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Original Article
miR-27a promotes cell proliferation and metastasis in renal cell carcinoma

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Abstract: miR-27a has been reported to exhibit abnormal expression in renal cell carcinoma (RCC), but the role of miR-27a in RCC remains unknown. In our study, up-regulation of miR-27a was validated by Real-time PCR analysis in 133 RCC samples. Overexpression of miR-27a promoted cell migration, invasion and proliferation in vitro, while its low expression exerted opposite effects. Kaplan-Meier analysis demonstrated that the patients with high expression of miR-27a had a worse overall and relapse-free survivals compared with those with low expression of miR-27a. Cox proportional hazards analyses showed that miR-27a expression was an independent prognostic factor for RCC patients. Collectively, our findings illustrate the promoting-cancer effect of miR-27a in RCC, suggesting that miR-27a could be a potential therapeutic target for RCC. Additionally, Kaplan-Meier analyses and Cox proportional regression analysis suggest that miR-27a may be a potential biomarker for predicting the survival of RCC patients.

Keywords: miR-27a, proliferation, metastasis, renal cell carcinoma, biomarker

Introduction

Renal cell carcinoma (RCC) represents the most common kidney malignancy and is the third common urological cancer after prostate and bladder cancer, but it has the highest mortality at 40%. RCC is heterogeneous and comprises several histological subtypes according to the differences in genetics, biology and behavior [1]. Clear cell carcinoma (CCC) accounts for about 70% of the cases [2]. Furthermore, approximately 30% of RCC patients develop invasive disease commonly metastasizing to bone, lung, brain and other vital organs [3]. Therefore, a better understanding of the molecular mechanism of RCC progression is needed to provide a rationale for the effective therapeutic measures of RCC [4].

microRNAs (miRNAs) were identified as an abundant class of small non-coding RNAs that acted a vital role in post-transcriptional regulation in different biological processes [5-8]. The involvements of miR-27a in the progression of malignancies have recently been reported. For instance, miR-27a promotes the growth, colony formation and migration of pancreatic cancer cells by targeting Sprouty2 [9]. Down-regulation of miR-27a might inhibit proliferation and drug resistance of gastric cancer cells [10]. MiR-27a functions as a tumor suppressor in acute leukemia by regulating 14-3-3θ [11].

In this study, we detected the expression of miR-27a in the RCC tissues and found that miR-27a was related to tumor size and metastasis of RCC patients. Then, we demonstrated that miR-27a could promote the migration, invasion and proliferation of RCC cell in vitro. Furthermore, Kaplan-Meier analysis proved that the patients with high expression of miR-27a had a worse overall and relapse-free survivals compared with those with low miR-27a expression. Cox proportional hazards analyses indicated that miR-27a expression was an independent prognostic factor for RCC patients.

Materials and methods

Clinical samples

A total of 133 patients with RCC had undergone routine surgery at Jinling Hospital, Nanjing University School of Medicine from August

Table 1. Correlation between miR-27a expression and clinicopathological characteristics of RCC patients (n = 133)

Characteristics	Number of patients	miR-27a high expression (≥ Median a)	miR-27a low expression (< Median)	*P*-value Chi-squared test
No.	133	67	66	
Age (years)				0.881
< 60	76	39	37	
≥ 60	57	30	27	
Gender				0.097
Male	78	44	34	
Female	55	23	32	
Tumor size				0.008[a]
T1 + T2	64	26	38	
T3 + T4	69	44	25	
Lymph node metastasis				0.014[a]
Negative	108	44	64	
Positive	25	17	8	
Distant metastasis				0.026[a]
Negative	107	44	63	
Positive	26	17	9	

Note: [a]$P < 0.05$, indicates statistical significance.

