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Prof. Mohamed Labib Salem, PhD

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## **Welcome letter from Editor-in-Chief**



Welcome to the Int J Cancer and Biomedical Research (IJCBR)!

It is with great pleasure that I write this editorial to welcome you to the IJCBR. This journal provides a platform for publication of original and reviews research articles, short communications, letter to editor, thesis abstract, conference report, and case studies. These types of publication are directed at the interface of the fields of cancer and biomedical research.

The IJCBR relies on a distinguished expert of the Advisory and Editorial Board Members from the top international league covering in depth the related topics. They timely review all manuscripts and maintain highest standards of quality and scientific methodology and ethical concepts. Meanwhile, we take all possible means to keep the time of the publication process as short as possible.

I take this chance to welcome your contributions to the IJCBR and have every expectation that it will soon become one of the most respected journals in both the fields of cancer and biomedical research.

A handwritten signature in blue ink that reads "Mohamed L. Salem". The signature is fluid and cursive.

**Mohamed L. Salem,**

Editor in Chief

## Measurement of plasma microRNA-500 could differentiate between liver fibrosis and cirrhosis

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### ABSTRACT

**Background:** A significant number of patients with hepatitis C virus (HCV) infection are at high risk of progressing to liver cirrhosis and hepatocellular carcinoma (HCC). The lack of noninvasive methods for assessment of the degree of liver damage is the main limitation in the clinical management of liver diseases. **Aim:** This study was conducted to evaluate the expression level of plasma miR-500 in HCV-induced fibrosis, cirrhosis and HCC patients. **Methods and results:** This prospective study involved 120 subjects, who were divided into 4 groups (n=30/group): healthy control, liver fibrosis patients, liver cirrhosis patients and HCC patients; all patients encountered in the work were clinically diagnosed as chronic HCV patients. The expression of miR-500 in plasma was assessed using quantitative real-time PCR, whereas serum alpha-fetoprotein (AFP) was measured using electrochemiluminescence immunoassay. **Results:** The fold change in expression of plasma miR-500 showed significant up-regulation in cirrhotic patients more than 8 fold change as compared to fibrosis group. No significant fold changes in plasma miR-500 expression were found between either (HCC versus cirrhosis group) or (HCC versus fibrosis group). The serum level of AFP was 28.09 higher in the HCC group than non-HCC group. Receiver operating characteristic curve (ROC) analysis indicated that the ideal cut-off values of the plasma miR-500 in both fibrosis and cirrhosis groups were 1.52 and 1.15, respectively and 8.2 and 4.55, respectively for AFP. **Conclusion:** Plasma miR-500 can be used as a novel biomarker for the differentiation between liver fibrosis and cirrhosis.

**Keywords:** Biomarkers; Hepatocellular carcinoma; Liver cirrhosis; Liver fibrosis; MicroRNA-500

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### INTRODUCTION

Hepatitis C virus (HCV) infection is a health disaster disturbing among 185 million individuals universally. Egypt has the topmost frequency for chronic HCV infection worldwide. HCV infected subjects constitute from 10%-30% would progress to more worsening pathogenesis such as cirrhosis and/or HCC (Shepard et al., 2005). Existing biomarkers for diagnosis of liver pathogenesis are lacking both sensitivity and specificity. It is mandatory to develop reliable biomarkers that are sensitive, specific, and non-invasive for early diagnosis and rapid intervention of hepatic fibrosis,

cirrhosis and HCC to increase the survival rate of patients. Circulatory microRNAs (miRs) constitute great attention as promising biomarkers for various diseases (Kim et al., 2016). Small non-coding RNA sequences, known as microRNAs (miRNAs), are 18-25 nucleotides. MiRNAs can posttranscriptionally regulate different cellular pathways including cellular differentiation, growth, proliferation, metabolism, angiogenesis, regeneration, survival, apoptosis, and tumorigenesis. Most miRNA genes are located on intergenic regions (non-coding regions) of the nuclear DNA and are expressed in the cytoplasm through the

following process. In the nucleus, RNA polymerase II transcribes a long primary miRNA (pri-miRNA) with a hairpin-like structure. Drosha then converts it to precursor miRNA (pre-miRNA), a 70- nucleotide stem loop, followed by its transport to the cytoplasm through the action of Exportin 5, where it meets Dicer and is cleaved into 22-nucleotide double-stranded miRNAs. Then, it is separated into two single-stranded miRNAs. The sense (passenger) strand is degraded while the antisense (guide) strand binds to RNA-induced silencing complex (RISC). The interaction between the 5' seed sequence of the miRNA and the 3'-untranslated region of the mRNA determines the effect of the miRNA, whether degradation of the mRNA or the inhibition of its translation (Elfar et al., 2019).

