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ORIGINAL ARTICLE

MicroRNA-10a suppresses cell metastasis by targeting BDNF and predicted patients survival in renal cell carcinoma

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Summary

Purpose: *Renal cell carcinoma (RCC) is the most common renal neoplasm and accounts for 3% of all cancers. Increasing studies reported that miR-10a, acting as tumor suppressor, was downregulated in several cancers. It has been reported that the proteins encoded by Brain-Derived Neutrophic Factor (BDNF) were members of the nerve growth factor family, and could promote neuronal survival in the adult brain. The purpose of this study was to explore how miR-10a worked in RCC on the metastasis.*

Methods: *The expression level of miR-10a and BDNF were calculated using RT-PCR and western blot. Transwell assay was utilized to evaluate the invasive ability. Kaplan-Meier method along with log-rank test were applied to evaluate the 5-year overall survival of RCC patients.*

Results: *miR-10a was significantly downregulated and BDNF was upregulated in RCC tissues and cell lines A498 and 786-O. The expression of miR-10a had a reverse correlation with BDNF in RCC tissues. Overexpression of miR-10a or interference of BDNF inhibited the invasion and epithelial-mesenchymal transition (EMT) of A498 cells. What's more, BDNF was demonstrated to be a target gene of miR-10a and miR-10a could mediate the expression of BDNF in RCC. In addition, low expression of miR-10a or overexpression of BDNF predicted poor prognosis of RCC patients.*

Conclusion: *Our results indicated that miR-10a inhibited the invasion and EMT by regulating BDNF in RCC. The newly identified miR-10a/BDNF axis provides novel insight into the pathogenesis of RCC.*

Key words: *miR-10a, BDNF, EMT, survival, renal cell carcinoma*

Introduction

Renal cell carcinoma (RCC) is the most frequent malignancy of the kidney, accounting for 80-90% of renal neoplasms and also 3% of all cancers [1,2]. The incidence rate is higher in men than in women, with a 5-year overall survival rate of 45% [3,4]. Therefore, there is an urgent need to investigate new biomarkers for early diagnosis of RCC. MicroRNAs (miRs) are a type of evolutionarily conserved small single-stranded non-coding RNAs with sequence lengths of 19-25 nucleotides. MiRs inhibit gene expression by directly binding to the 3'-untranslated region (3'-UTR) of target genes at post-transcriptional level [5, 6]. Accumulating evidence illuminated that miRs could play significant roles in tumorigenesis or tumor progression [7-9]. Previous studies have reported that miR-10a was downregulated and acted as tumor suppressor in several cancers, including gastric cancer, colorectal cancer, glioblastoma, ovarian failure and even in RCC [10-14]. In breast cancer, Kongliang et al have discovered that miR-10a inhibited cell proliferation, migration and promoted cell apoptosis [15]. Fan et al also demonstrated that miR-10a suppressed the proliferation and promoted cell apoptosis in diffuse large B-cell lymphoma [16]. What's more, miR-10a suppressed cell metastasis

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by modulating the EMT in colorectal cancer [11]. However, in RCC, few studies reported the roles of miR-10a, therefore, we wonder whether miR-10a suppresses the EMT in RCC.

BDNF encodes a member of the nerve growth factor family of proteins, which could promote neuronal survival in the adult brain through binding to its cognate receptor. BDNF is synthesized in a precursor form (pro-BDNF), whose mature form is generated through proteolytic cleavage of metalloproteinases [17,18]. BDNF was upregulated in a variety of tumors, including neuroblastoma, gastric cancer, bladder cancer and glioma [19-22]. Liu et al demonstrated that BDNF was upregulated and associated with the pathological grade and the astrocytoma location [23]. What's more, Tsai et al illuminated that BDNF was a key factor that regulated a network of metalloproteases and calmodulin and could increase cell migration in triple negative breast cancer [24]. Similarly, upregulation of BDNF promoted cell migration, invasion and the EMT in cervical cancer [25]. Additionally, BDNF was associated with poor prognosis and functional regulation in non-small cell lung cancer [26]. Therefore, we strongly believe that BDNF could promote the EMT and was associated with the survival of RCC patients.

Methods

Patients and clinical samples

From January 2015 to December 2017, 51 patients with RCC were collected at Weifang People's Hospital, and 51 pairs of clinical samples were obtained, including RCC and corresponding paracancer tissues. Fresh tissue was frozen in liquid nitrogen immediately and stored at 80°C after surgery. No patients had any therapy before surgery. The research protocol was approved by the Ethics Committee of Weifang People's Hospital, and all patients provided written informed consent before specimen collection.

Cell lines and culture condition

Two human RCC cell lines, A498 (HTB-44) and 786-O (CRL-1932), and a normal renal tubular epithelial cell line HK-2 (CRL-2190) were purchased from American Type Culture Collection (ATCC, Rockville, USA). The cells were maintained in RPMI 1640 medium (Gibco, Carlsbad, USA), submixed with 10% fetal bovine serum (FBS; Life Sciences, Logan, USA), 100 U/ml penicillin and 100 μg/ml streptomycin and grown at 37°C in an incubator with 5% CO_2.

