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Serum hsa-miR-299-5p and hsa-miR-379-5p are novel diagnostic markers for HBV-related hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) represents a leading cancer worldwide, especially in Southeast Asia, where hepatitis B virus (HBV) infection is common. Overall, the prognosis of HCC is poor due to an advanced tumor stage at the diagnosis. MicroRNAs (miRNAs) are involved in several important biological processes and disease pathogenesis. Recently, dysregulated miRNAs have been reported in HCC and appear to be promising tumor biomarkers. The expressions of hsa-miR-299-5p and hsa-miR-379-5p in liver tissues and serum samples were analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Paired cancerous and adjacent non-cancerous liver tissues collected from surgical resection of 24 patients with early-stage HBV-related HCC were recruited. In addition, serum samples of 20 patients with HBV-related HCC, 20 patients with non-malignant chronic HBV infection and 10 healthy controls were examined. The expression of hsa-miR-299-5p in cancerous tissues was significantly downregulated compared with non-cancerous specimens (1.34±0.23 vs. 1.46±0.41, P=0.046). The expression of hsa-miRNA-379-5p in the corresponding specimens were 1.30±0.23 and 1.39±0.39, respectively (P=0.210). In serum samples, the expression of hsa-miR-299-5p in the HCC group (0.52±0.03) was significantly lower than those of the non-HCC group (1.17±0.07) and healthy controls (1.05±0.13) (P<0.001). Similarly, the corresponding figures for hsa-miRNA-379-5p expression in serum samples were 0.28±0.05, 1.16±0.25 and 1.08±0.18, respectively (P=0.002). Our findings demonstrated that the expressions of hsa-miR-299-5p and hsa-miR-379-5p were downregulated in cancerous tissues and serum specimens of patients with HBV-related HCC. These data indicate that serum hsa-miR-299-5p and hsa-miR-379-5p may represent novel diagnostic markers for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the one of common cancer worldwide, especially in Southeast Asia [1]. Chronic viral hepatitis infection, including hepatitis B virus (HBV) and hepatitis C virus (HCV) are major causes of HCC [2]. Previous reports have revealed that more than 60% of HCC patients are diagnosed in advanced stages [3]. As a consequence, curative treatment such as surgical resection or liver transplantation are not possible for these patients [4]. Currently, alpha-fetoprotein (AFP) is the most widely used serum marker for the detection and monitoring of HCC. However, serum AFP is not constantly increased to a diagnostic level in all patients, particularly in early-stage HCC. Thus, novel biomarkers including microRNA (miRNA) are highly needed [5]. Many evidences have supported that miRNAs can transport from intracellular into circulation by vesicle such as high-density lipoprotein (HDL), apoptotic bodies, exosome and ribonucleoprotein complexes [6, 7]. Dysregulated of miRNA expression in body fluid or circulation might be able to help disease prediction or diagnosis.

MiRNAs is a class of short non-coding RNA approximately 18-22 nucleotides in length that plays important roles in many cellular processes by regulate gene expression at post-transcriptional steps [8]. Functions of miRNAs are initiated by denatured miRNA duplex and RNA helicase, then single stranded miRNA is combined with Argonaute2 (Ago2) and target mRNAs to form RNA-induced silencing complex (RISC complex). MiRNAs use base pairing at nucleotide position 2-8 from the 5'UTR, seed region, to guide and bind to mRNA target that induces mRNA degradation when miRNA is complementary with mRNAs. Translational repression is induced by miRNA which is partial complementary with mRNAs [9]. Several studies have reported the roles of miRNAs in the liver such as lipid metabolism [10, 11], inflammation [12], apoptosis [13], hepatocyte proliferation [14], liver fibrosis [15, 16] and metastasis [17, 18]. Not only oncogene but also miRNAs can act as tumor suppressor gene because one miRNA can bind many mRNA targets at the same time [3]. Dysregulation of miRNAs might be able to relate to pathogenesis including hepatocarcinogenesis [19].

miR-299-5p and miR-379-5p are located in the delta-like 1 homolog–deiodinase, iodothyronine 3 (DLK1-DIO3) on the chromosome 14q32.31 [20, 21]. The DLK1-DIO3 region contains 54 miRNAs [22] that is associated with organ development and disease pathogenesis, especially carcinogenesis [23]. Previous studies reported that dysregulated miR-299-5p expression was associated with colorectal cancer [20], gastric adenocarcinoma [24], breast cancer [25], oral squamous cell carcinoma [26] and prostate cancer [27]. Moreover, dysregulation of miR-379-5p expression was found in several cancers including breast cancer [21], non-small cell lung cancer [28] and bladder cancer [29]. However, miR-299-5p and miR-379-5p has never been reported in sera of patients with HCC. In this study, the expression of miR-299-5p and miR-379-5p in tissue and sera of patients with HBV-related HCC were investigated. In addition, their association with clinicopathological characteristics were examined.