2012 to December 2013. RCC samples and the corresponding adjacent tissues taken from the 133 patients were collected, immediately snap frozen in liquid nitrogen, and stored at -80°C until RNA extraction. This study was approved by the Ethical Committee of Nanjing General Hospital of Nanjing Command, PLA, and every patient had written informed consent

Cell lines and culture conditions

Human renal cell carcinoma cell lines ACHN and 786-O cell lines were purchased from the Shanghai Institutes for Biological Sciences or Chinese Academy of Sciences or American Type Culture Collection (ATCC, USA) and grown in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), penicillin (100 IU/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 at 37°C.

Transient transfection

Oligonucleotides miR-27a mimics (miR-27a), mimics control (miR-27a control), miR-27a inhibitors (anti-miR-27a), inhibitor control (anti-miR-control) were purchased from GenePharma (Shanghai, China). Transfection of cells with oligonucleotides was performed using Lipofectamine 2000 Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Transfection efficiency was monitored by real-time PCR.

Cell migration and invasion assay

For cell migration assay, a cell suspension of in 0.2 ml RPMI-1640 medium with 2% FBS was seeded into each well of the upper Transwell chamber (8 μm pore size, Corning, New York, USA). And, the upper Transwell chamber was pre-coated with Matrigel (BD Biosciences, NJ, USA) for invasion assay. In the lower chamber, 0.8 ml RPMI 1640 with 10% FBS was added. After incubating for 6 h (for migration) or 24 h (for invasion) 37°C in a humidified incubator with 5% CO_2, chambers were disassembled and the membranes were stained with 2% crystal violet for 10 min and placed on a glass slide. The number of cells penetrating across membrane was counted under a microscope in ten random visual fields. A set of images was acquired using NIS Elements image analysis software (Nikon, Tokyo, Japan).

Cell counting kit-8 (CCK-8)

Cells were collected 24 h following microRNA transfection and re-plated at the same density per well into 96-well plates. Following incubation for 24, 48, 72 and 96 h, cell proliferation was measured using the CCK-8 kit (Dojindo, Gaithersburg, MD). Briefly, 10 μl CCK-8 was added to each well and incubated for 1.5 h. The OD value at 405 nm was measured.

Cell apoptosis assay

The extent of apoptosis was evaluated by annexin V-fluorescein isothiocyanate (FITC)/

