Role of serum miRNA-500 as a biomarker of hepatocellular carcinoma (HCC) progression with hepatitis C virus infection

Alaa W. Elmetwalli[1], Ahmed Esmael[2] and Mahmoud M. Amer[2]

[1]Department of Clinical trial Research, Egyptian liver Research Institute and Hospital, Mansoura, Dakhliah, Egypt.
[2] Microbiology Department, Faculty of Science, Benha University, 13511, Egypt.
E-mail: Dr.prof2011@gmail.com

Abstract: Hepatitis C virus (HCV) infection is a significant health challenge affecting over 185 million individuals globally. Egypt has the highest rate for chronic HCV infection globally. Unresolved chronic HCV infection could progress to more deteriorating conditions such as cirrhosis and/or hepatocellular carcinoma (HCC). The latter is one of the most common malignant tumor in the world. Current conventional HCC biomarkers lack both sensitivity and specificity. Discovering novel and reliable biomarkers that are sensitive, specific and non-invasive is very decisive for early diagnosis and rapid intervention for HCV related liver HCC. Recently, miRNA have drawn great attention as promising non-invasive biomarkers for various diseases.
[Alaa W. Elmetwalli, Ahmed Esmaeland Mahmoud M. Ame. **Role of serum miRNA-500 as a biomarker of hepatocellular carcinoma (HCC) progression with hepatitis C virus infection.** *Nat Sci* 2019;17(9):29-36]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 4. doi:10.7537/marsnsj170919.04.

Keywords: Hepatocellular carcinoma, early diagnosis, Biological marker miRNA.

1. Introduction

Nowadays HCC is estimated to be the second leading cause for cancer related mortalities and the seventh most common cancer globally [1]. HCC is a highly lethal cancer as the majority of the cases are detected at a late stage in patients with underlying chronic hepatitis or cirrhosis in which there is continuous inflammation and regeneration of hepatocytes. HCV has been a primary cause of HCC in developed nations and is the first warning for hepatic transplantation for patients with HCC in the United States [2]. Pathogenesis of HCV-induced HCC involves multiple steps: establishment of acute HCV infection, prognosis to chronic infection, chronic hepatitis, gradual liver fibrosis, appearance of neoplastic clones associated with permanent somatic genetic/epigenetic changes, and finally progression of the malignant clones in an oncogenic tissue microenvironment which is usually referred to as "field cancerization". This cirrhotic oncogenic microenvironment accelerates genetic abnormalities and cellular transformation which in turn allows development and promotion of carcinogenic clones. HCV chronic infection, leads to hepatic damage which is manifested in the release of inflammatory mediators for instance: reactive oxygen species (ROS), cell death signals, nucleotides and hedgehog ligands; proteins that regulate cell growth and its fate. A complex series of events then occurs that leads to hepatic stellate cell activation. These events include the activation of intracellular inflammation factors, a family of transcription factors and other transcriptional events. Hepatic stellate cell activation stimulates liver scarring as a result of proliferation, fibro-genesis, matrix degradation and inflammatory signaling [1]. An increasing incidence expected in the next decades [3]. The high fatality rate demonstrates the need for specific diagnostic methods and effective therapeutic strategies for HCC [4]. Currently, the diagnosis of HCC is usually based on imaging (abdominal ultrasonography, contrast-enhanced computed tomography (CT), and magnetic resonance imaging (MRI)) and laboratory analysis (serum□-fetoprotein (AFP) levels), and it is sometimes verified by hepatic tissue biopsy [5]. Ultrasonography can detect large lesions but not small tumors, and the diagnostic accuracy of this operator-dependent procedure varies. Advances in MRI and CT have greatly improved the diagnostic imaging of small lesions of HCC. However, these procedures are costly, and they may not be readily available in some developing countries. Currently, AFP is the main laboratory analysis performed in the clinic for the diagnosis of primary HCC; however, its sensitivity (39–65%) and specificity (76-94%) are unsatisfactory [6]. Despite the substantial effort of doctors and investigators, a large proportion of patients with HCC are still diagnosed at an advanced stage when effective treatments are lacking [7]. Hence, the development of noninvasive biomarkers with high sensitivity and specificity that can be used for large-scale clinical investigations is greatly needed.

MicroRNAs (miRNAs), which are endogenous small noncoding RNAs consisting of 20–25 nucleotides, may regulate as much as 60% of the human genome [8]. Humans' express nearly 1000 miRNAs, each with the potential to bind multiple host miRNA molecules [9]. miRNA biogenesis, which is

important for various cellular and physiological processes, including cellular development, apoptosis, proliferation, and differentiation, has been well characterized and consists of several steps [10]. The development of various diseases may be due to alterations in any of the steps of miRNA biogenesis [11,12]. For instance, the deregulation of miRNAs may activate oncogenes and inactivate tumor suppressor genes in human carcinogenesis.

