Alternation in circARF3 (ADP-ribosylation Factor 3) and its Target Gene miR-103 Activity Promotes Hepatocellular Carcinoma in Obese Patients with Metabolic-Associated Fatty Liver Disease

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the fourth most common cancer-related cause of death worldwide and poses a severe threat to public health. In addition to being an underlying risk factor for HCC, obesity is one of the common causes of metabolic-associated fatty liver disease (MAFLD). **Objective:** Therefore, the current study aimed to investigate the expression levels of both circARF3 (ADP-ribosylation factor 3) and its target gene miR-103 in obese patients with MAFLD and to assess their relations to susceptibility and clinicopathological features of HCC.

Patients and methods: The current study was conducted on 100 subjects (50 control groups and 50 obese patients with MAFLD). The case group was subclassified to 39 patients without HCC and 11 patients with HCC. The expression levels of circARF3 and miR-103 were investigated by RT PCR. **Results**: Our results revealed statistically significant higher values of circARF3 in MAFLD (1.89±0.614) compared to control (0.72±0.341). In addition, the level of miR-103 was statistically significantly higher in MAFLD (2.41±0.82) compared to control (0.912±0.335), P <0.001. Also, there were statistically significant higher values of circARF3 in HCC (4.67±1.63) compared to non-HCC (1.44± 0.74). In addition, the level of miR-103 was statistically significantly higher in HCC (4.99±1.32) compared to non-HCC (1.512±0.45), P <0.001. Interestingly, circARF3 and miR-103 significantly correlated with obesity indices and metabolic and hepatic dysfunction biomarkers. Cut-off values 0.94, 1.2, 1.8, 2.98 were able to discriminate simple steatosis, steatohepatitis, cirrhosis, and HCC with AUC 0.78, 0.64, 0.77, 0.81 respectively. **Conclusion**s: The current study results detected upregulation of both studied epigenetic markers; circARF3 and miR-103 in obese MAFLD patients especially patients with HCC. Thus, they could be used as diagnostic biomarkers of MAFLD-associated HCC.

Keywords: MAFLD, HCC, circARF3, obesity, miR-103 relative expression.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major public health problem and the 4th leading cause of cancer related mortality worldwide [(1)]. Recently, a report demonstrated that metabolic-associated fatty liver disease (MAFLD) is emerging as the main etiology for chronic liver disease progressing HCC [(2)]. The diagnosis of MAFLD is based on the presence of hepatic steatosis plus one or more of other conditions such as overweight/obesity, type 2 diabetes mellitus (T2DM), or metabolic abnormalities with no additional exclusion criteria [(3)]. Regarding metabolic dysfunction, the definition includes at least two features from the following: increased waist circumference, arterial hypertension, elevated triglycerides, low high-density lipoprotein (HDL-C), prediabetes, insulin resistance, and subclinical inflammation [(4)].

Of interest, increased prevalence of treatment of hepatitis C virus (HCV) by antiviral therapy leads to a decrease HCV-related HCC [(5)]. Additionally, the increased prevalence of western dietary pattern and decrease physical activity has accelerated the incidence rates of MAFLD [(6,7)]. Obesity is one of the usual causes of MAFLD, which is also an underlying risk factor for HCC [(8)]. One must bear in mind that obesity is strictly associated with HCC [(9)]. There is a lot of evidence emphasizing the pathogenic role of increased reactive oxygen species, adipokines dysregulation, remodeling of fatty tissue, changes of gut microbiota, and dysregulated microRNA in increasing the relative risk of HCC among obese patients [(10,11)].

There is published data indicating that circRNAs in adipose tissues can gain access to the circulation inside microvesicles and have functions in target organs. In support of this hypothesis, a recent report indicated that adipose-derived exosomes, through regulating the deubiquitination-related miR-34a/USP7 axis, can mediate the delivery of circRNAs and promote the tumorigenesis of HCC [(12)].

