, JPN). A standard SYBR Green PCR kit (Takara, JPN) was used to perform qRT-PCR. The following forward and reverse primer sequences of HMGA1 were used: 5'-CAGCCGTCCTTTCACA:3' and 5'-TGC-CTTGCTTCC-3', respectively. The following forward and reverse primer sequences of β-actin were used: 5'-TGCAGGGTCCACCAC- ACTGTCCTCCCTGA-3' and 5'-CTGAAGCATTGG-CGGTGACGATTGGAGG-3', respectively. The reaction conditions used for mRNA detection were as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 30 s.

**Cell proliferation assays**

The cell counting kit-8 (CCK-8) was used for assessing cell proliferation. For the CCK-8 assay, 48 h subsequent to transfection, Caki-1 and Caki-2 cells were seeded in 96-well plates at the density of 2000 cells per well. CCK8 (Dojindo, JPN) was used to determine cell proliferation at 24, 48, 72 and 96 respectively after seeding. The absorbance was determined by an absorbance reader (Thermo Scientific, USA) at 450 nm.
Cell migration and invasion assays

For migration assay, a total of $5 \times 10^4$ cells resuspended in serum-free McCoy’s 5A (modified) Medium after transfection were placed in the upper chamber with 8 μm pore size PET track-etched membranes (Corning, Inc., Corning, NY, USA) and incubated at 37°C in 5% CO$_2$ continuously for 24 h. The bottom chamber contained 10% FBS as a chemotaxtractant. After the incubation, the cells on the upper surface were removed by a cotton swab, while the cells invaded on the bottom of membranes were fixed in 4% methanol solution for 1 hours and stained with 0.005% crystal violet for 2 hours and counted in 3 random fields under a microscope.

Cell cycle analysis

A flow cytometry assay was performed to assess the influence of change of cell cycle distribution of Caki-1 and Caki-2 cells. The cells were fixed in 70% ethanol overnight at 4°C and then resuspended in PBS and labeled with 50 μg/ml propidium iodide and 100 μg/ml RNase for 30 min. The percentages of G$_1$, G$_2$ and S cells were counted and compared.

Luciferase assays

We used TargetScan (www.targetscan.org) and miRanda (www.microma.org) to predict the binding site of miR-195 and HMGA1. The binding sequence was point mutated to form a control luciferase reporter plasmid. For the luciferase assay, luciferase reporters containing 3’-UTR were cotransfected with miR-195 mimics or miRNA control, and wild-type or mutant-type HMGA1 plasmid. The luciferase activity was measured by a luminometer after 48 hours.

RNA immunoprecipitation (RIP) assay

RIP experiments were carried out utilizing the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) and antibody against IgG or Ago2 following the manufacturer’s specification. Caki-1 cells were collected at about 90% confluency and then lysed in complete RNA lysis buffer. Next, the supernatant of RIP lysate was incubated with the RIP buffer supplemented with magnetic beads containing antibody against IgG or Ago2, followed by the purification of RNA. Then, the expression patterns of HMGA1 and miR-195 in IgG or Ago2 immunoprecipitated complex were detected using Real-time PCR.

Data analysis

The association of clinicopathological parameter and protein expression were assessed using Chi-square test. Kaplan-Meier curve and log-rank tests were used to assess the association of HMGA1 expression and clinicopathologic parameters. All data were analyzed using the SPSS 14.0.

Results

Expression of miR-195 is decreased in RCC tissues and cell lines

We compared the miR-195 expression levels between RCC tissues and paired adjacent noncancerous renal tissues from 2008 to 2011 individual patients by qRT-PCR. The results showed that miR-195 expression was significantly reduced ($P=0.002$) in RCC tissues compared with adjacent normal tissues (Figure 1A). Next we analyzed miR-195 expressions in two RCC cell lines (Caki-1, Caki-2) and normal renal epithelial cells (HK-2). As shown in Figure 1B, the expression level of miR-195 was significantly lower in Caki-1 and Caki-2 compared with HK-2. These results suggest that miR-195 may function as a suppressive gene in the progression of RCC.

MiR-195 overexpression significantly suppresses RCC cells proliferation and metastasis

To examine the potential role of miR-195 in tumorigenesis, we first evaluated the effect of miR-195 on cell proliferation of cancer cells. Two RCC cell lines (Caki-1, Caki-2) were transfected with NC or miR-195 mimics and verified by using qRT-PCR (Figure 1C). And then a CCK8 assay was performed. Notably, miR-195 transfected cells displayed significantly low proliferation rate in both Caki-1 and Caki-2 (Figure 1D and 1E), indicating a growth-inhibitory effect of miR-195 in RCC cells.

