

CIRCULATING MICRORNA-615-5P AS A DIAGNOSTIC MOLECULAR MARKER IN EGYPTIAN PATIENTS WITH LIVER CIRRHOSIS

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ABSTRACT

Background: Liver cirrhosis has emerged as a major cause of global health burden. The vast majority (80-90%) of HCCs develop in a cirrhotic liver. Liver cirrhosis and HCC due to chronic hepatitis C are among the main indications for liver transplantation. The confront in the 21st century is to prevent the need for liver transplantation in cirrhotic patients as much as possible. Due to the unique situation of hepatitis C virus (HCV) in Egypt, being plagued with the highest HCV prevalence in the world, a considerable increase was observed in the proportion of chronic liver disease in Egyptian patients. Liver biopsy has traditionally been the “gold standard” test; it is an invasive procedure with rare but potentially life-threatening complications. These limitations rapidly reduced its use to the sake of developing non-invasive methods as indices and imaging. Alpha fetoprotein (AFP) is seen as the commonest and feasible marker for assessing liver cirrhosis; however, it is not completely reliable because of its low specificity and sensitivity. In early cirrhosis, however, conventional imaging can lead to false-negative diagnosis so other strategies are urgently needed. Currently, lack of robust biomarkers still limits evaluation of hepatic failure and progression in chronic diseases, especially in HCV infection. **Aim:** the main objective of the present study was to evaluate the significance of a new, non-invasive molecular marker, microRNA-615-5p (miR-615-5p) in circulating blood for diagnosing liver cirrhosis arising from HCV infection. **Methods:** A total of 50 blood samples were collected; 30 samples from hepatic cirrhotic patients, 10 samples from chronic HCV hepatitis patients and 10 control samples. The level of circulating miRNA-615-5p was detected by RT-qPCR in all samples. Besides, miR-615-5p levels in relation to clinical laboratory parameters were explored. **Results:** The expression of circulating miR-615-5p was distinctly increased in hepatic cirrhosis with HCV chronic patients compared with control group (**mean ± SE: 5.44±0.02, P < 0.01 and 4.9±0.01, P < 0.01, respectively**). Receiver Operator Characteristic (ROC) curve analysis revealed much higher specificity and sensitivity for the circulating miR-615-5p in comparison to the traditional AFP. **Conclusions:** The results showed that miR-615-5p might be a potential molecular biomarker for diagnosing liver cirrhosis, implying that it could be even used as a novel non-invasive molecular biomarker for predicting HCV-related liver cirrhosis.

Key words: Liver cirrhosis, miR-615-5p, non-invasive marker, Egyptians.

INTRODUCTION

Following long-term liver injury, the evolution of liver fibrosis to cirrhosis is associated with (i) architectural disturbance; (ii) angiogenesis and hemodynamic changes (intra- and extra-hepatic) resulting in portal hypertension; (iii) a propensity for carcinogenesis [1]. Liver fibrosis is part of the

structural and functional alterations in most chronic liver diseases. It is one of the major prognostic factors as the amount of fibrosis is correlated with the risk of emergent cirrhosis and liver-related complications in viral and non-viral chronic liver diseases [2]. Cirrhosis can occur in consequence of

an exogenous/toxic, infectious, toxic/allergic, immunopathological/autoimmune, or vascular process or an inborn error of metabolism [3].

Cirrhosis is the final stage gained by various chronic liver diseases after years or decades of slow progression. There are, however, ways to avoid cirrhosis, because the diseases that most commonly lead to it progress slowly, and measures are accessible to prevent and treat them. Moreover, most cases of hepatocellular carcinoma (HCC) arise in a cirrhotic liver, so cirrhosis prevention is, in fact, also HCC prevention [4].

The epidemiology of liver cirrhosis has been evaluated widely in several developed countries in Europe and the Americas. There has been much less interest in mortality from the disease, though, in much of the developing world [5–8], in part because of poor data [9]. Liver cirrhosis deaths worldwide have increased progressively over the past 30 years [9]. In 2010, liver cirrhosis ranked as the 23rd cause of disease saddle worldwide [10]. On average, there were twice as many liver cirrhosis male deaths as female deaths. Age-standardized liver cirrhosis mortality rates and time trends in Europe followed a strong 'East-to-West gradient', as identified by Zatonski *et al.* [7].

