Original Article

MicroRNA 122 as adiagnostic biomarker for hepatitis C- related hepatocellular carcinoma

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Summary

***Aim**: To explore the potential usefulness of serum microRNA (miR-122) as non-invasive diagnostic marker for hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC). **Methods**: The study has been conducted on 100 participants and were divided into 4 equal groups: Group I; Twenty five normal, healthy individuals' age and gender-matched healthy volunteers (control group). Group II; Twenty five chronic HCV patients without cirrhosis. Group III; Twenty five HCV related cirrhosis without HCC. Group IV; Twenty five HCV-related HCC (proved radiologically by abdominal ultrasonography (US) & Triphasic abdominal computed tomography (CT). All participants underwent full clinical assessment and laboratory investigations in addition to the evaluation of the level of serum miR-122 by RT-PCR. **Results**: microRNA-122 displayed significant fold increase in expression level in chronic hepatitis group (9.85), and less significant fold increase in cirrhosis group (3.73) and significant fold increase in expression level in HCC group (7.46). Comparing serum miR-122 expression level between different studied groups displayed that, No significant fold change in miR-122 expression was found between different groups and that miRNA122 had no significant up-regulation in HCC patients in comparison to non-HCC patients (Hepatitis and Cirrhosis); (P value = 0.682). Specificity & sensitivity was 94% & 16% respectively, with AUROC (0.529). **Conclusion**: miR-122 could not be used as a biomarker for early detection of HCC in HCV related cirrhosis.*

Keywords: *miR-122, hepatocellular carcinoma, chronic hepatitis C and cirrhosis*

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Introduction

HCC is the most common primary liver cancer and the fifth most common cancer worldwide and considered the second leading cause of cancer-related mortality in the world and accounts for 7% of all cancers[1]. In Egypt, there is a remarkable increase in the annual proportion of HCC among chronic liver disease patients from 4% to 7.2%[2]. Chronic HCV accounted for 94% of HCC cases in Egypt in 2010, with 6000-7000 deaths/year due to HCC[3]. Early HCC stage is asymptomatic and clinical manifestations are obvious only in the advanced stage, resulting in an undesirable curative effect and very bad prognos[4]. Early diagnosis of HCC by good surveillance represents the best requirement for an effective treatment to extend the lifetime of HCC patients[5,6]. Alpha fetoprotein (AFP) has long been used for the diagnosis of HCC. It has also been part of surveillance algorithms. However, the AFP is insufficiently sensitive or specific for use as a surveillance assay[7]. Guidelines published by **EASL, 2012 & AASLD, 2010** rejected AFP whether for the surveillance or the diagnosis of HCC. So, the need for other methods that would be minimally-invasive, simple and reliable for the early detection of HCC is mandatory. MicroRNAs (miRNAs) are endogenous, evolutionally conserved, non-coding RNAs that have been identified as posttranscriptional regulators of gene expression. MiRNAs are known to play various roles in the carcinogenesis process, including metastasis, tumor invasion, cell proliferation, cell cycle, and apoptosis[8]. In HCC, several tumor-suppressing and oncogenic miRNAs may have potential application in early diagnosis

and prediction of prognosis[9,10]. MiR-122 is the most abundant hepatic miRNA, and it is involved in the proliferation and differentiation of hepatocytes[11]. MiR-122 is frequently reported to be down regulated in HCC; however, some investigators have reported up regulation of this miRNA, depending on the etiology of HCC. Serum levels of miR-122 were reported to be higher in patients with HCC or hepatitis than in the healthy controls[12]. The aim of our study is to evaluate the possibility of using serum miR-122 for early detection of HCC.

