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Original Article

Association of serum miR-205 with liver cirrhosis and cancer and its diagnostic significance

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Abstract: Objective: Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide. A sensitive and reliable biomarker for early-stage HCC detection would facilitate its diagnosis, thus improve the survival of HCC patients. Several recent studies have shown that microRNA was able to be detected in the systemic circulation and could be used as a biomarker for diagnosis and prognosis-prediction of malignancy. In our study, we examined the expression level of serum microRNA-205 (miR-205) in HCC patients and analyzed its potential diagnostic value in the treatment of HCC. Methods: Clinicopathological data and serum samples from 33 HCC patients (HCC group), 22 cirrhosis patients (Cirrhosisgroup), and 31 healthy controls were collected respectively. Quantitative real-time PCR was applied to detect the expression level of serum miR-205. The receiver operating characteristic (ROC) curve was used to predict the cutoff value of miR-205. Association between expression level of serum miR-205 and liver cirrhosis and cancer was explored. Results: Serum miR-205 level was remarkably decreased in patients with cirrhosis or HCC compared with healthy controls ($P<0.05$). ROC curve analysis showed that miR-205 had a better diagnostic accuracy with an area under curve (AUC) of 0.784 (95% CI: 0.661-0.906, $P<0.001$) compared with α-fetoprotein method with an AUC of 0.689 (95% CI: 0.548-0.830, $P<0.05$). Additionally, miR-205 could distinguish the patients with HCC or cirrhosis from healthy subjects with a sensitivity of 76% and a specificity of 73%. Conclusion: Expression of miR-205 was significantly downregulated in serum of patients with HCC or cirrhosis, which could be applied to robustly distinguish these patients from healthy control subjects with better sensitivity and specificity. Therefore, serum miR-205 might be utilized as a new and promising noninvasive circulating tumor biomarker in the diagnosis for HCC.

Keywords: miR-205, hepatocellular carcinoma, cirrhosis, diagnostic biomarker

Introduction

Hepatocellular carcinoma (HCC) is of high frequency and globally, its incidence and mortality ranked 5th and 3th, respectively, among various human malignant tumors [1]. About 80-90% of liver cancer cells develop from chronic liver diseases such as liver cirrhosis which caused by chronic hepatitis B and C virus infection, alcohol abuse and non-alcoholic fatty liver disease [2]. As the poor prognosis and a high mortality for patients diagnosed via radiological imaging and α fetoprotein (AFP) analysis method at present, identifying novel and non-invasive biomarkers are of great clinical significance to improve the diagnostic accuracy for HCC [3, 4].

Recent study has suggested that microRNA (miRNA) was detectable in the circulation and could be used as a biomarker for diagnosis and prognosis-prediction of malignancy [5]. miRNA is a small, evolutionarily conserved, and single-stranded RNA molecule which consists of 19-25 nucleotides. By binding to specific mRNA, miRNA can regulate expression of various genes which are involved in a variety of essential cellular processes including cell growth, differentiation, apoptosis, metabolism as well as pathological progression of HCC with tumor-suppressive effects [6, 7]. Meanwhile, most of cancer-associated miRNAs can be detected in blood [8, 9].

Various studies have suggested that miRNA-205 (miR-205) is an important tumor suppres-

Table 1. Clinicopathological characteristics of study participants

Variable	Healthy controls	Cirrhosis group	HCC group	*P*
Age (year)	51 ± 9	47 ± 11	54 ± 11	0.0705
Gender (M/F)	30/1	20/2	27/6	0.1476
ALT (U/L)	19.9 ± 6.37	72.27 ± 54.60	62.24 ± 65.89	0.0003
AST (U/L)	20.87 ± 4.121	97.59 ± 56.13	113.1 ± 123.5	<0.0001
GGT (U/L)	27.68 ± 11.26	75.14 ± 52.90	193.5 ± 250.2	0.0013
ALB (g/L)	46.69 ± 2.560	33.30 ± 4.511	36.63 ± 5.637	<0.0001
TBIL (μmol/L)	15.10 ± 3.273	211.2 ± 193.5	41.95 ± 73.51	<0.0001
Creatinine (mmol/L)	82.11 ± 19.08	73.14 ± 17.26	88.38 ± 29.64	0.0659
INR	1.010 ± 0.24	1.900 ± 0.8666	1.234 ± 0.3527	0.0002
Hemoglobin (g/dL)	151.2 ± 16.06	107.7 ± 23.71	125.7 ± 25.78	<0.0001
HBV/HCV infection	NA	20/2	31/2	NA
AFP	2.155 ± 4.019	61.72 ± 204.3	412.6 ± 517.9	<0.0001

Note: ALT, alanine transaminase; AST, aspartate aminotransferase; ALB, albumin; TBIL,total bilirubin; INR, International normalized ratio; NA, not applicable.

