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MiRNA-206 inhibits proliferation of renal clear cell carcinoma by targeting ZEB2

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Abstract. – OBJECTIVE: The purpose of this study was to investigate the effect of microRNA-206 on the malignant progression of renal clear cell carcinoma (RCC). In addition, whether microRNA-206 could regulate ZEB2 expression and the underlying mechanisms was also explored.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine microRNA-206 level in 46 tumor tissue specimens and adjacent ones of RCC patients. Also, the relationship between microRNA-206 expression and clinical indicators of RCC was analyzed. The negative control (NC) and microRNA-206 mimics were transfected into RCC cell lines, and the transfection efficiency was verified by qRT-PCR. The effects of microRNA-206 on the proliferation and apoptosis of RCC cells were analyzed by cell counting kit-8 (CCK-8), clone formation, and flow cytometry assays. Finally, the regulation of microRNA-206 on the downstream gene ZEB2 was indicated by Western Blot and cell recovery experiments.

RESULTS: qRT-PCR results showed that the expression level of microRNA-206 in tumor tissue samples of RCC patients was remarkably lower than that in adjacent normal tissues, and the difference was statistically significant. Meanwhile, compared with patients with high expression of microRNA-206, the pathological stage of patients with low expression of microRNA-206 was higher, and the overall survival rate was lower. In the RCC cell lines (Caki-1 and Caki-2), the cell proliferation ability of the microRNA-206 overexpression group was remarkably weakened, while the cell apoptosis rate was oppositely enhanced when compared with the NC group. In addition, this study demonstrated that ZEB2 expression was remarkably increased in RCC cells as well as tissues and was negatively correlated with microRNA-206 expression. At the same time, microRNA-206 mimics was found remarkably reduced in the expression of proteins in ZEB2-related signaling pathway, including ZEB2, β-catenin, cyclinD1, c-Myc, MMP-2, and MMP-9. In the cell reverse experiment, the overexpression of ZEB2 was found to be able to counteract the impact of microRNA-206 mimics on RCC cell proliferation and apoptosis and thus, participated in the malignant progression of RCC.

CONCLUSIONS: This study revealed that microRNA-206 was remarkably associated with the pathological stage and poor prognosis of RCC patients. In addition, microRNA-206 might inhibit the malignant progression of RCC by regulating the targeted ZEB2.

Key Words:
MicroRNA-206, ZEB2, Renal clear cell carcinoma, Proliferation.

Introduction

Renal cell carcinoma (RCC) is one of the most common malignancies of the urinary system, among which renal clear cell carcinoma is the most common, with the highest mortality rate among the tumors of the urinary system[1-3]. With the comprehensive application of ultrasound, Computed Tomography (CT), Magnetic Resonance Imaging (MRI) and other means, renal cancer can be preliminarily diagnosed[4,5]. Hematuria, low back pain and mass are the three recognized typical symptoms of renal cancer, which are mostly the manifestations of advanced renal cancer[5,6]. Currently, surgical resection of the tumor is the best treatment for renal cancer. However, due to the insensitivity of renal cancer patients to radiotherapy and chemotherapy after surgery, most patients with advanced renal cancer accompanied by metastasis are given the priority to conservative treatment, resulting in poor prognosis and low survival rate[7-9]. Therefore, it is particularly important to study the molecular mechanism

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of renal cancer to find out the methods for early diagnosis and early treatment, and inhibit the malignant progression of renal cancer[10,11].

MicroRNA (miRNA) is one of the most popular biological small molecules in recent years. It is an endogenous single-stranded small molecule RNA composed of 18-25 nucleotides, which is not involved in protein-coding and is highly conserved in evolution[12,13]. MiRNAs play a role mainly through base complementary pairing with the 3'-Untranslated Region (3'-UTR) of the target gene mRNA to inhibit translation at the post-transcriptional level[14,15]. Many studies[16,17] have confirmed that miRNA is closely related to the occurrence and development of renal cancer and thus is expected to be used for the early diagnosis and treatment of this disease. Currently, researches[18,19] have found that miRNA-206 is involved in a variety of physiological and pathological processes such as cell proliferation, differentiation, apoptosis, and aging in various tumor diseases. Recently, miRNAs may be new therapeutic targets for human tumors, and have been found to regulate ZEB2 signal transduction in a great number of tumor cells. Therefore, miRNA-206 might target ZEB2 in RCC and thus can serve as a potential therapeutic target.

