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Original Article

Prognostic value of serum exosomal microRNA-21 for patients with renal cell carcinoma

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Abstract: Background: Renal cell carcinoma (RCC) is the most common malignant cancer in the adult kidney. The aim of this study is to explore the prognostic values of exosomal miR-21 in patients with RCC. Methods: We retrospectively reviewed 62 patients with RCC. The expressions of exosomal miR-21 were detected by real-time PCR. Prognostic factors were evaluated using Kaplan-Meier curves and Cox proportional hazards models. Results: Of all 62 patients with RCC, miR-21 concentration was significantly higher in the exosomes than in the exosome-depleted supernatants and the whole plasma samples (P<0.001), which indicated that miR-21 mostly expressed in the exosomes. Moreover, through Kaplan-Meier survival analysis to compare OS and PFS according to miR-21 expression, we found that patients with low expression of exosomal miR-21 had a significantly better OS (P=0.03) and PFS (P=0.001) than patients with high exosomal miR-21 expression. Multivariable ananlysi showed that low exosomal miR-21 was also a favorable independent risk factor for both OS and RFS. Conclusions: In this study, we found that miR-21 concentration was significantly higher in the exosomes than in the exosome-depleted supernatants and the whole plasma samples. Furthermore, low exosomal miR-21 was also a favorable independent risk factor for both OS and PFS of patients with RCC.

Keywords: Exosome, renal cell carcinoma, miR-21

Introduction

Renal cell carcinoma (RCC) is the most common malignant cancer in the adult kidney and has three common types including clear cell RCC (80%-90%), papillary RCC (10%-15%), and chromophobe RCC (3%-5%) [1, 2]. Although a great deal of effort has been made and the prognosis of patients with RCC has been improved, the survival outcomes of patients with RCC may vary, as several factors are associated with the prognosis including TNM stage, Fuhrman grade and several integrated models like University of California Integrated of RCC, Staging System (UISS) and Mayo Clinic stage, size, grade and necrosis (SSIGN) score [3, 4]. Accurately finding effective means of diagnosis and prognosis prediction of patients with RCC is still difficult. Discovery of novel biomarkers would play a pivotal role in RCC diagnosing and prognostic improvement of patients with RCC [5].

MicroRNAs (miRNAs), existing naturally as the most biologically stable nucleic acid molecule with only about 19-23 nucleotides, act as fine-tuning regulators of gene expression at post-transcriptional level through a complicated miRNA-mRNA interaction [6]. MiRNAs are ideal candidates for biomarkers because of their resistance to endogenous RNase and high stability under different storage conditions. Recent studies have shown that human serum miRNAs are aberrantly expressed in many malignancies such as liver [7, 8], colorectal cancer [9], and pancreatic cancer [10]. Increasing evidence suggests that unique serum miRNA expression signatures may serve as new noninvasive biomarkers for cancer diagnosis including RCC [11].

Exosomes, which are released into circulation from all cell types, are lipid bilayer cup-shaped nanovesicles with 40-100 nm in diameter and provide membrane protection for inclusive

Table 1. Patient and tumor characteristics

Variables	Number	Percent(%)
Gender		
Female	26	41.9
Male	36	58.1
Age in years		
≤ 65	28	45.2
> 65	34	54.8
Diameter (cm)	7.6±2.7	2.7-10.4
Fuhrman grade		
1	16	25.80
2	31	50.00
3	12	19.40
4	3	4.80
ECOG PS		
0	37	59.70
≥1	25	40.30
Histology (main component)		
ccRCC	45	72.60
pRCC	10	16.10
chRCC	7	11.30
TNM Stage		
I	27	43.50
II	13	21.00
III	15	24.20
IV	7	11.30
Metastases		
Yes	14	22.60
No	48	77.40
Plamatic miRNA-21		
High expression	26	41.9
Low expression	36	58.1
Exosomal miRNA-21		
High expression	30	48.40
Low expression	32	51.60

RNAs and proteins [12, 13]. large amounts of exosomes can be secreted by tumor cells which is due to the influence of hypoxia, internal environmental changes and other factors. These exosomes are not easily degradable in either intercellular space or peripheral blood due to the protection by plasma membranes. Until now, emerging studies have suggested that tumor-derived exosomes quantitatively predominate in peripheral blood and exosome-mediated miRNA transduction plays a pivotal role in the dialogue between human tumors and their microenvironment [14].

