



## Associations of high expression of miR-106b-5p detected from FFPE sample with poor prognosis of RCC patients

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### ARTICLE INFO

#### Keywords:

microRNA  
miR-106b-5p  
Renal cell carcinoma  
Prognostic biomarker  
Prognosis  
Formalin-fixed paraffin-embedded tissue

### ABSTRACT

**Objective:** The present study aims to determine the association of microRNA-106b-5p (miR-106b-5p) expression with the clinicopathological features and prognosis of renal cell carcinoma (RCC).

**Patents and methods:** The formalin-fixed paraffin-embedded (FFPE) Tumor tissues were collected from 284 RCC patients. The expression of miR-106b-5p was examined by Reverse Transcription Real-time Quantitative Polymerase Chain Reaction (RT-qPCR), followed by correlation analysis with clinicopathological features and prognosis. Patient survival analysis was determined by the Kaplan-Meier method using the log-rank test was used for patient survival analysis, and the univariate and multivariate analysis was determined by a Cox's regression model.

**Results:** Kaplan-Meier analysis indicated that patients with high miR-106b-5p expression showed a significantly shorter overall survival, compared with patients with low miR-106b-5p expression ( $p = 0.001$ ). Univariate and multivariate analysis considered miR-106b-5p expression as an independent risk factor for predicting the prognosis of RCC patients. No significant evident showed that the expression level of miR-106b was related to gender, age, tumor size or tumor stage. Also, the results above were verified by analyzing the data of 506 cases from The Cancer Genome Atlas database (TCGA).

**Conclusions:** The results pointed out that the high expression of miR-106b-5p serves as an independent factor for predicting the worse prognosis of RCC patients.

### 1. Introduction

Renal cell carcinoma (RCC) is the 14th most common cancer in the world and the third most common urological cancer in the USA [1,2]. The incidence and mortality of RCC is increasing in developing countries while plateaued in Europe and North American in the past few years [3]. Worldwide, ~295,000 new cases and ~134,000 death cases of kidney cancer occur annually [4]. Stage I RCC patients usually show asymptomatic, while 25%–30% of them have already metastasized at the time of diagnosis [5]. Radical nephrectomy is considered as the main treatment for RCC, however, as literatures reviewed show 30–40% of patients after radical nephrectomy experienced metastatic

and recrudescence [6]. The 5-year survival rate of patients with stage III is ~5% [7]. Therefore, it is essential to evaluate prognosis of RCC. However, no corresponding effective biomarker for RCC prognosis exists currently.

MicroRNAs (miRNAs/miRs), a class of endogenous short non-coding single stranded RNAs consisting of ~22 nucleotides in length, regulate gene expression post-transcriptionally, which play important roles in the occurrence and development of tumors [8–10]. miRNAs can imperfectly or perfectly bind the 3' untranslated regions (UTRs) of targeted messenger RNAs (mRNAs) to fulfil their functions that suppress the expression of target gene and manipulate various biological processes including cell proliferation, differentiation, apoptosis, migration

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and body's homeostasis, physiological functions [11–13]. Emerging evidence revealed that more and more miRNAs are relative to the development and prognosis of RCC and other cancers. For instance, Ma et al. [14] suggested miR-19a acted as a potential prognostic biomarker for RCC for its function that miR-19a could promote RCC cell proliferation by targeting SMAD4 and PTEN. Chen et al. [15] reported that downregulation of miR-30a-5p promoted RCC cell metastasis and growth. Yang et al. [16] reported that miR-30c repressed MTA-1 expression to inhibit the RCC cell proliferation and enhance the sensitivity of RCC cells to anticarcinogen drugs. Therefore, miRNAs are expected to be useful markers for evaluating the prognosis of RCC.

Previous studies have reported that microRNA-106b (miR-106b) is significantly upregulated in RCC tissues and cell lines, compared with the normal controls [17,18], and the inhibition of miR-106b leads to the suppression of cell migration and proliferation [18]. Furthermore, multiple human diseases turned out worse prognosis associating with the upregulation of miR-106b, such as heart failure [19], breast tumor [20], p53-like bladder tumors [21], hepatocellular tumor [22] and gastric cancer [23]. Hence, we collected the information of RCC patients to explore the association between the expression of miR-106b-5p and the survival rate of patients with RCC.