MiRNAs depending on their inhibited targets can be either tumor-suppressing or oncogenic. Meaningfully, miR-21 causes stimulation, proliferation and cell invasiveness while inhibiting apoptosis. MiRNA expression is associated with clinical and biological characteristics of tumors, such as tissue of origin, differentiation, invasiveness and treatment responsiveness. MiRNAs can be utilized as diagnostic markers in human serum or plasma measurements. Thus, cancer and tumor miRNAs in serum or plasma can be measured noninvasively, excluding leukemias which is easily accessible to malignant cells (Babaei et al., 2020).

MiR-500 is of great interest in cancer therapies due to its association with various types of cancer, including gastric and prostate cancer (Bao et al., 2018). MiR-500 is an oncofetal miRNA, which is highly expressed in fetal liver, more than in adult normal liver, and aberrantly expressed in HCC tissue (Yamamoto et al., 2009). A study done by Guo et al. Reported that miR-500 may play an important part in the migration and invasion of HCC, and represents a novel therapeutic target for HCC. However, the precise biological effects of miR-500 in HCC are yet to be determined (Guo et al., 2017).

This study was undertaken in order to investigate miR-500 expression in HCV-infected patients with fibrosis, cirrhosis and HCC and to determine if the miR-500 could be used as novel

biomarkers for diagnosis of several stages of HCV-associated liver diseases as an alternative to existing biomarkers.

## SUBJECTS AND METHODS

### Patients

Ninety HCV-infected Egyptian patients were involved in the research. The patients included 55 males and 35 females, and; their ages ranged from 22 to 71 years. A Prospective randomized double-blinded study was carried out and the patients were recruited from the Egyptian Liver Research Institute and Hospital (ELRIAH) in Sherbin, Mansoura, Egypt during the period from May 2016 to September 2017. Exclusion criteria included pregnancy, cancer therapy (radiotherapy, chemotherapy and immunosuppressive therapy), renal disorders (serum creatinine concentration > 1.5 mg/dL), positive results on tests for human immunodeficiency virus (HIV) and hepatitis B virus (HBV), frequent blood transfusions, heart diseases (ECG abnormalities, ischemic or congestive diseases), muscle disorders, pancreatitis and bilharziasis. Univariate analysis was utilized to measure differences in demographic data among fibrosis, cirrhosis and HCC patients. Data were process using SPSS statistical package version 21, IBM corporation software group, USA. However, the controls were younger than HCC patients ( $p < 0.001$ ). Fibrotic and cirrhotic patients were age-matched with HCC patients. The demographic data of the patients are summarized in Table 1.

HCV-infected patients were classified into three groups based on ultrasonographic and elastographic criteria. The stage of fibrosis was diagnosed by FibroScan (Echosens®, Paris, France) and estimated according to METAVIR scoring, as follows (Wendum *et al.*, 2009): Fibrosis stages were arranged on a 0–4 scale. A score of 0 (F0) indicated no fibrosis, a score of 1 (F1) was identified as mild to moderate focal areas of fibrosis without septa, a score of 2 (F2) was identified as fibrous extensions of most portal areas, with short fibrous septa, the fibrous spreading of large portal areas with many septa without cirrhosis is scored as F3, and a score of 4 (F4) was categorized by manifested bridging and irregular nodules and definitive cirrhosis. Patients with F0/F1 score of

liver fibrosis were excluded. Thirty fibrotic patients were diagnosed with fibrosis stage F2/F3 and served as the fibrosis group. Thirty patients with cirrhosis were diagnosed with fibrosis stage F4 and served as the cirrhosis group. Thirty patients with HCC were included, as confirmed by the presence of focal lesions detected by Tomography (PHILIPS®, U.S.A), who served as the HCC group. The etiology of fibrosis, cirrhosis, and HCC for all patients engaged in this study was chronic HCV infection. Thirty infection-free cases were involved in the study and represented as the control group.

The informed consent forms were signed from all applicants before conduct the study. This research study was authorized by the Ethical Committee of the Faculty of Pharmacy, Tanta University.

