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*Original Article*

# Low expression of miR-99b promotes progression of clear cell renal cell carcinoma by up-regulating IGF1R/Akt/mTOR signaling

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**Abstract:** Objective: Dysfunctions of microRNAs have been implicated in the progression of clear cell renal cell carcinoma (ccRCC). Here, we investigated the roles of miR-99b and miR-99b* in ccRCC development. Methods: The expression levels of miR-99b and miR-99b* in tumor and tumor-adjacent tissues from ccRCC patients were quantified by quantitative Real-Time PCR (qRT-PCR). MicroRNA mimics and inhibitors were employed to evaluate the functions of miR-99b and miR-99b*. The effects of miR-99b on the proliferation and migration of ccRCC cells were analyzed by MTT and wound-healing assays, respectively. The effect of miR-99b on the expression of its target gene IGF1R and mTOR was determined by western blotting and qRT-PCR. Results: The abundances of miR-99b and miR-99b* were lower in ccRCC tissues than in the tumor-adjacent tissues from patients. Similarly, the expression of these two microRNAs was higher in the normal kidney HK-2 cells than in the ccRCC cell lines. Moreover, miR-99b and miR-99b* inhibited the proliferation and migration of ccRCC cells. MiR-99b was found to down-regulate IGF1R and mTOR expression, likely through targeting their mRNAs to induce degradation. Consistently, the mRNA levels of IGF1R and mTOR were higher in ccRCC tissues than in the tumor-adjacent tissues, and Akt, a downstream factor of IGF1R, was highly activated correspondingly in ccRCC tissues. Conclusion: The low expression of miR-99b and miR-99b* contributes to ccRCC development and miR-99b acts as an onco-suppressor by suppressing IGF1R and mTOR expression to down-regulate IGF1R/AKT/mTOR signaling.

**Keywords:** miR-99b, IGF1R, mTOR, Akt, clear cell renal cell carcinoma

## Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common subtype of RCC [1]. Worldwide, RCC is the sixth common cancer occurring in men and the tenth in women [2], and it is one of the fastest growing tumor types [3]. There are no specific therapies for RCC and the main treatment is based on radical nephrectomy [4, 5]. Unfortunately, many patients have recurrence and metastasis after surgery and do not respond to chemotherapy or radiotherapy, resulting in the adverse outcomes and high mortality rate [6]. Therefore, it is necessary to better understand the mechanisms involved in the progressions of ccRCC, in order to explore more effective therapeutic strategies.

MicroRNAs (miRNA) are a single-stranded and noncoding RNAs that are 19-24 nt in length [7, 8]. MiRNAs exert functions through negative regulation of gene expression. They generally bind to the complementary sequences in the 3' untranslated region (3' UTR) of targeting mRNAs, leading to selective inhibition of translation [9, 10]. More than 30% of human mRNAs are regulated by miRNAs, making them playing important roles in many fundamental processes, such as development, differentiation, cell proliferation, apoptosis, and stress response [11]. Many miRNAs function in human cancers, of which both loss and gain of their functions contribute to cancer development through a variety of mechanisms [12, 13].

MicroRNA-99b (miR-99b) gene is located on chromosome 19 [14, 15], and encodes two mature miRNA forms, miR-99b and miR-99b* [16]. In the miR-99 family, miR-99a/b and miR-100 have the same seed sequence, while miR-99a* and miR-99b* have the same seed sequence [16]. Previous studies suggested an inhibitory role of the miR-99 family in many human cancers, including lung cancer [17], breast cancer [18], cervical cancer [19], pancreatic cancer [20] and head and neck squamous cell cancer [21]. HOXA1, TRIB2 and FGFR3 have been suggested as targets of the miR-99 family [18, 19, 22]. However, the role of miR-99b in ccRCC pathogenesis remains unknown.