## 2. Materials and methods

### 2.1. Patients and samples

Clinical-pathological characteristics of 284 patients have been obtained, who received radical or partial nephrectomy from April 2003 to May 2013 at Peking University Shenzhen Hospital (Guangdong, China), but no any pre-operative radiotherapy or chemotherapy. The surgical specimens (RCC and matched adjacent normal tissues) from the patients above were treated as the formalin-fixed paraffin-embedded (FFPE) samples by professional pathologists from Department of Pathology, Peking University Shenzhen Hospital. All tissues were dissected, subsequently immersed in RNA later (Qiagen, Hilden, Germany) for over 30 min and store at  $-80^{\circ}\text{C}$  before analysis. Analyzed in accordance with the 2010 American Joint Committee on Cancer (AJCC) staging system [24], information of the clinical and pathological characteristics including gender, age, tumor size and tumor stage is presented in Table 1. Postoperative follow-up of all patients was kept regularly until May 2018 or until death. For this study, the Ethics Committees of Peking University Shenzhen Hospital provided Ethical Approval, and all patients provided written informed consent.

**Table 1**  
Association between miR-106b expression level<sup>1</sup> and Clinical information in FFPE renal cancer samples.

Variable	Total	No. of patients (%)		P-value <sup>2</sup>
		High	Low	
Gender				
Male	177	91	86	0.540
Female	107	51	56	
Age(Years)				
$\leq 60$	206	105	101	0.595
$> 60$	78	37	41	
Tumor size(cm)				
$\leq 4.0$	121	64	57	0.401
$> 4.0$	163	78	85	
Tumor stage				
I + II	175	88	87	0.903
III + IV	109	54	55	

<sup>1</sup> cut-off point: median.

<sup>2</sup> calculated using Fisher's Exact test or Pearson Chi-square test.

### 2.2. RNA extraction and RT-qPCR

Total RNA from FFPE specimens was extracted with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. After purified with the RNeasy Maxi kit (Qiagen GmbH), concentration of the RNA was measured with a NanoDrop 2000c (Thermo Fisher Scientific, Inc.). Then, total RNA (1  $\mu\text{g}$ ) with optical density (OD) ratios at 260/280 nm of 1.8–2.0 from each sample was reverse-transcribed into cDNA with the miScript Reverse Transcription kit (Qiagen GmbH) in accordance with the manufacturer's protocol. RT-qPCR was performed with miScript SYBR-Green PCR kit (Qiagen GmbH) using the Roche LightCycler 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland). The primers are shown as follows: miR-106b-5p forward: 5'-TAAAGTGCTGACAGTGCA GAT-3'; reverse: universal primers (miScript SYBR Green PCR kit); U6 forward: 5'-CTCGCTTCGGCAGCACACA-3'; reverse: 5'-ACGCTTCACGAAT TTGCGT-3'. The thermocycling conditions of RT-qPCR was set up as follows:  $95^{\circ}\text{C}$  for 15 min, followed by 40 cycles of  $94^{\circ}\text{C}$  for 15 s, subsequently by  $55^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 30 s. The  $2^{-\Delta\Delta\text{Ct}}$  method was used to calculate the expression levels of miR-106b in accordance with the 2-Cq method [25].

### 2.3. Statistical analysis

The SPSS statistical software program version 19.0 (IBM SPSS, Armonk, NY, USA) was used for all the statistical analyses above. The correlation between clinicopathological variables (gender, age, tumor size and tumor stage) and the expression level of miR-106b-5p was analyzed by Pearson Chi-square test. The univariate and multivariate levels between miR-106b-5p expression and clinical-pathological variables or survival were analyzed by using Cox proportional-hazard regression analysis. The effect of miR-106b-5p expression on patient survival or clinical-pathological variables was evaluated by the Univariate and multivariate Cox regression analyses. Kaplan-Meier method was used to analyze and construct the survival curves. The differences between these curves above were evaluated by the log-rank test. We also constructed the ROC curves to examine the prognostic efficiency of miR-106b-5p. P-value  $< 0.05$  was considered statistically significant.