Methodology

Studied population and sample specimens

Paired cancerous and adjacent non-cancerous liver tissues collected from surgical resection of 24 patients with early-stage HBV-related HCC were recruited. In addition, serum samples of 20 patients with HBV-related HCC were collected. These patients were diagnosed HCC for the first time at King Chulalongkorn Memorial Hospital from July 2012 to July 2017. In addition, serum samples of 20 patients with non-malignant chronic HBV infection and 10 healthy controls were obtained as the control groups.

Tissue samples were stored in liquid nitrogen and serum samples were stored at -80°C until further analysis. Clinical data of patients were collected which included sex, age, liver function tests, Child-Pugh classification, serum AFP level and HCC staging classified by the

Barcelona Clinic Liver Cancer (BCLC) system. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand and all participants had provided written informed consent.

MiRNA isolation

Small RNAs including miRNAs were extracted from liver tissues using modified protocol from microRNA Purification Kit (#21300, NORGEN, Canada). Briefly, tissues samples were homogenized in 1 ml TRIzol® reagent (Invitrogen, USA) followed by 200 µl of chloroform and incubated at room temperature for 5 minutes. Total RNA in aqueous phase was collected after centrifuged at 8,760 rpm for 15 minutes at 4°C and then 96-100% ethanol was added at half the volume of aqueous phase. The mixture was applied in Large RNA Removal Column and centrifuged at 14,000 rpm for 1 minutes. The filtrate was then added 1 volume of 96–100% ethanol and applied in microRNA Enrichment Column before centrifuged at 14,000 rpm for 1 minute. Finally, small RNAs were eluted in 30 µl of elution solution. In addition, miRNAs were extracted from 200 µl of serum using miRNA Isolation Kit (RMI050, Geneaid, USA) according to the manufacturer's protocol and miRNAs were eluted in 30 µl of elution solution. Nanodrop was used to measure concentration of purified small RNAs that were stored at -80°C until cDNA synthesis.

cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)

Complementary DNA (cDNA) synthesis was performed by 10 µl of purified small RNAs from liver tissue and purified miRNAs from serum incubated at 37°C for 10 minutes. Tailing of poly U was added by poly(U) polymerase (M0337S, New England Biolabs, UK) and then was reverse transcripted into cDNA (#EP0441, Thermo Scientific, USA) using SL-polyA primer [30]. Total 20 µl of reverse transcription mixture was incubated at 42°C for 60 minutes followed by heat inactivation at 70°C for 10 minutes and stored at -20°C until miRNA expression detection. miRNA expression was detected by SYBR Green real-time PCR (BR0500402, Biotechrabbit, Germany) using Applied Biosystem 7500 Real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Each sample was analyzed in triplicate in tissue specimens and duplicate in serum samples. Total volume of reaction is 12.5 µl that contain 1X qPCR Green Master Mix HRox, 0.2µM of forward, 0.2µM reverse primer and 1 µl of template. The sequences of the primers that used in this experiment are shown in Table 1. The conditions of qRT-PCR were pre-denatured at 95°C for 3 minutes followed by 50 cycles of denaturing at 95°C for 30 seconds, annealing 20 seconds and fluorescence detection at 72°C for 20 seconds, each running included positive and negative controls.

Table1 Primers and annealing temperature for detection of miRNA expression by qRT-PCR

Primer name	Sequence (5' → 3')	annealing temperature
U6	CTCGCTTCGGCAGCACA	55°C
miR-299-5p	GTTTACCGTCCCACATACAT	56°C
miR-379-5p	TGGTAGACTATGGAACGTAG	58°C
reverse	GCAGGGTCCGAGGTATTC	

Normalization and Data analysis

The relative expression of miRNA was normalized using housekeeping reference gene, U6, and ANCTs were used as a calibrator. The relative miRNA expression in HCC tissue compared with ANCTs was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The statistical analysis was applied using SPSS 23.0 (SPSS Inc., Chicago, IL) and GraphPad Prism v5.0 (GraphPad Software, San Diego, CA). Values were presented as mean ±

standard deviation (SD), and percentages as appropriate. The association between patient's characteristics and miRNA expression was tested by a non-parametric test. Comparisons between groups were analyzed by Student's t-test for quantitative variables. The specificity and sensitivity of miRNA expression levels were analyzed by the Receiver Operating Characteristic (ROC) analysis to discriminating between HCC and non-HCC. *P*-values < 0.05 were indicated statistically significant results.

Results and Discussion

Clinical characteristics

The clinical characteristics of all subjects enrolled in this study are shown in Table 2. Compared with the non-HCC group, patients with HCC exhibited significant difference in gender distribution, mean age and AFP level, as well as most liver function tests.

