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Expression of serum miR-221 in human hepatocellular carcinoma and its prognostic significance

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ABSTRACT

Objective: To investigate whether the serum miR-221 expression correlates with clinicopathologic features and the prognosis of hepatocellular carcinoma (HCC) patients.
Methods: Four miRNAs (miR-221, miR-222, miR-21 and miR-224) related to HCC were selected in the present study. Serum miRNA expression was investigated in 46 HCC patients and 20 healthy normal controls by using real-time PCR technique, and then correlations between miR-221 expression and the clinicopathological features and prognosis of HCC patients were evaluated.
Results: The four miRNAs were found to be differentially overexpressed in HCC serum samples, and high level of miR-221 expression was correlated with tumor size ($P < 0.001$), cirrhosis ($P = 0.003$) and tumor stage ($P = 0.016$). In addition, Kaplan–Meier survival analysis showed that the overall survival rate of the high miR-221 expression group (27.6%) was significantly lower than that of the low miR-221 expression group (62.3%, $P < 0.05$).
Conclusions: Serum miR-221, upregulated in HCC, can provide predictive significance for prognosis of HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is a global health problem, with over 700,000 cases worldwide each year [1]. In China, HCC is the second highest cancer killer since the 1990s, which alone accounts for 53% of all liver cancer deaths worldwide [2]. The prognosis of HCC patients remains poor, mainly resulting from the high recurrence rate [3,4]. Thus, it is still necessary to search better prognostic markers which accurately represent biological characteristics of tumors, screen for early-stage disease and predict the outcome so as to improve the clinical management of HCC patients.

miRNAs are small (19–25nt) regulatory RNAs that are frequently dysregulated in cancer and have shown promise as tissue-based markers for cancer classification and prognostication. Recently, these miRNAs were also identified in serum and plasma in a remarkably stable form that is protected from endogenous RNase activity [5]. The circulating miRNAs were first investigated as biomarkers for diagnosis of cancer and other diseases: Serum miR-21 has been reported to be elevated in lymphoma patients [6], serum miR-141 can distinguish patients with prostate cancer from healthy controls [7] and serum miR-122 can be used to detect liver injury [8].

miR-221 has been reported to be overexpressed in human tumor tissues, such as breast cancer, colorectal cancer and glioblastoma [9–13]. A recent report shows that miR-221 stimulates the onset of tumors and promotes tumor progression, significantly shortening the mean time to death in the mouse model of liver cancer [14]. However, the actual role of serum miR-221 expression in HCC has not been systematically studied yet, and it is not fully understood whether serum miR-221 has a similar clinicopathological influence in HCC. The present study was to investigate the feasibility of using serum miR-221 as a noninvasive prognostic biomarker for HCC.

2. Materials and methods

2.1. Patients and serum samples

Serum samples of HCC were obtained from 46 patients at Yinzhou People's Hospital (Ningbo, China) prior to definitive therapy. All 46 patients were clearly diagnosed as having HCC based on the clinicopathologic findings. The healthy sera were collected from 50 age-matched healthy individuals who volunteered to serve as normal controls (NC). The tumor type and the grade of cell

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differentiation were designated based on the criteria of World Health Organization (WHO), whereas the pathological stage of each tumor was determined by the International Union Against Cancer (UICC) TNM classification. Details of clinical and pathological characteristics of the patients were summarized in Table 1. Informed consent was obtained from all subjects and this study was approved by the Review Board of Hospital Ethics Committee.

2.2. RNA isolation

All serum samples were immediately stored in liquid nitrogen and kept frozen at −80 °C until RNA extraction. The frozen sera were thawed and transferred into Eppendorf tubes. In general, 50 fmol mmu-miR-295 mimics (Qiagen, USA) were added into 100 μl serum and incubated for 5 min. Then isolation of RNA was carried out using the Norgen's total RNA purification kit (Norgen Biotek Corporation, Canada) for sera according to the manufacturer's instructions. All serum RNA preparations were quantified using a DU 800 spectrophotometer (Beckman Coulter) and then pretreated with RNase-free DNase I (Promega) to eliminate potential DNA contamination.