In this study, we found that the expression of miR-99b and miR-99b* was decreased in human ccRCC tissues, and both exhibited an inhibitory effect on ccRCC cell proliferation and migration. Moreover, miR-99b targeted and down-regulated the mRNAs of mTOR and IGF-1R, thereby resulting in the down-regulation of IGF1R/Akt/mTOR signaling pathway. On the contrary, the decreased expression of miR-99b in ccRCC resulted in the upregulation of mTOR and IGF1R mRNAs as well as Akt activation.

## Materials and methods

### *Patients and ccRCC samples*

Tumor tissues and tumor-adjacent tissues of 20 patients with ccRCC were collected. The patients had not received any treatments before surgery. The tissues were stored in liquid nitrogen after surgical excision. All the specimens in this study were of the informed consent from patients and were approved by the Ethical Committee of Zhangzhou Affiliated Hospital of Fujian Medical University.

### *Cell lines and cell culture*

The human ccRCC cell line 786-O and human normal kidney cell line HK-2 were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). 786-O and HK-2 cells were respectively cultured in RPMI 1640 (Hyclone, UT, USA) and minimum Eagle's medium (Hyclone, UT, USA) supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% $CO_2$ at 37°C. The ccRCC cell line A498 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured in RPMI 1640.

### *RNA isolation and quantitative real-time PCR*

Total RNA of tissues and cells was extracted using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. For mRNAs, RNA was converted to cDNA by the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). The expression levels were then determined by real-time PCR using the Hieff qPCR SYBR Green Master Mix (Yeasen Biotech, Shanghai, China). Quantitation of mature miRNAs was performed by TaqMan MicroRNA Assays (Applied Biosystems). All real-time PCR reactions were performed on an AriaMx real-time PCR system (Agilent). Relative expression was calculated and quantified with the $2^{-\Delta\Delta Ct}$ method and normalized to U6 snRNA, 18S rRNA or GAPDH. The primer sequences in this study were as followed: GAPDH, F: 5'-CTCTGCTCCTCCTGTTCGAC-3', R: 5'-GCGCCCAATACGACCAAATC-3'; 18S rRNA, F: 5'-CGGCTTAATTTGACTCAACACG-3', R: 5'-TTAGCATGCCAGAGTCTCGTTC-3'; IGF-1R, F: 5'-GACAACCAGAACTTGCAGCA-3', R: 5'-GATTCTTCGACGTGGTGGTG-3'; mTOR, F: 5'-CTGAGCAGAACCAGGGTACA-3', R: 5'-GGACACAGCTGGGTAGAACT-3'.

### *Transient transfection of miRNA mimics and inhibitors*

The miRNA mimics/inhibitors and the negative control (NC) were purchased from RIBOBIO (Guangzhou, China). Cell transfection was performed using Liposomal Transfection Reagent (Yeasen Biotech, Shanghai, China) with a final concentration of 50 nM for mimics and 100 nM for inhibitors. Medium was changed 6 h after transfection, and cells were harvested 24 or 48 h later for further analysis.

### *Cell viability assays*

Cell viability was determined by MTT assay. Approximately $2\times10^3$ cells were plated into each well of a 96-well plate. Cells were cultured overnight and then transfected with the mimics/inhibitors for 24, 36 or 48 h. MTT was added to each well to the final concentration of 0.5 mg/mL and incubated for 4 h. The medium was removed and cells were lysed with DMSO. The absorbance was measured at 490 nm by a spectrophotometer.

*Wound-healing assay*

Cells were seeded onto 12-well plates. When the cells confluence reached about 80%~90% after transfection, scratch wounds at cell surface were made using the tip of a 10 μL pipette. After wounding, cells were washed by phosphate buffered saline (PBS) to romove the debris. Cell images were captured at 0 or 24 h after wound creation and the scratch acreage was calculated using Image J software to obtain cellular migration ability.

