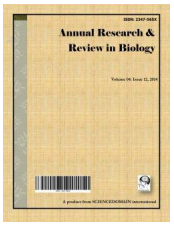

Annual Research & Review in Biology

18(5): 1-9, 2017; Article no.ARRB.37073
ISSN: 2347-565X, NLM ID: 101632869

Diagnostic Value of MicroRNA (miR-122) in Hepatitis C Virus (HCV)-related Hepatocellular Carcinoma (HCC) in Egyptian Patients

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2017/37073
Editor(s):
(1) Ibrahim Farah, Jackson State University, Mississippi, USA.
(2) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Lívia Garcia Bertolacci-Rocha, Universidade Federal de Goiás, Brasil.
(2) Simone Regina Alves De Freitas Barros, University Hospital Alberto Antunes de Alagoas, Brasil.
(3) Mohamed Ahmed Mohamed Nagy Mohamed, Beni-Suef University, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history/21581

Original Research Article

Received 29th September 2017
Accepted 19th October 2017
Published 26th October 2017

ABSTRACT

Background: In Egypt, the prevalence of chronic hepatitis C (CHC) infection is 14% of whole population and about 80% of the patients with hepatocellular carcinoma have underling hepatitis C. **Aim:** To explore the diagnostic value of serum miR-122 as non-invasive diagnostic markers of (HCV)- (HCC).

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Methodology: The study included 102 healthy controls (group I), in addition to 228 patients, they were divided into 2 groups: 120 CHC patients (group II), and 108 hepatitis C patients with HCC (group III). All samples underwent full clinical assessment and laboratory investigations additionally to the detection of the concentration of blood serum miR-122 expression by RT-PCR. Selected biomarkers were assessed, evaluated and correlated with degree of liver damage.
Results: Revealed that miR-122 had the highest efficiency in prediction of liver cell damage. Also, miR-122 was strongly correlated with vascular endothelial growth factor (VEGF) and alpha fetoprotein (α-FP) in HCC patients.
Conclusions: Plasma miR-122 can be considered as a potential biomarker, for detection of HCC combined with VEGF and α -FP.

Keywords: miR-122; MicroRNA; HCC; Egyptian patients.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer mortality [1,2]. HCC is defined as a primary tumorigenesis in the liver, mainly in patients suffering from chronic liver cirrhosis or hepatitis B or C [3,4].

In Egypt, the incidence of hepatocellular carcinoma has been doubled over the last 10 years [5]. Egypt has been troubled with the highest prevalence of Hepatitis C Virus in the world, ranging from 7% to 27% [5].

Several serum markers have been suggested and a number of them are actually used in frequent clinical practice for detection HCC, as Alpha-1 fetoprotein (AFP) and vascular endothelial growth factor (VEGF) [6,7].

MicroRNAs are small non-coding RNAs. MicroRNAs efficient block translation by binding to complementary sequences in the 3′ untranslated region (UTR) or promoting the degradation of target mRNAs. microRNA (miRNA) have gotten the best consideration over the previous years [8]. MiRNAs serve as post-transcriptional regulators of mRNA expression, where MiRNA interfere with translation to protein. MiRNA become a part of the so called RISC (RNA silencing complex) after common modifications steps to be functionally active [9].

In newly studies as in study of Vojtechova et al. [10], the correlation between tumors and microRNAs becomes a point of discussion.

miRNAs are important players in pathogenesis of HCV infection directly by controlling signaling pathways; they play a role in innate and adaptive immune response [11,12]. miR-122 is the most abundant miRNA in normal liver parenchyma, accounting for more than 70% of the total miRNA in hepatocytes [13]. The binding of miR-122 to the 5′-UTR of HCV genomic RNA is critical for viral replication; it stimulates viral protein translation and protects the uncapped HCV RNA from degradation [14,15]. Moreover, miR-122 is one of the first detected oncogenic miRNAs; it controls cell cycle and tumorigenesis [16,17]. The aim of the present study was to assess the diagnostic value of plasma miR-122 in Egyptian in early stages of hepatocellular carcinoma (HCC) in patients with CLD.