Materials and Methods

This cross sectional observational study was performed on patients selected from the outpatient hepatology clinics and HCC early detection clinic, in Egyptian Liver Hospital (ELH) and Specialized Medical Hospital (SMH), Mansoura University, Egypt, from April 2016 to April 2017. The study has been conducted on 100 participants and was divided into 4 equal groups: Group I; Twenty five normal healthy individuals age and gender-matched healthy volunteers (control group). Group II; Twenty five chronic HCV patients without cirrhosis. Group III; Twenty five HCV related cirrhosis without HCC. Group IV; Twenty five HCV-related HCC (proved radiologically by abdominal US & Triphasic abdominal CT). All patients were enrolled in our study after signing an informed written consent. Approval of the study protocol by the medical ethics research committee, Faculty of Medicine, Mansoura University was obtained. Exclusion criteria included: Previous treatment of HCC (surgical, interventional or medical), presence of other malignancies (e.g. cholangiocarcinoma, gastric, colorectal cancer), patients with HCC on top of other causes rather than HCV, and patients on direct acting antiviral therapy for chronic HCV. All participants were subjected to full medical history, complete clinical examination, and full basal laboratory and radiological investigations with collecting samples for serum miR-122 and serum AFP. **Serum sample collection:** Five milliliters of venous blood was obtained and the serum fraction was extracted and stored at -80 °C until used. **RNA extraction:** For the real-time PCR, RNA was extracted from serum using QIAzolLysis reagent according to the manufacturer's instructions. The RNA purity was assessed by the RNA concentration and quantified by NanoDrop ND-1000 (Nanodrop, United States). **Reverse transcription using miScript II RT Kit:** Only 5 x miScriptHiSpec Buffer had been used preparation of cDNA for real-time PCR with miScriptmiRNA PCR Arrays. Single-stranded cDNAs were generated using the RT kit according to the manufacturer's directions (miSCriptmiRNA PCR system, miRneasy mini kit for miRNAextraction, miScript RT Ⅱ formiRNA reverse transcription, miSCript Primer Assay and miScript SYBR Green PCR Kit for PCR amplification. **RNA quantification:** PCR quantification experiments were performed with PCR (Applied Biosystems; Foster City, CA) using the SYBR Green PCR Master Mix according to the manufacturer's protocol. The primers for miR-122 and housekeeping gene were supplied by Qiagene, Germany.

Statistical analysis

Patients were categorized into 4 groups; normal, Chronic hepatitis, Cirrhosis and HCC. Further comparisons were performed between HCC group and Non-HCC (chronic hepatitis and cirrhosis). Quantitative variables were expressed by mean ± SD or expressed by median and inter quartile range (IQR) for non-parametric data. They were compared by t-students t-test or ANOVA test when appropriate. Qualitative variables were compared by $\chi 2$ or Fischer's exact test when appropriate. Receiver operator characteristic (ROC) curves were constructed to assess the value of miRNA 122 in diagnosing HCC and to assess area under the curve (AU ROC). Spearmen and Pearson correlations were done for correlating quantitative variables. In all tests, P value was considered significant if equal or less than 0.05.

Results

The clinico-demographic &laboratory data of the studied participants are shown in tabs. (1 & 2). Table (1) shows that there was no significant statistical difference between cirrhosis & HCC groups regarding special habits & presence of hypertension. There was a significant difference between the diseased groups regarding age (P <0.001). Regarding gender difference; males were predominant in HCV related liver disease patients. Rural residency and presence of diabetes mellitus (DM) were statistically significant higher in HCC patients, and most cases of HCC were clinically asymptomatic discovered during their routine follow up.

Table (1) Demographic and clinical data

Characteristic	Cirrhosis N=25	HCC N=25	P value
Age (Mean ± SD**)**	57.3±5.7	58.9±6.9	**<0.001**
Gender			**0.042**
- Male, N (%)	12 (48.0%)	21 (84.0%)	
- Female, N (%)	13 (52%)	4 (16%)	
Residency			**0.000**
- Rural	12 (48.0%)	14(56%)	
- Urban	13 (52.0%)	11(44%)	
Special habits			**0.695**
- Non smoker, N (%)	18 (72.0%)	10(40%)	
- Smoker, N (%)	7 (28.0%)	15 (60%)	
DM, N (%)	5 (20.0%)	12 (48.0%)	**0.009**
Hypertension, N (%)	9 (36.0%)	4 (16%)	**0.203**
Symptoms at presentation			
- Asymptomatic	10 (40%)	15 (60.0%)	
- Symptomatic:	15 (60%)	10 (40%)	**0.000**
▪ jaundice	1 (4%)	5 (20.0%)	
▪ ascites	4 (16%)	2 (8.0%)	
▪ abdominal pain	0 (0%).	2 (8.0%)	
▪ mixed constitutional symptoms (fatigue, anorexia, weight loss)	10 (40%)	1 (4.0%)	