Several studies had demonstrated that expression of microRNA-21 (miR-21) was significantly different in many human cancers compared with the healthy people, and miR-21 level was identified as a promising biochemical marker [15-17]. During recent years, the role of miRNA-21 in RCC progression has been experimentally described. Down-regulation of miRNA-21 is found in RCC patients in comparison with healthy human cells, and a significant difference exists in miRNA-21 expression levels between ccRCC and pRCC subtypes, suggesting that the miRNA-21 expression level can be used as a diagnostic marker in distinguishing RCC subtypes [18]. In particular, the expression level of miRNA-21 is found to be correlated with a span of 5-year survival and pathological stage in RCC patients [19]. The findings above suggest that miRNA-21 might play a crucial role in the biological functions of RCC.

However, few studies focused on the significance of exosomal miR-21 in patients with RCC. In the study, we aimed to explore the prognostic values of exosomal miR-21 in patients with RCC. The results of our study shed new light on the identification of new prognostic biomarkers for RCC patients.

Materials and methods

Patients and blood samples

We prospectively collected data from consecutive patients with Tumor staging was determined according to the 7th edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. Ultimately, 62 patients enrolled in this study from TCM-Integrated Hospital, Southern Medical University. Written consents were obtained from all subjects prior to the recruitment. The study protocol was approved by the Research Ethics Committee of TCM-Integrated Hospital, Southern Medical University. The clinical characteristics of the subjects are listed in **Table 1**.

Extraction of exosomes from peripheral blood

Whole blood was centrifuged at 3000*g for 15 min to remove cells or cell debris, the supernatant liquid was then placed into a centrifuge tube, added with 63 μl of ExoQuick reagent per 250 μl of serum and allowed to stand at 4°C for 30 min. In a 4°C environment, the mixture was

centrifuged at 1500*g for 30 min (exosomes precipitated at the bottom of the centrifuge tube). Supernatant was aspirated completely and centrifuged at 1500*g, 4°C for 5 min. Supernatant was aspirated completely (during which there should be no shaking of the centrifuge tube), completely dissolved and precipitated with 20 μl of 1*PBS and stored at -20°C.

Extraction of total RNA from serum exosomes

200 μl of sample was dispensed and added with 200 μl of 2*denaturing solution, then the mixture was placed in ice for 5 min. Next, an equivalent volume of acid-phenol: chloroform was added, test tube was shaken for 50 sec, and the mixture was centrifuged at 10000*g for 5 min. The supernatant was transferred to a fresh tube, elution solution was preheated, supernatant was added with a 1.25-fold volume of absolute ethanol, and the mixture was placed into a Filter cartridge and centrifuged at 10000*g for 15 sec, then base solution was discarded. 700 μl of miRNA wash solution 1 was added onto the Filter cartridge, centrifuged at 10000*g for 15 sec, then base solution was discarded; 500 μl of wash solution 2/3 was added, centrifuged at 10000*g for 15 sec, then base solution was discarded (repeated twice). The Filter cartridge was placed into a fresh tube, added with 35 μl of 95°C elution solution, centrifuged at 10000*g for 1 min, then the eluate was collected and stored at -70°C. The extracted genomic DNAs were tested for purity and content with UV-Vis spectrophotometer.

Statistical analysis

Continuous variables were expressed as mean ± SD (standard deviation) and compared using a two-tailed unpaired Student's t test; categorical variables were compared using χ^2 or Fisher analysis. Life-table estimates of survival time were calculated according to the Kaplan and Meier methodology [20]. The Greenwood formula was used for the standard deviation. A Cox proportional hazards regression approach [21] was chosen for the evaluation of the prognosis. Potential prognostic variables were analyzed both univariately with one factor taken at a time, and then in a multivariate model combining all factors. Results were showed as hazard ratios (HR) and their 95% confidence intervals (CI) A HR>1 indicated an elevated risk with respect to the reference category. A confidence interval which did not include the value 1 indicated statistical significance at the 5% level. The primary end points were overall survival (OS) and progression-free survival (PFS). Overall survival (OS) was defined as the time between date of operation and date of death. Subjects still alive at the end of the study were censored. The Progression-free Survival (PFS) was defined as the time from randomisation to disease progression or death as assessed by the treating physicians in the study. Statistical analysis was conducted with the SPSS for Windows version 18.0 release (SPSS, Inc., Chicago, IL) and ROC curve analysis were computed using MedCalcV.11.0.3.0 (MedCalc software, Mariakerke, Belgium). A value of $P<0.05$ was considered significant in all the analysis.

