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RESEARCH ARTICLE

Circulating miR-375 and miR-199a-3p as potential biomarkers for the diagnosis of hepatocellular carcinoma

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Abstract Aiming to find novel non-invasive biomarkers with high accuracy for the detention of early-stage hepatocellular carcinoma (HCC), we examined the predictive power of two microRNAs (miR; miR-375 and miR-199a-3p) as potential biomarkers in early-stage HCC. A total of 234 serum samples (78 samples from HCC patients, 156 samples from healthy controls) were collected. We measured the levels of the two mature microRNAs (miRNAs) (miR-375 and miR-199a-3p) with probe-based stem-loop quantitative reverse-transcriptase PCR (RT-qPCR) in all subjects. In addition, the correlation between the expression levels of two miRs and clinicopathological factors was explored. Receiver operating characteristic curve (ROC) analyses revealed that the two serum miRs could be promising biomarkers for HCC, with relatively high area under the curve (AUC) values as follows: miR-375, 0. 637 with 95 % confidence interval (CI) of 0.560–0.741; miR-199a-3p, 0. 883 with 95 % CI of 0.827–0.938. Stratified analyses indicated that circulating miR-199a-3p showed better predictive value in patients with long-term drinking. Our data suggested that circulating miR-375 and miR-199a-3p could be a novel serum biomarker for HCC. Nevertheless, further validating and improving study with larger sample should be conducted to confirm our results.

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Keywords Hepatocellular carcinoma (HCC) · Biomarker · miR-375 · miR-199a-3p

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed malignancies and the third leading cause of cancer-related mortality worldwide. There are about 564,000 new cases reported worldwide each year [1], 75–80 % of whom are Asians [2]. Fifty-five percent of HCC cases worldwide are from China [3, 4]. Almost equal rates of morbidity and mortality in HCC patients are due to the diagnosis at an advanced stage [5–7]. Although early diagnosis can be helpful to decrease deaths rates, it is too difficult to conduct the early diagnosis and prognostic assessment of HCC as a result of the coexistence of inflammation and cirrhosis [8]. Therefore, the exploration of a reliable clinical diagnostic method appears crucial to improve the poor 5-year survival rate (lower than 5 %) [5].

Serum alpha-fetoprotein (AFP) level, a widely applied biomarker for HCC monitoring, has a sensitivity of 60 % at a cutoff value of 20 ng/ml. Another version of the popular non-invasive detection—ultrasonography—can reach to a sensitivity >65 % with a specificity of 90 % when applied as a screening test [9]. In addition, to improve the sensitivity and specificity for early prediction of the prognosis of HCC, more and more new specific markers have been identified, such as homeobox gene SMG-1 [10], GOLM1 [11], and Barx2 [12]. However, there is no confirmed evidence of diagnostic accuracy of these markers.

The lack of ideal biomarkers for diagnosis and therapy is the main challenge for management of HCC. Fortunately, there are a number of new advanced

technologies—microarray technologies [13, 14] and next-generation sequencing [15, 16], which make the examination or analysis of tumor genome [17, 18], proteome [19, 20], microRNA (miR) profile [21, 22], and many other molecular targets easier. Recently, miRs may reportedly be a useful, potential biomarker for diagnosis, prognosis, and individual therapy of human cancers [23–25]. Several previous studies have demonstrated circulating miRs (such as miR-122, miR-125b-5p, miR-223-3p, miR-15b, and miR-130b) might serve as potential biomarkers for the diagnosis and prognosis of HCC [26–29]. However, these discoveries are not enough to open a new era of HCC management, and further exploration of aberrant miR expression and the identification of novel miR biomarkers for HCC remain urgent.

Previous studies have reported the involvement of abnormal expression of miR-375 and miR-199a-3p in the diagnosis and prognosis of several other types of human cancers, such as pancreatic cancer [30], papillary thyroid carcinoma [31], and colorectal cancer [32]. The goals, in this present study, were to examine alternations of the expression of these two miRs in the serum of HCC patients and to evaluate whether the level of any specific miR could serve as a new biomarker.