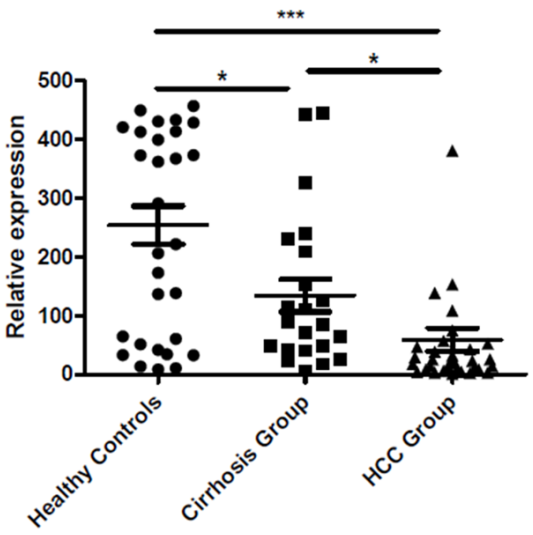

Figure 1. Relative expression of serum miR-205 in three groups. Levels of serum miR-205, represented as relative quantitation (RQ), were significantly lower in cirrhosis and HCC patients compared with healthy controls (P<0.05). Results are expressed as mean of RQ ± standard error. *P<0.05; ***P<0.001.

sor [10-12]. Its expression is drastically reduced in a variety of malignant lesions such as lung, breast, prostate, colon, and renal cancers compared with the corresponding normal adjacent tissues [13-16]. Previous studies have also implied the regulating role of miR-205 in cell cycle and apoptosis [17]. In addition, the decreased expression of miR-205 might be associated with a lower survival rate of patients with HCC [18].

In this study, we evaluated and compared the role of miR-205 as a circulating diagnostic marker in cirrhosis-associated HCC with AFP method in order to provide a novel insight for the clinic HCC therapy.

Materials and methods

Patients and samples

This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Human Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. A written informed consent was provided by each participant for clinical data documentation and sample collection.

Thirty-three HCC patients (HCC group) and 22 cirrhosis patients (Cirrhosis group) who received treatment in The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, from July 2014 to June 2015 were enrolled and followed up regularly. Meanwhile, 31 healthy volunteers (Healthy controls) who came to our hospital for regular physical checkups were also recruited. All the healthy subjects were negative in HBV, HCV, and HIV infections and had normal serum ALT and AST levels with reference ranges of 3-35 and 13-40 U/L, respectively.

Patients who received chemotherapy or radiotherapy or under immunosuppression medication were excluded to avoid any potential influences on miRNA analysis. All HCC patients accompanied with cirrhosis, while the healthy controls were not. Clinicopathological data were collected for all participants whose characteristics are described in **Table 1**.

5 mL venous blood was withdrawn from each participant and then centrifugation at 1,200 g for 10 minutes to separate the serum form the blood samples. The supernatant serum was collected and stored at -80°C for further analysis.

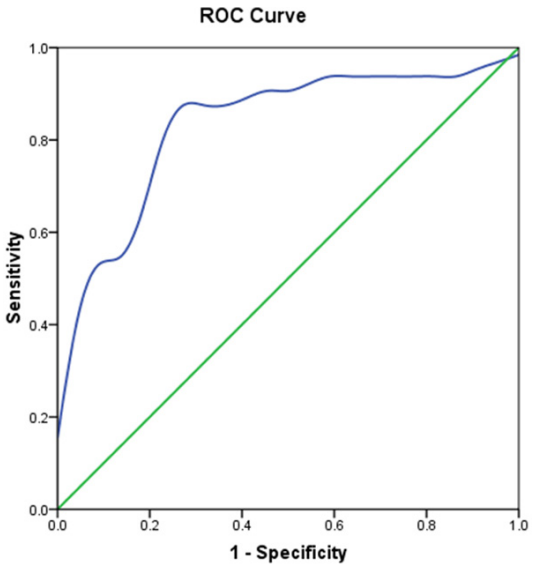

Figure 2. ROC-Curve for serum miR-205 as a diagnostic marker for HCC.

