European Review for Medical and Pharmacological Sciences 2020; 24: 5988-5995

MicroRNA-133a-5p inhibiting metastatic capacity of renal clear cell carcinoma through regulating MON2

P. LI[1], Q. WU[2], X.-R. YANG[1], T. SUN[1]

[1]Department of Urology, The First Affiliated Hospital of Nanchang University, Nanchang, China
[2]Department of Urology, The Sixth Affiliated Hospital of Wenzhou Medical University (The People's Hospital of Lishui), Lishui, China

Abstract. – OBJECTIVE: We aimed at analyzing the correlation between microRNA-133a-5p expression and clinical pathological parameters in patients with clear cell renal cell carcinoma (ccRCC) and exploring the mechanism by which microRNA-133a-5p affects the biological behavior of ccRCC cells.

PATIENTS AND METHODS: MicroRNA-133a-5p expression in ccRCC tissues and cell lines were examined by quantitative real-time polymerase chain reaction (qRT-PCR), and the relationship between ATG14 expression and clinicopathological parameters of ccRCC patients was analyzed. A control group (NC mimic) and a microRNA-133a-5p overexpression group (microRNA-133a-5p mimic) were set in the ccRCC cell lines ACHN and 786-O, respectively. The impacts of microRNA-133a-5p on the proliferation and invasion of ccRCC cells were evaluated through performing Cell Counting Kit-8 (CCK-8) and transwell tests, respectively. We further explored the interaction between microRNA-133a-5p and its downstream target gene MON2 by bioinformatics analysis and Luciferase assay.

RESULTS: Both in ccRCC tissues and cell lines, microRNA-133a-5p showed a significantly reduced expression, which could be used to predict poor prognosis of ccRCC patients. Upregulation of microRNA-133a-5p markedly blunted the proliferation and migratory capacities of HCC cells. Bioinformatics analysis suggested that microRNA-133a-5p can target MON2. In addition, qPCR assay indicated an increased expression of MON2 in ccRCC cell lines and tissues, which was negatively correlated with microRNA-133a-5p. Finally, *in vitro* cell reverse experiments suggested that overexpression of MON2 counteracted the inhibitory effects of overexpression of microRNA-133a-5p on the proliferation and metastatic capacity of ccRCC.

CONCLUSIONS: This study suggests that the reduced expression of microRNA-133a-5p in ccRCC tissue specimens can predict poor prognosis of ccRCC patients. At the same time, microRNA-133a-5p may suppress the proliferation capacity and metastasis of ccRCC cells by acting on MON2.

Key Words:
MicroRNA-133a-5p, MON2, Clear cell renal cell carcinoma, Metastasis.

Introduction

Clear cell renal cell carcinoma (ccRCC), is the most common renal tumor in adults, accounting for about 2%-3% of adult malignant tumors. The incidence of urinary tract tumors in developing countries is lower than that in developed countries[1,2]. The morbidity and mortality of ccRCC in different regions of China also vary greatly. Meanwhile, the incidence of male patients was higher than that of female ones (the ratio was about 2:1); the incidence of urban areas was higher than that of rural areas, with a maximum difference of 43 times[3,4]. According to statistics, 20%-35% of patients with ccRCC had metastasized when they were treated, and about 6%-15% of patients were treated for metastasis-related symptoms[5,6]. Different from urologic tumors such as prostate cancer, ccRCC lacks tumor biomarkers for early diagnosis[7,8]. Now, due to the rapid development of imaging technology and the popularity of physical examination, the detection rate of asymptomatic ccRCC is remarkably higher than before, the detection rate of asymptomatic ccRCC accounts for about 50%[8]. ccRCC is not sensitive to radiation or chemotherapy, and the only known therapy for ccRCC is performing surgery at an early stage, which includes radical surgery and nephron sparing surgery[9,10]. However, the

Corresponding Author: Ting Sun, MD; e-mail: suntingdr@163.com

incidence of postoperative tumor recurrence is still about 20%-40%, and patients with advanced metastatic ccRCC lost the chance of surgery, due to cancer progression or systemic metastasis[10]. In addition, despite the continuous research on immunotherapy and the advent of targeted drugs, the overall survival rate of patients with locally or metastatic ccRCC in advanced stage is still not ideal; therefore, it is urgent to uncover the pathogenesis of ccRCC and thus provide clues for the advancement of prognosis of ccRCC[11,12].

