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ORIGINAL ARTICLE

Potential biomarkers in NASH-induced liver cirrhosis with hepatocellular carcinoma: A preliminary work on roles of exosomal miR-182, miR-301a, and miR-373

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Abstract

Introduction: Recent studies have published the roles of exosomal miRNAs in the pathogenesis of various type of malignancies and can be developed as potential biomarkers for diagnostic, prognostic and therapeutic purposes. The aim of this study was to identify the expression level of selected miRNAs (miR-182, miR-301a, and miR-373) in exosomes of the serum and ascitic fluid in patients with non-alcoholic steatohepatitis (NASH)-related liver cirrhosis with or without hepatocellular carcinoma (HCC). *Materials and Methods:* A literature search was performed to identify potential miRNAs involved in the pathogenesis of HCC. Unpaired serum and ascitic fluid were obtained from 52 patients with NASH related liver cirrhosis (n=26 for each group of with and without HCC). Exosomal miRNA was isolated from all samples. Expression levels of miR-182, miR-301a and miR-373 were determined using quantitative real-time PCR. *Results:* 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. We identified 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 ($p <0.05$). There was poor correlation expression of all the selected exosomal miRNA between serum- and ascitic fluid-derived in HCC group. *Conclusions:* This preliminary data showed significant increase in the expression levels of exosomal miR-182, miR-301a and miR-373 in both serum and ascetic fluid suggesting the possible roles of these miRNAs as circulating biomarkers for NASH-induced liver cirrhosis with hepatocellular carcinoma.

Keywords: exosomes, miR-182 microRNA, miR-301 microRNA, miR-373 microRNA, hepatocellular carcinoma

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 increases 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 disease that eventually

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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 Malaysia population, 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 chemoembolisation (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 regular interval 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 translational inhibition or degradation.[11] The involvement of miRNAs in cancer pathogenesis is well-established as they can behave as either as an oncogene or tumour 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, tumour invasion, tumour 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 biomarkers 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 biomarkers 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 signalling 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 biomarkers for HCC, particularly in NASH-induced liver cirrhosis.

MATERIALS AND METHODS

Patients recruitment

Patients were recruited from the Gastroenterology clinic, Universiti Kebangsaan Malaysia (UKM) between April 2018 till December 2018 after the study was approved by the institutional ethical committee. Written informed consent was obtained from each patient enrolled in this study. The diagnosis of NASH-induced liver cirrhosis was based on clinical, supported by biochemical including liver function test (albumin level, ALT), coagulation profile and abdominal ultrasonography. The presence of hepatic steatosis by abdominal ultrasonography is defined as diffuse increase in hepatic

echogenicity, or "bright liver", due to increased reflection of USG from the liver parenchyma, which is caused by intracellular accumulation of fat vacuoles supporting the diagnosis of NASH in right clinical settings.[19] The presence of cirrhosis is defined as coarse echotexture with irregular margin and surface nodularity as well as evidence of portal hypertension in addition with transient elastography (i.e. Fibroscan®).[20] Other ultrasound findings, such as the presence of ascites, splenomegaly, and esophageal varices were noted.

HCC was diagnosed based on the computerised 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.[21] There was no tissue biopsy performed for this study as the diagnosis were made biochemically and radiologically. Those with cardiac cirrhosis, secondary liver tumour, chronic hepatitis B and C virus infection, fatty liver, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD) and those who have undergone any chemotherapy regime or tumour resection were excluded from this study. A total number of 26 patients with NASH-induced liver cirrhosis with HCC were selected into the disease group and a total number of 26 patients with NASH-induced liver cirrhosis without HCC were selected into the control group. The assessment of alanine aminotransferase (ALT), alkaline phosphatase, total bilirubin, albumin, serum alpha-fetoprotein (AFP) and abdominal ultrasonography were recorded in the enrolled patients.

MiRNA candidates selection

A literature search using PubMed was performed to identify potential miRNAs as biomarkers in HCC. The selected miRNA candidates, namely miR-182, miR301a and miR-373 had been previously validated in HCC tissues. We limited the target miRNAs to oncogenic miRNAs , as previous studies have revealed that high plasma levels of oncogenic miRNAs might be derived from tumour necrosis, apoptosis, and the active release of secretory vesicles, such as exosomes, from cancer cells, which might reflect the tumour dynamics of in cancer tissues.[16,22-24] However, to the best of our knowledge, there is no human studies involving these miRNAs among HCC in NASH-induced liver cirrhosis.

