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Plasma expression levels of microRNA-21 might help in the detection of HCV patients complicated by hepatocellular carcinoma

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HIGHLIGHTS

- Distinguishing complicated from non-complicated HCV is still a matter of debate.
- Differentiation of complicated cases depending on clinical scores may be misleading.
- Deregulated plasma expression levels of microRNA help in diagnosis of multiple diseases.
- Deregulated expression levels of microRNA 21 and 126 might be helpful for defining cases with complicated HCV.

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ABSTRACT – Objective – To investigate the ability of the estimated plasma gene-expression levels of microRNA (miR)-21 and 126 to define patients suspected to have hepatocellular carcinoma (HCC) among patients with complicated hepatitis-C virus (HCV) infection. **Methods** – Patients with uncomplicated (U-HCV) or complicated HCV underwent clinical and ultrasonographic (US) evaluations and assessment for the computerized hepatorenal index, hepatic steatosis index and fibrosis indices. Blood samples were obtained for estimation of serum levels of alpha-fetoprotein (AFP) and tumor necrosis factor- α (TNF- α), and plasma expression levels of miR-21 and miR-126 using the quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). **Results** – Serum levels of AFP and TNF- α were significantly higher in samples of HCV-HCC patients than controls and other HCV patients. Plasma levels of miR-21 were the highest, while miR-126 levels were the lowest in samples of HCV-HCC patients with significant differences in comparison to samples of controls and other HCV patients. The ROC curve analysis defined high plasma miR-21 levels as specific predictor for HCV infection, and could identify samples of complicated HCV, and samples of HCV-HCC patients, while estimated plasma levels of miR-126 could be applied to screen for HCV and its related complications. **Conclusion** – Deregulated plasma expression levels of miR-21 and miR-126 might distinguish cases of HCV complicated by HCC and define cases of HCV-LC, even those that showed low Fib-4 scores.

Keywords – Hepatitis-C virus; hepatocellular carcinoma; liver cirrhosis; hepatosteatosis; MicroRNAs; tumor necrosis factor- α ; alpha-fetoprotein.

INTRODUCTION

Hepatitis C virus (HCV) infection is a health threat that approximately 242 000 people died from hepatitis C, mostly from cirrhosis and hepatocellular carcinoma (primary liver cancer) according to the reports of WHO estimated in 2022⁽¹⁾. Egypt has the highest worldwide prevalence of HCV infection⁽²⁾. Chronic viral hepatitis is the main cause of liver cirrhosis (LC) and progression to liver decompensation and hepatocellular carcinoma (HCC); the most prevalent cancer in Egypt⁽³⁾.

Disrupted signaling pathways that affect a broad spectrum of cellular activities, such as proliferation, differentiation, invasiveness, and apoptosis are the characteristics of tumor microenvironment⁽⁴⁾. HCV can alter the host immune response through its evading capacity for the immune surveillance and induction of variations in immune-related genes leading to differential susceptibility to HCV infection as well as the development of hepatic fibrosis, LC, and HCC⁽⁵⁾.

Deregulated expression levels of microRNAs (miR) may underlie the development of multiple liver pathologies⁽⁶⁾ and are involved in inflammatory and fibrotic processes of chronic liver disease⁽⁷⁾. The miR-576-3p/MPP8 axis regulates the proliferation, migration, and invasion of liver cancer cells through the PI3K/Akt signaling pathway⁽⁸⁾. miR-155-5p can differentiate among the HCV patients' groups, while miR-574-3p and IL-1 β can discriminate between recurrent HCV and control groups⁽⁹⁾. miR may be used as biomarkers to diagnose the progression of liver fibrosis secondary to HCV infection disease⁽¹⁰⁾.

Objectives

The study aims to determine the discriminative ability of the estimated plasma gene-expression levels of miR-21 and 126 for HCV patients with suspected HCC among patients with complicated HCV.

Design

Prospective observational case-control comparative study.

Setting

Departments of Internal Medicine and Medical Biochemistry, Faculty of Medicine, Benha University.

Ethical considerations

The preliminary approval of the study protocol was obtained from by the Local Ethical Committee at Benha Faculty of Medicine by date 1-7-2021 and registered by ClinicalTrials.gov Identifier: NCT05449847. The study protocol was discussed with patients, and those who fulfilled the inclusion criteria and accepted to participate in the study, signed a written consent. After completion of case collection, the final outcomes of the study was approved by the number RC: 8-9-2024.

Blindness

The Internal Medicine Physicians were blinded about the results of the laboratory investigations, and the authors who were responsible for conducting lab investigations were blinded about the clinical diagnosis. At the end of the study period, both clinical and laboratory data were confronted, interpreted, and analyzed.

Patients

Patients who were previously diagnosed to have HCV disease and attend the Internal Medicine outpatient clinic from June 2022 to March 2023 were evaluated clinically and by abdominal ultrasonography for exclusion and inclusion criteria.

Exclusion criteria

The presence of pre-portal fibrosis secondary to previous bilharzial disease, alcoholic fatty liver disease, presence of hepatic malignancy other than HCC, decompensated cirrhosis, hepatorenal failure, or hepatic manifestations of congestive heart failure. Also, patients who were maintained on medical treatment were excluded.