MicroRNA (miRNA) is a non-protein-coding small molecule RNA, approximately 18-25 nt in length, which is widely found in various eukaryotic cells[13,14]. Its main function is to participate in the metabolism of messenger RNA (mRNA), which inhibits the translation of mRNAs through pairing with the bases of the 3' -end non-coding region (3'-UTR)[15,16]. MiRNAs regulate at least 1/3 of human genes, with an average of 200 different kinds of RNA regulated by each miRNA, and that certain specific target genes can be regulated by multiple miRNA coordinated activities separately or simultaneously[17,18]. Meanwhile, it plays an essential part in the progression of human tumors by acting as an oncogene or an anti-oncogene[19,20]. MicroRNA-133a-5p, a member of the microRNAs family, is involved in the biological functions of cancer, and thus might become a new target for the diagnosis and treatment of cancers[21,22].

According to search tests of miRNAs softwares (TargetScan, miRDB and StarBase), we found a base complementary pairing between microRNA-133a-5p and the 3'-UTR of MON2 mRNA, which provided a theoretical basis for microRNA-133a-5p to regulate the expression of MON2. Our study elaborated the interaction of microRNA-133a-5p and MON2 in the development of ccRCC, which might bring new ideas for the diagnosis and treatment of ccRCC.

Patients and Methods

Patients and ccRCC Samples

Tumor tissue specimens and adjacent ones of 56 ccRCC patients undergoing radical surgery were collected. All subjects had not received any radiotherapy or chemotherapy before surgery. Renal clear cell carcinoma pathological classification and staging criteria are implemented in accordance with the International Union Against Cancer (UICC) renal clear cell carcinoma staging criteria. Inclusion criteria of ccRCC patients were as follows, (1) no severe diseases in other organs; (2) none of patients had preoperative chemotherapy/radiotherapy or molecular targeted therapy. In addition, the exclusion criteria of ccRCC patients were as follows, (1) other malignancies; (2) mental disease; (3) myocardial infarction; (4) heart failure or those previously exposed to radioactive rays. Patients and their families signed informed consent. This study complies with the Helsinki Declaration Clinical Practice Guidelines. This investigation was approved by the Ethics Committee of The First Affiliated Hospital of Nanchang University.

Cell Lines and Reagents

Human-derived ccRCC cells (ACHN, Caki-1, 769P, Caki-2, 786-O, A498) and a normal renal tubular epithelial cell (HK-2) provided by American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) in an incubator with 5% CO_2 at 37°C.

Transfection

Transfection reagent was mixed with microRNA-133a-5p mimic (GenePharma, Shanghai, China) and then added into cells when cell density reached to 30-50%. 48 hours later, cells were collected for analysis.

Cell Counting Kit-8 (CCK-8) Test

Cells were plated in 96-well plates (2×10^3 cells/well) in 100 uL culture medium. CCK-8 test was performed according to the manufacturer's protocol (Dojindo Molecular Technologies, Kumamoto, Japan).

Transwell Assay

Cell migration or invasion was tested using a 24-well plate cell pre-coated or not coated with matrix gel according to the manufacturer's instructions.

Cell Wound Healing Test

After 48 h of transfection, cells were digested, centrifuged and resuspended in medium without FBS to adjust the density to 5×10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached

90% or more the next day. After the stroke, cells were rinsed gently with phosphate-buffered saline (PBS) for 2-3 times and observed again after incubation in low-concentration serum medium for 24 h.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells to extract total RNA from the tissue. Real-time PCR was performed according to the instructions of SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) kit on StepOne Plus Real-time PCR System, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 as internal reference. Primers used in the qPCR reaction: microRNA-133a-5p: forward: 5'-ACACTCCAGCTGGGAGCTGGT-3', reverse: 5'-CTCAACTGGTGTCGTGGAGT-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; MON2: forward: 5'-CCCTCTTCCAAGGCAACAAG-3'; reverse: 5'-AGGGTGGTATTCCAAATAGGC-3'; GAPDH: forward: 5'-CTCTGCTCCTCCTGTTCGAC-3', reverse: 5'-ACCAAATCCGTTGACTCCGA-3'.

Luciferase Assay

The transcription factor expression plasmid to be tested was co-transfected with the reporter plasmid into the ccRCC cell line. The Luciferase activity was measured using a luciferase reporter kit (Promega, Madison, WI, USA).

Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). χ^2 test and the exact probability Fisher test was used for Univariate analysis; COX regression analysis was performed for multivariate analysis. The survival analysis probability was analyzed by Kaplan-Meier curves and evaluated using the log-rank test. Data are presented as X ± SD (standard deviation), and p less than 0.05 was statistically significant.