Materials and methods

This study was approved by the Human Research Ethics Committee of The First Affiliated to Chinese PLA General Hospital, China. All participants provided written informed consents with inclusion/exclusion criteria.

Patients and samples

Between January 2012 and October 2013, we gathered a total of 234 serum samples, 78 out of which were from HCC patients, and the remainings were from healthy controls by 1:2 ratio matched pair based on age, gender, BMI, and the status of drinking and smoking. All patients enrolled in this study were newly diagnosed, histopathologically confirmed by liver biopsy, and all controls were diagnosed without any type of malignancy or other benign disease. We excluded those patients who suffered from secondary or recurrent tumors and a history of other malignant tumors.

Demographics of all subjects were collected using a questionnaire. The clinical characteristics including tumor differentiation, tumor size, metastasis, chemotherapy, and surgery were obtained by reviewing medical records. The clinical and pathological data were listed in Table 1.

RNA extraction

Blood samples (5 ml per subjects) for miR detection were drawn into EDTA-K2 tubes. RNA isolation was performed using a mirVana miRNA Isolation Kit (Ambion, Austin, Texas, USA) according to manufacturer's instructions. RNA yields obtained were about 250 ng per 400 ml of serum. Extracted RNA samples were reverse transcription to cDNA as soon as possible, using an All-in-One First-Strand cDNA Synthesis Kit (Genecopoeia). Concentration and purification of RNA were carried out using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA).

Real-time PCR quantification of miRs

The miRNA expression profiles were quantified by TaqMan miRNA assays (Applied Biosystems), according to manufacturer's instructions. Reactions were drawn onto a 96-well plate and run in duplicate using the 7900HT Fast RT-PCR System (Applied Biosystems). The incubation of reactions was performed at 50 °C for 20 s, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The expression level of miRNA was determined using the $\Delta\Delta$CT method. The snRNA U6 was chosen as the reference miRNA. The primer sequences for miR-375, miR-199a-3p, and U6 were UUUGUUCGUUCGGCUCGCGUGA, ACAGUAGUCUGCACAUUGGUUA, and CAAAGTCAGTGCAGGTAGGCTTA, respectively.

Statistical analysis

All statistical analyses were performed with SPSS software 17.0 (SPSS Inc., Chicago, USA). Differences between two groups were analyzed by a paired *t* test. The relationship between the above miR expression levels and various clinicopathologic characteristics was assessed using Wilcoxon-Mann-Whitney test. The receiver operating characteristic curve (ROC) analysis was applied to assess the diagnostic accuracy of each miR. The area under the ROC (AUC) was used to explore optimal sensitivity and specificity levels. A *P* value lower than 0.05 was considered statistically significant.

Results

General characteristic of included subjects

Demographic characteristics of all the participants are displayed in Table 1. A total of 78 HCC patients and 156 healthy controls were enrolled in this study. No significant difference was observed between the HCC patients and controls in the distribution of age, gender, BMI, and the status of drinking and smoking. All participants comprised 29 cases whose tumor size was greater than 5 cm and 49 patients with a tumor size of less than 5 cm. According to the tumor-node-metastasis (TNM) system, fifty-eight cases were in the

Table 1 Demographic and clinical characteristics of included subjects

Variables	HCC (n=78)	Healthy control (n=156)	P value
Gender (F/M)	29/49	54/102	0.699
Age (years) (n, %)	56.3±6.7	55.8±7.1	0.899
BMI (kg/m2)	23.4±5.5	22.9±6.1	0.782
Alcohol drinking (n, %)			0.703
Y/N	47 (60.3)/31 (39.7)	98 (62.8)/58 (37.2)	
Tobacco smoking (n, %)			0.642
Y/N	41 (52.6)/37 (47.4)	87 (55.8)/69 (44.2)	
AFP (ng/ml)			0.518
>20	53 (67.9)	–	
≤20	25 (32.1)	–	
HBsAg status (n, %)			
Y/N	49 (62.8)/29 (37.2)	–	–
TNM staging		–	-
TNM-I	32	–	–
TNM-II	26	–	–
TNM-III	12	–	–
TNM-IV	8	–	–
LN metastasis		–	–
Y/N	9/69	–	–
Distant metastasis		–	–
Y/N	4/74	–	–
Tumor size (cm)		–	–
≥5 cm	29	–	–
<5 cm	49	–	–

