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Exosomal miR-1821, miR-301a, and miR-373 as potential biomarker in NASH-induced liver cirrhosis with hepatocellular carcinoma.

Allia Najmie Muhammadi Y usuf[1,4] MD Raja Affendia Raja Ali[2,3], Khairul Najmia Muhammad Nawawi[2,3], Norfilza Mohd Mokhtar[1,3*]

[1] Department of Physiology, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia

[2] Department of Medicine, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia

[3] GUT Research Group, University Kebangsaan Malaysia, Kuala Lumpur, Malaysia

[4] Biomedical Sciences and Therapeutic Department, University Malaysia Sabah, Sabah, Malaysia

Abstract

Exosomal miRNAs was reported to play roles in the pathogenesis of various types of malignancies. This study aimed to identify the expression level of chosen miRNAs (miR-182, miR-301a and miR-373) in exosomes of serum and ascitic fluid in patients with non-alcoholic steatohepatitis (NASH)-related liver cirrhosis with or without hepatocellular carcinoma (HCC). A literature search was performed to identify potential miRNAs involved in this cancer. Unpaired serum and ascitic fluid were obtained from 52 patients with NASH-related liver cirrhosis with or without HCC. Exosomal miRNA was isolated from all samples. Expression levels of miR-182, miR-301a and miR-373 were determined using qPCR. Serum-derived exosomal mir-182, miR-301a and miR-373 were significantly up-regulated with fold-change of 1.77, 2.52, and 1.67 ($p<0.05$) respectively in NASH-induced liver cirrhosis with HCC as compared to NASH-induced liver cirrhosis without HCC. The expression levels of ascitic fluid-derived exosomal mir-182, miR-301a, and miR-373 were significantly up-regulated with fold-change of 1.6, 1.94 and 2.13 respectively in NASH-induced liver cirrhosis with HCC as compared to NASH-induced liver cirrhosis without HCC ($p<0.05$). High expression of the selected exosomal miRNAs in both serum and ascitic fluid suggesting the possible roles of these miRNAs as circulating biomarkers for NASH-induced liver cirrhosis with HCC.

Keywords: Exosomes, mir-182 microrna, Human:mir-301 microrna, Human:mir-373 microrna, Human:hepatocellular carcinoma.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the second most common cause of cancer-related deaths worldwide [1,2]. HCC is the third most common cancer-related death in the Asia-Pacific region [3]. In Malaysia, HCC is the second most common malignancy in the digestive tract following colorectal cancer in Malaysian men. According to the Malaysia National Cancer Registry, the incidence was predominantly in males and increasing with age. The lifetime risk was 1 in 144 for males and 1 in 418 for females [4]. Based on the previous epidemiological data, HCC may arise from multiple aetiologies and associated with many risk factors. The progressive chronic liver diseases that eventually leads to cirrhosis, and HCC were mainly caused by hepatitis B virus (HBV), hepatitis C virus (HCV), heavy alcohol consumption, and non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH) [5,6]. There are other risk factors such as hemochromatosis, aflatoxin, diabetes mellitus, obesity, and other metabolic disorders. Liver cirrhosis is one of the common risk factors for progression to HCC, where the cause of death from liver cirrhosis alone was estimated to cause over 1.2 million deaths (2% of global deaths) in 2013. In Malaysia, 16.4% of HCC patients were cryptogenic [7]. The increasing incidence of metabolic disorders, diabetes mellitus, as well as fatty liver among Malaysians, led to a dramatic increase in HCC associated with NAFLD/NASH [8,9]. At present, in Malaysia, mainly, the survival rate of HCC is abysmal as the majority of patients presented with an advanced stage of the disease. This was because most patients were asymptomatic on their first presentations [4,7]. The current treatments available in this clinical setting are curative surgical resection, supportive therapy such as non-curative transarterial chemoembolization (TACE), percutaneous ethanol injection, radiofrequency

ablation (RFA) and palliative care for the advanced stage of the disease.