RNA isolation and qRT-PCR

The total RNAs were extracted and purified by using the TRIzol reagent (Invitrogen; 15596026, Carlsbad, USA). Following this, reverse transcrip¬tion was performed using the PrimeScript™ Reverse Transcription Reagent Kit (RR036A, TaKaRa Bio, Otsu, Japan) and synthesized the first cDNA chain. The expression of miR-10a and BDNF were evaluated by the SYBR PrimeScript miRNA RT-PCR Kit (Takara, Otsu, Japan) and the SYBR Premix Kit (Takara). The small nuclear RNA U6 (U6) and glyceraldehyde-phosphate dehydrogenase (GAPDH) were used as internal controls for miR-10a and BDNF, respectively. The cycling conditions were as follows: 95°C for 10 min; 40 cycles at 95°C for 15 sec; and 60°C for 1 min. The results were analyzed by $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: miR-10a forward 5'-GGATACCCTGTAGATCCGAA-3' and reverse 5'-CAGTGCGTGTCGTGGAGT-3'; U6 forward 5'-CTCGCTTCGGCAGCACATATACT-3' and reverse 5'-ACGCTTCACGAATTTGCGTGTC-3'; BDNF forward, 5'-CTACGAGACCAAGTGCAATCC-3' and reverse, 5'-AATCGCCAGCCAATTCTCTTT-3'; GAPDH forward 5'-CTTCACCACCATGGAGAAGGC-3' and reverse 5'-GGCATGGACTGTGGTCATGAG-3'.

Protein extraction and western blotting

The specific cells were lysed with the RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) and isolated through centrifugation at 4°C for 20 min. Equal quantities of total proteins were loaded onto each lane of sodium dodecyl sulfate polyacrylamide gel electrohoresis (SDS PAGE), and quantified by the bicinchoninic acid (BCA) assay kit (P0012, Beyotime Institute of Biotechnology, Haimen, China). After electrophoresis, the proteins were electro-transferred onto PVDF membranes (Millipore, Billerica, USA). The blots were blocked by skimmed milk at room temperature for 1 h, followed by incubation with primary antibodies. The membranes were exposed to 4°C overnight with GAPDH as internal reference. The primary antibodies and diluted concentrations were: BDNF (1:1000, ab108319, Abcam, Cambridge, USA), E-Cadherin (1:1000, ab194982, Abcam), N-Cadherin (1:1000, ab18203, Abcam), Vimentin (1:1000, ab92547, Abcam) and GAPDH (1:3,000, sc-47724, Santa Cruz, CA, USA). The anti rabbit (1:3,000, sc-516721, Santa Cruz Biotechnology) and anti-mouse (1:4,000, sc-516102, Santa Cruz) HRP-conju¬gated antibody were utilized as the secondary antibodies, and they were incubated at room temperature for 2h. The membranes were visualized using Enhanced Chemiluminescence Film System (GE Healthcare Life Sciences, Little Chalfont, UK).

Transwell assay

Transwell inserts (8 μm pore size, Corning Incorporated, Corning, NY, USA) covered with polymerized Matrigel (BD Biosciences, San Jose, CA, USA) were added in 24-well plates, and generated the upper and lower chambers. For the invasion assay, 200ul cells suspension at a density of 8×10^4 cells/well were added into the upper chamber, while 500ul of normal medium containing 20% FBS was added to the lower chamber as chemoattractant. After 48 h of incubation at 37°C, the non-invaded cells were wiped off by cotton swabs. The invaded cells were fixed with 4% paraformaldehyde and then stained sequentially using 10% crystal violet, and the cells were counted under a high-power field microscope (Olympus Corporation, Tokyo, Japan).

Vectors and transfection

The A498 cells were seeded in 24-well plates at 3×10^5 cells/well and incubated overnight. The miR-10a mimic and the miR-10a inhibitor associated with the negative control (NC) were utilized to up- or down-regulate miR-10a, which were purchased from Ribo-Bio (Guangzhou, China). BDNF small interfering RNAs (siRNAs-BDNF) and overexpression plasmid (pcDNA3.1-BDNF) were purchased from GenePharma Company (Shanghai, China), and were used to silence or overexpress BDNF. One mg/ml lipofectamine 2000 (11668019, Thermo Fisher Scientific, Shanghai, China) and 300 nmol