Risk factors for MAFLD-associated HCC including obesity, diabetes, deposition of iron, genetic and epigenetic factors, microRNA, and gut microbiota. The aim of this work is to investigate the expression levels of both circARF3 and its target gene miR-103 in obese patients with MAFLD and assess their relations to susceptibility and clinicopathological features of HCC.

PATIENTS AND METHODS

The current study enrolled 50 healthy subjects as a control group and 50 obese patients with MAFLD; both groups were sex and age matched. For accurate assessment of body fat, a dual energy X-ray absorptiometry (DEXA) scan was done for estimation of fat mass index (FMI) and fat-free mass index (FFMI). The study design is shown in figure 1.

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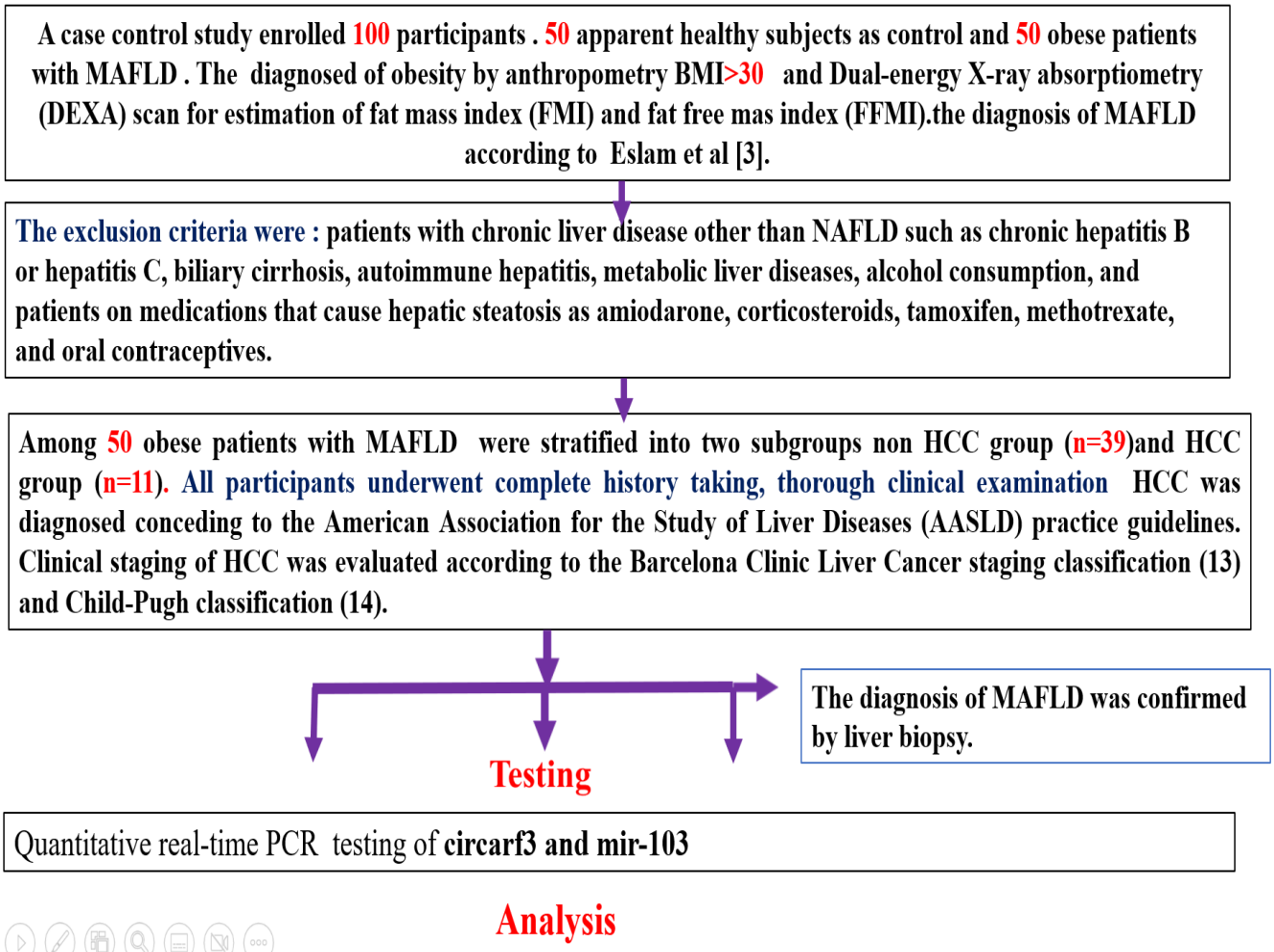