Liver transplantation is one of the recommended treatments for HCC; however, it is not well established in Malaysia [7]. Early detection and prompt treatment are vital for better prognosis. Currently, the screening modalities for HCC in the high-risk population at the regular intervals of six months are an abdominal ultrasound and serum alpha-fetoprotein (AFP). However, the limitations of the current existing screening modalities include operator-dependency for ultrasound detection of HCC at an early stage and reduced sensitivity and specificity for AFP. MicroRNA (miRNA) has been investigated or proposed as a potential biomarker for a screening tool in HCC patients [10]. MiRNAs are small, non-coding RNA molecules (19-25 nucleotides) that are important in post-transcriptional gene regulation whereby specific mRNA expression is suppressed by either translation inhibition or degradation [11]. The involvement of miRNAs in cancer pathogenesis is well-established as they can behave as either as an oncogene or tumor suppressor gene depending on their targets. The role of miRNA has been of interest, particularly in cancer research, especially in the development of non-invasive biomarker for screening, diagnostic, prognostic, and therapeutic purposes [12]. Previous studies have discovered that miRNAs in cancer were associated with increased cell proliferation, tumor invasion, tumor metastasis, angiogenesis, apoptosis, immune response, and cell metabolism [13].

The clinical applications of miRNAs have been studied in most cancers, namely blood, breast, lung, brain, ovarian, and prostate cancers [12,14,15]. To date, the study in circulating biofluid biomarker has been of interest because serum and plasma are natural to collect and reproducible. In the context of HCC, many studies have been conducted to determine the expression level of circulating miRNAs and some miRNAs have been reported as candidates for biomarker associated with the cancer [16]. Exosomes are complex (30-150 nm) vesicles that are generated from multivesicular bodies intracellularly, which released by many types of cells. The exosomes contain RNAs (mRNAs and miRNAs) as well as proteins with the potential of regulating signaling pathway of the recipient cells.

The critical roles of exosomes are to deliver the RNAs or proteins via cell to cell communication and modulating microenvironment. Given the complicated microenvironment of the peripheral blood, exosomal miRNAs have been studied as a potential biomarker because of its properties, which are stable in the blood due to the presence of protective membrane of exosomes against RNAse, an enzyme that is responsible for RNA degradation [17,18]. This study aimed to investigate the feasibility of using peripheral blood serum as well as ascitic fluid using exosomal miRNAs as potential diagnostic biomarker for HCC, particularly in NASH-induced liver cirrhosis.

Materials and Methods

Patients recruitment

Patients were recruited from the Gastroenterology clinic, University Kebangsaan Malaysia (UKM) between April 2018 until December 2018 after the study was approved by the institutional ethical committee. Written informed consent was obtained from each patient enrolled in this study. An equal number of patients, n=26 each, were selected from NASH induced liver cirrhosis with HCC as the disease group and NASH induced liver cirrhosis without HCC, which acts as the control. Alanine aminotransferase (ALT), alkaline phosphatase, total bilirubin, albumin, serum alpha-fetoprotein (AFP) and abdominal ultrasonography were checked in the enrolled patients. The diagnosis of liver cirrhosis was based on clinical symptoms and abdominal ultrasound. The presence of cirrhosis is defined as coarse echo texture with irregular margin and surface nodularity. Other ultrasound findings, such as the presence of ascites, splenomegaly, and esophageal varices were noted. HCC was diagnosed based on the computerized tomography-multiphase findings, described as the presence of arterial enhancement of a nodule with a size of 2 cm or more with subsequent washout on the portal or delayed phases.

Serum and ascitic fluid sample collection

Peripheral blood serum (6 ml) was collected in a plain tube (BD Vacutainer (Franklin Lakes, New Jersey, and United States) from each patient at the time of diagnosis. The whole blood was left for 1 hour at room temperature (15-25°C) for complete clotting. Then, the sample was subjected to a two-spin protocol (1,900 X g for 10 min and 16,000 X g for 10 min) to collect the serum phase for the first spin. The second spin was to remove additional cellular nuclear acids attached to cell debris. The serum collected will be stored in -80°C until further processing. Ascitic fluid (5 ml) was collected in a sterile specimen container and immediately subjected to the two-spin protocol as described earlier. The serum collected will be stored in -80°C until further processing.

Exosomal isolation and total RNA extraction

Isolation of the exosomes and the total RNA was extracted from 500 µL of peripheral blood serum and ascitic fluid using an exoRNeasy Serum/Plasma Midi Kit (Qiagen, Germany) according to the manufacturer’s protocol.