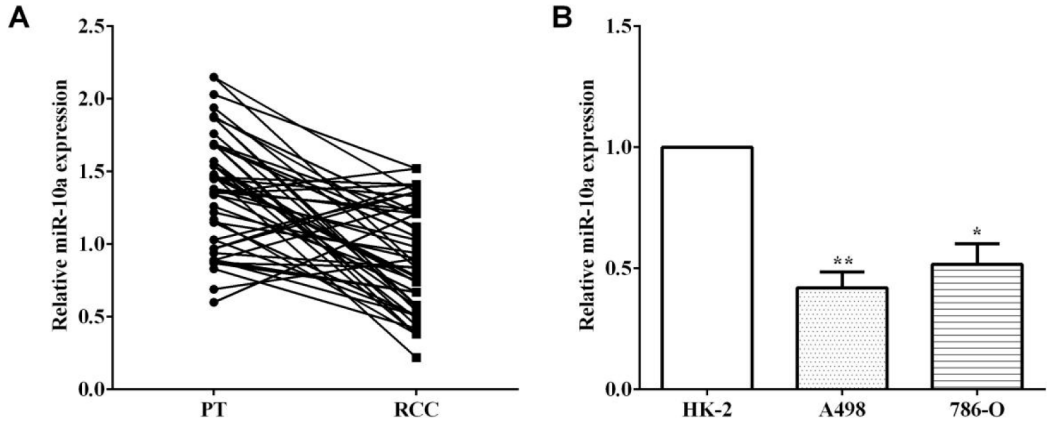

Figure 1. The expression of miR-10a in renal cell carcinoma (RCC) tissues and cells. **A:** Forty cases of RCC patients (78.4 %, 40/51) had lower levels of miR-10a compared with the corresponding paracancer samples. **B:** Low expression of miR-10a was calculated in RCC cell lines A498 and 786-O. *p< 0.05; **p<0.01.

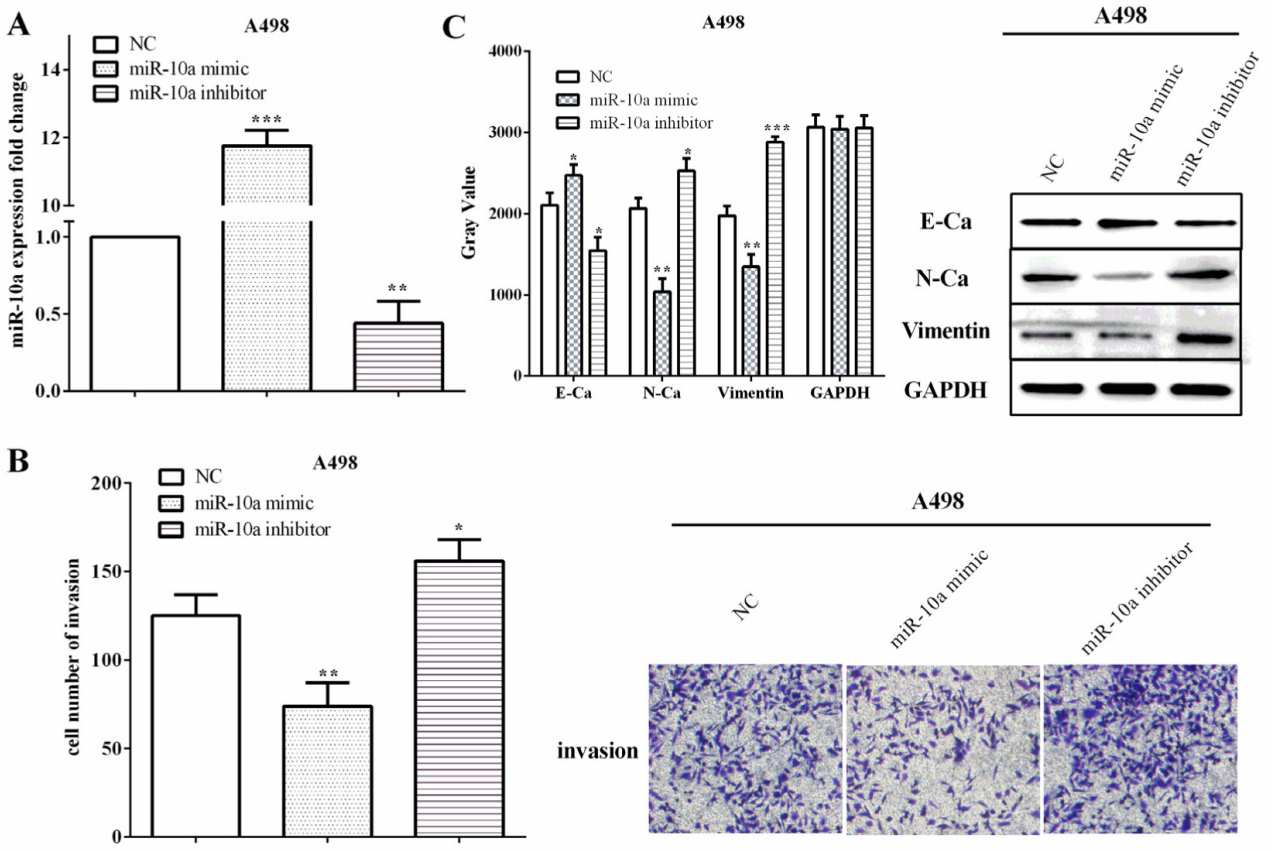

Figure 2. MiR-10a suppressed the invasion and EMT of renal cell carcinoma (RCC) cells. **A:** qRT-PCR demonstrated the transfection efficiency with the miR-10a mimic or the miR-10a inhibitor in A498 cells. **B:** After transfecting miR-10a mimic, the invasive ability of A498 cells was suppressed. On the contrary, Transfection of miR-10a inhibitor enhanced the invasive number of A498 cells. **C:** Transfection of miR-10a mimic promoted the expression of E-Ca, but inhibited the expression of N-Ca and Vimentin in A498 cells. However, transfection of miR-10a inhibitor inhibited the expression of E-Ca, while enhanced the expression of N-Ca and Vimentin. *p< 0.05; **p<0.01.

of vectors were both diluted by Opti-MEM/Reduced serum medium (31985070, Thermo Scientific, Shanghai, China) and then added into cells to carry out cells' transfection.