2. MATERIALS AND METHODS

2.1 Patients and Sample Collection

This study included 228 HCV serum-positive Egyptian patients (108 HCC patients and 120 with chronic liver hepatitis), in addition to 102 healthy subjects as control group (HCV-negative patients). Study subjects were recruited from the Clinical Hepatology Department, Gastroenterology Center, Mansoura University. Blood samples were collected according to the ethical standards for donor approval required by the national regulatory bodies under supervision of a physician and after having signed informed consent for the use of their blood in this study. All patients were pre-diagnosed HCV antibody positive by enzyme-linked immunosorbent assay. All samples were subjected to HCV antibody detection in serum. HCC was detected by ultrasound scan and confirmed using computed tomography (CT). Routine lab tests were performed for each sample. Patients with any hepatitis other than C, or malignancy other than caused by HCV, were excluded from the study. Sex distribution among healthy controls was 72 males and 30 females, with mean age of 36.3 ± 7.5 years. Control subjects had normal levels of

liver enzymes and alpha-fetoprotein (AFP) and vascular endothelial growth factor (VEGF).

2.2 Laboratory Investigation

2.2.1 Routine investigations

Complete blood count (CBC) was determined by automated cell counter (ERMA Inc., Tokyo, Model PCE-210). Liver profiles—aspartate transaminase (AST) and alanine transaminase (ALT), serum albumin, total bilirubin, plasma prothrombin time (PT), and INR—were measured.

Alpha-fetoprotein (α-FP) was performed by the method described by Chemiluminescence (Immulite 1000, Siemens, Germany).

Vascular endothelial growth factor (VEGF) was assayed by the method described in the commercial VEGF ELISA kit instructions (Peninsula Inc., USA).

2.2.2 miR-122 quantification by real-time qPCR (RT-qPCR)

Blood samples were collected in EDTA tubes, centrifuged at 4000 rpm for 10 min at 4°C, plasma separated, and stored at −70°C.

2.2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from 200 μl plasma using the miRNeasy extraction kit (Qiagen,Valencia, CA, USA).RNApurity was assessed by the RNA concentration and quantified by NanoDrop Spectrophotometer (NanoDrop ND-1000, United States), followed by analysis using agarose gel electrophoresis. Reverse transcription was performed for cDNA synthesis using the miRNeasy Plasma Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

2.2.4 Amplification and quantification

The expressions miR-122 was evaluated by RT-qPCR analysis according to the manufacturer's protocol. The housekeeping miRNA SNORD 68 was used as the endogenous control. For realtime PCR, the cDNA template was mixed with SYBER Green Master Mix (Qiagen, Valencia, CA, USA). PCR array plate was enriched with forward and reverse miRNA-specific primer. Real-time PCR were performed using an Applied Biosystems 7500 Real-Time PCR System (Foster city, CA, USA) with the following conditions: 95°C for 15 min, followed by 40 cycles at 94°C for 15s, 55°C for 30s, and 70°C for 34s. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in real-time PCR.

2.3 Statistical Analysis

2.3.1 Statistical methods

The data were statistically analyzed using SPSS (Statistical Program for Social Science version 16). The following statistical parameters were used:

* *Parametric analysis: It is used to compare between more than two quantitative variables (mean) that normally distributed.*
* *Non parametric analysis:*

The Kruskal Wallis nonparametric test:

Non-parametric comparisons of location (mean rank) are for three or more groups.

2.3.2 Chi-square analysis

It is used to compare between qualitative data and find out the relation between various qualitative data. Variables were cross tabulated in all possible combinations against each other.

P value =level of significance: *P*> 0.05 insignificant, *P* ≤ 0.05 significant, *P*<0.001 highly significant.

3. RESULTS

This study was conducted on 330 participants stratified into: 102 healthy volunteers (group I) with negatively hepatitis B and C by PCR and normal transaminases and hepatic ultrasound, and 120 non-cirrhotic patients with chronic hepatitis C (CHC) patients (group II) and 108 patients with HCV-related HCC (group III). The clinic-pathologic characteristics of the studied individuals are shown in Table 1.