cDNA conversion and quantification of miRNA by qRT-PCR

A reverse transcription reaction was performed using a miRCURY LNA RT Kit (Qiagen, Germany) in a 10 µL solution containing 2 µL of 5 x miRCURY RT reaction buffer, 1 µL of 10 x miRCURY RT Enzyme mix, 0.5 µL of UniSp6 RNA spike-in, 2 µL of RNA template and 4.5 µL of RNase-free water. To synthesize cDNA, the reaction mixtures were incubated for 60 min at 42°C and 5 min at 95°C, after which the reactions were held at 4°C. Next, 4 µL of cDNA was

amplified using 5 µL of miRCURY SYBR Green Master Mix and 1 µL of nuclease-free water in a final volume of 10 µL. Quantitative PCR was run on a Bio-Rad CFX, and the reaction mixtures were incubated at 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec and 56°C for 1 min. The miRNA expression from blood serum and ascitic fluid samples were normalized using 2-ΔΔCt method relative to miR-26a (endogenous control). The ΔCt was calculated after subtracting the Ct values of miR-26a from those of the miRNA of interest. The ΔΔCt was subsequently calculated after subtracting the mean of ΔCt of the serum of NASH-induced liver cirrhosis without HCC from the ΔCt of HCC [19,20].

Statistical analysis

All results except the miRNA values, gender, ethnicity, and underlying medical condition (i.e., were described as the median). The miRNA values were expressed as the mean ± S.E.M. The Mann-Whitney U-test was used to analyze differences between the two groups (serum LC vs. serum HCC and ascitic fluid LC vs. ascitic fluid HCC). The Spearman correlation coefficient, r, was used to evaluate the correlation of miRNA expression between peripheral blood serum and ascitic fluid exosomal miRNA in HCC. All data were statistically analyzed using GraphPad Prism 7 for Windows, Version 7.00 (GraphPad Software, La Jolla, CA, USA) Statistical significance was considered positive when $p<0.05$.

Results and Discussion

Clinical information of patients with HCC and non-HCC

The results are shown in Table 1. All patients (n=26) were diagnosed with liver cirrhosis with underlying NASH, was further divided to two groups either with HCC (group 1) or without HCC (group 2: control). In NASH-induced liver cirrhosis with HCC group, the ratio of men:women was 20:6 with a median age of 71.5. The majority of the patients were Chinese (42.3%), followed by Malays (38.5%) and Indian (19.2%). On the other hand, for the control group, the ratio of men:women was 18:8 with a median age of 60.5. In terms of ethnicity, this group consisted predominantly Malays (50%), followed by Chinese (38.5%) and Indian (11.5%). The serum albumin level was significantly lower for the HCC group than the control group ($p=0.023$). The serum AFP and ALT of the HCC group were significantly higher as compared with the control group ($p<0.0001$and $p<0.01$ respectively). There was no significant difference in the total bilirubin in the HCC as compared with the control group ($p=0.734$). The underlying medical conditions (diabetes mellitus, hypertension, and dyslipidemia) were comparable between both groups. The BMI was significantly higher in the control group as compared with the HCC group ($p<0.01$).

The expression level of serum exosomal miRNAs

The expression levels of three serum exosomal miRNA candidates between NASH-inducedliver cirrhosis with or

Table 1. *Clinical information on HCC patients and non-HCC patients (n=52).*

Clinical characteristics	HCC (n=26)	Non- HCC (n=26)
Age (years) (median)	71.5 (58-88)	60.5 (45-69)
Men	20 (69.2)	18 (76.9)
Women	6 (30.8)	8 (23.1)
Ethnicity (%)		
Malay	10 (38.5)	13 (50)
Chinese	11 (42.3)	10 (38.5)
Indian	5 (19.2)	3 (11.5)
Diabetes mellitus (%)		
Yes	23 (88.5)	24 (92.3)
No	3 (11.5)	2 (7.7)
Hypertension (%)		
Yes	21 (80.8)	19 (73.1)
No	5 (19.2)	7 (26.9)
Dyslipidemia (%)		
Yes	15 (57.7)	21 (80.8)
No	11 (42.3)	5 (19.2)
BMI (kg/m^2), (median)	23.15(18.9-33.2)	27 (19.5-46.7)
LFT (median)		
Albumin (g/l)	22.5 (15-25)	29.5 (19-37)
Total bilirubin (umol/L)	27.15 (6.8-342.2)	25.1 (7.6-316.2)
ALP (U/l)	132 (64-298)	95 (70-216)
ALT (U/l)	38.5 (19-235)	27 (7-89)
AFP (ng/ml)	16.9 (3.2-1332)	4.75 (0.8-9.9)
Child-Pugh score (%)		
A	11 (42.3)	
B	15 (57.7)	
C	-	
Tumor stage (BCLC) (%)		
0	9 (34.6)	
A	11 (42.3)	
B	6 (23.1)	
Tumor size (%)		
>2 cm	17 (65.4)	
<2 cm	9 (24.6)	

*AFP-alpha-fetoprotein; ALT, alanine aminotransferase-ALP, alkaline phosphatase; BMI-body mass index; HCC-hepatocellular carcinoma; LFT-liver function test; NS-not significant.

without HCC are demonstrated in Figure 1. The levels of serum exosomal miR-182, miR-301a and miR-373 were significantly increased in the NASH-induced liver cirrhosis with HCC group ($p<0.01$) as compared to the control group.