To further explore the roles of microRNA-206 and ZEB2 in RCC, this study elaborated the mechanism of microRNA-206 and ZEB2 in the occurrence and development of RCC, bringing new ideas for the diagnosis and treatment of RCC.

Patients and Methods

Patients and RCC Samples

Tumor tissues and para-cancerous tissues of 46 patients with radical clear cell carcinoma were collected. All patients did not receive any radiotherapy or chemotherapy before surgery. The pathological classification and staging criteria of renal clear cell carcinoma were performed according to the international association of cancer against cancer (UICC) renal clear cell carcinoma staging criteria. Patients and their families in this study had been fully informed, and the informed consent had been signed and approved by the Ethics Oversight Committee.

Cell Lines and Reagents

Five human RCC cells (ACHN, Caki-1, 769P, Caki-2, 786-O) and one normal renal tubular epithelial cell (HK-2) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). The cells were cultured with DMEM medium containing 10% FBS at 37°C with 5% CO_2.

Transfection

Negative control (NC) and microRNA-206 overexpression sequence (microRNA-206 mimics) were purchased from Shanghai Jima Company (Shanghai, China). The cells were plated in 6-well plates and grown to a cell density of 70%, and then transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 hours, the cells were collected for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and functional cell experiment.

Cell Proliferation Assay

The cells after 48 h of transfection were collected and plated into a 96-well plate at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h, the cells were added with cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent. After incubation for 2 hours, the optical density (OD) value of each well was measured using a microplate reader at 490 nm absorption wavelength.

Flow Cytometry Analysis of the Cell Apoptosis

The method of binding with Annexin V-FITC (fluorescein isothiocyanate) (Merck, Billerica, MA, USA) and Propidium Iodide (PI) was used, and cell apoptosis was detected using flow cytometry. The procedure was as follows. First, when the cell density reached about 1×10^6 cells/mL, the medium was removed, and the cells were washed twice with Phosphate-Buffered Saline (PBS). Second, the cells were gently resuspended with 0.5 mL of pre-cooled 1 × binding buffer, and 1.25 μL of Annexin V-FITC was added and incubated at room temperature for 15 min in the dark. Subsequently, centrifugation was performed at 1000 × g for 5 min at room temperature, and then the cell supernatant was removed. After cells were resuspended with 0.5 mL of pre-cooled l × binding buffer, 10 μL of PI was added in. Lastly, the sample was placed on ice and stored in the dark, and immediately analyzed by flow cytometry (BD Biosciences, Detroit, MI, USA).

QRT-PCR

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and the total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and purify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, and real-time PCR was performed according to the SYBR® Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan) instructions. The PCR reaction was performed using the StepOne Plus real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used: microRNA-206: F: 5'-ATCCAGTGCGTGTCGTG-3'; R: 5'-TGCTTGGAATGTAAGGAAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'; ZEB2: forward: 5'-TCTCGGGGTGATCGA-CAAG-3', R: 5'-CCCTTTGTTCATTCGTTCCT-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'. Bio-Rad PCR instrument was used to analyze and process the data with software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). The β-actin and U6 genes were used as internal parameters, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 x g for 15 minutes at 4°C. The total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The monoclonal antibodies against ZEB2, β-catenin, cyclinD1, c-Myc, MMP-2, and MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the horseradish peroxidase-labeled goat anti-rabbit secondary antibody was purchased from GenScript Company (Piscataway, NJ, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference control. After being separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, the protein samples were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), which was then blocked in skim milk for 1 hour and incubated with primary antibodies overnight at 4°C. In the next day, the membrane was incubated with secondary antibody for 1 hour. Subsequently, enhanced chemiluminescence (ECL) coloration was performed, and the image was semi-quantitatively analyzed by Alpha SP Image Analysis software.

Statistical Analysis

The statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA). The univariate analysis was performed using the χ^2 test and the exact probability Fisher test. The multivariate analysis was performed using the COX regression analysis, while the patient survival was analyzed using the Kaplan-Meier method, and the Intergroup curves were compared using the log-rank test. The data were expressed as mean ± standard deviation, and $p<0.05$ was considered to be statistically significant.