Invasiveness and migratory capacity of the cancer cells are essential for tumor progression. To investigate whether miR-195 worked on migratory and invasive capabilities of RCC cells,
Micro-195 inhibits RCC via HMGA1

Figure 1. Expression of miR-195 is significantly lower in RCC tissues and cell lines and miR-195 overexpression inhibited RCC cells function. (A) The expression of miR-195 was compared between RCC and adjacent normal tissues. Further, the expression in RCC was significantly lower than the adjacent normal tissues. (B) The expression of miR-195 in normal renal cells (HK-2) was much higher than RCC cells (Caki-1 and Caki-2). (C) qRT-PCR was performed to validate the efficacy of overexpressing miR-195 in Caki-1 and Caki-2. (D and E) Proliferation effect of Caki-1 cells and Caki-2 cells with control or overexpression of miR-195 were analyzed by CCK-8 assays. This result indicated that miR-195 transfected cells displayed significantly low proliferation rate in both Caki-1 and Caki-2. (F and G) Representative results of migration and invasion of (F) (Caki-1 cells) and (G) (Caki-2 cells) were analyzed by transwell assays. The migration and invasion rate were significantly lower in Caki-1 and Caki-2 cells transfected with miR-195 compared with NC-transfected cells. (H and I) Effect of miR-195 overexpression on cell cycle in (H) (Caki-1) and (I) (Caki-2). The percentage of cells in S and G2 phase was significantly increased upon treatment with miR-195 mimics in both Caki-1 and Caki-2. *P<0.05.
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Transwell and Matrigel invasion assays were performed to assess the significance of miR-195 in RCC cells. As shown in Figure 1F and 1G, the migration and invasion rate were significantly lower in Caki-1 and Caki-2 cells transfected with miR-195 compared with NC-transfected cells. This suggested that miR-195 may function as a suppressor role in RCC metastasis.

**MiR-195 induces cell cycle arrest in RCC cells**

To explore more about the intrinsic mechanism of miR-195 on cell proliferation, we measured the effects of miR-195 on cell cycle distribution. As showed in Figure 1H and 1I, the percentage of cells in S and G2 phase was significantly increased upon treatment with miR-195 mimics in both Caki-1 and Caki-2.

**HMGA1 is a direct target of miR-195**

Analysis of the 3'-UTR sequence of HMGA1 using TargetScan and MiRanda revealed one potential binding site for miR-195 (Figure 2A). To verify the influence of miR-195 in HMGA1 expression, qRT-PCR and western blot were performed both in Caki-1 and Caki-2 transfected with miR-195 mimics. As shown in Figure 2B and 2C, the mRNA and protein expression levels of HMGA1 were significantly lower in Caki-1 and Caki-2 cells transfected with miR-195 compared with NC. To further validate whether HMGA1 is a direct target of miR-195, we fused the predicted HMGA1 3'-UTR target site, including binding site for miR-195, to the downstream of the firefly luciferase gene. As shown in Figure 2D, miR-195 overexpression significantly suppressed the luciferase activity of the wild type HMGA1 plasmid but not that of the mutant one. (E) Relative amount of HMGA1 following transfected with miR-195 or miR-NC bound to IgG or Ago2 tested by Real-time PCR. Results showed that HMGA1 and miR-195 were substantially enriched in Ago2 immunoprecipitated of Caki-1 cells compared with control group. *P<0.05.

MiR-195 functions as a tumor-suppressive gene by inhibiting HMGA1 expression

To determine whether miR-195 suppresses RCC progression by reducing HMGA1 expression, we first investigated whether HMGA1 could affect the RCC progression. HMGA1
Figure 3. Knockdown of HMGA1 inhibited RCC cell functions. (A and B) qRT-PCR (A) and western blot (B) were performed to validate the efficacy of knocking down HMGA1 in Caki-1 and Caki-2 cells. (C and D) Proliferation effect of (C) (Caki-1) and (D) (Caki-2) cells with control or downregulation of HMGA1 were analyzed by CCK-8 assay. The capabilities of cell proliferation of (C) (Caki-1) and (D) (Caki-2) cells were suppressed following treatment with transient knockdown of HMGA1. (D and E) Representative results of migration and invasion of (E) (Caki-1) and (F) (Caki-2) cells with control of downregulation of HMGA1 were analyzed by transwell assays. The capabilities of cell migration and invasion of (E) (Caki-1) and (F) (Caki-2) cells were suppressed following treatment with transient knockdown of HMGA1. (G and H) Effect of control or knockdown of HMGA1 on cell cycle of (G) (Caki-1) and (H) (Caki-2). Compared with si-NC transfection, si-HMGA1 induced a statistically significant decrease of G1-cells and increase of G2-cells both in Caki-1 and Caki-2. *P<0.05.
Micro-195 inhibits RCC via HMGA1
Micro-195 inhibits RCC via HMGA1