Results

Reduced Expression of MicroRNA-133a-5p in ccRCC

We first detected microRNA-133a-5p expression in ccRCC and adjacent control tissues by qPCR. Figure 1A shows a significantly reduced expression of microRNA-133a-5p in ccRCC tissue samples. Consistently, in comparison to HK-2, a normal renal cell line, microRNA-133a-5p was also decreased in ccRCC cell lines, especially ACHN and 786-O, the two of which were hence subjected to subsequent experiments (Figure 1B). We then divided the 56 pairs of tissue samples into high and low microRNA-133a-5p expression group. Table I shows that low microRNA-133a-5p level was positively correlated with the incidence of metastasis, but not with age and pathological stage. Meanwhile, Kaplan-Meier survival curve revealed that low microRNA-133a-5p expression was significantly relevant to overall survival ($p<0.05$; Figure 1C) and disease-free survival of ccRCC patients ($p<0.05$; Figure 1D), suggesting that microRNA-133a-5p may be a new biological index for predicting the prognosis of ccRCC.

Overexpression of MicroRNA-133a-5p Inhibited Proliferation Rate and Migration Capacity of ccRCC Cells

To specify the impact of microRNA-133a-5p on ccRCC cell functions, we constructed microRNA-133a-5p overexpression models in ACHN and 786-O cell lines and verified the transfection efficiency by qPCR (Figure 2A). Subsequently, CCK-8 test, transwell experiment and cell wound healing assay demonstrated that upregulation of microRNA-195-5p markedly reduced the proliferation rate (Figure 2B). and attenuated the migrating (Figure 2C) as well as the crawling ability (Figure 2D) of ccRCC cells.

MicroRNA-133a-5p Can Bind to MON2

Bioinformatics software analysis revealed that microRNA-133a-5p could target MON2. Luciferase assay was then performed to further prove the binding relationship between the two (Figure 3A). After overexpression of microRNA-133a-5p in ACHN and 786-O cell lines, the results of qPCR and Western Blot experiments indicated a reduction in the expression of MON2 at both gene and protein levels (Figure 3B). In addition, we found an increased MON2 expression in tumor tissues of ccRCC patients (Figure3C) and a negative correlation between microRNA-133a-5p and MON2 (Figure 3D). Consistently, MON2 also showed a lower expression in ccRCC cells than in HK-2 cells (Figure 3E). We next constructed MON2 overexpression models in ccRCC cell lines and found microRNA-133a-5p levels were remarkably increased, measured by qPCR assay (Figure 3F).

