



Serum miRNA-27a and miRNA-18b as potential predictive biomarkers of hepatitis C virus-associated hepatocellular carcinoma

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Abstract

Hepatitis C virus (HCV) infection remains the main risk factor for chronic hepatitis (CHC), liver cirrhosis, and hepatocellular carcinoma (HCC). Changes in microRNA (miRNA) profiles can be associated with HCV infection and may either favor or inhibit the virus and/or its complication. Moreover, miRNAs have emerged as key regulators of various cancers including HCC. The aim of this work was to investigate the potential role of miRNA-27a and miRNA-18b expression levels as non-invasive predictive biomarkers of hepatitis C virus-associated HCC. Furthermore, we aimed to explore potential association of these miRNAs expressions with HCC clinicopathological features in Egyptian cases. This case control study included 200 participants [60 CHC patients, 39 post-HCV cirrhosis patients, 51 HCC cases], and 50 healthy volunteers. The serum miRNA-27a and miRNA-18b expression profiles were measured using quantitative real time-polymerase chain reaction (qRT-PCR). miRNA-27a and miRNA-18b expression levels were significantly increased in post-hepatitis C cirrhosis cases compared to control and CHC groups. In HCC group, only miRNA-27a expression levels were significantly increased. Moreover, miRNA-27a and miRNA-18b expression levels were positively correlated with distant metastasis, Child–Pugh grade, and lymph node metastasis. Logistic regression analysis revealed that miRNA-27a expression was an independent predictor of cirrhosis among CHC. Receiver operating characteristic (ROC) analyses showed that miRNA-27a and miRNA-18b expression levels were useful biomarkers discriminating cirrhosis from CHC (AUC were 0.672 and 0.487, respectively), and in differentiating HCC from post-hepatitis C cirrhosis (AUC were 0.897 and 0.723, respectively). By combined ROC analysis, power of miRNA-27a and miRNA-18b expression levels as discriminator between HCC from post-hepatitis C cirrhosis was high (AUC = 0.821). Serum microRNA-27a and miRNA-18b expression levels are promising diagnostic and non-invasive biomarkers of CHC, post-CHC cirrhosis, and HCC.

Keywords Hepatitis C virus · MicroRNAs · Hepatocellular carcinoma · Liver cirrhosis · Chronic hepatitis C · Real-time-PCR

Introduction

Worldwide, the incidence of individuals with hepatocellular carcinoma (HCC) is increasing. Moreover, HCC mortality has increased markedly over the past decades [1]. Mounting evidence indicates that among the various risk factors for HCC; hepatitis C virus (HCV) is the main risk factor. In Egypt, HCC mortality prevalence is 14.8% of cancer

mortality [2]. Similarly, the HCC incidence has been nearly doubled (23.8%) [3, 4].

Recently, non-coding RNAs are epigenetic regulator of gene expression including short non-coding RNA. microRNAs (miRNAs) is example of short single-stranded non-coding RNA consists of 19–24 nucleotides which can regulate gene expression at the post-transcriptional level. They bind with 3' untranslated regions (UTRs) of target mRNAs through base pairing [5]. Noteworthy, every miRNA might regulate few to thousands of genes and one target gene could also be regulated by hundreds of miRNAs. The miRNA-mRNA interactions can play important roles in many physiological processes including development, apoptosis, differentiation, and metabolism. The aberrant miRNAs expression proposed to be closely related to the development and progression of various human diseases,

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including cancers [6]. Thus, identifying novel microRNAs as diseases' biomarkers contributes to a better understanding of pathogenicity mechanisms of different diseases and could be promising therapeutic targets. Over the last few years, detailed observations have been made on microRNAs (miRNAs) and its association with different diseases. Even more interestingly, HCC the suspected pathological effect of miRNAs including host-viral interactions and different stages of carcinogenesis [5, 6].

Notably, efforts were done by viruses using miRNAs to control their host cell; reciprocally, host cells use miRNAs to target essential viral functions. However, recent researches revealed that host miRNAs inhibit or stimulate HCV replication in almost all stages of the viral life cycle by targeting viral RNAs and/or host mRNAs [7]. One of liver-abundant miRNAs is miRNA-27a; whose role in HCV pathogenesis is not clarified up till now [8, 9]. It is implicated in atherosclerosis through its role in regulating lipid metabolism in adipocytes, hepatocytes and macrophages [10–12].

Emerging evidence demonstrated that microRNA-18b (miRNA-18b) is another hepatic miRNA located at chromosome X within the miR-106a-363 cluster that is constituted by six different microRNAs [13, 14]. Notably, miR-18b is highly expressed in various cancers including stomach cancer [15], ovarian cancer [16], basal cell carcinoma of the skin [17], and in colon cancer [18]. However, few researches exploring the expression pattern of miRNA-27a & miRNA-18b in HCV related disease progression including cirrhosis and HCC reflecting the potential role of these novel biomarkers.

Unfortunately, there are no reliable biomarkers for diagnosis of HCC, and most cases were diagnosed at terminal stages. There is no debate as to whether liver biopsy procedure is hazards and the absence of a reliable biomarker for early HCC accentuate the need for an alternative sensitive, reliable and non-invasive biomarker is an enormously important clinical consideration. HCV infection alters the miRNAs expression profiles in the host cells which can, in turn, affect the virus life cycle. To date, very limited data exist concerning alterations of miRNA-27 and miRNA-18b in HCV-related disease progression; cirrhosis and HCC. Therefore, we conducted this work, for the first time, to investigate the possible role miRNA-27 and miRNA-18b expression levels as diagnostic biomarkers predicting HCC among CHC patients. Additionally, we explored the association between miRNA-27a and miRNA-18b expression levels with clinicopathological features of HCC.

