Serum miR-483-5p and miR-133a as Biomarkers for Diagnosis of Hepatocellular Carcinoma Post-Hepatitis C Infection in Egyptian Patients

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Hepatocellular Carcinoma (HCC) is one of the most common malignant tumors with high incidence and mortality rate. Identification of new, reliable and non invasive biomarkers are important to improve early detection of hepatocellular carcinoma. Circulating micro-RNAs are abundant and play a central role in different biological process of hepatocellular carcinoma. We aimed to evaluate miR-483-5p and miR-133a as potential biomarkers for diagnosis of hepato-cellular carcinoma in cirrhotic patients post chronic hepatitis C virus infection and compare their sensitivity and specificity to currently used alpha fetoprotein test. The study included 20 patients with HCC on top of hepatic cirrhosis post chronic hepatitis C viral infection, 20 patients with hepatic cirrhosis post chronic hepatitis C viral infection, and 20 age and sex matched healthy controls. Serum miRNAs 483 5p and 133a were measured by quantitative real-time polymerase chain reaction (qRT-PCR). Expression levels of miR-483-5p and miR-133a were higher in HCC patients than in patients with liver cirrhosis (*P* < 0.00).miR-483-5p could predict HCC with sensitivity 100%, specificity 75%, accuracy 0.907, and cut off value > 3.89 while, miR-133a could predict HCC with sensitivity 70%, specificity 90%, accuracy 0.84, and cut off value >4.79. However, the sensitivity and the specificity of AFP were 80% and 100% respectively. In detecting HCC, combining a-fetoprotein (AFP) and serum miR-483-5p (sensitivity = 95%) and miR 133 a (sensitivity = 90%) were better than AFP alone (sensitivity = 80%). In conclusion; serum miR- 483-5p and miR-133a might serve as noninvasive diagnostic biomarkers for hepatocellular carcinoma. Combination of serum miR-483-5p and miR-133a with AFP might complement the role of AFP in detecting hepatocellular carcinoma.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that control gene expression by inhibiting translation or inducing cleavage of target mRNAs. They are known to regulate diverse biologic processes including DNA repair, cell proliferation, differentiation, apoptosis and metastasis [1-2]. Aberrant miRNA expression contributes to tumor-genesis, cancer progression and involved in various biological processes that underlie hepatic tumor formation [1-2]. Several miRNAs with oncogenic characteristics are significantly up-regulated in hepatocellular carcinoma tumor tissues [3]. One of these miRNAs is miR-483-5p which found to be significantly up-regulated in the serum of individuals with hepatitis C virus infection, detected in the serum of patients with liver cirrhosis and up-regulated in HCC patients [4-5]. Also, MiR-133 which specifically expressed in muscles, however, it has been involved in cancer and identified as a key factor in cancer development, including bladder cancer, prostate cancer and so on [6]. Tumor-site–specific miRNAs can be extracellularly released into the bloodstream via active secretion from tumor tissues in a protein-bound complex or as membrane-bound vesicles [7-8]. Also, circulating miRNAs are consistently shown to have high stability- under severe conditions, such

as boiling, very low or high pH levels, extended storage, and multiple freeze–thaw cycles -due to their protection from Rnases. These data indicate that miRNAs are abundant in circulation, and may be useful for early diagnosis of hepatocellular carcinoma [9-10-11]

Serum a-fetoprotein (AFP) has long been used as an early diagnostic biomarker of hepatocellular carcinoma (HCC), but the sensitivity (39%–65%) and specificity (76%–94%) are wide and poor [12, 13].

We aimed to evaluate miR-483-5p and miR-133a as potential biomarkers for diagnosis of hepatocellular carcinoma in cirrhotic patients post chronic hepatitis C virus infection and compare their sensitivity and specificity to currently used alpha fetoprotein test.

