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Expression of miR-205 in renal cell carcinoma and its association with clinicopathological features and prognosis

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Abstract. – OBJECTIVE: The aim of the present study was to examine the expression of miR-205 in renal cell carcinoma (RCC) tissue and carcinoma cells; also, we aimed to determine the association of miR-205 expression with the clinicopathological features and prognosis of RCC, and to explore the mechanism of miR-205.

PATIENTS AND METHODS: Carcinoma tissue and adjacent normal tissue were collected from 60 patients with RCC, and the expression of miR-205 was determined by semi-quantitative PCR, followed by correlation analysis of miR-205 with clinicopathological features and prognosis. Subsequently, the human RCC line, ACHN, was transfected with miR-205, and the effect of miR-205 overexpression on the growth of RCC was examined by MTT assay. Moreover, the effect of miR-205 on the migration of colon cancer cells was studied by transwell assay. Additionally, immunohistochemistry and Western blot were used to investigate the epithelial-mesenchymal transition in renal cancer tissue.

RESULTS: The expression of miR-205 was downregulated in RCC tissue compared with adjacent non-cancerous tissue ($p < 0.01$). The expression of miR-205 was closely related to the infiltration and recurrence of tumors ($p < 0.01$), but was not correlated with a pathological grade or clinical stage ($p > 0.05$). We also found that overexpression of miR-205 in RCC significantly inhibited the growth of cancer cells ($p < 0.01$) and significantly reduced the migration ability ($p < 0.01$). The epithelial-mesenchymal transition occurs in RCC, and miR-205 might inhibit cell proliferation and migration by blocking the epithelial-mesenchymal transition.

CONCLUSIONS: The expression of miR-205 is low in RCC, and may play an important role throughout the progression of RCC. Further study of miR-205 may promote the development of a novel therapeutic approach for the treatment of RCC.

Key Words:
miR-205, Renal cell carcinoma, Clinical pathology, Prognosis.

Introduction

Renal cell carcinoma (RCC) is a common malignant tumor of the urinary system. The incidence of kidney cancer in China is increasing annually[1]. Currently, nephrectomy remains the primary method of treatment for RCC in China. However, the survival rate of patients with renal cancer after surgery is low because of an extremely high rate of cell migration and deterioration[2]. At present, RCC is believed to be caused by multiple factors. The imbalance between various tumor suppressor genes and oncogenes affects the development of RCC[3,4]. Owing to rapid advancements in molecular biology, biogenetic methods can be used to study the mechanism underlying tumor development for the early diagnosis and treatment of RCC.

In recent years, the study of miRNA has gradually progressed. Single-stranded small RNA molecules, as endogenous non-coding regulatory elements, not only participate in various physiological processes, but also regulate the onset and progression of multiple pathological processes[5]. Several studies have shown that miRNAs are involved in cancer. Gadducci et al[6] found that miR-205 was expressed at a lower level in ovarian cancer cells, and its expression was significantly reduced in patients with advanced ovarian cancer[6]. However, no reports from home or abroad on the relationship between miR-205 and RCC have been published. In this study, we investigated the expression of miR-205 in RCC and adjacent normal tissues, and compared the correlations of clinical stage and pathological grade of RCC with the expression of miR-205, with the aim of understanding the mechanism of miR-205 in the regulation of RCC, to identify new mechanisms for preventing the development of RCC and to provide theoretical evidence for the clinical treatment of RCC.

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Patients and Methods

Patients

Samples in this study were collected from patients with RCC admitted to the Department of Urology in Weifang People's Hospital from February 2010 to February 2014. All patients underwent partial nephrectomy, and pathological diagnosis was performed on samples collected during surgery to confirm the diagnosis of RCC. This investigation was approved by the Ethics Committee of Weifang People's Hospital. Signed written informed consents were obtained from all the participants. Patients with consumptive diseases were excluded from the study, and informed consent was obtained from all patients. Adjacent tissue over 3 cm away from the edge of the tumor site was harvested from patients, and the absence of cancer cells was confirmed by pathological examination. A total of 60 selected patients were followed-up in our hospital, and a comprehensive treatment program was provided for each patient. All samples were stored in liquid nitrogen immediately after harvesting[7].