Inclusion criteria

Patients had HCV disease with compensated liver function and were free of exclusion criteria were enrolled in the study.

Grouping

Patients had HCV were divided into two broad groups: uncomplicated (U-HCV) or complicated HCV. Patients with complicated HCV were categorized according to US findings into HCV with hepatosteatosis (HCV-HS), HCV liver cirrhosis (HCV-LC),

or HCC on top of HCV (HCV-HCC). For comparative purposes, 16 volunteers free of HCV and exclusion criteria and accepted to give blood samples as control samples were considered as Control group.

A) Evaluation tools

1. The abdominal US using an ACUSON S3000 Ultrasound System (Siemens) with a 4V1 (1–4.5 MHz), 6C2 (2–6 MHz), and 18L6 (5.5–18 MHz), and/or 9L4 (4–9 MHz) transabdominal transducer. Images were interpreted for the presence of:
 - Cirrhotic nodules appear as uneven, undulating liver surface and/or diffuse alteration in hepatic parenchymal echotexture with small nodular areas⁽¹¹⁾.
 - Findings suggestive of the presence of portal hypertension as the presence of ascites or varices; patients with portal hypertension were excluded from the study⁽¹¹⁾.
 - Liver Steatosis was graded using the computerized hepatorenal index (HRI) with a cutoff point of 1.49 for the prediction of >5% steatosis, 1.86 for the prediction of >25% steatosis, and 2.23 for the prediction of >60% steatosis⁽¹²⁾.
 - Foci of HCC appear as small foci of <30 mm appearing as hypoechoic lesions in the gray-scale US, but echogenicity increases on fatty tissue inclusion. Heterogeneous lesions indicate HCC with degenerative changes. Hyper-echoic focus within a larger hypoechoic mass suggests the development of HCC within a dysplastic nodule⁽¹¹⁾. According to previous studies, the US showed up to 96% sensitivity⁽¹³⁾ and >90% specificity for the detection of HCC foci⁽¹⁴⁾.

2. Clinical scorings

- The hepatic steatosis index (HSI) was calculated according to the equation: $HIS = ([ALT/AST \text{ ratio}]^{*8}) + BMI + 2$ (if the patient was diabetic) + 2 (in the case of the female patient), and the $HIS \geq 36$ is suggestive of non-alcoholic steatohepatitis (NASH)⁽¹⁴⁾.
- Fibrosis indices: Liver fibrosis was noninvasively graded using the APRI and FIB-4 scores.
 - The APRI index evaluated the effects of liver

fibrosis on serum aspartate transaminase (AST) level and platelet count and was calculated as the AST to platelet count ratio index (APRI), which was found to predict significant fibrosis and cirrhosis within the area under the ROC curve of 0.80 and 0.89, respectively⁽¹⁵⁾.

- The Fibrosis-4 (FIB-4) scores were calculated using the following formula: $\{age \text{ (yr)} \times AST \text{ [U/L]} / \{((PLT \text{ [10}^9\text{/L)}) \times (ALT \text{ [U/L]}) \times (1/2)\}^{(16)}$.
- Interpretation of fibrosis indices: APRI <0.5 &/or FIB-4 <1.5 indicates the absence of fibrosis, APRI =0.5–1.5 &/or FIB-4=1.5–3.25 indicates moderate fibrosis and APRI >1.5 &/or FIB-4 >3.25 indicates significant fibrosis⁽¹⁷⁾.

B) Laboratory investigations

1. Blood samples were collected under complete aseptic conditions from the antecubital vein and were divided into two parts:
 - The 1st part was collected in two EDTA tubes one for immediate quantification of complete blood count and the other was freeze at -20°C for quantification of the expression levels of micro-RNA 21 and 126 using the qRT-PCR technology.
 - The 2nd part was centrifuged at 1000 rpm for 10 min and serum was separated and divided into two parts: one for estimation of liver enzyme levels, total bilirubin, and albumin levels at the hospital lab. Another part was put in an Eppendorf tube and freeze at -20°C till ELISA estimation of AFP⁽¹⁸⁾ and TNF- α ⁽¹⁹⁾ using Abcam ELISA kits (catalogue no. ab193765 & ab179886, Abcam Inc., San Francisco, USA) according to the manufacturer instructions and results were read using a 96 well microplate ELISA reader (Dynatech, MR 7000).

2. The qRT-PCR for plasma levels of miR-21 and miR-126.

Total RNA including microRNA was extracted from blood samples using the miRNeasy Mini Kit (QIAGEN, Germany). The procedure briefly was described as reverse transcription of complementary DNA (cDNA)

from total RNA samples using miScript II RT Kit (Qiagen, Hilden, Germany) to be used as a template for real-time PCR analysis using SuperReal PreMix Plus (SYBR Green; Tiangen, Shanghai). The thermocycling conditions included an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. GAPDH served as an internal reference. The relative number of copies (RQ) of miR was determined using the $\Delta\Delta CT$ method and the levels of miR expression were calculated using the cycle threshold (CT) as the difference between the CT value of the target miR and the average CT value of the reference genes, per sample. The relative expression (fold change) for each miR of each group was then calculated as follows: in each sample, the ΔCT for each miR sample equals the CT for the miR minus that of GAPDH. Then the $\Delta\Delta CT$ was calculated as ΔCT for patients' minus ΔCT for controls⁽²⁰⁾. The primers' sequences are presented in TABLE 1.