There was a significant difference between the studied groups regarding transaminases (P < 0.001). Also, there was a significant difference between the diseased groups regarding age (P < 0.001).

3.1 Differential Expression of Serum miRNA Levels in HCC Patients

The expression levels of miR-122 in sera of patients compared to the normal control group, showed that miR-122 was significantly higher in CHC (2.7±1.7) and HCC (4.8±2.3) in comparison to healthy controls (0.81±0.25), (P= 0.0022)

(Table 2), in which miR-122 showed more significant increase with HCC group.

3.2 Detection of Both α-fetoprotein and VEGF Levels in the Studied Groups

The concentrations of both α-fetoprotein and VEGF in sera of patients compared to the healthy control group, showed that both α-fetoprotein and VEGF were significantly higher in HCC (265±152, 238±95, p≤0.001) in comparison to healthy controls (4.3±1.8, 36±16) respectively (Tables 3 & 4). Also, α-fetoprotein was significantly higher in CHC (41±29, p≤0.001) in comparison to healthy controls (4.3±1.8) (Table 3) while VEGF was insignificantly in CHC (42±18) in comparison to healthy controls (36±16) (Table 4).

3.3 Correlation between miR-122 & Both *α-fetoprotein* and *VEGF*

There were statistically significant positively correlation between serum expression level of studied miR-122 & both α-fetoprotein and VEGF levels in HCC group (0.63, 0.003*), (0.51, 0.006*) respectively. No significant correlation between studied serum miR-122 and both α-fetoprotein and VEGF levels in CHC group (0.21, 0.07), (0.3, 0.055) respectively, (Table 5).

3.4 Diagnostic Performance of Studied Serum miR-122

The diagnostic performance for the studied miR-122 panel was evaluated using ROC analysis. ROC curve was designed for discriminating CHC patients from other groups, and results revealed that miR-122 had 84% sensitivity, 80% specificity, and 81% accuracy (Fig. 1, Table 6).

4. DISCUSSION

The occurrence of HCC diversifies widely throughout the world, with rising rate in Egypt. Most HCC develop in patients suffering from hepatitis infection, which is most prevalent in Middle East region and Africa [18]. Also, liver pathology develops silently without any side effects (symptoms), and more than one-third of patients (33%) are diagnosed with end-stage liver failure/HCC and die within few months [19]. HCC is one of the most common and aggressive human malignancies worldwide. Hepatocellular carcinoma represents an extremely poor prognostic cancer. Over the last 2 decades, the performance of tumor markers for HCC diagnosis has not been optimal [20,21]. Early diagnosis is required for the prevention and treatment of liver cirrhosis and HCC.

Currently, the identification of HCC-specific miRNA profiles in the circulation is an emerging field of particular interest and has been implicated affecting cellular proliferation and oncogenesis [22]. The availability of miRNAs in the circulation makes them a potential target for early tumor detection [23]. The aim of the present research was to estimate the potential utility of serum miR-122 as a novel non-invasive marker for HCV diagnosis related hepatocellular carcinoma in Egyptian patients.

The expression levels of miR-122 in sera of patients compared to the normal control group, showed that miR-122 was significantly higher in CHC (2.7±1.7) and HCC (4.8±2.3) in comparison to healthy controls (0.81±0.25), (P= 0.0022)

(Table 2), in which miR-122 showed more significant increase in serum expression level with HCC group.

In this study, the expression level of serum miR-122 was significantly higher in CHC and HCC groups compared to healthy controls, with more expression among HCC patients. Increased expression of serum miR-122 in patients with HCC was similar to results reported by other studies; Laterza et al. reported that it might be due to its down regulation in HCC tissues and subsequent elevation in circulation of HCC patients [24]. Furthermore, increased expression of miR-122 in HCC group would be due to the fact that miR-21 functions as an oncogene and its up regulation promotes malignant cell proliferation and invasion and contributes to evasion of the host immune system [25].

These findings were in agreement with other studies. They concluded that hepatocytes are primary source of miR-122, and thus it exists

copiously in hepatocytes with much lower levels in serum of healthy individuals. Hepatocyte injury due to HCV infection results in enhanced miR-122 release into the circulation and serum levels upsurge [26,27].