Figure (1): Flowchart of the study

Blood samples and laboratory tests:

Samples were drawn from all after overnight fast. Fasting plasma glucose (FPG), fasting lipid profile, and liver function were done (Cobas 8000/ c702, ROCHE diagnostics, Germany). The original HOMA model (HOMA1)- as described by **Matthews and colleagues** [13] was used to assess insulin resistance (IR). Hepatic steatosis index- as described by **Lee and colleagues**[14] was used as a non-invasive method to assess fatty liver.

RNA Extraction and Quantitative PCR (qPCR):

Total RNA was isolated from blood using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's manual. Reverse transcription was carried out using Prime Script™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) following the manufacturer's manual. qPCR was consequently carried out using SYBR® Premix Ex Taq™ II (Takara) on the ABI Step One Plus system (Applied Biosystems, CA, USA) following the manufacturer's manual. The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an endogenous control.

The primers sequences were as follows:

	Primer Sequence 5′ to 3′	
	Forward	**Reverse**
Circ.ARF3	GGAACAAGCCCCAACCGG	CTAAAATCAGGGGTC-CCAACTG
miR-103	TCGGACCACCTCGCCTTACA	CTGGGCTCCTTCCCTCATCG
GAPDH	CCGGGAAACTGTGGCGTGATGG	AGGTGGAGGAGTGGGTGTCGCTGTT

Ethical consent:

This study was ethically approved by the Institutional Review Board of the Faculty of Medicine, Zagazig University and the reference number was IRB (Ethics number. 8055).

Written informed consent was taken from all participants. The study was conducted according to the Declaration of Helsinki.

Statistical analysis

The statistical analysis was carried out using IBM SPSS statistics program version 26. Quantitative data were presented as means and standard deviation (SD) and compared by independent t-test.

Qualitative data were presented as frequency and percentage and were compared by chi-square test. Correlation analysis were performed using the Pearson correlation test and linear regression to assess the relations between circARF3 and miR-103 relative expression levels. Receiver operation coefficient (ROC) curve analysis was used to detect the predictive accuracy. P-was considered significant if <0.05.

RESULTS

The current research enrolled 50 healthy control (35 male and 15 female); their mean age was 50.12. ±9.37 years and 50 obese patients (37 male and 13 female) with MAFLD (liver biopsy proved steatosis in addition to metabolic risk factors), their mean age was 51.33 ±8.21years.

Clinical and biochemical characteristics of the MAFLD patients

To compare MAFLD subgroups, we found that the MAFLD group without HCC had highly significant higher values of alanine aminotransferase (ALT), aspartate transaminase (AST), albumin, hemoglobin, and platelet compared to the HCC group.

On the other hand, HCC subgroup had significantly higher values of total bilirubin, direct bilirubin, alpha-fetoprotein, and creatinine compared to the non-HCC group (Table 1).