Results

Knockdown of MicroRNA-206 in Primary Renal Cancer Tissues and Cell

MicroRNA-206 expression in the renal clear cell carcinoma tissues and cell lines were verified by qRT-PCR, and the results showed that compared with the para-cancer tissues, microRNA-206 expression in the tumor tissues was found remarkably lower, and the difference was statistically significant (Figure 1A). In addition, compared with HK-2, microRNA-206 was found remarkably lowly expressed in RCC cell lines, especially in Caki-1 and Caki-2, so these two were selected for subsequent experiments (Figure 1B). According to microRNA-206 expression in tumor tissues and adjacent tissues of 46 RCC patients, these tissues specimens were divided into high expression and low expression group to explore the interplay between the expression of microRNA-206 and the prognosis of RCC patients. The Kaplan-Meier survival curve revealed that low expression of microRNA-206 was remarkably correlated with poor prognosis of RCC ($p<0.05$; Figure 1C).

Subsequently, we further analyzed the association between microRNA-206 expression and RCC patients' age, gender, pathological stage, lymph node metastasis, and distant metastasis. As shown in Table I, the low expression of microRNA-206 is positively correlated with the pathological stage of RCC, but not related to age, gender, lymph node metastasis, and distant metastasis. Therefore, the above results suggested that microRNA-206 might be a new biological indicator for the prediction of malignant progression of this cancer.

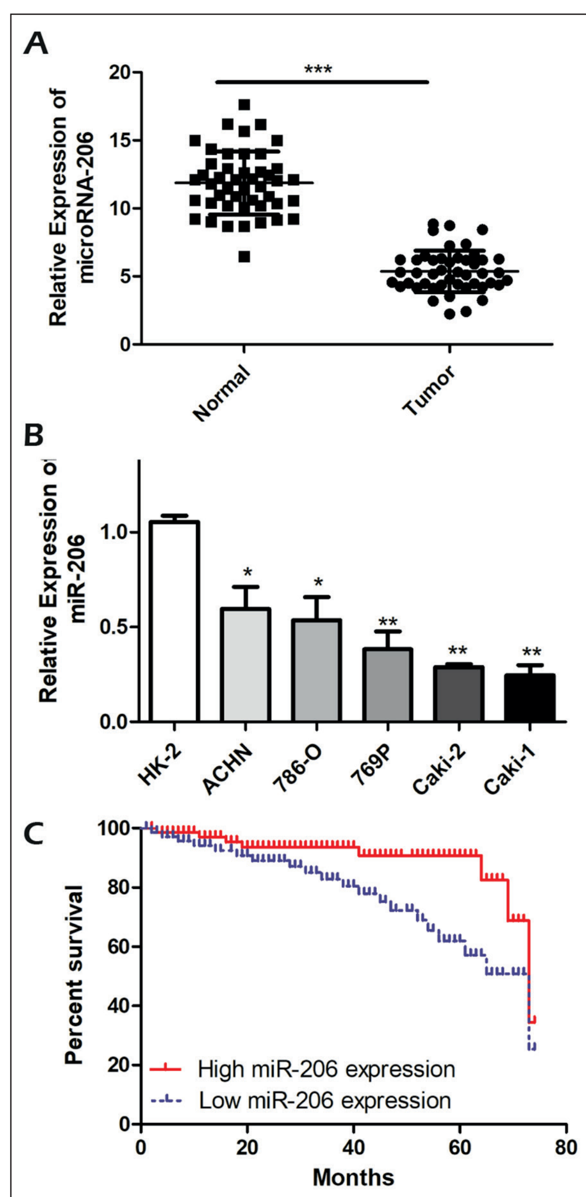

Figure 1. MiR-206 was underexpressed in renal clear cell carcinoma tissues and cell lines. **A-B,** qRT-PCR was used to detect the differential expression of miR-206 in tumor tissues and adjacent tissues of renal clear cell carcinoma. **C,** qRT-PCR was used to detect the expression level of miR-206 in renal clear cell carcinoma cell lines. **D,** The Kaplan Meier survival curve of renal clear cell carcinoma patients based on miR-206 expression. The data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Over-expression of MicroRNA-206 Inhibited Cell Proliferation and Promoted Cell Apoptosis

In order to explore the impact of microRNA-206 on the cellular function of RCC cells, the microRNA-206 overexpression model was firstly successfully constructed (Figure 2A). Subsequently, we conducted CCK-8 and flow cytometry experiments in Caki-1 and Caki-2 cell lines. Both the result of CCK-8 test and clonal formation experiment revealed that the cell proliferation ability of the microRNA-206 mimics group was remarkably decreased when compared with the NC group (Figures 2B, 2C). On the contrary, the flow cytometry assay indicated that the cell apoptosis of microRNA-206 mimics group was remarkably enhanced (Figure 2D).

