Assessment of miR-223 fold change in Chronic Hepatitis C Egyptian Patients

EL-Shahat A. Toson, Gamal E. Shiha, Mahmoud Al kazaz

Abstract— Chronic hepatitis C (CHC) infection is one of the causative factors of hepatocellular carcinoma (HCC) which is the third leading cause of all cancer-related death. A dozen of microRNAs have been reported as promising HCC biomarkers**.** Aim: To evaluate the effectiveness of plasma miR-223 as non-invasive diagnostic markers of CHC and HCV-related HCC. Correlation with α-fetoprotein (AFP) will also be evaluated. Methods, This study was conducted on 100 participants which were subdivided into; 25 HCC on top of HCV, 25 with HCV-related cirrhosis, 25 with chronic HCV without cirrhosis , and 25 healthy volunteers. The level of alpha-fetoprotein (AFP) was measured using Cobas Integra E 411 and that of miRNA-223 with real-time quantitative PCR (RT-PCR). HCV antibodies, hepatitis B surface antigen (HBsAg), HCV- RNA, routine liver function tests and platelets count were also done for each patient and control subject. Results: demonstrated that the mdian and (IQR) of AFP level in different groups was 4.05 (5.00), 6.90 (14.52) and 23.09 (88.48) in fibrotics, cirrhotic and in sera of patients with HCC, respectively. The differences between these values and that of the healthy control were significant (P<0.001). Comparing serum AFP fold changes in the HCC group versus that of the non HCC groups, there was a significant fold increase in their serum AFP levels (P= <0.001 and the AUC was 0.824), Surprisingly, on comparing miRNA fold changes in sera of the HCC patients group's with that of the non HCC group, the difference showed no significant fold decrease (P= 0. 827 and the AUC was 0.484) serum miRNA-223 was not significantly differ among the individuals of these two groups. In addition, the median values and (IQR) of miRNA-223 in sera of the different pathological groups of the liver included herein were 0.57 (12.99), 0.27 (2.44), and 0.31 (2.87) in fibrotics, cirrhotics and in patients with HCC, respectively. The differences between these values and that of the healthy control showed no statistical significancy among groups (P<0.238). AFP ROC curve gave 52% sensitivity but with 94% specificity in discriminating HCC patients from non-HCC one ((p <0.001 and AUC of 0.824).Therefore, our results suggested that AFP serum level can still be used to identify patients with liver disorders from those of the healthy control, its tendency to discriminate HCC patients from those with non HCC cannot be neglected, even it was previously questionable. Compared with AFP, plasma miR-223 was not able to be a useful biomarker for early detection of HCV-related HCC. Whether the small size of HCC patients is a contributing factor or not is still be an area of investigation.

Key words—HCC diagnosis, chronic HCV infection, plasma miR-223, RT-PCR.

1 INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer mortality [1,2]. Hepatocellular HCC is defined as a primary tumor genesis 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]. Hospital-based studies have reported an overall increase in the relative frequency of all liver-related cancers from approximately 4% in 1993 to 7.3% in 2003 [6]. 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) Also, its use as a diagnostic test is less specific than was thought, as it was found to be elevated in intrahepatic cholangiocarcinoma (ICC) and in some metastases from colon cancer [7]. The noninvasive Fibroscan is becoming a diagnostic tool. However, it does not predict the progression of liver disease. Thus, identifying individuals witb high risk for developing HCC can generates new opportunities for surveillance, therapeutic intervention, and patient management. Thus, early diagnosis of HCC is still requires specific biomarkers.

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. They have gotten the best consideration over the previous years [8]. Also, they serve as posttranscriptional 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]. Vojtechova et al [10] considered the correlation between HCC and microRNAs a point of

discussion. Generally, miRNAs are important players in pathogenesis of HCV infection. Controlling signaling pathways may be one of the players, functions. They, also, play a role in innate and adaptive immune response [11]. Therefore, the aim of the present study was to assess the diagnostic value of plasma miR-223 in chronic hepatitis C Egyptian patients; especially in patients with HCC. The discriminant power of AFP will also be included.