*Western blotting*

Total lysates were obtained by lysing cells or tissues using RIPA buffer (Tris 50 mM; NaCl, 150 mM; NP-40, 1%; sodium deoxycholate, 0.5%; pH 7.4) containing proteinase and phosphatase inhibitors. The protein concentration was determined by the BCA protein assay kit (Thermo Scientific). Equal-amount protein was loaded in gel lanes, electrophoresed by 8% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk in TBST (NaCl, 150 mM; Tris, 10 mM; Tween-20, 0.1%) for 1 h, and then incubated with primary antibodies and secondary antibodies followed by detection using ECL system (Thermo Scientific). The dilutions of the primary antibodies were as follows: anti-β-actin (A5441, Sigma) in 1:5000, anti-p-Akt (#4060, Cell Signaling Technology) in 1:1000, anti-Akt-1/2/3 (H-136, Santa Cruz) in 1:1000, anti-mTOR (H-266, Santa Cruz) in 1:1000 and anti-IGF1Rβ (#8521, Cell Signaling Technology) in 1:1000.

*Statistical analysis*

All statistical analysis was performed by GraphPad Prism 8 software (GraphPad Prism, Inc.), and all data were expressed as the mean ± SEM. The comparison between two groups was analyzed by unpaired *t*-test. The one-way ANOVA was used to compare the differences among more than two groups while the two-way ANOVA was used to analyze the effect of miR-99b and miR-99b* in time series. The association between miR-99b expression level and clinicopathologic characteristics of ccRCC was evaluated using Chi-square test. $P<0.05$ was considered significant.

## Results

*MiR-99b and miR-99b* were low expressed in ccRCC tissue samples and cell lines*

To determine the expression levels of miR-99b and miR-99b* in ccRCC, we collected 20 pairs of tumor tissues and the corresponding tumor-adjacent tissues from ccRCC patients. The results from our qRT-PCR experiments showed that compared with the adjacent tissues, the expression of both miR-99b and miR-99b* were very low in tumor tissues, and the difference was statistically significant (**Figure 1A**, **1B**). The correlation between clinicopathologic features of ccRCC and the expression level of miR-99b was evaluated (**Table 1**). The data showed that the expression level of miR-99b was not correlated to gender, age, tumor size or Fuhrman grade, most likely owing to the limited number of samples and, especially, the high-grade samples. In addition, the expression levels of miR-99b and miR-99b* were higher in the human normal kidney HK-2 cell line than in the ccRCC 786-0 and A498 cell lines (**Figure 1C**, **1D**). These results indicated that miR-99b and miR-99b* are lowly expressed in ccRCC.

*MiR-99b and miR-99b* inhibited the proliferation and migration of ccRCC cells*

To determine the role of miR-99b and miR-99b* in ccRCC, both gain-of-function and loss-of-function assays were conducted using the mimics and inhibitors of miR-99b and miR-99b*, respectively. MTT assay was performed to detect the effect of the transfected mimics on cell proliferation. As shown in **Figure 2A** and **2B**, both mimics of miR-99b and miR-99b* significantly suppressed the proliferation of A498 cells at 48 h after transfection. Moreover, both miR-99b and miR-99b* mimics inhibited the migration of 786-0 cells (**Figure 2C**), while their inhibitors promoted cell migration (**Figure 2D**). Together, our results revealed the inhibitory effect of miR-99b and miR-99b* on ccRCC cell proliferation and migration.

*IGF1R and mTOR are direct targets of miR-99b*

MicroRNAs exert their functions by binding to their targeting mRNAs to decrease translation. We found that both mTOR and IGF1R were potential targets of miR-99b based on the prediction from TargetScan (**Figure 3A**). Moreover,