Subjects and Methods

Patients and specimens

A cross sectional study was conducted on 40 patients who were recruited from the outpatient clinics and inpatient departments of Tropical and Internal Medicine at Al-Zahraa University Hospital during the period from November 2017 to May 2018 along with 20 ages and sex matched healthy subjects as a control group. The patients were categorized into two groups: group 1: HCC group (n= 20) related to post chronic hepatitis C infection liver cirrhosis, and group 2: liver cirrhosis post hepatitis C virus infection group (n= 20). This study was approved by Ethical Committee of Faculty of Medicine for Girls, Al-Azhar University and carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. Informed written consent was obtained from all participants prior to enrollment in the study. Patients with other cancers or metastatic liver cancer were excluded. All patients were submitted to detailed history and clinical assessment. Liver cirrhosis was diagnosed on the basis of history, clinical examination, laboratory findings, and abdominal ultrasonography. Hepatocellular carcinoma cases were diagnosed by abdominal ultrasonography, abdominal triphasic computed tomography and serum Alpha fetoprotein (AFP).

Fasting venous blood samples (10 mL) were collected from each subject. Three ml were collected in EDTA tubes for RNA extraction and miRNA; 2 ml of whole blood was collected in EDTA tube for determination of HCV by PCR, and another 2 ml of blood was collected in tube containing sodium citrate for Prothrombin time measurement. The remaining was centrifuged and serum was separated for estimation of liver function tests, creatinine, HCV Abs, HBsAg and AFP.

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total bilirubin levels and creatinine were done using (Cobas C311, Roche, Germany). Viral status (HCV-Ab and HBs Ag) were estimated by ELIZA system (reader; A1851 Das, Italy, and washer; 16041412 Bio Tek, USA), kits were supplied by (Axiom - *in vitro* dignosticum/Germany with Lot No C20180403 and ADALTIS/Italy with Lot No. 17310 respectively). Prothrombin time measurements were performed on diagnostic full automated coagulation analyzer (Stago, France). HCV was determined by Real time PCR on (QIAcube for Extraction and Rotor- Gene Q for Detection) using Kit from QIAGEN/Germany according to manufacturer's instructions.

Serum (AFP) Measurement

Serum level of AFP from all enrolled subjects were estimated by electrochemilumeniscience technique using IMMULITE 1000 from SIEMENS and kit for AFP (From SIEMENS, Catalog LKAP1, Lot No. 0214).Test principle is a solid phase, two site sequential chemilumiescent immunoassay immunometric assay. Materials supplied are AFP test units (Each barcode labeled unit contains one bead coated with murine monoclonal anti-AFP; AFP Reagent wedges (one wedge 7.5 ml of a protein buffer/non human serum matrix and the other one contain 7.5 ml of alkaline phosphatase conjugated to polyclonal rabbit anti-AFP in buffer), AFP adjustor (two vials Low and high of AFP in a bovine serum matrix).Assay procedure was carried out according to the manufacturer’s instructions. In each experiment, a quality control (Lot NO.54651 &54652, bio-rad) was used as instructed. A satisfactory level of performance is achieved when the analyte values obtained are within the acceptable range for the system, Then 200 µL of serum samples were added in sample cups and placed in the machine. The whole procedure is fully automated on the IMMULIE 1000.

miRNA Expression Measurement

- RNA Extraction

RNA was isolated using RNeasy Mini Kit (QIAGEN, Hilden, Germany, Lot No. 154041481)which contains(QIAamp Spin Columns (clear), Collection Tubes (1.5 ml), Collection Tubes (2 ml), Qiazole lysis reagent 50 ml, Buffer RWT 45ml, Buffer RPE 45ml, RNase-free Water 10 ml) according to the manufacturer's instructions for purification of miRNA, Briefly 200 µL of plasma was mixed with 1000 µL (5 volumes) Qiazol lysis reagent and incubated for 5 minutes at room temperature. In brief, 200 µL (1 volume) of chloroform was added, and the tube was vortexed vigorously for 15 seconds. After 3 min incubation at room temperature, the tube was centrifuged at 12,000 xg for 15 min, in which the sample was separated into 3 phases; an upper colorless aqueous phase containing RNA, a white interphase and a lower red organic phase. 600 µL of the upper aqueous phase was transferred into a 2 ml sample tube and placed in Qiacube (Qiagen, USA.cat N: 9001292) for automated extraction of miRNA using RNeasy Mini Spin column according to manufacturer's instruction. The RNA purity and concentration in the extract was assessed by the Nano Drop ND-1000 (Nano drop, USA).

