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MicroRNA-187, down-regulated in clear cell renal cell carcinoma and associated with lower survival, inhibits cell growth and migration though targeting B7-H3

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ABSTRACT

Aberrantly expressed microRNAs (miRNAs) are frequently associated with the aggressive malignant behavior of human cancers, including clear cell renal cell carcinoma (ccRCC). Based on the preliminary deep sequencing data, we hypothesized that miR-187 may play an important role in ccRCC development. In this study, we found that miR-187 was down-regulated in both tumor tissue and plasma of ccRCC patients. Lower miR-187 expression levels were associated with higher tumor grade and stage. All patients with high miR-187 expression survived 5 years, while with low miR-187 expression, only 42% survived. Suppressed in vitro proliferation, inhibited in vivo tumor growth, and decreased motility were observed in cells treated with the miR-187 expression vector. Further studies showed that B7 homolog 3 (B7-H3) is a direct target of miR-187. Over-expression of miR-187 decreased B7-H3 mRNA level and repressed B7-H3-3′-UTR reporter activity. Knockdown of B7-H3 using siRNA resulted in similar phenotype changes as that observed for overexpression of miR-187. Our data suggest that miR-187 is emerging as a novel player in the disease state of ccRCC. miR-187 plays a tumor suppressor role in ccRCC.

1. Introduction

Renal cell carcinoma (RCC) is the most lethal genitourinary tumors with an incidence of approximately 5–10 per 100,000 and accounts for 2–3% of all adult malignancies [1]. Clear cell RCC (ccRCC) is the most common subtype (70–80%) of renal cell carcinoma [2]. Although many genetic and epigenetic changes are found to be correlated with ccRCC, the carcinogenesis remains poorly understood.

microRNAs (miRNAs) are a family of single-stranded non-coding RNAs (ncRNAs) that play critical roles in carcinogenesis [3]. Many miRNAs are abnormally expressed in both solid and haematopoietic tumors [4]. Deregulated miRNAs have generated considerable interest in using them as molecular targets for cancer diagnosis and therapy [5–6]. miRNAs as predictive biomarkers or therapeutic targets in ccRCC are still needed to be explored.

Based on the deep sequencing data [7], we selected miR-187 as the possible tumor marker for ccRCC. We hypothesized that miR-187 may play an important role in malignant behaviors of ccRCC.

As a potential prognostic factor, miR-187 has been shown to be aberrantly expressed in several types of tumors, such as breast cancer [8] and ovarian cancer [9]. Previous work has also demonstrated that miR-187 decreased cancer cell migration through down-regulation of disabled homolog-2 (Dab2), which caused an inhibition of epithelial–mesenchymal transition [10]. However, no studies apparently have been conducted to investigate the role of miR-187 in ccRCC.

In the present study, the expression patterns of miR-187 in ccRCC were investigated by using Real-Time qPCR. Correlation between the expression of miR-187 and survival in patients with ccRCC was also determined by Kaplan–Meier method. In vitro cell proliferation, migration and in vivo tumor growth were measured after overexpression of miR-187 in 786-O and ACHN cells. The direct target of miR-187 was also identified using both Real-Time qPCR and luciferase reporter assay. To the best of our knowledge, this is the first report showing the correlation of miR-187 expression with poor prognosis and the background of its functional role in ccRCC.

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2. Materials and methods

2.1. Clinical sample collection

One hundred and eight patients with ccRCC and 50 age-matched healthy people participated in the study. Blood samples were got before any treatment and immediately stored at −80 °C. These patients received radical nephrectomy. Clear cell renal cell carcinoma was diagnosed histopathologically. Paired samples of ccRCCs and adjacent normal tissues were snap-frozen in liquid nitrogen immediately after resection. Written informed consent was obtained from each patient and this study was approved by Institutional Review Board of Foshan Maternal and Child Health Care Hospital (Foshan, China). The clinical data of the patients with ccRCC were shown in Table 1.

2.2. Cell lines and cell culture

The human clear cell renal cell carcinoma derived cell lines 786-O and ACHN were purchased from the Institute of Cell Research, Chinese Academy of Sciences, Shanghai, China. Cells were cultured in RPMI 1640 medium (GIBCO Invitrogen, USA) supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C in a 5% CO_2 atmosphere.