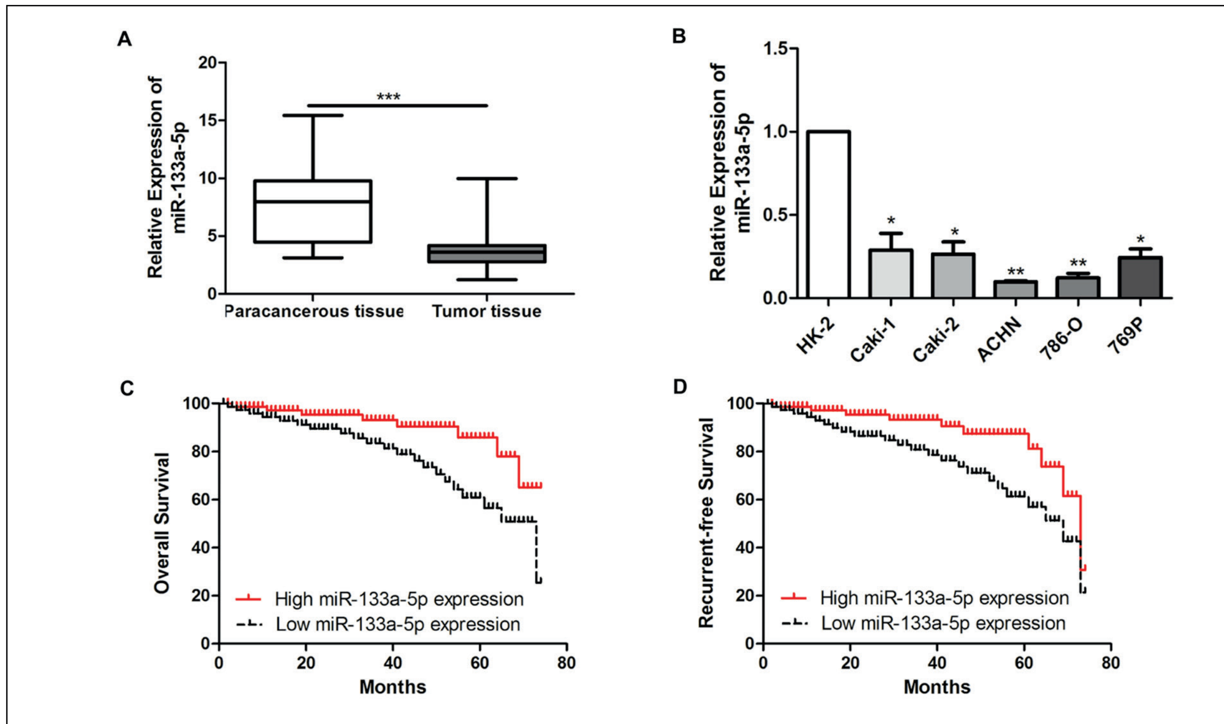

Figure 1. MiR-133a-5p is underexpressed in renal clear cell carcinoma tissues and cell lines. **A,** qRT-PCR detection of miR-133a-5p expression in tumor tissues and adjacent tissues of renal clear cell carcinoma. **B,** qRT-PCR detection of miR-133a-5p expression level in renal clear cell carcinoma cell lines (**C**) Kaplan Meier survival curve showed the overall survival of patients with renal clear cell carcinoma based on miR-133a-5p expression. **D,** Kaplan Meier survival curve showed disease-free survival of patients with renal clear cell carcinoma based on miR-133a-5p expression. Data are average ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

MicroRNA-133a-5p Exactly Inhibited MON2 in ccRCC Cell Lines

Furthermore, microRNA-133a-5p and MON2 overexpression vectors were co-transfected in ccRCC cell lines to explore whether microRNA-133a-5p suppresses the malignant progression of ccRCC *via* regulating MON2 expression. Consequently, this co-transfection remarkably

Table I. Association of miR-133a-5p expression with clinicopathologic characteristics of renal cell cancer.

Parameters	No. of cases	miR-133a-5p expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.760
< 60	23	13	10	
≥ 60	33	20	13	
Gender				0.554
Male	27	17	10	
Female	29	16	13	
T stage				0.159
T1-T2	33	22	11	
T3-T4	23	11	12	
Lymph node metastasis				0.014
No	35	25	10	
Yes	21	8	13	
Distance metastasis				0.032
No	36	25	11	
Yes	20	8	12	