ZEB2 Was Highly Expressed in RCC Tissues and Cell Lines

In RCC cell lines, the ZEB2 expression was found remarkably inhibited after the overexpression of microRNA-206 (Figure 3A). QRT-PCR revealed that ZEB2 expression in RCC tumor tissues was remarkably higher than that in adjacent tissues (Figure 3B). In addition, ZEB2 was found highly expressed in RCC cells compared with that in HK-2 (Figure 3C). Therefore, we detected the expression of microRNA-206 and ZEB2 in renal clear cell carcinoma by qRT-PCR, and the results showed that microRNA-206 and ZEB2 were negatively correlated in RCC patients (Figure 3D).

Overexpression of MicroRNA-206 Decreased the Expression of ZEB2 Related Signaling Pathway

In order to further explore the pathways by which microRNA-206 inhibits the malignant progression of renal clear cell carcinoma, we detected the expression of the key proteins in ZEB2 related pathways after the overexpression of microRNA-206 by Western blot. The results showed that the protein levels of ZEB2, β-catenin, cyclinD1, c-Myc, MMP-2, and MMP-9 were remarkably decreased after the overexpression of microRNA-206, and that the progression of this cancer was weakened at the same time (Figure 4).

MicroRNA-206 Exactly Inhibited ZEB2 Gene Expression

In order to further explore whether microRNA-206 inhibits the malignant progression of renal clear cell carcinoma through ZEB2, we overexpressed ZEB2 in the RCC cells transfected with NC and microRNA-206 mimics, and the transfection efficiency was tested by qPCR (Figure 5A). Subsequently, CCK-8, clonal formation, and flow cytometry assay confirmed that

Table I. Association of miR-206 expression with clinicopathologic characteristics of renal cell cancer.

Parameters	Number of cases	miR-206 expression High (%)	miR-206 expression Low (%)	*p*-value
Age (years)				0.828
< 60	18	11	7	
≥ 60	28	18	10	
Gender				0.489
Male	22	15	7	
Female	24	14	10	
T stage				0.007
T1-T2	28	22	6	
T3-T4	18	7	11	
Lymph node metastasis				0.181
No	30	21	9	
Yes	16	8	8	
Distance metastasis				0.166
No	35	24	11	
es	11	5	6	