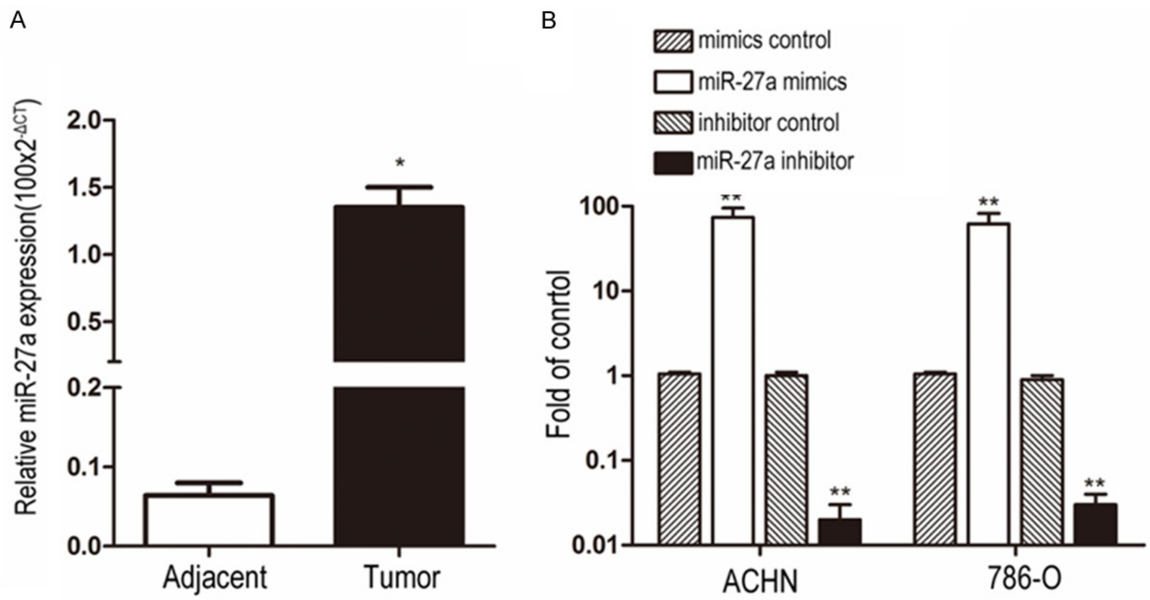

Figure 1. MiR-27a is up-regulated in RCC patients. A. The expression levels of miR-27a in human RCC tissues and corresponding adjacent tissues relative to U6 were determined by Real-time PCR (n = 133, $P < 0.05$). B. MiR-27a expression levels in cell lines transfected with miR-27a mimics, control for miR-mimics (mimics control), miR-27a inhibitor and control for miR-27a inhibitor (inhibitor control) were verified by Real-time PCR. Data are represented as mean ± SEM. *Indicates $P < 0.05$, **Indicates $P < 0.01$. Every independent experiment was performed 3 times.

propidium iodide (PI) detection kit (Invitrogen, CA, USA). ACHN and 786-O cell lines were respectively transfected with miR-27a mimics, mimics control, miR-27a inhibitor and inhibitor control. Cells were collected, washed twice with pre-chilled PBS and re-suspended in 1 × binding buffer, 48 h after treatment. Aliquots were mixed with 5 μl annexin V-FITC and 5 μl PI at room temperature for 15 min. The apoptosis assay was performed using a flow cytometry (EPICS, XL-4, Beckman, CA, USA).

miRNA isolation and Real-time PCR

Total RNAs were extracted from the fresh cells using TRIZol reagent (Life Technologies, MD, USA). The concentration and quality of RNA were measured by the UV absorbance at 260 nm and 280 nm and guaranteed RNA equal amount from each sample. The miRNA isolation was carried out from the total RNA using mirVanaTM miRNA Isolation Kit (Ambion Inc. Austin, Texas, USA) according to manufacturer's instructions. For the quantitative detection of miR-27a, the templates and primer sets were mixed with SYBR qPCR master mix (TaKaRa, Dalian, China), and real-time PCR was performed an initial step of denaturation at 95°C for 5 min, 40 cycles of amplification, denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, elongation at 72°C for 30 sec on a 7500 Fast Real-time PCR System (Applied Biosystems, California, USA). All reactions were performed in a 20 μl volume in triplicate. The primer sequences were as below: miR-27a, forward primer: 5'-TTCACAGTGGCTAAG-3', reverse primer: 5'-GTGCAGGGTCCGAGGT-3' and U6, forward primer: 5'-CTCGCTTCGGCAGCACA-3', reverse primer: 5'-AACGCTTCACGAATTTGCGT-3'. U6 snRNA levels were used as the internal control. Data analyses for the miRNA expression were performed using the $2^{-\Delta\Delta Ct}$ method.

Statistical methods

Statistical analysis was performed using Graphpad prism software (GraphPad Software, California, USA). The results obtained from experiment in vitro assays are presented as mean ± SEM from five separate experiments in triplicates per experiment. Comparison between groups was made by Student's t test (two tailed) or one-way ANOVA. Kaplan-Meier survival curves were plotted and log rank test was done. The significance of various variables for survival was analyzed by Cox proportional hazards model in a multivariate analysis. Results were considered statistically significant at $P < 0.05$.