Table (2) shows that, in HCC group, there were statistically significant higher levels of ALT, AST, ALP, bilirubin and on the other hand, there were significant lower levels of albumin, RBCs, WBCs & platelets. Most of cirrhosis& HCC patients were within Child-Pugh A and B classifications and most cases of HCC presented as single focal lesions and most cases were stage A, followed by B & C on Bacelona clinic liver cancer (BCLC) scoring system. **Serum miR 122 level:** analysis of median fold change in expression level of miR-122 in patients' sera in comparison to the normal control group presented in table (3) shows that miR-122 displayed significant fold increase in expression level in chronic hepatitis group (9.85), and less significant fold increase in cirrhosis group (3.73) and significant fold increase in expression level in HCC group (7.46). Comparing serum miR-122 expression level between different studied groups displayed that, No significant fold change in miR-122 expression was found between either (HCC vs Hepatitis groups) or (HCC vs cirrhosis groups) nor (Hepatitis vs cirrhosis).

Table (2) Laboratory and clinical Characteristics

Characteristic	Cirrhosis N=25	HCC N=25	P value
Age (Mean ± SD**)**	57.3±5.7	58.9±6.9	**<0.001**
Gender			**0.042**
- Male, N (%)	12 (48.0%)	21 (84.0%)	
- Female, N (%)	13 (52%)	4 (16%)	
Residency			**0.000**
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▪ abdominal pain	0 (0%).	2 (8.0%)	
▪ mixed constitutional symptoms (fatigue, anorexia, weight loss)	10 (40%)	1 (4.0%)	

Table (3) Comparison between all patients groups regarding miR-122 fold difference relative to controls and AFP

Variable	Hepatitis N=25	Cirrhosis N=25	HCC N=25	P value			
				All	HCC vs. Hepatitis	HCC vs. Cirrhosis	Hepatitis vs. Cirrhosis
Fold difference relative to control miR-122, median (IQR)	9.85 (49.84)	3.73 (66.31)	7.46 (53.33)	0.842	0.892	0.567	0.684
AFP, median (IQR)	4.05 (5.00)	6.90 (14.52)	23.09 (88.48)	**<0.001**	**<0.001**	**0.003**	**0.014**

Table (4) shows that MiRNA122 had non significant up-regulation in HCC patients in comparison to non-HCC patients (Hepatitis and Cirrhosis); (P value = 0.682). **Serum AFP level:** There was significant difference in serum AFP level between different groups with higher level in HCC groups in comparison to non HCC group (P value <0.001), as shown in table (3 & 4). Table (5) and fig. (1) show that the best cut off levels of serum miR 122 for distinguishing HCC from non HCC was 294.07 (p value 0.683), specificity & sensitivity was 94% & 16% respectively with AUROC (0.529). As regards AFP, cut off levels of 23.09 ng/ml had highly significantly distinguished patients having HCC from those cirrhotic non-HCC (p value <0.001) with specificity and sensitivity in diagnosis of HCC was 94% & 52% respectively and AUROC (0.824).

Table (4) Comparison between HCC and non HCC patients groups regarding miR-122 fold difference relative to controls and AFP

Fold difference relative to control	Non-HCC N=50	HCC N=25	P value
miR-122, median (IQR)	7.07 (48.94)	7.46 (53.33)	0.682
AFP, median (IQR)	5.25 (9.31)	23.09 (69.83)	**<0.001**

Table (5) Validity of serum levels of miR 122 and AFP in diagnosing HCC

	MiR122	AFP
Best Cut-off	294.07	23.09
Sensitivity	16.0	52.0
Specificity	94.0	94.0
PPV	57.1	81.2

NPV	69.1	79.7
AUROC	0.529	0.824
95% C.I.	0.389-0.669	0.725-0.823
Significance	**0.683**	**<0.001**

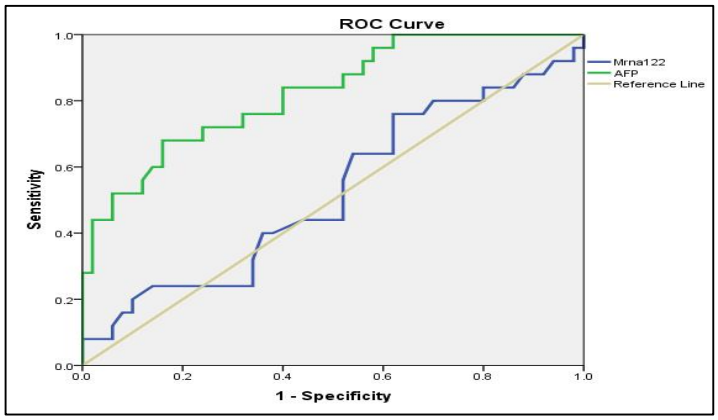

Figure (1) Receiver Operating Characteristics (ROC) curves of miR 122 and AFP for diagnosis of HCC