Clinical Data

In this study, 60 cancer tissue and 60 adjacent tissue samples from patients with RCC were collected. The clinical data of patients with RCC were analyzed and classified. There were 28 well differentiated, 15 moderately differentiated, and 17 poorly differentiated specimens according to the pathological grade. Moreover, there were 35 stage I, 13 stage II, and 12 stage III specimens according to the clinical stage. There were no significant differences in age or sex between the two groups ($p > 0.05$). Follow-up lasted until October 2016, with an average follow-up time of 5 years.

Instruments and Reagents

The ACHN cell line was from Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China); thiazolyl blue (MTT), dimethyl sulfoxide (DMSO), rabbit anti-human U6 antibody, and rabbit anti-human GAPDH antibody were from Sigma-Aldrich (St. Louis, MO, USA); transwell chambers were from Millipore (Billerica, MA, USA); fetal calf serum, trypsin, and propidium iodide (PI) dye were from Gibco (Grand Island, NY, USA); Rabbit anti-human E-cadherin antibody and rabbit anti-human Vimentin antibody were from Cell Signaling Technology (Boston, MA, USA); Lipofectamine™ 2000 was from Invitrogen; fluorescence inverted microscope was from Thermo (Waltham, MA, USA); cell culture flasks were from Corning Incorporated (Corning, NY, USA); pipettes were from Eppendorf (Hamburg, Germany); gel electrophoresis imaging analysis system was from Alpha Innotech, San Leandro, CA, USA; PCR instrument was from ABI (Waltham, MA, USA).

Semi-quantitative PCR Analysis of miR-205 Expression

Total RNA was extracted from RCC tissue and adjacent tissues using a TRIzol kit (Invitrogen, Carlsbad, CA, USA), and then reverse transcribed with a reverse transcription kit (Invitrogen) to obtain cDNA. Using U6 as an internal reference, the transcriptional levels of miR-205 from all samples were measured. The primers were synthesized by Shanghai Jima Pharmaceutical Co., Ltd, and the sequences are shown in Table I. The PCR reaction conditions were as follows: initial activation of Taq polymerase at 95°C for 5 min, followed by 40 cycles of 94°C for 45 s, 53°C for 30 s, and 72°C for 45 s. Agarose gel electrophoresis of PCR products was performed, and the results were analyzed with a UV imaging system.

Establishment of miR-205 Overexpressing Cells

ACHN cells in the exponential growth phase were seeded in 6-well plates at a concentration of 6×10^4/ml and transfected by mixing with Lipofectamine™ 2000 and the miR-205 complex. Transfection efficiency was assessed after 48 h, and successfully transfected cells were placed in an incubator for subsequent experiments.

Table I. PCR primers.

	Sequences
DMTF1v4	Forward primer: 5'-TCCTTCATTCCACCGGAGTCTG-3' Reverse primer: 5'-TGGTGTCGTGGAGTCG-3'
U6	Forward primer: 5'-GCTTCGGCAGCACATATACTAAAAT -3' Reverse primer: 5'-CGCTTCACGAATTTGCGTGTCAT-3'

MTT Assay

MTT assay was performed to determine the effect of miR-205 overexpression on the growth of RCC cells. Briefly, ACHN cells were seeded at 3×10^4 cells /ml in 96-well plates, and cultured for 48 h. MTT was added (5 mg/ml) and cells were incubated for an additional 4 h. The culture medium was then completely aspirated, and DMSO was added as a solvent to cells, with shaking. The absorbance values were measured at 490 nm using a microplate reader.

Transwell Migration Assay

Transwell migration assay was carried out to investigate the effect of miR-205 overexpression on the migration of RCC cells. ACHN cells were starved for 24 h. Cell concentration was then adjusted to 5×10^5 cells/ml, and cells were placed in small Transwell chambers. After staining and fixation, cell migration across the small chamber was assessed[8].