- Reverse Transcription

A miScript II RT Kit (Lot. No. 218160,218161)–(QIAGEN) was used, which contains (5x miScript Hi spec buffer [specific to miRNA], Reverse transcriptase, Nucleic mix) according to the manufacturer's instruction. Briefly, 2 µL of miScript reverse transcriptase Mix, 2 µL of 10 x miScript Nucleics Mix, 4 µL of 5 x miScript Hispec buffer, and the template RNA was added to each tube with a total volume is 20 µL/reaction. After gentle mix and brief centrifugation, the tubes were placed in Rotor gene cycler and run conditions were adjusted as follows: incubation for 60 min at 37°C, then incubation for 5 min at 95°C to inactivate miScript reverse transcriptase mix then placed on ice.

- Quantitative real-time PCR

MicroRNAs expression (miR-483-5p and miR-133a) were determined by applying the miScript primers of miR-483-5p and miR-133a (QIAGEN, Germany with Cat No. MS00009758 and MS00031423 respectively), Master Mix of miScript SYBR Green PCR Kit with Lot No. 157013666 (QIAGEN) was applied for real-time PCR.). SNORD68 (Cat. No. MS00033712) was used as housekeeping gene (endogenous reference control cDNA) for all micro RNA in this study. The reaction was run on rotor gene Q 5 plex (Qiagen, USA) according to the manufacturer's instruction. Briefly, RNase free water, Templet cDNA and The reaction mix were dispensed into the individual wells of the PCR plate which was placed in the real time cycler with the following cycling conditions (initial activation step at 95 C for 15 min followed by 40 cycles of denaturation at 94 C for 15 s., annealing at 55 C for 30 s., and extension at 70 C for 30 s.). Fluorescence measurements were made in every cycle.

Melting curve analysis was performed after the thermal profile to ensure specificity in the amplification. Temperature increased very slowly (from 65°C to 95°C) with monitoring of fluorescence signal. Melting curve analysis resulted in detection of single sharp peak for each target.

- Calculation of q-PCR results

The ΔC_T was calculated by subtracting the C_T values of SNORD 68 from the C_T values of the target miRNAs. The resulting normalized ΔC_T values were used in calculating relative expression values of each miRNA by using $2^{-\Delta\Delta CT}$ method please insert reference. These values are directly related to the miRNA expression levels (Fold-Change of expression).

Statistical Analysis

Data were analyzed by the Statistical Package for Social Science (IBM SPSS) version 20. Qualitative data were presented as number and percentages while quantitative data were presented as mean, standard deviations and ranges when parametric. The comparison between two independent groups with quantitative data and parametric distribution was done by using Independent t-test while the comparison between more than two independent groups with quantitative data and parametric distribution was done by using One Way ANOVA test. Pearson correlation coefficients were used to assess the correlation between two quantitative parameters in the same group. Receiver operating characteristic curve (ROC) was used to assess the best cut off point with its sensitivity, specificity, positive and negative predictive values and area under curve (AUC). AUC was interpreted as follow 0.9-1 mean excellent biomarker, 0.8-0.9 good 0.70 – 0.80 fair and 0.60 – 0.70 poor. The *P*-value was considered significant at the level of < 0.05.

Results

The demographic and clinical characteristics of patients and control groups are shown in (Table 1). There is a male predominance among HCC patients and Cirrhotic patients (80.0% and 75.0% respectively) with a highest mean values of age were found in patients with HCC (mean =61.60).

Serum levels of (AST), (ALT), total bilirubin, direct bilirubin, INR and (AFP) show a highly statistically significant increase in both HCC and Cirrhotic groups compared to controls. Also, there are highly statistically significant increases in the serum levels of AST, ALT, total bilirubin, direct bilirubin, INR and (AFP) in HCC group compared with Cirrhotic group. Serum albumin and Total protein were significantly decreased in both Cirrhotic and HCC groups compared to control group. Furthermore, the serum levels of albumin and Total protein were significantly lower among HCC patients than in Cirrhotic patients.