TABLE 1. List of the primers used for qRT-PCR.

Name	Sequence (5'-3')
miR-126F	ACACTCCAGCTGGGCATTATTACTTTTGGT
miR-126R	TGTCGTGGAGTCGGCAATTC
miR-21F	TGAGACTGATGTTGACTGTTGAA'
miR-21R	TGTCAGACAGCCCATCGAC
GAPDH F	CCACCCATGGCAAATTCATGGCA
GAPDH R	TCTAGACGGCAGGTCAGGTCCAC

C) Study outcome

The ability of the quantitated plasma levels of miR-21 and/or miR-126 to distinguish cases of HCV-HCC from other HCV complicated in comparison to other diagnostic lab parameters and clinical scorings is the target outcome.

D) Statistical analyses

The intergroup variance of the obtained results was conveyed using the One-way ANOVA test and chi-square test for nonparametric results. The relation between the studied variates was assessed by the Pearson's correlation analysis and the predictability for the defined target was evaluated using the receiver operating characteristic (ROC) curve as judged by the area under the curve (AUC) and its relation to the area under the reference line (0.5). The significance of the comparisons was expressed as the *P* value at the cutoff point of 0.5. Statistical analyses were conducted using the software of IBM SPSS Program (IBM, USA, Ver. 22).

RESULTS

Clinical evaluation of 129 chronic HCV patients excluded nine patients; five had decompensated cirrhosis, three patients had hepatorenal failure and one patient had alcoholic fatty liver. Among the enrolled 120 patients, 27 patients had uncomplicated HCV (U-HCV) and 93 patients had complicated HCV as shown in FIGURE 1.

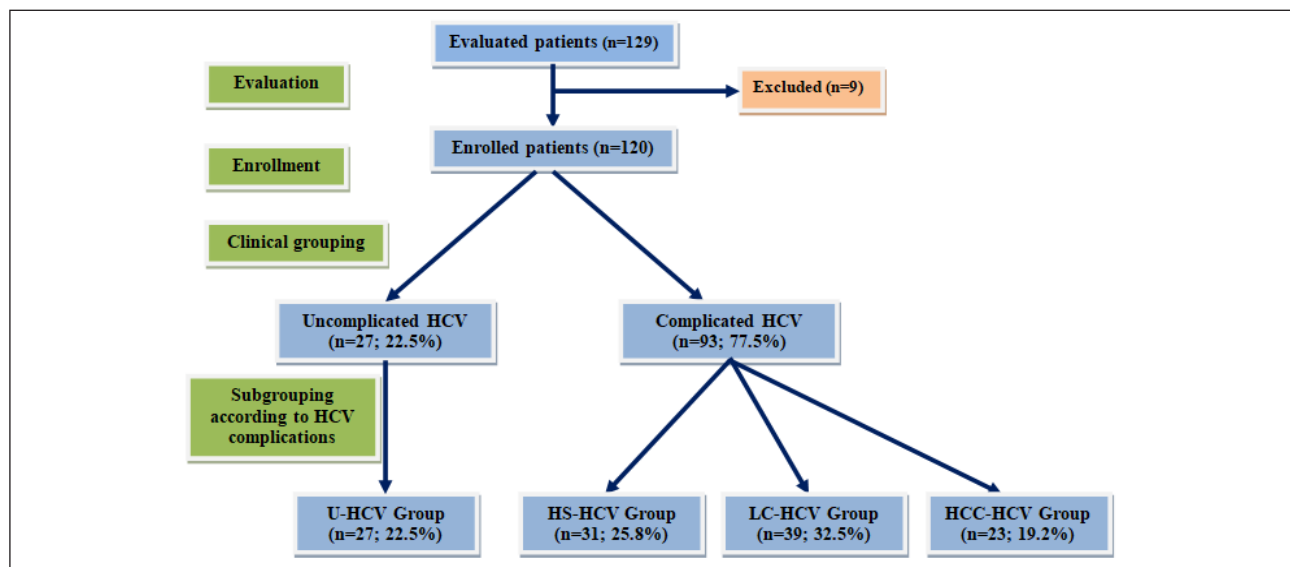


FIGURE 1. Study flow chat.

Patients who had HCV-LC and HCV-HCC were significantly older than patients who had HCV-HS. Patients of the HCV-HS group had significantly higher body mass index than other HCV patients. Moreover, patients of the HCV-HS and HCV-LC groups showed significantly higher fasting blood glucose than patients of U-HCV with a significant difference between HCV-LC and HCV-HCC patients. The calculated AST/ALT ratio in the serum of HCV-LC patients was significantly higher than the ratio calculated in the serum of HCV-HS and HCV-HCC patients (TABLE 2).

The mean HSI value was significantly higher in HCV-LC and HCV-HCC than in HCV-HS and U-HCV patients and in HCV-HS than in U-HCV patients. The mean APRI index and FIB-4 score were significantly higher in HCV-LC patients than in other patients and in HCV-HCC than HCV-HS patients. In comparison to U-HCV patients, HCV-HS patients had significantly lower APRI index and non-significantly lower FIB-4 score, while HCV-HCC patients had non-significantly higher APRI index (TABLE 3).