Results

Patients' characteristics

62 patients with RCC were recruited into this study. The median follow-up was 4.7 years (range 4.1 months-9.5 years). The baseline characteristics of patients at diagnosis were summarized in **Table 1**. Overall, among these patients. the gender distribution was roughly equal (M:F=1.38:1). The most patients were included in Fuhrman grade 1 (25.8%) and 2 (50.0%) and TNM stage I (43.5%) and II (21.0%), respectively. Most patients had no metastases (77.4%).

Comparing of plasmatic and exosomal miR-21 expression in RCC patients

In order to determine if miRNAs in plasma of patients with RCC are enclosed in exosomes and/or are circulating freely, we firstly extracted RNA from both exosome pellets isolated from 10 control plasma samples and the exosome-depleted plasma supernatants. MiR-21 expression was examined by qRT-PCR. Notably, miR-21 concentration was significantly higher in the exosomes than in the exosome-depleted supernatants (P<0.001, **Figure 1A**). To examine miR-21 expression in whole plasma, we extracted RNA directly from the 10 serum samples used above and quantified miR-21 expression. The concentration of miR-21 in the whole plasma samples was lower than in the exosomes, but higher than in the exosome-free supernatants (**Figure 1A**). Furthermore, we performed comparison of plasmatic and exosomal miR-21 expression in all 62 RCC patients. We demon-

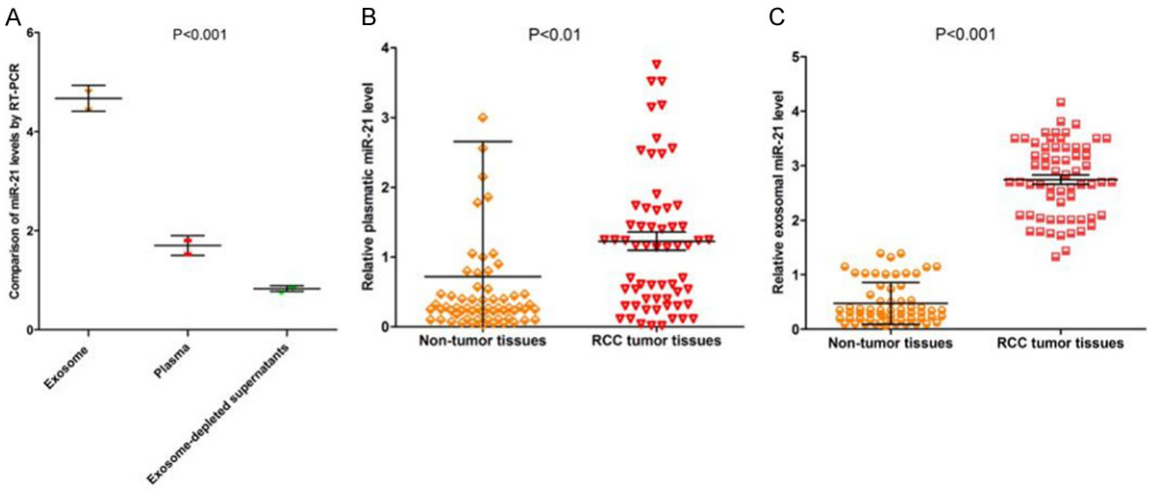

Figure 1. Serum exosomal miR-21 expression is significantly higher in RCC patients. A: Serum miR-21 predominantly exists in exosomes in RCC patients. MiR-21 levels in serum exosomes, exosome-depleted supernatants, and whole serum were determined by qRT-PCR; B: The plasmatic miR-21 expression levels were significantly higher in tumor tissues with RCC compared with non-tumor tissues (P<0.001); C: The exosomal miR-21 expression levels were significantly higher in tumor tissues with RCC compared with non-tumor tissues (P<0.001).