This study was conducted in accordance with the World Medical Association's Declaration of Helsinki. It was accepted by the Research Ethics Committee of Tanta University with registration number: PR/CTU/09018. The aim of the study was fully clarified to the cases, and all cases signed an informed consent prior to enrolment in the study. The data were reserved secured and utilized only for purposes of research.

## Methods

A venous blood sample (8 mL) was collected from each case using a sterile BD vacutainer butterfly needle. Each blood sample was divided into four portions (2 mL each). The first portion was collected in a tube containing 4 mg of potassium-ethylene diamine tetraacetic acid (K<sub>2</sub>EDTA) for complete blood count (CBC). The second portion was delivered in a tube containing 4 mg of K<sub>2</sub>EDTA; plasma was separated after centrifugation and stored in a sterile RNase-free Eppendorff tube at -80 °C until analysis of miR-500. The third portion was collected into a citrate-tube, and the plasma was separated for determination of prothrombin time. The fourth portion was collected in a BD vacutainer serum separator tube, and serum samples were separated after centrifugation and stored at -80 °C until liver biomarkers were assessed.

## Biochemical assays

CBC were assayed using automated hematology analyzer (Sysmex® XT-1800i, Japan). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assessed spectrophotometrically by the kinetic method (Bergmeyer *et al.*, 1986). The activity of serum gamma glutamyl transferase (GGT) was measured spectrophotometrically using the enzymatic colorimetric method (Szasz, 1969). Serum alkaline phosphatase (ALP) was measured using the colorimetric method (Tietz *et al.*, 1983). Serum albumin was measured by the immunoturbidimetric method (Gentilini *et al.*, 2005). Serum total bilirubin was measured using the colorimetric method (Parviainen, 1997). Serum direct bilirubin was measured according to the diazo method (Westwood, 1991). Prothrombin time was determined by coagulation technique described by Matsumoto *et al.* (Matsumoto *et al.*, 2014). Serum AFP was measured using an electrochemiluminescence immunoassay (ECLIA) method (Guder *et al.*, 1996). The kits were purchased from Roche® Diagnostics (Germany).

## RNA isolation, reverse transcription and quantitative real time PCR

Total RNA (including miRs) was extracted from K<sub>2</sub>EDTA blood samples using a miRNeasy Mini Kit (Qiagen®, USA) according to manufacturer's guidelines. The reverse transcription of total RNA, then quantitative real-time polymerase chain reaction (qPCR) were performed. The concentration of RNA was measured by measuring by Nonodrop method using Immuline. By measuring the ratio of absorbance at 260 nm and 280 nm (A<sub>260</sub>/A<sub>280</sub>) and this provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. Pure RNA has an A<sub>260</sub>/A<sub>280</sub> ratio of 1.9–2.1.

First, a reverse transcription miScript II RT Kit, Catalog no. 218161 kit (Qiagen, Germany) was used to reverse transcript the extracted RNA (200 µL) into complementary DNA (cDNA). The obtained cDNA was amplified using a TaqMan® miR assay kit (Applied Biosystems Inc®, USA) as described by the manufacturer. The PCR program was customized to begin with an initial activation for 2 min at 95 °C, then 40 cycles (95° C for 15 s and 60 °C for 60 s) using RT-qPCR

system (DNA technology<sup>®</sup>, Russia). Each sample was analyzed and normalized to the level of the reference gene (SNORD 68) and expressed as the relative copy number (RCN). The threshold cycle (Ct) values of the samples were calculated through DT master software. The relative quantity of each miR-500 to Snord 68 was calculated by using the following equation:  $\Delta\Delta Ct = \Delta Ct (\text{target gene}) - \Delta Ct (\text{reference gene})$  (Guan *et al.*, 2016).

The sequence of the MiR-500 primers (Invitrogen Co<sup>®</sup>, USA) utilized in RT-qPCR were as following: miR-500a forward reverse transcription primer: 5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TCT CAC CC-3'; quantitative PCR forward primer: 5'-ACA CTC CAG CTG GGT AAT CCT TGC TAC CTG G-3' and reverse primer: 5'-TGG TGT CGT GGA GTC G-3'. The primer sequence for the housekeeping miRNA SNORD 68 was as following: forward primer, 5'-CTCGCTTCGGCAGCACA-3', reverse primer, 5'-AACGCTTCACGAATTTGCGT-3'.

### Statistical Analysis

The expression levels of plasma miR-500 were analyzed using fold change. Data were processed using SPSS statistical package version 21.0, IBM Corporation Software Group, USA. Quantitative results were examined for normality using one sample Kolmogorov-Smirnov test.