2 MATERIALS AND METHODS

2 .1 Patients and Blood sampling

2.1.1 Patients

This cross sectional observational study was performed on patients selected from the outpatient hepatology and HCC early detection clinic (Egyptian Liver Research institute and Hospital (ELRIAH), Sherpin, El-Dakahlia Governorate, Egypt) from April 2016 to April 2017. The study has been conducted on 100 participants and were divided into 4 equal groups: Group I; Twenty five normal healthy individuals age and gender-matched healthy volunteers (control group). Group II; Twenty five chronic HCV patients without cirrhosis. Group III; Twenty five HCV related cirrhosis without HCC. Group IV; Twenty five HCV-related HCC (proved radiologically by abdominal US &Triphasic abdominal CT). All patients were enrolled in our study after signing an informed written consent. Approval of the study protocol by the medical ethics research committee, Faculty of Medicine, Mansoura University was obtained. Exclusion criteria included: Previous treatment of HCC (surgical, interventional or medical), presence of other malignancies (e.g. cholangiocarcinoma, gastric, colorectal cancer) , patients with HCC on top of other causes rather than HCV and patients on direct acting antiviral therapy for chronic HCV. Also, the healthy volunteers were with negative HCV antibodies, negative hepatitis B surface antigen (HBsAg) as well as negative HCV-RNA by PCR. In addition, their transaminases activities and hepatic ultrasound were normal, All participants were subjected to full medical history, complete clinical examination, and full basal laboratory and radiological investigations with collecting samples for serum miRNA-223 and serum AFP.

2.1.2 Blood sampling

Five ml venous blood sample were withdrawn from each individual; of them 3.0 ml were left to clot, centrifuged and the serum fraction was extracted and either freshly used or stored at−80 °C until its use. The remaining 2.0 ml were collected on sodium citrated tube.

2.2.Biochemical and hematological investigations

2.2.1 Routine liver function tests

Aspartate transaminase (AST) and alanine transaminase(ALT), serum albumin, total bilirubin, and alkaline phosphatase (ALP) were measured using Sysmex auto-analyzer (Japan).

2.2.2. Hematological markers

They include counting of red blood cells (RBCs, 109/mm3), white blood cells (WBCs, 103/mm3), and platelets (plt, 103/mm3) using automated hematology analyzer (Sysmex XT1800i) .Besides, hemoglobin (Hgb) level (gm/dl) was coloumetrically analyzed. Furthermore, plasma prothrombin time (PT, autoanalyzer; Roche Cobas Integra-800), and thence INR was calculated .

HCC was detected by ultrasound scan and confirmed using computed tomography (CT).

2.3 Serological and tumor markers

2.3.1 Serological markers

Serologically, hepatitis C virus antibodies [HCV Abs] and HBsAg were determined by ELISA (Abbottci 4100).

2.3.2 Tumor markers

Alpha-fetoprotein (α-FP) was measured using autoanalyzer (Cobas Integra, E 411) for its in vitro quantitative determination in human plasma.

2.4 Molecular biology assays

2.4.1 Quantitative HCV RNA assay

HCV RNA was quantized using RT-PCR (QIAamp viral RNA extraction kit, Qiagen USA cat #52906).

2.4.2 Quantitative MiR-223 assay

2.4.2.1 MiR-223 quantification using real-time polymerase chain reaction (RT-PCR)

2.4.2. 1.1 RNA extraction and cDNA synthesis

Total RNA was extracted from 200 µl plasma using QIAzol according to the manufacturer's instruction. The RNA purity was assessed by the RNA concentration which was quantified by NanoDrop (Nanodrop: ND-1000, United States). Single-stranded cDNAs were generated using the RT kit (Qiagen, Valencia, CA United States) according to the manufacturer's directions (miSCript miRNA PCR system, miRneasy mini kit for miRNA extraction, miScript RT for miRNA reverse transcription, miSCript primer assay and miSCript SYBR Green PCR Kit for PCR amplification using RT- PCR.

2.4.2. 1.2 Amplification and quantification

PCR quantification experiments were performed with PCR (Applied Biosystems; Foster City, CA) using the SYBR Green PCR Master Mix according to the manufacturer's protocol. The primers for microRNA-223 and housekeeping gene were supplied by Qiagene, Germany (catalog numbers 3416, 3857 and 33712). 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.