Trebicka et al. [28] studied hepatic miR-122 expression in 43 HCV related HCC in comparison to 3 healthy liver samples using q RT-PCR; He found that miR-122 was strongly up-regulated in malignant liver nodules compared to healthy liver. They presented that miR-122 lead to further tumor growth where miR-122 may down regulate target mRNA of unknown tumor suppressor genes [28].

Xu et al. [29] in a research on hepatitis B patients suggested that cancer-induced hepatocyte damage would release the abundant intracellular miR-122 into the circulation, the miRNA stability would be reflected by easily detectable high blood levels [30].

In contrast to our results, Meng et al. [31], Wang et al. [32] and Huang et al. [33] reported significant down regulation of miR-122 in HCC compared to normal liver tissue. They compared miR-122 expression profile of three different pairs of tumor and normal human liver-derived RNA and twenty HCC liver tissues (mixed etiologies) to normal tissues respectively using microarray [31,32,33]. Similarly, Connolly et al. [34] and Ladeiro et al. [35] reported a significant down regulation in miR-122 in HBV related HCC liver tissues (mixed etiologies) in comparison to paired healthy liver by next-generation sequencing.

In 13.4% of recruited HCC patients, Alfa fetoprotein level was normal, less than 10 ng/dL. Tateishi et al. [36] and Chen et al. (1984) observed the same finding; they suggested that serum levels are normal in up to 40% of small HCCs because not all tumors secrete AFP. So, α-Fetoprotein alone is not recommended for the HCC diagnosis [36]. Researchers reported that α-Fetoprotein cut off value should be set at 200 ng/mL.

VEGF was significantly higher in HCC group compared to both CHC and healthy control groups; also, there was no significant difference of VEGF between both CHC and healthy groups. These results were in agreement with results that were obtained by El-Mezayen and Darwish; they found no significant difference in VEGF levels between control group and chronic hepatitis Cpatients; they explained it by the possibility that the two groups had benign liver tissue with no hypoxia and thus is no need for expression of angiogenic factors [37]. On the other hand, Mukozu et al. [38] stated that VEGF was significantly higher in patients with CHC and HCC patients than controls. Mukozu et al. [38] concluded that VEGF are produced by hepatocytes and contribute to progressive hepatic fibrosis through induced proliferation of HSCs.

Our results showed that, There were statistically significant and positive correlation between serum expression level of studied miR-122 & α-fetoprotein level in HCC group (0.63, 0.003*), while, No, significant correlation between them in CHC group (0.21, 0.07).

In our study, no statistically significant correlation could be verified between serum miR122 expression level and CHC patient characters, age and liver synthetic functions tests (Albumin, bilirubin and INR) while there was significant and positive correlation between serum expression level of studied miR-122 and HCC patients characters, age and liver synthetic functions tests.

However, in both CHC and HCC groups, serum miR-122 was significantly and positively correlated with higher AST and ALT levels compared to healthy group.

Significant correlation was reported by Köberle et al. [39] between serum miR-122 expression level and necro-inflammatory markers (ALT, AST) in HCC patients [39]. Bihrer et al. [40] found that serum miR-122 levels were comparable between CHC patients with normal ALT levels and healthy controls.

In this study, we confirm that serum miR122 can be measured from a relatively small amount of serum. A few studies reported altered levels of circulating miRNAs in association with HCC [41]. Serum miR-122 was strongly correlated with VEGF and α-FP. Serum miR-122 could be used as an early diagnostic marker for HCC, or maybe combined with VEGF and α-FP for a better and early diagnosis.