Plasmid construction and luciferase reporter assay

TargetScan was used to predict the potential target genes for miR-10a. The binding sequences were mutated from ACAGGGU to UGUCCCA, performed by the Quik-Change Multi Site-Directed Mutagenesis Kit (210513, Santa Clara, USA). The wild type (WT) BDNF 3'-UTR sequences and mutant sequences (MUT) were amplified by PCR and cloned into pmirGlo vector (Promega, Madison, WI).

A498 cells in 24-well plates at 3×10^5 cells/well were co-transfected with the miR-10a mimic and WT or MUT BDNF using Lipofectamine 2000 (Thermo Fisher Scientific, Shanghai, China). The luciferase activity was calculated using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) after 48-h transfection, which was normalized by renilla luciferase activity.

Statistics

Data are presented as the mean±standard deviation (SD). Statistical analyses were performed using SPSS 16.0 software (SPSS, Inc, Chicago, USA). The Student's *t*-test was used to perform comparisons between two groups. The comparisons between more or three groups were calculated using Tukey-Kramer *post hoc* test after ANOVA. The 5-year overall survival of patients with RCC was assessed by Kaplan-Meier method and the log-rank test. A $p<0.05$ was considered as statistically significant difference.

Results

The expression of miR-10a in renal cell carcinoma tissues and cell lines

The mRNA level of miR-10a was firstly calculated in RCC tissues and corresponding paracancer tissues by qRT-PCR. As a result, the level of BDNF of 40 cases (78.4%, 40/51) RCC tissue samples showed to be lower compared with the corresponding paracancer samples (Figure 1A). In addition, the expression of miR-10a was also evaluated in normal renal tubular epithelial cell line HK-2 and two RCC cell lines A498 and 786-O. Low expression of miR-10a was detected in RCC cell lines A498 and 786-O versus the normal renal tubular epithelial cell line HK-2 (Figure 1B).