Only 23 patients had moderate hepatic fibrosis

TABLE 2. Patients' enrolment and routine lab data.

		U-HCV	Complicated HCV			
			HCV-HS	HCV-LC	HCV-HCC	
Number (%)		27 (22.5%)	31 (25.8%)	39 (32.5%)	23 (19.2%)	
Age (years)		38 (5.8)	48 (9.6)*	54 (6)*†	55.5 (6.8)*†	
Gender	Males	16 (59.3%)	13 (41.9%)	22 (56.4%)	13 (56.5%)	
	Females	11 (40.7%)	18 (58.1%)	17 (43.6%)	10 (43.5%)	
Average		0	0	3 (7.7%)	2 (8.7%)	
Body mass index (kg/m²)	Overweight	10 (37%)	5 (16.1%)	21 (53.8%)	12 (52.2%)	
	Obese grade-I	17 (63%)	22 (71%)	13 (33.3%)	7 (30.4%)	
	Obese grade-II	0	4 (12.9%)	2 (5.2%)	2 (8.7%)	
	Mean (SD)	29.87 (1.8)†	32.12 (2.3)	29.2 (3)†	29.7 (3.3)†	
Lab data	FBG (mg/dl)	Mean (SD)	115 (12)	130 (15.7)*	138 (19.5)*	120 (16.9)‡
		DM	5 (18.5%)	11 (35.5%)	12 (30.8%)	6 (26.1%)
		Hemoglobin conc. (g %)	12.85 (1.7)	13 (2.1)	13 (1.4)	12.7 (1.6)
		Total leucocyte count (10³/cc)	6.3 (1.5)	7 (1.6)	6.1 (1.7)	7.3 (1.7)
		Platelet count (10³/cc)	176 (48.5)	192 (39.4)	179 (23.5)	173 (19.7)
		Total bilirubin (mg/ml)	1.22 (0.31)	1.13 (0.23)	1.25 (0.24)	1.2 (0.12)
		Serum albumin (g/ml)	3.9 (0.44)	4.2 (0.23)	4.1 (0.46)	4.3 (0.37)
		Serum AST (U/L)	65 (36.1)	60 (17.1)	74 (12.4)	68 (10.4)
		Serum ALT (U/L)	78 (33.4)	65 (35.7)	59 (32.2)	67 (29.6)
		AST/ALT ratio	0.82 (0.36)	1.09 (0.47)	1.41 (0.37)*†	1.19 (0.47)*

FBG: fasting blood glucose; AST: aspartate transaminase; ALT: alanine transaminase; *significant difference versus U-HCV; †significant difference versus HCV-HS; ‡significant difference versus HCV-LC, significance was at $P < 0.05$.

TABLE 3. Mean values and patients' distribution according to the finding on US imaging of liver.

Group Test		U-HCV	Complicated HCV		
			HCV-HS	HCV-LC	HCV-HCC
Mean (SD) of US detected hepatosteatosis index		36 (1.52)	38.3 (2.24)*	42.4 (3.36)*†	43 (2.97)*†
Mean (SD) of US detected fibrosis indices	APRI index	1.144 (0.77)	0.347 (0.1)*	1.62 (0.36)*†	1.284 (0.34)†‡
	Fibrosis-4 scores	1.68 (0.77)	1.12 (0.43)	5.1 (0.98)*†	4.39 (1.07)*†‡
Fibrosis grade	No	18 (66.7%)	17 (54.8%)	0	0
	Moderate	9 (33.3%)	14 (45.2%)	6 (15.4%)	4 (17.4%)
	Severe	0	0	33 (84.6%)	19 (82.6%)
Hepatorenal index	<5%	12 (44.4%)	5 (16.1%)	7 (17.9%)	3 (13%)
	>5-25%	8 (29.6%)	15 (48.4%)	15 (38.5%)	5 (21.7%)
	>25-60	5 (18.5%)	8 (25.8%)	13 (33.3%)	15 (65.3%)
	>60	2 (7.5%)	3 (9.7%)	4 (10.3%)	0

US: ultrasonographic; U-HCV: uncomplicated; *significant difference versus U-HCV; †significant difference versus HCV-HS; ‡significant difference versus HCV-LC; significance was at $P < 0.05$.

among patients of U-HCV and HCV-HS groups with insignificant differences between both groups. On the contrary, 52 patients had severe fibrosis among patients of HCV-LC and HCV-HCC with insignificant differences between both groups. Patients' distribution according to fibrosis grades showed significant ($P<0.05$) differences between patients of HCV-LC and HCV-HCC groups in comparison to patients of U-HCV and HCV-HS groups. The incidence of high hepatorenal index was significantly higher among patients of HCV-HCC than among patients of groups U-HCV and HCV-HS but was insignificantly higher than among patients of HCV-LC. However, the incidence of high HRI showed an insignificant difference from the incidence among patients of other groups (TABLE 3).

Mean values of serum levels of AFP and TNF- α in samples of HCV-HCC patients were significantly higher than levels estimated in samples of controls and other HCV patients and in samples of HCV-LC patients than in control and U-HCV patients' samples. Moreover, mean values of serum AFP and TNF- α levels estimated in samples of U-HCV and HCV-HS patients were significantly higher than in control samples with significantly higher levels of TNF- α and insignificantly levels of AFP in samples of HCV-HS patients than in samples of U-HCV patients (TABLE 4).