Figure 4. Re-expression of HMGA1 reestablished RCC cell functions and miR-195 regulates HMGA1 expression through EMT process and HMGA1 expression is correlated with RCC prognosis. (A) Re-expression of HMGA1 was confirmed by Western blot following co-transfection of miR-195 mimics and pL-HMGA1. The protein level of HMGA1 was significantly increased following co-transfection of HMGA1 plasmid compared with miR-195 mimics alone. (B and C) Proliferation effect of (B) (Caki-1) and (C) (Caki-2) cells co-transfected with miR-195 mimics and pL-HMGA1. Abilities of proliferation were restored by restoration of HMGA1 in miR-195-overexpressed RCC cells. (D and E) Representative results of migration and invasion of Caki-1 and Caki-2 (E) co-transfected with miR-195 mimics and pL-HMGA1 were analyzed by transwell assays. Abilities of migration and invasion were restored by restoration of HMGA1 in miR-195-overexpressed RCC cells. (F and G) Effect of control or co-transfected with miR-195 mimics and pL-HMGA1 on cell cycle of (F) (Caki-1) and (G) (Caki-2). There were no significant differences in G1-, G2- and S cells. (H) Western blot was performed to test EMT associated protein such as E-cadherin, N-cadherin and Vimentin in Caki-1 and Caki-2 transfected with miR-NC or miR-195 mimics. (I) EMT associated protein expression after transfection with si-HMGA1 or NC in Caki-1 and Caki-2. Western blot results showed that the expression of E-cadherin was increased while N-cadherin and Vimentin were decreased after inhibiting HMGA1 in Caki-1 and Caki-2 cells. (J) EMT associated protein expression in Caki-1 and Caki-2. Restoration of HMGA1 in miR-195-overexpressed RCC cells significantly increased the abilities of EMT process. (K) To investigate the expression pattern of HMGA1 in RCC, Immunohistochemical images of HMGA1 expression in RCC tissues. (L) Kaplan-Meier/Log-rank analysis for overall survival from 133 RCC patients according to HMGA1 expression in tumor tissues. The Kaplan-Meier curve indicated a significant shorter overall survival for those patients with high HMGA1 expression. (M) Kaplan-Meier/Log-rank analysis for overall survival from TCGA database. *P<0.05.
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Table 1. Characteristics of the 133 RCC patients involved in this study

<table>
<thead>
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<th>Value</th>
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<td>Age</td>
<td>Mean ± SD, year</td>
</tr>
<tr>
<td></td>
<td>&lt;60</td>
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<tr>
<td></td>
<td>≥60</td>
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<tr>
<td>Gender</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>Female</td>
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<tr>
<td>Tumor size</td>
<td>Mean ± SD, cm</td>
</tr>
<tr>
<td></td>
<td>≤4</td>
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<tr>
<td></td>
<td>&gt;4</td>
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<tr>
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<tr>
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</tr>
<tr>
<td></td>
<td>II</td>
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<td></td>
<td>III</td>
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<td></td>
<td>T3</td>
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<td></td>
<td>T4</td>
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<tr>
<td>Survival</td>
<td>Mean ± SD, month</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>HMGA1 expression</td>
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<tr>
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</table>

SD, standard deviation.

siRNA was transfected into Caki-1 and Caki-2 cells to decrease endogenous HMGA1 expression and verified by qRT-PCR and western blot (Figure 3A, 3B). We observed that capabilities of cell proliferation (Figure 3C and 3D), migration and invasion (Figure 3E and 3F) were suppressed following treatment with transient knockdown of HMGA1. Compared with si-NC transfection, si-HMGA1 induced a statistically significant decrease of G1-cells and increase of G2-cells both in Caki-1 and Caki-2 (Figure 3G and 3H). These results were consistent with the previous data when cells were transfected with miR-195 mimics. To further explore the relationship between miR-195 and HMGA1, we co-transfected HMGA1 overexpression plasmids and miR-195 mimics to RCC cells and observed whether cell functions could be reversed by overexpression of HMGA1. As shown in Figure 4A, the protein level of HMGA1 was significantly increased following co-transfection of HMGA1 plasmid and miR-195 mimics compared with miR-195 mimics alone. Moreover, restoration of HMGA1 in miR-195-overexpressed RCC cells significantly restored abilities of proliferation (Figure 4B and 4C), migration and invasion (Figure 4D and 4E). Moreover, no significant differences were observed in G1-cells, G2-cells and S cells between cells co-transfected with miR-195 mimics and HMGA1 plasmid and cells transfected with miR-NC and control plasmids (Figure 4F and 4G). These data suggest that miR-195 may suppressed RCC progression through the downregulation of HMGA1 expression.