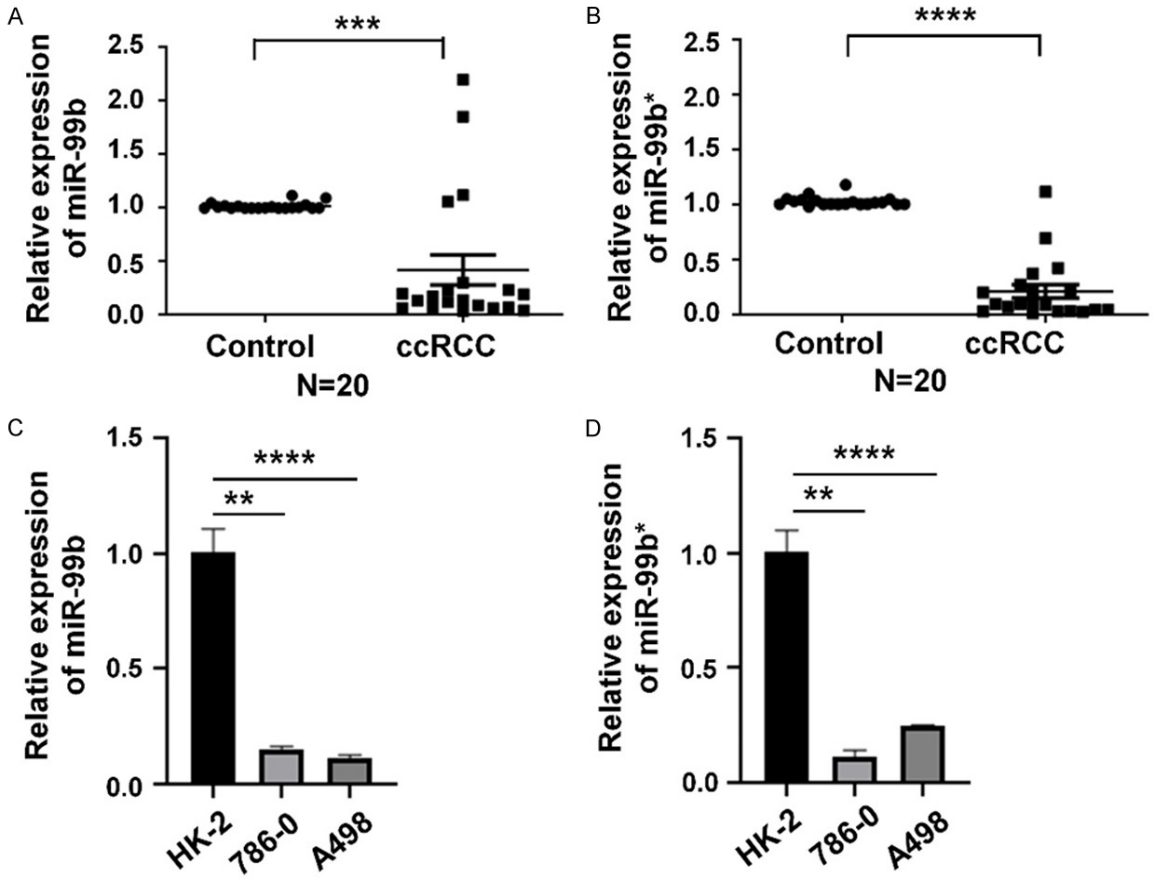


**Figure 1.** miR-99b and miR-99b* have low expression in ccRCC tissues and cell lines. A, B. The relative expression levels of miR-99b and miR-99b* were determined by qRT-PCR in paired adjacent and ccRCC tissues. Each dot represents one sample. C, D. The qRT-PCR analysis of miR-99b and miR-99b* expression in ccRCC cell lines (786-O and A498) and a renal normal cell line (HK-2). Data were normalized to U6 and presented as mean ± SEM. **P<0.01, ***P<0.001, ****P<0.0001.

**Table 1.** Association of miR-99b expression with clinical information in 20 ccRCC samples

| Finding | Cases | miR-99b levels | | *P*-value |
|---|---|---|---|---|
| | | Low | High | |
| Sex | | | | 0.178 |
| Male | 11 | 7 | 4 | |
| Female | 9 | 3 | 6 | |
| Age (years) | | | | 0.178 |
| <60 | 9 | 6 | 3 | |
| ≥60 | 11 | 4 | 7 | |
| Tumor size (cm) | | | | 0.3613 |
| <5 | 8 | 3 | 5 | |
| ≥5 | 12 | 7 | 5 | |
| Fuhrman grade | | | | 0.7625 |
| I | 12 | 6 | 6 | |
| II | 5 | 2 | 3 | |
| III | 3 | 2 | 1 | |

we found that the expression of mTOR and IGF1R was significantly decreased at both mRNA and protein levels by transfection of miR-99b mimics in the A498 cell (**Figure 3B** and **3C**). To further confirm this, we investigated the effect of the mimic and inhibitor of miR-99b on the mRNA expression of mTOR and IGF1R in HK-2 cells. We found that miR-99b mimic and inhibitor significantly and respectively decreased and increased the mRNA levels of mTOR and IGF1R (**Figure 3D**). Therefore, our results suggested that mTOR and IGF1R are the direct targets of miR-99b in both the normal kidney and ccRCC cells.