2.5 Statistical analysis and interpreting data

Patients were categorized into 4 groups; normal, chronic hepatitis, cirrhosis and HCC. Further comparisons were performed between HCC group and Non-HCC (chronic hepatitis and cirrhosis).Quantitative variables were expressed by mean ± SD or expressed by median and inter quartile range (IQR) for non-parametric data. They were compared by student's T-test or ANOVA test when appropriate. Qualitative variables were compared by $\chi 2$ or Fischer's exact test when appropriate. Receiver operator characteristic (ROC) curves were constructed to assess the value of miR-223 in diagnosing of HCC and to assess area under the curve (AUROC). Spearmen and Pearson correlations were done for correlating quantitative variables. In all tests, P value was considered significant if equal or less than 0.05

3. RESULTS

This study was conducted on 100 participants stratified into; 25 HCC on top of HCV, 25 with HCV-related fibrosis, 25 with chronic HCV with cirrhosis , and 25 healthy volunteers, were included in this study. Healthy volunteers with negative hepatitis B (ELISA) and C (ELISA and PCR) and normal transaminases and hepatic ultrasound,. The Biochemical characteristics of the studied individuals are shown in (Table1).There were significant differences among the diseased groups regarding the parameters of liver function testes and AFP ($P < 0.001$).

In table 2, log10 HCV RNA was significantly differ among groups $p<0.048$. Also, the count of red blood cells ,white blood cells and platelets showed significant difference among groups ($p<0.006$ or less).

TABLE 1: BIOCHEMICAL AND ALPHA-FETOPROTEIN CHARACTERISTICS OF THE STUDIED INDIVIDUALS.

Variable	Control N=25	Fibrosis N=25	Cirrhosis N=25	HCC N=25	P value
ALT (U/L, median IQR)	19.0 (17.5)	44.16 (33.85)	41.0(39.5)	51.0 (24.0)	<0.001
AST (U/L, median (IQR)	21.0 (9.50)	34.67 (17.87)	61.0 (42.0)	77.0 (33.5)	<0.001
ALK (/L, median (IQR)	68.0 (27.5)	74.62 (33.35)	61.0 (32.5)	99.0 (49.0)	0.004
Albumin (g/dl, median (IQR)	4.39 (0.37)	4.31 (0.53)	4.20 (0.90)	3.50 (0.80)	<0.001
Total Bilirubin (mg/dl, median (IQR)	0.50 (0.20)	0.62 (0.29)	0.70 (0.50)	1.20 (0.83)	<0.001
Direct Bilirubin (mg/dl, median IQR)	0.10 (0.00)	0.25 (0.13)	0.30 (0.24)	0.50 (0.58)	<0.001
Creatinine (mg/dl, median (IQR)	0.88 (0.20)	0.94 (0.55)	0.70 (0.20)	0.90 (0.35)	0.528
AFP (ng/ml, median (IQR)	2.38 (1.94)	4.05 (5.0)	6.9 (14.52)	23.09 (88.48)	<0.001

Variables were expressed as median and inter quartile range (IQR) for non-parametric data Reference values: alanine aminotransferase (ALT) 40 U/l ; aspartate aminotransferase (AST) up to 40 U/l; alkaline phosphatase (ALK) level is 20 to 140 IU/L; albumin 3.8–5.5 g/dl; bilirubin up to 1 mg/dl and creatinine up to 1.2mg/dl; α-fetoprotein (AFP) up to 10 U/L.

TABLE 2: HCV-RNA CLINICAL AND HEMATOLOGICAL CHARACTERISTICS OF THE STUDIED INDIVIDUALS

Variable	Control (n=25)	Fibrosis (n=25)	Cirrhosis (n=25)	HCC (n=25)	P value
HCV RNA (log_{10} IU/ml, median (IQR)	Negative	5.11 (0.99)	5.67 (1.19)	4.99 (1.54)	0.048
Child-Turcotte-Pugh Classification:					
- A			19 (76.0%)	18 (72.0%)	0.747
- B			6 (24.0%)	7 (28.0&)	
Age (yrs, mean±SD)	33.8±6.0	51.2±8.1	57.3±5.7	58.9±6.9	<0.001
Sex (male, n %))	15 (60.0%)	13 (52.0%)	12 (48.0%)	21 (84.0%)	0.042
Hb (g/dl, mean±SD)	13.82±1.14	13.46±0.77	12.83±2.20	13.33±1.67	0.163
RBCs ($\times10^3/\mu L$, mean±SD)	4.86±0.47	5.18±0.51	4.60±1.12	4.47±0.68	0.006
WBCs ($\times10^3/\mu L$, mean±SD)	6.99±2.01	7.53±2.70	5.82±2.69	5.16±1.67	0.002
Plt ($\times10^3/\mu L$, mean±SD)	253.28±55.47	191.36±50.96	120.6±55.53	96.44±40.22	<0.001