MiR-195 overexpression significantly suppresses EMT process of RCC cells

Initiation of tumor metastasis requires invasion, which is supported by EMT [16, 17]. Additionally, miR-195 has been proven to be involved in EMT process in multiple cancers [15, 18, 19]. Based on the association between miR-195 expression and the metastasis of RCC cells, western blot assay was performed to assess the EMT-associated protein expression including E-cadherin, N-cadherin and vimentin. After transfection with miR-195 mimics, we observed the increased expression of E-cadherin and decreased of N-cadherin and vimentin in both Caki-1 and Caki-2 (Figure 4H). These results indicated that miR-195 overexpression reversed the EMT process in RCC cells.

MiR-195 regulates HMGA1 expression through EMT process

Western blot results showed that the expression of E-cadherin was increased while N-cadherin and vimentin were decreased after inhibiting HMGA1 in Caki-1 and Caki-2 cells (Figure 4I). In addition, restoration of HMGA1 in miR-195-overexpressed RCC cells significantly increased the abilities of EMT process (Figure 4J), which suggested that miR-195 suppress RCC progression by inhibiting HMGA1 expression possibly through EMT process.
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Table 2. Relationship of HMGA1 expression and clinico-pathologic characteristics of patients

<table>
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HMGA1 may act as a prognostic indicator for RCC

To investigate the expression pattern of HMGA1 in RCC, immunohistochemical staining for HMGA1 was used to detect the expression pattern and subcellular localization of HMGA1 protein in 133 RCC tissues (Figure 4K). The characteristics of 133 RCC patients were shown in Table 1. As shown by the chi-square test results (Table 2), higher HMGA1 protein expression was significantly associated with tumor size (P<0.005), histological grade (P<0.005) and tumor stage (P=0.005). In addition, the Kaplan-Meier curve indicated a significant shorter overall survival for those patients with high HMGA1 expression (P<0.0001) (Figure 4L). These findings were validated in an independent microarray data set from TCGA (Figure 4M). These results indicated that high expression of HMGA1 resulted in the poor prognosis in RCC patients.

Discussion

This is the first study to show that HMGA1 is negatively regulated by miR-195. We also showed that miR-195 inhibit HMGA1 expression through EMT process.

Emerging evidence has shown that microRNAs play a crucial role in the development, progression and metastasis of RCC [20-22]. miR-195 has been reported as tumor suppressor, metastatic inhibitor and novel therapeutic targets in many types of cancers [18, 23, 24]. However, further investigations on the functional effects and the molecular mechanisms of specific of miR-195 in RCC are still unknown.

In this study, we confirmed that miR-195 was frequently down-regulated in both RCC tissues and cell lines, which may lead to a poor prognosis of RCC. Ectopic expression of miR-195 suppressed RCC cell lines to growth in vitro. Moreover, our studies revealed that miR-195 could induce cell cycle arrest in RCC cells. We propose that reduced expression of miR-195 may disrupt cell cycle control, then promote cell proliferation, and consequently facilitate the progression of RCC.

We found that overexpression of miR-195 significantly downregulated HMGA1 protein expression in RCC cells. HMGA1 proteins are thought to play an important role in various cancers [25, 26]. For example, hepatocellular carcinoma was promoted by HMGA1 through ILK/Akt/GSK3β pathway [27]. HMGA1 induced thyroid cancer proliferation and invasion possibly through TGF-β1 pathway [28]. In addition, HMGA1 was reported to promote metastatic processes in breast cancer by regulating EMT [29]. In the present study, we confirmed that miR-195 could reverse the epithelial phenotype and repress a mesenchymal phenotype by decreasing the expression of HMGA1 in RCC. We further demonstrated HMGA1 was not only the downstream of miR-195 in RCC, but also regulated by promoting effects of miR-195 on the EMT of RCC cells. Many studies have proved that induction of EMT is the first mechanism by which epithelial cancer cells acquire malignant phenotypes that promote tumor metastasis, chemoresistance and poor prognosis [30-32]. In summary, our findings provided a theoretical basis for future research of mechanism between miR-195 and RCC and suggest that miR-195 may be a new therapy target to combat aggressive RCC.
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Conclusion
Our data reveal that miR-195 contributes to the regulation of the EMT process by targeting HMGA1 at the post-transcriptional level in RCC cell lines. Our data suggest a significant role of miR-195 in the molecular etiology of RCC and explore its potential application in RCC therapy.

Acknowledgements
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Disclosure of conflict of interest
None.

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