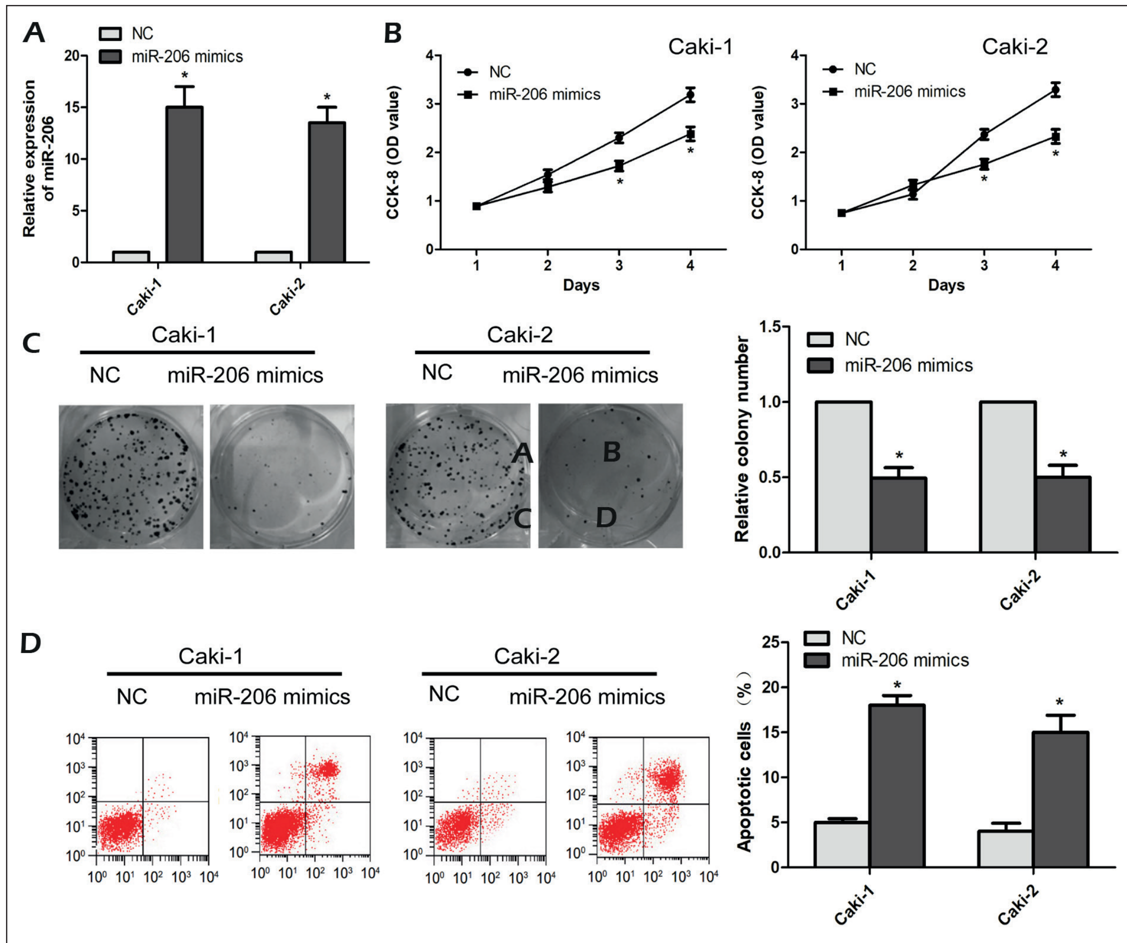

Figure 2. MiR-206 mimics inhibited the ability of RCC cells to proliferate and inhibit their apoptosis. **A,** qRT-PCR verified the transfection efficiency of miR-206 after transfection of NC and miR-206 mimics in Caki-1 and Caki-2 cell lines. **B,** The CCK-8 assay detects the effect of Caki-1 and Caki-2 cell lines on the proliferation of renal clear cell carcinoma cells. **C,** The clonal formation assays tested the ability of Caki-1 and Caki-2 cell lines to positive for renal clear cell carcinoma cells (magnification: 10×). **D,** The flow cytometry assay was used to detect the apoptosis ability of CKI-1 and Caki-2 cell lines in renal clear cell carcinoma cells. The data are mean ± SD, *p<0.05.