2.3. Quantitative RT-PCR of mature miRNAs

Assays to quantify the mature miRNAs were conducted as previously described [15,16] with minor modification. Briefly, 20 μl reverse transcriptase reactions contained 40 ng of total RNA, 5× RT buffer, 10 mM of each dNTPs (Takara), 5 U/μl of RNase Inhibitor (Takara), and 0.25 μl of antisense looped primer. The mixture was incubated at 16 °C for 15 min, 42 °C for 60 min, and 85 °C for 5 min. Subsequently, real-time quantification was performed as described in the method of Quantitect SYBR Green PCR Kit (Qiagen, Hilden, Germany) with 7300 sequence detection system (Applied Biosystems). mmu-miR-295 was selected as the internal normalization control [7,17]. The relative expression levels of miRNAs were calculated using the comparative $\Delta\Delta C_t$ method as described previously [18,19]. The fold changes in miRNAs were calculated by the equation $2^{-\Delta\Delta C_t}$. The primer design was according to Chen et al. [15]. All primers used are listed in Supplementary Information, Table S1.

Table 1
Patient characteristics and clinicopathologic correlation of serum miR-221 expression levels.

Characteristics	All cases	Serum miR-221		
		High expression	Low expression	*P* value
Age				0.895
<50	18	8	10	
⩾50	28	13	15	
Gender				0.593
Male	33	16	17	
Female	13	5	8	
Alcohol abuse				0.747
Yes	12	5	7	
No	34	16	18	
HBsAg				0.850
Postive	30	14	16	
Negtive	16	7	9	
Live cirrhosis				0.003*
Presence	24	16	8	
Absence	22	5	17	
Tumor size (cm)				0.000*
<5	24	4	20	
⩾5	22	17	5	
Tumor differentiation				0.997
Well	9	4	5	
Moderate	24	11	13	
Poor	13	6	7	
Tumor-node-metastasis stage				0.016*
I	16	3	13	
II	19	10	9	
III–IV	11	8	3	

* $P < 0.05$.

2.4. Statistical analysis

The Mann–Whitney test or Kruskal–Wallis was performed to determine the significance of serum miRNA levels. Overall survival rates were calculated actuarially according to the Kaplan–Meier method and survival curves were plotted; statistical differences were analyzed using the log-rank test. Multivariate analysis of the prognostic factors was performed with Cox regression model. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using SPSS software (version 11.0).

3. Results

3.1. miR-221 is upregulated in HCC serum samples

A stem-loop reverse transcription polymerase chain reaction (RT-PCR) assay [15,16] was adapted to screen mature miRNA expression in serum. The results showed that among the 46 HCC samples analyzed, miR-221 was significantly upregulated 2-fold or more (2-fold to 18.7-fold) in 35 samples compared with NC (Table 2). Moreover, there miRNAs (miR-222, miR-21 and miR-224) were found to be differentially overexpressed in HCC serum samples compared with NC (Table 2). These miRNAs have been revealed differences between HCC and normal tissues [14,20–21].

3.2. Serum miR-221 correlates with clinicopathological features of HCC

For better understanding of the potential roles of serum miR-221 in HCC development and progression, The Mann–Whitney test or Kruskal–Wallis test was performed to determine the relationship of the miR-221 with various clinical features of HCC. In the present study, the average fold change of miR-221 (miR-221 in serum of HCC compared with NC) was 4.8-fold. The average fold

Table 2
miRNAs expression profile in serum samples of HCC.