Cirrhosis and HCC due to chronic hepatitis C are amongst the main indications for liver transplantation in Western manufacturing countries. From 1988 to 2010, viral hepatitis was the underlying cause of liver disease in 39% of liver transplant recipients—hepatitis B in one-third of cases, and hepatitis C in two-thirds [11].

In Egypt, cure for schistosomiasis until the late 1960s led to widespread transmission of the hepatitis C virus [12]. Currently, Egypt has the highest prevalence of chronic hepatitis C infection in the world [13, 14]. This is reflected in the very high liver cirrhosis death rates, particularly in males. In 2010, the age-standardized cirrhosis mortality rate in Egypt was the highest internationally at 72.7 (57.2 to 87.2) per 100,000. During the same year, 18% of deaths in males between ages 45 to 54 years were due to liver cirrhosis [9].

Cirrhosis has long been known by the development of acute deterioration of liver and/or renal function, hepatic encephalopathy and high risk of hospital mortality in connection to a precipitating event, commonly an infection [15]. For several years, liver biopsy has been considered the “gold standard” test [16]. Liver biopsy analysis has several limitations. It is an invasive procedure with rare but potentially life-threatening complications and prone to

sampling errors and to intra- and inter-observer variation [17]. These limitations have rapidly decreased the use of liver biopsy and led to the development of non-invasive methodologies. Among the currently accessible non-invasive methods, there are two distinct approaches: (i) a “biologic” approach based on the quantity of serum biomarkers of fibrosis; (ii) a “physical” approach based on the measurement of liver stiffness using transient elastography (TE) [18]. Although complementary, these two approaches are based on differing rationale and conception: TE measures liver stiffness related to elasticity, which corresponds to physical property of liver parenchyma, whereas serum biomarkers are combinations of several not strictly liver-specific blood parameters optimized to mimic fibrosis stages as assessed by liver biopsy [19]. Overall, biomarkers are less accurate in detecting intermediate stages of fibrosis than cirrhosis [20].

Among the proposed markers, the so-called **direct markers** reveal the deposition or removal of extracellular matrix in the liver. These include glycoproteins such as serum hyaluronate, laminin, and YKL-40, collagens such as procollagen III N-peptide and type IV collagen, collagenases, and their inhibitors such as matrix metalloproteases and tissue inhibitory metalloprotease-1. So-called **indirect markers** include factors that can be calculated in routine blood tests, such as the prothrombin index, platelet count, and ratio of aspartate transaminase to alanine transaminase (AST : ALT), which indicate alterations in hepatic function. Outcome from measurements of direct and indirect markers can be combined and used in diagnosis; the FibroTest® (BioPredictive, Paris, France) was the first algorithm that combined these data [21]. Several other scores have been proposed – four are sheltered by patents- and commercially available [22– 31].

When compared and validated externally in patients with hepatitis C virus infection, the different patented scores have similar performances for the diagnosis of cirrhosis [32– 36]. In the largest study to date (n=1307 patients with viral hepatitis) comparing prospectively the most popular patented scores (FibroTest®, FibroMeter®, HepaScore®) with the non-patented score (APRI), the mean area under the receiver operating characteristic curve (AUROC) ranged from from 0.77 to 0.86 with no significant difference between the scores [36]. Although non-patented scores such as FIB-4 and APRI may have slightly lower performance, they are cost-free, easy to calculate, and available almost everywhere [37–

40]. However, none are liver specific, their results can be influenced by comorbid conditions and they require critical explanation of the results [41].

In early cirrhosis, however, usual imaging can lead to false-negative diagnosis (42), and TE applicability is not as good as that of serum biomarkers (43). Combining TE with biomarkers does not increase diagnostic accuracy for cirrhosis in patients with persistent hepatitis C virus infection [44- 46], so other strategies are needed.

Emerging evidence indicates that the newly discovered non-coding short RNAs (microRNAs) deregulation have contributed greatly to the epigenetic-based liver diseases [47] that function by regulating the activity of mRNA targets and thus play important roles in a wide range of physiological and pathologic processes. Some miRNAs are formed in cell- or tissue-specific manners. Consequently, miRNA changes within a tissue type might correlate with certain disease states. Moreover, miRNAs are present in the peripheral blood in a remarkably stable form; thus, they might serve as potential blood-based biomarkers [48].