Variables were expressed as by mean ± SD or expressed by median and inter quartile range (IQR) for non-parametric data Reference values: Haemoglobin (g/dl); Red blood count 4.5 – 6.5 $\times10^3/\mu L$; White blood count 4.00 – 11.00 $\times10^3/\mu L$; platelet count 150–450 × $\times10^3/\mu L$.

3.1 Differential Expression of Plasma MiRNA Levels in Cirrhotics and fibrotics patients and HCC patients

The median (IQR) of serum miRNA-223 was 0.57 (12.99), 0.27 (2.44), and 0.31 (2.87) in fibrotics, cirrhotic and in patients with HCC respectively. The differences between these values and that of the healthy control were not significant with (P < 0.238, **and table 3).**

The expression levels of serum miRNA-223 was not significantly (P<0.827) differs between the individuals of HCC group [0.31 (2.87)] and those of the non-HCC [0.41 (3.18)]. (p <0.827and the AUC was 0.484, **Figure 1and table 4).**

.

TABLE 3: MIR-223 LEVELS IN THE DIFFERENT GROUPS

Variable	Fibrosis (n=25)	Cirrhosis (n=25)	HCC (n=25)	All	P value HCC vs. Fibrosis	P value HCC vs. Cirrhosis	P value Fibrosis vs. Cirrhosis
miR-223,median (IQR)	0.57(12.99)	0.27 (2.44)	0.31 (2.87)	0.238	0.362	0.59	0.103

□ *: miR-223 fold difference relative to control and in each case the values were expressed as median and inter quartile range (IQR) (n= number, p= probability (Significance Level), NS= non-significant.*

TABLE 4: DIFFERENTIAL EXPRESSION OF PLASMAMIRNA LEVELS IN HCC PATIENTS

Fold difference relative to control	Non-HCC (n=50)	HCC (n=25)	p value
miR-223, median (IQR)	0.41 (3.18)	0.31 (2.87)	P< 0.827

* *: miR-223 fold difference relative to control and in each case the values were expressed as median and inter quartile range (IQR) (n= number, p= probability (Significance Level), NS= non-significant.*

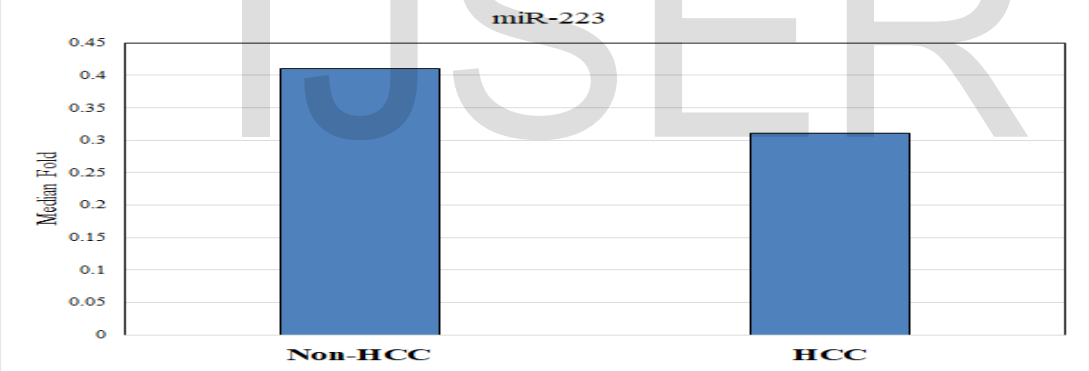

Figure (1): Comparison between HCC and non HCC patients groups regarding miR 223 fold difference.