Discussion

Most of our HCC patients have statistically significant male predominance (84%), this has been concomitant with the results published[13-17]. The possible explanation may be higher male prevalence of HCV infection and other risk factors for HCC as sex hormones and other x-linked genetic factors that may also be important. Age was statistically significantly higher in HCC patients, a finding similar to that was observed before[13,14,16,17], This could be explained by natural history of HCC on top of HCV related cirrhosis. DM was found in 48% of HCC patients which is higher than that observed by Abdel-Wahab et al, which was 13.6%[16]. This discrepancy may be explained by the increase in prevalence of DM in Egypt that was 15 % in 2015 versus 8% in 2007[18]. A positive correlation between the history of DM and its duration with HCC was also confirmed by Lagiouet al[19]. Like many previous studies, about 56% of HCC patients in our study were from rural areas[16,20]. This might be due to increased rates of HCV infection with a general trend toward higher rates among male gender, older age, rural communities, and the regions of the Nile Delta and Middle. Also, the risk of environmental factors exposure such as aflatoxin in stored seeds and agricultural pesticides may play a role[21,22]. Most of our HCC patients were asymptomatic (60%), a finding that was already proven in previous studies[4,23]. In our study, ALT, AST, ALP, bilirubin was statistically significantly higher in HCC patients which might be explained by continued hepatocellular damage in HCV-related HCC. This observation in accordance with several studies[24,25]. The platelets count was significantly lower in HCC patients and these results were similar to the results of Gopal et al. Platelets play a crucial role in predicting liver fibrosis and cirrhosis; being lower with further progression of cirrhosis, so lower platelets count is found in HCC cases. Most of our HCC patients were within Child-Pugh A and B classifications (72%, 28% respectively), and most cases were staged 0, A and B (12%, 36%, 24% respectively) according to BCLC scoring system, a result that was in agreement with manu previous studies[13,16,26]. This could be explained by the fact that, most of them were recruited while being assessed for the possibility of interventional treatment. Another possible explanation for rather good liver condition seen in HCC series could be attributed to the implementing surveillance programs, allowing detecting tumors at an early stage in well compensated patients, but disagreeing with the result of Ziada et al, where 91.3% of HCC cases were Child B or C[27]. In HCC group the incidence of single lesion was more than multiple lesions (72% and 28% respectively), and 56.5% of HCC cases were found as small sized HCC. This result was