### 2.4. Validation from TCGA

For validating our finding, data of 506 RCC patients was gathered from The Cancer Genome Atlas ([www.cancergenome.nih.gov](http://www.cancergenome.nih.gov)) [26], and analyzed to plot Kaplan-Meier curves just like we did above with our data. The expression level of miR-106b-5p and clinical variables including survival time, tumor stage, tumor size were enrolled in our study. Clinical variables including survival time, tumor size, tumor stage were collected, together with miR-183-5p expression counts.

## 3. Result

### 3.1. Clinicopathological features of RCC and expression of miR-106b-5p

As Table 1 shown, the patients we collected consisted of 107 women and 177 men with median age of 50 years. 175 patients are at stage I–II RCC while 163 at stage III–IV. The median of follow-up period is 44 months (range at 4–163 months). At the end of our study follow-up, 189 patients have kept alive. According to the median of miR-106b-5p expression level we detected, each group of clinicopathological features of all cases was categorized into the high group and low group. We had not found any significant association between miR-106b-5p expression level and age, gender, tumor size or tumor stage.

**Table 2**  
miR-106b expression and patients' survival.

Variable	Univariate analysis		Multivariate analysis	
	HR(95% CI)	P-value	HR(95% CI)	P-value
Gender(Female vs Male)	0.803(0.518-1.243)	0.324		
Age(≤60y vs > 60y)	0.912(0.573-1.451)	0.697		
Tumor size(≤4.0 cm vs > 4.0)	1.131(0.755-1.693)	0.550		
Tumor stage(I + II vs III + IV)	0.583(0.390-0.872)	0.009	0.568(0.379-0.850)	0.006
miR-106b-5p (low vs high)	0.507(0.335-0.768)	0.001	0.496(0.327-0.752)	0.001

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

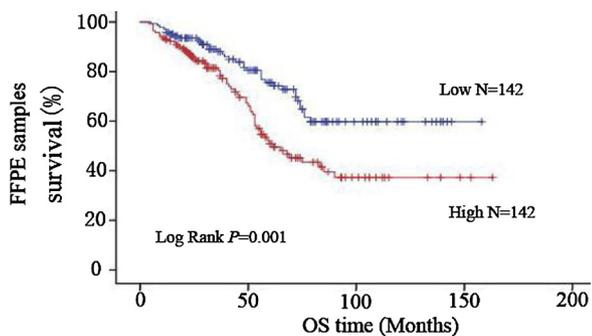
### 3.2. Patients survival and miR-106b-5p expression

In univariate analysis, the survival of RCC patient was significantly associated with tumor stage (HR = 0.583, 95%CI, 0.390–0.872; P = 0.009), irrespective of gender, age or tumor size, as Table 2 shown. Moreover, high miR-106b-5p expression level resulted in the poorer survival rate (HR = 0.507, 95%CI, 0.335–0.768; P = 0.001). In the multivariate analysis, after controlling for gender, age, tumor size and tumor stage, subsequently analyzed, patients with high expression level of miR-106b-5p were considered to have a lower overall survival (HR = 0.496, 95%CI, 0.327–0.752, P = 0.001).

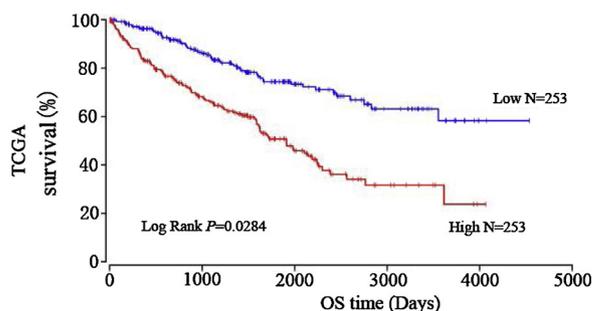
In Kaplan-Meier analysis, we separately analyzed the data of FFPE sample and the data from TCGA. As the survival curves shown in Fig. 1, patients we collected with high expression of miR-106b-5p had a remarkable poor prognosis (P = 0.001), which indicated that miR-106b-5p could have an independent prognostic potential to predict worse prognosis for RCC. Subsequently, after treating the data from TCGA into high expression group and low expression group by the dividing line of the median expression of miR-106b-5p on the data from TCGA, and analyzing as the way above, we found that the high miR-106b-5p expression ones, compared to the patients with low expression, had remarkable lower overall survival (P = 0.0284). The Kaplan-Meier curves are shown in Fig. 2.