Several miRNAs have been linked to the initiation and progression of HCC, which may present a new way for the study of the molecular mechanisms, diagnosis, and implementation of novel therapeutic targets in HCC. In 2008, Chen et al. reported an exciting discovery that human serum/plasma contains a large amount of stable miRNAs and that the unique expression profile of serum miRNAs could serve as a fingerprint for various diseases [13]. Subsequently, numerous important studies have demonstrated the potential of serum/plasma miRNAs as novel noninvasive biomarkers for the diagnosis of HCC, and most of these studies have been conducted in Egypt. In this paper, we discuss recent evidence related to the value of serum microRNAs-500 as biomarker for HCV-related HCC in Egypt.

2. Material and methods

2.1 Study Design and sample collection.

One hundred twenty HCV infected patients (positive for anti-HCV antibodies and HCV RNA) attending The Egyptian Liver Research Institute and Hospital, Mansoura, Dakhliah were included in this prospective study. They were divided into three groups: group 1 = Patients with uncomplicated chronic hepatitis C infection and fibrosis (n = 30), group 2 = Patients with chronic hepatitis C infection and cirrhosis (n = 30), group 3 = Patients with chronic hepatitis C infection and HCC (n = 30). A control group (n = 30) of healthy volunteers (with normal liver enzymes, normal hepatic ultrasound and negative for HBV, HCV and HIV) was included in the miRNAs expression levels analysis by RT-PCR. Exclusion criteria included patients with: decompensated liver disease, malignancy other than HCC, organ transplantation, co-infection with HIV or HBV, immunosuppression, renal disorder and autoimmune co-morbidities. All patients were anti-HCV positive with detectable serum HCV RNA by PCR **(GenXpert PCR)**. Child-Pugh score was used to assess the severity of liver cirrhosis. Grading and staging of chronic hepatitis were evaluated by fibroScan [14]. All HCC patients were on top of HCV cirrhosis and HCC diagnosis was made upon the presence of hepatic focal lesions diagnosed by abdominal ultrasound and confirmed by triphasic computed tomography (CT) according to American Association for the Study of Liver Diseases (AASLD) 2011 guidelines [15]. A written informed consent for specimen use was obtained from all study subjects and the study protocol was approved by the ethics review committee of Egyptian Liver Research Institute and Hospital. Data and sample collection thorough history taking and full clinical assessment. Ultrasound data and fibroScan data for all study participants were collected from their medical files (grading and staging of liver disease, viral load, HCC U/S data). Blood sampling, 10 mL of peripheral blood was collected from each patient. Three mL were put on gel tubes to separate serum samples (used for RT-PCR), and 2 mL put on potassium ethylenediamine tetra-acetic acid (K3 EDTA) tubes for CBC. Five mL were collected into plain tubes to separate serum samples (used in biochemical analysis). Serum samples for PCR were stored at −80˚C until further processing.

2.2. Biochemical Investigations

Routine lab investigations including liver function tests (ALT, AST, alkaline phosphatase (ALP), total bilirubin, direct bilirubin, and albumin) creatinine, prothrombin activity and prothrombin international normalized ratio (INR), as well as complete blood counts (CBC) including hemoglobin (Hb), platelets and total leukocyte counts (TLC) were performed.

2.3. Detection of miRNA Expression Levels by Quantitative Real-Time Reverse Transcription (RT)-PCR).

Real-time quantitative RT-PCR for miRNA was performed to detect the expression levels of miRNA-500.

2.3.1. RNA Extraction

Total RNA with preserved miRNAs was extracted from 200 µl serum with the miRNA easy extraction kit (Qiagen, Valencia, CA, and USA) using 1000 µl QIAzol lysis reagent as per manufacturer protocol. The eluted miRNAs were then stored at –80^0 C for further investigations.

2.3.2. Reverse Transcription

The specific cDNA of miRNA-500, were synthesized using miRNA-500 specific primers according to the TaqMan® MicroRNA reverse transcription kit **(Applied Biosystems, Foster City, CA, USA).** Each Reverse transcriptase reaction (20 µl reaction volume) consisted of: 10 µl Master Mix (100 mM dNTPs, 50 U/µl MultiScribe Reverse transcriptase, 10× Reverse transcriptase buffer, 20 U/µl RNase inhibitor), 3 µl primer, 2 µl H2O and 5 µl RNA sample. The 20-µl reaction volumes were mixed very well and incubated for 1 hour in DT-lite 4 Real-Time PCR system.