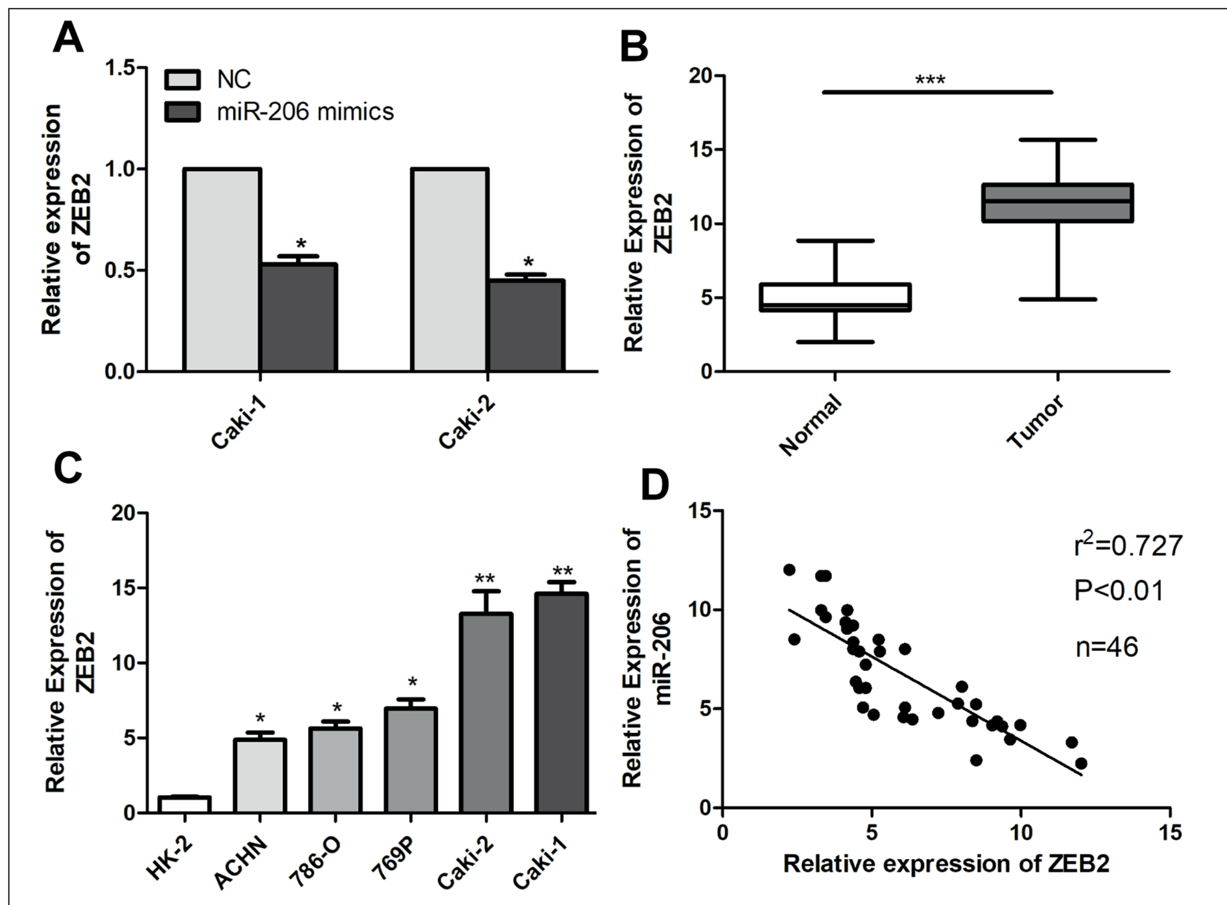

Figure 3. ZEB2 was highly expressed in renal clear cell carcinoma tissues and cell lines. **A,** qRT-PCR verified the expression level of ZEB2 after transfection of miR-206 mimics in Caki-1 and Caki-2 cell lines. **B,** qRT-PCR was used to observe the difference in the expression of ZEB2 in renal clear cell carcinoma tumor tissues and adjacent tissues. **C,** qRT-PCR was used to detect the expression level of ZEB2 in renal clear cell carcinoma cell lines. **D,** There was a significant negative correlation between miR-206 and ZEB2 expression in renal clear cell carcinoma. The data are mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

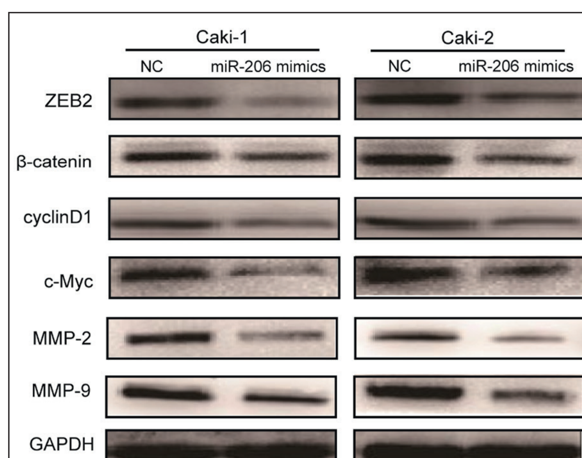

Figure 4. MiR-206 mimics down-regulated ZEB2-related pathway protein levels. The Western blotting verified the protein expression levels of ZEB2, β-catenin, cyclinD1, c-Myc, MMP-2, and MMP-9 in the Caki-1 and Caki-2 cell lines after miR-206 overexpression.

the overexpression of ZEB2 could counteract the influence of the overexpressed microRNA-206 on the proliferation and apoptosis ability of RCC cells (Figures 5B and 5D).