AS for conditioned Kaplan-Meier analyses of tumor stage, tumor size, gender and age, miR-106b-5p showed significant prognostic value for smaller size(≤4.0 cm) tumors (P = 0.0041), larger size (> 4.0 cm) tumors (P = 0.010), older (> 60 years) patients (P = 0.003), male (P = 0.010) and the high(III-IV) tumor stage (P = 0.002) cases separately, but not significantly for younger (< 60 years) patients (P = 0.069), female (P = 0.055) and the early(I-II) tumor stage (P = 0.070) cases. The results are shown in Fig. 3.

For the area under ROC curves, tumor stage alone was 0.591 (95% CI, 0.520–0.662) with a sensitivity and specificity of 50.5% and 32.3%, miR-106b-5p expression alone was 0.591 (95% CI, 0.521–0.661) with a sensitivity and specificity of 62.1% and 43.9%. The combination of miR-106b-5p expression and tumor stage was 0.633 (95% CI, 0.563–0.702) with a sensitivity and specificity of 80.0% and



**Fig. 1.** Kaplan-Meier survival curves. Kaplan-Meier analysis of overall survival (OS) for patients with RCC for the predictive value of miR-106b in the overall patients. N = 284, P-value was calculated by log-rank test.



**Fig. 2.** Kaplan-Meier survival curves. Kaplan-Meier analysis of overall survival (OS) for patients with RCC for the predictive value of miR-106b, data from TCGA. N = 506, P-value was calculated by log-rank test.

64.0%. The results were shown in Fig. 4.

### 4. Discussion

RCC is one of the most common urological cancers with an increasing incidence in the past 20 years [27,28] and a high mortality, especially when metastatic tumor happens [29]. ~30–40% of early-stage RCC who had received partial or radical nephrectomy had relapse [6], which meant a poor prognosis. New effective method to predict the disease progression of RCC is a pressing need. Emerging evidence revealed that novel biomarkers was vital to improve the prognosis of patients with RCC for the function to predict RCC patients' prognosis accurately [30].

Moreover, an increasing number of microRNAs were reported to be prognostic marker for cancers, such as miR-221 and miR-221 for breast cancer [31], miR-132 and miR-212 for hepatocellular carcinoma [32], miR-221 for chronic lymphocytic leukemia [33], miR-222 for pancreatic cancer [34], miR-147b-5p for ovarian cancer [35]. However, to our best knowledge, the prognosis value of micrRNAs in RCC was less to be reported. In our study, we payed attention to the association between miR-106b-5p in FFPE samples with RCC.

MiR-106b, belonging to the miR-17 family, is located on chromosome 7. Previous studies have demonstrated that the miR-106b was upregulated in multiple human tumors [21–23]. Chow et al. [17] reported that miR-106b-5p was significantly in RCC cell lines and tissues. Slaby et al. [36] had indicated that miR-106b-5p might service as a promising biomarker to predict poor survival of patients with early metastasis following nephrectomy. We independently came to the same conclusion in the field of potential prognostic function of miR-106b-5p. What made it different was that Slaby et al extracted miR-106b-5p from postoperative specimens directly while we made the postoperative specimens further processed to FFPE specimens and extracted miR-106b-5p from FFPE specimens. FFPE samples are easily accessible and suitable to be used to estimate the prognosis of cancer. Moreover, it provides a new train of thought and new evidence to explore the prognosis function of miR-106b-5p and it may also value for the future studies that we could explore the prognosis value of miR-106b-5p in the serum and urine, which may be equally efficient and more convenient.