In this study, we investigated: the expression of circulating miR-615-5p in patients with cirrhosis on top of HCV-chronic infection, the potential role of miR-615-5p as a diagnostic non-invasive molecular marker for liver cirrhosis and assessed its sensitivity and specificity in comparing with the conventional marker AFP in a trial to weave the need for biopsy.

SUBJECTS AND METHODS

This study was conducted on 50 Egyptian subjects who were divided into 30 patients (20 are males) with liver cirrhosis on top of HCV with a mean age (\pm SE) of (58.37 \pm 0.9) years, 10 patients (5 males) of chronic HCV without fibrosis, with a mean age of (55.3 \pm 1.4) years, and blood samples were also collected from 10 age- and gender-matched healthy volunteers (defined as those with normal liver function test (LFT) and negative for HBs Ag, HBe-Ab and HCV Ab with a mean age of (55.1 \pm 1.6) years. All subjects were recruited from the Hepatology & Gastroenterology and Infectious Diseases Department, Benha University Hospital, Benha University.

Liver cirrhosis with HCV diagnosis was based on standard clinical, biochemical, serological, and ultrasonography. Diagnosis of HCV-associated chronic liver disease (CLD) was based also on standard clinical, biochemical and serological criteria. These group members were proved to be not fibrotic by applying FIB-4 index (28).

All patients and controls were subjected to full history taking. Exclusion criteria included: cases of chronic HBV infection, autoimmune hepatitis, metabolic liver diseases (haemochromatosis, Wilson's disease, nonalcoholic steatohepatitis), history of alcohol consumption, previous treatment for HCC or antiviral therapy and any associated malignancies other than HCC. The study was approved by Benha University Hospital research ethics committee and has been performed in accordance with the ethical standards of the Declaration of Helsinki. An informed consent was obtained from all individual participants included in the study.

Clinical Laboratory tests

For all participants, the following was conducted: clinical evaluation (medical history and physical examination), complete blood count, liver function tests, and prothrombin time, AFP, abdominal US examination- except for control group-, testing for hepatitis B surface antigen (HBs Ag) and hepatitis C antibody (HCV-Ab).

Molecular test for miR-615-5p

Total RNA, including low molecular weight RNAs, were extracted from all participants using RNeasy Protect Animal Blood kit (QIAGEN, Germany) according to the manufacturer's instructions. The quality of RNA was determined using NanoDrop2000 (Thermo scientific, USA). Then single-stranded cDNAs were generated on 10 ng of total RNA in a final volume 15 μ l RT reactions (16°C for 30 min, 42 °C for 30 min and 85 °C for 5 min) using TaqMan reverse transcription kit; TaqMan® Small RNA Assays (Applied Biosystems, California, USA) according to the manufacturer's instructions. Then cDNA was preserved at 4°C till needed.

Expression levels of microRNA-615-5p was evaluated using TaqMan® Small RNA Assays (Applied Biosystems, California, USA) as follows: 1.3 μ l from its corresponding RT reaction (cDNA) were mixed in a mixture of 10 μ l TaqMan Universal PCR Master Mix II with 1.00 μ l ready-made primer (20X) and 7.7 μ l nuclease-free water in a total volume of 20 μ l per reaction. The expression levels were normalized to RNU6B levels. All reactions were run on 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) using the following conditions: 95°C for 10 minutes once, then 40 cycles of: denaturation (95°C for 15 sec.), annealing (60°C for 60 sec.) and extension (60°C for 60 sec.). Relative expression of miRNA-615-5p was calculated using the comparative cycle threshold (Ct) method. Δ Ct

was calculated by subtracting the Ct values of RNU6B from the Ct values of the selected miRNA-615-5p samples. $\Delta\Delta^{Ct}$ was then calculated by subtracting mean ΔCT of the control samples from ΔCT of tested samples. Fold change of miRNA expression levels was calculated using the formula $2^{-\Delta\Delta^{Ct}}$ using healthy controls as calibrators.