Table 2 Baseline characteristics of all subjects in the study

Baseline Characteristics	**Liver tissue**	**Serum**			
	HCC	**Healthy**	**Non-HCC**	**HCC**	***P*-value**
N	**24**	**10**	**20**	**20**	
Gender					
Male	20 (83.33%)	4 (40%)	8 (40%)	16 (80%)	0.02*
Female	4 (16.67%)	6 (60%)	12 (60%)	4 (20%)	
Age (years)	59 (37-77)	50 (43-58)	48 (32-74)	57 (44-86)	0.013*
AFP (ng/mL)	7.4 (1.1-51924)		1.5 (0.9-3.8)	1589(2.12-149421)	< 0.001*
AST (IU/L)	184.5 (15-677)		24 (13-54)	120.5 (23-562)	< 0.001*
ALT (IU/L)	117 (15-518)		22.5 (11-114)	56.5 (10-209)	0.005*
ALP (IU/L)	88.5 (40-714)		55.5 (0-88)	153.5 (64-482)	< 0.001*
ALB (g/dL)	3.1 (1.9-4.3)		4.4 (3.7-5.1)	3.8 (2.3-4.5)	< 0.001*
TB (mg/dL)	1 (0.29-4.11)		1 (0-2)	1 (0.2)	0.146
PLT count (10^3/L)	182.5 (72-368)		209 (0-296)	228 (114-557)	0.954
Tumor size (cm)	4.5 (1.4-26)			5.85 (1.5-23.1)	
Child-Pugh					
A				16 (80%)	
B-C				4 (20%)	
BCLC stage					
0-A				4 (20%)	
B				4 (20%)	
C-D				12 (60%)	

Data are presented as median (range) or n (%); *, *P*-value<0.05

HCC, Hepatocellular carcinoma; AFP, alpha-fetoprotein; AST, aspartate amino transferase; ALT, alanine transaminase; ALP, alkaline phosphatase; ALB, albumin; TB, total bilirubin; PLT, platelet; BCLC, Barcelona Clinic Liver Cancer staging.

MiRNA expression in HCC and adjacent non-cancerous tissues

The expression of miR-299-5p was significantly lower in cancerous tissue than in the paired non-cancerous tissue (1.34±0.23 vs. 1.46±0.41, *P*=0.046) (Figure 1A). However, no significant difference was found regarding the expression of miR-379-5p in liver tissue (1.30±0.23 vs.1.39±0.39, *P*=0.210) (Figure 1B).

Previous studies have demonstrated that dysregulation of miRNAs is involved in pathogenesis of cancers. The DLK1-DIO3 is the paternal imprinting gene that is located on

chromosome 14q32.31. The domain contains miRNA cluster, 53 miRNAs on forward strand and only one miRNA on reverse strand [22]. The correlation between DLK1-DIO3 miRNA cluster and HCC tumor-initiating stem cell markers were previously investigated in HCC cell line and human HCC tissue specimens. Moreover, overexpression of DLK1-DIO3 miRNA cluster in HCC patients such as miR-154*, miR-154 and miR-376b were related to high levels of AFP and low survival rate [31]. In contrast, miR-1188, the one member of DLK1-DIO3, was reported as a tumor suppressor and was decreased in HCC cells[32]. Therefore, the functional roles of miRNAs located on DLK1-DIO3 are still inconclusive. However, these data suggest that miR-299-5p and miR-379-5p, the member of DLK1-DIO3 miRNA cluster, might play an important role in HCC development.

The present study demonstrated that downregulation of miR-299-5p and miR-379-5p were observed in HCC tissues compared with non-cancerous tissue, which was similar to other types of cancers [20, 21, 24, 25, 27-29]. It has been shown that osteopontin (OPN) regulated by miR-299-5p. It is secretory protein that involves in cell proliferation, tumor survival and metastasis [20, 24]. Moreover, miR-379-5p, is tumor suppressor and its expression is found relatively low in cancerous tissues [21, 28]. In HCC, this miRNA was reported to be associated with tumor invasion and metastasis by targeting FAK/AKT signaling *in vitro* and *in vivo* [33].

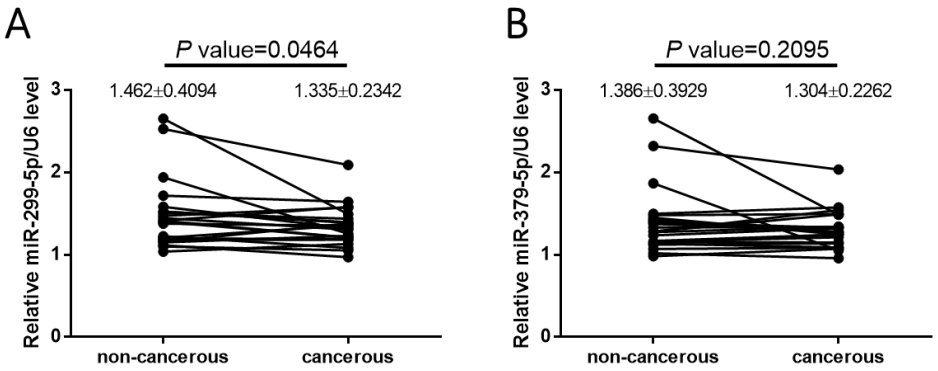

Figure 1 The relative expression of (A) miR-299-5p and (B) miR-379-5p in liver tissue specimens