Table (1): Clinical, anthropometric and laboratory characteristics of studied patients' subgroups

Variables	**MAFLD Group without HCC (mean ± SD) (n=39)**	**MAFLD group with HCC (mean ± SD) (n=11)**	P
Age (years)	45.02±4.37	57.4±8.91	0.768
Systolic blood pressure (mm Hg)	131.3±15.07	127.3±13.1	0.124
Diastolic blood pressure (mm Hg)	89.1±4.96	86.9±7.30	0.221
Body mass index (kg/m^2)	33.6±6.94	32.3±3.126	0.690
Waist/hip ratio	1.26±0.09	1.35±0.108	0.137
FMI (kg/m^2)	6.5±1.52	6.6±0.94	0.690
FFMI (kg/m^2)	26.1±3.5	26.4±4.34	0.686
Total cholesterol (mg/dL)	178.3±27.34	156.6±21.48	0.180
Triglycerides (mg/dL)	165.6±12.51	153.6±22.01	0.181
LDL cholesterol (mg/dL)	175.9±22.9	173.2±42.13	0.371
HDL cholesterol (mg/dL)	33.1±6.67	30.9±6.775	<0.05*
Fasting plasma glucose (mg/dL)	178.9±13.97	160.1±21.1	0.213
Fasting serum insulin (lU/mL)	17.3±4.35	17.9±4.21	0.827
HOMA-IR	7.57±1.51	7.9±1.62	0.837
AST(IU/L)	78.23±13.1	35.5±8.43	<0.001*
ALT (IU/L)	99.16±12.5	44.5±10.31	<0.001*
PT (seconds)	11.8±0.68	17.9±2.34	<0.001*
Total bilirubin (mg/dl)	0.89±0.13	1.9±0.33	<0.001*
Direct bilirubin (mg/dl)	0.26±0.03	0.75±0.08	<0.001*
Albumin (g/dl)	4.3±0.5	3.5±0.71	<0.001*
GGT (IU/L)	92.7±21.50	89.5±15.6	0.181
CRP (mg/dL)	4.82±1.11	8.92±2.13	<0.001*
Serum ferritin (ng/ml)	18.02±3.06	17.02±4.06	0.902
Hepatic steatosis index	44.9±1.28	45.4±3.65	*0.821
Alpha-fetoprotein(ng/ml)	11.7±2.43	149.5±35.6	<0.001*
WBC count (cell×10^3/µl)	5.7±1.32	6.34±1.31	0.654
Hemoglobin (g/dl)	10.8±1.12	9.31±1.21	<0.001*
Platelet(cell×10^3/µl)	200.7±31.50	69.76±6.61	<0.001*
Creatinine (mg/dl)	0.69±0.13	1.89±0.31	<0.001*

FMI, fat mass index; FFMI, fat free mass index; HOMA-IR, homeostasis model assessments of insulin resistance, HCC: hepatocellular carcinoma; BMI: body mass index; PT: prothrombin time; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; WBC: white blood cell; *: Significant P

Comparison of relative expression of circARF3 and miR-103 in studied patients.

Our results revealed statistically significant higher values of circARF3 in MAFLD (1.89±0.614) compared to control (0.72±0.341), (Figure 2a). In addition, the level of miR-103 was statistically significantly higher in MAFLD (2.41±0.82) compared to control (0.912±0.335) (Figure 2b); P < 0.001.