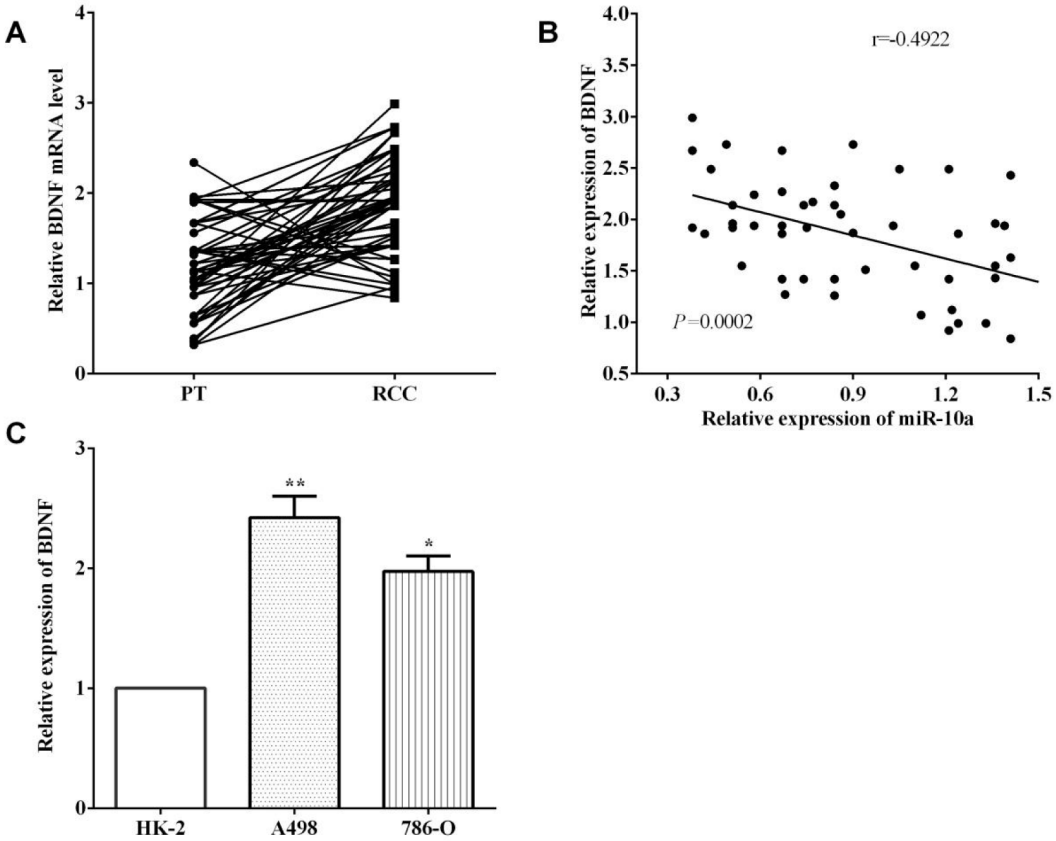

Figure 3. The expression of BDNF and the correlation with miR-10a in renal cell carcinoma (RCC) tissues. **A:** 39 cases of RCC patients (76.5%, 39/51) had higher level of BDNF compared with their corresponding paracancer samples. **B:** Spearman's correlation analysis demonstrated reverse correlation between the expression of miR-10a and BDNF. **C:** The expression of BDNF was higher in RCC cells A498 and 786-O than normal renal tubular epithelial cell HK-2. *p< 0.05; **p<0.01.

MiR-10a suppressed the invasion and EMT of renal cell carcinoma cells

To verify the roles of miR-10a in RCC on the invasion, A498 cells were transfected with miR-10a mimic or miR-10a inhibitor to overexpress ($p<0.0001$) or knockdown ($p=0.0023$) miR-10a, and the transfection efficiency was demonstrated by qRT-PCR (Figure 2A). Subsequently, transwell assay was conducted to evaluate the invasive ability. The number of invaded cells was reduced ($p=0.0075$) when transfected with miR-10a mimic, while it was increased ($p=0.0341$) when transfected with miR-10a inhibitor (Figure 2B), which indicated that miR-10a suppressed RCC cell invasion.

Subsequently, miR-10a inhibited the RCC EMT of A498 cells by increasing the expression of epithelial marker E-cadherin ($p=0.0346$) while suppressed the expression of mesenchymal markers N-cadherin ($p=0.0010$) and Vimentin ($p=0.0051$). On the other hand, transfection of miR-10a inhibitor reduced the expression of E-cadherin ($p=0.0123$) whereas promoted the expression of N-cadherin ($p=0.0164$) and Vimentin ($p=0.0003$) (Figure 2C).

The expression of BDNF in renal cell carcinoma tissues

Thirty-nine cases of RCC tissue samples (76.5%, 39/51) had higher level of BDNF than their corresponding paracancer samples (Figure 3A). Because of the low expression of miR-10a in RCC tissues, we wondered whether a correlation existed between the expression of miR-10a and BDNF in RCC tissues. Spearman's correlation analysis demonstrated that miR-10a and BDNF had a reverse correlation in RCC tissues ($p=0.0002$, $r=-0.4922$) (Figure 3B). Moreover, the expression of BDNF in RCC cell lines A498 and 786-O were higher than in normal renal tubular epithelial cell line HK-2 (Figure 3C).

Knockdown of BDNF inhibited invasion and EMT

Since BDNF was upregulated in RCC cells, an interference plasmid (siRNA-BDNF) was used to silence the expression of BDNF in A498 cells and the efficiency was evaluated by RT-PCR ($p=0.0023$) (Figure 4A) and western blot ($p=0.0152$) (Figure 4B).

Subsequently, the invasive capacity was evaluated after transfection of siRNA-BDNF to silence