Immunohistochemistry

Tissue samples were embedded in paraffin, sectioned, and dehydrated. After washing with PBS (phosphate buffer saline) and blocking for 15 min, the primary antibody was added to sections and they were placed in a humid chamber for incubation at 4°C overnight, followed by a 20 min incubation with secondary antibody. After washing with PBS, DAB staining, counterstaining with hematoxylin, dehydration, and mounting were performed. Sections were then observed by fluorescence microscopy. E-cadherin staining presented as brown-yellow granules in the cell membrane, while Vimentin staining presented as brown granules in the cytoplasm. The results were determined according to the percentage of positive cells.

Western Blot

Total protein was extracted with a total protein extraction kit (Millipore, Billerica, MA, USA). Protein lysates were quantified and adjusted to equal concentrations before loading on the gel. The protein bands corresponding to E-cadherin and Vimentin were obtained according to standard Western blot methods, scanned, and analyzed.

Statistical Analysis

All data are presented as mean ± standard deviation. SPSS19.0 software (IBM, Armonk, NY, USA) was used for data analysis. Quantitative data were analyzed by the *t*-test, while categorical data between groups were analyzed by the χ^2-test. The Kaplan-Meier Log-rank test was carried out for survival analysis. $p \leq 0.05$ was considered statistically significant.

Results

miR-205 Expression in Renal Cell Carcinoma

Sixty RCC tissue samples and 60 adjacent tissue samples were analyzed. The expression of miR-205 in RCC was significantly lower than in adjacent normal tissue ($p < 0.01$) (Figure 1).

The Relationship Between the Expression of miR-205 and Clinicopathological Features as Well as Prognosis

The expression of miR-205 in tumor tissues was measured by semi-quantitative PCR. The correlations of the expression of miR-205 with the clinicopathological features and prognosis of RCC were investigated. There was no significant correlation between miR-205 expression and

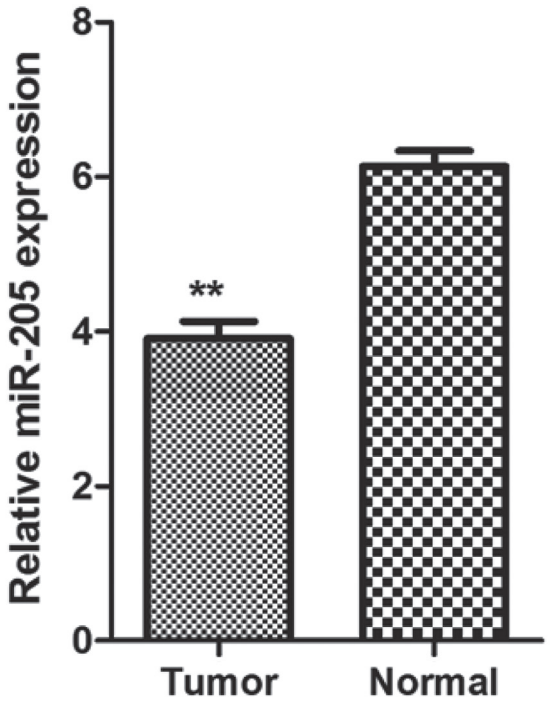

Figure 1. Semi-quantitative PCR analysis of miR-205 expression in RCC and adjacent normal tissue. The differential expression of miR-205 between the two groups was statistically significant, $^{**}p < 0.01$.

Table II. Relationship between miR-205 expression and clinicopathological features.

Parameter	n	miR-205	p
PT stage			
T1	28	3.38 ± 1.83	0.735
T2-4	32	3.67 ± 2.14	
Clinical stage			
I stage	35	3.98 ± 2.37	0.856
II-III stage	25	3.85 ± 2.21	
Metastasis			
Yes	43	3.29 ± 2.32	0.0075**
No	17	4.21 ± 2.56	
Recurrence			
Yes	12	3.06 ± 2.52	0.022*
No	48	3.86 ± 2.09	

Note: miR-205 expression was not significantly correlated with pathological grade or clinical stage ($p > 0.05$), but was significantly correlated with tumor migration ($p = 0.0075$) and recurrence (*$p < 0.05$ and **$p < 0.01$, respectively).

pathological grade ($p = 0.735$) or clinical stage ($p = 0.856$). However, there was a significant correlation between miR-205 expression and tumor migration ($p = 0.0075$) and recurrence ($p = 0.022$). The expression of miR-205 was closely related to the migration and recurrence of RCC (Table II). The patients were grouped according to the level of miR-205 expression, and statistical analysis of survival time showed that mean survival time was significantly lower in patients with reduced miR-205 expression (33.22 ± 8.25 months) compared with patients with normal or upregulated miR-205 expression (48.28 ± 7.63 months) ($p < 0.01$). Survival curves are shown in Figure 2.