Table 1. Demographic data and laboratory parameters of the studied group.

		HCC group	Cirrhotic group	Control group	*P*-value
		No. = 20	No. = 20	No. =20	
Gender	Females	4 (20.0%)	5 (25.0%)	6 (30.0%)	0.021
	Males	16 (80.0%)	15 (75.0%)	14(70.0%)	
Age(years)	Mean ± SD	61.60 ± 6.39	59.05 ± 7.18	42.20 ± 13.11	0.000
	Range	52 – 78	45 – 72	27 – 61	
Alb (g/dL)	Mean ± SD	2.21 ± 0.34	2.97 ± 0.74	4.55 ± 0.26	0.000
	Range	1.7 – 2.7	2.1 – 4.4	4.2 – 5	
Bil.T (mg/dL)	Median(IQR)	3.1 (1.2 – 6.5)	1 (0.7 - 3.4)	0.3 (0.2 - 0.4)	0.000
	Range	0.3 – 21.7	0.2 – 25	0.2 – 0.7	
Bil.D (mg/dL)	Median(IQR)	1.55 (0.35 - 4.8)	0.3 (0.3 - 2.2)	0.17 (0.09 – 0.22)	0.001
	Range	0.08 – 18.8	0 – 21.3	0.08 – 0.3	
T.P (g/dL)	Mean ± SD	6.16 ± 0.64	7.00 ± 0.83	7.09 ± 0.41	0.000
	Range	4.5 – 7.2	5.4 – 8.3	6.7 – 7.8	
Urea (mg/dL)	Median(IQR)	33 (19 - 76)	33 (18.5 - 101)	25 (23 - 33)	NS
	Range	15 – 198	10 – 256	16 – 34	
Creatinine. (mg/dL)	Mean ± SD	1.01 ± 0.51	1.11 ± 0.59	0.69 ± 0.19	NS
	Range	0.3 – 2.3	0.4 – 2.2	0.5 – 1	
INR	Mean ± SD	1.79 ± 0.43	1.44 ± 0.13	1.03 ± 0.02	0.000
	Range	1.3 – 2.95	1.13 – 1.59	0.99 – 1.06	
AST (U/L)	Median(IQR)	67.5 (49 - 151)	40 (36 - 52)	19 (16 - 20)	0.000
	Range	17 – 577	14 – 159	15 – 25	
ALT (U/L)	Median(IQR)	43.5 (27 - 96)	26 (21.5 - 29)	19.5 (17 - 23)	0.003
	Range	6 – 336	16 – 53	15 – 26	
AFP (ng/mL)	Median(IQR)	88.5 (50 - 785)	15 (9.8 - 24)	3 (1.7 – 3.4)	0.000
	Range	6.8 – 50000	4.4 – 34	1.5 – 6.8	
HCV-RNA	Negative	0 (0.0%)	0 (0.0%)	20 (100.0%)	0.000
	Positive	20 (100.0%)	20 (100.0%)	0 (0.0%)	

*:Chi-square test; •: One Way ANOVA test ‡: Mann Whitney test. *P*>0.05 is not significant (NS)

The results of miRNAs expression showed that miRNA 483-5p & miR-133a were significantly higher in HCC group than Cirrhotic group and Control at $P<0.00$ as demonstrated in (Table 2).

Table 2. Micro RNA 483-5p and RNA 133a Expression among HCC & Cirrhotic and control groups.