Patients and methods

Patients

This case–control study was conducted in Internal Medicine and Medical Biochemistry Departments, Faculty of Medicine, Zagazig University. A total 150 patients were enrolled; group I: CHC patients ($n=60$), group II: post-CHC cirrhosis without HCC ($n=39$), and group III: post CHC cirrhosis with HCC ($n=51$). In addition to the 50 controls and they were age ethnic, sex, and smoking matched to the cases. HCC was diagnosed according to American Association for the Study of Liver Diseases practice guidelines. Clinical staging of HCC was evaluated according to Barcelona Clinic Liver Cancer staging classification [19] and Child–Pugh classification [20, 21]. Clinical parameters of HCC cases including tumor number, size, site, presence of metastasis, and portal vein thrombosis were recorded. None of patients received neoadjuvant chemotherapy, radiotherapy, or immunotherapy. Liver cirrhosis diagnosis was established on basis of clinical, laboratory, and imaging investigations. Patients suffering from diabetes mellitus (DM), obesity, dyslipidemia, chronic HBV infection or any other identifiable cause for chronic hepatitis other than HCV were excluded from this study. Additionally, heart failure, renal failure, proteinuria, active bacterial infections, and evidence of endocrine disorder or receiving hormone replacement therapy were also excluded. The study protocol was approved by the Ethics Committees of Faculty of Medicine, Zagazig University. Informed written consent was obtained from each individual.

Laboratory assays

Blood samples were drawn from all subjects after an overnight fast. We divided blood sample into 2 portions: 1 ml of whole blood was collected into tubes containing EDTA for complete blood count (CBC) including hemoglobin, platelet, and total leukocyte counts. Sera were separated immediately from the remaining portion and stored at $-20\text{ }^{\circ}\text{C}$ until analysis for miRNA extraction and biochemical analyses. Prothrombin time and international normalized ratio (INR) were performed for all patients. ALT, aspartate aminotransferase (AST), albumin, total bilirubin, and creatinine were measured in serum by routine enzymatic methods (spinreact). Serum alpha foetoprotein (AFP) concentration was measured by ELISA (kit provided by Biosource Europe S.A, Belgium). All patients were tested for anti-HCV antibodies in sera by ELISA, using third generation kits (DiaSorin, Italy) according to the manufacturer's instructions.

MiRNA extraction from sera

Total RNA was extracted from sera using miRNEasy RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was eluted by 30 μ L of ribonuclease-free water. The RNA quality was then determined using UV/visible spectrophotometer. The ratio of absorbance at 260 and 280 nm was used to assess the purity of RNA. The quality of total RNA was detected by A260 to A280 ratio and 1.2% agarose gel electrophoresis.

qRT-PCR analysis of miRNA-27a and miRNA-18b expressions levels

One microgram miRNA was used in reverse transcription with a miScript II RT Kit (Qiagen/SABiosciences Corporation, Frederick, MD, USA). Then, the cDNA was kept in -80°C till the real-time-polymerase chain reaction (RT-PCR) analyses.

Quantitative real-time-polymerase chain reaction (qRT-PCR) was carried out by StepOnePlus™ System (Applied Biosystems Inc., Foster, CA, USA). Small RNA (SNORD-68) was used as the internal control (catalogue no. MS00033712, Qiagen). The miRNA-specific primers [miR-27a: catalogue no. MS00003241, Qiagen; and miR-18b: catalogue no. MS00031521, Qiagen] were used. SYBR Green Master Mix (Qiagen/SABiosciences Corporation, USA) was used in the (RT-PCR) reaction according to the manufacturer's suggested protocol, along with the manufacturer-provided miScript Universal primer and miRNA-specific forward primer. The miRNA-specific primers, miRNA-27a and miRNA-18b, were chosen based on the miRNA sequences obtained from the miRbase database (<http://microrna.sanger.ac.uk/>). The relative gene expression (fold change) of serum microRNAs expressions levels were analyzed using the comparative threshold cycles method [22].

Statistical analysis

Data analysis was performed using Statistical Package for the Social Sciences Software (SPSS Version 20, Chicago, Illinois). Data were expressed as mean \pm standard deviation (SD), or number (percentage) when appropriate. Comparisons between two groups were performed using chi-squared (X^2), or Fischer exact test was performed for non-parametric parameters. One-way analysis of variance (ANOVA) test was used to compare between more than 2 groups. The correlation of miRNAs expression levels with clinical and biochemical characteristics was tested with the Pearson rank correlation. We explored the main independent variables of miRNA-27a among HCC group by multiple stepwise linear regression analysis. Receiver operating characteristic (ROC) analysis was performed to assess the potential diagnostic

accuracy of circulating miRNAs expression levels, the area under the curve (AUC), and the cutoff values. Logistic regression analysis was performed to identify predictors of cirrhosis and HCC. We considered P to be significant at <0.05 with a 95% confidence interval (CI).