Discussion

Renal cell carcinoma is one of the most common malignant tumors in the urinary system. With the rapid development of modern medical imaging, it is possible to make a preliminary clinical diagnosis of most early renal carcinoma. However, for remote rural areas, it is still difficult to detect early renal carcinoma due to unsound medical conditions and a lack of awareness of physical examination[1-4]. Currently, for the treatment of renal cancer, the surgical resection of the tumor is the main method in clinical practice,

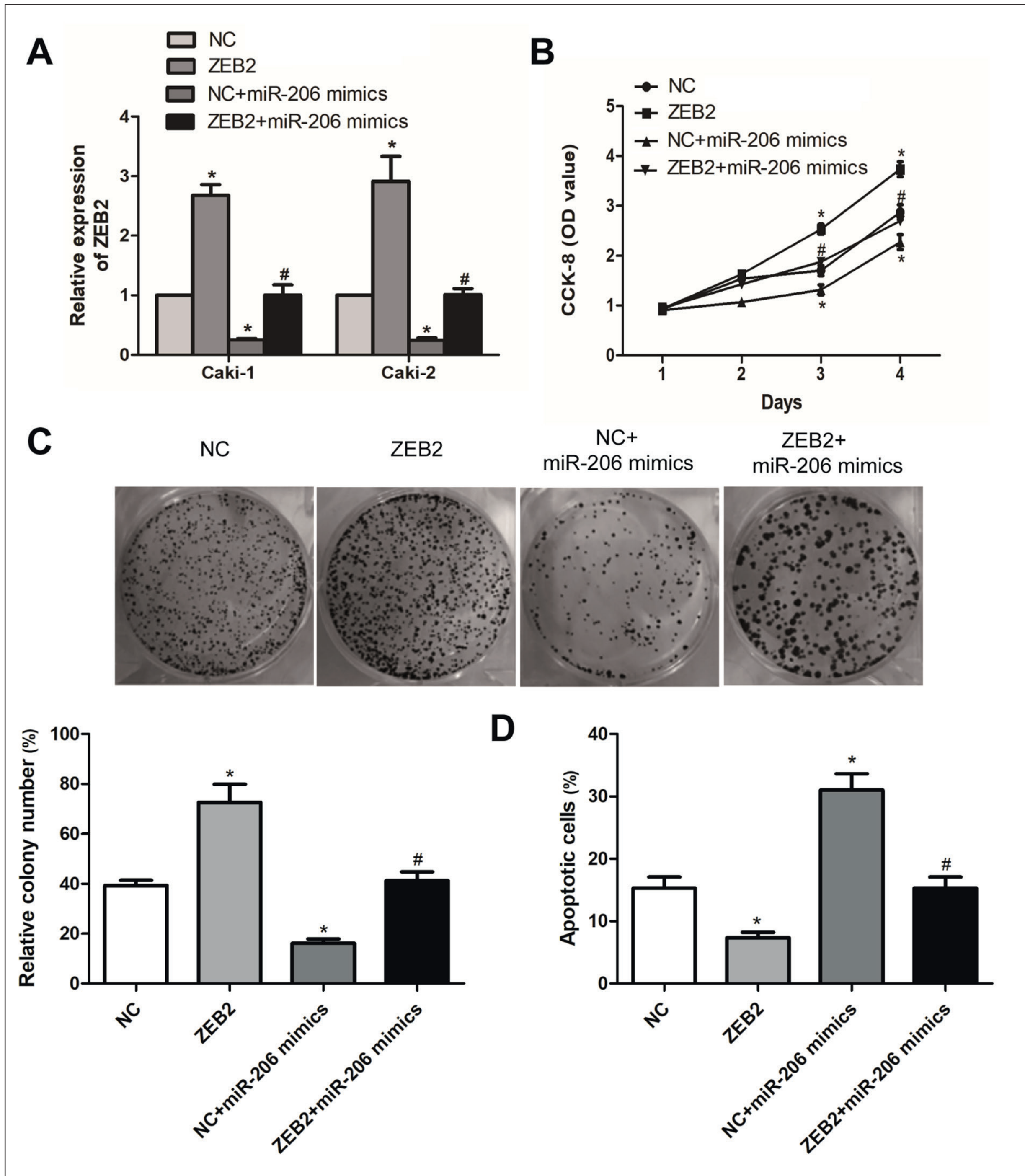

Figure 5. MiR-206 regulated the inhibiting effect of ZEB2 on the development of renal clear cell carcinoma. **A,** ZEB2 expression levels in miR-206 and ZEB2 co-transfected cell lines were observed by qRT-PCR. **B,** The CCK-8 cell proliferation assay detected the proliferation of RCC cells after co-transfection of miR-206 and ZEB2. **C,** The colony formation assay detected the ability of miR-206 and ZEB2 co-transfection to regulate the formation of renal clear cell carcinoma cells (magnification: 10×); **D,** Flow cytometry assay was used to detect the apoptosis of RCC cells after co-transfection of miR-206 and ZEB2. The data are mean ± SD, *$p<0.05$.