Plasma levels of miR-21 were significantly higher in samples of U-HCV and HCV-HS patients than in control samples with insignificant differences between U-HCV and HCV-HS patients, while plasma levels of miR-126 showed insignificant differences between samples of controls, U-HCV and HCV-HS patients. Regarding samples of HCV-LC patients, estimated levels of miR-21 were significantly higher than in samples of controls and U-HCV patients and were insignificantly higher than levels estimated in samples of HCV-HS patients, while plasma levels of miR-126 in HCV-LC patients were significantly lower than levels estimated in control and HCV-HS patients. Plasma levels of miR-21 were the highest, while plasma levels of miR-126 were the lowest in samples of HCV-HCC patients with significant differences in comparison to levels estimated in samples of controls and other HCV patients (TABLE 4).

Estimated serum levels of TNF- α and plasma expression levels of miR-21 were significantly correlated with HSI ($r=0.532$ & 0.400 , respectively; $P<0.001$) and FIB-4 scores ($r=0.474$ & 0.446 , respectively; $P<0.001$) and with the estimated serum levels of AFP ($r=0.457$ & 0.548 , respectively; $P<0.001$). Further, estimated serum levels of TNF- α showed a positive significant correlation with plasma expression levels of miR-21 (FIGURE 2A), while the relation

TABLE 4. Mean serum AFP and TNF- α and plasma expression levels of miR-21 and miR-126 estimated in control and HCV patients.

Group Biomarker	Control	U-HCV	Complicated HCV		
			HCV-HS	HCV-LC	HCV-HCC
AFP (ng/mL)	19 (4.2)	119 (24.8)*	125 (18.3)*	237 (68.4)*†‡	471 (215.8)*†‡€
TNF- α (ng/mL)	13 (3.2)	27 (6.5)*	46 (9)*†	52 (10.6)*†	61 (14.8)*†‡€
miR-21 (FC)	4765 (1127)	6427 (904)*	6896 (955)*	7409 (843)*†	9380 (835)*†‡€
miR-126 (FC)	4492 (984)	3920 (996)	4235 (1129)	3409 (718)*†	3017 (811)*†‡

TNF- α : tumor necrosis factor- α ; AFP: alpha-fetoprotein; miR-MicroRNA; FC: fold change; HCV: hepatitis-C virus; U-HCV: uncomplicated; *indicates significant difference versus control samples; †versus samples of U-HCV patients; ‡versus samples of HCV-HS patients; €versus samples of HCV-LC patients.

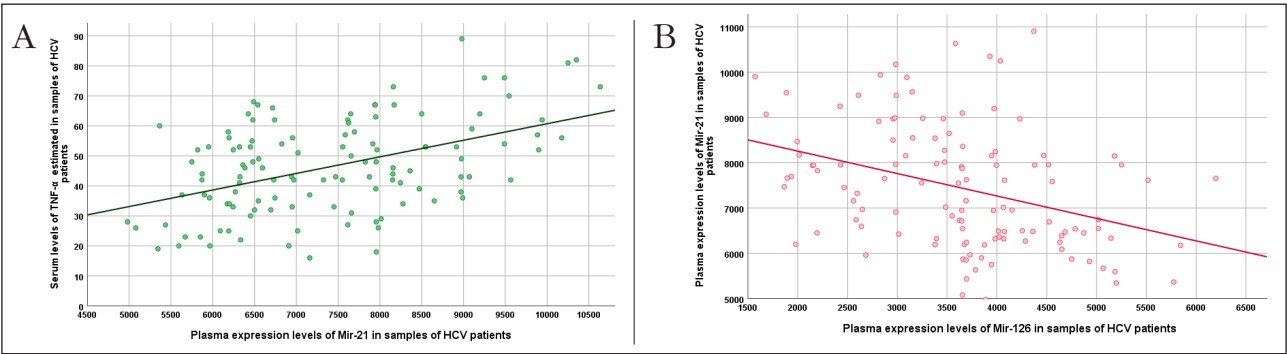


Figure 2. A) Shows the relation between levels of serum TNF- α and plasma expression of miR-21. B) Shows the relation between plasma expression levels of miR-21 and miR-126.

with the plasma expression levels of miR-126 was negative and insignificant. The relation between the expression levels of miR-21 and miR-126 was negative and significant ($r=-0.374$, $P<0.001$) as shown in FIGURE 2B.

The ROC curve analysis for estimated levels of miR-21 and 126 and serum levels of TNF- α to distinguish between samples of HCV patients and control samples defined high plasma miR-21 as a specific predictor for HCV infection than serum TNF- α with significant AUC difference in favor of miR-21 (FIGURE 3A). Moreover, plasma miR-21 levels could identify samples of complicated HCV with significantly

($P=0.007$) higher AUC than that of TNF- α (FIGURE 3B). Also, plasma miR-21 levels could differentiate between samples of HCV-HCC patients out of samples of patients had HCV complicated by HS or LC (FIGURE 3C) than serum levels of TNF- α with significant AUC difference ($P=0.025$). Furthermore, the levels of miR-21 could differentiate between samples of HCV-LC patients and samples of HCV-HS patients (FIGURE 3D) with significant ($P=0.022$) difference between the AUCs of both parameters. On the contrary, estimated plasma levels of miR-126 could be applied as a screening test for HCV and its related complications (TABLE 5).