Results

Clinical and laboratory characteristics of the studied groups

The main finding of our study is that serum miRNA-27a and miRNA-18b expression levels were significantly increased in HCC, post-hepatitis C cirrhosis, CHC patients compared to control group (Fig. 1a, b). In post-CHC cirrhosis and CHC case groups, we found significant higher levels of ALT, AST, AFP, PT, INR, creatinine, total & direct bilirubin levels, and WBC compared to control group ($P < 0.001$). On the contrary, we detected significant lower hemoglobin, platelet and albumin values in CHC and post-CHC cirrhosis patients compared to controls group. Furthermore in HCC cases, our results revealed significant higher values of ALT, AST, PT,

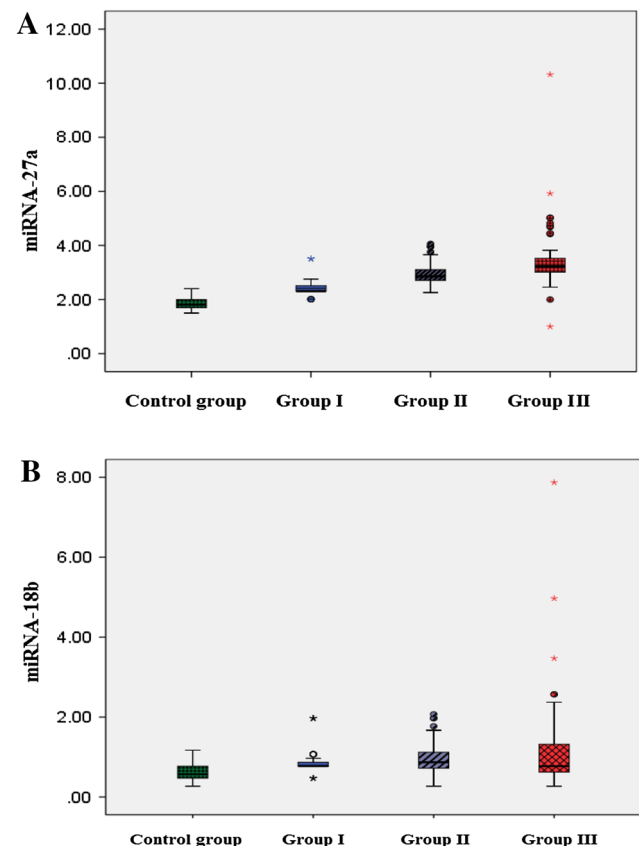


Fig. 1 a Differential microRNA-27a expression in the studied groups. b Differential microRNA-18-b expression in the studied groups

AFP, total, and direct bilirubin compared to CHC cases ($P < 0.001$) (Table 1).

Clinical and laboratory characteristics of post-CHC cirrhosis patients with or without HCC

Among cirrhosis cases with HCC, our results revealed significant increase of miRNA-27a expression levels compared to cirrhosis group without HCC ($P < 0.001$). While, we did not observe any significant differences in miRNA-18b expression levels between two groups. There were significant higher levels of AST, AFP, INR, creatinine, total and direct bilirubin, WBC count in cases with HCC group compared to those without HCC ($P < 0.001$). On the contrary, we detected significant lower hemoglobin and albumin values in cirrhosis patients with HCC group compared to those without HCC ($P > 0.05$) (Table 2).

Clinicopathological features of HCC patients as summarized in Table 3

In HCC group, there were 30 patients (58%) with stage III/IV, 25 patients (49.1%) with Child–Pugh grade C, tumor size more than 5 cm was detected in 31 cases (60.8%). Lymph node metastasis was found in 22 patients (43.2%), distant metastasis presents in 21 cases (42%), portal vein thrombosis found in 11 patients (21.6%), multiple tumor lesion in 25 patients (49.1), and finally both lobes affection was detected in 28 cases (55%) (Table 3).

Pearson correlations between miRNA-27a and miRNA-18-b expression levels with laboratory and clinicopathological features of HCC

In HCC group, miRNA-27a expression levels were positively correlated with creatinine, Child–Pugh grade, lymph node and

Table 1 Clinical and laboratory characteristics of studied groups

Parameters	Healthy subjects ($n=50$)	CHC patients ($n=60$)	Post-CHC cirrhosis ($n=90$)	<i>P</i> value
Age (years)	45.55 ± 3.8	45.28 ± 5.72	46.96 ± 5.94	0.698
Sex(male/female)	(27/23)	(32/28)	(49/41)	0.90
Residence				
Rural	37 (75%)	46 (77%)	68 (76%)	0.97
Urban	13 (25%)	16 (23%)	22 (24%)	
Smoking status				
Smoker	22 (44%)	27 (45%)	43 (48%)	0.89
Non-smoker	28 (56%)	33 (55%)	47 (42%)	
INR	1.2 ± 0.05	1.20 ± 0.084	1.6 ± 1.45 ^{*#}	< 0.001
Prothrombin time	11.7 ± 0.7	14.1 ± 0.90 [#]	16.92 ± 3.6 [#]	< 0.001
Albumin (g/dl)	4.5 ± 0.23	3.31 ± 0.229 [#]	2.59 ± 0.711 [#]	< 0.001
AST (IU/L)	21.56 ± 1.7	34.8 ± 5.2 [#]	100.5 ± 85.1 [#]	< 0.001
ALT(IU/L)	18.3 ± 3.37	42.5 ± 10.3 [#]	84.5 ± 64.35 [#]	< 0.001
Total bilirubin (mg/dl)	0.9 ± 0.13	1.6 ± 0.18 [#]	3.78 ± 5.1 [#]	< 0.001
Direct bilirubin mg/dl)	0.25 ± 0.07	0.81 ± 0.76 [#]	2.7 ± 4.82 [#]	< 0.001
Alpha fetoprotein (ng/ml)				
Mean ± SD	8.86 ± 1.68	13.3 ± 3.9	381.4 ± 251.5 ^{*#}	< 0.001
min–max	8.20–10.21	9.01–27.40	11–1234	
WBC count (cell × 10 ³ /μl)	6.3 ± 0.9	7.7 ± 2.4 [#]	8.5 ± 4.81 [#]	< 0.001
Hemoglobin (g/dl)	13.6 ± 0.71 [#]	11.5 ± 2.56 [#]	11.01 ± 2.42 [#]	< 0.001
Platelet(cell × 10 ³ /μl)	236.8 ± 38.1	131.4 ± 46.7 [#]	138.04 ± 70.8 [#]	< 0.001
Creatinine (mg/dl)	0.74 ± 0.23	1.2 ± 1.08	1.44 ± 1.38 [#]	< 0.001
MiRNA-27a expression	1.84 ± 0.23	2.83 ± 0.63 [#]	3.8 ± 1.327 ^{*#}	< 0.001
MiRNA-18-b expression	0.61 ± 0.234	0.88 ± 0.324 [#]	1.16 ± 1.024 ^{*#}	< 0.001