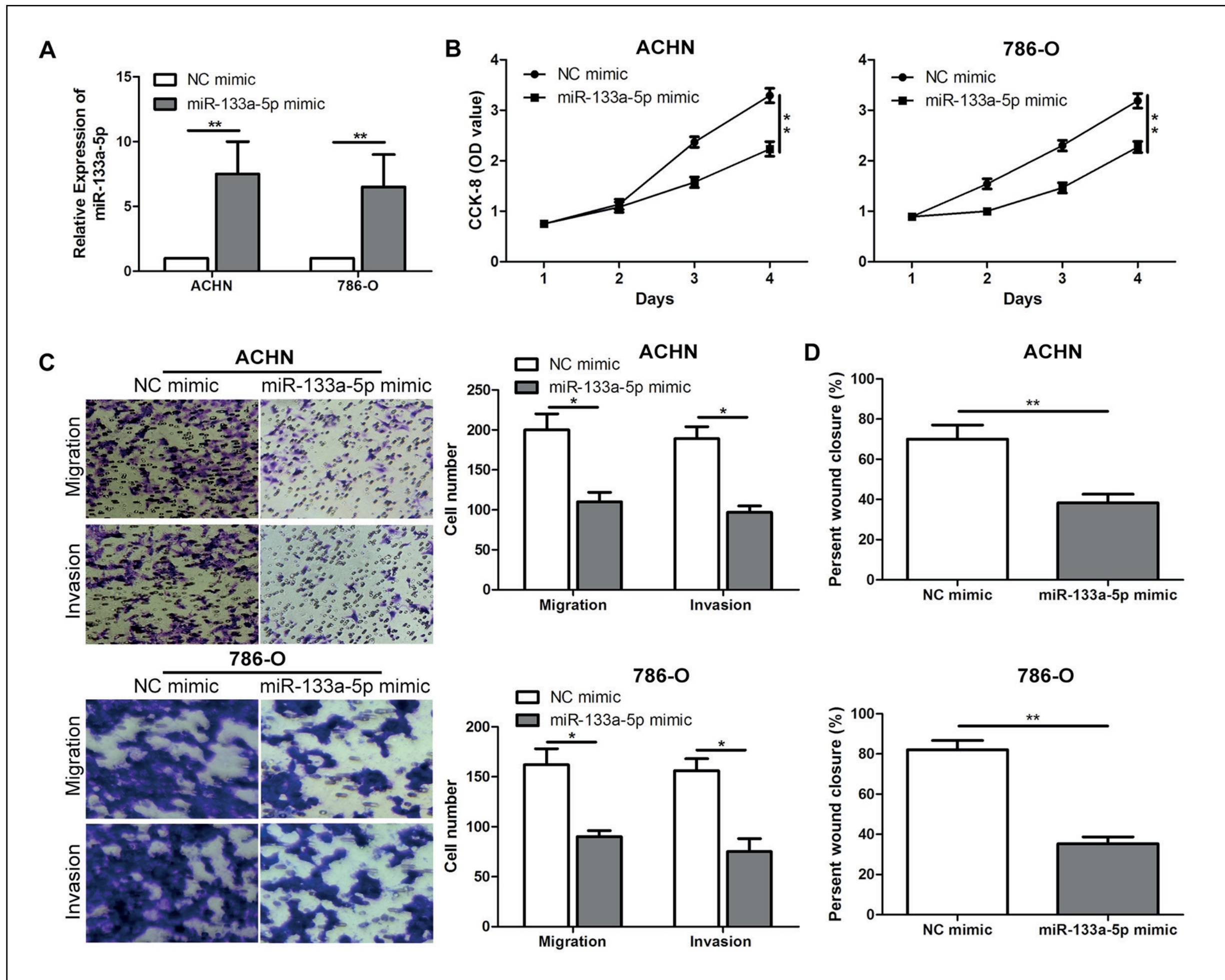

Figure 2. Overexpression of miR-133a-5p inhibits ccRCC cell proliferation and metastatic ability. **A,** qRT-PCR verified the transfection efficiency of miR-133a-5p overexpression vector in ACHN and 786-O cell lines. **B,** CCK-8 detected the proliferation rate of renal clear cell carcinoma cells after transfection of miR-133a-5p overexpression vector in ACHN and 786-O cell lines. **C,** Transwell assay detected the ability of renal clear cell cancer cells to invade and migrate after transfection of miR-133a-5p overexpression vector in ACHN and 786-O cell lines (magnification: 40×). **D,** Cell wound healing assay detected the crawling ability of renal clear cell cancer cells after transfection of miR-133a-5p overexpression vector in ACHN and 786-O cell lines. Data are average ± SD, * $p<0.05$, ** $p<0.01$.

enhanced MON2 expression compared to single transfection of microRNA-133a-5p overexpression vector (Figure 4A). Subsequently, CCK-8 and transwell assays proved that over-expressing MON2 can counteract the inhibitory effect of microRNA-133a-5p upregulation on the proliferative and metastatic capacity of ccRCC cells (Figure 4B and 4C).

Discussion

With the rapid development of modern medical imaging, it is possible to make a preliminary clinical diagnosis of most early ccRCCs. However, for remote rural areas, early detection of ccRCC is still difficult due to incomplete medical conditions and lack of physical examination awareness[1-4]. At present, the effective treatment of ccRCC is mainly surgery combined with chemotherapy. However, for ccRCC with metastasis, the treatment is just limited to conservative therapy or palliative resection[5-7]. Early detection and treatment can effectively inhibit the metastasis of ccRCC, which is conducive to improving the survival period, the prognosis of patients and the quality of life[8-10]. In recent years, gene therapy for ccRCC has received much attention from many scientific researchers, and the research on pathogenesis of ccRCC has become a hot topic[11,12].