2.3.3. MiRNA-500 Amplification.

After we getting the cDNA after the reverse transcription, tubes were prepared one for miRNA-500

and SNORD68 control tube. Master Mix prepared by add 12.5 ul SYBER Green, 2.5 ul universal primer, 2.5 primary assay and 5 micro water. Take 22.5 ul to cDNA tubes. For 2 hours the tubes put in Slan instrument for obtain Ct for miRNA 500.

2.3.4. Relative Quantitation of Target MiRNA Expression

It was evaluated using the comparative cycle threshold (CT) method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to Control. PCR quantification experiments were performed with PCR **(Gene eXpert, USA)** using the SYBR Green PCR master mix according to the manufacturer's protocol. Primers for microRNA-500 and the housekeeping gene were supplied by **Qiagene, Germany** (catalog numbers 218073, 218075 and 218076). The housekeeping miRNA SNORD68 was used as the endogenous control. Fluorescence measurements were made in every cycle and the cycling conditions used were: 95°C for 30 s, and 40 cycles of 95°C for 5 s and 60°C for 34 s.

Expression of miRNAs was reported as ΔCt value. The ΔCt was calculated by subtracting the Ct values of miRNA SNORD from the Ct values of the target miRNAs. As there is an inverse correlation between ΔCt and miRNA expression level, lower ΔCt values were associated with increased miRNA. The resulting normalized ΔCt values were used in calculating relative expression values by using 2-Δ (Ct), these values are directly related to the miRNA expression levels. The 2-ΔΔ (Ct) method was used to determine relative-quantitative levels.

3. Statistical analysis

The statistical analysis was performed using Statistical Package for Social Sciences **(SPSS Inc, Chicago, IL, V 15.0)** and MedCalc **(MedCalc Software, Ostend, Belgium, V 18.0)**. Patients were categorized into 3 groups; chronic HCV (fibrosis), cirrhosis and HCC. Further comparisons were performed between HCC group and non-HCC groups (fibrosis, cirrhosis and control). The relative expression of miRNA in our samples demonstrated a normal distribution, so statistically significant differences in miRNA levels between the cases and controls were determined by the independent samples test. Quantitative variables were expressed by mean ± standard deviation (SD) or expressed by median for non-parametric data. Data on the relative expression of miRNA is shown as the mean± SD. Comparison of percentages was done using the Spearman Chi-square test. Diagnostic values for the different classifiers (miRNAs or AFP) were determined by calculating the area under the receiver operating characteristic (ROC) curve. In all tests, P- value, was considered to be statistically significant if less than 0.05.

1. Results.

4.1. Demographic Parameters and Laboratory results of Patients

Table 1. Comparison between patients and control groups regarding demographic Parameters

Group / Variable	Control N=30	Fibrosis N=30	Cirrhosis N=30	HCC N=30	P value
Age, yrs., mean±SD	33.8±7.3	48.8±9.8	57.0±6.2	58.1±7.3	<0.001
Sex, male, n (%)	15 (50.0%)	15 (50.0%)	17 (56.6%)	22 (73.3%)	0.121
Smoking, n (%)		10 (33.3%)	10 (33.3%)	9 (30.0%)	0.521
DM, n (%)		3 (10.0%)	11 (36.6%)	16 (53.3%)	<0.001
Hypertension, n (%)		4 (13.3%)	13 (43.3%)	6 (20.0%)	0.020
Child-Turcotte-Pugh Classification:					
A			23 (76.66%)	20 (66.6 %)	0.351
B			7 (23.33%)	10 (33.3 %)	

Demographic and clinical data records are summarized in **(Tables 1, 2)** and **(Figures 1, A: f).** There was no significant differences in gender distribution among the four groups (*P*=0.121) with male predominance in diseased groups and they represented 56.6% & 73.3% in cirrhosis groups, and HCC groups, respectively. However the patients showed significant bearing of elder age with progression of liver disease from chronic HCV to HCC (*P*<0.001). there was no significant differences in Child-Turcotte Pugh classification within four groups (*P*=0.351). Cirrhotic and HCC patients had significant increased levels in ALT (*P*<0.0001 and *P*<0.0001 respectively) and AST (*P*<0.0001 and *P*<0.0001 respectively) compared to healthy control. Direct bilirubin and total bilirubin levels were significantly elevated among the three diseased groups compared to healthy control subjects (*P*<0.0001, *P*<0.0001 respectively). On the other hand, Albumin was significantly reduced in the three diseased groups compared to healthy control (*P*<0.0001). There were significant difference in prothrombin activity between the four categories and tended to decrease significantly (*P*<0.0001) during liver disease progression among studied groups. Regarding blood picture, HCC and cirrhotic patients showed significantly lower platelets

count versus uncomplicated HCV patients ($P = 0.004$). Total leucocyte count was significantly lower in HCC group versus the cirrhotic group, while, the hemoglobin level was significantly higher in fibrotic groups versus the 2 other groups ($P = 0.001$). Both the HCV RNA level (viral load) and AFP were significantly different between studied groups ($P < 0.0001$).

