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Original Article

MiR-378a-5p acts as a tumor suppressor in renal cell carcinoma and is associated with the good prognosis of patients

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Abstract: Renal cell carcinoma (RCC) is a common cancer that accounts for about 1.6% of all malignancies. Accumulating evidence has shown that miRNAs may play important roles in the development of cancers and that these same miRNAs may serve as diagnostic and prognostic biomarkers. The role of the miRNA miR-378a-5p in RCC, however, has been largely unexplored. In our study, we have demonstrated that miR-378a-5p expression was decreased in renal tissues and in RCC cell lines compared with corresponding expression levels in normal renal tissues and in the 293-T cell line. Functional studies in two RCC cell lines (ACHN and 786-O) have indicated that miR-378a-5p overexpression attenuated cell proliferation, migration, and invasion while promoting cell apoptosis. Inhibition of miR-378a-5p expression, on the other hand, promoted cell proliferation, migration, and invasion while reducing cell apoptosis. Additionally, in 42 cases of renal cancer formalin-fixed paraffin-embedded specimens, patients with higher expression levels of miR-378a-5p had significantly longer overall survival rates ($P<0.05$) than patients with lower miR-378a-5p expression levels. Thus, in this study, we have shown that miR-378a-5p can serve as a tumor suppressor and a potential prognostic biomarker in RCC.

Keywords: MicroRNA, miR-378a-5p, renal cell carcinoma, oncogene, prognosis biomarker

Introduction

Renal cell carcinoma (RCC) is the fourteenth-most commonly diagnosed cancer in China, accounting for ~1.6% of all cancer cases and causing an estimated 23,400 deaths in 2015 alone [1]. Surgery is the only recommended management strategy for localized RCC, and no evidence has shown that patients benefit from adjuvant therapy after surgery [2]. Unfortunately, 20-30% of patients experience recurrence after surgery [3]. Furthermore, kidney cancer is insensitive to chemotherapy [4]. Thus, novel therapeutic targets and biomarkers are urgently needed to improve RCC treatment.

miRNAs are single-stranded RNAs that are ~22 nucleotides long. These miRNAs post-transcriptionally regulate gene expression by binding to the 3'-UTR of target mRNAs [5]. Emerging evidence has identified miRNAs involved in cell metabolism, differentiation, angiogenesis [6], drug resistance and radiation-resistance [5, 7, 8], cancer cell proliferation, invasion, metastasis, and apoptosis [9, 10]. miR-378, specifically, has been reported as having functions related to cell proliferation, migration, invasion, and apoptosis in several cancers [11-13]. The function of miR-378 in RCC, however, is still largely unexplored.

In the present study, we have observed that miR-378a-5p was significantly down-regulated in RCC tissues and cell lines. Overexpression of miR-378a-5p promoted cell apoptosis and repressed cell proliferation, migration, and inva-

Table 1. Clinicopathological features in RCC patients

Characteristics	Number of cases
Mean age range (years)	45 (24-87)
Sexual distinction	
Male	28
Female	17
Tumor stage	
T1	23
T2	18
T3 + T4	4
Fuhrman grade	
I	17
II	15
III	9
IV	4
AJCC clinical stages	
I	22
II	16
III + IV	7

pT, primary tumor; AJCC, American Joint Committee on Cancer.

Table 2. Association between miR-378a-5p expression level[1] and Clinical information in FFPE renal cancer samples

Variable	Total	No. of patients (%)		*P*-value[2]
		High	Low	
Gender				
Male	26	11	15	0.341
Female	16	10	6	
Age (Years)				
≤60	33	17	16	1.000
>60	9	4	5	
Tumor size (cm)				
≤4.0	17	7	10	0.530
>4.0	25	14	11	
Tumor stage				
I + II	27	13	14	1.000
III + IV	15	8	7	

[1]cut-off point: median; [2]calculated using Fisher's Exact test or Pearson Chi-square test.

sion. Knockdown of miR-378a-5p, which reduced expression levels, resulted in opposite phenotypic effects when compared to those of the overexpression lines. Further studies, however, are needed to explore the potential mechanism of how miR-378a-5p functions as a tumor suppressor gene.

Materials and methods

Clinical specimens

Forty-five pairs of RCC tissues with adjacent normal renal tissues were obtained from Peking University Shenzhen Hospital from January 2010 to December 2015. The clinicopathological parameters are shown in **Table 1**. Forty-two formalin-fixed paraffin-embedded (FFPE) RCC tissue samples were collected from the Department of Pathology of Peking University Shenzhen Hospital. The corresponding patient characteristics are listed in **Table 2**. Written informed consent was obtained from all patients, and the study was approved of by the Ethics Committee of Peking University Shenzhen Hospital.

Cell culture and transfection

Renal cancer cell lines (ACHN, 786-O, and Caki-1) and the human embryonic kidney cell line, 293-T, were obtained from The Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics. 293-T cells and ACHN cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 μl/ml penicillin, and 100 mg/ml streptomycin sulfates. The supplemented ACHN cells were maintained in a humidified incubator with 5% CO_2 at 37°C. The 786-O cells were cultured in RPMI1640 medium. The Caki-1 cells were cultured in McCoy's 5A medium.