Quantitative data were presented as mean ± standard deviation; qualitative data were presented as numbers and frequency

CHC chronic hepatitis C, HCV hepatitis C virus, INR international normalized ratio, ALT alanine aminotransferase, AST aspartate; aminotransferase, WBC white blood cell count, F fibrosis

*Significant *P* values ($P < 0.05$) when compared with CHC group

#Significant *P* values ($P < 0.05$) when compared with control group

Table 2 Clinical and laboratory characteristics of post-CHC cirrhosis patients with or without HCC

Parameters	Cirrhosis without HCC group (n = 39)	Cirrhosis with HCC group (n = 51)	P value
Age (years)	48.28 ± 5.04	45.94 ± 6.42	0.118
Sex (male/female)	(18/21)	(31/20)	0.24
Residence			
Rural	32 (82%)	36 (70%)	0.31
Urban	7 (18%)	15 (30%)	
Smoking status			
Smoker	24 (62%)	19 (37%)	0.04*
Non-smoker	15 (38%)	32 (63%)	
INR	1.42 ± 0.33	1.75 ± 1.9	0.106
Prothrombin time	16.37 ± 3.34	17.33 ± 3.88	0.078
Albumin (g/dl)	2.88 ± 0.68	2.37 ± 0.65	<0.001*
AST (IU/L)	83.75 ± 53.4	113.2 ± 101.7	<0.05*
ALT (IU/L)	92.8 ± 125.4	78.13 ± 85.7	0.346
Total bilirubin (mg/dl)	3.61 ± 2.59	5.68 ± 4.89	<0.001*
Direct bilirubin (mg/dl)	1.60 ± 2.99	3.62 ± 5.73	<0.001*
Alpha fetoprotein (ng/ml)			
Mean ± SD	301.7 ± 275.5	442.3 ± 213.6	<0.001*
min–max	11–1234	58–1035	
WBC count (cell × 10 ³ /μl)	7.92 ± 2.49	8.95 ± 5.99	0.158
Hemoglobin (g/dl)	11.77 ± 2.65	10.42 ± 2.06	<0.001*
Platelet (cell × 10 ³ /μl)	126.02 ± 46.13	147.2 ± 84.29	0.065
Creatinine (mg/dl)	1.13 ± 0.46	1.87 ± 1.62	<0.001*
miRNA-27a	2.99 ± 0.46	3.50 ± 1.265	<0.001*
miRNA-18b	1.01 ± 0.46	1.27 ± 1.29	0.082

Quantitative data were presented as mean ± standard deviation; qualitative data were presented as numbers and frequency

CHC chronic hepatitis C, HCV hepatitis C virus, INR international normalized ratio, ALT alanine aminotransferase, AST aspartate; aminotransferase, WBC white blood cell count

*Significant differences were detected between HCV with cirrhosis patients investigated subgroups at $P < 0.05$ using Chi-square test

distant metastasis, and tumor size. Regarding miRNA-18b, the expression levels were positively correlated with creatinine, PT, WBC, total and direct bilirubin levels, Child–Pugh grade, as well as distant and lymph metastasis (Table 4).

A stepwise multiple linear regression analysis in HCC

In HCC group, stepwise multiple linear regression analysis revealed that Child–Pugh grade and distant metastasis were main predictors of serum miRNA-27a expression level among other clinical and laboratory biomarkers ($P = 0.004$, $P = 0.02$, respectively) (Table 5).

Accuracy of serum miRNA-27a and miRNA-18b expression levels for predicting liver cirrhosis by logistic regression analysis

Logistic regression analysis was performed to clarify whether these serum miRNAs can predict with cirrhosis among CHC patients. MiRNA-27a was an independent predictor of cirrhosis among CHC with odds ratio of 45.517, while miRNA-18b expression levels were non-significant (Table 6).

Table 3 Clinicopathological features of HCC patients

	HCC patients (n=51) n (%)	P value
Clinical stage		
Stage I/II	21 (42)	0.208
Stage III/IV	30 (58)	
Tumor size		
< 5 cm	20 (39.2)	0.258
> 5 cm	31 (60.8)	
Lymph node metastasis		
Absent	29 (56.8)	0.327
Present	22 (43.2)	
Distant metastasis		
Absent	30 (58)	0.208
Present	21 (42)	
Child–Pugh grade		
A	9 (17.6)	<0.001*
B	17 (33.3)	
C	25 (49.1)	
Portal vein thrombosis		
Negative	40 (78.4)	<0.001*
Positive	11 (21.6)	
Number of tumor lesions		
Single	26 (50.9)	0.889
Multiple	25 (49.1)	
Site of lesions		
Right lobe	13 (25.4)	0.327
Left lobe	10 (19.6)	
Both	28 (55)	

*Significant *P* value (*P*<0.05)

Accuracy of serum miRNA-27a and miRNA-18b for predicting HCC by logistic regression analysis

Logistic regression analysis was performed to explore the predictors of HCC among CHC patients with cirrhosis. MiRNA-27a was an independent predictor with odds ratio of 3.010 (*P*=0.03) (Table 7).