HCC hepatocellular carcinoma, *NCHD* non-cancerous hepatic disease, *F* female, *M* male, *BMI* body mass index, *Y* yes, *N* no, *AFP* alpha-fetoprotein, *LN* lymph nodes

relatively early stages (32 in TNM-I and 26 in TNM-II) while 12 cases of TNM-III and 8 cases of TNM-IV. The lymph nodes and distant metastasis were examined in 9 and 4 cases, respectively. A total of 49 HCC patients were diagnosed with HBsAg infection.

The expression of miR-375 and miR-199a-3p in the serum of all subjects

A decreased expression of serum miR-375 was distinctly observed in HCC patients, compared with controls (P<0.001). The miR-199a-3p expression was significantly downregulated in the HCC patients than that in the healthy controls (P<0.001) (Fig. 1). Subsequently, the correlation between miR-375 and miR-199a-3p levels and the clinicopathological features of the HCC patients was investigated (Table 2). HCC patients were divided into two groups, based on the expression levels of the two miRs. There was no correlation between miR-375 expression levels and clinicopathological features of the patient. Similar results were observed after examining the relationship of expression levels of miR-199a-3p and characteristics of the patients, except alcohol drinking habit. Patients with alcohol drinking habit presented a significant down-expression of miR-199a-3p (P=0.037).

Diagnostic accuracy of serum miR-375 and miR-199a-3p for HCC

The ROC curve was analyzed to verify the diagnostic accuracy of serum miR-375 and miR-199a-3p. The results displayed that both serum miR-375 and miR-199a-3p could serve as valuable

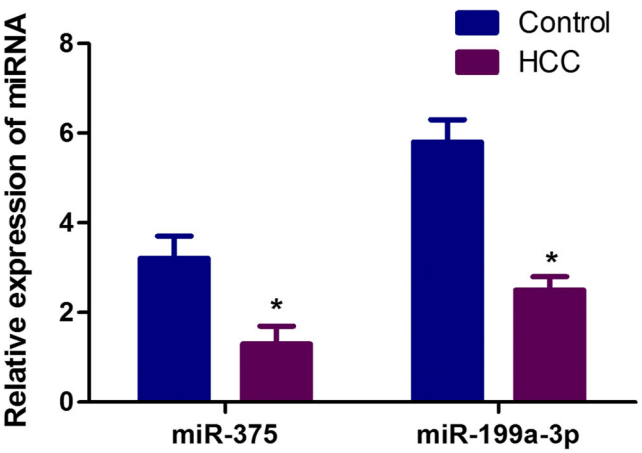

Fig. 1 Relative expression levels of miR-375 and miR-199a-3p in 156 controls and 78 HCC patients. The expression levels were determined using a qRT-PCR assay, and the relative expression data were analyzed using the $2^{-\Delta\Delta CT}$ method. All of the assays were performed in triplicate. U6 was used as a reference miRNA

Table 2 Correlation between the expression of miR-375 and miR-199a-3p and clinicopathological factors in HCC patients