The miR-378a-5p mimics, miR-378a-5p inhibitor, negative control, and inhibitor negative control (Genepharma, China) were transfected at a concentration of 50 nM using Lipofectamine 2000, according to manufacturer guidelines. The transfection efficiency was determined using qRT-PCR. The sequences are shown in **Table 3**.

RNA extraction and RT-PCR assay

Total RNA samples were isolated from cells with Trizol (Invitrogen) and purified with an RNeasy Maxi kit (Qiagen GmbH) following the manufacturer's protocols. Total RNA of FFPE was extracted using the miRNeasy FFPE kit (Qiagen). RNA quality and quantity were verified with a NanoDrop 2000/2000c. The miScript Reverse Transcription kit (Qiagen GmbH) was used to synthesize cDNA from total RNA following the manufacturer's protocols.

Table 3. Sequences of primers and microRNAs

Primer/microRNA	Sequence
miR-378a-5p	Forward: 5'-CTCCTGACTCCAGGTCCTGTGT-3'
	Reverse: Universal primers (miScript SYBR Green PCR kit)
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3'
	Reverse: 5'-ACGCTTCACGAATTTGCGT-3'
miR-378a-5p mimic	Forward: 5'-CUCCUGACUCCAGGUCCUGUGU-3'
	Reverse: 5'-ACAGGACCUGGAGUCAGGAGUU-3'
miR-378a-5p inhibitor	5'-ACACAGGACCUGGAGUCAGGAG-3'
NC	Forward: 5'-UUCUCCGAACGUGUCACGUTT-3'
	Reverse: 5'-ACGUGACACGUUCGGAGAATT-3'
NC inhibitor	5'-CAGUACUUUUGUGUAGUACAA-3'

miR, microRNA; NC, negative control; PCR, polymerase chain reaction.

Quantitative PCR (qPCR)

The expression levels of miR-378a-5p were determined using qPCR with a miScript SYBR® Green PCR kit (Qiagen GmbH) on the Roche Lightcycler 480 Real-Time PCR system, according to the manufacturer's specifications. U6 served as an internal control. The reaction conditions were as follows: 95°C for 2 minutes, 40 cycles at 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The primers used in this assay are listed in **Table 3**. Expression of miR-378a-5p was quantified using the $2^{-\Delta\Delta Cq}$ method [14].

Wound healing assay

Renal cancer cells were seeded onto 12-well plates (3×10^5 cells per well) and incubated for 24 h to achieve a monolayer of about 80% confluence. Then, the cells were transfected with miR-378a-5p mimics, negative control, inhibitor, or inhibitor negative control. Wounds were created using a sterile 200 µl pipette tip on the confluent cells after transfection. The cells were rinsed with phosphate-buffered saline (PBS) twice to wash away the cell debris. Then, the cells were maintained in the incubator. The images were recorded at 0 h and 24 h following wound creation.

Cell proliferation assay

A Cell Counting Kit-8 (CCK-8) assay was conducted to identify the proliferative ability of renal cancer cells. Cells were seeded onto 6-well plates (3×10^5 cells per well) and maintained in the incubator. After transfection for 24 h, cells were reseeded onto 96-well plates (4×10^3 cells/well). At 0, 24, 48 and 72 h, the absorbance values of experimental wells were read at 450 nm in a microplate reader.

Transwell assay

The migration and invasion ability of renal cancer cells was measured using a transwell assay. Chambers with or without matrigel were used to determine the invasion ability and migration ability of RCC cells, respectively. After 24 h of transfection, 100 µl of serum-free medium containing 3×10^4 renal cancer cells was added into the upper chambers, and 500 µl of medium supplemented with 10% FBS was added to the lower chamber. The cells were cultured in a humidified incubator with 5% CO_2 at 37°C for 24 h. Then, cells that migrated to or invaded the bottom chambers were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Finally, stained cells were counted under a microscope.

Apoptosis analysis

Cells were grown in 6-well plates, and when the cells had achieved a monolayer of about 50% confluence, they were transfected with miR mimics, miR inhibitor, negative control, or inhibitor negative control. After 48 h of incubation, the cells were collected and washed with PBS twice. Next, the cells were resuspended in 100 µl of 1 × binding buffer and treated with 5 µl of Annexin V-fluorescein isothiocyanate (Invitrogen; Thermo Fisher Scientific, Inc.) and 5 µl propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc.) in the dark for 15 min at room temperature. Then, 400 µl of 1 × binding buffer was added to the resuspended cells, and the apoptosis rate was immediately analyzed using flow cytometry (EPICS, XI-4; Beckman Coulter, Inc., Brea, CA, USA).

Bioinformatic analyses

Target prediction was done using starBase v2.0 (http://starbase.sysu.edu.cn/). Only predictions that were independently repeated by at least three programs were included.