2.3. Cell transfection

miR-187 expression vector (Cell Biolabs, Inc.) and empty vector were transfected into cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Short interfering RNA (siRNA) targeting human B7-H3 and the negative control siRNA were purchased from Sigma–Aldrich and transfected into cells according to the supplier's protocols.

2.4. RNA extraction and real-time quantitative PCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacture's protocol. The cDNA strand was synthesized from total RNA with with M-MLV Reverse Transcriptase (Promega, USA) in a 25 μl volume. Real time PCR was carried out with the reagents of a Sybr green I mix (Takara, China) in a 20 μl reaction volume on an ABI PRISM 7000 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). The PCR cycling parameters were: 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Relative expression fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 1
Clinical and pathologic characteristics of 54 patients.

Clinical-pathologic variables	*N* (%)
All cases	54
Male	40(74%)
Female	14(26%)
Age	
>50	50(93%)
⩽50	4(7%)
Histological type	
Clear cell renal cell carcinoma	54(100%)
Others	0
Pathological grade (WHO)	
G1	25(46%)
G2	21(39%)
G3	8(15%)
AJCC clinical stage	
T_1	26(48%)
T_2	21(39%)
T_3, T_4	7(13%)

2.5. Luciferase reporter assay

The 3′-UTR of B7 homolog 3 (B7-H3) was PCR amplified and inserted into the digested psicheck-2 plasmid (Promega, USA) between the *XhoI* and *NotI* sites. B7-H3-3′-UTR target site mutations were generated using the KOD-plus mutagenesis kit (Toyobo, Japan) according to the manufacturer's introductions. Forty-eight hours after transfection, luciferase activity was detected using the Dual-Glo luciferase assay kit (Promega, USA) according to the manufacturer's introductions. Assays were performed in duplicate and repeated three times.

2.6. MTT assay

In vitro cell proliferation was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were grown in a 96-well plate for 24 h and cultured in normal medium. Cells were then incubated in 0.1 mg/ml MTT at 37 °C for 5 h and lysed in dimethyl sulfoxide (DMSO) at room temperature for 10 min at 0, 24, 48, and 72 h after transfection. The absorbance in each well was then measured at 490 nm, and at 630 nm as a reference, with a microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was performed at least three times.

2.7. Tumourigenicity assay

All experiments involving animals were approved by Institutional Review Board of Foshan Maternal and Child Health Care Hospital (Foshan, China). Stable transfection cell lines were constructed in our lab by transfecting the miR-187 expression vector or empty vector into 786-O and ACHN cells and selecting with G-418. In detail, 10^7 ccRCC cells were suspended in 100 μl PBS and injected subcutaneously into either side of the posterior flank of the same male BALB/c athymic nude mice at 4 weeks of age. Tumor growth was monitored weekly, and tumor volume was determined according to the formula: volume = length × width^2 × 0.5.

2.8. Scratch assay

Cells were cultured and transfected as described above. Cells were scratched in the monolayer and cultured in normal medium. Photographic images were taken from each well immediately and again after 24 h by a digital camera system. Cell migration distance (μm) was calculated by the software program HMIAS-2000. Each experiment was done at least three times.

2.9. Statistical analyses

Data analyses were performed by ANOVA test or Student's *t*-test, respectively, using the SPSS (Version 17.0 SPSS Inc.). Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. miR-187 was down-regulated in ccRCC

The relative expression levels of miR-187 were determined using Real-Time qPCR in a total of 108 patients with ccRCC. miR-187 was down-regulated in ccRCC compared to matched histologically normal tissue. The miR-187 expression fold change (ccRCC/

matched histologically normal tissue) was 0.42 (Fig. 1A). Compared with the control, expression of microRNA-187 was also decreased in plasma of 30 ccRCC patients (Fig. 1B). We further analyzed expression differences between tissue samples according to grading and staging. We found significant stepwise decreases in miR-187 expression with advancing tumor grade (Fig. 1C) and stage (Fig. 1D).

3.2. Correlation of miR-187 expression with survival in ccRCC

Low expression level of miR-187 in the tissue sample appears to be associated with low survival in patients with ccRCC. For patients with high miR-187 expression (T/N > 1), all survived 5 years after surgery, while for those with low miR-187 expression (T/N < 0.42), only 42% survived (Fig. 1E). The survival curve was based on the number of patients (86) for whom we had the survival information.