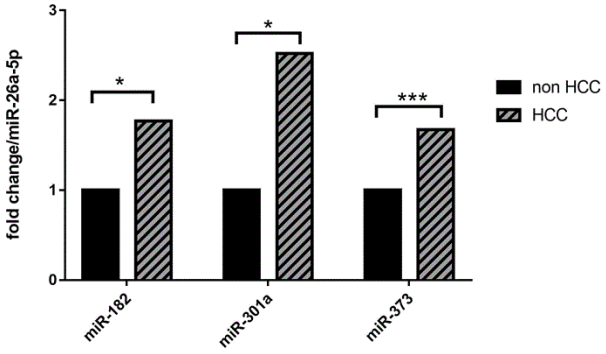

Figure 1. *Serum exosomal microRNAs from NASH-induced liver cirrhosis patients with and without HCC. The levels of serum exosomal miRNA were measured by RT-qPCR. The values of the relative gene expression for target microRNA were normalized to miR-26a-5p and calculated using the 2-ΔΔCT method. The level of serum exosomal miR-182, miR-301a, and miR-373 were up-regulated in the HCC group compared to non-HCC group ($^{*}p<0.05$, $^{***}p<0.001$). $p<0.05$ was considered statistically significant.*

The expression level of ascitic fluid exosomal miRNAs

The expression levels of three ascitic fluid exosomal miRNA candidates between NASH-induced liver cirrhosis with or without HCC are demonstrated in Figure 2. Compared with the control group, the levels of serum exosomal miR-182, miR-301a, and miR-373 were significantly increased in the NASH-induced liver cirrhosis with HCC group ($p<0.05$). The correlation of miRNA expression (miR-182, miR-301a and miR-373) between serum exosomal and ascitic fluid exosomal miRNAs were investigated in each group. There was poor correlation of the expression of miR-182 ($r=0.213$, $p=0.444$), miR-301a ($r=-0.242$, $p=0.422$) and miR-373 ($r=0.24$, $p=0.427$) between serum exosomal and ascitic fluid exosomal in the HCC group (Table 2).

Table 2. *Correlation of miRNAs expression between serum exosomal and ascitic fluid exosomal miRNAs in HCC group.*

MicroRNA	Correlation coefficient (r)	P-value	Confidence interval (CI)
miR-182	0.213	0.444	-0.351 to 0.663
miR-301a	-0.242	0.422	-0.709 to 0.372
miR-373	0.24	0.427	-0.375 to 0.708

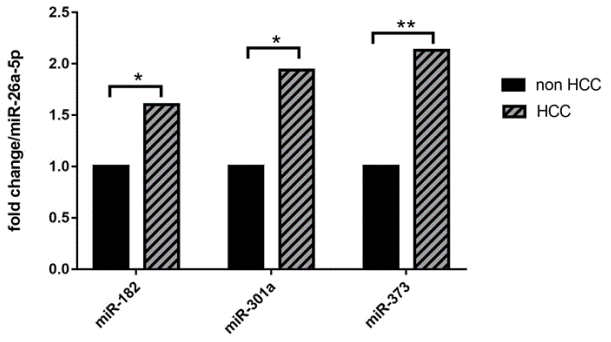

Figure 2. *Ascitic fluid exosomal microRNAs from NASH-induced liver cirrhosis patients with and without HCC. The levels of ascitic fluid exosomal miRNA were measured by RT-qPCR. The values of the relative gene expression for target microRNA were normalized to miR-26a-5p and calculated using the 2-ΔΔCT method. The level of ascitic fluid exosomal miR-182, miR-301a, and miR-373 were up-regulated in the HCC group compared to non-HCC group ($^{*}p<0.05$, $^{**}p<0.01$). $p<0.05$, was considered statistically significant.*