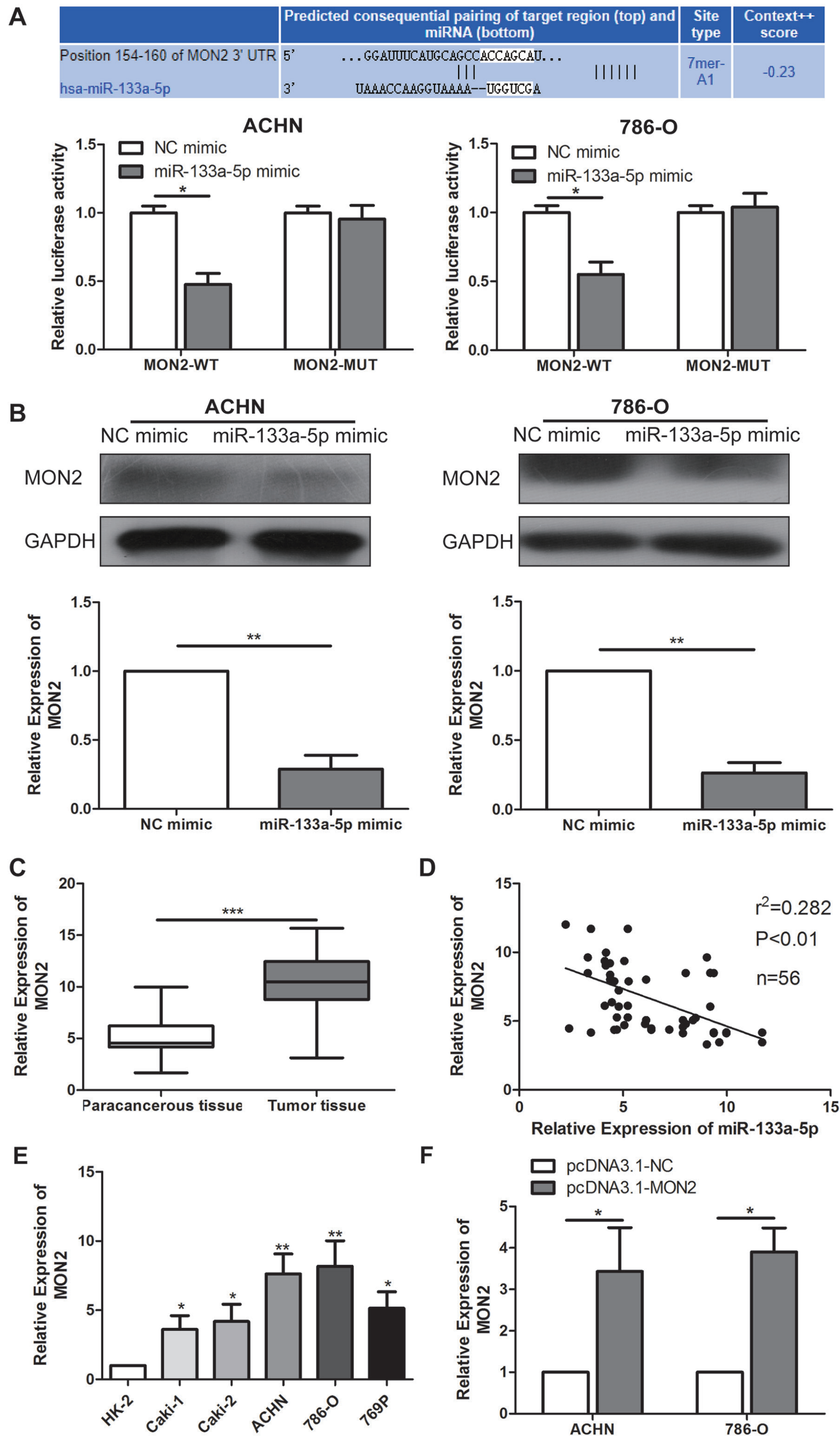

Figure 3. MiR-133a-5p directly targets MON2. **A,** Dual-Luciferase reporter gene experiment verified the direct targeting of miR-133a-5p and MON2. **B,** qRT-PCR and Western Blot detected the expression level of MON2 after overexpression of miR-133a-5p. **C,** qRT-PCR detected the MON2 expression in tumor tissues and adjacent tissues of renal clear cell carcinoma cells. **D,** miR-133a-5p and MON2 expression levels were significantly negatively correlated in renal clear cell carcinoma tissues. **E,** qRT-PCR detected the MON2 expression level in renal clear cell carcinoma cell lines. **F,** qRT-PCR detected the expression of miR-133a-5p after transfection of MON2 overexpression vectors in ACHN and 786-O cell lines. Data are average ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