*MiR-99b inhibited the IGF1R/Akt signaling pathway*

IGF1R/Akt signaling exerts a potent effect on promoting ccRCC progression. We then investi-

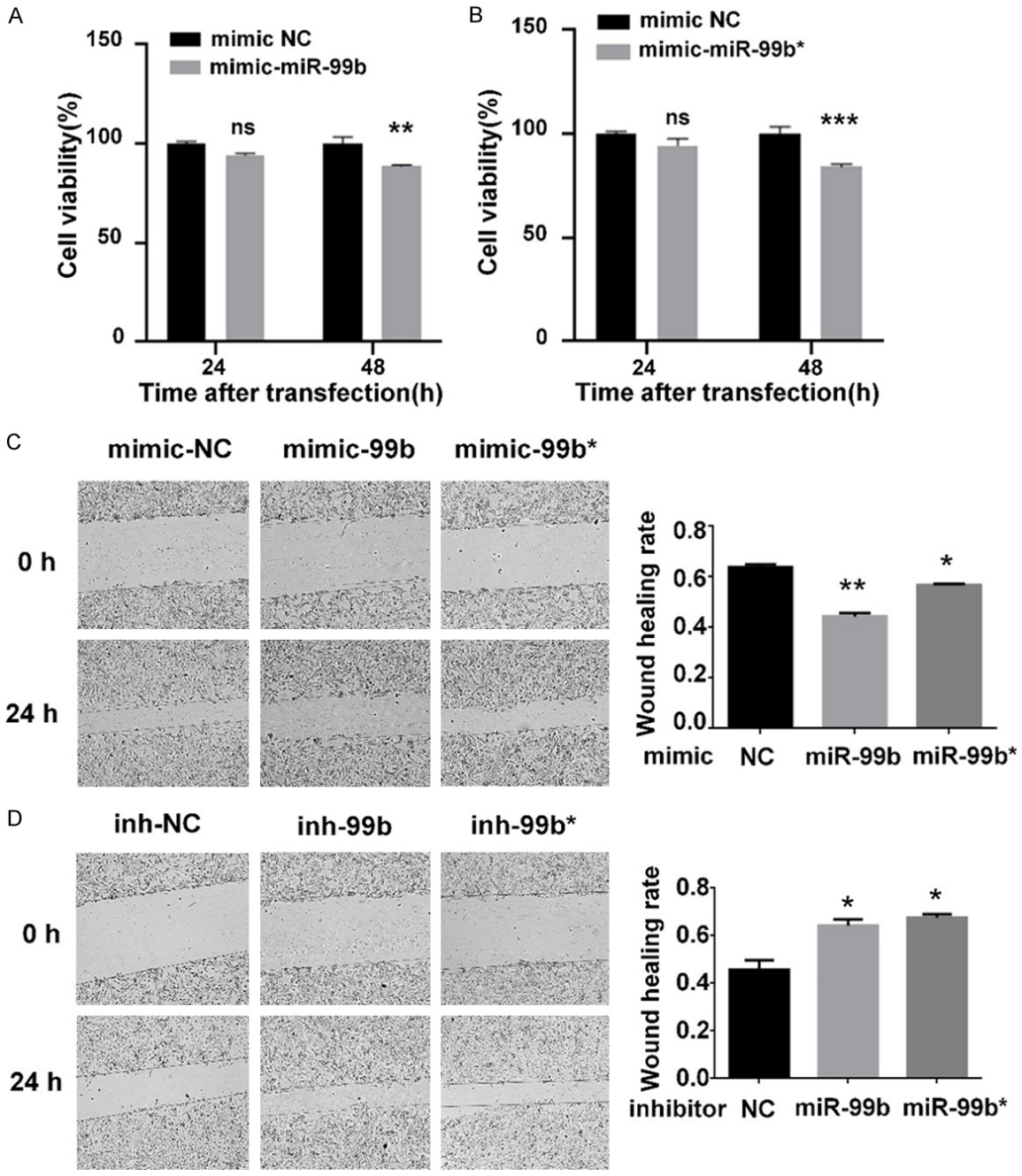


**Figure 2.** MiR-99b and miR-99b* inhibit the proliferation and migration of ccRCC cells. (A, B) The effects of mimics of miR-99b (A) and miR-99b* (B) on A498 cell proliferation were examined by MTT assay. Mimic NC was a negative control of mimic. (C, D) The effects of miR-99b and miR-99b* mimics (C) and inhibitors (D) on 786-0 cell migration. Left panel: representative micrographs of wound healing assay were recorded at 0 and 24 h. Right panel: the wound healing rate was calculated and presented. Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ns: non-significant.

gated the effect of miR-99b on this signaling pathway. As shown in **Figure 3E**, treatment with IGF1, an active ligand of IGF1R, strongly induced the phosphorylation of Akt, a potent tumor promotor and a downstream factor of IGF-1R, in A498 cells. MiR-99b mimic substantially inhibited IGF1-induced Akt phosphorylation. Moreover, the promoting effect of IGF1 on cell proliferation was also blocked by miR-99b mimic in A498 cells (**Figure 3F**). Therefore, miR-