3.2 Detection of α-fetoprotein Levels in the Studied Groups

The median (IQR) of serum α-fetoprotein was 4.05 (5.00), 6.9 (14.52), and 23.09(88.5) in fibrotics, cirrhotic and in patients with HCC respectively. The differences between these values and that of the healthy control were significant with (P < 0.001, **and table 4).**

The expression levels of serum α-fetoprotein was significantly (P<0.001) differs between the individuals of HCC group [23.09 (69.83)] and those of the non-HCC [5.25 (9.31)]. (p <0.001 and the AUC was 0.824 , **Figure 2 and table 5).**

TABLE 4: AFP LEVELS IN THE DIFFERENT GROUPS

Variable	Fibrosis (n=25)	Cirrhosis (n=25)	HCC (n=25)	All	P value HCC vs. Fibrosis	P value HCC vs. Cirrhosis	P value Fibrosis vs. Cirrhosis
AFP, median (IQR)	4.05 (5.00)	6.9 (14.52)	23.09(88.5)	<0.001	<0.001	<0.003	<0.014

AFP values are fold difference relative to control and in each case the values were expressed as median and inter quartile range (IQR). (n= number, p= probability (Significance Level), NS= non-significant).

TABLES 5: A-FETOPROTEIN LEVELS IN THE DIFFERENT GROUPS.

Fold difference relative to control	Non-HCC (n=50)	HCC (n=25)	p value
AFP, median (IQR)	5.25 (9.31)	23.09 (69.83)	p <0.001

AFP values are fold difference relative to control and in each case the values were expressed as median and inter quartile range (IQR). (n= number, p= probability (Significance Level), NS= non-significant).

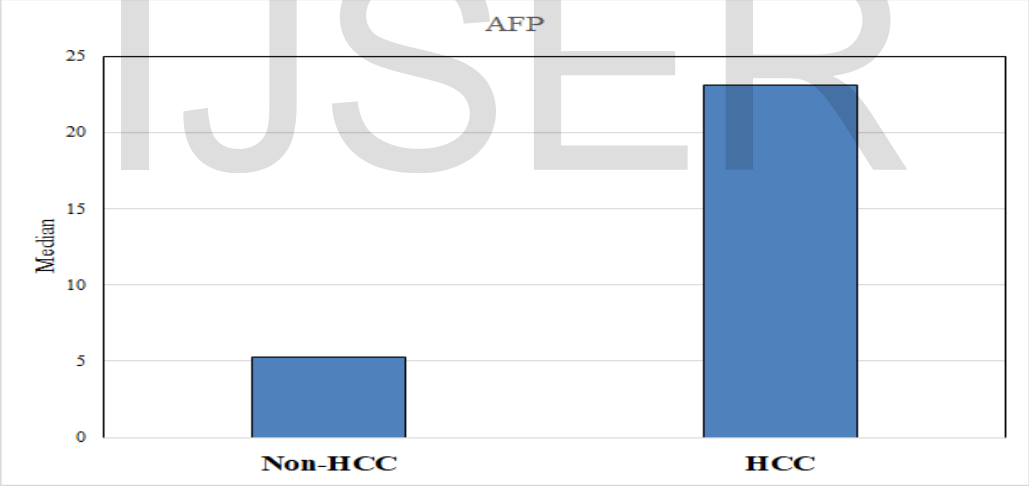

Figure. (2): Comparison between HCC and non HCC patients groups regarding AFP fold difference.

3.3 Correlation between miR-223 & α-fetoprotein.

There was no significant correlation between miR-223 and α-fetoprotein levels in HCCgroup (r=0.019, p=0.869 and **figure3).**