Table 2. Comparison between patients and control groups regarding laboratory data.

Group / Variable	Control N=30	Fibrosis N=30	Cirrhosis N=30	HCC N=30	P value
ALT, U/L, mean±SD	24.366±14.53	57.933±33.562	54.667±38.127	65.300±40.636	<0.0001
AST, U/L, mean±SD	21.200±7.165	44.566±16.747	67.433±41.629	88.800±37.913	<0.0001
GGT, U/L, mean±SD	26.266±5.976	39.133±17.756	53.266±29.779	145.433±162.158	<0.0001
Albumin, g/dl, mean±SD	4.570±0.174	4.132±0.202	3.676±0.532	3.413±0.484	<0.0001
T. Bilirubin, mg/dl, mean±SD	0.572±0.263	0.638±0.215	0.999±0.672	1.841±1.077	<0.0001
Direct Bilirubin, mg/dl, mean±SD	0.113±0.034	0.249±0.101	0.472±0.831	0.696±0.585	<0.0001
INR, Seconds, mean±SD	1.01±0.016	1.114±0.125	1.279±0.315	1.246±0.219	<0.001
AFP, ng/ml, mean±SD	3.182±1.208	7.246±3.211	13.639±13.102	27.433±29.653	<0.0001
Creatinine, mg/dl, mean±SD	0.831±0.137	0.988±0.190	0.915±0.135	2.715±10.066	0.014
Hb, g/dl, mean±SD	13.42±0.856	12.45±0.828	11.655±1.835	11.634±1.224	<0.001
WBCs $\times10^3/\mu L$, mean±SD	7.087±1.945	7.424±2.51	5.916±2.597	5.058±1.578	0.002
Platelets, $\times10^3/\mu L$, mean±SD	252.50±52.841	194.866±49.704	140.433±40.307	91.866±27.298	0.004
HCV RNA, log_{10} IU/ml, mean±SD	Negative	5.79±5.9	6.23±6.36	5.81±5.96	<0.0001

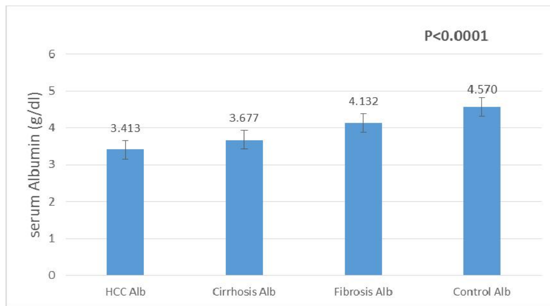


Figure 1, a. Serum Albumin levels in healthy controls (n=30), fibrosis (n=30), Cirrhosis (n=30) and HCC (n=30). Data are expressed by means ± standard error.

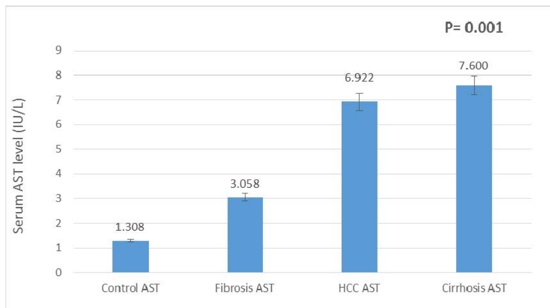


Figure 1, b. Serum AST levels in healthy controls (n=30), fibrosis (n=30), Cirrhosis (n=30) and HCC (n=30). Data are expressed by means ± standard error.

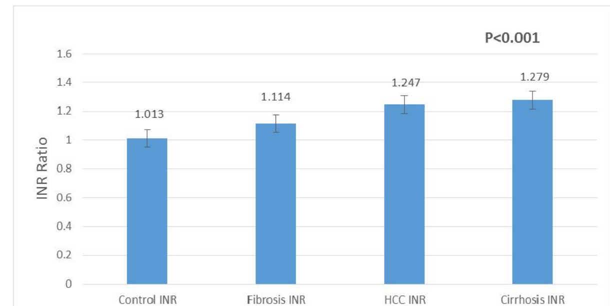


Figure 1, c. Prothrombin time (INR) in healthy controls (n=30), fibrosis (n=30), Cirrhosis (n=30) and HCC (n=30). Data are expressed by means ± standard error.