Establishment of miR-205 Overexpressing Cells

After 48 h of transfection of cells with miR-205, the transfection efficiency was examined by fluorescence microscopy. The cells with a transfection efficiency of over 80% were considered successfully transfected and were selected for subsequent experiments. The results of transfection are shown in Figure 3.

Effect of miR-205 Overexpression on Cell Proliferation

The effect of miR-205 on cell proliferation was analyzed by MTT assay. The survival rate of the miR-205 group was significantly lower than that of the control group ($p < 0.01$), while there was no difference in survival rate between the negative control group and untreated control group, indicating that overexpression of miR-205 decreased cell proliferation.

Effect of miR-205 Overexpression on Cell Migration

Transwell assay was conducted to determine the effect of miR-205 overexpression on cell migration. There were no significant changes in cell migration between the negative control group and untreated control group. However, compared with the control group, the miR-205 transfected cells exhibited significantly lower migration ability ($p < 0.01$), indicating that miR-205 overexpression significantly decreased the migration ability of ACHN cells.

The Expression of E-cadherin and Vimentin Protein in RCC Tissue and Adjacent Tissue

To determine whether the epithelial-mesenchymal transition occurred in RCC, the expression of E-cadherin and Vimentin was detected by immu-

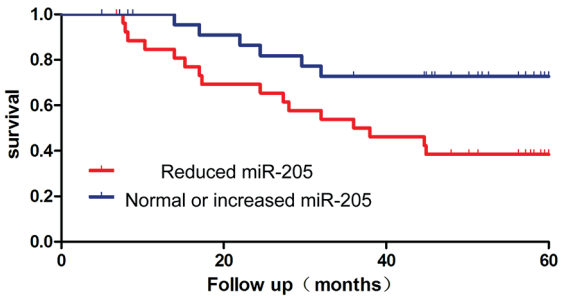

Figure 2. Relationship between miR-205 expression and survival time of patients. Patients with reduced miR-205 expression had a significantly shorter survival time compared with those with normal or increased miR-205 expression.