		HCC group	Cirrhotic group	Control group	*P*-value
		No. = 20	No. = 20	No. = 20	
Micro RNA 483 5p fold change	Median(IQR)	23 (9.29 - 56.11)	2.85 (1.85 - 8.06)	0.18 (0.11 – 0.43)	0.000
	Range	3.9 – 97.98	0.31 – 42.95	0.055 – 0.942	
Micro RNA133a fold change	Median(IQR)	6.97 (3.5 - 9.96)	1.65 (0.44 - 4.06)	0.17 (0.15 – 0.46)	0.000
	Range	2.13 – 12.73	0.11 – 8	0.012 – 0.818	

‡: Mann Whitney test.. *P*<0.05 is significant

MiR483-5p expression showed significant direct correlation with miR-133a in both HCC group and cirrhotic group. While, it was significantly inversely correlated with age in cirrhotic group (Table 3). On the other hand, miR-133a expression showed inverse significant correlation with age and AFP in cirrhotic group (Table 4).

Table 3.Correlation of Micro RNA 483-5p Expression with clinical variables in HCC and cirrhotic groups.

	Micro RNA 483 5p fold change			
	HCC group		Cirrhotic group	
	r	P-value	r	*P*-value
Micro RNA133 a fold change	0.717**	0.000	0.611**	0.004
Age	0.207	NS	-0.589**	0.006
Alb. (g/dL)	-0.112	NS	0.180	NS
Bil. T (mg/dL)	0.085	NS	0.105	NS
Bil. D (mg/dL)	0.152	NS	0.075	NS
T.P (g/dL)	-0.083	NS	0.175	NS
Urea (mg/dL)	0.357	NS	0.082	NS
Creatinine (mg/dL)	0.230	NS	0.045	NS
INR	0.382	NS	-0.015	NS
AST (U/L)	-0.124	NS	-0.045	NS
ALT (U/L)	0.081	NS	-0.036	NS
AFP (ng/mL)	-0.035	NS	-0.146	NS

P>0.05 is not significant (NS)

Table 4. Correlation of Micro RNA 133a Expression with clinical variables in HCC and Cirrhotic groups.

	Micro RNA133 a fold change			
	HCC group		Cirrhotic group	
	r	*P*-value	r	*P*-value
Micro RNA 483 5p fold change	0.717**	0.000	0.611**	0.004
Age	0.146	NS	-0.499*	0.025
ALB (g/dL)	-0.271	NS	0.360	NS
BIL T (mg/dL)	-0.079	NS	0.057	NS
BIL D (mg/dL)	0.014	NS	-0.092	NS
T.P (g/dL)	-0.306	NS	0.028	NS
Urea (mg/dL)	0.072	NS	0.089	NS
Creat (mg/dL)	-0.114	NS	0.080	NS
INR	0.151	NS	-0.034	NS
AST (U/L)	-0.010	NS	-0.167	NS
ALT (U/L)	0.065	NS	-0.067	NS
AFP (ng/mL)	0.001	NS	-0.554*	0.011

P>0.05 is not significant (NS)

ROC curve analysis performed on the data to assess whether serum miRNAs have diagnostic value in (Fig. 1) and (Table 5) which showed that serum miR-483-5p and miR-133a levels had the potential to discriminate HCC patients from cirrhotic group; the areas under the ROC curves were 0.907 and 0.84 respectively, compared with an AUC for AFP of 0.908.

MiR-483-5p could predict HCC with sensitivity 100%, specificity 75%and accuracy 0.907 at cut off value > 3.89 while, miR-133a could predict HCC with sensitivity 70%, specificity 90% and accuracy 0.84, at cut off value >4.79. However, the sensitivity and the specificity of AFP were 80% and 100% respectively as shown in (Table 5).

Moreover, AUC of combination of miR-483-5p and AFP were 0.975 with sensitivity and specificity 95% in diagnosis of HCC. Also, AUC of combination of miR- 133a and AFP were 0.982 with sensitivity and specificity 90% and 100% respectively in diagnosis of HCC.

These results further strengthened the conclusion that miR- 483-5p and miR-133a has an equal or even greater efficient in diagnosing HCC.

We, thus, assumed that we could use miR-483-5p and miR-133a as additional diagnostic index to increase specificity and the sensitivity of AFP.