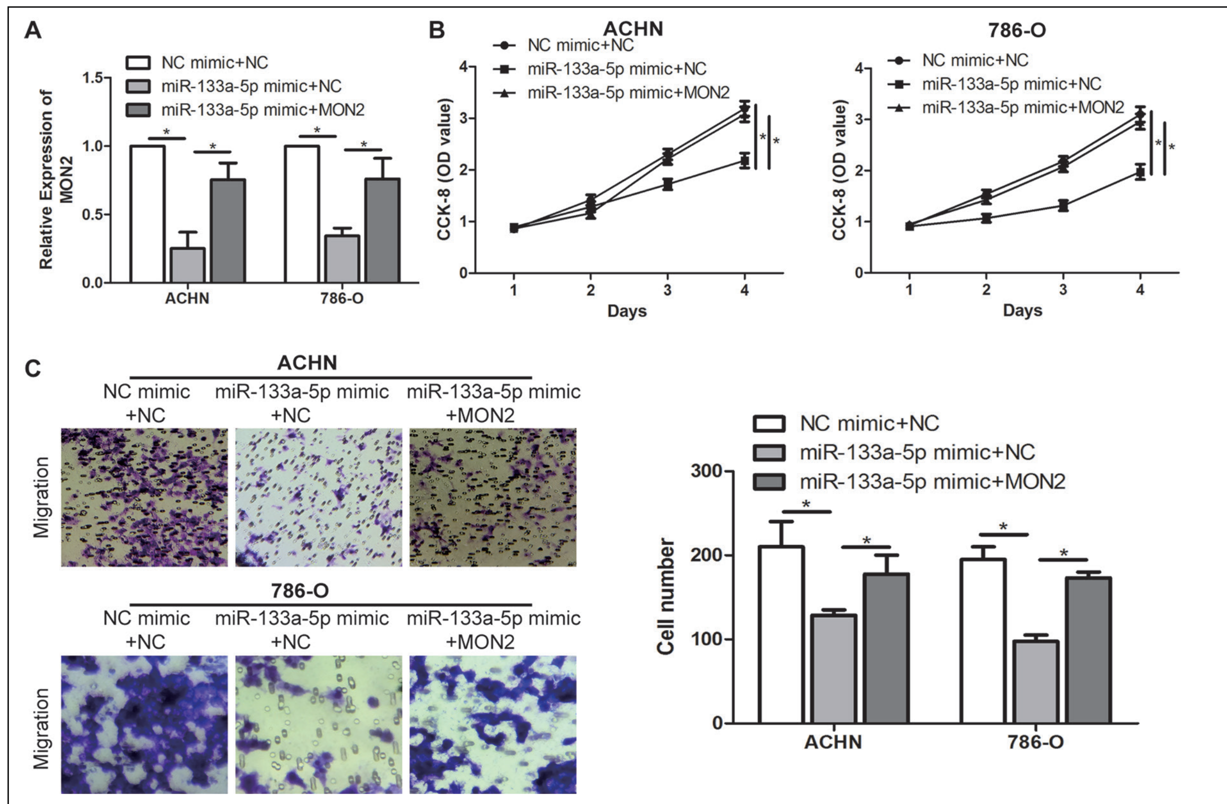

Figure 4. MiR-133a-5p inhibits the development of renal clear cell carcinoma through regulating MON2. **A,** qRT-PCR detection of MON2 expression levels after co-transfection of miR-133a-5p and MON2 overexpression vectors in ACHN and 786-O cell lines. **B,** CCK-8 assay detected the proliferation of renal clear cell carcinoma cells after co-transfection of miR-133a-5p and MON2 overexpression vectors in ACHN and 786-O cells. **C,** Transwell migration assay detected the invasion and migration ability of renal clear cell cancer cells after co-transfection of miR-133a-5p and MON2 overexpression vectors in ACHN and 786-O cell lines (magnification: 40×). Data are average ± SD, $*p<0.05$.

MiRNA, encoded by genomic DNA and widely exists in animals, is one of the small biomolecules discovered in recent years that plays a pivotal role in the development of tumors, the study of which is expected to provide a new target for gene therapy of tumors[19,20].

MicroRNA-133a-5p has been observed to be lowly expressed in a variety of tumor tissues, and its expression showed a close association with clinicopathological characteristics and prognosis of tumor patients, suggesting that microRNA-133a-5p may serve as an oncogene in a variety of malignant tumors including ccRCC[21,22]. However, the association between microRNA-133a-5p and ccRCC is unclear. This study aimed to clarify the role of microRNA-133a-5p in the development of ccRCC, microRNA-133a-5p expression was detected and a significant reduction of collected ccRCC tissue samples was found. Meanwhile, this microRNA indicated a correlation with the incidence of metastasis in ccRCC patients, suggesting that microRNA-133a-5p may act as a cancer-inhibiting gene in ccRCC. Furthermore, to explore the impact of microRNA-133a-5p on ccRCC cell functions, we transfected microRNA-133a-5p mimics in ccRCC cell lines and found the proliferative capacity and metastasis ability of ccRCC cells were markedly attenuated.

In addition, search tests performed by miRNAs software revealed that microRNA-133a-5p could pair with 3'-UTR of MON2 mRNA through base complementation to regulate its expression. We then verified the direct binding of microRNA-133a-5p to MON2 through Luciferase assay and showed that overexpression of microRNA-133a-5p remarkably down-regulated the mRNA level of MON2. MON2 gene, a newly discovered oncogene, mainly regulates cell growth through the crosstalk mechanism in cancer cell lines. In this study, MON2 was

confirmed to be highly expressed in ccRCC tumor tissues and promoted proliferation ability and metastasis of ccRCC cells. Meanwhile, we proved *in vitro* that overexpression of MON2 could counteract the inhibitory effect of overexpression of microRNA-133a-5p on proliferation rate and migration ability of ccRCC cells, suggesting that MON2 can reverse the impact of microRNA-133a-5p on biological functions of ccRCC, thus jointly affecting the malignant progression of ccRCC.

Conclusions

In summary, microRNA-133a-5p was found to be remarkably correlated with the incidence of metastasis and poor prognosis of ccRCC patients. In addition, it may suppress the malignant progression of ccRCC *via* regulating MON2.