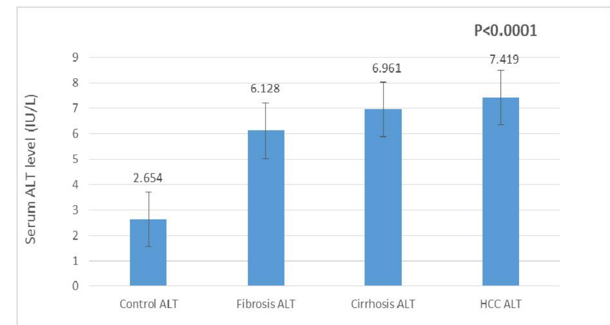


Figure 1, d. Serum ALT levels in healthy controls (n=30), fibrosis (n=30), Cirrhosis (n=30) and HCC (n=30). Data are expressed by means ± standard error.

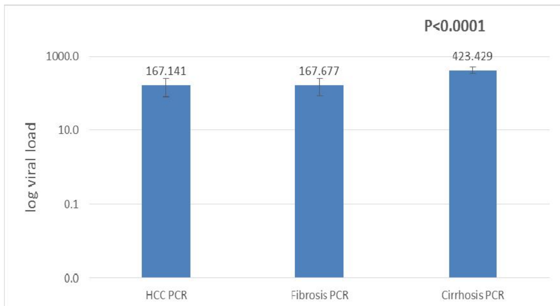


Figure 1, e. Log10 viral load levels in fibrosis (n=30), Cirrhosis (n=30) and HCC (n=30). Data are expressed by means ± standard error.

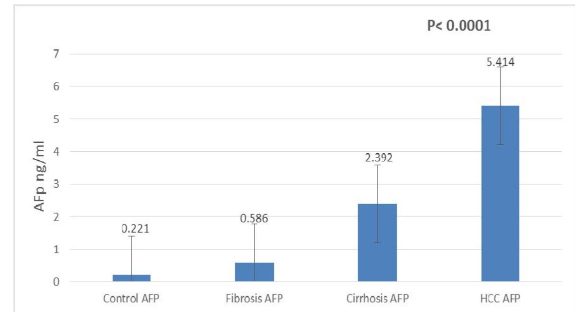
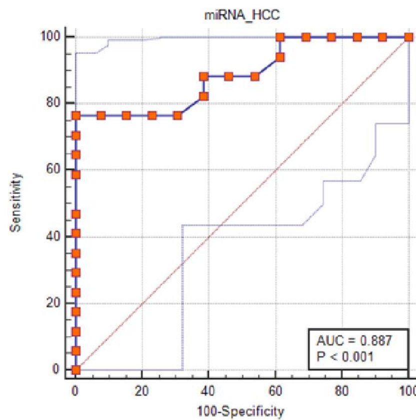
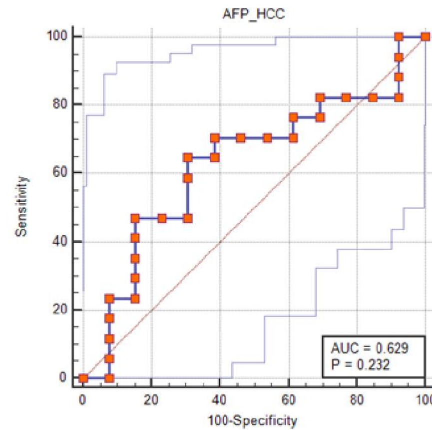


Figure 1, f. Alpha feto protein levels in fibrosis (n=30), Cirrhosis (n=30) and HCC (n=30). Data are expressed by means ± standard error.



(A)



(B)

Figure 2: (A) Receiver operator characteristic curve for miRNA-500 as a discriminant between hepatocellular carcinoma vs. non-hepatocellular carcinoma; (B) Receiver operator characteristic curve for AFP as a discriminant between hepatocellular carcinoma vs. non-hepatocellular carcinoma.