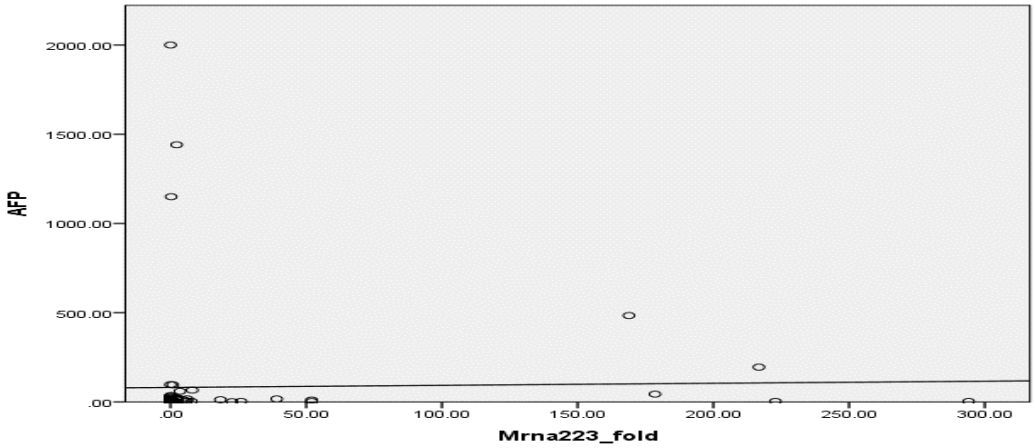

Figure 3. Correlation between miR-223 & a-fetoprotein.

4- Diagnostic Performance of Studied miR-223 and AFP for diagnosis of HCC

The diagnostic performance for the studied miR-223 panel was evaluated using ROC analysis. ROC curve was designed for discriminating HCC patients from other groups, and results revealed that miR-223 had 88% sensitivity but with 20%specificity and AUC of only 0.484. surprisingly, ROC curve which was designed for AFP to discriminate HCC patients from non-HCC one revealed that AFP had 52% sensitivity but with 94% specificity and AUC of 0.824 **(**p <0.001, **Figure 4 and table 6).**

TABLE 6.DIAGNOSTIC PERFORMANCE OF STUDIED MIR-223 AND AFP FOR DIAGNOSIS OF HCC

Factor	MiR-223	AFP
Best Cut-off	0.004	23.09
Sensitivity	88.0	52.0
Specificity	20.0	94.0
PPV	35.5	81.2
NPV	76.9	79.7
AUROC	0.484	0.824
95% C.I.	0.342- 0.627	0.725- 0.823
Significance	p <0.827	p <0.001

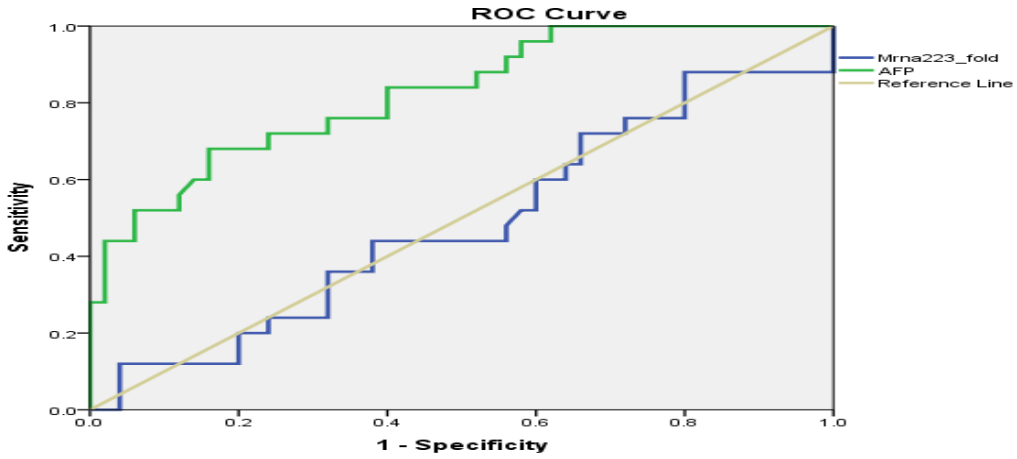

Figure 4: Receiver Operating Characteristics (ROC) curves of miR 223 and AFP for diagnosis of HCC.

4. Discussion.

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers worldwide, and it is predominant in Asia and Africa (Fathy *et al* [12]. Most HCC develops in patients with history of cirrhosis and chronic hepatitis as there is continuous inflammation and regeneration of hepatocytes (Gao *et al* [13].

Most of our HCC patients have statistically significant male predominance (84%,), this has been concomitant with the results published by El-Garem *et al* [14]. The possible explanation may be higher male prevalence of HCV infection and other risk factors for HCC. These may include sex hormones.

Age was statistically significantly higher in HCC patients (58.9±6.9, p value <0.001). This finding was similar to that observed by Gopal *et al* (15). This is because, age could be included in the natural history of HCC on top of HCV related cirrhosis. From the previous results one can expect that both male gender and age may be contributing factors of HCC.