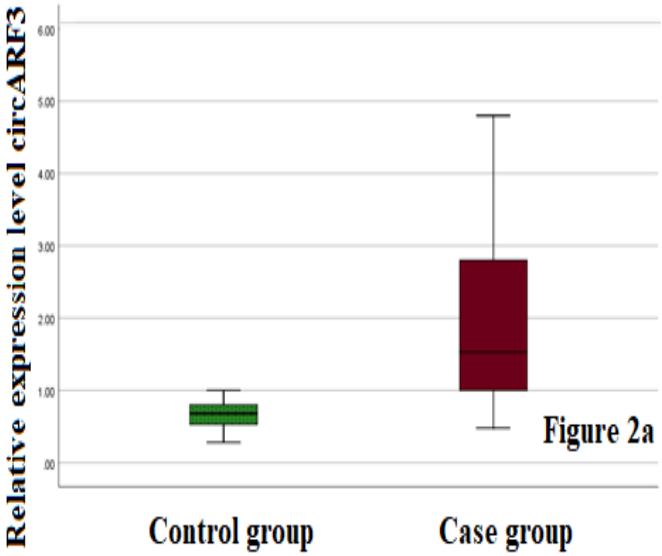

Figure (2a): Relative expression levels of circARF3 in studied groups

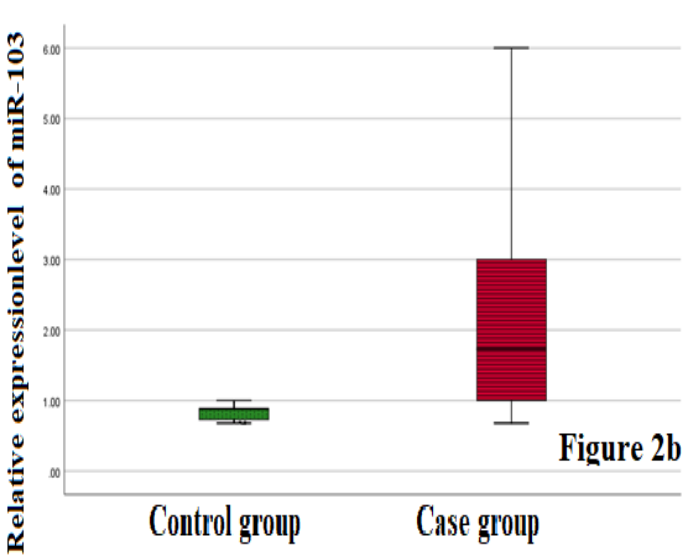

Figure (2b): Relative expression levels of miR-103 in studied groups.

Comparison of relative expression of circARF3 and miR-103 in MAFLD patients with or without HCC

There was a statistically significant higher values of circARF3 in HCC (4.67±1.63) compared to non-HCC (1.44± 0.74), (Figure 3a). In addition, the level of miR-103 was statistically significantly higher in HCC (4.99±1.32) compared to non-HCC (1.512±0.45), (Figure 3b). P <0.001.

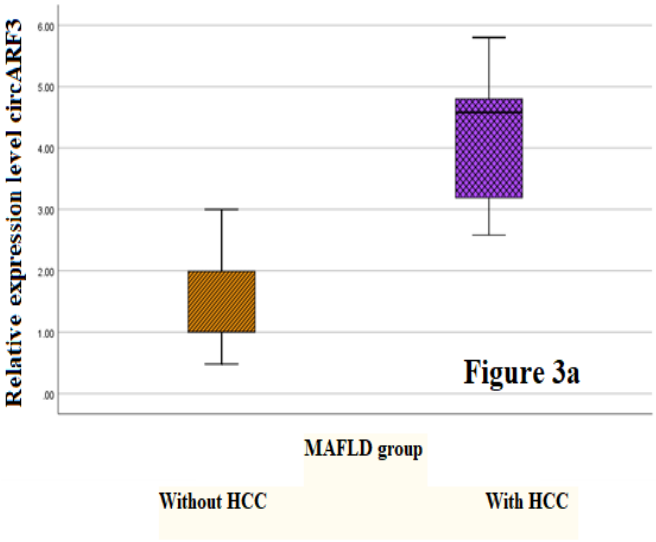

Figure (3a): Relative expression levels of circARF3in MALFLD groups

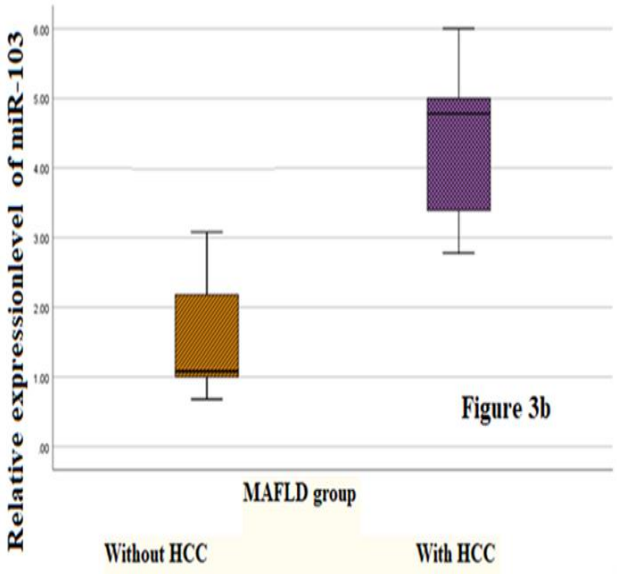

Figure (3b): Relative expression levels of miR-103 in MAFLD groups

Clinicopathological features of the HCC subgroup.