Qualitative data was presented as frequency and percentage, normally distributed data as mean  $\pm$  SD, and non-normal distributed data as median and interquartile range (IQR). Chi-square test was used to compare qualitative data, ANOVA was used to compare normally distributed quantitative data while Kruskal-Wallis test was used to compare non-normally distributed quantitative data. Comparison of any two groups was done using Chi-square for qualitative data, student's t-test or normally distributed quantitative data or Mann-Whitney U test for non-normally distributed quantitative data. Spearman correlation coefficient was used to assess strength and direction of association that exists between two variables. All p values were two-tailed and  $p \leq 0.05$  was considered statistically significant. ROC curve was designed to assess the diagnostic values of miR-500 and AFP in different groups.

### RESULTS

The results are summarized in Table 2. The following variables were found to be normally distributed: Age, Hg, RBCs, WBCs, Plt. The remaining numeric variables were not normally distributed: HCV RNA, ALT, AST, GGT, ALP, Albumin (total, direct), creatinine, uric acid, INR, AFP, miR-500. The activities of ALT, AST and GGT were significantly increased in all patients in the fibrosis, cirrhosis and HCC groups ( $P < 0.0001$ ), in compare with those of the control healthy cases.

The HCC group showed significant elevations in ALP activity by 44% and 47% ( $P \leq 0.001$ ), in comparison with those of the fibrosis and cirrhosis groups, respectively. The patients in the HCC group showed the lowest serum albumin levels compared to those of the healthy control, fibrosis and cirrhosis groups (by 18%, 17% and 11%, respectively). The other hepatic biomarkers (total bilirubin and INR) were significantly increased in the cirrhosis and HCC groups, when compared to control healthy and fibrosis groups. There were no significant changes in the concentrations of uric acid, creatinine and urea among the studied groups.

Analysis of median fold change in the expression level of plasma miR-500 showed that miR-500 level decreased in fibrosis group (0.76) and there was a significant fold increase in the cirrhosis group (8.29) relative to control healthy group (Table 2). Furthermore, results of comparing plasma miR-500 expression level between different studied groups showed that there was statistically significant increase in the miR-500 expression level in the fibrosis group in comparison to liver cirrhosis group ( $P$  value 0.02).

No significant fold changes in plasma miR-500 expression were found between either (HCC *versus* cirrhosis group) or (HCC *versus* fibrosis group). A non-significant negative correlation was found between miR-500 and AFP in all studied groups (Table 3). Characteristically, receiver operator characteristic (ROC) analysis was utilized to characterize the ideal cut-off value for the studied diagnostic markers.

**Table 1.** Demographic data for patients and healthy controls

Variable	Group	Healthy Control Group (n=30)	Fibrosis Group (n=30)	Cirrhosis Group (n=30)	HCC Group (n=30)	P-value			
						All	HCC vs. Fibrosis	HCC vs. Cirrhosis	Fibrosis vs. Cirrhosis
Age, yr, mean±SD		33.8 ± 7.3	48.8 ± 9.8	56.6 ± 6.02	58.3 ± 7.3	<0.001	<0.001	0.520	<0.001
Sex (M: F)		15:15	16:14	16:14	23: 7	0.131	0.058	0.058	1.000
Smoking (%)		0	7 (23.3%)	10 (33.3%)	10 (33.3%)	0.621	0.390	1.000	0.390
DM (%)		0	3 (10.0%)	9 (30.0%)	17 (56.7%)	<0.001	<0.001	0.037	0.053
HTN (%)		0	3 (10.0%)	12 (40.0%)	6 (20.0%)	0.020	0.278	0.091	0.007
HCV RNA log <sub>10</sub> IU/mL; median (IQR)		Negative	5.11 (0.89)	5.91 (1.10)	5.19 (1.49)	0.009	0.953	0.019	0.003
Focal lesion (Single: Multiple)		0	0	0	16: 14	0.400			
Tumor size (<3 cm: ≥3cm)		0	0	0	15:15	0.870			

HCC: Hepatocellular carcinoma; DM: Diabetes mellitus; F: Female; HTN: Hypertension; HCV: Hepatitis C virus; M: Male. Significance was set at a P < 0.05.