Variables	Relative expression of miR-375			Relative expression of miR-199a-3p		
	High	Low	*P* value	High	Low	*P* value
Age (years)						
>60/≤60	10/21	15/32	.975	3/13	20/42	.291
Gender (M/F)	13/18	16/31	.480	7/10	16/45	.232
BMI (kg/m2)						
>24.9/≤24.9	7/24	12/35	.766	7/16	12/43	.419
Alcohol drinking (Y/N)	17/14	30/17	.427	18/5	29/26	.037
Tobacco smoking (Y/N)	17/14	24/23	.744	9/14	32/23	.124
AFP (ng/ml)						
>20/≤20	20/11	33/14	.598	18/5	35/20	.207
HBsAg status (Y/N)	17/14	32/15	.236	15/8	34/21	.777
TNM staging						
Low (I–II)/High (III–IV)	24/7	34/13	.433	19/4	39/16	.281
LN metastasis (Y/N)	6/25	3/44	.079	1/22	8/47	.199
Distant metastasis (Y/N)	2/39	2/45	.889	0/23	4/51	.184
Tumor size (cm)						
≥5 cm/<5 cm	10/21	19/28	.465	10/13	19/36	.457

biomarkers for differentiating HCC patients from healthy controls with the AUC of 0.637 (95 % CI 0.560–0.741, P=0.008) and 0.883 (95 % CI 0.827–0.938, P<0.0001), respectively (Fig. 2). At the cutoff value of 1.192 for miR-375, the sensitivity and the specificity were 52.3 and 72.7 %, respectively. In respect to miR-199a-3p, the sensitivity was 71.8 % and the specificity was 86.1 % in discriminating HCC from the non-tumor control subjects when a cutoff point was set at 2.133.

Discussion

The purpose of the present study was to explore novel serum biomarkers as minimally invasive tools for the diagnosis of HCC. In this study, we analyzed the expression of two miRs (miR-375 and miR-199a-3p) in HCC and found diminished expressions of miR-199a-3p in HCC patients with alcohol consumption. After the ROC curve analysis, our data also

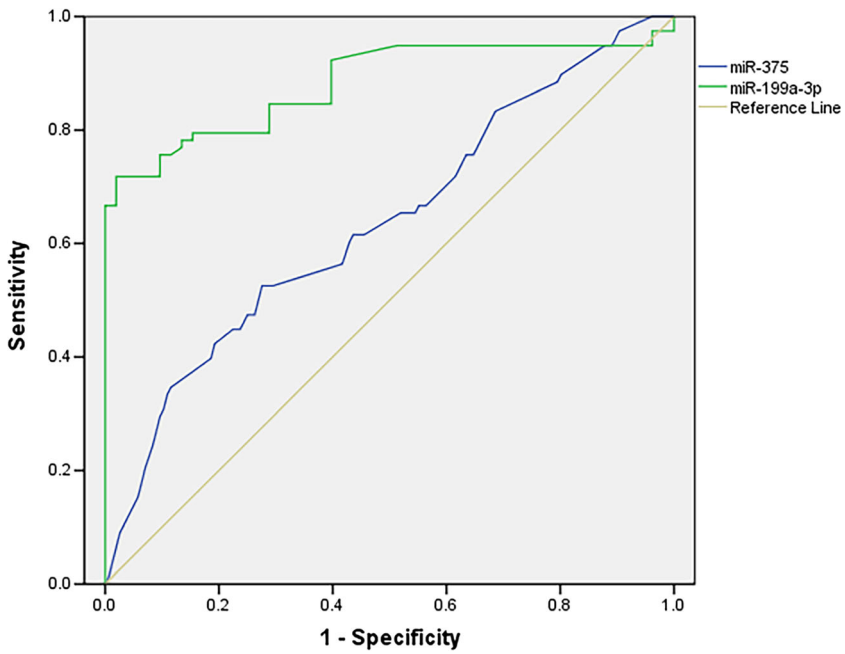

Fig. 2 Receiver operating curve (*ROC*) analysis of miR-375 and miR-199a-3p expression levels

indicated that both the two miRs might be independent diagnosed factors for HCC.