We investigated our research on the clinicopathological features of HCC among patients with MAFLD and the findings are shown in Table 2.

Table (2): Clinicopathological features of hepatocellular carcinoma patients

Variable	HCC (n =11)- n (%)	p value
Stage Stage I/II Stage III/IV	 5 (45.5%) 6 (54.5%)	0.763
Tumor size <5 cm >5 cm	 3 (27.2%) 8 (72.8%)	0.132
Lymph node metastasis -Absent -Present	10 (90.9%) 1(9.1%)	0.007*
Distant metastasis – Absent -Present	 9(81.8%) 2(18.2%)	0.035*
Child-Pugh grade A B C	 5 (45.5%) 4(36.3%) 2(18.2%)	0.529
Portal vein thrombosis -Negative Positive	7(63.7%) 4 (36.3%)	0.366
Number of tumor lesions Single Multiple	6(54.5%) 5(45.5%)	0.763
Site of lesions Right lobe Left lobe Both	 5(45.5%) 4(36.3%) 2(18.2%)	0.529

*: Significant P

Pearson correlation between relative expression of circARF3 and miR-103 with clinical and laboratory parameters among MAFLD patients

There were significant positive correlations between both epigenetic markers circARF3 and miR-103 with body composition parameters; BMI, waist/hip ratio, FMI%, HOMA-IR, triglycerides (TG), and alpha-fetoprotein. On the contrary, relative expression of circARF3 and miR-103 levels were significantly negatively correlated with HDL, platelets, and hemoglobin (Table 3).

Table (3): Pearson correlation of expression levels of circARF3 and miR-103 with clinical, anthropometric, and biochemical characteristics in MAFLD groups

Variables	CircARF3		MR-103	
	r	p	r	p
Body mass index	0.71	<0.001*	0.62	<0.001*
Waist/hip ratio	0.61	<0.001*	0.59	<0.001*
FMI (kg/m^2)	0.61	<0.001*	0.60	<0.001*
Triglycerides	0.68	<0.001*	0.73	<0.001*
HDL cholesterol	-0.53	<0.001*	-0.61	<0.001*
HOMA-IR	0.71	<0.001*	0.80	<0.001*
Total bilirubin	0.2	0.572	0.23	0.472
HSI	0.15	0.369	0.60	<0.001*
ALT (IU/L)	0.07	0.653	0.744	<0.001*
Alpha-fetoprotein	0.33	<0.001*	0.72	<0.001*
WBC count	0.35	<0.05*	0.41	<0.05*
Hemoglobin	-0.65	<0.001*	-0.76	<0.001*
Platelet	-0.64	<0.001*	-0.87	<0.001*
Creatinine	0.23.	0.571	0.631	<0.001*

FMI, fat mass index; HOMA-IR, homeostasis model assessments of insulin resistance, HSI, Hepatic steatosis index ;ALT; alanine aminotransferase, *: Significant P

Linear regression analysis in the HCC group to test the influence of the main independent variables against circARF3 and miR-103

Only alpha-fetoprotein and HOMA-IR were the key predictors of the relative expression levels of circARF3 and miR-103 among other laboratory biomarkers in the HCC subgroup (Table 4).