Statistical analysis:

All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 17 (SPSS Inc., Chicago, IL, USA). Data are presented as means with corresponding standard error (SE). Comparisons between different groups were performed by one-way analysis of variance (ANOVA). Tukey's test was used as a posthoc test. Correlation among variables was determined using Pearson's correlation test. The performance of a noninvasive diagnostic method is evaluated by calculation of the area under the receiver operator characteristic curve (AUROC) taking AFP as the

reference standard (MedCalc v. 16.4). In all tests, the level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Population and clinical studies:

Demographic, biochemical, and hematological characteristics of the patients are summarized in table (1). The mean ages (\pm SE) of control, chronic non-cirrhotic hepatitis C patients and cirrhotic liver patients were (55.1 \pm 1.6, 55.3 \pm 1.4, 58.37 \pm 0.9) years, respectively and proved to be nonsignificant ($P > 0.05$). Gender was nonsignificant between studied groups ($P > 0.05$). A positive correlations were observed between ALT, AST, GGT, ALP, total bilirubin, direct bilirubin, INR, AFP, miR-615-5p and the progress of the disease to cirrhosis. Inversely, there were negative correlations between: albumin, platelet count, hemoglobin level and progression of the disease to cirrhosis, while WBCs was nonsignificantly correlated to cirrhotic group.

Table (1): Demographic, biochemical, and hematological parameters of the three studied groups.

Variables	Mean \pm SE			P value
	Control (n=10)	Non-Cirrhotic (n=10)	Cirrhotic (n=30)	
Demographic Data				
Age: Range (Mean)	48-63(55.1)	49-64(55.3)	49-68(58.37)	N.S.
Gender				N.S.
Male	5 (50%)	5 (50%)	20 (66.7%)	
Female	5 (50%)	5 (50%)	10 ((33.3%)	
Biochemical parameters				
ALT (up to 40 U/L)	(21.9 \pm 1.2)	(45.2 \pm 6.8) ^{1*}	(53.5 \pm 3.8) ^{1**}	<0.01
AST (up to 40U/L)	(23.7 \pm 1.4)	(36.8 \pm 4.4)	(72.5 \pm 4.9) ^{1**, 2**}	<0.01
GGT (up to 50 U/L)	(15.1 \pm 1.03)	(52.2 \pm 11.7)	(147.3 \pm 12) ^{1**, 2**}	<0.01
ALP (up to 115 U/L)	(52 \pm 5.1)	(98.5 \pm 5.7) ^{1**}	(104.63 \pm 3.4) ^{1**}	<0.01
Total Bil. (up to 1 mg/dL)	(0.89 \pm 0.03)	(0.53 \pm 0.08)	(4.13 \pm 0.5) ^{1**, 2**}	<0.01
Direct Bil. (up to .25 mg/dL)	(0.18 \pm 0.01)	(0.17 \pm 0.02)	(1.91 \pm 0.29) ^{1**, 2**}	<0.01
Albumin (3.5:5.5 gm/dL)	(4.42 \pm 0.17)	(4.64 \pm 0.09)	(2.54 \pm 0.06) ^{1**, 2**}	<0.01
Hematological parameters				
Plat. (150:450x10 ³ / mm ³)	(228.6 \pm 12.4)	(207 \pm 11.4)	(104.9 \pm 7.6) ^{1**, 2**}	<0.01
INR	(0.98 \pm 0.03)	(0.98 \pm 0.04)	(1.49 \pm 0.07) ^{1**, 2**}	<0.01
WBCs (3.9: 11x10 ³ /mm ³)	(6.2 \pm 0.5)	(7.8 \pm 0.6)	(8.2 \pm 0.6)	N.S.
Hgb (11.5:16.5gm/dL)	(13.86 \pm 0.3)	(14.68 \pm 0.6)	(9.87 \pm 0.3) ^{1**, 2**}	<0.01

¹ denotes statistically significant difference from control. ² denotes statistically significant difference from non-cirrhotic group. NS: nonsignificant. * = statistical significant difference <0.05 and **= statistical significant difference <0.01

