Int Urol Nephrol (2013) 45:373–379
DOI 10.1007/s11255-012-0374-y

UROLOGY - ORIGINAL PAPER

Overexpression of microRNA-100 predicts an unfavorable prognosis in renal cell carcinoma

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Received: 15 October 2012 / Accepted: 24 December 2012 / Published online: 3 February 2013

Abstract
Purpose Dysregulation of microRNA-100 (miR-100) has been reported to be involved in tumorigenesis and tumor progression of several cancer types. However, its expression patterns in tumors are controversial. The aim of this study was to investigate the expression and clinical significance of miR-100 in renal cell carcinoma (RCC).
Methods Real-time quantitative PCR was performed to detect the expression levels of miR-100 in 96 paired samples of RCC and adjacent non-cancerous renal tissues. Then, statistical analysis was performed to determine the associations of miR-100 expression with the clinical features and the prognosis of RCCs.
Results miR-100 expression was significantly higher in RCC tissues compared with adjacent non-cancerous renal tissues (5.3 ± 2.2 vs. 1.9 ± 0.8, $P < 0.001$). In addition, high miR-100 expression in RCC tissues was significantly associated with advanced tumor T stage ($P = 0.005$) and grade ($P = 0.01$), and the presence of metastasis ($P = 0.008$). Moreover, Kaplan–Meier analysis showed the significant differences in 5-year overall (50.0 vs. 83.3 %, $P = 0.006$) and tumor-specific survival (58.3 vs. 83.3 %, $P = 0.008$) for patients with high and low miR-100 expression, respectively. Furthermore, multivariable Cox regression analysis identified high miR-100 expression in RCC tissues as an independent poor prognostic marker of both overall ($P = 0.01$) and tumor-specific survival ($P = 0.02$) in patients with RCCs.
Conclusion Our data offer convincing evidence that miR-100 overexpression strongly associates with advanced tumor progression and unfavorable clinical outcome of patients with RCC. miR-100 expression may be a useful prognostic marker for this disease.

Keywords microRNA-100 · Renal cell carcinoma · Real-time quantitative PCR · Prognosis

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Background

Renal cell carcinoma (RCC) represents the most common renal malignancy with the highest mortality rate and the steadily increasing incidence [1]. Worldwide, the incidence of RCC is over 200,000 new cases annually, with over 100,000 deaths per year [2]. Human RCCs arise from various specialized cells located along the length of the nephron [3]. RCC is

heterogeneous and comprises several histological types with different genetic and clinicopathologic features which determine clinical course and outcome. The most common histological type is clear cell carcinoma, also called conventional RCC, which represents approximately 80 % of RCC. Papillary (~15 %), chromophobe (5 %) and other more rare forms such as collecting duct carcinoma (<1 %) comprises the remainder [4]. In clinics, pathologic stage and tumor grade, based on the size of the tumor and the extent of invasion, are both important prognostic indicators. However, it seems to have limited value in predicting the clinical outcome of individual patients because nearly half of RCC patients who undergo surgery with curative intent for less advanced disease can be expected to develop a distant recurrence [5]. Thus, there is an urgent need to identify valuable diagnostic and prognostic markers to improve the clinical outcome and to develop the effective individual therapeutic strategies for patients with RCC.

MicroRNAs (miRNAs) are a class of naturally occurring small non-coding, single stranded RNAs that regulate protein coding gene expression at the post-transcriptional level through targeting the 3′ untranslated region (3′UTR) of specific mRNAs to regulate mRNA degradation or translational inhibition [6]. MiRNAs have been demonstrated to be involved in many developmental processes, including cell cycle, cell proliferation, differentiation, metabolism and apoptosis [7]. Dysregulated miRNAs have been reported in many human cancers, and they often play important roles in tumorigenesis and tumor progression [8]. MiRNAs function as oncogenes or tumor suppressors according to the functions of their targeting genes. Some highly expressed miRNAs act as oncogenes by repressing tumor suppressors, whereas low-level miRNAs act as tumor suppressors by negatively regulating oncogenes. A growing body of evidence has recently identified a considerable number of dysregulated miRNAs which may play a pivotal role in the pathogenesis of RCC. In the present study, we focus on miR-100, which has been reported to be involved in tumorigenesis and tumor progression of several cancer types, including glioma, prostate cancer, bladder cancer, lung cancer, leukemia, breast cancer and epithelial ovarian cancer [9–16]. However, its roles in RCC are still unclear. The aim of this study was to investigate the expression and clinical significance of miR-100 in RCC.