MiRNA expression in serum specimens

The expression of miRNAs was also compared in serum samples of HCC, non-HCC and healthy controls. As shown in Figure 2A, significant downregulation of miR-299-5p was observed in the HCC group compared with the non-HCC group (0.52±0.03 vs 1.17±0.07, $P<0.0001$) and healthy controls (0.52±0.03 vs 1.05±0.13, $P<0.0001$). Similarly, the relative expression of miR-379-5p was significantly downregulated in the HCC group compared with the non-HCC group (0.28±0.05 vs 1.16±0.25, $P=0.0015$) and healthy controls (0.28±0.05 vs 1.08±0.18, $P<0.0001$) (Figure 2B).

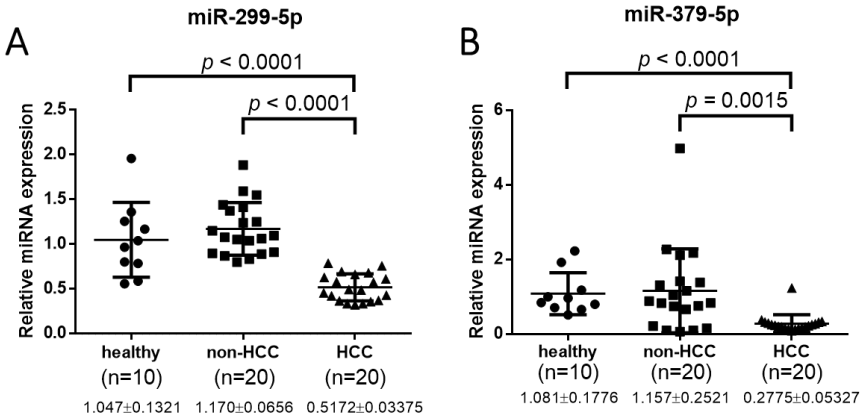

Figure 2 The relative expression of (A) miR-299-5p and (B) miR-379-5p in serum specimens of HCC compared with non-HCC and healthy controls

To our knowledge, this is the first report demonstrating the circulating expressions of miR-299-5p and miR-379-5p in patients with HCC. Our findings clearly showed that the expression of these miRNAs was significantly decreased in HCC compared with non-HCC and healthy controls. In fact, serum miR-299-5p and miR-379-5p represent novel non-invasive diagnostic markers for HCC with high sensitivities and specificities as shown in the ROC curves below (Figure 3). Of noted, miR-299-5p and miR-379-5p exhibited areas under the ROC (AUROC) of 0.97 and 0.83, respectively. The sensitivity and specificity of miR-299-5p were 95% and 93%, respectively, while the corresponding figures of miR-379-5p were 95% and 84%, respectively.

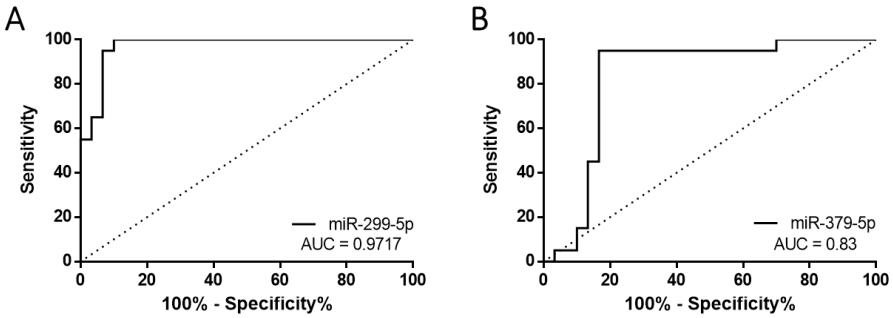

Figure 3 Receiver operating characteristic (ROC) curve of (A) miR-299-5p and (B) miR-379-5p

Conclusion

Our findings demonstrated that the expressions of hsa-miR-299-5p and hsa-miR-379-5p were downregulated in cancerous tissues and serum specimens of patients with HBV-related HCC. This data indicates that serum hsa-miR-299-5p and hsa-miR-379-5p may represent novel diagnostic markers for HCC with high sensitivity and specificity. Further studies are required to investigate target genes and molecular mechanisms of hsa-miR-299-5p and hsa-miR-379-5p.

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