In this study, the activities of ALT, AST, and ALP as well as bilirubin levels were statistically significantly higher in the different pathological stages of the liver; specially in HCC patients. This might be explained by continued hepatocellular damage in HCV-related disorders which were ended with HCC. A somewhat similar results were reported by Elbedewy *et al* [16].

The platelets count was significantly lowered in the blood of not only HCC patients but also in the blood of other liver disorders. This is because platelets play a crucial role in predicting liver fibrosis and cirrhosis; being more lowered with the progression of cirrhosis including HCC patients. These results confirm those of Gopal *et al* [15]**.**Our study showed that there were a significant difference in serum AFP level between different groups; especially in sera of HCC group (23.09 ng/ml) (P value <0.001) These results are in agreement with those of Montaser *et al* [17] who found highly significant increase in the median serum AFP in the HCC cases (31.67 ng/ml). Beside, ROC curve which was designed for AFP to discriminate HCC patients from non-HCC gave 52% sensitivity but with 94% specificity and AUC of 0.824 **(**p <0.001)

Our study showed that miR-223 could not be used as a biomarker for early detection of HCC in HCC related-HCV. This is because, such micRNA displayed less significant fold decrease not only in its expression level in HCC group [0.31 (2.87)] but also in hepatic fibrosis(0.57) and in cirrhosis group [0.27 (2.44)]. Also, in comparing plasma miR-223 expression level between the different studied groups, no significant differences were displayed. Further, ROC curve of miR-223 had 88% sensitivity but with 20%specificity and AUC of only 0.484 for discriminating HCC patients from other groups, Therefore, the result of the present study confirm that of Nasser *et al* [18], who stated that the expression level of serum miR-223 showed no significant difference across their studied groups including HCC versus fibrosis groups, or HCC versus cirrhosis groups nor fibrosis versus cirrhosis.

The same trend of the results ROC curve was also reported by Xu *et al* [19] who found that, serum miR-223 levels were measured in sera of patients with HCC without significant difference.
The results of the present study showed that all measured liver functions as well as AFP showed higher significant differences not only between HCC patients and control group but also can differentiate the individual patients, groups from each others. Therefore, they confirm the result of Fathy *et al* [13] .

In contrast to the results of the present study, significant down regulation of miR-223 in HCC patients compared to those with normal liver tissues and serum was reported by Bhattacharya *et al* [20] in fact, their HCC patients were with mixed etiologies. Also and in contrast to our results miR-223 discriminate healthy populations from patients with HCC on top of HBV with high diagnostic accuracy Zhu *et al* [21]. The possible explanation may include the etiological differences of our and their HCC patients. Therefore, the results of the present study suggested that AFP serum level can still be used to identify patients with liver disorders from those of the healthy control. Its tendency to discriminate HCC patients from those with non HCC cannot be neglected, even it was previously questionable. Compared with AFP, plasma miR-223 was not able to be a useful biomarker for early detection of HCV-related HCC. Whether the small size of HCC patients is a contributing factor or not is still be an area of investigation.