Accuracy of serum miRNA-27a and miRNA-18b in discriminating between CHC patients and controls by ROC analysis

We investigated the potential diagnostic value of the two selected miRNAs (miRNA-27a and miRNA-18b) by ROC curves and the area under the curve (AUC) values through all patients with CHC and control group. ROC curves are presented in Fig. 2a, b; the cutoff values of miRNA-27a and miRNA-18b were 2.2 and 0.92 and the AUC were 0.945 (95% CI 0.896–0.994) and 0.704 (95% CI

Table 4 Pearson correlations of circulating miRNA-27a and miRNA-18b expression levels with laboratory and clinicopathological features of HCC

Parameters	MiRNA-27a expression		MiRNA-18b expression	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
INR	−0.011	0.942	0.007	0.960
Prothrombin time (PT)	0.156	0.274	0.379	<0.001*
Albumin (g/dl)	−0.069	0.629	−0.172	0.227
AST (IU/L)	0.067	0.641	0.137	0.336
ALT (IU/L)	0.233	0.100	0.263	0.062
Total bilirubin (mg/dl)	0.096	0.501	0.375	<0.001*
Direct bilirubin (mg/dl)	0.112	0.435	0.396	<0.001*
WBC count (cell × 10 ³ /μl)	0.140	0.326	0.449	<0.001*
Hemoglobin (g/dl)	−0.034	0.812	−0.093	0.517
Platelet (cell × 10 ³ /μl)	−0.203	0.154	−0.085	0.555
Creatinine (mg/dl)	0.588	<0.001*	0.787	<0.001*
α-Fetoprotein (ng/ml)	0.092	0.522	0.123	0.391
Portal vein thrombosis	0.155	0.277	0.183	0.200
Lymph node metastasis	0.396	<0.001*	0.375	<0.001*
Number of tumor lesions	0.173	0.224	0.236	0.101
Clinical stage	0.167	0.242	0.276	0.102
Tumor size	0.307	0.03*	0.201	0.157
Distant metastasis	0.601	<0.001*	0.561	<0.001*
Child–Pugh grade	0.467	<0.001*	0.441**	<0.001*

*Significant *P* value (*P*<0.05)

0.605–0.804), respectively, *P*<0.001 for each. Additionally, the sensitivities and the specificities of miRNA-27a were 93.3 and 94%, and of miRNA-18b were 95.3 and 16%, respectively. Thus, miRNA-27a more powerful than miRNA-18b and these miRNAs could be useful diagnostic biomarkers discriminate CHC from healthy controls.

Accuracy of serum miRNA-27a and miRNA-18b for differentiating cirrhosis among CHC patients

The diagnostic power of miRNA-27a in differentiating post-CHC cirrhosis among CHC patients, the optimum cutoff value was 2.79; the sensitivity and specificity of miRNA-27a were 83.1 and 42.9%, respectively, and the AUC was 0.672 (95% CI 0.555–0.789, *P*<0.001). On the other hand, the optimum cutoff value of miRNA-18b was 0.82; the sensitivity decreased (54.2%), and the specificity decreased significantly to 41.9%, and the AUC decreased to 0.487 (95% CI 0.366–0.608, *P*=0.839 (Fig. 3a, b). Therefore, our results shed the light, for the first time, that miRNA-27a could be potential promising diagnostic biomarkers of cirrhosis among CHC patients.

Table 5 Multiple stepwise linear regression analysis for miRNA-27a expression as dependent variable among other laboratory and clinicopathological of HCC (independent variables)

	Model	Unstandardized Coefficients		Standardized coefficients β	t	P value	95% C.I.	
		β	Std. error				Lower bound	Upper bound
1	(Constant)	3.275	3.903	–	0.839	0.406	–4.569	11.119
	Child–Pugh grade	4.952	1.626	0.399	3.046	0.004*	1.685	8.219
2	(Constant)	9.882	4.758	–	2.077	0.043*	0.315	19.449
	Child–Pugh grade	5.802	1.607	0.467	3.611	0.001*	2.571	9.032
	Distant metastasis	–5.584	2.475	–0.292	–2.256	0.029*	–10.560	–0.607

95% C.I. 95% confidence interval

*significant P value ($P < 0.05$)**Table 6** Accuracy of serum miRNA-27a and miRNA-18b expressions levels for predicting liver cirrhosis by logistic regression analysis

	Unstandardized coefficients		Odds ratio	95% C.I.		t	P value
	β	SE		Lower bound	Upper bound		
Constant	–10.912	1.863	0.000	–	–	34.310	<0.001*
MiRNA-27a	3.818	0.696	45.517	11.638	178.01	30.109	<0.001*
MiRNA-18-b	1.247	0.680	3.480	0.919	13.18	3.367	0.067
Alpha fetoprotein	0.067	0.053	1.070	1.070	0.964	1.593	0.207

Table 7 Accuracy of serum miRNA-27a and miRNA-18b expression levels as predictors HCC by logistic regression analysis

	Unstandardized coefficients		Odds ratio	95% C.I.		t	P value
	β	SE		Lower bound	Upper bound		
Constant	–2.932	1.379	0.053	–	–	4.524	<0.001*
MiRNA-27a	1.102	0.520	3.010	1.087	8.334	4.499	0.034*
MiRNA-18-b	–0.302	0.503	0.739	0.276	1.983	0.360	0.549
Alpha fetoprotein	–0.021	0.033	0.979	0.917	1.045	0.404	0.525

*Significant P value ($P < 0.05$)

Accuracy of serum miRNA-27a and miRNA-18b for predicting HCC among cirrhosis patients

The cutoff values of miRNA-27a and miRNA-18b expression levels were 2.38 and 0.62, respectively, and the AUC were 0.897 (95% CI 0.839–0.956), $P < 0.001$, and 0.723 (95% CI 0.643–0.802), $P < 0.001$, respectively. Additionally, the sensitivities and the specificities of miRNA-27a were 96.7 and 71.7%, respectively, and of miRNA-18b were 75.6 and 46.7%, respectively. Notably, the diagnostic power of miRNA-18b increased in discriminating HCC among cirrhosis patients. Thus, these miRNAs could be useful diagnostic biomarkers discriminating HCC cases among cirrhosis patients (Fig. 4a, b).