Patient No.	Average fold change				Patient No.	Average fold change			
	miR-221	miR-222	miR-21	miR-224		miR-221	miR-222	miR-21	miR-224
1	2.2	1.6	2.4	1.9	24	6.4	2.9	3.1	0.3
2	2.8	3.0	0.5	1.2	25	1.3	2.1	5.2	1.2
3	3.6	1.1	3.9	1.2	26	1.9	1.3	2.0	1.1
4	4.3	2.1	7.9	0.8	27	2.8	9.6	0.9	5.4
5	6.5	5.2	1.2	4.3	28	5.9	2.7	6.4	0.9
6	0.8	1.5	3.8	3.6	29	7.8	2.7	2.2	0.6
7	12.9	4.9	1.9	0.6	30	1.6	1.6	5.0	1.7
8	1.2	0.8	2.1	2.3	31	4.2	3.1	1.2	1.1
9	4.7	3.9	0.1	0.4	32	0.3	1.1	3.8	1.2
10	0.5	0.7	18.4	1.6	33	6.0	0.5	3.2	1.2
11	2.3	5.5	2.3	0.5	34	7.9	3.7	0.1	0.7
12	6.6	2.8	0.2	0.8	35	2.3	4.1	0.3	0.7
13	5.8	2.3	3.0	4.9	36	4.6	2.4	3.2	1.9
14	5.5	4.5	2.7	3.1	37	5.3	2.8	2.4	1.9
15	18.7	8.9	0.7	0.1	38	5.7	2.7	2.7	5.3
16	1.2	1.1	5.6	2.1	39	6.1	3.3	2.4	4.2
17	2.7	0.8	1.0	1.0	40	1.9	1.4	3.8	2.1
18	3.4	2.1	0.3	4.9	41	6.8	4.2	0.9	0.5
19	6.6	3.4	5.7	0.4	42	11.3	7.2	2.8	0.5
20	4.7	3.7	0.8	1.1	43	5.5	2.3	0.2	0.1
21	11.7	6.1	2.7	0.9	44	6.5	3.1	0.4	3.2
22	7.2	2.8	2.7	7.2	45	2.3	2.8	1.8	2.2
23	1.3	1.5	4.3	1.1	46	0.5	0.7	3.1	2.4

Date show the means from three independent analyzes. Fold change was calculated as described previously [11,12] and is presented as $2^{-\Delta\Delta Ct}$.

change was used as the threshold, and patients were separated into high expression (above 4.8-fold) and low expression (below 4.8-fold) groups. The relationship between serum miR-221 expression and clinicopathological features was summarized in Table 1. The results revealed that high level of miR-221 expression was correlated with tumor size ($P < 0.001$), cirrhosis ($P = 0.003$) and tumor stage ($P = 0.016$). There was a tendency for miR-221 expression to increase with the progression of TNM stage (stage I < stage II < stage III–IV) (Fig. 1). However, there was no correlation of miR-221 expression with other clinical features, such as age, gender, alcohol abuse and HBV infection.

3.3. Serum miR-221 correlates with prognosis of HCC patients

The level of serum miR-221 expression was correlated with tumor size, cirrhosis and tumor stage, so we concluded that serum miR-221 might affect the prognosis of HCC patients. Therefore, according to the average fold change of miR-221 (4.8-fold) in all HCC serum samples, we divided the 46 patients into two groups: a high expression group and a low expression group. As shown in Fig. 2, the overall survival rate (OS) of HCC patients with high miR-221 expression group (27.6%) was significantly lower than that of patients with low miR-221 expression group (62.3%, $P < 0.05$). To determine the possibility of serum miR-221 as an independent risk factor for poor prognosis, both clinicopathological factors and the level of serum miR-221 expression were evaluated by multivariate Cox regression analysis. Results showed that tumor size, cirrhosis and high level of serum miR-221 were independent factors in predicting the OS of HCC patients (Table 3).

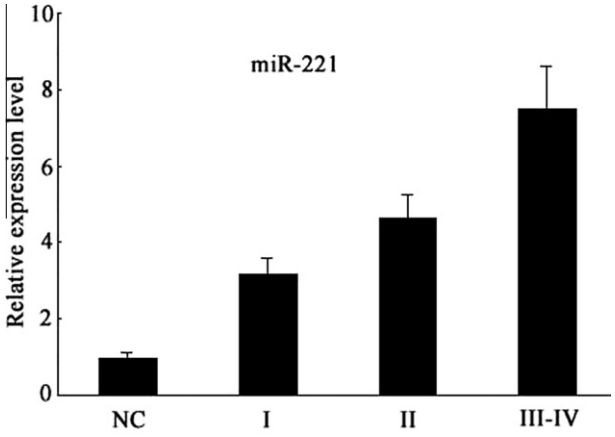

Fig. 1. Expression pattern of miR-221 in normal controls and in different clinical stages of HCC serum samples. Significant differences were observed between each pair of the four groups ($P < 0.05$).