**Table 2.** Biochemical and hematological variables of healthy control and patient groups

Variable	Group	Healthy Control Group (n=30)	Fibrosis Group (n=30)	Cirrhosis Group (n=30)	HCC Group (n=30)	P-value			
						All	HCC vs. Fibrosis	HCC vs. Cirrhosis	Fibrosis vs. Cirrhosis
ALT (U/L), median (IQR)		19.0 (16.0)	42.34 (25.98)	41.00 (39.25)	51.00 (28.50)	< 0.001*	0.403	0.234	0.375
AST (U/L), median (IQR)		20.00 (8.75)	35.39 (17.30)	60.50 (45.00)	77.00 (37.75)	< 0.001*	<0.001*	0.029*	0.047*
GGT (U/L), median (IQR)		24.50 (12.0)	30.82 (17.47)	39.0 (44.0)	85.0 (62.75)	< 0.001*	<0.001*	<0.001*	0.119
ALP (U/L), median (IQR)		68.50 (22.75)	72.02 (33.31)	64.50 (39.50)	99.00 (54.50)	0.001*	0.007*	0.002*	0.367
Albumin (g/dL), median (IQR)		4.40 (0.33)	4.31 (0.53)	4.20 (0.90)	3.59 (0.83)	< 0.001*	<0.001*	0.004*	0.300
Total Bilirubin (mg/dL), median (IQR)		0.50 (0.23)	0.60 (0.31)	0.75 (0.60)	1.20 (0.78)	< 0.001*	<0.001*	0.003*	0.003*
Direct Bilirubin (mg/dL), median (IQR)		0.10 (0.00)	0.24 (0.14)	0.28 (0.13)	0.50 (0.59)	< 0.001*	<0.001*	0.001*	0.402
Creatinine (mg/dL), median (IQR)		0.87 (0.21)	0.88 (0.50)	0.80 (0.20)	0.79 (0.27)	0.640	0.355	0.891	0.283
Uric Acid (mg/dL), median (IQR)		4.85 (1.40)	4.85 (1.25)	4.75 (1.80)	5.25 (1.65)	0.527	0.290	0.482	0.784
INR (seconds)		1.01 (0.00)	1.01 (0.16)	1.12 (0.25)	1.25 (0.37)	< 0.0001*	0.341	0.682	0.338
AFP, median (IQR)		2.54 (1.98)	4.31 (5.25)	6.50 (12.83)	28.90 (86.84)	<0.001*	<0.001*	0.003*	0.019*
miR-500, median (IQR)		1.00 (0.00)	0.758 (4.024)	8.291 (42.14)	1.503 (13.82)	0.050*	0.178	0.188	0.020*
Hb (g/dL), mean ± SD		13.72 ± 1.08	13.42 ± 0.77	12.83 ± 2.25	13.41 ± 1.63	0.160	0.960	0.258	0.175
RBCs ×10 <sup>3</sup> /μL, mean ± SD		4.80 ± 0.47	5.08 ± 0.53	4.58 ± 1.07	4.50 ± 0.70	0.013	0.001*	0.742	0.027*
WBCs ×10 <sup>3</sup> /μL, mean ± SD		7.09 ± 1.95	7.42 ± 1.95	5.92 ± 2.60	5.06 ± 1.58	< 0.001*	<0.001*	0.128	0.026*
Plt ×10 <sup>3</sup> /μL, mean ± SD		252.50 ± 52.84	194.87 ± 49.70	115.36 ± 56.98	95.03 ± 37.64	< 0.001*	<0.001*	0.108	<0.001*

HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transferase; INR: International normalized ratio; AFP: Alpha-fetoprotein; Hb: Hemoglobin; RBCs: Red blood cells; WBCs: White blood cells; Plt: Platelet.

\*: Significance at a P 0.05.

First of all, ROC analysis for fibrotic group showed AUC = 1, and  $P < 0.0001$  for AFP, while plasma miR-500 failed to do so, with AUC=0.67 and  $P=0.21$ . Furthermore, the calculated cut-off showed that miR-500 had 71.4% sensitivity, 78.3% specificity, and 49.6% accuracy. On the other hand, the AFP ROC curve showed 100% sensitivity, 100% specificity, and 100% accuracy (Figure 1A). On the other hand, ROC analysis bared AUC=0.843, and  $P < 0.001$  for AFP which distinguish between HCC and non-HCC patients, whereas plasma miR-500 revealed AUC=0.5 and  $P=0.98$ . Moreover, the calculated cut-off showed that miR-500 had 86.7% sensitivity, 23.3% specificity, and 55% accuracy. On the other hand, the AFP ROC curve showed 70% sensitivity, 86.7% specificity, and 78.3% accuracy (Figure 1C).