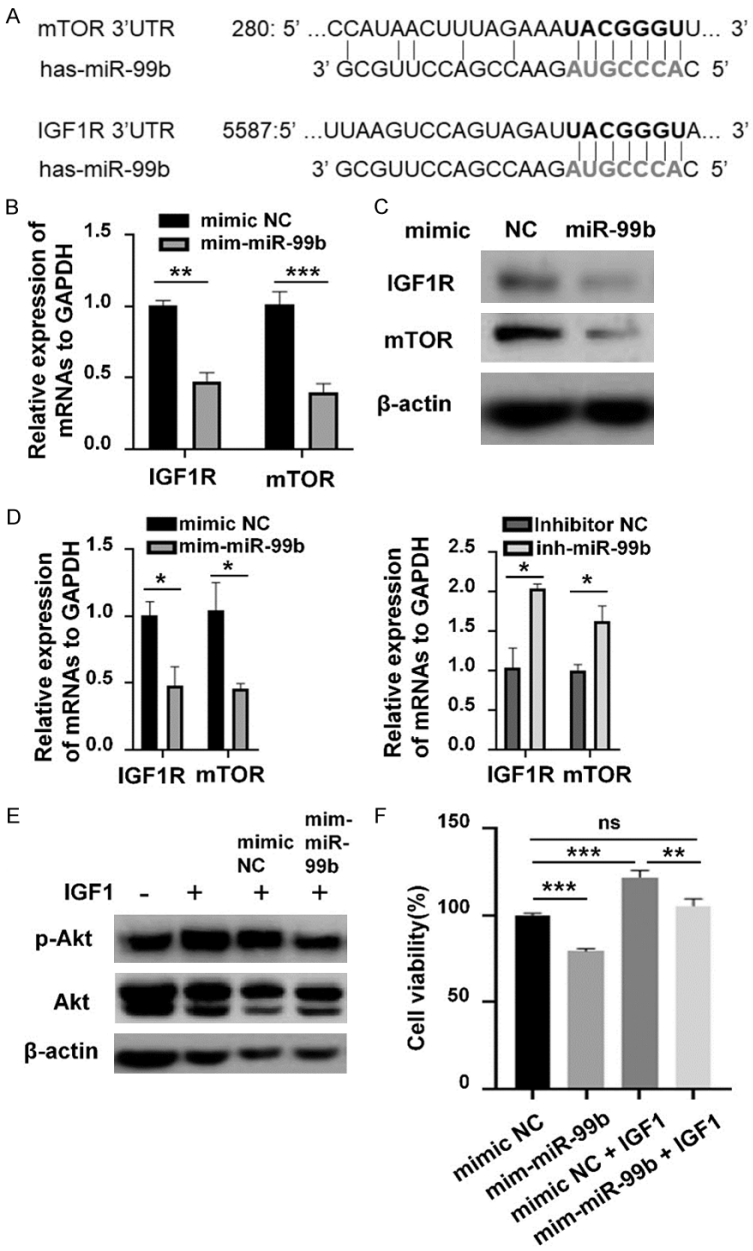


**Figure 3.** MiR-99b targets IGF1R and mTOR mRNAs for degradation. (A) The complementary sequences of miR-99b with 3'-UTR of mTOR and IGF1R mRNAs were obtained using TargetScan. (B, C) The effects of miR-99b mimic on the expression of mTOR and IGF1R in A498 cells were examined at mRNA level by qRT-PCR (B) and at protein level by western blotting (C). (D) The effects of miR-99b mimic and inhibitor on the expression of mTOR and IGF1R were examined at mRNA level by qRT-PCR in HK-2 cells. (E) A498 cells were transfected with control or miR-99b mimics for 24 h and then treated with IGF (10 μM) for 0.5 h. Protein expressions were examined by western blotting. (F) A498 cells were transfected with control or miR-99b mimics for 24 h and then treated with IGF (10 μM) for 48 h. Cell viability was examined by MTT assay. Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ns: non-significant.

99b inhibits IGF1R/Akt signaling pathway, likely through its down-regulation of IGF1R expression.

*The IGF1R/Akt/mTOR signaling pathway is activated in ccRCC*