Recently, there has been a growing interest in understanding the roles of exosomal miRNAs in cancer. It has been reported that miR-182, miR-301a, and miR-373 were involved in the pathogenesis of HCC [3,21,22]. However, there have been limited reports on exosomal miRNAs concerning NASH-related liver cirrhosis with HCC. We investigated the levels of serum and ascitic fluid exosomal microRNAs in NASH-related liver cirrhosis with HCC and compared them with the levels observed in NASH-related liver cirrhosis without HCC. Compared with the previous studies, the current results were mostly consistent in tissue miRNAs from HCC. From our study, the levels of serum exosomal miR-182, miR-301a, and miR-373 were significantly higher in patients with NASH-related HCC than in a patient without HCC. Meanwhile, similar findings were noted in the levels of ascitic fluid exosomal of all chosen miRNA i.e. miR-182, miR-301a, and miR-373 in HCC as compared to non-HCC patients. This suggested that serum and ascitic fluid of the chosen exosomal miRNAs as potential biomarker for NASH-related liver cirrhosis with HCC. MiRNAs are essential regulators of gene expression and affect mRNA stability and functions. Differentially expressed miRNAs are responsible in a variety of disease processes such as lipid metabolism, inflammation, cell growth and differentiation, and also apoptosis [14,15,23-25]. MiRNAs are abundantly expressed in the liver where they regulate a diverse array of functions, in which the development of NASH-associated HCC has been closely

related with the dysregulation of miRNA expression, lipid metabolism, NF-KB, and PI3K-AKT-PTEN pathway [25,26].

Previous study revealed that there was an alteration in hepatic miRNA expression associated with NASH. For example, miR-122 was responsible for lipid metabolism, and its expression was found to be low in human liver samples from patients with NASH [27]. There are many other studies related to the progression and development of cancer based on the actions of miRNAs. Some miRNAs were down regulated while others were up regulated in cancer tissues as compared with non-tumor tissues. However, due to the highly invasive procedure in obtaining the liver tissues, circulating miRNAs, primarily exosomal miRNAs, are currently studied in various disease mechanisms, especially in cancer. Serum exosomal miRNAs are meanwhile investigated as a potential resource of biomarker because of its relatively stable nature [18]. Apart from that, exosomes can be regarded as one form of intercellular communication. Cancer cell-derived exosomes are essential in determining the tumor progression, including in HCC [17].

Our findings were also in consistent with the previous published data on circulating miRNAs. A study in 2012 demonstrated that up-regulation of miR-301a induced proliferation, migration, invasion, and apoptosis of HCC cells [28]. While miR-182 up-regulation contributed to the intrahepatic metastasis and early recurrence of HCC [21]. Additionally, miR-373 acted as the tumor oncogene and facilitated cell proliferation in HCC [22]. The exosomes derived from cancer cells are also found in the ascitic fluid. We have taken a step forward to study the exosomal miR-182, miR-301a, and miR-373 expression levels in NASH-related liver cirrhosis patients with HCC. We found out that the expression levels of these miRNAs were consistent with the previous study. These findings indicated that the ascitic fluid exosomal miRNAs are also could act as a potential resource of biomarker. There were several limitations identified in the current study. First, healthy subjects were not included as a control in this study. It is almost impossible to get ascitic fluid from healthy subjects. The risk of HCC is higher in a patient with underlying liver cirrhosis. Therefore, we mainly concentrate on the patients with underlying NASH-related liver cirrhosis with or without the presence of HCC. Second, the relationship between tissue and serum as well as ascitic fluid was not investigated because no liver biopsy was done. This is because the procedure is considered to be an invasive procedure and the risk of bleeding, is higher especially for a non-expert specialist. Third, this study was based on a relatively low number of subjects. Further large-scale study clarifying the role of serum and ascitic fluid exosomal miRNAs in NASH-related liver cirrhosis with HCC is needed.

Conclusion

In conclusion, there was a significant difference between the levels of serum exosomal miRNAs in patients with NASH-related liver cirrhosis with HCC compared to patients without HCC. The same pattern can be seen in the ascitic fluid exosomal miRNAs where there was a significant difference between the levels in patients with NASH-related liver cirrhosis with HCC. Our work represents an advance in biomedical science because it suggests that serum, and ascitic fluid exosomal miRNAs may be potential resources of biological marker for NASH-related liver cirrhosis with HCC.

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Conflict of Interest

All authors declare that they have no financial and non-financial conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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*Correspondence to

Norfilza Mohd Mokhtar

Department of Physiology

University Kebangsaan Malaysia

Kuala Lumpur

Malaysia