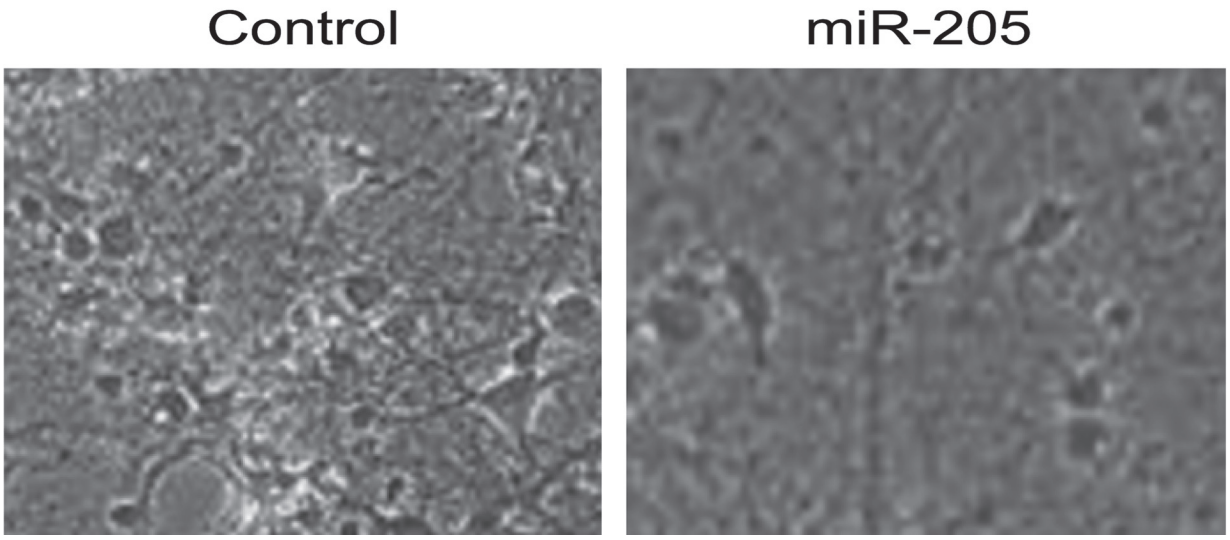

Figure 3. ACHN cells were transfected with miR-205. The transfected cells were compared with control cells. When the transfection efficiency reached over 80%, the cell line overexpressing miR-205 was successfully established and was used for subsequent experiments.

nohistochemistry. Figure 6 shows that E-cadherin was mainly localized in the cytoplasm and cell membrane. In addition, E-cadherin expression was higher in RCC compared with adjacent tissues. Vimentin was mainly expressed in the cytoplasm, and Vimentin expression in adjacent tissue was significantly higher than in RCC tissue. The expression of E-cadherin and Vimentin protein in RCC cells was also measured by Western blot as shown in Figure 7. The expression of E-cadherin in normal cells was significantly higher than in RCC cells ($p < 0.01$), and the expression of Vimentin in RCC cells was significantly higher than in normal kidney cells ($p < 0.01$). These observations were consistent with the results obtained by immunohistochemistry.

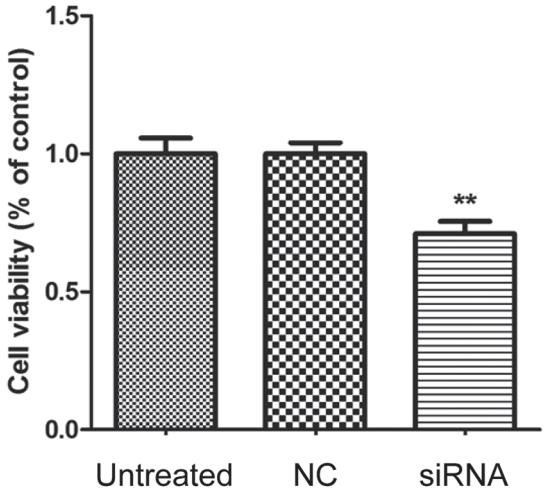

Figure 4. There was no significant difference in cell viability between the untreated control group and negative control (NC) group ($p > 0.05$). However, compared with the siRNA-control group, the cell viability of the siRNA group was significantly lower (**$p < 0.01$).

Discussion

The progression of RCC is associated with a variety of factors, among which is the deregulation of tumor suppressor genes and oncogenes. MiR-205 is a common tumor suppressor gene that is specifically expressed in a variety of tumors. Thomas et al[9] found that the expression of miR-205 was significantly reduced in prostate cancer tissue and prostate cancer cells by studying the expression of miR-205 in pancreatic tissue. Saito et al[10] reported that the expression of miR-205 in non-muscle invasive bladder cancer tissue was significantly higher than in muscular invasive bladder cancer tissue. Rauhala et al[11] also reported a significant decrease in miR-205 expression in metastatic breast cancer tissue compared with non-metastatic breast cancer tissue. The dysregulated expression of miR-205 in the aforementioned cancerous tissues seriously affected their development, progression, and recurrence, as well as the prognosis of patients[12].