REFERENCES

[1] Alison MR. Liver stem cells: Implications for hepatocarcinogenesis. Stem Cell Rev. 2005;1(3):253-60.
[2] Freedman LS, Edwards BK, Ries LAG. Cancer incidence in four member countries (Cyprus, Egypt, Israel, and Jordan) of the Middle East cancer consortium (MECC) compared with US SEER. Bethesda: National Cancer Institute; 2006.
[3] Lehman EM, Wilson ML. Epidemiology of hepatitis viruses among hepatocellularcarcinoma cases and healthy people in Egypt: A systematic review and metaanalysis. Cancer. 2009;124(3):690–7.
[4] Altekruse SF, McGlynn KA, Reichman ME.Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. J Clin Oncol. 2009;27(9):1485–91.
[5] Khattab MA, Eslam M, Sharwae MA, Hamdy L. Seroprevalence of hepatitis C and B among blood donors in Egypt: Minya Governorate, 2000-2008. Am J Infect Control. 2010;38(8):640–1.
[6] El-Zayadi A-R, Badran HM, Barakat EM, Attia ME-D, Shawky S, Mohamed MK, Selim O, Saeid A. Hepatocellular carcinoma in Egypt: a single center study over a decade. World journal of gastroenterology: WJG. 2005; 11(33): 5193.
[7] Bruix J. and Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma.Hepatology. 2002; 35(3): 519-524.
[8] Berindan-Neagoe I, Monroig Pdel C, Pasculli B, Calin GA. MicroRNAome genome: A treasure for cancer diagnosis and therapy. CA Cancer J Clin. 2014;64: 311-336.
[9] Link A, Kupcinskas J, Wex T, Malfertheiner P. Macro-role of microRNA in gastric cancer. Dig Dis. 2012;30:255-267.
[10] Vojtechova Z, Zavadil J, Klozar J, Grega M, Tachezy R. Comparison of the miRNA expression profiles in fresh frozen and formalin-fixed paraffin-embedded tonsillar tumors. Journal. Plos One. 2017;12(6):1–
[11] Shelbaya A, Kuznik M, Salem W, Mankola, Sadik K. P1265: Estimating the epidemiologic and economic impact of different treatment rates for hepatitis C virus (HCV) in Egypt. Journal of Hepatology. 2015;6(2):S832-S83
[12] Fathy WM, Montaser BA, and El-Assal M.Angiotensin-Converting Enzyme Gene Polymorphism (insertion/deletion) and The Risk of Hepatocellular Carcinoma in Egyptian HCV and HBV Patients.Journal of American Science, 2016;12(3): 116-124.
[13] GaoJ, Xie L, Yang WS, et al. Risk factors of hepatocellular carcinoma--current status and perspectives. Asian Pac J Cancer Prev. 2012; 13: 743-752
[14] El-Garem H. Circulating microRNA, miR-122 and miR-221 signature in Egyptian patients with chronic hepatitis C related hepatocellular carcinoma. World Journal of Hepatology. 2014; 6(11): 818.
[15] Gopal P, Yopp AC, Waljee AK, Chiang J, Nehra M, Kandunoori P, Singal AG. Factors That Affect Accuracy of α-Fetoprotein Test in Detection of Hepatocellular Carcinoma in Patients With Cirrhosis. Clinical Gastroenterology and Hepatology. 2014; 12(5): 870-877.
[16] Elbedewy TA, El-Feky S, El Sheikh MA, El Shimy A, El-Wakeel BM, Hussein G, Daoud SA. Serum Levels of Soluble Fas and Soluble Fas Ligand as Markers for Hepatocellular Carcinoma in Hepatitis C Virus Patients. International Journal. 2014; 2(9): 911-919.
[17] Fawzy Montaser M, Amin Sakr M, Omar Khalifa M. Alpha-l-fucosidase as a tumour marker of hepatocellular carcinoma. Arab Journal of Gastroenterology. 2012; 13(1): 9-13
[18] Mona Zaky Nasser, Ahmed Mahmoud Mohamed, Naglaa Ali Zayed and Gamal Esmat Circulating microRNAs (miR-21, miR-223, miR-885-5p) along the clinical spectrum of HCV-related chronic liver disease in Egyptian patients
[19] Xu J, Wu C, Che X, et al (2011). Circulating MicroRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol Carcinog, 50, 136-42
[20] Bhattacharya S, Steele R, Shrivastava S, Chakraborty S, Di Bisceglie AM, and Ray RB. Serum miR-30e and miR-223 as Novel Noninvasive Biomarkers for Hepatocellular Carcinoma.Am J Pathol. 2016; 186(2):242-247.
[21] Zhu K, Da Z and Zhoul J. Biomarkers for hepatocellular carcinoma: progression in early diagnosis, prognosis, and personalized therapy. Biomarker Research, 2013; 1: 10.

• EL-Shahat A. Toson, *Professor of Biochemistry*, *Department of Chemistry; faculty of science Damietta University*, *Damietta*, *Egypt. eatoson@yahoo.com*
• Gamal E. Shiha, *Department of Internal Medicine*, *Mansoura university*, *Faculty of Medicine*, *Mansoura University*, *Mansoura*, *Egyptian Liver Research Institute and Hospital*, *Egypt.*
• Mahmoud Al kazaz, *Department of Chemistry; faculty of science*, *of science Damietta University*, *Damietta*, *Egypt*,*mahmoudramadan2051@yahoo.com*