3.3. Overexpression of miR-187 inhibited in vitro cell proliferation and in vivo tumor growth

To investigate the possible impacts of miR-187 on the in vitro proliferation and in vivo growth of ccRCC cells, the cell growth changes of 786-O and ACHN cells were determined by MTT assay (Fig. 2A) and tumourigenicity assay (Fig. 2B). Cell growth inhibitions were obtained in both cell lines. Significant differences were demonstrated between the miR-187 expression vector and empty vector-transfected cells.

3.4. Overexpression of miR-187 inhibited cell migration

To investigate the possible impact of miR-187 on the migration of ccRCC cells, the cell motility changes of 786-O and ACHN cells were determined by in vitro scratch assay. Decreased cell motility was observed in 786-O and ACHN cells (Fig. 2C).

3.5. B7-H3 is a direct target of miR-187

Real time PCR analysis revealed that the expression of B7-H3 mRNA was decreased by the treatment with miR-187 expression vector in 786-O and ACHN cells (Fig. 3A). The luciferase reporter assay indicated that the activity of the reporter containing the 3′-UTR of the B7-H3 gene was decreased following treatment with miR-187 expression vector, whereas the reporter containing the mutated sequences was not obviously altered (Fig. 3B and C).

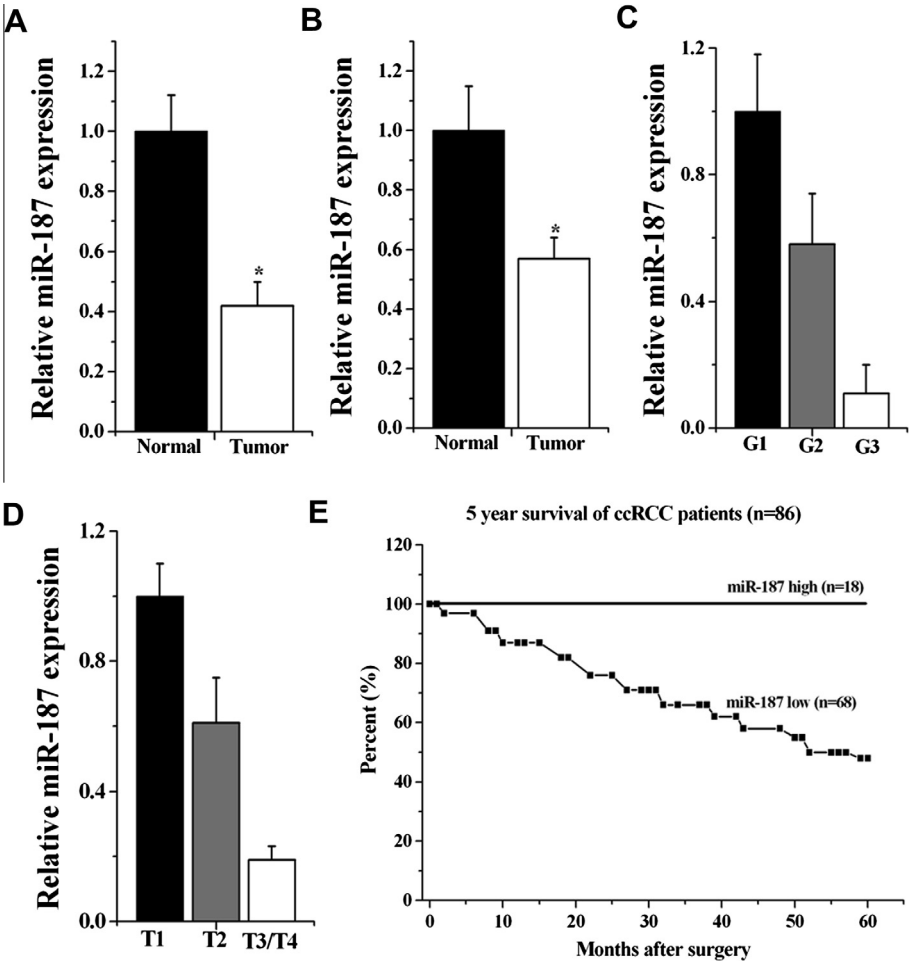

Fig. 1. The expression of miR-187 in ccRCC. The relative miR-187 expression levels were determined using Real-Time qPCR. Data are shown as mean ± SEM. (A) miR-187 was down-regulated in ccRCC compared to paired histologically normal tissue in 108 patients ($p < 0.01$). (B) The expression level of miRNA-187 in the plasma of ccRCC patients was lower than that in normal control ($p < 0.01$). (C) Lower miR-187 expression level was associated with higher tumor grade ($p < 0.01$). (D) Lower miR-187 expression level was associated with higher tumor stage ($p < 0.01$). (E) Correlation of miR-187 expression with 5 year survival of ccRCC patients. The survival rate of the group with low expression of miR-187 was lower than that of the group with high expression of miR-187 (log-rank test, $p < 0.01$).