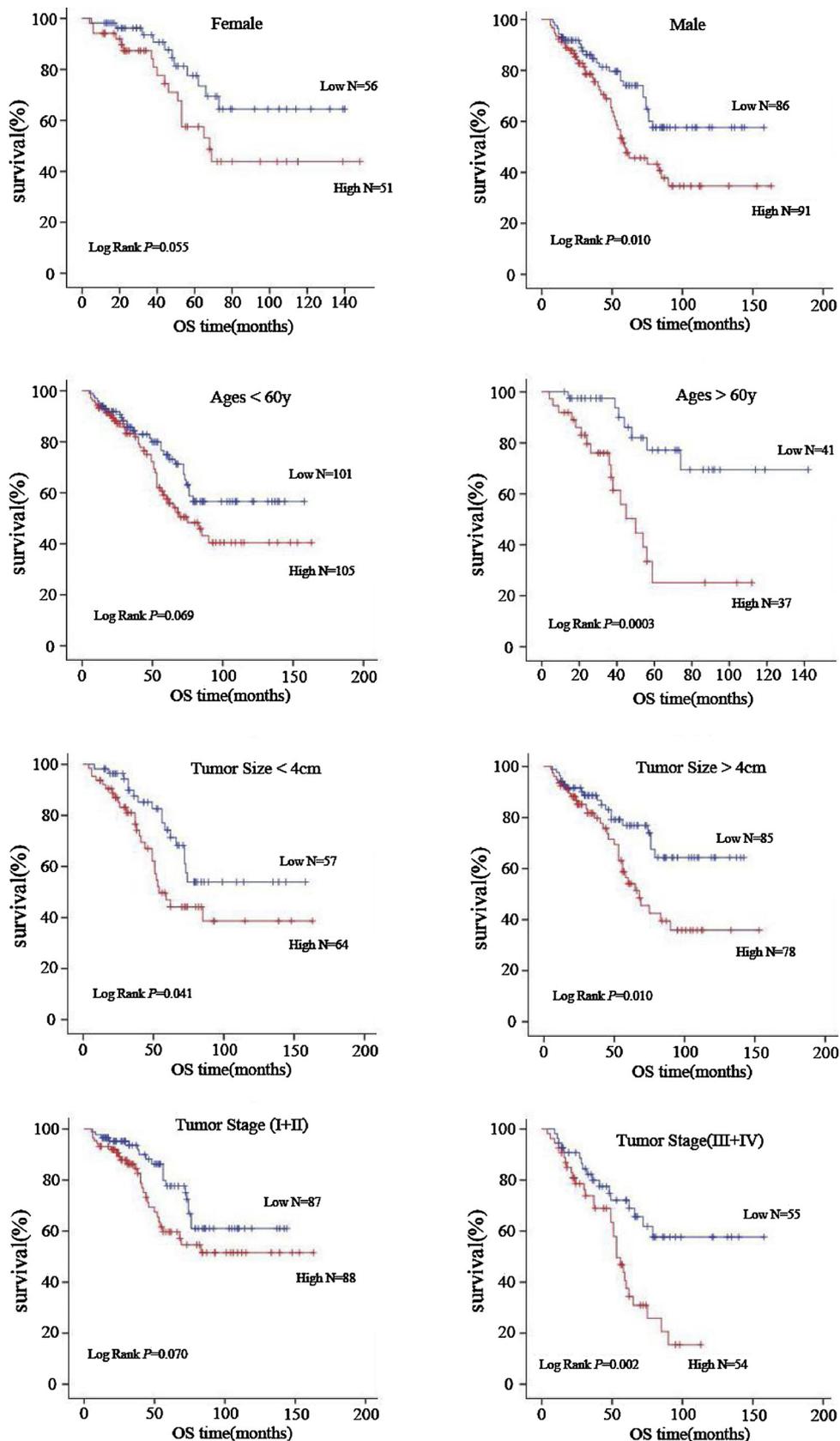
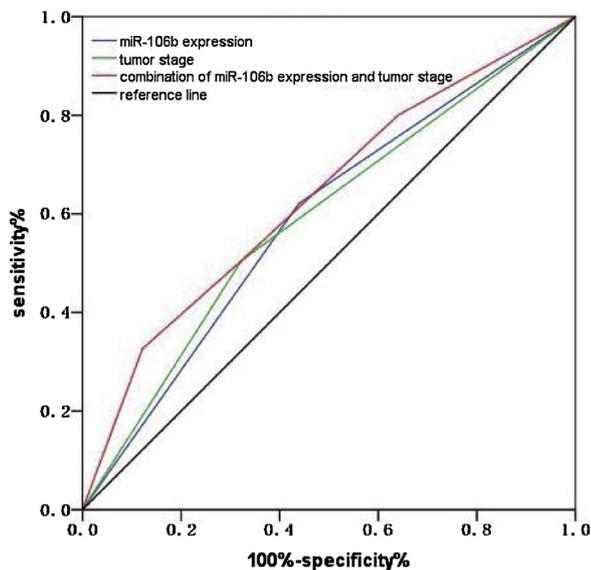