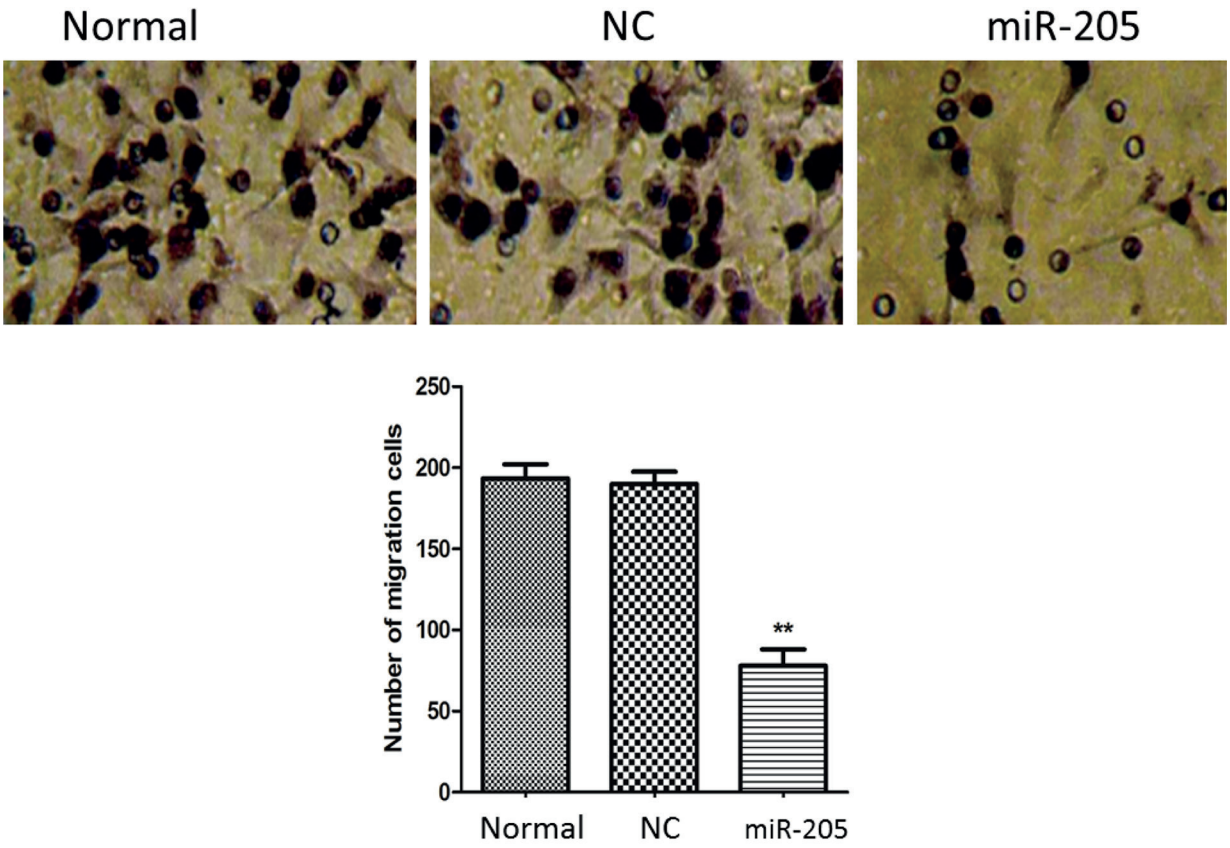

Figure 5. Cell migration ability as measured by transwell assay. Figure A shows cells at 50× magnification. Cells from the miR-205 group showed a significant reduction in migration compared with both the negative and untreated control groups; Figure B. Statistical analysis shows that the migration ability of the miR-205 group was significantly lower than that of both control groups ($p < 0.01$), while no significant difference was observed between the negative control group and untreated control group ($p > 0.05$).

The aim of this work was to explore the role of miR-205 in RCC though comparison of the expression of miR-205 in renal cancer tissue and adjacent tissue. The expression of miR-205 in adjacent normal tissue was higher than in RCC tissue, which was consistent with the functions of miR-205 as reported in other tumor types such as breast cancer and bladder cancer[13-15]. By analyzing the expression of miR-205 as well as the pathological grade and clinical stage of patients, we found that the expression of miR-205 was not correlated with either pathological grade or clinical stage. However, miR-205 was closely related to the migration and recurrence of RCC. The epithelial-mesenchymal transition plays an important role in the progression of malignant tumors. When cells are transformed from mesenchymal cells into epithelial cells, the adhesion between cells decreases because of changes in protein structure. This results in decreased cell contact and cell adhesion, making cells prone to migration. If this occurs in tumor cells, cells from the primary site can migrate to adjacent tissue. Furthermore, systemic metastasis and infiltration can occur[16,17]. As a mesenchymal marker, Vimentin is generally expressed in mesenchymal tissue, but not in mature epithelial cells. Therefore, it can be used to determine whether the epithelial-mesenchymal transition has occurred in cells[18,19]. In this study, we observed the epithelial-mesenchymal transition in RCC tissue or cells by both immunohistochemistry and Western blot analysis. The expression of E-cadherin in adjacent tissue and normal renal cells was significantly lower than in RCC tissue or cells. In contrast, the expression of Vimentin protein in RCC tissue and RCC cells was significantly lower than in adjacent normal RCC tissue and normal renal cells ($p < 0.01$). These observations suggest that the epithelial-mesenchymal transition occurs in the development and progression of RCC. In this study, we also transfected ACHN cells with miR-