Materials and methods

Patients and tissue samples

Prior informed consent was obtained from the patients for the collection of specimens in accordance with the guidelines of Huai'an First People's Hospital, Nanjing Medical University, China, and the study protocols were approved by Ethics Committee of Huai'an First People's Hospital, Nanjing Medical University. All specimens were handled and made anonymous according to the ethical and legal standards.

Ninety-six pairs of tissue specimens (tumor and adjacent non-cancerous renal tissue) from 96 patients with RCC were obtained and histologically confirmed by two pathologists at Huai'an First People's Hospital, Nanjing Medical University. All patients underwent curative surgical resection at Huai'an First People's Hospital, Nanjing Medical University from February 2002 to October 2009. No patients had received adjuvant treatments including radiotherapy or chemotherapy prior to surgery, in order to eliminate potential treatment-induced changes to expression profiles of miRNAs. After surgical resection, tumor specimens and corresponding adjacent non-cancerous tissues were collected and stored in liquid nitrogen until use. Information on gender, age, tumor location, tumor size, tumor stage, tumor grade and the status of metastasis was abstracted from the medical records. Among these, tumor stage was determined according to the 2009 TNM (tumor, node, metastasis) staging classification system, and tumor grade was determined according to the Fuhrman classification system (well differentiated = grade 1 and 2, moderately differentiated = grade 3 and poorly differentiated = grade 4). Sixty-eight of these 96 patients were men and 28 were women. The median age of the patients was 52 years (range, 30–78 years); 30, 26, 20 and 20 presented with pT1, pT2, pT3 and pT4 cancer, respectively. Tumor grade showed that 40, 36, 20 suffered from well, moderately and poorly differentiated tumors. Furthermore, 46 patients presented with metastasis. The clinical characteristic of 96 patients with RCC was summarized in Table 1.

Complete follow-up information was obtained from all 96 RCC patients. The duration of the follow-up was calculated from date of surgery to the date of death or last follow-up. Death was classified into cancer-related and -unrelated groups. The median

Table 1 Associations of miR-100 expression with clinicopathological features of 96 renal cell carcinoma (RCC) patients

Clinical variables	No. of patients (%)	MiR-100 expression (*n*, %)		*P*
		Low	High	
Gender				
Male	68 (70.8)	26 (38.2)	42 (61.8)	0.8
Female	28 (29.2)	10 (35.7)	18 (64.3)	
Age (year)				
>66*	40 (41.7)	13 (32.5)	27 (67.5)	0.2
≤66	56 (58.3)	23 (41.1)	33 (58.9)	
Tumor location				
Upper pole	46 (47.9)	20 (43.5)	26 (56.5)	0.09
Middle	20 (20.8)	6 (30.0)	14 (70.0)	
Lower pole	30 (31.3)	10 (33.3)	20 (66.7)	
Tumor size				
<40 mm	26 (27.1)	12 (46.2)	14 (53.8)	0.1
40–70 mm	50 (52.1)	17 (34.0)	33 (66.0)	
>70 mm	20 (20.8)	7 (35.0)	13 (65.0)	
Tumor stage				
T1	30 (31.3)	20 (66.7)	10 (33.3)	**0.005**
T2	26 (27.1)	11 (42.3)	15 (57.7)	
T3	20 (20.8)	5 (25.0)	15 (75.0)	
T4	20 (20.8)	0 (0)	20 (100.0)	
Tumor grade				
Well differentiated	40 (41.7)	21 (52.5)	19 (47.5)	**0.01**
Moderately differentiated	36 (37.5)	12 (33.3)	24 (66.7)	
Poorly differentiated	20 (20.8)	3 (15.0)	17 (85.0)	
Metastasis				
Present	46 (47.9)	6 (13.0)	30 (87.0)	**0.008**
Absent	50 (52.1)	20 (40.0)	30 (60.0)	