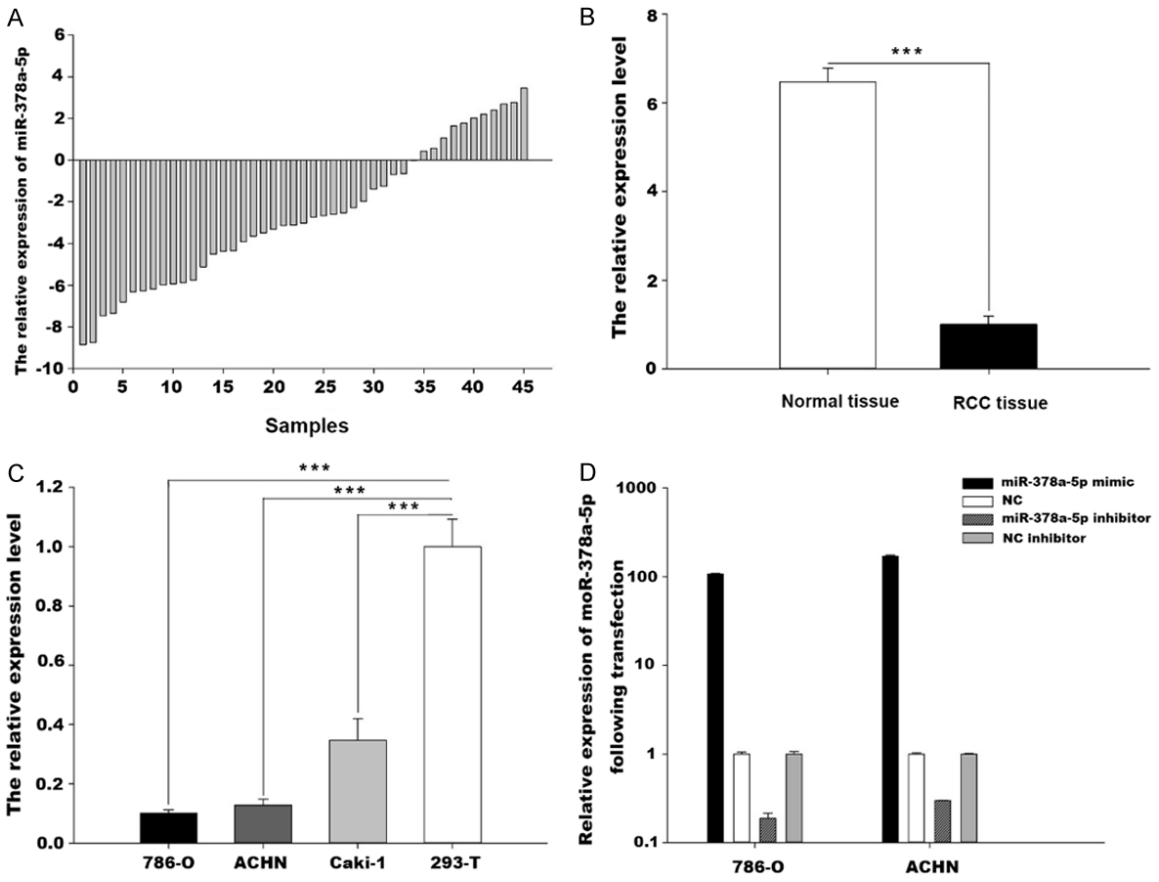


Figure 1. miR-378a-5p expression levels in tissues and cell lines. A. The relative expression level of miR-378a-5p in 45 paired RCC tissues and normal renal tissues. B. The relative expression level of miR-378a-5p in RCC tissues was 6.472 times that of normal renal tissues. C. Relative expression level of miR-378a-5p in cell lines. miR-387a-5p was reduced by 89.99% in 786-O cells, 87.18% in ACHN cells, and 65.29% in Caki-1 cells when compared to that of 293-T cells. D. Transfection efficacy of miR-378a-5p into RCC cells. miR-378a-5p of 786-O and ACHN cells were 106.89 times and 168.90 times higher, respectively, in the mimics group than in the negative control group. miR-378a-5p expression levels of 786-O and ACHN cells were reduced by 81.23% and 70.06%, respectively, in inhibitor groups when compared to the expression levels in the inhibitor negative control group. *P<0.05, **P<0.01, ***P<0.001; RCC, renal cell carcinoma; miR, microRNA; NC, negative control.

Statistical analyses

All statistical analyses were done using SPSS 18.0 statistical software. The expression of miR-378a-5p in RCC tissues and adjacent normal renal tissues and cell lines was analyzed with a Student's paired t-test. Patients were separated into either the higher miR-378a-5p expression group or the lower miR-378a-5p expression group based on whether the patient had an miR-378a-5p expression level either above or below the median value of the 42 FFPE specimens. Either the Fisher's exact test or the Pearson Chi-square test was used to analyze the associations between miR-378a-5p status and clinicopathological variables. Cox proportional hazard regression analysis (univariate and multivariate) was conducted to analyze the association between miR-378a-5p expression levels and overall survival rates. The Kaplan-Meier survival curve test was conducted to further analyze the association between miR-378a-5p expression level and overall survival, and the Log-rank test was used to evaluate the difference. Differences were considered to be statistically significant when the *P* value was less than 0.05.

Results

miR-378a-5p is down-regulated in tissues and cell lines

Real-time PCR was performed to determine the miR-378a-5p expression level in 45 paired RCC tissues and adjacent normal renal tissues.