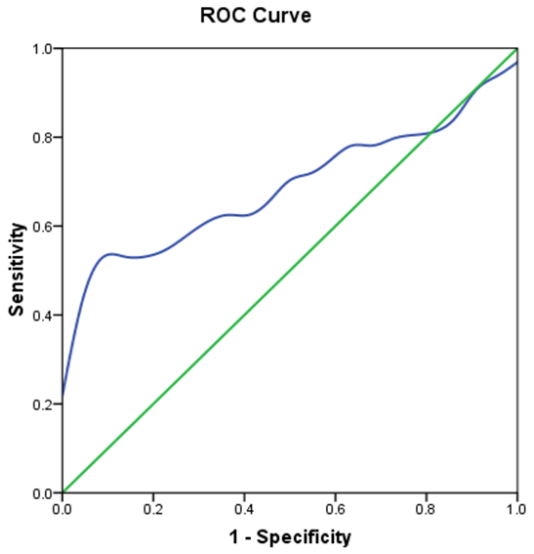

Figure 3. ROC-Curve for serum AFP as a diagnostic marker for HCC.

RNA extraction

Total RNAs were isolated from serum samples using TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quality of RNA was assessed by the spectrophotometer. RNA concentration and purity were measured using RNA 6000 Nano/Pico LabChip (Agilent Tech., Boeblingen, Germany). The extracted total RNA which included miRNA from both tissue and serum was subjected to reverse transcription.

RT-qPCR

Total RNA was reverse-transcribed using miScript II RT kit (Qiagen, Valencia, CA, USA), followed by real-time quantitative PCR (RT-qPCR) using miScript SYBR Green Kit (Qiagen, Valencia, CA, USA) and a LightCycler 480 system (Roche, Basel, Switzerland) according to the manufacturer's instructions. For quantification of miR-205 expression in serum, the U6 small nuclear RNA was used as the reference to normalize the data. The PCR was performed in 96-well plates with a volume of 15 μL for each reaction. The cycling conditions included an initiation step at 50°C for 2 minutes, a denaturation step at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds and 85°C for 5 minutes. All reactions were performed in triplicate. Template controls were used to evaluate the background signal in 96-well plates. Relative expression of miR-205 was calculated using the Livak $2^{-\Delta\Delta Ct}$ (cycle threshold) method [19].

Indicators

The predicted probability of diagnosis for patients with cirrhosis or HCC was used to construct the receiver operating characteristic (ROC) curve, which was then performed to select the most appropriate cut-off values for miR-205 to diagnose and stratify patients. Area under the ROC curve (AUC) with its corresponding 95% confidence interval (CI) was used to determine the specificity and sensitivity of serum miR-205 as a diagnostic marker for HCC.

Statistical analysis

Statistical analysis was performed using the SPSS software (version 18, SPSS Inc., Chicago, IL, USA). Continuous data were expressed as mean ± standard deviation and differences between two groups were evaluated using Student's t-tests. One-way ANOVA was used for comparing the continuous variables. A *P* value less than 0.05 was considered statistically significant.

Results

Clinicopathological characteristics of study participants

The HCC group consisted of 33 patients (27 males and 6 females), with a mean age of 54 ±

Table 2. Diagnostic performance of serum miR-205 and α-fetoprotein for hepatocellular carcinoma

Predictor	AUC	95% CI	*P* value[a]	Cutoff value	Sensitivity (%)	Specificity (%)
miR-205	0.784	0.661-0.906	0.000	47.43	0.76	0.73
AFP	0.689	0.548-0.830	0.019	20	0.59	0.68

Note: [a]The *P* value for AUCs regarding diagnostic values of miR-205 and AFP on HCC. AFP, α fetoprotein; AUC, area under ROC curve; ROC, receiver operating curve; CI, confidence interval.

11 years. The cirrhosis group consisted of 22 patients (20 males and 2 females), with a mean age of 47 ± 11 years. Thirty-one participants (30 males and 1 female), with a mean age of 51 ± 9 years were recruited as healthy controls (**Table 1**).

As shown in **Table 1**, HBV infection and HCV infection are the most common risk factors of chronic liver diseases in both the HCC and cirrhosis groups. Moreover, no significant differences were observed between the HCC and the cirrhosis groups in terms of liver function. While AFP, a traditional marker for HCC, was significantly increased in HCC patients. In addition, there were increasing trends in ALT, AST, GGT, ALB, INR, hemoglobin, and AFP levels in cirrhosis and HCC patients compared with healthy controls.