* Mean age of 96 RCC patients

The bold values refer to the differences with statistical significance

and mean follow-up times were 81.8 months (interquartile range [IQR], 25.2–133.6 months) and 81.6 months. Until the last follow-up examination, 40 patients were alive, 36 patients had died from progressive RCC and 20 patients due to other causes.

Real-time quantitative RT-PCR for miRNA

Real-time quantitative PCR (qRT-PCR) was performed to detect the expression levels of miR-100 in 96 paired samples of RCC and adjacent non-cancerous renal tissues. Total RNA was isolated using the RNeasy Maxi Kit (Qiagen, Germany), according to the manufacturer's protocol. QRT-PCR was performed using the miScript Reverse Transcription and miScript SYBR Green PCR Kit, according to the manufacturer's protocol (Qiagen, Germany). RNU6B was used as a normalization control. The following primers were used: for RNU6B, forward primer, 5′-CGC TTC GGC AGC ACA TAT AC-3′; reverse primer, 5′-TTC ACG AAT TTG CGT GTC AT-3′; for MiR-100, 5′-AAC CCG TAG ATC CGA ACT TG-3′; reverse primer: 5′-TAC CTA TAG ATA CAA GCT TGT GCG-3′. 0.1 μg total RNA was reverse transcribed into cDNA using the miScript Reverse Transcription Kit (Qiagen, Germany). The 20-μl PCR system consisted of 10 μl of 2 x QuantiTect SYBRGreen PCR Master Mix, 2 μl of 10 x miScript Universal Primer, 300 nM of forward primers, 1 μl of cDNA template and RNase-free water. The reactions were incubated in 96-well optical plates at 95 °C for 10 min and then followed by 40 cycles of 95 °C for 15 s and 60 °C for 10 min. We used human RNU6B to normalize the data for quantification of miR-100. The delta–delta Ct method was employed to calculate the fold change. For each sample, all experiments were done in triplicate. Mean normalized miRNA expression ± SE was calculated from three independent experiments.

Statistical analysis

The software of SPSS version 13.0 for Windows (SPSS Inc, IL, USA) was used for statistical analysis. Differences in miR-100 expression in RCC and adjacent non-cancerous renal tissues were assessed by paired samples *t* test. Associations of miR-100 expression with clinicopathological features of RCC were assessed by the nonparametric Mann–Whitney *U* test. Kaplan–Meier survival times were calculated, and subgroups were compared by the log-rank test statistic. Multivariate Cox regression models were used to assess the association of miR-100 expression with survival adjusted for different clinical and patient covariates. Differences were considered statistically significant when *P* was less than 0.05.

Results

Overexpression of miRNA-100 in RCC patients

QRT-PCR analysis was performed to demonstrate the differences of miR-100 expression in RCC and adjacent non-cancerous renal tissues. The results showed that the expression of miR-100 was significantly increased in RCC tissues compared with adjacent non-cancerous renal tissues (5.3 ± 2.2 vs. 1.9 ± 0.8, $P < 0.001$, Fig. 1). RCC patients expressing miR-100 at levels less than the median expression level (5.5) were assigned to the low-expression group (mean expression value 3.4, $n = 36$), and those samples with expression equal or above the median value were assigned to the high-expression group (mean expression value 6.5, $n = 60$).

miR-100 overexpression associates with advanced clinicopathological features of RCC patients

The relationships between miR-100 expression and clinicopathological features of RCCs are summarized in Table 1. Overexpression of miR-100 in RCC tissues was significantly associated with advanced tumor T stage ($P = 0.005$) and grade ($P = 0.01$), and the presence of metastasis ($P = 0.008$). However, there were no significant associations of miR-100 expression with patients' age and gender, tumor location and tumor size (all $P > 0.05$). Collectively, these findings suggested that miR-100 overexpression was relevant to tumor differentiation, invasion and metastasis, which are involved in tumorigenesis and tumor progression of RCC.