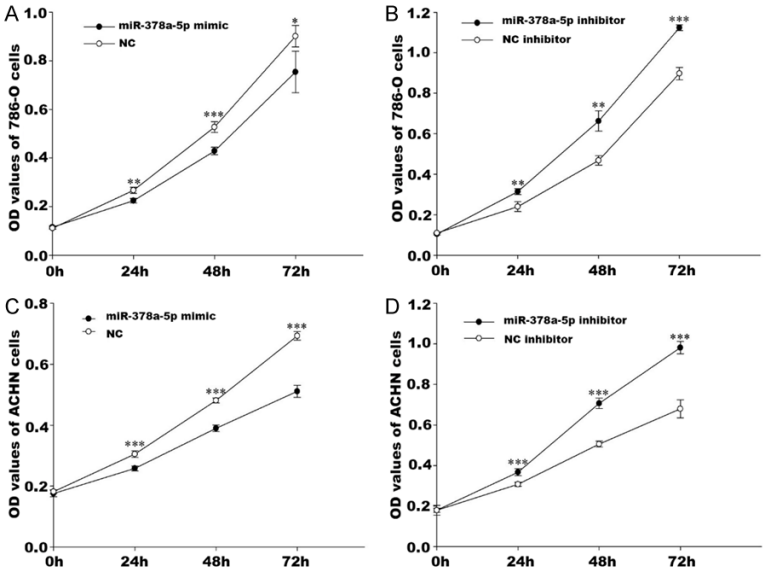


Figure 2. miR-378a-5p suppressed the proliferation of RCC cells. A and B. Proliferation of 786-O and ACHN cells transfected with miR-378a-5p mimics or with the negative control. The cell proliferation rate in the mimics-transfected group of 786-O cells was reduced by 15.96% at 24 h, 18.66% at 48 h, and 16.32% at 72 h; and, in ACHN cells, by 15.39% at 24 h, 19.00% at 48 h, and 26.27% at 72 h after transfection compared with the negative control group. C and D. Proliferation of 786-O and ACHN cells transfected with miR-378a-5p inhibitor and inhibitor negative control. The cell proliferation rate in the inhibitor-transfected group of 786-O cells was increased by 31.17% at 24 h, 41.57% at 48 h, and 25.20% at 72 h; and the rate in ACHN cells was increased by 19.27% at 24 h, 39.64% at 48 h, and 44.36% at 72 h after transfection compared with the inhibitor-transfected negative control group. *P<0.05, **P<0.01, ***P<0.001. OD, optical density; miR, microRNA; NC, negative control.

Expression of miR-378a-5p was lower in RCC tissues than in adjacent normal renal tissues (P<0.001) (**Figure 1B**). The relative expression of miR-378a-5p is shown in **Figure 1A**. Also, the expression level of miR-378a-5p in RCC cell lines and in human embryonic kidney cell line was determined, and miR-378a-5p expression was significantly down-regulated in RCC cell lines compared to expression in the 293-T cell line (P<0.001) (**Figure 1C**). The dysregulation of miR-378a-5p is most prominent in the 786-O and ACHN cell lines, so both cell lines were selected for further *in vitro* assays. The transfection efficacy of miR-378a-5p into RCC cells was verified by qRT-PCR (**Figure 1D**).

miR-378a-5p suppresses cell proliferation in RCC cell lines

The CCK-8 assay showed that proliferative ability was suppressed in 786-O and ACHN cells transfected to overexpress miR-378a-5p but was unaffected in the same cell lines transfected with negative control (**Figure 2A** and **2B**). On the other hand, inhibition of miR-378a-5p facilitates the proliferation of 786-O and ACHN cells in which miR-378a-5p is inhibited have increased proliferation rates compared to that of cells transfected with the inhibitor negative control (**Figure 2C** and **2D**).

miR-378a-5p reduced migration and invasion of RCC cells in vitro

A wound healing assay and a transwell assay were performed to determine the effect of miR-378a-5p on cell migration and invasion. The results of the wound healing assay revealed that 786-O and ACHN cells with increased miR-378a-5p expression levels migrated more slowly than control cells. Knockdown of miR-378a-5p in 786-O and ACHN cells, however, promoted migration (**Figure 3**). The transwell assay revealed similar results, wherein forced up-regulation of miR-378a-5p attenuated the migration and invasion abilities of 786-O and ACHN cells compared to those of cells transfected with the negative control. Down-regulation of miR-378a-5p, however, resulted in acceleration of migration and invasion in 786-O and ACHN cells compared with that of cells transfected with the inhibitor negative control (**Figure 4**).

miR-378a-5p suppressed apoptosis of RCC cells in vitro

We performed flow cytometry to analyze the effect of miR-378a-5p on cell apoptosis. The results suggested that the apoptosis rate was lower in 786-O and ACHN cells transfected with miR-378a-5p mimics than in cells transfected with the negative control. Furthermore, the apoptosis rate of RCC cells in the miR-378a-5p inhibitor group was significantly higher than the