To further analyze the effect of low miR-99b level on ccRCC, we detected the relative mRNA expression of mTOR and IGF1R in ccRCC tissues from patients. As expected, the mRNA expression of mTOR and IGF1R was increased in ccRCC tissues compared with the adjacent tissues (**Figure 4A**, **4B**). Consistently, a remarkable increase of Akt phosphorylation was observed in the ccRCC tissues. These results suggest that a low expression of miR-99b may result in the abnormally increased activation of the IGF1R/Akt/mTOR signaling pathway, contributing to ccRCC progression.

## Discussion

Evidence has shown that overexpression of tumor promoting miRNAs or loss of tumor suppressive miRNAs are tightly associated with the development of human cancers [13, 23]. Introduction of tumor suppressive miRNAs and the use of antagomirs of tumor promoting miRNAs may produce therapeutic effects [24, 25]. Previous studies have disclosed important roles of some miRNAs in RCC development. For example, miR-204 was found to act as a tumor suppressor and regulate the autophagic process in ccRCC by targeting LC3B [26]. MiR-182-5p suppresses the proliferation and tumorigenicity of RCC cells by targeting FLOT1 [27]. MiR-141 represses RCC proliferation and aggressive behavior by controlling EphA2 expression [28]. Contrarily, the upregulation of miR-452-5p is associated with poor prognosis in RCC [29]. In this study, we disclosed the tumor suppressive role of miR-99b and miR-99b* in ccRCC.