Combination of serum miRNA-27a and miRNA-18b for predicting HCC among cirrhosis patients

ROC analysis revealed that combined miRNA-27a and miRNA-18b expression levels were useful discriminating biomarkers of HCC cases from cirrhosis patients (AUC=0.821, 95% CI 0.748–0.895, $P < 0.001$) with sensitivity 91.1% and specificity 71.7 (Fig. 4c).

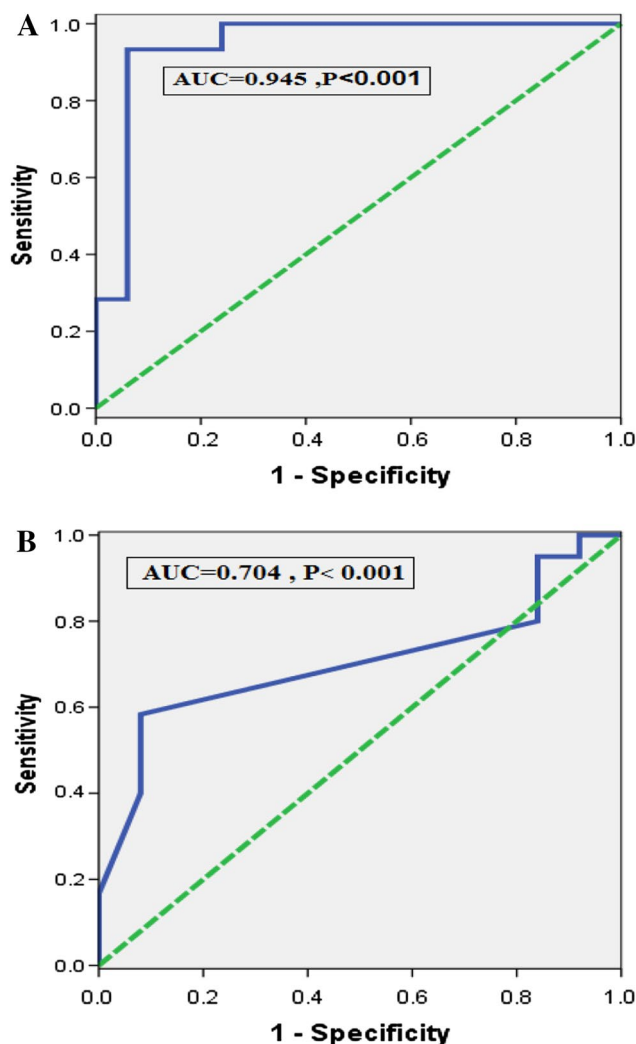


Fig. 2 **a** Receiver operating characteristic (ROC) curve for serum miRNA-27a expression in discriminating between CHC patients and controls. **b** Receiver operating characteristic (ROC) curve for miRNA-18b in differentiating between CHC patients and controls

Discussion

HCC is a multifaceted and mixed tumor with several genomic alterations [6]. Despite significant progress in diagnosis and treatment options, the incidence of HCC is still increasing. MiRNAs could be used as diagnostic biomarkers for liver injury including HCV, CHC, and HCC [23]. These miRNAs expressed only by the host to modulate the HCV infection as no evidence of viral encoded miRNAs [24].

Early diagnosis of HCC allows curative treatment such as resection or liver transplantation, or local ablative therapies can be applied with intent to cure. To the best of our knowledge, very few reports investigating the potential value of miRNA-27a and miRNA-18b as diagnostic biomarkers of CHC and its complications. To address this

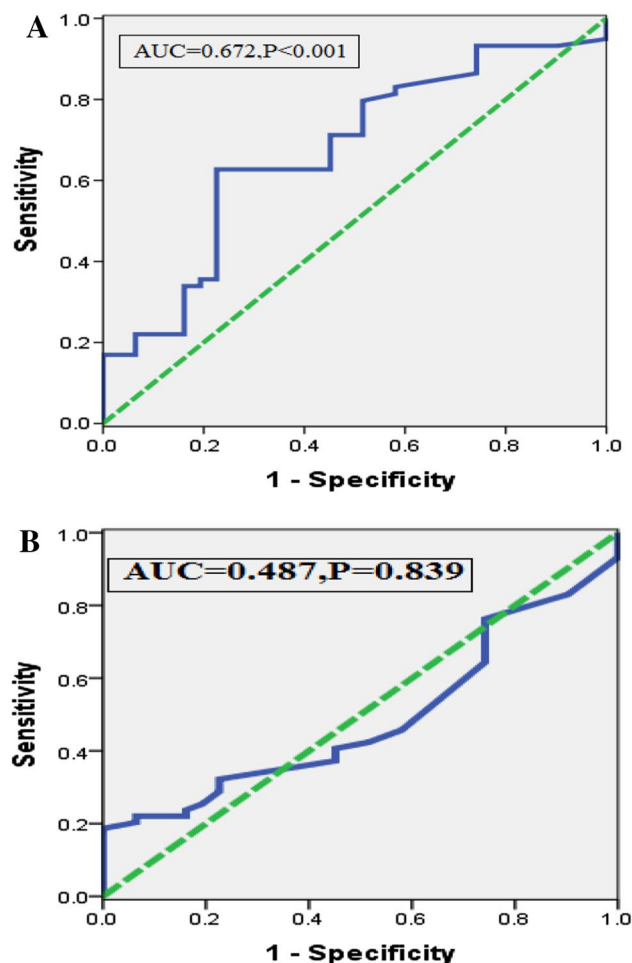


Fig. 3 **a** Receiver operating characteristic (ROC) curve for miRNA-27a in differentiating between CHC and post-CHC cirrhosis. **b** Receiver operating characteristic (ROC) curve for miRNA-18b in differentiating between CHC and post-CHC cirrhosis

need, we chose miRNA-27a and miRNA-18b among a total numbers of 277 microRNAs expressed in the liver, and they are implicated in HCV-infection [22].