Table 3: Comparison between patients' groups regarding miRNA-500-fold difference relative to controls and AFP

Group / Variable	Fibrosis N=30	Cirrhosis N=30	HCC N=30	P value			
				All	HCC vs. Fibrosis	HCC vs. Cirrhosis	Fibrosis vs. Cirrhosis
Fold difference relative to control miR-500, median (IQR)	0.76 (3.16)	1.5 (12.91)	8.29 (40.5)	<0.00671	<0.002	0.280	0.147
AFP, median (IQR)	6.43 (10.2)	8.33 (18.2)	17.25 (33.5)	<0.00001	<0.0035	0.067	0.095

4.2 miRNA-500 serum levels

Analysis of median fold change in expression level of miR-500 in participants' sera in comparison to the normal control group showed that miRNA-500 showed significant fold decrease in expression in fibrosis group (0.76) and significant fold increasing in expression level in both Cirrhosis (1.5) and HCC (8.29) groups ($P \leq 0.00671$), **(Table 3).** Comparing serum miRNA-500 expression level between different studied groups showed an increasing tendency towards statistical significant fold elevation in expression of miRNA-500 in serum of HCC patients (8.29) in comparison to liver cirrhosis (1.5) with P value 0.28. No significant fold change in miR-500 expression was found between either (HCC vs. Cirrhosis groups) or (fibrosis vs. cirrhosis groups). MiRNA-500 showed significant up-regulation in HCC patients in comparison to non-HCC patients (fibrosis and Cirrhosis); ($P = 0.002$)

Receiver operator characteristic (ROC) analysis was used to define the optimum cut-off value for the studied diagnostic markers. ROC analysis (**Figure 2**)

revealed AUC = 0.887, and $P < 0.001$ for miRNA-500 thus distinguish between HCC and non-HCC patients, while AFP failed to do so, with AUC = 0.629 and P = 0.232, a sensitivity of only 64.7% and specificity of 69.3 % at a cutoff of 14.2. The calculated cut-off of miRNA-500 that showed the highest sensitivity (76.47%) and specificity (100%) was 0.1. Finally, when comparing ROC curves of miRNA-500 and that of AFP, there was no statistically significant difference in diagnostic accuracy between miRNA-500 (AUC = 0.887) and AFP (AUC = 0.629), (P = 0.232).

4.3. Relation of Circulating MiRNA and AFP of HCC groups.

To verify the correlation between the expression levels of miRNA-500 and with AFP of HCC group (**Table 4**), regression correlation was performed. It was found that miRNA-500 expression levels were significantly positively correlated with AFP levels (r = 0.54, P = 0.002). **(Figure 3)**

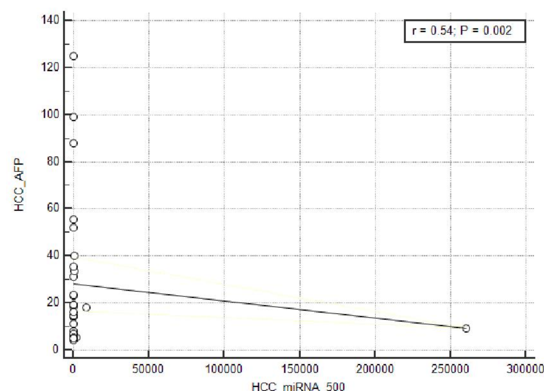


Figure 3: regression correlation between AFP in HCC group vs. miRNA-500 in HCC group.

Table 4: Diagnostic performance of miR-500 for discriminating patients with hepatocellular carcinoma from those without vs. AFP

Marker	Cutoff	Se (%)	Sp (%)	AUROC	95% C.I.	Sig.
miRNA-500	0.100	76.47	100	0.887	0.718 - 0.973	<0.0001
AFP	14.2	64.7	69.3	0.629	0.434 - 0.797	0.232

2. Discussion:

Over the last few years, it has become common practice to use tumor markers, mainly AFP, for the screening of HCC. However, performance of tumor markers has not been optimal with the sensitivity and specificity of AFP in the range of 64.7%-69.3%, respectively [16,17]. Several studies have examined the expression levels of certain miRNAs which have been implicated in roles affecting cellular proliferation and oncogenesis [18]. Cellular miRNAs have been linked with HCC. Their availability in the circulation makes them a tempting target for early tumor detection. The aim of the present study was to explore the potential usefulness of serum miR-500 as novel noninvasive markers for diagnosis of HCV related hepatocellular carcinoma in Egyptian patients.

HCC patients were within Child-Pugh A and B classifications (66.6%, 33.3% respectively). This could be explained by the fact that most of them were referred for interventional treatment. Another possible explanation is implementation of surveillance programs; allowing detection of tumors at an early stage in well compensated patients. Moreover, serum AFP level was normal (<14.2 ng/dL) in 26.66% of recruited HCC patients. Similar finding was observed by Chen et al. (1984) who suggested that not all tumors secrete AFP, and serum levels are normal in up to 40% of small HCCs. It was also showed that α-Fetoprotein alone is not recommended for the diagnosis of HCC and studies showed that its cut off value should be set at 200 ng/mL. Thus, AFP alone is not recommended for the diagnosis of HCC [19].