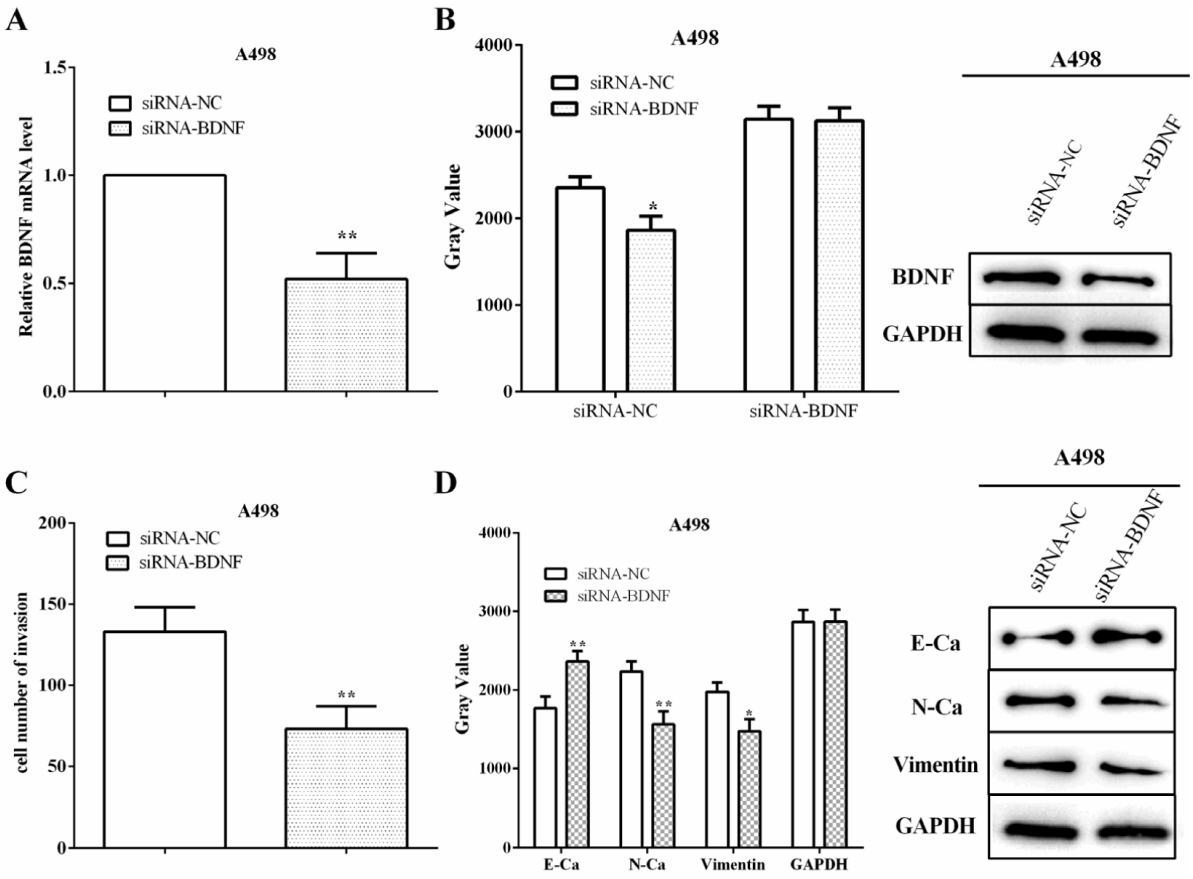

Figure 4. Knockdown of BDNF inhibited cell invasion and EMT(**A** and **B**) RT-PCR and western blot demonstrated the interference efficiency when transfected with siRNA-BDNF in A498 cells. **C:** Knockdown of BDNF inhibited the invasive capacity in A498 cells. **D:** Silencing of BDNF promoted the expression of E-Ca, but inhibited the expression of N-Ca and Vimentin in A498 cells. *p< 0.05; **p<0.01.

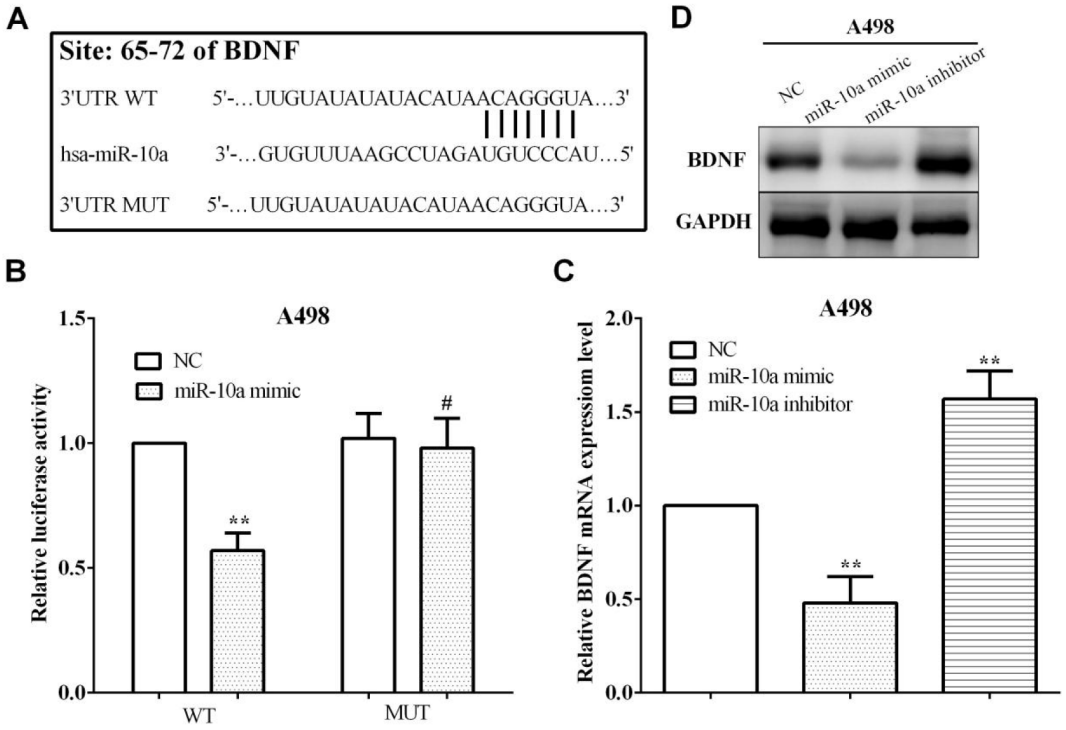

Figure 5. MiR-10a targeted to BDNF and regulated the expression of BDNF in renal cell carcinoma (RCC) cells. **A:** The miR-10a binding site on BDNF mRNA 3'-UTR. **B:** The luciferase activity was decreased in A498 cells co-transfected with the miR-10a mimic and wild type BDNF. **C:** Transfection of miR-10a mimic or miR-10a inhibitor could mediate the mRNA level of BDNF in A498 cells. **D:** The protein level of BDNF was reduced after transfecting miR-10a mimic, whereas it was enhanced by transfecting miR-10a inhibitor. *p< 0.05; **p<0.01; #p>0.05.