Funding
This work was supported by the Key Research and Development Project (No. 2017ZDYF11), Zhejiang University Cooperation Project (No. 2018ZDHZ12) of Lishui, Zhejiang Public Welfare Technology Application Research Project (No. LGF20H050003) and Zhejiang Medical and Health Science and Technology Plan Project (No. 2020382934) and Traditional Chinese medicine of Zhejiang province science and technology plan project (2020ZB309).

Conflict of Interest
The Authors declare that they have no conflict of interests.

References

1) Rini BI, Campbell SC, Escudier B. Renal cell carcinoma. Lancet 2009; 373: 1119-1132.
2) Inamura K. Renal cell tumors: understanding their molecular pathological epidemiology and the 2016 WHO classification. Int J Mol Sci 2017; 18: 2195.
3) Pang C, Guan Y, Li H, Chen W, Zhu G. Urologic cancer in China. Jpn J Clin Oncol 2016; 46: 497-501.
4) Zabell J, Demirjian S, Lane BR, Derweesh IH, Isharwal S, Suk-Ouichai C, Wu J, Palacios DA, Campbell SC. Predictors of long-term survival after renal cancer surgery. J Urol 2018; 199: 384-392.
5) Choueiri TK, Motzer RJ. Systemic therapy for metastatic renal-cell carcinoma. N Engl J Med 2017; 376: 354-366.
6) Walz AL, Fernandez CV, Geller JI. Novel therapy for pediatric and adolescent kidney cancer. Cancer Metastasis Rev 2019; 38: 643-655.
7) Maher ER. Hereditary renal cell carcinoma syndromes: diagnosis, surveillance and management. World J Urol 2018; 36: 1891-1898.
8) Rossi SH, Klatte T, Usher-Smith J, Stewart GD. Epidemiology and screening for renal cancer. World J Urol 2018; 36: 1341-1353.
9) Barata PC, Rini BI. Treatment of renal cell carcinoma: current status and future directions. CA Cancer J Clin 2017; 67: 507-524.
10) Garje R, An J, Greco A, Vaddepally RK, Zakharia Y. The future of immunotherapy-based combination therapy in metastatic renal cell carcinoma. Cancers (Basel) 2020; 12: 143.
11) Kelsey R. Kidney cancer: PDL1 as a biomarker in high-risk RCC. Nat Rev Urol 2018; 15: 202.
12) Moch H, Srigley J, Delahunt B, Montironi R, Egevad L, Tan PH. Biomarkers in renal cancer. Virchows Arch 2014; 464: 359-365.
13) Akkoc Y, Gozuacik D. MicroRNAs as major regulators of the autophagy pathway. Biochim Biophys Acta Mol Cell Res 2020; 1867: 118662.
14) Lekchnov EA, Zaporozhchenko IA, Morozkin ES, Bryzgunova OE, Vlassov VV, Laktionov PP. Protocol for miRNA isolation from biofluids. Anal Biochem 2016; 499: 78-84.
15) Varela N, Lanas F, Salazar LA, Zambrano T. The current state of microRNAs as restenosis biomarkers. Front Genet 2019; 10: 1247.
16) Park JH, Jeong GH, Lee KS, Lee KH, Suh JS, Eisenhut M, van der Vliet HJ, Kronbichler A, Stubbs B, Solmi M, Dragioti E, Koyanagi A, Shin JI, Gamerith G. Genetic variations in MicroRNA genes and cancer risk: A field synopsis and meta-analysis. Eur J Clin Invest 2020; 50: e13203.
17) Afonso-Grunz F, Muller S. Principles of miRNA-mRNA interactions: beyond sequence complementarity. Cell Mol Life Sci 2015; 72: 3127-3141.
18) Conti I, Varano G, Simioni C, Laface I, Milani D, Rimondi E, Neri LM. MiRNAs as influencers of cell-cell communication in tumor microenvironment. Cells 2020; 9: 220.
19) Tutar Y. miRNA and cancer; computational and experimental approaches. Curr Pharm Biotechnol 2014; 15: 429.
20) Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017; 16: 203-222.
21) Zheng L, Kang Y, Zhang L, Zou W. MiR-133a-5p inhibits androgen receptor (AR)-induced proliferation in prostate cancer cells via targeting FUsed in Sarcoma (FUS) and AR. Cancer Biol Ther 2020; 21: 34-42.
22) Wang T, Wang X, Du Q, Wu N, Liu X, Chen Y, Wang X. The circRNA circP4HB promotes NSCLC aggressiveness and metastasis by sponging miR-133a-5p. Biochem Biophys Res Commun 2019; 513: 904-911.