Serum and ascitic fluid sample collection

Peripheral blood serum (6ml) was collected in a plain tube (BD Vacutainer (Franklin Lakes, New Jersey, 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 and to remove additional cellular nuclear acids attached to cell debris for the second spin. The serum collected was stored in -80°C until further processing.

Ascitic fluid (5ml) was collected in a sterile specimen container and immediately subjected to the two-spin protocol as described earlier. The serum collected was stored in -80°C until further processing.

Exosomal isolation and total RNA extraction

Isolation of exosomes and the total RNA was extracted from 500uL 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 10μL solution containing 2μL of 5x miRCURY RT reaction buffer, 1μL of 10x miRCURY RT Enzyme mix, 0.5μL of UniSp6 RNA spike-in, 2μL of RNA template and 4.5μL of RNase-free water. To synthesise 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, 4μL of cDNA was amplified using 5μL of miRCURY SYBR Green Master Mix and 1μL of nuclease-free water in a final volume of 10μL. 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 normalised using $2^{-\Delta\Delta Ct}$ 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.[25,26]

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 analyse 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 analysed using GraphPad Prism 7 for Windows, Version 7.00 (GraphPad Software, La Jolla, CA, USA). Statistical significance was considered positive when p-value <0.05.

RESULTS

Table 1 shows the clinical information of the patients with HCC and non-HCC in this study. All patients (n=26) were diagnosed with liver cirrhosis with underlying NASH, was further divided into 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 were 20:6 with a median age of 71.5. 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

TABLE 1: Clinical information on HCC patients and non-HCC patients. (n=52)

Clinical characteristis	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)	19 (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 (%)		
> 2cm	17 (65.4)	
< 2cm	9 (24.6)	

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; ALP, alkalinephosphatase; BMI, body mass index; HCC, hepatocellular carcinoma, NS, not significant.

were 18:8 with a median age of 60.5. In term of ethnicity, this group consisted predominantly Malays (50%), followed by Chinese (38.5%) and Indian (11.5%). The serum albumin level was significantly lower in the HCC group than the control group ($p = 0.023$). The serum AFP and ALT of the HCC group were significantly higher as compared to 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 to 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 to the HCC group ($p < 0.01$).

The expression level of serum exosomal miRNAs

The expression levels of three serum exosomal miRNA candidates between NASH-induced liver cirrhosis with or without HCC are demonstrated in FIG. 1. The level of serum exosomal miR-182 were significantly increased in HCC group by 1.77 (±0.13) as compared to the control group ($p = 0.045$). On the other hand, the expression level for serum exosomal miR-301a was also found to be significantly raised by 2.52 (±0.26) in HCC group as compared to control group ($p=0.016$). The same pattern can be found in serum exosomal miR-373 where it was significantly increased by 1.67 (±0.22) in the HCC group as compared to the control group ($p=0.0008$).

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 FIG. 2. The level of ascitic fluid exosomal miR-182 were significantly increased in HCC group by 1.60 (±0.208) as compared to the control group ($p = 0.042$). On the other hand, the expression level for ascitic fluid exosomal miR-301a was also found to be significantly raised by 1.94 (±0.327) in HCC group as compared to control group ($p=0.023$). The same pattern can be found in ascitic fluid exosomal miR-373 where it was significantly increased by 2.13 (±0.332) in the HCC group as compared to the control group ($p=0.0092$).

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).

DISCUSSION

MiRNAs are essential regulators of gene expression and affect mRNA stability and functions. Differentially expressed miRNAs are