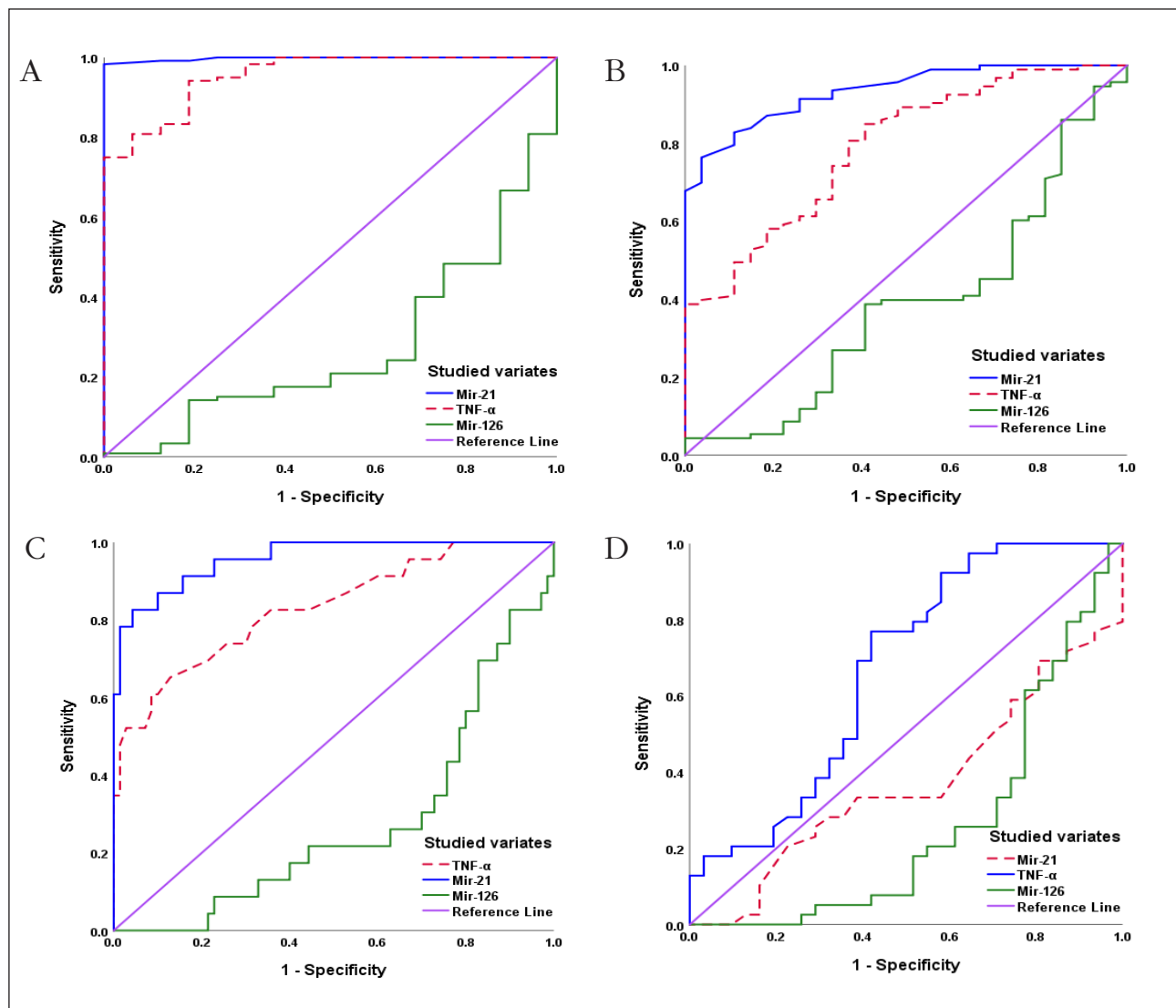


FIGURE 3. A) ROC curve analysis for the studied variates for differentiation between samples of HCV patients and control samples. **B)** ROC curve analysis for the studied variates for differentiation between samples of U-HCV from complicated HCV. **C)** ROC curve analysis for differentiation between samples of HCC-HCV and samples of HS-HCV and LC-HCV patients. **D)** ROC curve analysis for the studied variates for differentiation between samples of HCV-HS and HCV-LC.

TABLE 5. Statistical analysis of the estimated levels of serum TNF- α and plasma expression of miR-21 and miR-126 for differentiation between samples of HCV patients.