Expression of circulating miRNA-205

Serum miR-205 was significantly decreased in the serum of patients with HCC or cirrhosis compared to healthy controls ($P<0.05$). Furthermore, miR-205 was significantly decreased in the serum of HCC patients compared to that of cirrhosis patients ($P<0.05$, **Figure 1**).

Evaluation of miRNA-205 as a diagnostic serum marker

As significantly lower circulating miR-205 expression in patients with cirrhosis-associated HCC has been observed, we further evaluated the diagnostic sensitivity, and specificity of serum miRNA-205 as a biomarker to differentiate HCC patients from cirrhosis patients and healthy controls by plotting ROC curves. The level of miR-205 expression could distinguish patients with cirrhosis-associated HCC from healthy controls as shown in **Figure 1**. We next examined the efficacy of miR-205 and AFP detection in differentiating patients with HCC from controls. miR-205 had a greater AUC (0.784, 95% CI: 0.661-0.906) than AFP (0.689, 95% CI: 0.548-0.830), suggesting that miR-205 is superior to AFP in diagnosing HCC patients with cirrhosis (**Figures 2**, **3**). With the optimal diagnostic cut-off value of 47.43, miR-205 had a sensitivity of 0.76 and a specificity of 0.73based on the ROC curve. However, with a cut-off value of 20 ng/μL, the sensitivity of AFP was only 0.59 and the specificity was only 0.68 (**Table 2**). Subsequently, we then investigated serum miR-205 level in AFP-normal (<20 IU/mL) HCC patients (34%, 12/33) and 75% (9/12) of them showed declining miR-205 levels using the optimal diagnostic cut-off value of 47.43.

Discussion

Patients with HCC have a poor prognosis and a high mortality, which is partially due to the lack of methods for specific detection [20]. Current diagnostic methods based on radiological imaging and serum AFP analysis remain deficient for HCC patients with liver cirrhosis at surgically manageable stages [21]. Therefore, it is of vital importance to identify a novel and noninvasive biomarker to improve the diagnostic accuracy in HCC treatment [9, 22].

Emerging evidence has suggested that miRNAs synthesized by tumor tissues are usually released into blood [23]. Hence, the circulating miRNA might reflect the pathological conditions of tumors (such as tumor burden, and metastasis). A recent study indicated that miRNA is detectable in both blood and tumor tissue of patients with HCC [24], suggesting miRNA could be applied as a potential noninvasive diagnostic biomarker.

Research has confirmed that miR-205 could suppress cell proliferation, migration, invasion, and promote apoptosis [25]. For example, miR-205 is decreased at the early stages of liver regeneration, suggesting the role of miRNA plays in hepatocyte proliferation [26, 27]. Thus, all of these functions further underscore that miRNA-205 plays an essential role in the development and progression of HCC, which may be the reason for the lower level of miR-205 in serums of HCC group and cirrhosis group.

Additionally, this study demonstrated that serum miR-205 level was significantly decreased in HCC patients compared to the healthy controls. Compared to AFP, miR-205 is a better biomarker to distinguish HCC patients from others based on the ROC curve analysis.

miRNA acts through silencing genes which has been confirmed that miRNA could target and silence phosphatase and tensin homolog (PTEN) in HCC cells to regulate the AKT/mTOR pathway [28]. Briefly, as a tumor-suppressor gene correlates with poor prognosis, the reduction of PTEN leads to phosphorylation of mTOR, which will suppress cell proliferation [29, 30].

In summary, our results indicate that serum miR-205 was gradually decreased in cirrhosis and cirrhosis-associated HCC patients. Meanwhile, compared to AFP analysis, serum miR-205 is another biomarker for the diagnosis of cirrhosis-associated HCC patients with higher specificity and sensitivity and better application potentiality. Nevertheless, trials with larger sample sizes and samples from other ethnic populations are needed for further confirmation before any application in clinic.

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Disclosure of conflict of interest

None.

Abbreviations

miRNA, microRNA; 3' UTR, 3' untranslated region; AFP, α fetoprotein; AUC, area under curve; HCC, hepatocellular carcinoma; PTEN, phosphatase and tensin homolog; ROC, receiver operating curve; CI, confidence interval; RT-qPCR, quantitative reverse transcriptase polymerase chain reaction; ALT, alanine transaminase; AST, aspartate aminotransferase; ALB, albumin; TBIL, total bilirubin; INR, International normalized ratio.

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