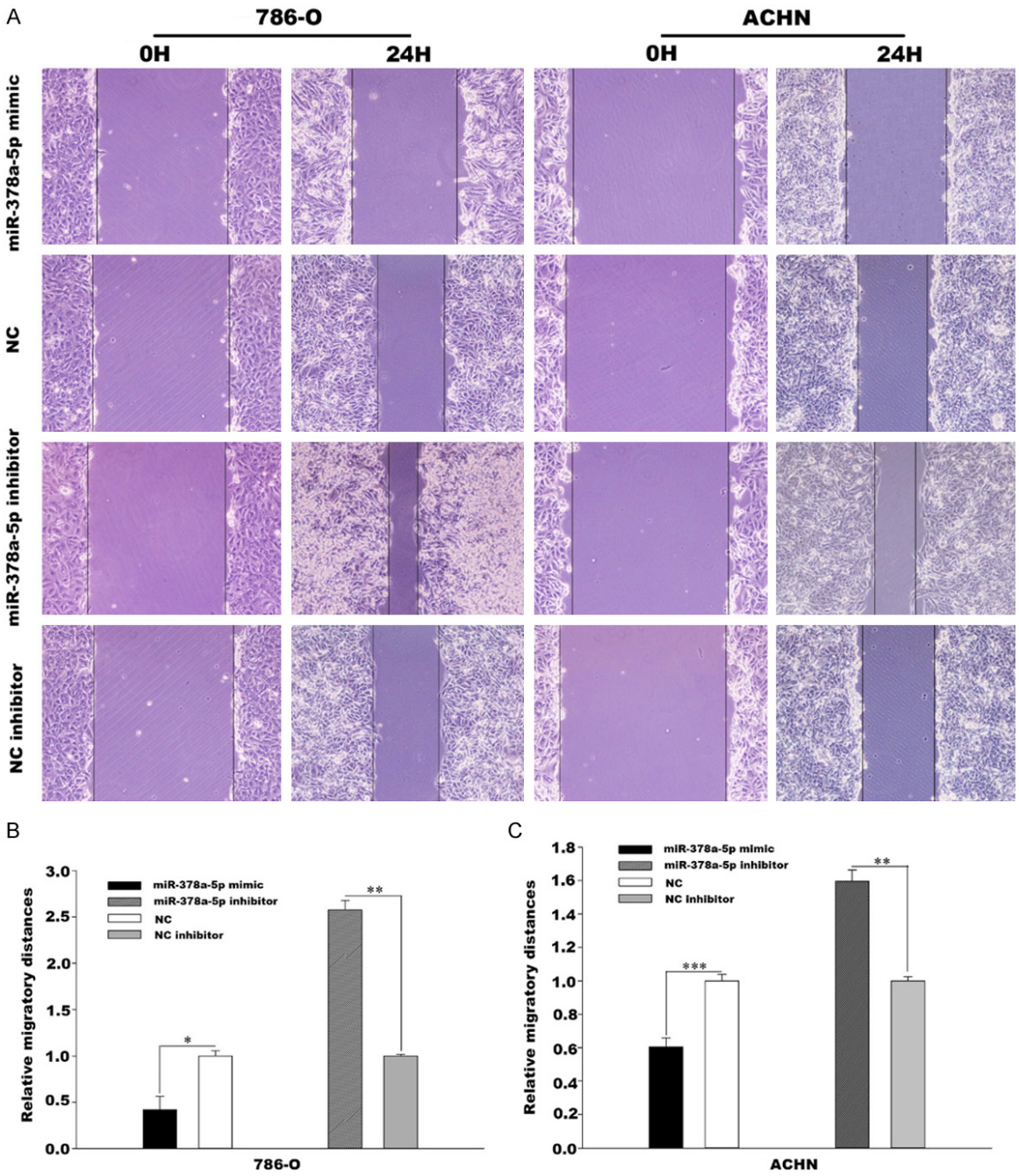
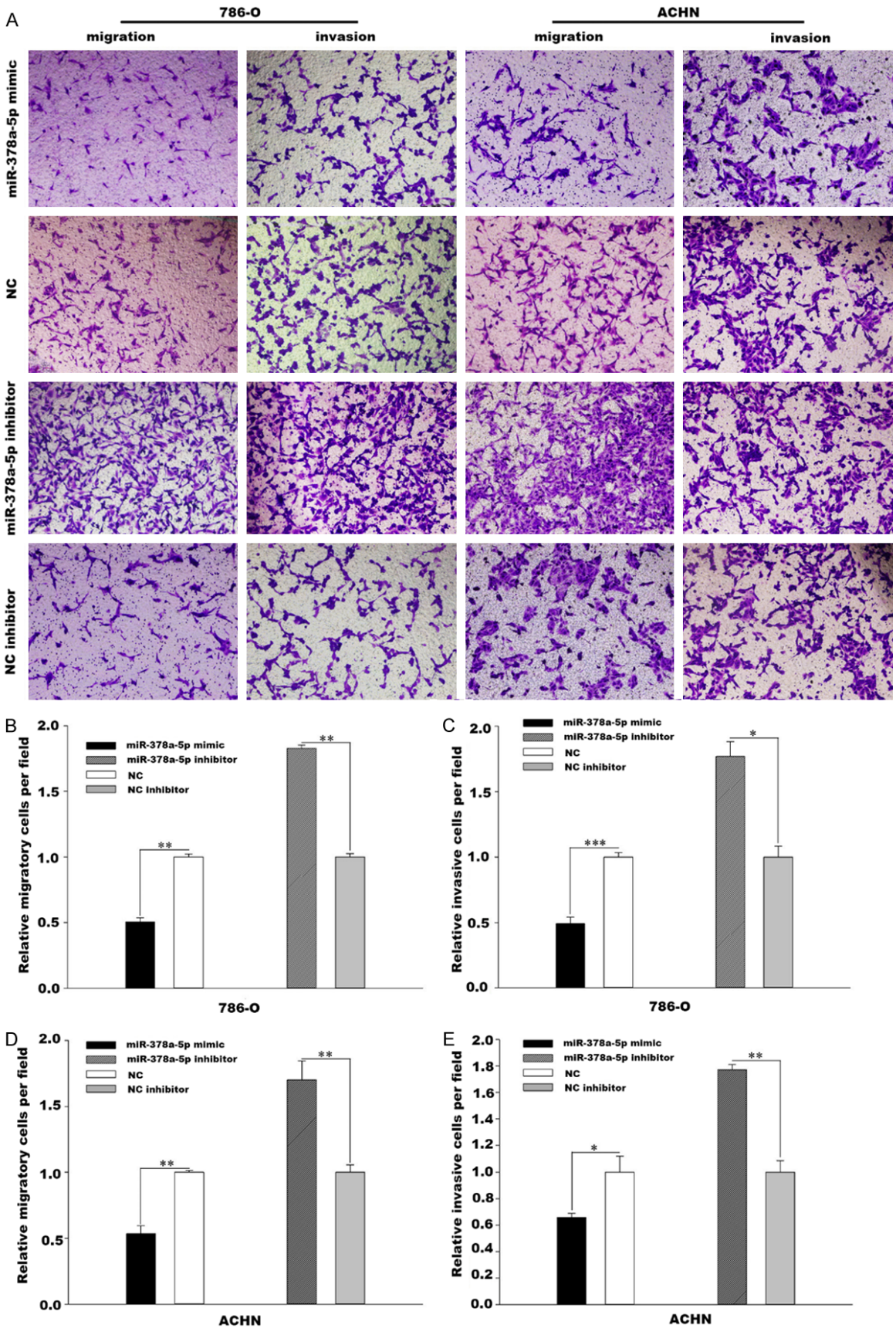