Differentiation between samples of	Analysis	Receiver operating characteristic (ROC) curve				Paired-sample analysis of AUCs-difference under the ROC curves		
	Variates	AUC	Std. E	P	95% CI	AUC diff	P	95%CI
HCV patients and controls	TNF- α	0.954	0.022	<0.001	0.911-0.997	0.043	0.048	0-0.087
	miR-21	0.998	0.002	<0.001	0.993-1.00			
	miR-126	0.271	0.062	0.003	0.149-0.393			
Complicated HCV and U-HCV patients	TNF- α	0.790	0.047	<0.001	0.698-0.882	0.144	0.007	0.039-0.248
	miR-21	0.934	0.022	<0.001	0.891-0.977			
	miR-126	0.386	0.063	0.027	0.263-0.506			
HCC-HCV and both HCV-HS and HCV-LC patients	TNF- α	0.832	0.052	<0.001	0.730-0.934	0.127	0.025	0.016-0.238
	miR-21	0.959	0.021	<0.001	0.918-1.00			
	miR-126	0.275	0.059	0.001	0.159-0.391			
HCV-HS and HCV-LC patients	TNF- α	0.371	0.066	0.064	0.240-0.501	0.298	0.002	0.105-0.490
	miR-21	0.668	0.068	0.016	0.535-0.801			
	miR-126	0.259	0.063	0.001	0.136-0.382			

HCV: hepatitis-C virus; U-HCV: uncomplicated.

DISCUSSION

The obtained results and the detected correlations spotlight on a vicious circle between the dysregulated levels of plasma microRNAs and serum proinflammatory cytokines, liver injury caused by HCV infection with deterioration of hepatic functions and the progression of HCV disease to the complicated levels up to the development of HCC. In line with this assumption, experimental studies detected severe inflammation and degeneration of hepatic tissues with downregulation of the levels of the suppressor of cytokine signaling-1 leading to the release of TNF α and interleukins through dysregulation of miR-33 and 155⁽²¹⁻²³⁾. Clinically, Neuman & Cohen⁽²⁴⁾ reported that changes in serum cytokines' levels in HCV patients help the clinician to monitor the severity of HCV-induced liver damage and Ferreira et al.⁽²⁵⁾ attributed the resolution of hepatitis C with improved liver damage, decreased liver fibrosis/inflammation and normalization of liver injury biomarkers after direct-acting antiviral (DAA) treatment to the suppression of the pro-inflammatory response. Moreover, De Sousa et al.⁽²⁶⁾ attributed this vicious circle to the experimentally detected upregulation of B-cell activating factor by the HCV-induced GU-enriched microRNAs through exosome transmission and Toll-like receptor seven activations; a mechanism which is implicated in HCV-infected hepatocyte-immune

system communication, microRNAs and development of HCV complications.

Concerning the reported progressive increases of the expression levels of miR-21 with increasing severity of HCV complications and its ability to differentiate between these patients support the study previously detected increased plasma levels of miR-21 by 1.74-fold in HCC patients than control levels and concluded that plasma miR-21 might be potential biomarker as an oncogenic microRNA in HCC subjects⁽²⁷⁾. Also, these data are in line with Khairy et al.⁽²⁸⁾ who reported significant upregulation of miR-21 in the HCV-related HCC patients compared to control samples and found these increases were significantly decreased with DAA therapy than in HCC patients who did not receive DAA therapy and concluded that miR-21 is a noninvasive biomarker for discrimination of HCC cases and can differentiate patients who received and responded to DAA therapy.

Statistical analyses defined high miR-21 levels as a significant predictor of the presence of complicated HCV especially HCC with high accuracy as shown by AUCs 0.934 and 0.959, respectively. Similarly, previously it was documented that the upregulated plasma miR-21 is more diagnostic for HCC⁽⁸⁾, can be used as a reliable biomarker for the detection of HCC with high diagnostic accuracy⁽²⁸⁾, and can be used as a biomarker to discriminate cases of HCC from chronic hepatitis C and HCV-LC⁽²⁹⁾.

In support of the differentiating ability of miR-21 levels for complicated HCV, Malik et al.⁽³⁰⁾ evaluated 26 microRNAs for differentiation between HCC and chronic liver diseases and found only miR-21, -320d and -423 could significantly distinguish between these cases with AUC of 0.87 and found combination of microRNAs with AFP did not perform better than any of these microRNAs solely. Thereafter, Rusu et al.⁽³¹⁾ explored the molecular mechanisms underlying HCC progression in nonalcoholic fatty liver disease (NAFLD) patients and detected dysregulated expression profile of miR panel of miR-21, 34a, 130a and 155 between NAFLD, adjacent non-tumoral liver tissue and HCC tissue specimens.

In a trial to explore the mechanisms for the relation between miR-21 and HCC, experimental studies found overexpression of miR-21 suppresses the fructose-1,6-bisphosphatase, so promotes HCC growth and metastasis⁽³²⁾ or significantly promotes *hepatic* stellate cells proliferation with reduction of cellular apoptosis and increased collagen deposition through activation of the ERK and TGF- β 1/Smads pathways⁽²⁹⁾. Moreover, upregulation of hepatic miR-21 was found to induce the full spectrum of the NAFLD up to HCC possibly via activation of the PI3K/AKT, induction of hepatic inflammation secondary to increased expression of inflammatory genes via STAT3 signaling pathways, and induction of liver fibrosis through activation of hepatic stellate cell and collagen deposition and finally oncogenic activation of Smad3/Stat3 signaling pathways induces HCC⁽³³⁾. Further, in a rat model of HCC, the extract of astragalus and salvia miltiorrhiza inhibited HCC progression via down-regulating miR-21 expression⁽³⁴⁾. Moreover, Ratnasari et al.⁽³⁵⁾ attributed the resistance to sorafenib therapy of advanced HCC, to the induction of nuclear translocation of miR-21, which activates the Akt pathway through small nucleolar RNA host gene-1 dependent and independent mechanisms.