In this study, we examined the expression level of mi RNA-500 in 30 patient with HCC compared with cirrhotic and fibrotic groups to assess whether miRNA acts as an oncofetal miRNA and found that it increases more in HCC group. We confirm that some miRNAs can be measured from a relatively small amount of serum. A few studies reported altered levels of circulating miRNAs in association with HCC [20].

In our study, analysis of fold changes in expression level of miR-500 showed significant fold increase in expression level in HCC group (8.3±40.5) in comparison to non-HCC groups (0.87±11.3) (P<0.002, AUC= 0.88). miRNA-500 is considered a tumor-specific miRNA, contributing to the carcinogenesis and deterioration of HCC and circulating in the peripheral blood and can be a novel diagnostic marker. This was evaluated as oncofetal miRNA, by analyzing the expression levels of human miRNA-500 via RT-PCR in both non HCC and HCC groups. Differences in the miRNA-500 expression level were statistically significant (P=0.0035) between HCC and non HCC group (**Table 5, Figure 4).**

Table 5: Difference between HCC group and non-HCC group regarding miRNA-500

Group / Variable	Non-HCC N=60	HCC N=30	P value
Fold difference relative to control miR-500, median (IQR)	0.87 (11.3)	8.3 (40.5)	0.0035

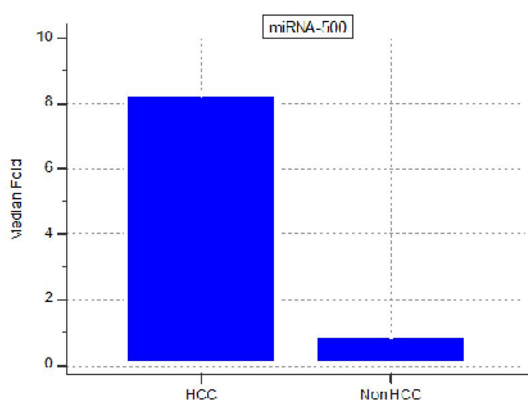


Figure 4: Comparison between HCC group and Non-HCC group that were analyzed by PCR of Human miRNA-500. The data represent the median ± IQR, *p*=0.0035.

Some of the samples exhibited remarkably high levels of expression of miRNA-500, and 43.33 % (13/30 patients) of the samples showed 3.5-13.5 fold higher up-regulation in the cancerous samples than in each cirrhotic and chronic samples and 8 patients showed more than 2 fold higher expression (26%). Substantially, significant differences in miRNA-500 expression was found between normal liver, liver fibrosis and liver cirrhosis samples (*p*=0.00671) for all groups. We found that significant differences between HCC group and fibrosis (*p*=0.002) but there's no significance between HCC and cirrhosis alone means, miRNA-500 is highly expressed in a fetal liver and down regulated in the developmental process and then up-regulated in the process of liver chronicity**.**

As several groups have reported that levels of certain circulating miRNA are associated with clinical characteristics in diseases [21,22]**.** Our data suggest that miRNA-500 was circulating in the sera of the HCC patients. Although our results are promising for miRNA-based HCC screening, there are several limitations in this study and we suggest: (1) as the sample size is quite small, further validation that miR-500 could be a reliable marker for HCC in a large cohort is necessary; (2) use of better controls to determine whether or not serum miR-500 levels are changed due to the trauma of surgery; (3) it is desirable to examine whether serum miR-500 levels change in patients with chronic hepatitis and liver cirrhosis; (4) it is necessary to compare if serum miR-500 could be better than earlier diagnostic methods such as serum Alpha-fetoprotein [23]**.**

Better results could be obtained if combined with other sero-markers and testing a panel of miRNA's collectively could ultimately serve as a reliable diagnostic test for HCC. These promising results should be validated in a larger patient cohort; nevertheless, clinical relevance of serum miRNAs as potential diagnostic, prognostic tools, therapeutic targets and biomarkers of treatment efficacy should be evaluated.

3. Conclusion

In conclusion, studied serum miRNAs; particularly serum miR-500 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.