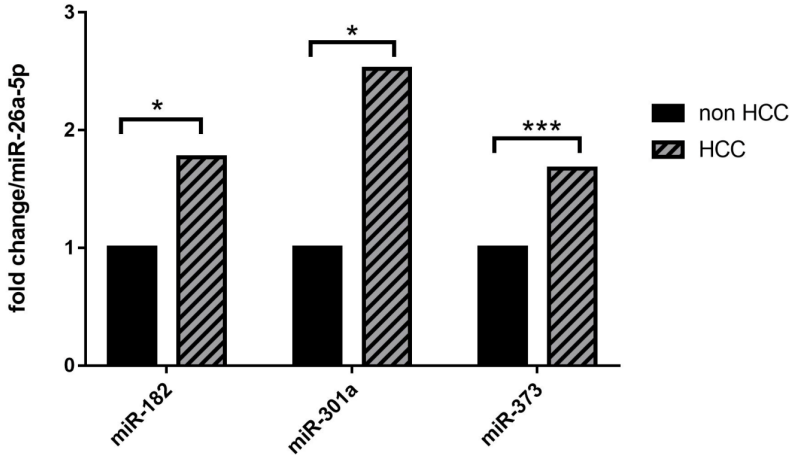

FIG. 1: Serum exosomal microRNAs from NASH-induced liver cirrhosis patients with and without HCC. The levels of serum exosomal miRNA were measured by RT-qPCR. The values of the relative gene expression for target microRNA were normalised to miR-26a-5p and calculated using the 2- ΔΔCT method. The level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

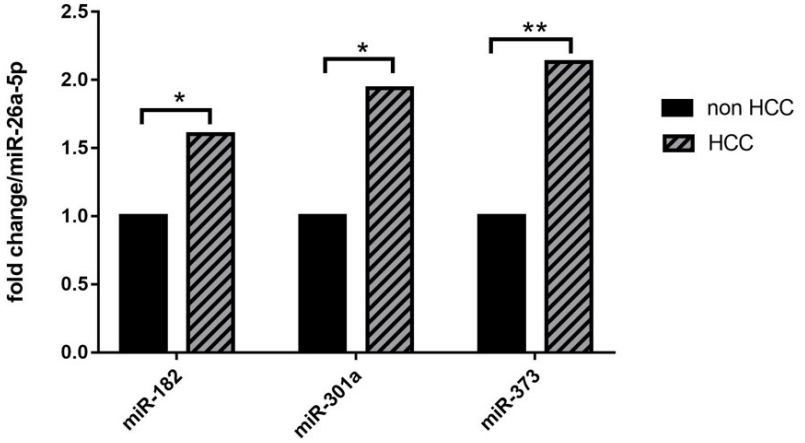

FIG. 2: Ascitic fluid exosomal microRNAs from NASH-induced liver cirrhosis patients with and without HCC. The levels of ascitic fluid exosomal miRNA were measured by RT-qPCR. The values of the relative gene expression for target microRNA were normalised to miR-26a-5p and calculated using the 2- ΔΔCT method. The level of ascitic fluid exosomal miR-182, miR-301a, and miR-373 were upregulated in the HCC group compared to non-HCC group

responsible in a variety of disease processes such as lipid metabolism, inflammation, cell growth and differentiation, and also apoptosis.[14,15,27-29] 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.[27,30] 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.[31]

Recently, there has been a growing interest in understanding the roles of exosomal miRNAs in cancer. There are many 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-tumour 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 at present investigated as a potential resource of biomarker because of its relatively stable nature.[17] Apart from that, exosomes can be regarded as one form of intercellular communication. Cancer cell-derived exosomes are essential in determining the tumour progression, including in HCC.[18]

It has been reported that miR-182, miR-301a, and miR-373 were involved in the pathogenesis of HCC.[22,24,32] Recently, miR-182 was found to be upregulated thus repressed its target gene FOXO3a and activating the AKT/FOXO3a pathway to promote HCC cells' proliferation in HCC cell lines. In addition, repressed FOXO3a also activating the Wnt signaling pathway by inhibiting the degradation of β-catenin and enhancing the interaction between β-catenin and TCF4. Thus, upregulation of miR-182 leads to the development of HCC.[33] In addition, miR-182 upregulation also contributed to the intrahepatic metastasis and early recurrence of HCC.[24]

On the other hand, the upregulation of miR-301a associated with pathogenesis of HCC by

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

negatively regulating its target gene, growth arrest-specific homeobox (Gax) gene in HCC tissue. Gax was shown to inhibit angiogenesis by targeting NF-κB gene and also promotes apoptosis. MiR301a was also found to modulate proliferations, migrations and invasion of HCC cell. Therefore, the upregulation of miR-301a is associated with tumourigenesis of HCC.[23]

Apart from that, miR-373 acts on its target gene protein phosphatase six catalytic subunit (PPP6C).[22] PPP6C induces cell cycle arrest at the G_1 / S checkpoint in cancer cells.[34] The upregulation of miR-373 negatively regulated PPP6C, thus promote HCC cells' proliferation in cell lines.[22] However, to the best of our knowledge, there was no study on the expression of these miRNAs (miR-182, miR-301a, and miR-373) in serum or ascitic fluid among HCC patients.