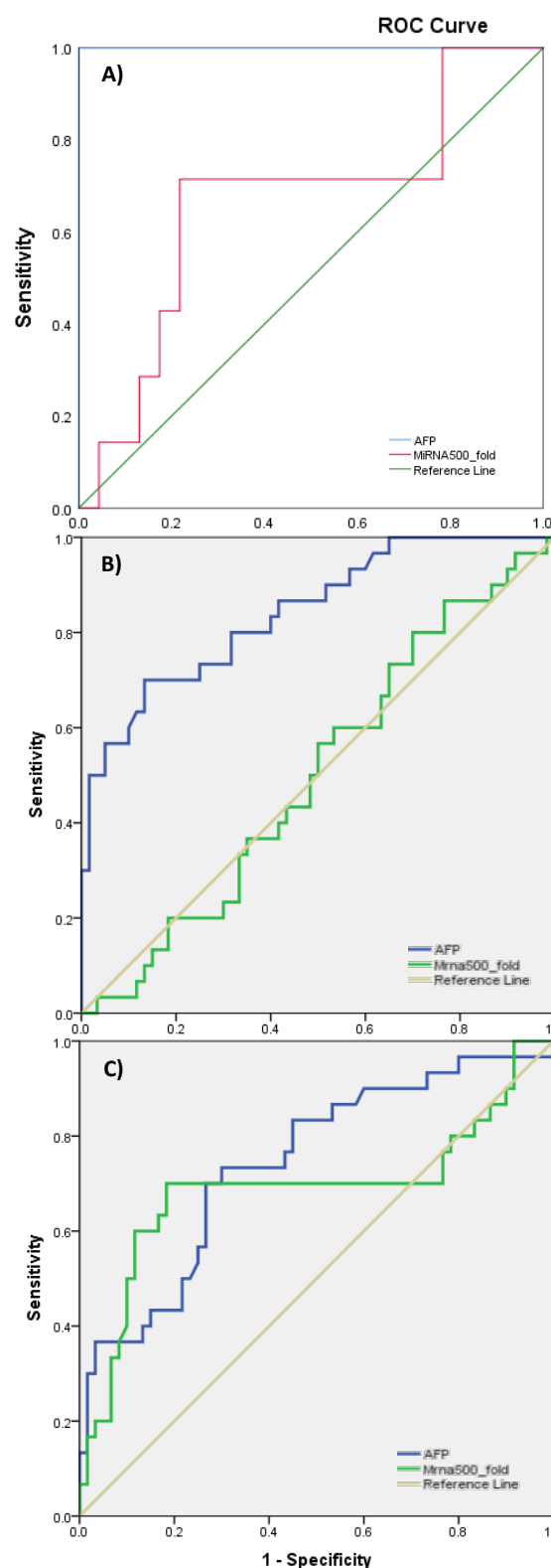
**Table 3.** Correlation between miR-500 and AFP in different patient groups

Variable	Groups	Fibrosis n=30	Cirrhosis n=30	HCC n=30
r (miR-500 and AFP)		-0.016	-0.064	-0.058
P value		0.933	0.736	0.761

Data are presented as r (Pearson correlation coefficient), HCC: Hepatocellular carcinoma; Alpha-fetoprotein; miR-500: micro RNA-500; Negative correlation.

## DISCUSSION

The prevalence of HCC is increased globally. It is the second most common reason of cancer death worldwide in males and the fifth most common cause of cancer-related death worldwide in females (Gomaa *et al.*, 2014). In Egypt, which has the greatest incidence of HCV worldwide, HCC is the most common cancer (Gomaa *et al.*, 2014). MiRs can be used as a diagnostic biomarker for several diseases. For example, overexpression of miR-205 and miR-21 in ductal adenocarcinoma has been reported to precede phenotypic changes in the ducts (Du Rieu *et al.*, 2010), supporting the possibility to use them for early detection of cancers. In addition, a study interrogated plasma miR-10b and miR-373 levels, which are mediators of metastasis in breast cancer, and they uncovered association of these miRNAs for detecting the lymph node metastasis, thereby showing potential as prognostic biomarkers (Chen *et al.*, 2013).