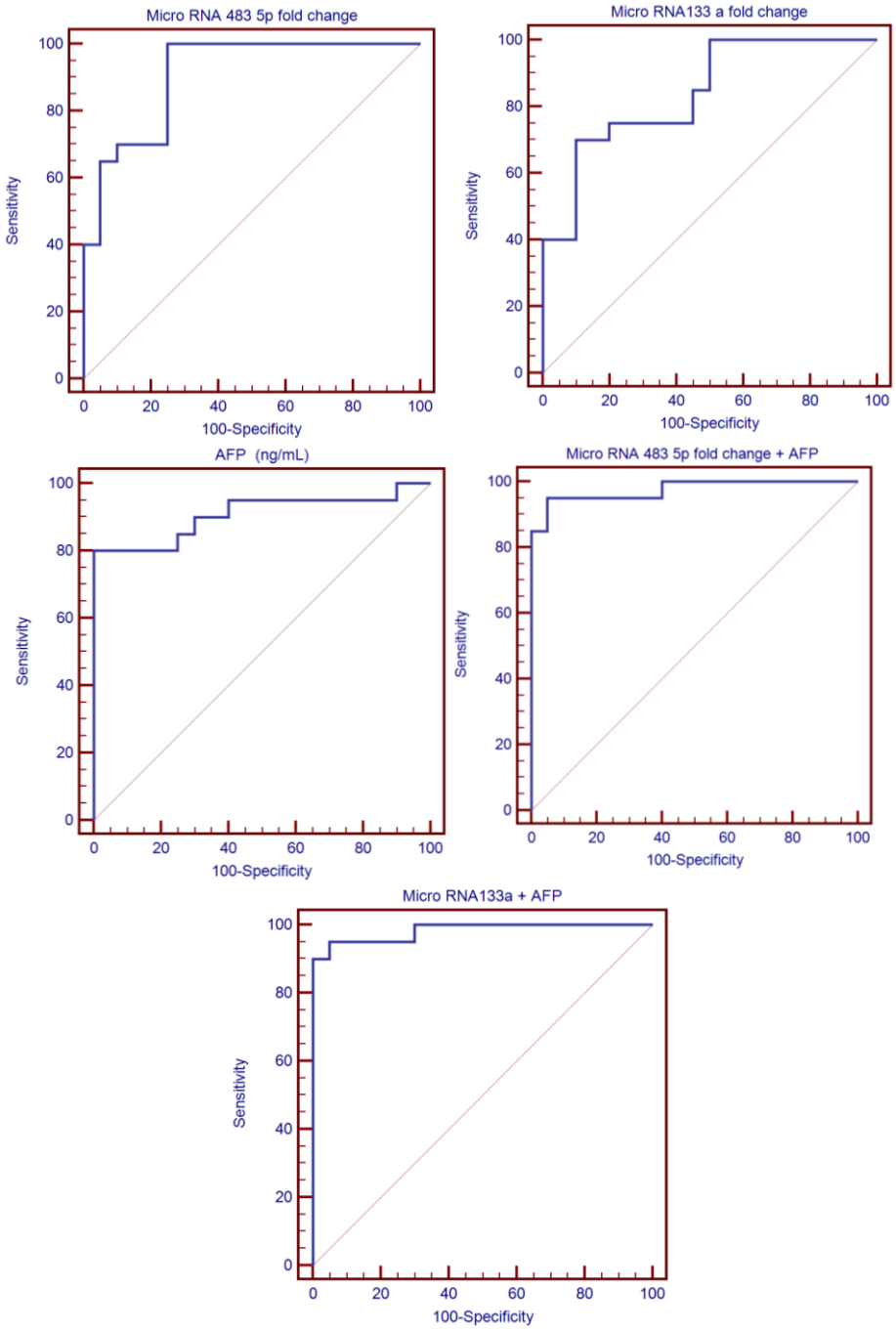

Figure 1. The ROC curve analysis of serum miR-483-5p, miR-133a, AFP, miR-483-5p + AFP, and miR-133a + AFPin discriminating HCC patients from Cirrhotic patients.

Table 5. Diagnostic accuracy of miRNAs (483 5p and 133a)and AFP in discriminating HCC patients from Cirrhotic patients.