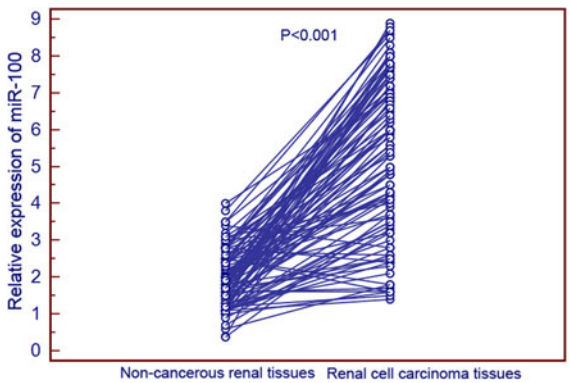

Fig. 1 MiR-100 expression in 96 paired samples of renal cell carcinoma (RCC) and adjacent non-cancerous renal tissues. After normalization to RNU6B expression levels, the expression of miR-100 was significantly increased in RCC tissues compared with adjacent non-cancerous renal tissues (5.3 ± 2.2 vs. 1.9 ± 0.8, $P < 0.001$). P values were calculated using the paired samples t test

miR-100 overexpression associates with unfavorable prognosis of RCC patients

The median 5-year overall and tumor-specific survival rates of all 96 RCC patients were, respectively, 62.5 and 67.7 % months. Kaplan–Meier analysis showed the significant differences in 5-year overall and tumor-specific survival for patients with high and low miR-100 expression, respectively. The 5-year survival rates for patients with high vs. low miR-100 expression were 50.0 vs. 83.3 % for overall survival ($P = 0.006$, log rank) and 58.3 vs. 83.3 % for disease-specific survival ($P = 0.008$, log rank), respectively (Fig. 2). In addition, using univariate analysis, high miR-100 expression significantly predicted shorter overall and tumor-specific survival than low miR-100 expression during the follow-up ($P = 0.006$ and 0.008, respectively, Table 2). Besides, tumor stage (both $P = 0.01$, Table 2) and grade ($P = 0.01$ and 0.02, respectively, Table 2), and metastasis status (both $P = 0.008$, Table 2) were also associated with both overall and tumor-specific survival of RCC patients. Furthermore, applying multivariable Cox regression analysis, including tumor stage and grade, metastasis status, and miR-100 expression patterns, high miR-100 expression (for overall survival: $P = 0.01$, HR 3.6, 95 % CI 1.8–5.2; for tumor-specific survival: $P = 0.02$, HR 2.4, 95 % CI 1.4–4.9), advanced tumor stage (for overall survival: $P = 0.02$, HR 2.2, 95 % CI 1.2–4.5; for tumor-specific survival: $P = 0.03$, HR 1.6, 95 % CI 0.7–2.7) and the presence of metastasis (for overall survival: $P = 0.02$, HR 2.4, 95 % CI 1.5–4.9; for tumor-specific survival: $P = 0.02$, HR 2.1, 95 % CI 1.0–4.2) could be identified as independent poor prognostic markers for both overall and tumor-specific survival in RCC patients (Table 3).