**Figure 1.** Receiver operating characteristics (ROC) curve comparing the specificity *versus* sensitivity of microRNA-500 and AFP. (A) Fibrosis group, (B) Cirrhosis group, (C) HCC group. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value, AUROC: Area under the ROC curve, CI: Confidence interval, Sig: Significant level P-value, AFP: Alpha-fetoprotein.



The miRNAs have emerged as a powerful biomarker of hepatic diseases. The miR-122 is major miRNA in liver accounting up to 70% of the total hepatic miRNAs. The levels of miR-122 can also be correlated with the stage and severity of HBV and HCV infection and are also helpful in the measurement of treatment response (Köberle *et al.*, 2013). The expression levels of 4 miRNAs (miR-122, miR-134, miR-424-3p, and miR-629-5p) were up-regulated during HCV infection (Zhang *et al.*, 2015).

Regarding HCC, Various studies have reported the up-regulation of miR-21, miR-221 and miR-222 while down-regulation of miR-122a, miR-145, miR-199a and miR-223 in HCC in comparison to the normal tissue in human (Musaddaq *et al.*, 2018).

MiRs are stable and resist degradation by RNase (Cancer, 2012). In various liver diseases, miRs can be considered to be promising biomarkers for diagnosis (Chuanhao *et al.*, 2012). This study was done using the plasma level of circulating miR-500 as a diagnosing marker in detecting liver fibrosis, cirrhosis or HCC. The aim of the current study was to evaluate the expression level of plasma miR-500 in fibrosis, cirrhosis and HCC patients infected with HCV and to compare the plasma level of miR-500 and serum concentration of AFP in different patient groups. The results revealed that cirrhotic samples had significantly increased levels of miR-500 compared to healthy controls and fibrotic patients. Analysis of ROC curve demonstrated that miR-500 had better performance than AFP in distinguishing cirrhotic patients from fibrotic patients (AUROC: 0.688 *versus* 0.67, sensitivity: 70.0% *versus* 71.4%, specificity: 81.7% *versus* 78.3%, significance: 0.004 *versus* 0.212, respectively). AFP was more effective than plasma miR-500 in diagnosing of HCC (AUROC: 0.843 *versus* 0.501, sensitivity: 70.0% *versus* 86.7%, specificity: 86.7% *versus* 23.3%, significance: <0.001 *versus* 0.986, respectively). MiR-500 showed no advantage over AFP in diagnosing HCC. Based on these results, we discovered that plasma miR-500 is a novel biomarker of liver cirrhosis. Plasma miR-500 is considered an independent diagnostic biomarker as non-significant correlation was found between miR-500 and AFP in fibrotic,

cirrhotic and HCC patient groups (P value: 0.933, 0.736, 0.761, respectively).

There was some age and sex-relations with the development of HCC and HCV in the Egyptian patients. The age of the studied HCC cases was  $58.3 \pm 7.3$  years. This finding was agreed with a study conducted by Holah *et al.*, who reported that the age of HCC patients fluctuated from 42 to 70 years (mean  $58.70 \pm 5.76$  years) (Holah *et al.*, 2015). These findings can be explained by the long time from the beginning of the hepatic inflammatory process to the occurrence of HCC. The majority of HCV infections among Egyptian individuals aged 30 years and older can be explained by parenteral therapy and other iatrogenic exposures [30]. Our study showed that there were 23 (76.67%) males and 7 (23 %) females in the HCC group. This finding is consistent with the study performed by EL Zayadi *et al.* who found that HCC was significantly more frequent in males than females (77.7% and 23.3%, respectively) (El-Zayadi *et al.*, 2001). The precise reason for this male predominance is not known, but it may be elucidated by higher contact of males to different environmental carcinogenic factors (aflatoxins, agricultural pesticides, bilharziasis and anti-bilharzial injection treatment), or by greater DNA production in male cirrhotic cases than in females due to the role of sex hormones (Darbari *et al.*, 2003).

Regarding to smoking history, the difference between the studied groups was not significant (P-value = 0.621). This finding is consistent with the study performed by Ezzat *et al.*, 2005, who reported that the difference between HCC subjects and controls with regard to history of smoking and alcohol abuse was not statistically significant (Ezzat *et al.*, 2005) This may be related to physicians' advice to stop smoking in presence of liver disease. But this is not agreed with some studies that considered smoking as an independent risk factor for HCC (Koh *et al.*, 2011). The liver metabolizes and transform more than 40 tobacco-related active compounds, in which some considered carcinogens such as polycyclic aromatic hydrocarbons, nitrosamines, and aromatic amines. These tobacco-related carcinogens have been directly implicated in the hepatocarcinogenesis (Koh *et al.*, 2011).