Table 1. miR-10a expression and clinicopathological features in 51 renal cell carcinoma patients

Clinicopathological features	*Cases (n=51)*	*miR-10a expression*		*p value (x^2 test)*
		24 High n (%)	*27 Low n (%)*	
Gender				
Male	25	13 (52.0)	12 (48.0)	0.488
Female	26	11 (42.3)	15 (57.7)	
Age (years)				
<50	23	14 (60.9)	9 (39.1)	0.073
≥50	28	10 (35.7)	18 (64.3)	
Tumor size (cm)				
≤5.0	22	14 (63.6)	8 (36.4)	0.039
>5.0	29	10 (34.5)	19 (65.5)	
TNM stage				
I-II	25	16 (64.0)	9 (36.0)	0.017
III-IV	26	8 (30.8)	18 (69.2)	
Lymph-node metastasis				
0-2	28	17 (60.7)	11 (39.3)	0.031
>2	23	7 (30.4)	16 (69.6)	
Metastasis				
Absent	38	20 (52.6)	18 (47.4)	0.084
Present	15	4 (26.7)	11 (73.3)	
BDNF				
Low expression	23	15 (65.2)	8 (34.8)	0.019
High expression	28	9 (32.1)	19 (67.9)	

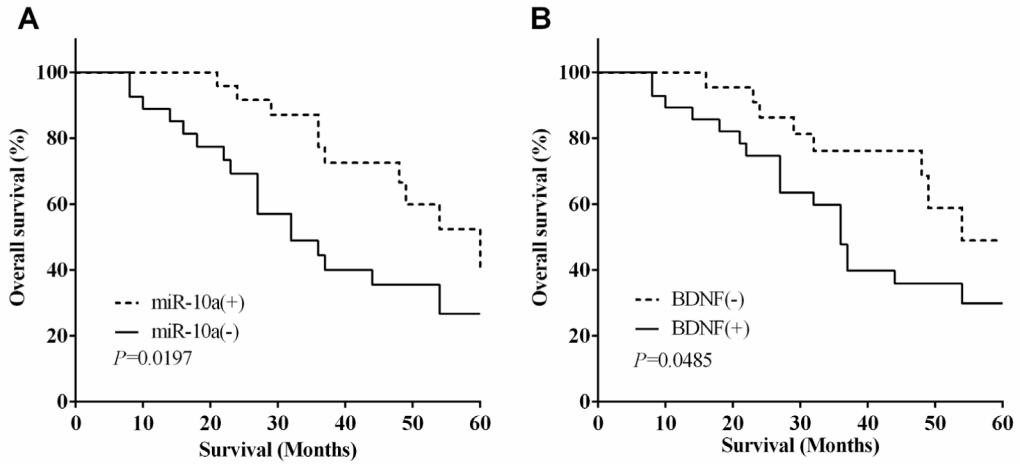

Figure 6. Identification of miR-10a and BDNF associated with poor survival in renal cell carcinoma (RCC). **A:** MiR-10a(-) group showed lower 5-year overall survival than miR-10a(+) group. **B:** Contrary to miR-10a, the 5-year overall in BDNF(-) group was higher than in BDNF(+) group.

BDNF expression, which showed that the number of invasive cells was reduced (p=0.0072) in A498 cells, which implied knockdown of BDNF inhibited the invasive ability of RCC cells (Figure 4C). What's more, it was also discovered that knocking down BDNF suppressed the EMT ability by increasing the expression of N-cadherin (p=0.0051) and Vimentin (p=0.0116) while reducing the expression of E-cadherin (p=0.0070) in A498 cells (Figure 4D).

MiR-10a directly targeted BDNF and regulated the expression of BDNF in renal cell carcinoma cells

TargetScan was performed to identify potential targets of miR-10a and BDNF was identified as a candidate target gene for miR-10a. To verify whether miR-10a directly binds to BDNF, the binding sequences on the 3'-UTR of BDNF mRNA were mutated from ACAGGGU to UGUCCCA (Figure 5A). Subsequently, the luciferase reporter gene assay was performed to calculate the luciferase activity after co-transfection with miR-10a mimic and the WT or the MUT 3'-UTR of BDNF mRNA. The luciferase activity was decreased (p=0.0046) in A498 cells co-transfected with the miR-10a mimic and the WT BDNF, while it had no obvious difference (p= 0.6803) when co-transfected with the MUT BDNF and miR-10a mimic (Figure 5B). In addition, the mRNA and protein levels of BDNF were reduced (p=0.0030) when transfected with the miR-10a mimic, whereas they were increased (p=0.0028) when transfected with the miR-10a inhibitor (Figures 5C,D).