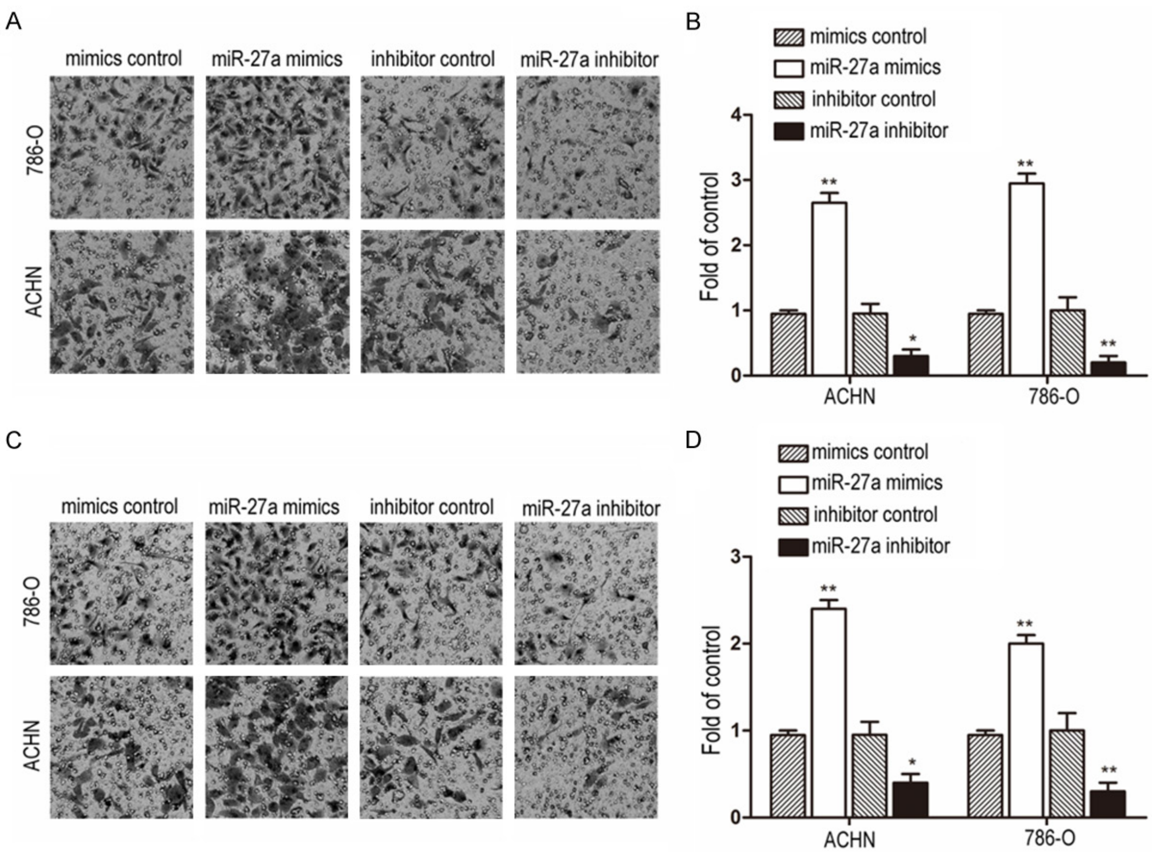

Figure 2. Effects of miR-27a on migration and invasion of RCC cells. A. High level of miR-27a enhanced the ability of RCC cell migration, while the low expression of miR-27a had an opposite effect. B. The quantifications of cell migration were presented by the histogram. C. Transwell chamber with Matrigel assay indicated that overexpression of miR-27a promoted ACHN and 786-O cell lines invasion, whereas the suppressed miR-27a attenuated the invasion of RCC cell lines. D. The quantifications of cell invasion were presented by the column chart. Data are representatives of three independent experiments. All experiments were performed in triplicate and presented as mean ± SEM. *Indicates significant difference compared with control group ($P < 0.05$). **Indicates $P < 0.01$. Every independent experiment was performed 3 times.