Figure 3. Wound healing assays were used to measure the effect of miR-378a-5p on cell migration. A. Wound healing assays were used to identify the effect of miR-378a-5p on 786-O and ACHN cells. B. miR-378a-5p suppressed the migration ability of 786-O cells. The cell migratory distance in the mimics group of 786-O cells was reduced by 58.29% compared with the negative control group. The cell migratory distance in the inhibitor group of 786-O cells was increased 1.58-fold compared with that of the inhibitor negative control group. C. miR-378a-5p suppress the migration of ACHN cells. The cell migratory distance in the mimics group of ACHN cells was reduced by 39.55% compared with that of the negative control group. The cell migratory distance in the inhibitor group of ACHN cells was increased by 59.59% compared with that of the inhibitor negative control group. *P<0.05, **P<0.01, ***P<0.001; miR, microRNA; NC, negative control.

apoptosis rate of cells in the inhibitor negative control group. These results have demonstrated that miR-378a-5p suppressed apoptosis of RCC cells (**Figure 5**).


A
786-O
ACHN
migration
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miR-378a-5p mimic
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NC inhibitor
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Relative migratory cells per field
miR-378a-5p mimic
miR-378a-5p inhibitor
NC
NC inhibitor
786-O
C
Relative invasive cells per field
786-O
D
Relative migratory cells per field
ACHN
E
Relative invasive cells per field
ACHN

Figure 4. A transwell assay was used to measure the effect of miR-378a-5p on cell migration and invasion. A. The effect of overexpression or knockdown of miR-378a-5p on migration or invasion ability in 786-0 or ACHN cells. B. Effect of miR-378a-5p on cell migration of 786-0 cells. The cell migration index in the mimics group of 786-0 cells was reduced by 49.32% compared with that of the negative control group. The cell migration index in the inhibitor group of 786-0 cells was promoted by 82.65% compared with that of the inhibitor negative control group. C. Effect of miR-378a-5p on cell invasion of 786-0 cells. The cell invasion index in the mimics group of 786-0 cells was reduced by 51.01% compared with that of the negative control group. The cell invasion index in the inhibitor group of 786-0 cells was promoted by 76.91% compared with that of the inhibitor negative control group. D. Effect of miR-378a-5p on cell migration of ACHN cells. The cell migration index in the mimics group of ACHN cells was reduced by 46.59% compared with that of the negative control group. The cell migration index in the inhibitor group of ACHN cells was increased by 70.13% compared with that of the inhibitor negative control group. E. Effect of miR-378a-5p on cell invasion of ACHN cells. The cell invasion index in the mimics group of ACHN cells was reduced by 33.13% compared with that of the negative control group. The cell invasion index in the inhibitor group of ACHN was promoted by 77.09% compared with that of the inhibitor negative control group. *P<0.05, **P<0.01, ***P<0.001; miR, microRNA; NC, negative control.

miR-378a-5p is a potential prognostic marker of RCC

The association between miR-378a-5p expression level and different clinicopathological characteristics was analyzed. The results showed that there was no correlation between miR-378a-5p expression level and age, gender, tumor size, or tumor stage.