Discussion

RCC is commonly asymptomatic in early stages, and the main therapeutic strategies for this disease are surgical operation, radiotherapy and chemotherapy. However, approximately 30 % of patients develop

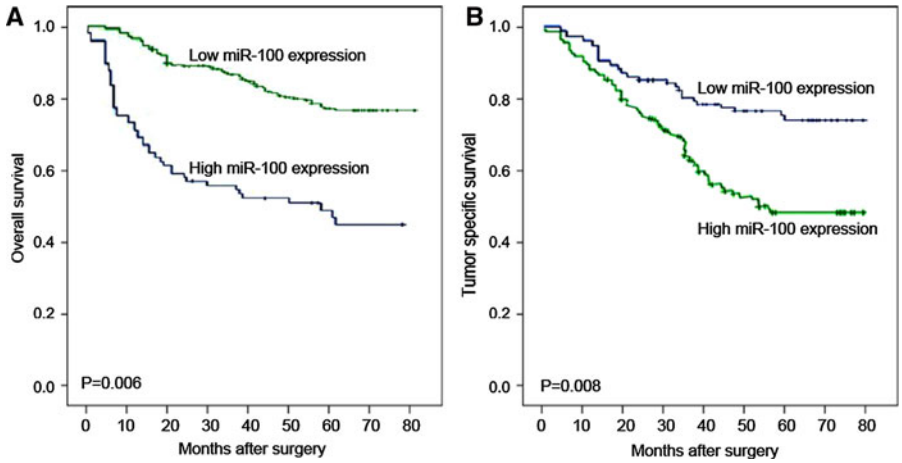

Fig. 2 Kaplan-Meier curves of overall survival (**a**) and tumor-specific survival (**b**) of patients with renal cell carcinoma (RCC) stratified by the level of miR-100 expression. High miR-100 expression was associated with shorter 5-year overall ($P = 0.006$) and tumor-specific survival ($P = 0.008$) in RCC patients, respectively

Table 2 Univariate analysis of the impact of variables on overall survival and tumor-specific survival in renal cell carcinoma (RCC) patients

Variable	Overall survival	Tumor-specific survival		
	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Gender				
Male vs. female	1.0 (0.9–1.2)	0.6	1.0 (0.9–1.1)	0.6
Age (year)				
>66* vs. ≤66	1.3 (0.8–2.3)	0.2	1.2 (0.7–2.2)	0.2
Tumor location				
Upper pole vs. middle vs. lower pole	1.1 (0.8–1.9)	0.6	1.0 (0.8–1.7)	0.6
Tumor size				
≤70 mm vs. >70 mm	1.5 (0.2–3.9)	0.1	1.4 (0.2–3.6)	0.1
Tumor stage				
T1–2 vs. T3–4	2.3 (1.0–5.8)	**0.01**	2.1 (1.0–5.5)	**0.01**
Tumor grade				
Well–moderately differentiated vs. poorly differentiated	2.2 (0.9–5.6)	**0.01**	1.9 (0.9–5.2)	**0.02**
Metastasis				
Present vs. absent	4.6 (2.3–8.8)	**0.008**	4.4 (2.0–9.1)	**0.008**
miR-100 expression				
High vs. low	5.0 (2.6–10.2)	**0.006**	4.3 (2.0–8.7)	**0.008**

The bold values refer to the differences with statistical significance

metastatic disease after the curative nephrectomy [17]. Patients with metastatic disease often face a dismal prognosis and have limited therapeutic options. Therefore, it is of great significance to identify specific molecular biomarkers for RCC in order to implement early diagnosis and early treatment. To the best of our knowledge, this is the first report about the expression of miR-100 in RCC tissues. In the present study, we detected miR-100 in 96 pairs of fresh human RCC and non-cancerous renal tissues with qRT-PCR. There are three points of finding according to our results: Firstly, miR-100 expression in RCC was higher than that in the corresponding non-cancerous renal tissues; secondly, overexpression of miR-100 more frequently occurred in RCC tissues with advanced tumor stage and grade, and with metastasis and finally, overexpression of miR-100 significantly predicts poor prognosis in patients with RCC. These findings indicated that miR-100 may be associated with the carcinogenesis and cancer development of RCC.