Results

miR-27a was up-regulated in RCC tissues

To investigate basal level of miR-27a in RCC tissues, we performed Real-time PCR to detect the microRNA in 133 RCC tissues and their corresponding adjacent tissues. It was found that the expression of miR-27a was significantly increased in RCC tissues as compared with the corresponding adjacent tissues ($P < 0.05$) (**Figure 1A**). All RCC samples were divided into miR-27a low-expression group (n = 66) and high-expression group (n = 67), median was used as cut off. The correlation between the miR-27a expression and clinicopathological characteristics is exhibited (**Table 1**). No positive correlation with age, gender, however, there was obviously correlation with tumor size ($P = 0.008$), lymph node metastasis ($P = 0.014$) and distant metastasis ($P = 0.026$). The abnormal expression of miR-27a indicated that miR-27a might act a vital role in RCC development. Therefore, based on the expression pattern of miR-27a, ACHN and 786-O cell lines were chosen to validate the effect of miR-27a on the development of RCC.

miR-27a affected RCC cell invasion and migration

To elucidate the functional role of miR-27a in RCC, ACHN and 786-O cell lines were transfected with miR-27a mimics, control for miR-mimics, miR-27a inhibitor and control for miR-27a inhibitor, respectively. The efficiency of trans-

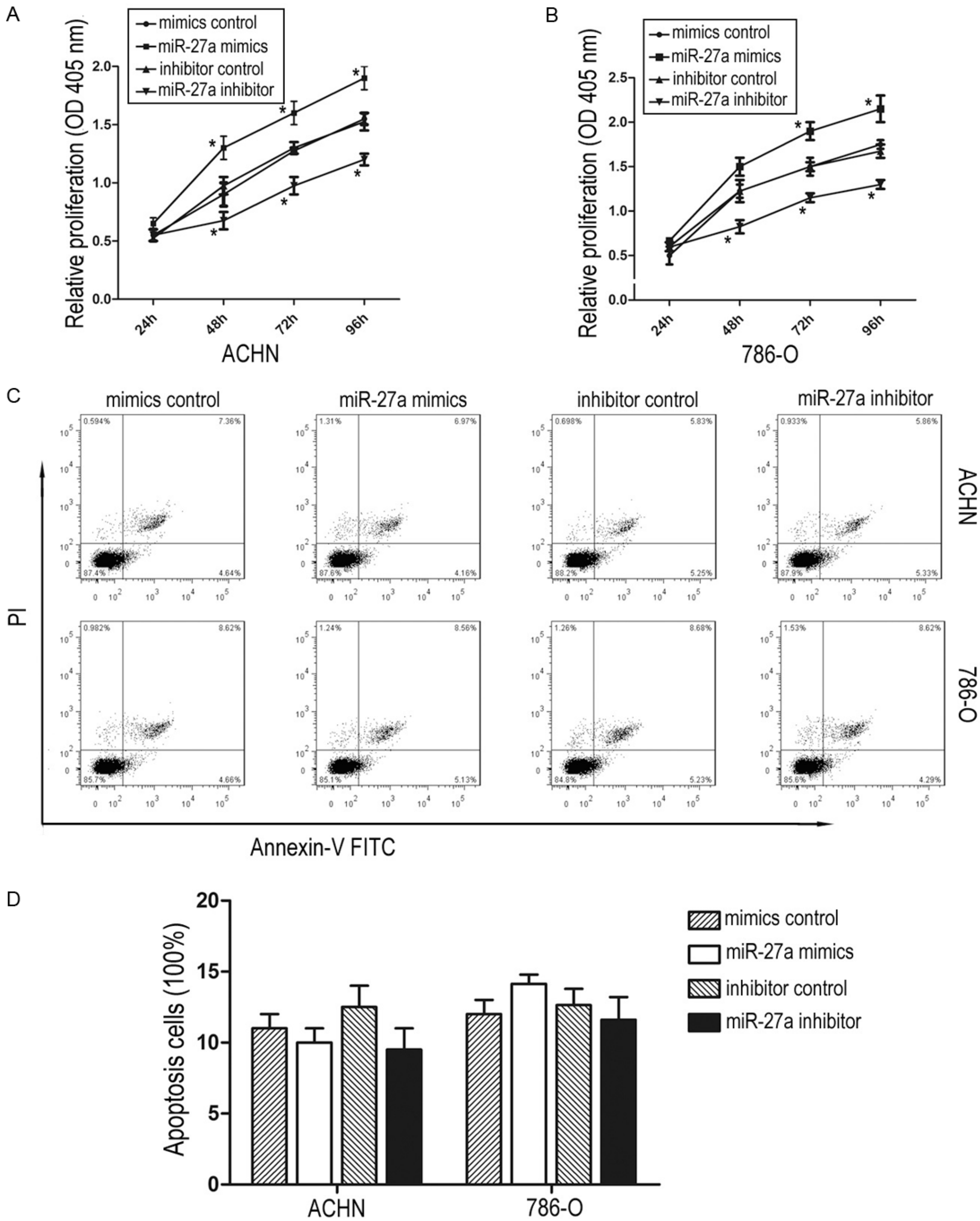