Circulating miR-615-5 and AFP levels in cases and controls

Blood level of miR-615-5p expression was detected in cirrhotic cases and other groups normalized to RNU6B as an internal control. As shown in (Fig. 1, table 2), we found that the expression of miR-615-5p was not significantly increased in “non-cirrhotic” chronic HCV group comparing to the control group (mean±SE: 4.94±0.03, 4.9±0.01 respectively); whereas the blood level of miR-615-5p expression was significantly increased in cirrhotic group in comparison to the control group (mean±SE: 5.44±0.02 vs. 4.9±0.01) and also to the non-cirrhotic groups (mean±SE: 5.44±0.02 and 4.94±0.03), respectively. This progressive increase in miR-615-5p expression observed in the cirrhotic patients was more than 5-fold enhancement than the control. There was also a significant difference in AFP levels also increased in progression to liver cirrhosis in a positive way (Fig. 2, table 2).

3.3. Correlation between circulating miRNA-615-5p, AFP and other parameters:

Pearson’s correlation between both: miR-615-5p, AFP levels and the liver function test (LFT) parameters in the cirrhotic group (table: 3). There were significant correlations between AST (r= 0.57 & P < 0.01), total bilirubin (r= 0.5 & P < 0.01), direct bilirubin (r= 0.37 & P < 0.05) and miR-615-5P gene-expression, while in the same group, there was only a significant positive correlation between AFP and AST (r= 0.434 & P < 0.05).

3.4. Accuracy of circulating miRNA-615-5p in Cirrhotic patients

Accuracy of using miR-615-5p and AFP as a diagnosing tool for liver cirrhosis was deduced by applying the ROC curve analysis (MedCalc v.16.4), shown in fig (3). It was obviously denoted that our tested microRNA was more accurate than AFP; possessed more area under curve (AUC). This was translated into higher specificity and more sensitivity (98.5% and 93.33% respectively) than the traditionally requested AFP (90% and 86.7%, respectively). PPV and NPV also showed much more values to the sake of the newly tested miR-615-5p (table: 4).

Table (2) Comparison of miR-615-5P and AFP expression levels in all studied groups

Variables	Mean ± SE			P value
	Control	Non-cirrhotic	Cirrhotic	
Log RU for miR-615-5P Fold	(4.9±0.01)	(4.94±0.03)	(5.44±0.02) ^{1**, 2**}	<0.01
AFP (up to 10 ng/ml)	(6±0.88)	(32.07±3.7)	(105.21±12.3) ^{1**, 2**}	<0.01

¹ denotes statistically significant difference from control. ² denotes statistically significant difference from non-cirrhotic group. **= statistical significant difference <0.01

Table (3): Pearson’s correlation between laboratory investigated parameters in the cirrhotic group.

Variables	AFP	miR-615-5P (Log RU)
	<i>r</i>	
ALT (~ 40 U/L)	0.325	0.341
AST (~ 40U/L)	0.434*	0.57**
GGT (~50 U/L)	0.200	0.215
ALP (~115 U/L)	0.066	0.25
Total Bil. (~1 mg/dL)	0.102	0.503**
Direct Bil. (~.25 mg/dL)	0.031	0.371*
Albumin (3.5:5.5 gm/dL)	-0.232	-0.096
Plat. (150:450x10 ³ / mm ³)	-0.351	-0.262
INR	0.183	0.141
WBCs (3.9: 11x10 ³ /mm ³)	0.115	0.049
Hgb (11.5:16.5gm/dL)	-0.128	-0.165

r = Correlation coefficient, negative values intended negative correlation
 * show statistical significant difference ($P<0.05$) while ** show statistical significant difference ($P<0.01$) in the cirrhotic group.