hand in hand with other studies[16,26] demonstrated that, most of patients have single focal lesion. Also, this also may be attri-buted to effective surveillance strategies for early diagnosis of HCC. In this study, there were significant differences in serum AFP level between different groups. It was significantly higher in HCC group (23.09 ng/ ml). These results are in agreement with results of other studies[15,25,28]. Also, consistent with Montaser et al, who stated highly significant increase in the serum AFP in the HCC cases (31.67 ng/ml)[29]. Our study proved that miR-122 could not be used as a biomarker for early detection of HCC in HCV related cirrhosis, as it was found that miR-122 displayed significant fold increase in expression level in chronic hepatitis group[9], and less significant fold increase in cirrhosis group[3], and significant fold increase in expression level in HCC group[7]. Comparing serum miR-122 expression level between different studied groups displayed that; No significant change in miR-122 expression was found between either (HCC vs hepatitis groups) or (HCC vs cirrhosis groups) nor (hepatitis vs cirrhosis) and that miR 122 had non significant up-regulation in HCC patients in comparison to non-HCC patients (hepatitis and cirrhosis), with specificity and sensitivity (94% & 16% respectively). The explanation is that miR-122 is a liver specific miRNA, and its serum level is increased with hepatocyte injury induced by HCV. Our result was hand in hand with Ghoneimet et al, who stated that the expression level of serum miR-122 displayed a significant fold increase in serum[30]. In our study, miR-122 in the HCV group and cirrhosis as compared with the control group, but patients with HCC showed a non significantly higher expression level of miR-122 as compared with controls. Our results also were in agreement with El-Garem et al, who found that, miR-122 was up regulated in chronic hepatitis and non significant up-regulation in HCC patients in comparison to non-HCC patients (chronic hepatitis and cirrhosis), but show fold decrease in cirrhosis in contrary to our results[13]. Our results match also with Miquelestorena et al, who showed that higher levels of miR-122 were found in non HCC HCV positive cases compared to HCV related HCC and control groups[31]. Our results also similar to that of Balkan et al, who found that comparison of serum miR-122 levels between patients with HCC and control group diagnosed with non alcoholic fatty liver disease (NAFLD) showed no statistically significant difference[32]. Our results also in agreement with Motawi et al, who found that, the serum levels of miR-122 was significantly elevated in HCV patients, but not in liver cancer patients, compared with controls and concluded that serum miR-122 is elevated in Egyptian patients with chronic HCV, and these miRNAs have a strong potential to serve as novel biomarkers for liver injury but not specifically for liver cancer[33]. EL-Abd et al[34] in agreement with our study showed that, no significant difference was observed for miR-122 between the HCV and HCC groups. Our results cope also with Ezzat et al[35] who showed that expression level of miR-122 was significantly higher in both HCV and cirrhotic groups as compared to control group, patients with HCC showed non significant higher expression level of miR-122 as compared to cases with cirrhosis alone. Also our results cope with Qi et al[36] who reported that serum miR-122 was significantly higher in HCC patients than healthy controls but not specifically for detection of HCC in chronic HBV infection patients. Also in agreement with Xu et al[12] found that the levels of miR-122 was significantly higher in patients with HCC than healthy controls and also elevated in patients with chronic hepatitis suggesting that the elevated serum miR-122 may reflect liver injury but not tumor itself similar to our results. Zekri et al[37] found that, miR-122 was significantly up regulated in HCC, and provided high diagnostic accuracy in contrast to our results, but this may be due to using combinations of different panels of miRNAs in addition to miR-122. Also, Demerdash et al[38] proved that plasma miR-122 was significantly higher in chronic HCV and HCC in comparison to healthy controls, but in contrary to our result there was more significant increase in HCC compared to hepatitis group. Against our results, El-Shennawy et al[39] found that, the highest expression of plasma level of miR-122 was in HCC in comparison to cirrhosis and ROC curve analysis yielded 73% sensitivity and 96 % specificity, this may be due to their limited small sample size. In contrast to our result, Wang et al[40] found that miR-122 expression in liver was significantly down-

regulated. But this may be explained by study was conducted on HBV cases in contrast to our HCV patients. Also in contrast to our results**,** Huang et al[41] performed a systematic review and meta-analysis and revealed that miR-21 and miR-122 individually distinguished patients with HCC from healthy controls, with an AUC of 0.77 for miR-122. In contrast to our results, significant down regulation of miR-122 in HCC compared to normal liver tissue was reported by others[42-44], but this may be attributed to mixed etiologies of HCC. Also, Ladeiro et al[45] have established significant down expression of miR-122 in 28 HCC liver tissues (mixed etiologies). similarly a significant down regulation in miR-122 in 19 HBV related HCC liver tissue reported by Connolly et al[46]**.**

Conclusion

miR-122 could not be used as a biomarker for early detection of HCC in HCV related cirrhosis, as it showed non-significant up-regulation in HCC patients in comparison to non-HCC patients.

List of abbreviations

* **AFP**: *Alfa Fetoprotein*
* **AUROC**: *area under the ROC curve*
* **BCLC**: *Bacelona clinic liver cancer*
* **CT**: *computed tomography*
* **DM**: *diabetes mellitus*
* **ELH**: *Egyptian Liver Hospital*
* **HBV**: *hepatitis B virus*
* **HCC**: *hepatocellular carcinoma*
* **HCV**: *hepatitis C virus*
* **IQR**: *inter quartile range*
* **miR**: M*icroRNA*
* **miRNAs**: *MicroRNAs*
* **NAFLD**: *non alcoholic fatty liver disease*
* **PCR**: *polymerase chain reaction*
* **ROC**: *Receiver operator characteristic*
* **SMH**: *Specialized Medical Hospital*
* **US**: *ultrasonography*.

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