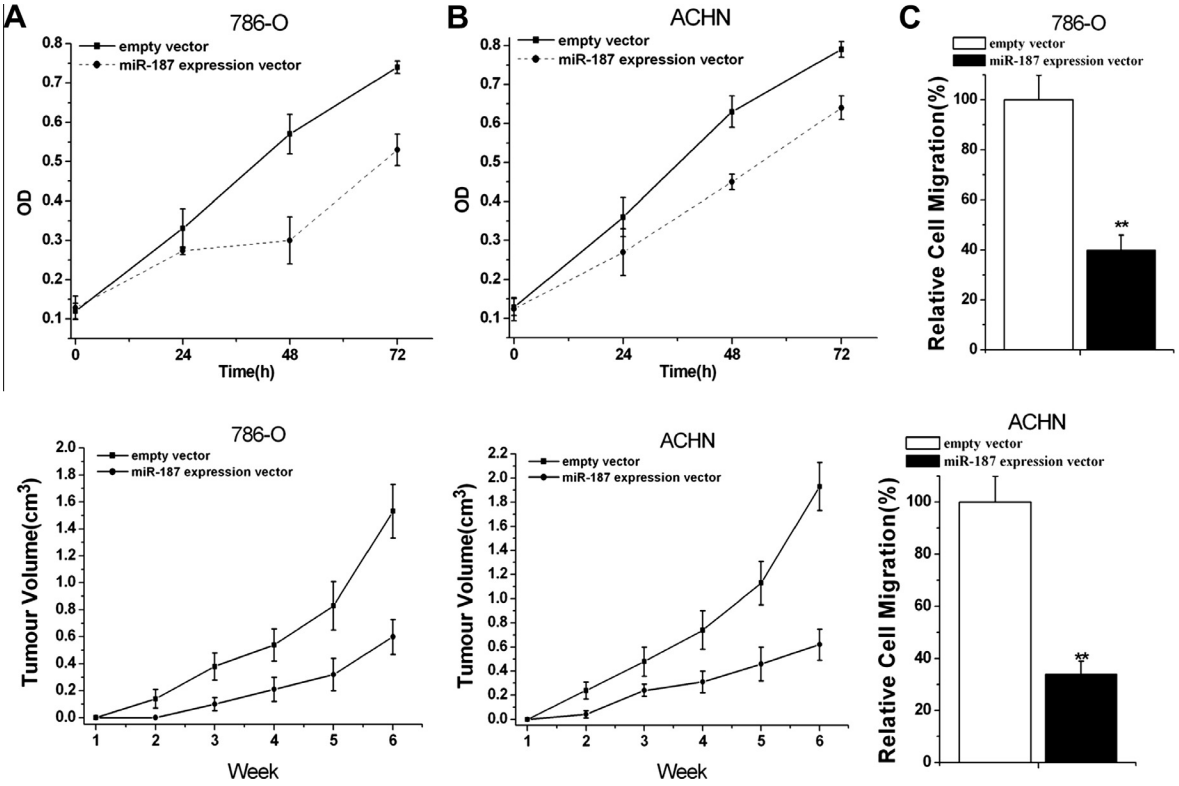

Fig. 2. Involvement of miR-187 in cell growth and migration. (A) Overexpression of miR-187 inhibited cell proliferation in vitro ($p < 0.01$, and $p < 0.01$ respectively). (B) Overexpression of miR-187 inhibited tumor growth in vivo ($p < 0.01$, and $p < 0.01$ respectively). (C) Overexpression of miR-187 inhibited cell migration ($p < 0.01$ and $p < 0.01$, respectively). Data are shown as mean ± SEM.