Figure 3. Effection of miR-27a on cell lines proliferation and apoptosis. A. CCK-8 cell proliferation assays show that miR-27a significantly enforced RCC cell proliferation in ACHN cells. B. MiR-27a significantly enforced RCC cell proliferation in 786-O cells, as demonstrated by CCK-8 assay. All experiments were performed in triplicate and presented as mean ± SEM. *Indicates significant difference compared with control group ($P < 0.05$). C. Representative flow cytometric plots of cell apoptosis. Cells were stained with both Annexin V and PI before analysis by flow cytometry. Numbers represent the frequency in each quadrant. D. Frequencies of apoptotic cells. Means ± SEMs of three independent experiments with statistical analysis.

fection was verified by real-time PCR (**Figure 1B**). Transwell chamber assay indicated that overexpression of miR-27a promoted ACHN and 786-O cell lines migration, whereas the sup-

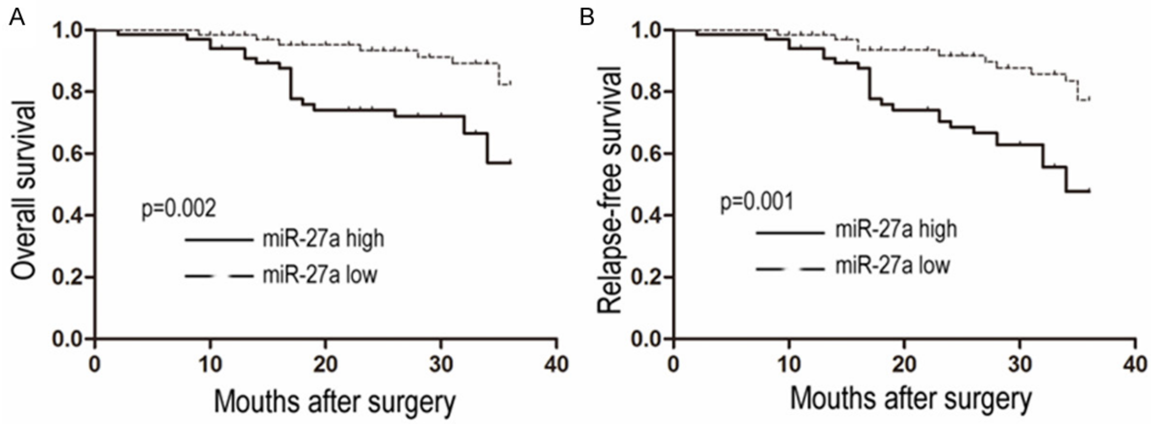

Figure 4. Association of miR-27a expression with Overall survival and Relapse-free survival of the patients with RCC. A and B Overall survival (OS) and Relapse-free survival (RFS) according to the microRNA signature in the training data set.

pressed miR-27a attenuated the migration of RCC cell lines (**Figure 2A** and **2B**). Additionally, Transwell chamber with Matrigel assay demonstrated that high level of miR-27a enhanced the ability of RCC cell invasion, while the low expression of miR-27a had an opposite effect (**Figure 2C** and **2D**). Taken together, the results showed that miR-27a may act a vital role in promoting metastasis of RCC *in vitro*.