Table 1. Demographic and clinical parameters of the studied groups

Parameters	Healthy control (n=102)	CHC (n=120)	HCC (n=108)	P
Age (yr)	36.3±7.5[a]	43±8.2[a]	56±9.1[b]	<0.001
Gender (male) n,%	72(70.6%)	89(74.2%)	83(77)	0.25
Hb (gm/dl)	13.8±1.6[b]	11.5±2.0[a]	11.4±2.1[a]	<0.001
Platelets (10^3/mm^3)	255±65[b]	191±71[a]	156±79[a]	<0.001
Albumin (g/dl)	4.3±0.91[a]	3.82±1.51[a]	2.85±1.15[b]	0.004
INR	1.1±0.25[a]	1.3±0.58[a]	1.6±0.66[b]	0.003
AST (IU/ml)	22±8.1[a]	58±12.2[b]	112±21[c]	<0.001
ALT (IU/ml)	27±8.5[a]	66±13.4[b]	110±20[c]	<0.001
Bil T. (mg/dl)	0.66±0.24[a]	1.1±0.38[a]	2.2±1.9[b]	<0.001

[A,b,c] *Groups with different letters show significant difference, p<0.05*

Table 2. Plasma miR-122 in the different groups

	Healthy control	CHC	HCC	P
miR-122	0.81±0.25[a]	2.7±1.7[b]	4.8±2.3[c]	0.0022

[a,b,c] *Groups with different letters show significant difference, p<0.05*

Table 3. α-fetoprotein (ng/ml) in the different groups

	Healthy control	CHC	HCC	P
α-fetoprotein (ng/ml)	4.3±1.8[a]	41±29[b]	265±152[c]	≤0.001

[a,b,c] *Groups with different letters show significant difference, p<0.05*

Table 4. Serum VEGF (Pg/ml) in the different groups

	Healthy control	CHC	HCC	P
VEGF (Pg/ml)	36±16[a]	42±18[a]	238±95[b]	≤0.001

[a,b,c] *Groups with different letters show significant difference, p<0.05*

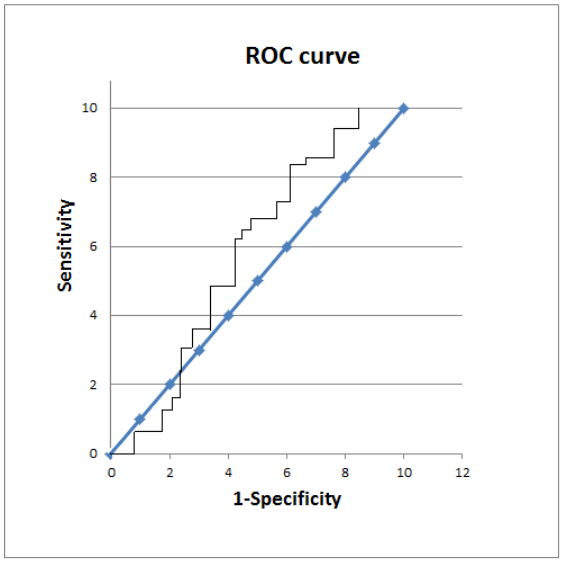

Fig. 1. Receiver operator characteristic curve of miR-122 as a discriminant of hepatocellular carcinoma vs CHC

Table 5. Correlation between miR-122 and both α-fetoprotein and VEGF

	CHC		HCC	
	r	p	r	p
α-fetoprotein	0.21	0.07	0.63	0.003*
VEGF	0.3	0.055	0.51	0.006*

significant difference, p<0.05
r Correlation Coefficient
p p-value

Table 6. Diagnostic performance of miR-122 for discriminating patients with Hepatocellular Carcinoma from those without

Area under the curve (AUC)	P-value	Cut-off	Sensitivity	Specificity	Accuracy
0.68	0.035	<1.78	84%	80%	81%

5. CONCLUSION

In conclusion, studied serum miR122 could distinguish HCV-related HCC from HCV-associated Liver disease and healthy control subjects suggesting their potential usefulness as HCC biomarkers and clinical utility in diagnosis of HCV-related HCC with 84% sensitivity and 80% specificity. Its detection in serum can clearly define the progression of the disease. Thus, miR122 may be able to serve as a promising non-invasive diagnostic marker for HCC. Better results could be obtained if combined with other markers to serve as a reliable diagnostic test for HCC.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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41. Borel F, Konstantinova P, Jansen PL. Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma. J Hepatol. 2012;56:1371-83.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/21581