MiR-100, localized to chromosome 11, has been reported to be aberrantly expressed in a number of human malignancies. However, its expression in tumor cells is controversial, and it is up- and downregulated in different types of tumors. On the one hand, miR-100 is upregulated in prostate cancer, acute myeloid leukemia and human glioma. For example, Leite et al. [9] found that high miR-100 expression was related to biochemical recurrence of localized prostate cancer in patients treated with radical prostatectomy; Feng et al. [10] demonstrated that miR-100 overexpression could

Table 3 Multivariate analysis of the impact of variables on overall survival and tumor-specific survival in renal cell carcinoma (RCC) patients

Variable	Overall survival	Tumor-specific survival		
	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Tumor stage				
T1–2 vs. T3–4	2.2 (1.2–4.5)	**0.02**	1.6 (0.7–2.7)	**0.03**
Tumor grade				
Well–moderately differentiated vs. poorly differentiated	1.2 (0.9–3.0)	0.07	1.1 (0.8–2.7)	0.08
Metastasis				
Present vs. absent	2.4 (1.5–4.9)	**0.02**	2.1 (1.0–4.2)	**0.02**
miR-100 expression				
High vs. low	3.6 (1.8–5.2)	**0.01**	2.4 (1.4–4.9)	**0.02**

The bold values refer to the differences with statistical significance

resensitize docetaxel-resistant human lung adenocarcinoma cells; Zheng et al. [11] also detected the overexpression of miR-100 in acute myeloid leukemia patients at the time of diagnosis, and they further confirmed that miR-100 could regulate tumor cell differentiation and survival of acute myeloid leukemia patients. Consistent with these reports, our data showed the increased expression of miR-100 in RCC tissues, which was associated with the aggressive clinicopathological features of patients with this cancer. In contrast to miR-100 overexpression observed in above cancers, miR-100 has been reported to be downregulated in ovarian cancer, breast cancer, hepatocellular carcinoma and oral squamous cell carcinoma. For example, Peng et al. [12] indicated that the low expression of miR-100 was significantly correlated with advanced FIGO stage, higher serum CA125 expression level, lymph node involvement and shorter overall survival of epithelial ovarian cancer patients; low miR-100 expression has been described as an important miRNA alteration in bladder tumors, and enforced miR-100 expression may decrease cell proliferation and colony formation capacity of human bladder cancer cell lines [13]; Henson et al. [14] also found that miR-100 was downregulated in oral squamous cell carcinoma tissues and cell lines, and that transfecting cells with exogenous miR-100 significantly reduced cell proliferation of cancer cells. These findings may indicate different and potentially tissue-specific roles for miR-100.

Our data mentioned above showed that miR-100 expression is higher in RCC compared to non-cancerous renal tissue, and increases with tumor stage, which prompted us to investigate the prognostic value of miR-100 in RCC patients. In the present study, we used the large study cohort with a sufficient follow-up period. Kaplan–Meier analysis disclosed significant differences in overall and tumor-specific 5-year survival for patients with higher and lower than median level of miR-100 expression. This supports the hypothesis that miR-100 plays a potential role in renal carcinogenesis or at least RCC progression. Furthermore, high miR-100 expression could be identified as an independent poor prognostic marker for RCC patients.

Until now, there was few RCC-specific biomarker which has been used for diagnosis and prediction of prognosis in clinic. Although miR-100 seems not to be specific marker for RCC, it can probably benefit RCC patients due to its significance in prediction of tumor prognosis. From the viewpoint of clinic, miR-100 upregulation might be considered as a risk factor of tumor aggressive progression. A regular detection of miR-100 expression after operation might improve prognosis.

In conclusion, our data offer the convinced evidence that miR-100 overexpression strongly associates with advanced tumor progression and unfavorable clinical outcome of patients with RCC. miR-100 expression may be a useful prognostic marker for this disease. Therefore, this study encourages further investigations to disclose the mechanism via which miR-100 is involved in tumor progression and to assess its function as prognostic marker for clinical use.

Conflict of interest None.

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