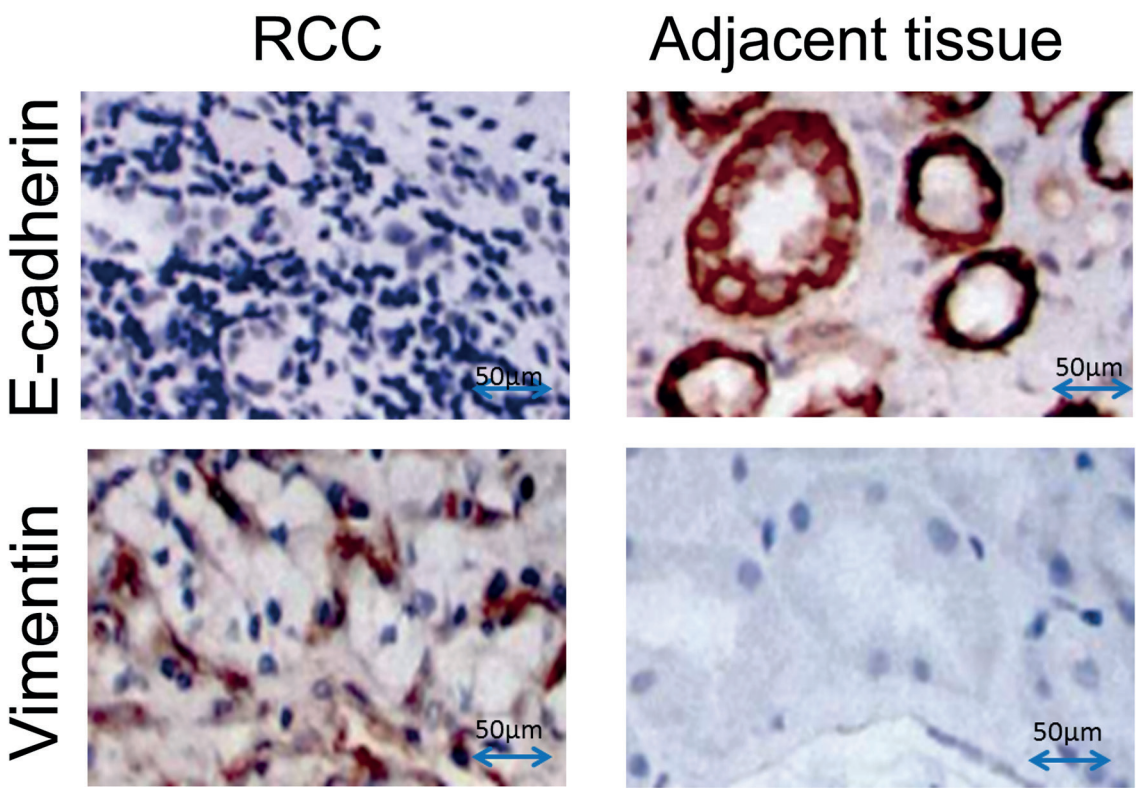

Figure 6. The expression of E-cadherin and Vimentin by immunohistochemistry in RCC and adjacent tissue. The expression of E-cadherin in RCC was significantly higher than in adjacent tissue; the expression of Vimentin in RCC tissue was significantly higher than in adjacent tissue.