In the present study, diabetes mellitus (DM) was significantly found in 56.7% of HCC patients (compared to 10% among patients with fibrosis and 30% among patients with liver cirrhosis). The results are in accordance with a meta-analysis performed by Wang *et al.*, which concluded that diabetes is associated with a moderately increased risk of HCC prevalence and HCC mortality (Wang *et al.*, 2012).

By analyzing laboratory biomarkers of our patients, we found a highly significant differences between the patient and control groups regarding liver function tests (albumin, bilirubin, ALT, AST, ALP, GGT), AFP and HCV RNA quantitative PCR. These changes may have occurred because HCC is a result of liver cirrhosis consequences, and these parameters are used to stage chronic liver disease. These results run parallel to many studies that concluded that liver cirrhosis is the most important causative factor for the development of HCC, which is present in 80% to 90% of HCC patients (El-Serag, 2012).

Our study focused on the plasma level of circulating miR-500 as a diagnosing marker in detecting the degree of liver damage. The study of Guo *et al.* (Guo *et al.*, 2017), who revealed that the expression level of miR-500 was higher in the HCC tissues (n= 17) compared with the normal liver tissues (n= 7) and was closely correlated with poor prognosis suggesting that miR-500 may act as an oncogene in HCC. To overcome the limited sample size performed in previous studies we compared the expression level of plasma miR-500 in larger patient groups. Our study showed that the plasma level of miR-500 in HCC cases (n= 30) was non-significantly higher (1.503-fold) than that of the normal control group (n=30).

On the other hand, our results exhibited that there is a significant elevation in the expression level of plasma miR-500 in the cirrhotic group, which was 8.291-fold increase relative to the control group with 70.0% sensitivity, 81.7% specificity, and 76.7% overall accuracy. These results were consistent with the results of Yamamoto *et al.*, (Yamamoto *et al.*, 2009), who reported that a significant difference was found in the expression level of miR-500 between normal liver tissues and liver tissues with

cirrhosis (n=17), proposing that the expression of miR-500 was increased during the development stage of cirrhosis.

Also, the level of expression of plasma miR-500 was decreased in fibrotic patients infected with HCV without cirrhosis, the level of miR-500 was 0.758-fold difference relative to the healthy control cases with 71.4% sensitivity, 78.3% specificity, and 49.6% overall accuracy. Our results were in accordance with the study performed by Yamamoto *et al.*, (Yamamoto *et al.*, 2009), who found that no significant difference in miR-500 expression was found between normal liver tissues (n= 11) and patients infected with HCV without cirrhosis (n=19). Several studies showed different cut-off values of AFP levels in diagnosing patients with HCC, a study conducted on one hundred and thirty nine cases with HCC with optimum cut-off value of 20.85 ng/ml showed 72.2% sensitivity, 86.2% specificity, 89.9% positive predictive value, 64.7% negative predictive value and 77.4% overall accuracy in diagnosing patients with HCC (Sarwar *et al.*, 2014).

Our results are also supported by the study of Gopal and colleagues, in which a cut-off value of serum AFP greater than 20 ng/mL had 70.4% sensitivity and 82.6% specificity, 76.9% overall accuracy for HCC detection, AFP level of 59 ng/mL or more with 59.6% sensitivity and 93.9% specificity, 78.0% overall accuracy accurately detecting HCC in patients with HCV-associated cirrhosis (Gopal *et al.*, 2014).

There are several limitations in this study including the sample size that was small and needed to be larger. The age of the control group was also required to be matched with the experimental groups. We suggest combining our results with other sero-markers and testing a panel of miRs that could ultimately serve as a reliable diagnostic tests for liver impairment.

## CONCLUSION

Plasma miR-500 could distinguish HCV induced cirrhosis from HCV-associated liver fibrosis and healthy control subjects suggesting their potential usefulness as a promising biomarker for the diagnosis of cirrhosis in HCV-infected patients. It is crucial to authenticate this study in ethnically different populations, with diverse

genotypes and other risk factors for HCC to survey the real noteworthiness of microRNAs in HCC.

### CONFLICT OF INTEREST

Authors declare that they have no conflicts of interest.

### FUDING

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EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (<http://acdd.tanta.edu.eg>). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: <https://jcbjournals.ekb.eg>) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: [www.ekb.eg](http://www.ekb.eg)).

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