We investigated the levels of serum and ascitic fluid exosomal miRNAs 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 selected 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 selected exosomal miRNAs as a potential biomarker for NASH-related liver cirrhosis with HCC. Thus, our results showed that the selected miRNAs (miR-182, miR-301a, and miR-373) were upregulated and consistent with the previous published data on HCC tissue and cell lines.[22,23,33] However, among these three miRNAs (miR-182, miR-301a and miR-373) only miR-182 has been described significantly upregulated in NASH-induced liver cirrhosis as well as HCC in mice studies.[35] Hence, our results might provide insight on actions of miR-301a and miR-373 in progression to HCC among NASH-induced liver cirrhosis.

Interestingly, the exosomes derived from cancer cells are also found in the ascitic fluid. We found out that the expression levels of these miRNAs were similarly u pregulated consistent with its serum counterpart and there was no significant difference between its serum expression and its ascitic fluid expression. These findings indicated that the ascitic fluid exosomal miRNAs could also 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. 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. 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, the levels of serum exosomal miR-182, miR-301a and miR-373 were significantly up regulated in patients with NASH-related liver cirrhosis with HCC as compared to patients with NASH-related liver cirrhosis without HCC. The same pattern can be seen in the ascitic fluid exosomal miRNAs where there was a significant difference in 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 markers for NASH-related liver cirrhosis with HCC.

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Authors' contribution: NMM & RARA secured the grant, design the study and supervision. KNMN helped in the data analysis, ANMY conduct the experiment and responsible for the first draft of the manuscript.

Conflict of interest: The authors declare they have no conflict of interest.

REFERENCES

1. Bertuccio P, Turati F, Carioli G, *et al*. Global trends and predictions in hepatocellular carcinoma mortality. J Hepatol. 2017; 67(2): 302-9.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012.