	Cut off point	AUC	Sensitivity	Specificity	PPV	NPV
Micro RNA 483 5p fold change	>3.89	0.907	100	75	80	100
Micro RNA133a fold change	>4.79	0.84	70	90	87.5	75
AFP (ng/mL)	>34	0.908	80	100	100	83.3
Micro RNA 483 5p fold change + AFP	--	0.975	95.0	95.00	95.0	95.0
Micro RNA133a + AFP	--	0.982	90.00	100.00	100.0	90.9

Discussion

The hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide and known to be one of the major liver-related mortalities in cirrhosis, mainly in patients suffering from chronic hepatitis B or C [14-15-16]. The serum level of alpha-fetoprotein (AFP) is widely used to detect primary HCC but its sensitivity and specificity are undecided [17-18].

Anomalous expressions of miRNAs have been involved in a wide variety of cancers, including (HCC) [19-20].As, both miR-483-5p and miR-133a is expressed in (colorectal cancer, malignant pheochromocytoma), (bladder cancer and prostate cancer) respectively [21 -6], also, each one of them was previously studied with other members of miRNAs in diagnosis of HCC [4-6].

We aimed to study the combination of miR-133a and miR-483-5p in diagnosis of hepato-cellular carcinoma in comparison to alphafetoprotein.

Up to our knowledge, it is the first time to use this combination in diagnosis of HCC in patients with liver cirrhosis post chronic hepatitis C viral infection.

Our results revealed that miRNA- 483-5p and miR-133a were significantly higher in HCC group than Cirrhotic group by (23 and 2.8 fold change) and (6.97 and 1.6 fold change) respectively. Also, miR-483-5p could predict HCC with sensitivity 100%and specificity 75% at cut off value > 3.89 while, miR-133a could predict HCC with sensitivity 70% and specificity 90% at cut off value >4.79

These results are in agreement with Shen *et al*., [22] study who found that miR-483-5p was statistically significantly over expressed in hepatocellular carcinoma cases compared with controls (3.20 vs. 0.82, $P < 0.0001$).

The combination of plasma miR-483-5p level and hepatitis C virus status can significantly differentiate hepatocellular carcinoma cases from controls with an area under the curve of 0.908 ($P<0.0001$).The sensitivity and specificity were 75.5% and 89.8% respectively. Also, they found that, miR-483-5p alone had a sensitivity of 55.1% and specificity of 85.7 %.miR-483-5p.

Moreover, Li *et al*., [23] stated that miR-483-3p was up regulated in HCC tissue; these data suggest that circulating miR-483-5p may be a useful biomarker for HCC diagnosis.

Also, Zhang *et al* [4] study found that, median level of miR-483-5p was higher in HCC patients than in patients with chronic hepatitis B and in healthy controls ($P<0.0001$). The area under receiver operating characteristic curve of miR-483-5p was 0.74 (cut off value = 2.824, sensitivity = 74%, and specificity= 66%) for the prediction of HCC. Moreover, combining of

AFP and serum miR-483-5p (sensitivity = 81% and specificity = 83%) was better than AFP alone (sensitivity= 78%, specificity = 70%).

Also, Lin *et al*., [24]classified miR-133a as early predictor of HCC before clinical diagnosis and has sensitivity and specificity better than alpha-fetoprotein in patients with post chronic hepatitis B liver diseases and early hepatocellular carcinoma patients.

In conclusion, micro RNA 483-5p and 133a were sensitive and specific in diagnosis of hepatocellular carcinoma (100% and 70%) and (75% and 90%) respectively. Combination of both Micro RNA 483 5p and miR- 133a and alpha-feto-protein has sensitivity and specificity of (95% - 95%) and (90% -100%) respectively in diagnosis of hepatocellular carcinoma. So our study suggests that circulating Micro RNA 483-5p and 133a could be useful as a potential biomarkers for detection of hepatocellular carcinoma. However, further well-designed studies with larger sample size of both cases and controls are needed to confirm the conclusion.

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