The results presented herein are innovative, as this study performs a robust evaluation of miRNA-27a and miRNA-18b as diagnostic biomarkers of CHC, and their potential role in prediction of cirrhosis and HCC in patients with CHC. Noteworthy, our results revealed that the expression levels of miRNA-27a and miRNA-18b were significantly increased in post-hepatitis C cirrhosis. Moreover, in HCC group, there was significant increase of miRNA-27a expression levels compared to cirrhosis group. Furthermore, both miRNAs using ROC analyses could be useful diagnostic biomarkers detecting HCC among CHC patients with cirrhosis ($P < 0.001$ for each).

In the same line with our results, El-Guendy et al.; Shirasaki et al.; and Murakami et al. found that circulating

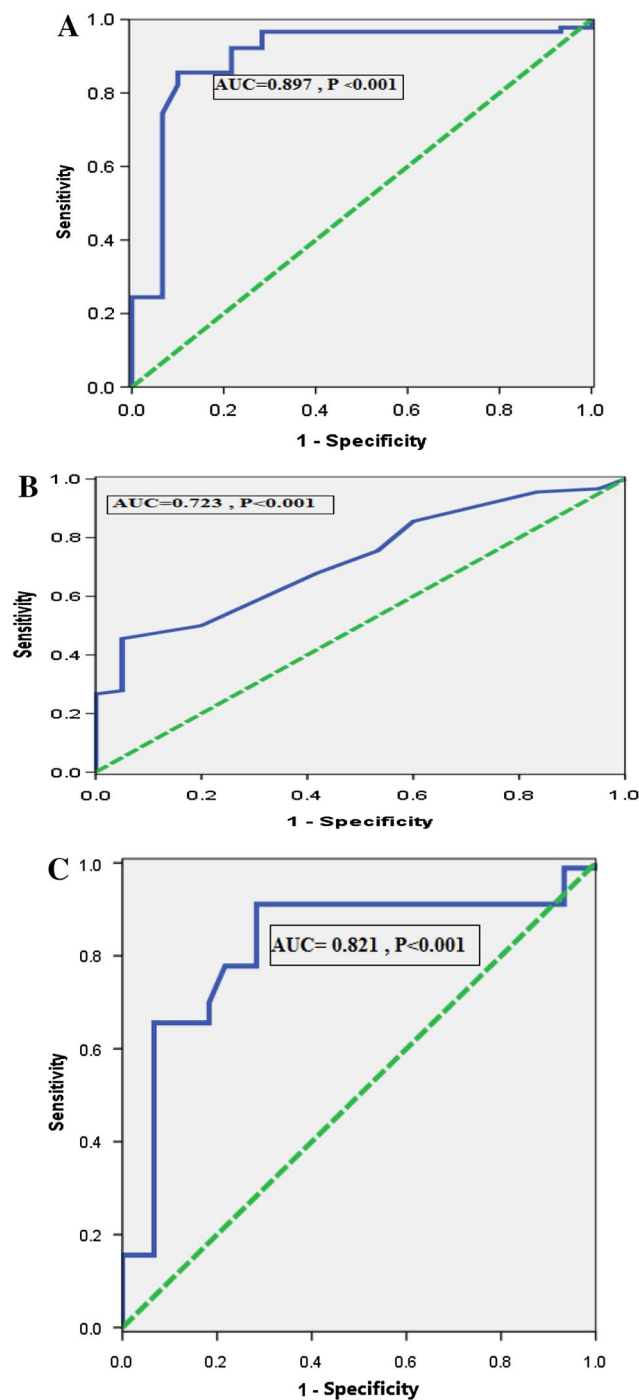


Fig. 4 **a** Receiver operating characteristic (ROC) curve for miRNA-27a in differentiating between post-CHC cirrhosis and HCC. **b** Receiver operating characteristic (ROC) curve for miRNA-18b in differentiating between post-CHC cirrhosis and HCC. **c** Receiver operating characteristic (ROC) curve for combined serum miRNA-18b and miRNA-27 expression levels in differentiating between post-CHC cirrhosis and HCC

miRNA-27a expression levels in CHC patients were higher than those in healthy controls [25–27].

Similar to our results, Wu et al. and Huang et al. found that miRNA-27a was significantly up-regulated in HCC tissues and cell lines. Moreover, they suggested that miRNA-27a plays important roles in mediating cancer cell proliferation, cell cycle, apoptosis, migration, and drug resistance [28, 29].

Tian et al. found that there was significant up-regulation of miRNA-27a in the tissues of laryngeal tumor as compared to the neighboring non-tumor tissues suggesting its role as an oncogene in the laryngeal squamous cell carcinoma through down-regulation of Polo-like kinase (PLK) (PLK2) [30]. In the same context, similar reports demonstrated that miRNA-27a was significantly increased in renal cell carcinoma [31], cancer cervix [32], gastric adenocarcinoma [33], and breast cancer [34]. Several reports showed also that miRNA-27a exhibited oncogenic activity through direct suppression of ZBTB10/RINZF expression, which leads to up-regulation of transcriptional factor specificity protein (Sp), vascular endothelial growth factor (VEGF), and VEGF receptor 1 (VEGFR1) [35].