HCC is a complex, multifactorial disease caused by multiple susceptibility genes and a variety of environmental factors. Not only hepatitis B virus infection but also alcohol consumption, cigarette smoking, obesity, and chronic viral hepatitis are widely recognized as important causal factors [33, 34]. Due to poor prognosis of HCC and diagnosis at a terminal stage, early diagnosis and treatment are essential for HCC management [9]. Although pathologic confirmation is the gold standard for HCC diagnosis, the test of serum AFP level combined with imaging techniques, including ultrasonography, magnetic resonance imaging, and computerized tomography [35, 36], is also widely applied in early-stage HCC. However, the sensitivity and specificity of serum AFP level are relatively unsatisfactory. Moreover, several new specific markers have been identified, but there was no confirmed evidence of accuracy; AFP has been the only standard serum marker for HCC detection by now. Thus, there is an urgent need to investigate a novel biomarker, which should be easily operable, non-invasively reliable, and highly accurate, for HCC diagnosis.

The miRs are small, non-coding single-strand RNAs with a length of 18–25 nucleotides. The miRs are involved in many biological processes, such as proliferation, apoptosis, differentiation, and cell cycle [37]. Increasing numbers of studies provide evidence for influence of miRs on human cancers, including HCC [26–29]. Therefore, miRs are considered as potential diagnostic biomarkers in the cancer detection [38]. Due to the lack of reliable biomarkers with high sensitivity and specificity currently, we conducted this study to identify whether two miRs (miR-375 and miR-199a-3p) could have potential diagnostic value in HCC detention.

The miR-375 gene is located at the human chromosome 2q35 region. The miR-375, which has key roles in expression of insulin gene and regulation of β-cell and pancreatic carcinoma cell growth, is well known as a pancreatic islet-specific miR [39, 40]. Recently, several studies have reported dysregulated expression of miR-375 in many human cancers, such as HCC [41], gastric cancer [42], adenocarcinoma of the esophagus [43], breast cancer [44], and non-small cell lung cancer [45]. Accumulating evidence has indicated that miR-375 acts as a tumor suppressor by suppressing many critical oncogenes [46]. Besides, miR-375 could reportedly have a role in a variety of tumors as a prognostic biomarker [43, 47–50]. Hu et al. [51] reported that laryngeal squamous cell carcinoma patients with low expression of miR-375 had poorer survival rate than those with high expression of miR-375. Chang et al. [48] also pointed out that downregulation of miR-375 expression in human gliomas may play an inhibitory role during the tumor development, and the miR might function as a candidate unfavorable prognostic marker. The potential diagnosis accuracy of miR-375 remains unclear yet. In this study, we observed miR-375 could act as a novel biomarker for HCC detention with the AUC of 0.637.

Human miR-199a-3p has an effect on the progression of several human cancers, including HCC, by downregulating phosphorylated-S6 protein [52–54]. Fornari et al. [54] in their study found the miR-199a-3p levels were inversely correlated with mammalian target of rapamycin protein expression in human HCC samples and concluded that miR-199a-3p may play a role in the invasion capability of HCC cells. In the present study, clinicopathological survey showed that the serum miR-199a-3p level was associated with drinking consumption, suggesting a decreased expression of miR-199a-3p in the patients with long-term drinking. After the ROC curve analysis, the results revealed that miR-199a-3p may be a potential serum biomarker to distinguish cancer patients from non-cancer patients.

Some limitations should be considered when interpreting the results of this study. First, relatively small sample size may cause significant fluctuations in statistics. Second, we did not replicate the results in another group. Third, only two miRs were examined and some potentially relevant markers could not be taken into account.

Conclusions

In the current study, the results showed the expression levels of miR-375 and miR-199a-3p in HCC patients were lower than those of the two miRs in healthy controls. And the ROC results displayed significant diagnostic accuracy of the two miRs. The findings indicated serum miR-375 and miR-199a-3p appeared to be potentially useful biomarkers for HCC detection. Further study with larger sample involving in validation and optimizing improvement should be conducted to confirm our results.

Conflicts of interest None.

Funds None.

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