miR-27a enforced RCC cell proliferation but had no effect on apoptosis

To identify whether miR-27a could regulate proliferation of RCC cell in vitro, CCK-8 assay was respectively performed in the ACHN and 786-O cell lines. The CCK-8 assay indicated that upregulation of miR-27a could promote the proliferation ability of the two RCC cell lines, whereas down-regulation of miR-27a exerted an opposite effect (**Figure 3A** and **3B**). To explore the role of miR-27a in cell apoptosis, we performed the cell apoptosis assay. However, the results of cell apoptosis assay indicated that miR-27a might have no effect on RCC cell apoptosis (**Figure 3C** and **3D**). In brief, these data showed that miR-27a significantly promoted proliferation ability of RCC cells but had no effect on cell apoptosis.

Expression of miR-27a was significantly associated with prognosis of RCC

We then performed Kaplan-Meier analyses to determine whether the expression of miR-27a was associated with overall survival and relapse-free survival of the RCC patients. 133 RCC samples were divided into miR-27a high expression group (n = 67) and low expression group (n = 66), median was used as cut off. Patients with low miR-27a expression (n = 66) had a mean 3-year overall survival of 34.3 months, whereas the high miR-27a expression group (n = 67) had a mean overall survival of 29.7 months. The difference was statistically significant (P = 0.002, **Figure 4A**). Likewise, a statistically significant association of miR-27a with relapse-free survival was also verified (P = 0.001, **Figure 4B**). Furthermore, Cox proportional regression analysis indicated that the expression levels of miR-27a were independent prognostic factor for the RCC patients (hazard ratio = 3.236; 95% CI: 1.478~7.084; P = 0.003).

Discussion

Despite the great advancement of cancer therapy, major limitations were still existed in the treatment of RCC. RCC is characterized by its resistance to current standard therapies [12-15]. Seeking alternative therapy strategies is still top priority.

It is well known that microRNAs (miRNA) have been shown to have critical regulatory role in malignancy biology [16-20]. Among them, miR-27a is one of the most frequently studied miRNAs in human malignancy, including pancreatic, gastric, breast cancer and acute leukemia [9-11, 21-23]. Besides acute leukemia, various studies of malignancies indicate that miR-27a may have an important vital role as tumor promotor in RCC [19]. Interestingly, there were few researches focusing on the correlation between expression of miR-27a and RCC. Therefore, we performed a Real-time PCR on 133 paired RCC samples and found that miR-27a expression was significantly up-regulated in the tumor tissues compared with corresponding adjacent tissues, which was consistent with most studies of miR-27a. Based on these data, we assumed that miR-27a may have a vital role as a tumor promotor in RCC. Therefore, we investigated the regulatory function of miR-27a in the progression of RCC and found that miR-27a significantly promoted RCC cell migration, invasion and proliferation. Collectively, our research clearly illustrates the promoting -cancer effect of miR-27a, providing sufficient evidence to support the tumor promotor role of miR-27a in RCC. Although the molecular mechanism under the promoting-cancer effect of miR-27a has been in part elucidated [9-11, 23-28], there are few studies concerning the correlation between miR-27a expression and prognosis of the RCC patients. Hence, we preformed the Kaplan-Meier analyses and Cox proportional regression analysis to verify the role of miR-27a in the RCC prognosis. Kaplan-Meier analyses suggested that miR-27a was distinctly associated with prognosis of RCC patients; meanwhile miR-27a was demonstrated as independent prognostic factor for RCC patients by Cox proportional regression analysis.

In conclusion, we firstly illustrate the promoting -cancer effect of miR-27a in RCC, suggesting that miR-27a can be a potential therapeutic target for RCC. Additionally, Kaplan-Meier analyses and Cox proportional regression analysis suggest that miR-27a should be further evaluated as a biomarker for predicting the survival of RCC patients.

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Disclosure of conflict of interest

None.

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