Fig. 3. Kaplan-Meier survival curves. Kaplan-Meier analysis of overall survival (OS) for patients with RCC for the predictive value of miR-106b in the sex set, age set, tumor size set and tumor stage set. P-value was calculated by log-rank test.



**Fig. 4.** ROC curve. ROC curve analysis of tumor stage alone (AUC: 0.591, sensitivity: 50.5%, specificity: 32.3%,  $p < 0.05$ , miR-106b expression alone (AUC: 0.591, sensitivity: 62.1%, specificity: 43.9%,  $p < 0.005$ ) and the combination of miR-106b expression and tumor stage (AUC: 0.633, sensitivity: 80.0%, specificity: 64.0%,  $p < 0.05$ ).

In our study, we find that high expression of miR-106b-5p can predict the shorter overall survival of RCC patients by studying 284 RCC cases. The result of multivariate analysis shows that high miR-106b-5p expression serves as an independent prognosis factor on overall survival controlling for gender, tumor size, age and tumor stage. Subsequently, the Kaplan-Meier survival curves of the research data from both TCGA and FFPE sample of our study further demonstrated the conclusion above. Moreover, we further examined the correlation between miR-106b-5p levels with clinical-pathological factors. The result showed that miR-106b-5p showed significant prognostic value for smaller tumor size tumor, larger size tumors, older patients, male and the stage III-IV ( $P = 0.002$ ) cases separately, but not significantly for younger patients, female and the early (I-II) tumor stage cases, which indicated that it was viable for miR-106b-5p to predict the overall survival independently. The result of Pearson Chi-square test showed no significant association between the expression level of miR-106b-5p and tumor stage, which indicated that patients with different stages of RCC might need equal attention. The mechanism of miR-106b-5p affecting the RCC prognosis remains unknown, which may value for the future studies.

The results of ROC curve explained that tumor stage, miR-106b-5p expression have predictive efficiency for RCC patients, and the combination of them could improve the predictive efficiency, to some extent. However, the numbers of our samples are not enough, which limited us to get a more convincing conclusion. Hence, it is a pressing need to research further to prove its value.

In these years, it's popular to combine several miRNAs to prove the prognostic efficiency of disease. For instance, the combination of miR-10b, miR-139-5p, miR-130b and miR-199b-5p affected the metastasis and prognosis in clear cell renal carcinoma [37]. It inspires us to study more miRNAs for their prognostic efficiency and try to combine some of them and invent prognosis kits in our latter work.

## 5. Conclusion

In summary, the study demonstrated that high expression of miR-106b-5p detected from FFPE sample is associated with poor prognosis in RCC patients after controlling for gender, age, tumor size and tumor stage, after controlling for gender, age, tumor size and tumor stage.

## Funding

This study was supported by Basic Research Project of Peking University Shenzhen Hospital (JCYJ2017001, JCYJ2017004, JCYJ2017005, JCYJ2017006, JCYJ2017007, JCYJ2017012), Clinical Research Project of Peking University Shenzhen Hospital (LCYJ2017001), Science and Technology Development Fund Project of Shenzhen (no. JCYJ20180507183102747) and Clinical Research Project of Shenzhen Health Commission (no. SZLY2018023).

## Competing interests

None.

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