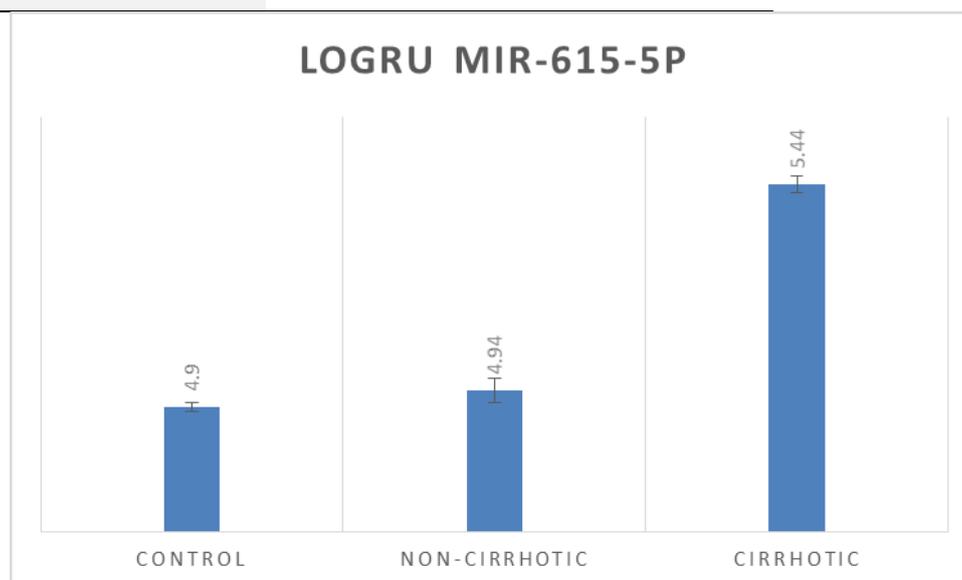


Fig. (1): Expression level of “miR-615-5p” in the three studied groups.

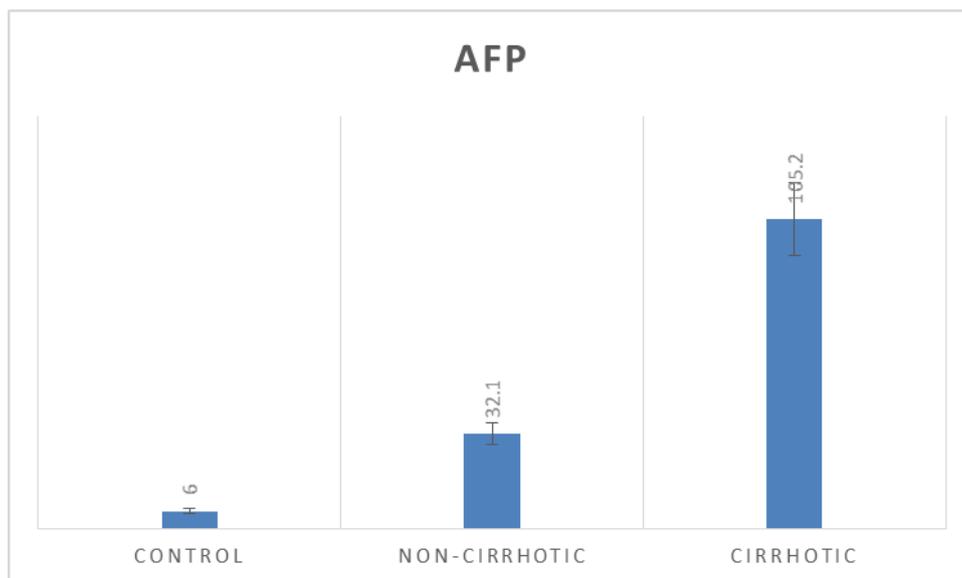


Fig. (2): Expression level of “AFP” in the three studied groups.

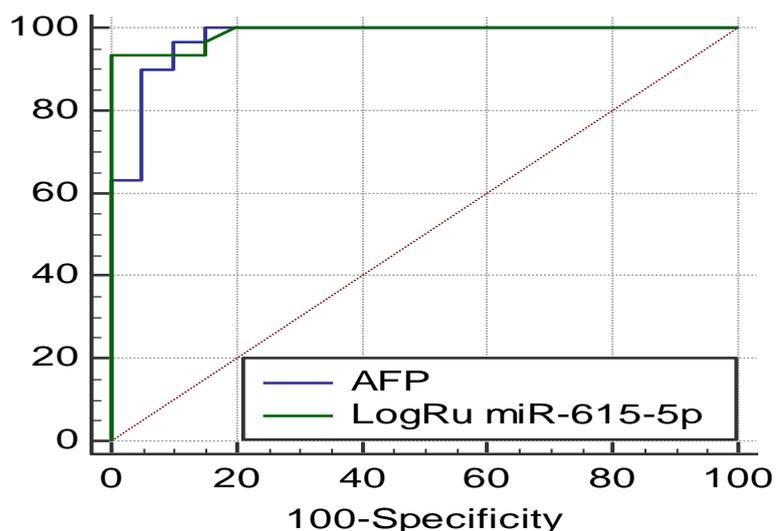


Fig. (3): ROC curve for performance of the studied miRNA-615-5p and AFP in early detection of liver cirrhosis.