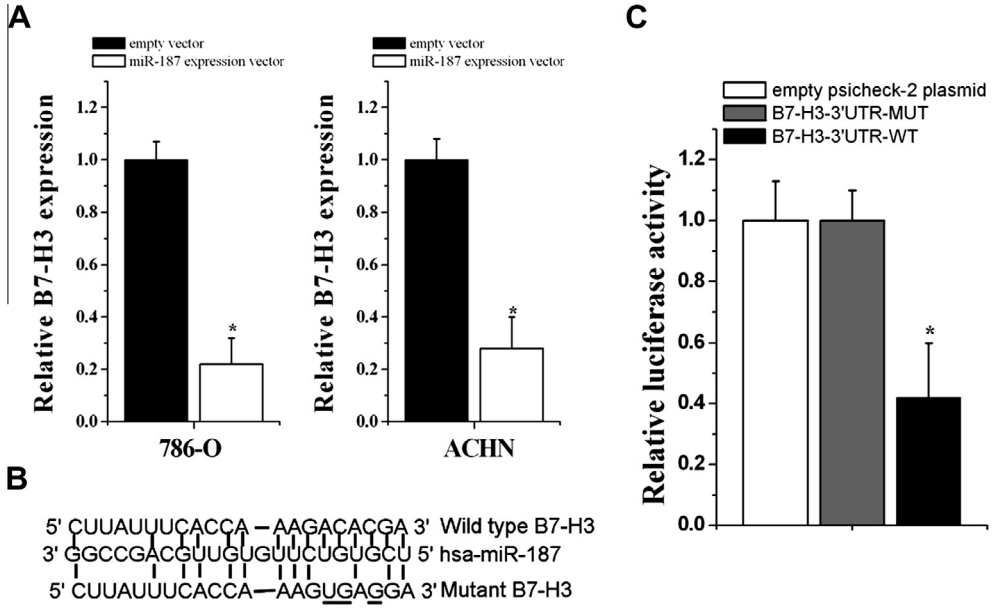

Fig. 3. B7-H3 is a target of miR-187. (A) Total RNA was extracted from ccRCC cells and real time PCR was performed 48 h after transfection ($p < 0.05$, and $p < 0.05$ respectively). (B) One miR-187 binding site in the 3′-UTR of the B7-H3 mRNA. A mutation was generated in the complementary site for the seed region of miR-187 as indicated. (C) HEK 293T cells were co-transfected with miR-187 expression vector or the empty vector and B7-H3 3′-UTR reporter plasmid or its mutant form. Luciferase activity was detected 48 h after transfection ($p < 0.05$). Data are shown as mean ± SEM.

3.6. RNAi knockdown of B7-H3 inhibited cell proliferation and migration

The results shown in Fig. 4A demonstrated that treatment with B7-H3 siRNA resulted in a statistically significant inhibition of viability of 786-O and ACHN cells. In vitro scratch assay in 786-O and ACHN cells showed that the cell migration distance is significantly reduced in B7-H3 siRNA-treated cells compared with non-targeting control (Fig. 4B). These results are similar to those observed in cells transfected with miR-187 expression vector.