Identification of miR-10a and BDNF associated with poor survival in renal cell carcinoma

In order to explore the correlation between the expression of miR-10a and the clinicopathological characteristics of RCC, we separated 51 patients into high and low groups, based on the expression of miR-10a, gender, age, tumor size, TNM stage, lymph node metastasis, metastasis and the expression of NOB1, respectively. Chi-square test was performed to calculate the p value and illuminated that miR-10a expression had negative association with tumor size (p=0.039), TNM stage (p=0.017), lymph node metastasis (p=0.031) and the expression of BDNF (p=0.019) (Table 1).

Moreover, Kaplan-Meier analysis and log-rank test were performed to evaluate the 5-year overall survival of RCC patients on the basis of the expression of miR-10a and BDNF. The results demonstrated that the 5-year overall survival of miR-10a(-) group was lower (log-rank p=0.0197) than that of miR-10a(+) group (Figure 6A). On the contrary, the 5-year overall survival (p=0.0485) was higher in the BDNF(-) group than in the BDNF(+) group (Figure 6B).

Discussion

Renal cell carcinoma is the most frequent malignancy in the kidney, accounting for 80-90% of renal neoplasms with a 5-year overall survival rate 45% [1,3,4]. Therefore, it is urgent to investigate new biomarkers for early diagnosis.

MiRs can inhibit gene expression post-transcriptionally by binding to the 3'-UTR of the target genes [5,6]. Accumulating evidence illuminated that miRs could play significant roles in tumorigenesis or tumor progression, and miR-10a played a tumor suppressive role in several cancers including RCC [7,8,14]. Mir-10a inhibited cell proliferation, migration and promoted cell apoptosis in breast cancer and diffuse large B-cell lymphoma

[15,16]. But so far, the specific functions and underlying biological mechanisms of miR-10a in RCC have remain unclear. In this study, we proved that miR-10a was downregulated in RCC tissues and cell lines. Low expression of miR-10a suppressed the invasion of RCC, while its overexpression promoted the RCC invasion, which were consistent with the above reports. What's more, our results were consistent with the findings of Liu et al in colorectal cancer [11], where miR-10a inhibited the EMT in RCC, which was the first time to propose the connection between miR-10a and the EMT in RCC. In addition, we have also demonstrated that miR-10a was inversely related to tumor size, TNM stage, nodal metastasis and the expression of NOB1. Low expression of miR-10a predicted a poor prognosis for RCC, which was contrary to the findings of low-grade gliomas [27]. In addition, miR-10a promoted the invasiveness and metastatic behavior of pancreatic cancer [28], thus, we proposed that the expression of miR-10a may be tissue-specific.

As miRs perform their functions through directly binding to the target genes, we explored the target genes of miR-10a using TargetScan. BDNF was selected as a candidate target gene of miR-10a. BDNF encodes a member of nerve growth factor family of proteins, which could promote the survival of neurons in the adult brain through binding to its cognate receptor [17,18]. BDNF was a key factor that regulated a network made up of metalloproteases and calmodulin and could increase cell migration [24]. Liu et al demonstrated that BDNF was upregulated and was associated with the pathologic grade and the astrocytoma location in astrocytomas [23]. Our results were consistent with the views of Tsai and Liu that BDNF was upregulated in RCC tissues and cells. What's more, we discovered that the expression of BDNF had a reverse connection with the expression of miR-10a in RCC tissues. Since the functions of BDNF in RCC are still unclear, we knocked down BDNF in RCC cell line A498 to explore the roles of BDNF in RCC. Our results were consistent with the results in cervical cancer [25], and we found that knockdown of BDNF inhibited RCC cell invasion and the EMT abilities. Moreover, through performing luciferase reporter gene assay, we first proposed that BDNF was a direct target gene of miR-10a and its expression was regulated by exogenous altering miR-10a. All of our results were consistent with Zhai et al study [29] that miR-10a suppressed cervical cancer proliferation and division by directly targeting BDNF. Additionally, we found that overexpression of BDNF predicted a poor prognosis for RCC, which was consistent with the results reported by Shen et al [26]. Since BDNF can affect the expression of extracellular proteases such as MMP, our next experiments will detect the expression of MMP-2 and MMP-9 and analyze the activity of MMP. It has not been explored how BDNF affects the process of RCC, which is also a deficiency of this article.

In conclusion, miR-10a was downregulated and BDNF was upregulated in RCC tissues and cell lines. Overexpression of miR-10a inhibited the invasion and the EMT via directly targeting BDNF in RCC cells A498. The miR-10a was negatively correlated with the expression of BDNF in RCC tissues. This miR played a tumor suppressive role in RCC by targeting BDNF, which indicated that miR-10a may provide a promising therapeutic target for the treatment of RCC. The current findings promote our understanding of the molecular pathogenesis of RCC and support the potential of miR-10a as an effective new therapeutic target for the treatment of this malignancy.

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Conflict of interests

The authors declare no conflict of interests.

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