followed by other adjuvant treatments. For renal cancer with metastasis, conservative treatment is the main method in clinical practice, or palliative resection is used, which has great limitations[5-7]. Early detection and early treatment can effectively inhibit the metastasis of renal cancer, which is beneficial to improve patients' survival rate and their quality of life, and can even help them to achieve a complete remission[8,9]. In recent years, gene therapy for renal cancer has received much attention from many researchers, and the research on the molecular mechanism of renal cancer has become a hot topic. MicroRNA (miRNA) is one of the small biomolecules discovered in recent years. Many studies[12,13] have found that microRNA plays a pivotal role in tumor development and thus provides new targets for gene therapy of tumors.

MiRNA, which is encoded by genomic DNA and widely exists in animals, is an oligonucleotide chain. It is an endogenous non-coding single-stranded small molecule RNA composed of 18-25 nucleotides and does not participate in protein coding[12]. One miRNA may be involved in the regulation of multiple target genes, and one gene may be regulated by multiple miRNAs. This interaction provides a new way to explore the molecular mechanism of tumorigenesis[16,17], MicroRNA-206 has been indicated to be lowly expressed in various tumor tissues such as liver cancer and gastric cancer, and its level is relevant to clinicopathological characteristics and clinical prognosis, suggesting that microRNA-206 can act as an oncogene in various malignant tumors including RCC[18,19]. In order to explore the role of microRNA-206 in the occurrence and development of renal clear cell carcinoma, we detected the expression of microRNA-206 in RCC tissue specimens and found that it was higher than that in the para-cancerous counterpart. Therefore, we believe that microRNA-206 might play a role in RCC tumor inhibition. Subsequently, in order to further explore the effect of microRNA-206 on the biological function of RCC, we constructed the microRNA-206 overexpression model. And the results of CCK8, clonal formation, and flow cytometry assays revealed that microRNA-206 could inhibit RCC cell proliferation but enhance cell apoptosis. In addition, the levels of ZEB2, β-catenin, cyclinD1, c-Myc, MMP-2, and MMP-9 were remarkably decreased after the overexpression of microRNA-206, thereby inhibiting the occurrence and development of RCC.

Through miRNA software search and test, it was found that microRNA-206 could complement with ZEB2 3'-UTR. Based on this, we explored whether microRNA-206 could target ZEB2 and inhibited the proliferation of renal cancer cells. The results of this study showed that the increase in the content of microRNA-206 in renal cancer cell lines could inhibit the proliferation of renal cancer cells and promote apoptosis, which was consistent with the role of microRNA-206 in other tumor studies. At the same time, microRNA-206 could inhibit the expression of ZEB2 in renal cancer cells, and this inhibitory effect had a dose-response relationship. This means that the expression of ZEB2 was decreased with the increased content of microRNA-206. In this study, microRNA-206 and ZEB2 expression were found negatively correlated, suggesting that ZEB2 might be the target gene of microRNA-206. In order to investigate the effect of the interaction between microRNA-206 and ZEB2 on the occurrence and development of renal clear cell carcinoma, we further verified the mRNA level of ZEB2 after microRNA-206 was overexpressed and we found that ZEB2 was remarkably down-regulated. Subsequently, the cell rescue assay demonstrated that the overexpression of ZEB2 could counteract the influence of microRNA-206 mimics on the proliferation and apoptosis ability of RCC cells. The above findings suggested that there might be a feedback loop regulatory circuit, indicating that ZEB2 might reverse the biological effect of microRNA-206 on RCC cells, thus jointly affecting the malignant progression of renal clear cell carcinoma.

Conclusions

MicroRNA-206 expression was remarkably associated with the pathological stage and poor prognosis of RCC. In addition, microRNA-206 may inhibit the malignant progression of RCC by regulating ZEB2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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