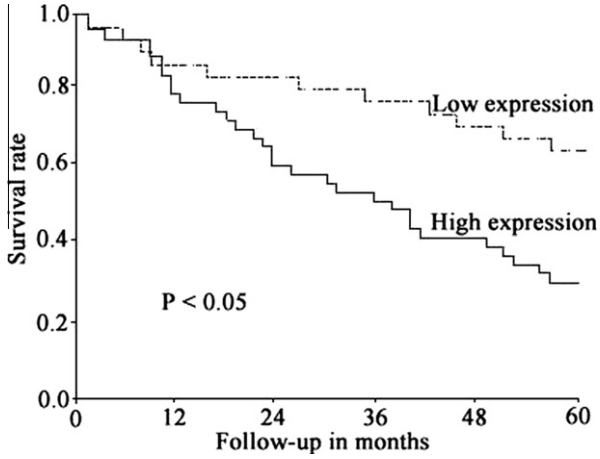

Fig. 2. Kaplan–Meier survival curves of patients with HCC. The 5-year overall survival rate of HCC patients with high serum miR-221 expression (27.6%) was significantly lower than that of HCC patients with low serum miR-221 expression (62.3%, $P < 0.05$).

Table 3
Multivariate analysis for prognostic factors.

Variate	Subset	Relative risk (95% CI)	*P* value
HBsAg	Positive/negative	0.689 (0.283–1.679)	0.412
Cirrhosis	Yes/no	1.914 (1.193–3.074)	0.008*
Tumor differentiation	Well/moderate/poor	1.020 (0.974–1.069)	0.399
Tumor stage	I/II/III–IV	1.417 (0.748–2.683)	0.285
Tumor size (cm)	<5/⩾5	1.243 (1.091–1.415)	0.062
miR-221	Low/high	1.903 (1.235–2.981)	0.018*

95% CI: 95% confidence interval.
* $P < 0.05$.

4. Discussion

Recent findings have exposed circulating miRNAs as potential biomarkers for several disease conditions including human cancer [6–8,22–25]. In this report, we show that individuals with HCC had significantly elevated levels of serum miR-221, and high level of miR-221 expression was correlated with tumor size, cirrhosis and tumor stage. We first reported that patients with high serum miR-221 levels had a significantly lower survival rate than those with low expression levels ($P < 0.05$) and serum miR-221 was an independent risk factor for poor prognosis. These results suggest that serum miR-221 can be used as a potential predictor of prognosis in HCC.

Normalization of circulating miRNAs was crucial for objective evaluation of their expression level. However, there is no current consensus on the use of endogenous control for quantitative RT-PCR analysis. miR-16, one commonly used reference miRNA, is inconsistent in our serum tests. In the present study, mmu-miR-295 was chosen as the normalized internal control due to its absence in humans [7,17]. We first selected four miRNAs miR-221, miR-222, miR-21 and miR-224 in the pilot study due to their elevated expression and abundance in HCC tissues [14,20–21]. miR-221/222, miR-21 and miR-224 are separately located in the genome [14,20], there seems to be no correlation between the expression of miR-221 and miR-21 and miR-224. However, in Table 2, the few cases with unusually low miR-221 instead appear to have exceptionally large miR-21, it might be interesting to investigate whether these miRNAs have cooperative functions in the development of HCC.

Circulating miRNAs are stably present and reproducible among individuals [7,25]. There are no significant gender differences in circulating miRNA expression [26]. As shown in Table 1, there is no correlation between gender and miR-221 expression ($P = 0.593$). Recently, advances in circulating miRNAs research [6–7,22–24] have generated the concept that tissue or organ-specific intracellular miRNAs may be released into the circulation during processes accompanying cellular destruction or pathological injury. It has been suggested that comprehensive investigations aimed at elucidating the correlations between circulating miRNAs and various diseases may provide new opportunities to use circulating miRNAs as indicators in a clinical setting [5,7]. Here, the confirmation that miR-221 is upregulated in the sera of patients with HCC could reflect a metabolic imbalance of this miRNA in vivo.

In conclusion, serum miR-221, upregulated in HCC, correlates with tumor size, cirrhosis and tumor stage. Most importantly, high

level serum miR-221 is associated with poor prognosis of HCC patients.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.111.

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