Table 4: Predictive performance of both AFP and miR-615-5p for liver cirrhosis.

VariableT	Cutoff	Sens.%	Spec.%	PPV%	NPV%	Accuracy	AUC	95%CI	P
AFP (ng/mL)	>49	86.7%	90%	93.3%	78%	88%	0.89	0.87-0.94	<0.01
miR-615-5p (Fold-expressed)	>5.2	93.3%	98.5%	97%	83.3%	95%	0.99	0.9-1.00	<0.01

Liver cirrhosis has emerged as a major cause of worldwide health burden [10, 49]. It represents the strongest predisposing factor, as about 80% of HCCs develop in cirrhotic livers [50].

Serum AFP was dropped out from guidelines due to its poor sensitivity (39–65%) [51], and since repeating MRI and CT scans may represent a problem in terms of

economic and personnel resources, the availability of reliable biomarkers, to be assayed over time, would represent an aid to the assessment of the malignant potential of liver nodules on cirrhosis. For these reasons, this field of research has been highlighted by both EASL-EORTC and AASLD guidelines as a priority. In cases not characterized by imaging techniques, biopsy is recommended even though it involves the risks of invasive procedures; no circulating biomarker contributing to the early detection is recommended at the moment [52, 53].

Circulating microRNAs have been demonstrated to be highly stable in serum and plasma due to their protection from RNase activity, therefore representing a possible source of diagnostic and prognostic biomarkers to be explored [54], especially for detection of early stage, presymptomatic diseases [55]. **Gui et al** [56] identified a panel of miRNAs up-regulated in patients with liver cirrhosis. For example, it was mentioned that miR-155 was markedly elevated in cirrhotic tissues; miR-155 showed increased expression in cirrhotic tissues compared to healthy tissues [57] and miR-595 and miR-765 were shown to be among the most recent identified miRNAs in cirrhotic patients [54].

miR-615-5p is a very novel microRNA that was once analyzed in prostate cancer cell lines where it showed high expression [58]. **El-Tyebi et al**, (57) were the first who investigated miR-615-5p in liver cirrhotic patients in Egypt in an approach to screen for the presence of it in healthy, cirrhotic and cancerous liver tissues as well as HCC cell lines, and they proved its expression in the cirrhotic tissues. The expressed level of miR-615-5p in circulating blood has never been studied before in liver cirrhotic patients, that makes it a potential target for further analysis. The present study is in agreement with **El-Tyebi et al**, (57) in that circulating miR-615-5p is highly expressed in cirrhotic patients compared to healthy persons. In terms of the expression status of miR-615-5p in cirrhotic patients compared with normal control, our study was in contrast with **El Tayebi et al**. [57] where miR-615-5p was detected as significantly expressed in normal tissues in comparison with cirrhotic cases.

ROC curve was conducted and the AUC was calculated to explore the predicting and/or diagnostic value of the studied miRNA (miR-615-5p) for differentiating cirrhotic patients from the non-cirrhotic ones. AUC for miR-615-5p was higher (0.99) than that of AFP (0.89). The concluded result was near perfect in diagnosing cirrhosis in comparison to the conventional requested AFP. The specificity and sensitivity of both miR-615-5p

and AFP were (98.5% vs. 90% and 93.3% vs. 86.7%, respectively) which enable us to apply the cut-off value of miR-615-5p (>5.2 expression fold) as a predicting marker for liver cirrhosis among Egyptians especially those of high risk population.

In conclusion, our data suggests -for the first time- that miR-615-5p might potentially serve as novel, sensitive molecular biomarker for diagnosing or even predicting cirrhosis of the liver before progressing to the most serious disease HCC, which would have a better outcome on the health of those suffering from this silent disease.

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