On the other side, plasma miR-126 levels showed a progressive decrease with advanced complications of HCV and were significantly lower in all samples of HCV patients than in controls. Moreover, plasma miR-126 levels were found to significantly screen for patients of HCV especially those with complicated disease. These findings supported the previously reported significant reduction of the expression levels of

several microRNAs including miR-126 in samples of chronic HCV patients than control samples^(36,37) and go in hand with Ando et al.⁽³⁸⁾ who found the expression levels of microRNAs were associated with the stage and grade of liver disease. Moreover, the obtained data are coincident with the experimentally detected significantly decreased miR-126 expression levels in samples of HCC patients⁽³⁹⁾ and HCC cell lines and tissues relative to their corresponding healthy tissues⁽⁴⁰⁾.

The reported decreasing slope of miR-126 levels with increasing severity of HCV complications and being the least with HCC indicated a possible relation between miR-126 and liver tumorigenesis and pointed to the failure of the anti-tumorigenic action of miR-126 if there was any. In line with this assumption, Gong et al.⁽⁴¹⁾ using HCC cell lines reported that miR-126 might inhibit tumor angiogenesis in HCC by inhibiting EGFL7 via down-regulating the ERK signaling pathway, decreasing cell proliferation and inducing apoptosis through increased Fas/FasL and Caspase-3, and its overexpression in nude mice resulted in reduced tumor weight and less neo-angiogenesis. Also, Huang et al.⁽⁴²⁾ detected a negative relationship between miR-126 and TMPO antisense RNA-1, an oncogene, in HCC specimens and Zailaie et al.⁽⁴⁰⁾ found decreased miR-126 relieved the suppression of epidermal growth factor receptor expression, which contributes to HCC tumorigenesis.

CONCLUSION

The reported deregulated plasma expression levels of miR-21 and miR-126 might help to distinguish cases of HCV complicated by HCC from other HCV cases complicated by LC or HS. Further, the estimation of plasma levels of miR-21 and miR-126 could define cases of HCV-LC, even those that showed low Fib-4 scores.

Multicenter studies are required to establish the obtained results and to define cutoff points for both microRNAs solely and in combination to distinguish between cases of complicated HCV.

Authors' contribution

El-Hammady AM: Conceived the research idea, designed experiments, and analyzed data. Marei YM: Conducted experiments, collected data, and contri-

buted to manuscript writing. Mohammed RR: Provided critical feedback, supervised the project, and reviewed the manuscript. Rahman SMAE: Analyzed results, contributed to discussions, and revised the manuscript. Marei YM: Assisted with data interpretation and manuscript preparation. Sarhan RS: Contributed to the theoretical framework and provided technical expertise.

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El-Hammady AM, Marei YM, Mohammed RR, Rahman SMAE, Marei YM, Sarhan RS. Os níveis de expressão plasmática de microRNA-21 podem ajudar na detecção de pacientes com HCV complicados por carcinoma hepatocelular. *Arq Gastroenterol.* 2024;61:e24025.

RESUMO – Objetivo – Investigar a capacidade dos níveis estimados de expressão gênica plasmática de microRNA (miR)-21 e 126 para identificar pacientes suspeitos de ter carcinoma hepatocelular (CHC) entre pacientes com infecção complicada pelo vírus da hepatite C (HCV). **Métodos** – Pacientes com HCV não complicado (U-HCV) ou complicado foram submetidos a avaliações clínicas e ultrassonográficas (US) e avaliação do índice hepatorenal computadorizado, índice de esteatose hepática e índices de fibrose. Amostras de sangue foram obtidas para estimativa dos níveis séricos de alfa-fetoproteína (AFP) e fator de necrose tumoral- α (TNF- α), e níveis de expressão plasmática de miR-21 e miR-126 usando a reação em cadeia da polimerase quantitativa com transcrição reversa (qRT-PCR). **Resultados** – Os níveis séricos de AFP e TNF- α foram significativamente mais altos em amostras de pacientes com HCV-CHC do que nos controles e outros pacientes com HCV. Os níveis plasmáticos de miR-21 foram os mais altos, enquanto os níveis de miR-126 foram os mais baixos em amostras de pacientes com HCV-CHC, com diferenças significativas em comparação com amostras de controles e outros pacientes com HCV. A análise da curva ROC definiu altos níveis plasmáticos de miR-21 como preditores específicos para infecção por HCV, e poderia identificar amostras de HCV complicado e amostras de pacientes com HCV-CHC, enquanto os níveis plasmáticos estimados de miR-126 poderiam ser aplicados para rastrear HCV e suas complicações relacionadas. **Conclusão** – Níveis desregulados de expressão plasmática de miR-21 e miR-126 podem distinguir casos de HCV complicados por CHC e definir casos de HCV-Cirrose hepática (HCV-LC), mesmo aqueles que apresentaram baixos escores de Fib-4.

Palavras-chave – Vírus da hepatite C, carcinoma hepatocelular, cirrose hepática, hepatosteatoze, MicroRNAs, Fator de necrose tumoral- α , Alfa-fetoproteína.

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