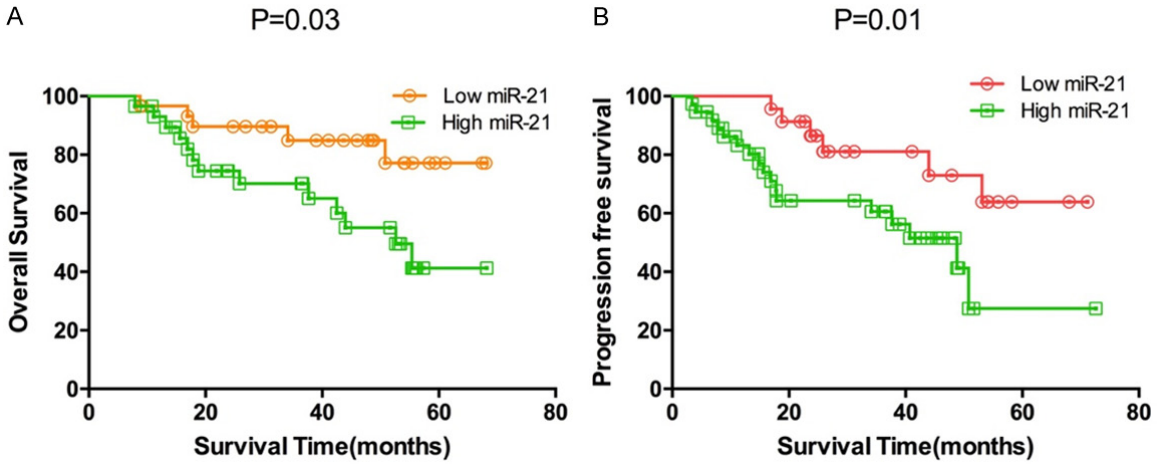

Figure 2. Overall and progression survival estimates according to the expression of exosomal miR-21 levels. High exosomal miR-21 level was defined as that the exosomal miR-21 was 2.5 times in tumor tissues compared with non-tumor tissues: A: The overall survival curves stratified by exosomal miR-21 expression levels (P=0.030); B: The progression survival curves stratified by exosomal miR-21 expression levels (P=0.010).

strated that expression of miR-21 in plasma and exosomes was significantly higher in tumor tissues of RCC compared with non-tumor tissues (P<0.001, **Figure 1B**, **1C**).

Low expression of exosomal miR-21 is associated with better prognosis of patients with RCC

Kaplan-Meier survival analysis was performed to compare OS and PFS according to miR-21 expression. Patients with low expression of exosomal miR-21 had a significantly better OS (P=0.03) and PFS (P=0.01) than patients with high exosomal miR-21 expression (**Figure 2A** and **2B**). Plasmatic miR-21 expression showed no significant difference related with prognosis of patients with RCC (data not shown). We then performed univariate and multivariate analyses to further assess whether exosomal miR-21 expression was an independent prognostic factor of OS and PFS. Univariate analysis showed that tumor size, TNM stage, tumor metastases and low exosomal miR-21 were significantly associated with OS and PFS. Furthermore, after adjusting for competing risk factors, we identified that low exosomal miR-21 was also a favorable independent risk factor for both

Table 2. Cox proportional hazard regression analyses in the patients with RCC

Variable	Univariate (OS)				Multivariate (OS)				Univariate (PFS)				Multivariate (PFS)			
	B value	HR	95% CI	*P* value	B value	HR	95% CI	*P* value	B value	HR	95% CI	*P* value	B value	HR	95% CI	*P* value
Age >65	0.153	1.032	0.845-1.135	0.562					0.156	1.004	0.832-1.143	0.637				
Male	0.332	1.083	0.932-1.539	0.436					0.226	0.936	0.845-1.527	0.375				
Diameter >4 cm	0.645	1.674	1.331-2.657	0.001	0.105	1.173	0.935-1.529	0.074	0.206	1.743	1.395-2.843	0.002	0.107	1.158	0.563-1.618	0.082
Fuhrman grade 3, 4/1, 2	0.301	1.136	0.903-1.304	0.372					0.462	1.136	0.903-1.304	0.372				
ECOG PS ≥1	0.361	1.128	0.938-1.847	0.089					0.671	1.082	0.818-1.320	0.096				
Histology (main component)	0.152	1.134	0.457-2.164	0.219					0.105	0.928	0.836-2.417	0.392				
Metastases: Yes/No	0.573	2.335	1.955-3.546	<0.001	0.554	1.765	1.255-2.563	0.005	0.554	2.054	1.887-4.157	0.001	0.617	1.824	1483-2.734	0.003
TNM III, IV/I, II	0.688	1.852	1.421-2.635	<0.001	0.673	1.921	1.572-2.465	0.011	0.671	1.672	1.443-2.866	0.004	0.633	1.847	1.659-3.184	0.001
Plamatic miRNA-21	0.721	1.157	1.011-2.021	0.117					0.662	1.105	0.997-2.104	0.071				
Exosomal miRNA-21	0.587	1.434	1.257-2.766	0.03	0.775	2.071	1.511-3.035	0.001	0.814	1.627	1.358-2.734	0.01	0.712	1.504	1.418-2.625	0.015

Abbreviations: CI, confidence interval; OS: overall survival; PFS: progression-free survival.