In contrast to our results, the study of Zhou et al. suggested that 4 down-regulated miRNAs (miRNA26a, miRNA27a, miRNA122, and miRNA223) may be used to distinguish HCC patients from healthy subjects, chronic hepatitis B, and cirrhosis patients [36]. On the contrary to our findings also, Bao et al. found that miRNA-27a was significantly decreased in colorectal cancer tissues and cell lines. However, they proposed that reduced miRNA-27a expression was associated with distant metastasis and lower clinical pathological stages at III/IV [37]. In another side, miRNA-27a expression was down-regulated in esophageal cancers [38], oral squamous cell carcinoma [39], acute leukemia [40], which sheds light on possible tumor suppressor roles. Putting these data together necessitates further future researches clarifying the role of this miRNA in cancer and its gene's targets.

In the current research, miRNA-18b expression levels were significantly increased in post-hepatitis C cirrhosis. However, the miRNA-18b expression levels were non-significant increase in HCC group. In contrast to our findings, El-Guendy et al. showed decreased miRNA-18b expression in CHC patients than in healthy controls [25]. It is an interesting note that miRNA-18b can promote cell proliferation and invasion and contribute to evasion of the host immune system [25]. Zhang et al. observed lower expression of miRNA-18b in chronic HBV infection [17].

Similarly, Murakami et al. showed that miRNA-18b expression levels were significantly increased in HCC group [41]. Furthermore, Fonseca-Sánchez demonstrated that miRNA-18b was up-regulated in breast cancer cell lines and in a set of clinical specimens. They showed that

knocking-down of miRNA-18b induces up-regulation of 55 olfactory receptor (OR) genes and nine genes (NLRP7, KLK3, OLFM3, POSTN, MAGED4B, KIR3DL3, CRX, SEMG1, and CEACAM5) with key roles in cell migration and metastasis [42]. Several reports also showed a higher expression of miRNA-18b in gastric cancer [18], cancer ovary [19], basal cell carcinoma of the skin [20], and cancer colonic [21].

On the other hand, Dar et al. showed that the overexpression of miRNA-18b has a potent suppressing tumor activity which evidenced by suppressed melanoma cell viability, induction of apoptosis, and reduced tumor growth in vivo [43]. Additionally, they proposed that overexpression of miRNA-18b leads to down-regulation of proto-oncogene MDM2 gene which play an important role in cancer progression [44].

Interestingly, the main finding of the present study that miRNA-27a expression levels were positively correlated with distant metastasis, creatinine, Child–Pugh grade, lymph node metastasis, and tumor size in HCC group. By further analysis of results, stepwise multiple linear regression analysis revealed that Child–Pugh grade and distant metastasis were the main predictors of miRNA-27a expression levels among in HCC patients. Wu et al. found that overexpression of miRNA-27a accelerate proliferation, migration, and invasion of both HepG2 and Huh7 cell lines, indicating the oncogenic role of miRNA-27a in HCC cells lines [28]. In agreement with our reports, Shen et al., found several miRNAs including and miRNA-27a-3p were related to carcinogenicity and played vital roles in HCC screening [44].

In our research, the results revealed that miRNA-18b, the expression levels were positively correlated with distant metastasis, lymph node metastasis, Child–Pugh grade, as well as other markers of severity of liver cirrhosis. These results agree with those reported by Shen et al., who found that among up-regulated microRNAs in HCC tissues and cell lines miR-18b-5p was the most up-regulated and significantly correlated with invasion and metastasis [44].

Concordance with our finding, Murakami et al. evaluated the expression of 12 miRNAs in HCC and they observed that miR-18b expression in poorly differentiated HCC was significantly higher than in well-differentiated HCC. They explained that over-expression of miR-18b or down-regulation of TNRC6B accelerated cell proliferation and loss of cell adhesion ability. Likewise, they observed that after surgical resection, HCC patients with high miR-18b expression had a significantly shorter relapse-free period than those with low expression [41].

In the current study, the results of ROC revealed that miRNA-27a and miRNA-18b expression levels were useful biomarkers discriminating cirrhosis from CHC (AUC were 0.672 and 0.487, respectively). as well as the diagnostic power of ROC in differentiating HCC from post-hepatitis

C cirrhosis (AUC were 0.897 and 0.723, respectively). The power of combined miRNA-27a and miRNA-18b expression levels in discriminating between HCC from post-hepatitis C cirrhosis was high (AUC = 0.821). Thus, the diagnostic power of miRNA-27a in differentiating HCC from post-hepatitis C cirrhosis, moreover combination of both miRNA-27a and miRNA-18b expression levels, improved their diagnostic utility.

Interestingly, divergent roles of miRNA-18b in carcinogenesis have been reported which could complicate therapeutic approaches. For example, lower miRNA-18b has been shown to predict melanoma progression and short survival [43]. This dual role of miRNA-18b in cancer is not surprising, considering that different functions have been previously attributed to individual miRNAs in different cellular settings [45]. Indeed, high expression of miR-18b is characteristic of human embryonic stem cells [46].

A potential limitation in current work, the sample size is relatively small. Future multicenter reports including large number of patients and clarifying target genes for each miRNA and also, taking in consideration the different treatment protocols available for CHC, post-CHC cirrhosis, and HCC.

In conclusion, the expression levels of both miRNA-27a and miRNA-18b were significantly increased in patients with post-CHC cirrhosis compared to CHC and controls groups. While in HCC group, only miRNA-27a expression levels were significantly overexpressed.

Compliance with ethical standards

Conflict of interest All authors declared that they do not have any conflict of interest.

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