Univariate analysis showed that low miR-378a-5p expression is associated with lower overall survival (HR = 5.318, 95% CI = 1.115-25.365, P = 0.046) (**Table 4**). Also, multivariate analysis revealed that patients with low miR-378a-5p expression had a statistically significant association with lower overall survival rates (HR = 5.894, 95% CI = 1.083-32.076, P = 0.040) (**Table 4**). As is shown in **Figure 6**, the Kaplan-Meier survival curves show that patients with higher miR-378a-5p expression levels had significantly longer overall survival than those with lower miR-378a-5p expression levels. The results have thus indicated that miR-378a-5p is a potential predictor for a good prognosis of RCC patients.

miR-378a-5p targets prediction

We looked for potential targets of miR-378a-5p by searching the database. The results indicated that potential targets include RBM14, KPNA6, PTGES3, KIAA1522, DAZAP2, C1orf9, and ZDHHC9. These potential targets will be our focus during future studies.

Discussion

In this study, we have demonstrated that miR-378a-5p was down-regulated in renal cancer tissues and RCC cell lines, and that miR-378a-5p acts as a tumor suppressor. Previous studies have shown that miR-378 is down-regulated in many cancers including glioma [15], colorectal cancer [12], prostate cancer [16], and gastric cancer [17]. On the other hand, some researchers have demonstrated that miR-387 is up-regulated in cervical cancer [18], acute myeloid leukemia [19], and nasopharyngeal carcinoma [20]. Thus, miR-378 may serve as an onco-miR or tumor suppressor in different types of cancer. Browne *et al.* reported that miR-378 represses breast cancer cells' aggressiveness by suppressing Runx1 [21]. Chen *et al.* revealed that miR-378 is associated with NSCLC brain metastasis, acceleration of tumor angiogenesis, cell migration, and invasion [22]. Chen *et al.* also suggested, however, that miR-378 may reverse chemoresistance to cisplatin by targeting sCLU in lung adenocarcinoma [23]. In gastric cancer, miR-378 is down-regulated and functions as a tumor suppressor via the repression of the CDK6 and VEGF signaling pathway [17]. It has been reported that miR-378a-3p enhances the sensitivity of breast cancer cells to tamoxifen by targeting GOLT1A [24]. Furthermore, ectopic expression of miR-378 was identified as accelerating tumor growth and angiogenesis by targeting the tumor suppression genes SuFu and Fus-1 [25]. Recently, a similar phenomenon was observed in liver cancer [26]. In addition, Li *et al*. found that miR-378 promotes cervical cancer cell proliferation and represses apoptosis through the ST7L/Wnt-β/catenin axis [18].

miR-378 has also been identified as an effective biomarker of many diseases. Zanutto *et al.* reported that miR-378 in serum could be used to discriminate colorectal cancer patients from healthy individuals, and miR-378 can thus