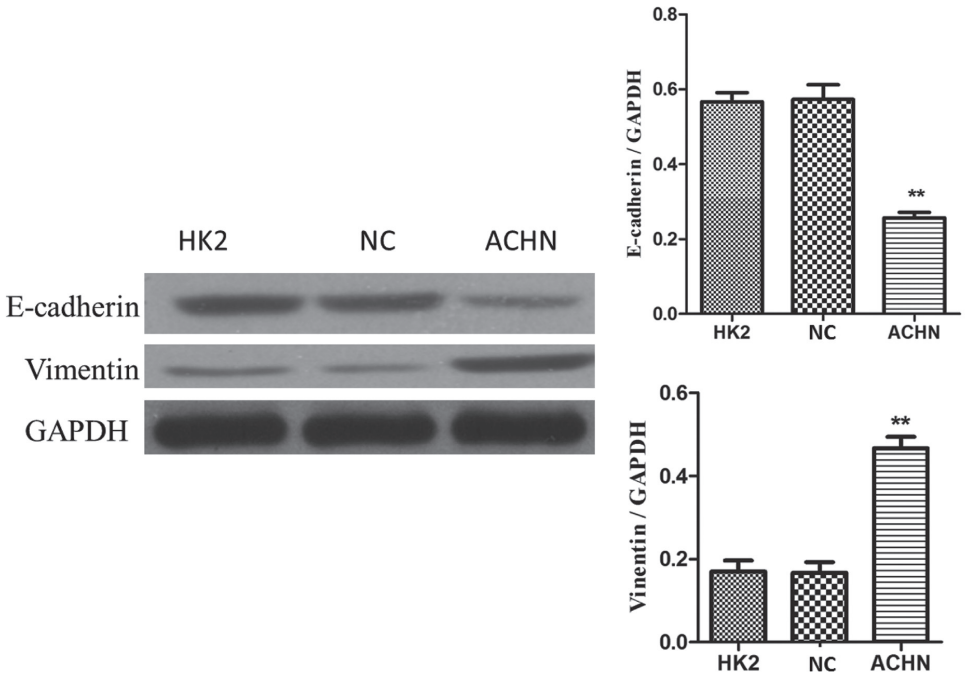

Figure 7. The expression of E-cadherin and Vimentin by Western blot. Figure 7A. Western blot analysis. Figure 7B. Statistical analysis of Western blot results showed that there was no significant difference in the expression of E-cadherin and Vimentin between the untreated control group and human kidney-2 (HK2) cells control group ($p > 0.05$). However, compared with the untreated control group, the expression of both E-cadherin and Vimentin protein was significantly increased in the miR-205 group ($p < 0.01$).

205 using liposomes. The expression of miR-205 in transfected cells was observed by fluorescence microscopy[20]. Compared with the untreated control group, the relative expression of miR-205 in the transfected cells was significantly higher than in the untreated control group, indicating that the transfection was successful, and appropriate conditions for subsequent analyses were established. By exploring the relationship between miR-205 and migration ability as well as the growth of RCC, we found that the overexpression of miR-205 inhibited the growth and migration ability of cells, which indirectly indicates that high rates of infiltration, metastasis, and relapse in RCC are mainly because of decreased expression of miR-205. By analyzing the correlation between the expression of miR-205 and the survival rates of patients, we found that, compared with adjacent tissue, the survival rates of patients with normal or high levels of miR-205 were significantly higher than those with low expression of miR-205, suggesting that high expression of miR-205 can significantly improve the survival of patients with RCC. This study had limitations regarding elucidating how miR-205 regulates the epithelial-mesenchymal transition, although we observed the epithelial-mesenchymal transition in RCC and found that miR-205 is closely related to the transition. Currently, it is largely unknown which pathway is involved in the regulation of the epithelial-mesenchymal transition by miR-205. Our group will continue to explore these mechanisms to further understand the signaling pathway through which miR-205 exerts its regulatory effects on the progression of RCC.

Conclusions

MiR-205 is expressed at low levels in RCC. As a tumor suppressor, it inhibits the growth and migration of RCC cells. Although the expression of miR-205 is not correlated with either the pathological grade or clinical stage of tumors, it correlates closely with RCC invasion and recurrence. In addition, miR-205 regulates the epithelial-mesenchymal transition in RCC. These findings may provide new strategies for early diagnosis and treatment of RCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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