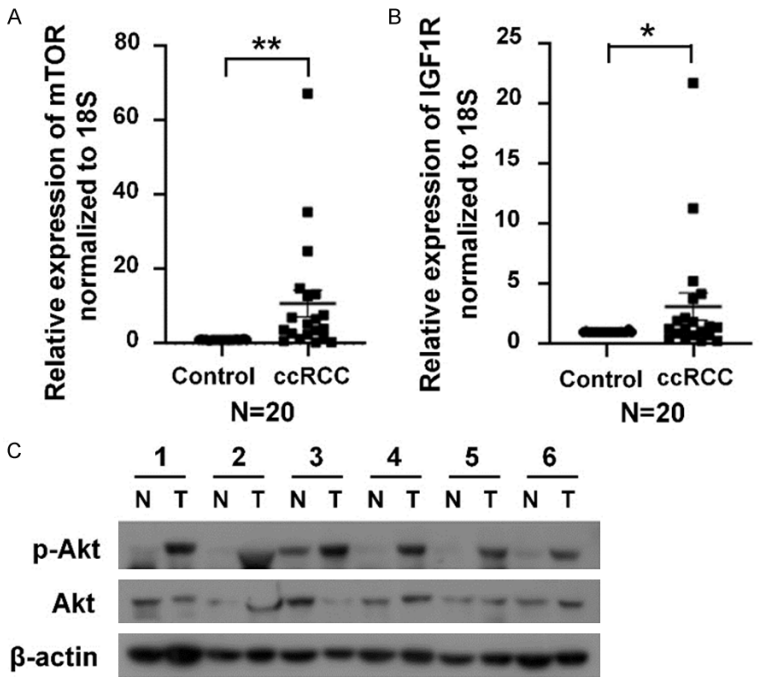


**Figure 4.** The expression of IGF1R and mTOR and the activation of Akt are high in ccRCC tissues. A, B. The relative expression of mTOR and IGF1R mRNAs in paired adjacent (control) and ccRCC tissues were determined by qRT-PCR. Each dot represents one sample. C. The protein expression and Akt phosphorylation in paired adjacent (N) and ccRCC (T) tissues were examined by western blotting. Data are presented as mean ± SEM. *P<0.05, **P<0.01.

We found a significantly low expression of miR-99b and miR-99b* in ccRCC tissues and cell lines (**Figure 1A**, **1B**), implying a loss-of-function of these two miRNAs in ccRCC. The tumor suppressive effects of miR-99b and miR-99b* were evidenced by their inhibitory effect on ccRCC cell proliferation and migration (**Figure 2**). Notably, both miR-99b and miR-99b*, originating from the same gene and transcript, exhibited a tumor suppressive effect. Thus, through inhibiting the miR-99b gene transcription or the maturation process, tumor cells can simultaneously inhibit these two tumor suppressive miRNAs. The dysregulation of miRNA expression in cancers results from several mechanisms such as genetic mutations, epigenetic dysregulation, tumor promoters and suppressors, and the alterations in core components of miRNA biogenesis [13, 30]. Further studies should be carried out to unravel the underlying mechanisms of miR-99b downregulation in ccRCC.

An individual miRNA can target different mRNAs, and an individual mRNA can be coordinately suppressed by multiple different miRNAs [31]. According to bioinformatic analyses and miR-99b mimic and inhibition assays, we determined that IGF1R and mTOR were targets of miR-99b in ccRCC (**Figure 3**). It is well-established that the IGF1R/Akt/mTOR signaling pathway plays a major role in cancer initiation and progression. Therefore, this pathway also represents one of the most attractive drug targets for cancer therapy. Interestingly, miR-99b targets both IGF1R and mTOR, the two essential components in this onco-promotive pathway. Thus, it is conceivable that miR-99b should be potent to suppress the IGF1R/Akt/mTOR signaling pathway. Consequently, this signaling pathway is strongly activated in ccRCC that had low miR-99b expression (**Figure 4**).

In a previous study, Li and colleagues drew a similar conclusion that miR-99a was markedly downregulated in RCC, and induced G1-phase cell cycle arrest [32]. Since miR-99a and miR-99b share the same seed sequence, miR-99a should also target the IGF1R/Akt/mTOR signaling pathway. It is amazing that ccRCC cells downregulate both miR-99a and miR-99b to efficiently upregulate the IGF1R/Akt/mTOR signaling pathway for ccRCC promotion. Intriguingly, how ccRCC achieves the low expressions of both miR-99a and miR-99b remains to be defined.

Taken together, our findings showed that the low expression of miR-99b and miR-99b* is closely associated with ccRCC development, and IGF1R and mTOR might be targets of miR-99b in ccRCC.

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## Disclosure of conflict of interest

None.

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