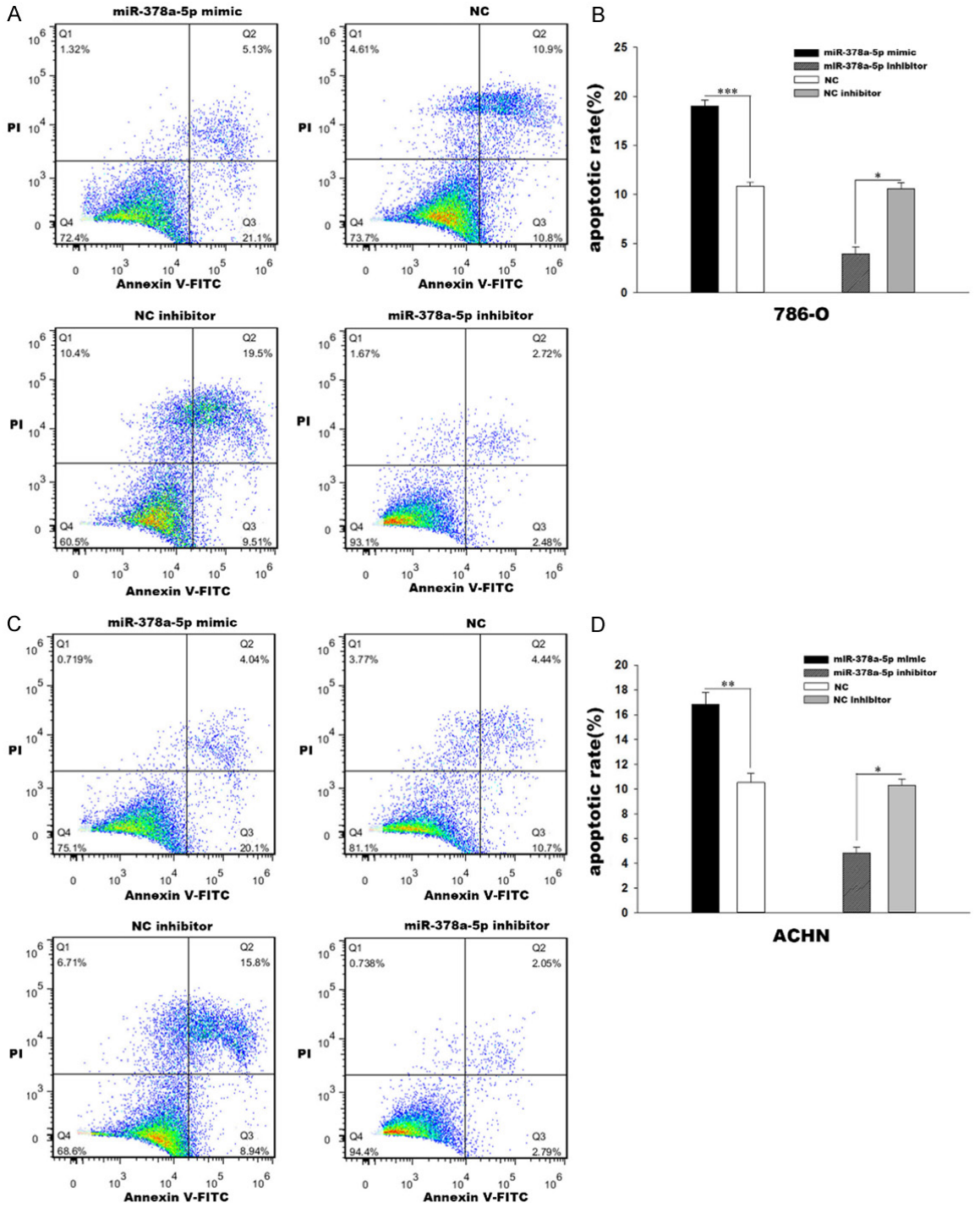

Figure 5. Cell apoptosis of 786-O and ACHN cells transfected with miR-378a-5p mimics or inhibitors, or the corresponding negative control, was measured by flow cytometry. A and B. The apoptosis rate of 786-O cells transfected with miR-378a-5p mimics or inhibitors, or the rate of the corresponding negative control. The apoptosis rate of 786-O cells in the mimics group was increased by 75.69% compared to that of the negative control group. In the inhibitor group, the apoptosis rate was reduced by 62.46% compared with that of the inhibitor negative control group. C and D. The apoptosis rate of ACHN cells transfected with miR-378a-5p mimics or inhibitors, or the rate of the corresponding negative control. The apoptosis rate of ACHN cells in the mimics group was increased by 59.81% compared to that of the negative control group. In the inhibitor group, the apoptosis rate was reduced by 53.07% compared with that of the inhibitor negative control group. *P<0.05, **P<0.01, ***P<0.001. PI, propidium iodide; FITC, fluorescein isothiocyanate; miR, microRNA; NC, negative control.

Table 4. miR-378a-5p expression and patients' survival

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Gender (Female vs Male)	0.375 (0.079-1.769)	0.215	0.417 (0.079-2.201)	0.303
Age (≤60 y vs >60 y)	0.295 (0.083-1.050)	0.060	0.464 (0.120-1.787)	0.264
Tumor size (≤4.0 cm vs >4.0)	1.576 (0.454-5.468)	0.473	3.096 (0.732-13.097)	0.125
Tumor stage(I + II vs III + IV)	0.184 (0.048-0.716)	0.015	0.132 (0.028-0.631)	0.011
miR-378a-5p (low vs high)	5.318 (1.115-25.365)	0.036	5.894 (1.083-32.076)	0.040

HR, Hazard ratio; 95% CI, 95% Confidence interval.

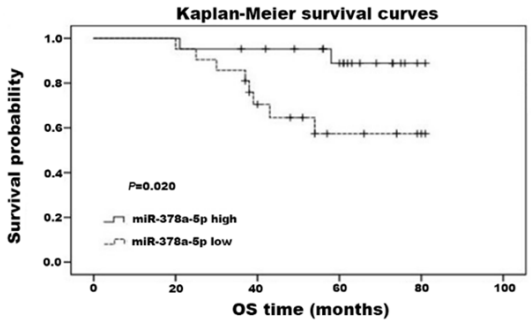

Figure 6. Kaplan-Meier curves showing significantly better prognosis in patients with higher expression levels of miR-378a-5p. OS, overall survival; miR, microRNA; NC, negative control.

serve as a biomarker to predict the efficacy of vaccine treatment [27-29]. In ovarian cancer, miR-378 may act as a biomarker to estimate the response to anti-angiogenic therapy.

In this study, we found that miR-378a-5p was down-regulated and acted as a tumor suppressor in RCC. Moreover, higher miR-378a-5p expression was correlated with longer overall survival of RCC patients. Further studies have shown that overexpression of miR-378a-5p suppresses proliferation, migration, and invasion of RCC cell lines while promoting apoptosis of RCC cell lines. Inhibition of miR-378a-5p, on the other hand, increases cell proliferation, migration, and invasion while reducing cell apoptosis. Thus, miR-378a-5p may serve as a tumor suppressor and a potential prognostic biomarker in RCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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