OS and PFS (OS, HR, 2.071, 95% CI, 1.511-3.035, P=0.001; PFS, HR, 1.504, 95% CI, 1.418-2.625, P=0.015) in multivariate analysis (**Table 2**).

Discussion

The prognosis of RCC can vary widely. Detecting recurrences early can improve patient outcomes because the likelihood of a favorable response to systemic treatment is greater when the metastatic burden is limited, and surgical resection of a single or limited number of metastases can result in longer survival [22]. The anatomical extent, or stage, of disease is the most useful prognostic factor for patients with RCC, but this is not always accurate. The most commonly used prognostic models for patients with metastatic disease are based on clinical parameters [23]. A more accurate assessment of RCC prognosis is urgently needed to better guide patient management.

Many studies showed that exosome could be presented in the urine, pleuroperitoneal fluid and exosome had pleiotropic biological functions, including antigen-presenting, intracellular communication and transmission of signals and transferring of RNAs and miRNAs [24, 25]. Similarly, miRNAs were repeatedly reported as diagnostic indicator and prognostic factor in various cancers. The potential pathogenesis of specific miRNAs promoting or blocking tumorigenesis is still unclear. Researchers had found Myc-driven reprogramming of miRNAs expression patterns contributes to the aggressive phenotype of liver tumors originating from hepatic progenitor cells [26]. They also showed that interplay of Wnt/β-catenin and Myc signaling played a critical role in poorly differentiated aggressive tumors and identified a 16-gene signature with strong prognostic significance [27]. miRNAs have an important role in the regulation of cellular activities such as cell growth, proliferation, and differentiation. miRNA-21 was first noticed for its apoptotic effects in various cell lines [28]. Upregulated miRNA-21 promoted tumor proliferation and inhibited cell apoptosis in breast cancer cells in vitro [29]. miRNA-21 silencing could inhibit the capacity of proliferation, migration, and invasion, and arrest the cell cycle and induce apoptosis of tongue squamous cell carcinoma cell lines [30]. miRNA-21 mimic-transfected cells exhibited increased cell proliferation and transformation capacity, whereas miRNA-21 inhibitor-transfected cells exhibited the opposite phenomenon in renal cancer cell lines (A498, 786-0, and caki-1) [31]. In addition, overexpression of miRNA-21 significantly decreased antiproliferative effects and apoptosis induced by paclitaxel, while knock-down of miRNA-21 dramatically increased antiproliferative effects and apoptosis induction by paclitaxel in human gastric cancer cells [32].

In present study, miR-21 concentration was significantly higher in the exosomes than in the exosome-depleted supernatants and the whole plasma samples, which indicated that miR-21 mostly expressed in the exosomes. Moreover, through Kaplan-Meier survival analysis performed to compare OS and PFS according to miR-21 expression, we found that patients with low expression of exosomal miR-21 had a significantly better OS (P=0.03) and PFS (P=0.01) than patients with high exosomal miR-21 expression. Multivariable ananlysi showed that low exosomal miR-21 was also a favorable independent risk factor for both OS and RFS.

To the best of our knowledge, while there are many recognized prognostic and predictive markers for RCC, including several clinicopathological features and serum biomarkers, the present study is the first to explore the potential implications for exosomal miR-21 related to RCC prognosis. Meanwhile, there are limitations of this study: (1) the study was based on the data of Chinese patients, whether the expression of exosomal miR-21 is downregulation in patients of other races remains to be determined; (2) this is a retrospective study and the sample size is small, further studies with larger samples or multi-centers are needed.

In conclusion, this study showed that miR-21 concentration was significantly higher in the exosomes than in the exosome-depleted supernatants and the whole plasma samples. Furthermore, low exosomal miR-21 was also a favorable independent risk factor for both OS and PFS of patients with RCC.

Disclosure of conflict of interest

None.

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