References

1 N. Goossens and Y. Hoshida, "Hepatitis C virus-induced hepatocellular carcinoma," *Clin. Mol. Hepatol.*, vol. 21, no. 2, pp. 105–114, Jun. 2015.
2 J. D. Yang and L. R. Roberts, "Hepatocellular carcinoma: a global view," *Nat. Rev. Gastroenterol. & Amp; Hepatol.*, vol. 7, p. 448, Jul. 2010.
3 M. Ahmedin Jemal, DVM, PhD1; Freddie Bray, PhD2; Melissa M. Center, MPH3; Jacques Ferlay and P. Elizabeth Ward, PhD5; David Forman, "Global cancer statistics. CA Cancer J Clin. 2011;," *Ca Cancer J Clin*, vol. 61, no. 1, pp. 69–90, 2011.
4 S. K. Ono, F. J. Carrilho, R. M. Abreu, L. O. O. Kikuchi, P. D. Nasser, and C. S. Ferreira, "Hepatocellular Carcinoma: The Final Moments of Life," *J. Cancer Ther.*, vol. 04, no. 02, pp. 377–383, 2013.
5 J. Qi, J. Wang, H. Katayama, S. Sen, and S. Liu, "Circulating microRNAs (cmiRNAs) as novel potential biomarkers for hepatocellular carcinoma," *Neoplasma*, vol. 60, no. 2, p. 135, 2013.
6 N. T. Zinkin *et al.*, "Serum proteomics and biomarkers in hepatocellular carcinoma and chronic liver disease," *Clin. Cancer Res.*, vol. 14,

no. 2, pp. 470–477, 2008.
7 J. Bruix and J. M. Llovet, "Prognostic prediction and treatment strategy in hepatocellular carcinoma," *Hepatology*, vol. 35, no. 3, pp. 519–524, 2002.
8 R. C. Friedman, K. K. H. Farh, C. B. Burge, and D. P. Bartel, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Res.*, vol. 19, no. 1, pp. 92–105, 2009.
9 B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.
10 C.-Z. Chen, L. Li, H. F. Lodish, and D. P. Bartel, "MicroRNAs modulate hematopoietic lineage differentiation," *Science (80-.).*, vol. 303, no. 5654, pp. 83–86, 2004.
11 X. Chen *et al.*, "CpG island methylation status of miRNAs in esophageal squamous cell carcinoma," *Int. J. cancer*, vol. 130, no. 7, pp. 1607–1613, 2012.
12 V. Libri, P. Miesen, R. P. Van Rij, and A. H. Buck, "Regulation of microRNA biogenesis and turnover by animals and their viruses," *Cell. Mol. Life Sci.*, vol. 70, no. 19, pp. 3525–3544, 2013.
13 X. Chen *et al.*, "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases," *Cell Res.*, vol. 18, no. 10, p. 997, 2008.
14 G. Shiha and K. Zalata, "Ishak versus METAVIR: terminology, convertibility and correlation with laboratory changes in chronic hepatitis C," in *Liver Biopsy*, IntechOpen, 2011.
15 J. Bruix and M. Sherman, "Management of hepatocellular carcinoma: An update," *Hepatology*, vol. 53, no. 3, pp. 1020–1022, 2011.
16 H. Okuda *et al.*, "Serum levels of des‐γ‐carboxy prothrombin measured using the revised enzyme immunoassay kit with increased sensitivity in relation to clinicopathologic features of solitary hepatocellular carcinoma," *Cancer*, vol. 88, no. 3, pp. 544–549, 2000.
17 J. A. Marrero and A. S. F. Lok, "Newer markers for hepatocellular carcinoma," *Gastroenterology*, vol. 127, no. 5, Supplement 1, pp. S113–S119, 2004.
18 N. Bushati and S. M. Cohen, "microRNA Functions," *Annu. Rev. Cell Dev. Biol.*, vol. 23, no. 1, pp. 175–205, Oct. 2007.
19 R. Tateishi, H. Yoshida, Y. Matsuyama, N. Mine, Y. Kondo, and M. Omata, "Diagnostic accuracy of tumor markers for hepatocellular carcinoma: a systematic review," *Hepatol. Int.*, vol. 2, no. 1, pp. 17–30, 2008.
20 F. Borel, P. Konstantinova, and P. L. M. Jansen, "Diagnostic and therapeutic potential of miRNA signatures in patients with hepatocellular carcinoma," *J. Hepatol.*, vol. 56, no. 6, pp. 1371–1383, 2012.
21 S. Gilad *et al.*, "Serum microRNAs are promising novel biomarkers," *PLoS One*, vol. 3, no. 9, p. e3148, 2008.
22 C. H. Lawrie *et al.*, "Detection of elevated levels of tumour‐associated microRNAs in serum of patients with diffuse large B‐cell lymphoma," *Br. J. Haematol.*, vol. 141, no. 5, pp. 672–675, 2008.
23 Y. Yamamoto *et al.*, "MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma," *Biomarkers*, vol. 14, no. 7, pp. 529–538, 2009.

6/10/2019