CA Cancer J Clin. 2015; 65(2): 87-108.
3. Zhu RX, Seto WK, Lai CL, Yuen MF. Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region. Gut Liver. 2016; 10(3): 332-9.
4. Azizah AM., Hashimah B., Nirmal K, *et al*. National Cancer Registry Report 2012-2106. Malaysia: Ministry of Health 2019.
5. Abubakar I, Tillmann T, Banerjee A. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2015; 385(9963): 117-71.
6. Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. Lancet. 2014; 383(9930): 1749-61.
7. Goh KL, Razlan H, Hartono JL, *et al*. Liver cancer in Malaysia: epidemiology and clinical presentation in a multiracial Asian population. J Dig Dis. 2015; 16(3): 152-8.
8. Chan WK, Tan ATB, Vethakkan SR, Tah PC, Vijayananthan A, Goh KL. Non-alcoholic fatty liver disease in diabetics–prevalence and predictive factors in a multiracial hospital clinic population in Malaysia. Journal of gastroenterology and hepatology. 2013; 28(8): 1375-83.
9. Jan Mohamed HJ, Yap RW, Loy SL, Norris SA, Biesma R, Aagaard-Hansen J. Prevalence and determinants of overweight, obesity, and type 2 diabetes mellitus in adults in Malaysia. Asia Pac J Public Health. 2015; 27(2): 123-35.
10. Singal AG, Pillai A, Tiro J. Early detection, curative treatment, and survival rates for hepatocellular carcinoma surveillance in patients with cirrhosis: a meta-analysis. PLoS Med. 2014; 11(4): e1001624.
11. Farazi TA, Hoell JI, Morozov P, Tuschl T. MicroRNAs in human cancer. MicroRNA cancer regulation. Springer. 2013; 1-20.
12. Nana-Sinkam SP, Croce CM. Clinical applications for microRNAs in cancer. Clin Pharmacol Ther. 2013; 93(1): 98-104.
13. Anwar SL, Lehmann U. MicroRNAs: Emerging Novel Clinical Biomarkers for Hepatocellular Carcinomas. J Clin Med. 2015; 4(8): 1631-50.
14. Reddy KB. MicroRNA (miRNA) in cancer. Cancer Cell Int. 2015; 15(1): 38.
15. Xu X, Tao Y, Shan L, *et al*. The Role of MicroRNAs in Hepatocellular Carcinoma. J Cancer. 2018; 9(19): 3557-69.
16. Okajima W, Komatsu S, Ichikawa D, *et al*. Circulating microRNA profiles in plasma: identification of miR-224 as a novel diagnostic biomarker in hepatocellular carcinoma independent of hepatic function. Oncotarget. 2016; 7(33): 53820.
17. Devhare PB, Ray RB. Extracellular vesicles: Novel mediator for cell to cell communications in liver pathogenesis. Mol Aspects Med. 2018; 60: 115-22.
18. Abudoureyimu M, Zhou H, Zhi Y, *et al*. Recent progress in the emerging role of exosome in hepatocellular carcinoma. Cell Prolif. 2019; 52(2): e12541.
19. Lee SS, Park SH. Radiologic evaluation of nonalcoholic fatty liver disease. World journal of gastroenterology: WJG. 2014; 20(23): 7392.
20. Procopet B, Berzigotti A. Diagnosis of cirrhosis and portal hypertension: imaging, non-invasive markers of fibrosis and liver biopsy. Gastroenterology report 2017; 5(2): 79-89.
21. Marrero JA, Kulik LM, Sirlin CB, *et al*. Diagnosis, staging, and Management of Hepatocellular Carcinoma: 2018 practice guidance by the American Association for the Study of Liver Diseases. Clinical Liver Disease. 2019; 13(1): 1.
22. Wu N, Liu X, Xu X, *et al*. MicroRNA-373, a new regulator of protein phosphatase 6, functions as an oncogene in hepatocellular carcinoma. The FEBS journal 2011; 278(12): 2044-54.
23. Zhou P, Jiang W, Wu L, Chang R, Wu K, Wang Z. miR-301a is a candidate oncogene that targets the homeobox gene Gax in human hepatocellular carcinoma. Digestive diseases and sciences. 2012; 57(5): 1171-80.
24. Wang J, Li J, Shen J, Wang C, Yang L, Zhang X. MicroRNA-182 downregulates metastasis suppressor 1 and contributes to metastasis of hepatocellular carcinoma. BMC Cancer. 2012; 12(1): 227.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2– ΔΔCT method. Methods. 2001; 25(4): 402-8.
26. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic acids research. 2001; 29(9): e45.
27. Szabo G, Csak T. Role of MicroRNAs in NAFLD/NASH. Dig Dis Sci 2016; 61(5): 1314-24.
28. Mohamad M, Wahab NA, Yunus R, *et al*. Roles of MicroRNA21 and MicroRNA29a in Regulating Cell Adhesion Related Genes in Bone Metastasis Secondary to Prostate Cancer. Asian Pac J Cancer Prev. 2016; 17(7): 3437-45.
29. Ibrahim FF, Jamal R, Syafruddin SE, *et al*. MicroRNA-200c and microRNA-31 regulate proliferation, colony formation, migration and invasion in serous ovarian cancer. J Ovarian Res. 2015; 8(1): 56.
30. Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. Nat Rev Gastroenterol Hepatol. 2013; 10(11): 656-65.
31. Otsuka M, Kishikawa T, Yoshikawa T, *et al*. MicroRNAs and liver disease. J Hum Genet. 2017; 62(1): 75-80.
32. Zhu HT, Dong QZ, Sheng YY, *et al*. MicroRNA-29a-5p is a novel predictor for early recurrence of hepatitis B virus-related hepatocellular carcinoma after surgical resection. PloS One. 2012; 7(12): e52393.
33. Cao MQ, You AB, Zhu X-D, *et al*. miR-182-5p promotes hepatocellular carcinoma progression by repressing FOXO3a. Journal of hematology & oncology. 2018; 11(1): 1-12.
34. Stefansson B, Brautigan DL. Protein phosphatase PP6 N terminal domain restricts G1 to S phase progression in human cancer cells. Cell cycle. 2007; 6(11): 1386-92.
35. Tessitore A, Cicciarelli G, Del Vecchio F, *et al*. MicroRNA expression analysis in high fat diet-induced NAFLD-NASH-HCC progression: study on C57BL/6J mice. BMC Cancer. 2016; 16(1): 1-14.