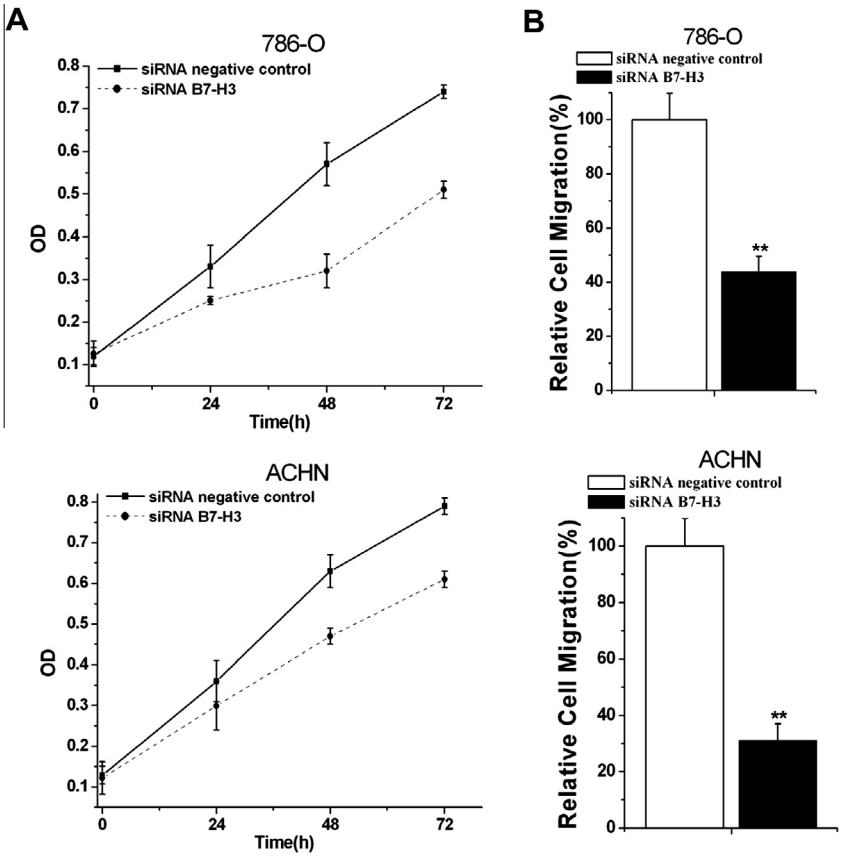

Fig. 4. Involvement of B7-H3 in cell growth and migration. (A) Knockdown of B7-H3 inhibited cell proliferation in vitro ($p < 0.01$, and $p < 0.01$ respectively); (B) Knockdown of B7-H3 inhibited cell migration ($p < 0.01$ and $p < 0.01$, respectively). Data are shown as mean ± SEM.

4. Discussion

miRNAs are emerging as endogenous triggers of the RNA interference pathway and play a key role in crucial biological processes, including development, differentiation, proliferation and apoptosis. Recent studies have identified a number of miRNAs as potential tumor targets for human cancers [11]. Some of them function either as tumor suppressors or as tumor promoters. On the basis of the miRNA expression profile, several miRNAs have been found to be up-regulated in ccRCC, such as miR-122-5p, miR-149, miR-210-5p, miR-224-5p, miR-638, and miR-1915. In contrast, miR-10b, miR-27b, miR-196a, and miR409-3p have been shown to be down-regulated in ccRCC [12–13]. Although these miRNAs have already been reported to be involved in the development of ccRCC, the results of the genome-wide miRNA profiling studies are usually inconsistent. So there is still a growing need for novel predictive and prognostic markers to improve the outcomes of ccRCC patients [14].

It appears that miR-187 may act in a cell or organ-specific manner, because the published data in this regard are conflicting. Higher levels of miR-187 are associated with better OS and recurrence-free survival in patients with ovarian cancer. In the later stages of tumorigenesis, upregulated miR-187 inhibits the tumor invasiveness [9]. Overexpression of miR-187 in HeLa cells can also cause a decrease in cell viability and an induction of cell apoptosis [15]. On the contrary, miR-187 expression in breast cancer results in a more aggressive, invasive phenotype [8]. These studies provide evidences for the complex role of miR-187 in the pathogenesis of human cancers. However, its function in ccRCC remains largely unknown.

B7-H3, a novel member of the B7 family [16], is overexpressed in several human cancers, including lung, breast, pancreatic, urothelial cell, prostate, and renal cell cancer. A correlation between high expression of B7-H3 and a poor outcome in patients with these types of cancer has been demonstrated in the previous work [17–22].

In the present study, we found that miR-187 was down-regulated in ccRCC tissue compared to paired histologically normal tissue. The expression level of miR-187 in plasma of ccRCC patients was also significantly lower than that in normal individuals. Lower levels of miR-187 in ccRCC tissues were associated with higher tumor grade and stage. Down-regulation of miR-187 in ccRCC was related to lower survival of kidney cancer patients. Thus we believe that miR-187 can be used as a tumor marker for ccRCC.

The differential miR-187 expression pattern may be helpful in developing gene therapy for ccRCC. To prove this possibility, we determined the possible impact of miR-187 on ccRCC cells. Cell growth inhibition (both in vitro and in vivo), and decreased motility were observed in miR187-transfected 786-O and ACHN cells. We also found that B7-H3 is one of the targets of miR-187 expression. Knockdown of the B7-H3 gene both inhibited ccRCC cell proliferation and inhibited cell migration. These observations suggest that down-regulated miR-187 may play roles in carcinogenesis via deregulating cancer-related gene B7-H3 in ccRCC. In vivo studies are still needed to further elucidate the effects of miR-187 on in vivo metastasis of ccRCC cells.

In summary, miR-187 may play a tumor suppressor role in ccRCC though targeting B7-H3. The differential expression patterns of miR-187 may have implications in guiding diagnosis and treatment of ccRCC.

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