Table (4): Linear regression analysis in HCC group to test the influence of the main independent variables against circARF3 and miR-103 *(*dependent variable)

Model		Unstandardized Coefficients		Standardized Coefficients	t	P value	95% C.I.	
		B	Std. Error	Beta			Lower Bound	Upper Bound
CircARF3	Constant	6.885	1.546		4.452	0.000	3.777	9.993
	HOMA-IR	0.009	0.003	0.278	3.017	<0.001*	0.003	0.015
	Triglycerides	0.011	0.006	0.075	1.817	0.075	-0.001	0.023
	Alpha-fetoprotein	1.026	0.042	0.977	24.560	<0.001*	0.942	1.110
	BMI	0.038	0.090	0.067	0.424	0.673	-0.143	0.220
	ALT	-0.039	0.179	-0.034	-0.215	0.830	-0.398	0.321
Mir-103	Constant	4.987	0.946		5.272	0.000	3.109	6.865
	HOMA-IR	0.192	0.090	5.466	1.925	<0.05*	-0.005	0.350
	Triglycerides	0.000	0.000	0.072	0.655	0.514	00.000	00.001
	BMI	0.053	0.034	0.148	1.532	0.129	-0.016	0.121
	Alpha-fetoprotein	0.520	0.183	0.526	2.840	<0.001*	0.152	0.888
	ALT	0.055	0.034	0.153	1.599	0.113	-0.013	0.122

*: Significant P

The accuracy of studied epigenetic markers for diagnosis of MAFLD by ROC analysis

As regards relative expression of circARF3, the AUC was 0.895 (95% CI = 0.831-0.958) with sensitivity = 86.7%, specificity = 85%, and the cutoff values (0.91), P <0.001. Regarding relative expression of miR-103 the AUC was 0.833 (95% CI =0.754-0.913) with sensitivity = 80%, specificity = 97.5%, and the cutoff values (0.94), (Fig. 4a), P <0.001.

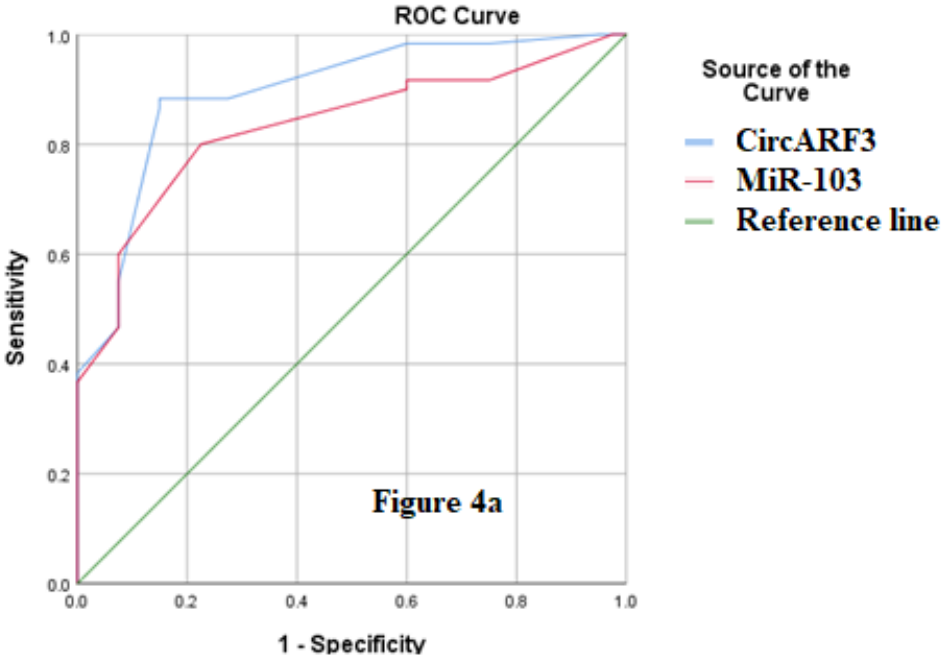

Figure (4a): Receiver operating characteristic curve of the relative expression levels of studied epigenetic markers for diagnosis of MAFLD

The accuracy of studied epigenetic markers for diagnosis of HCC among MAFLD by ROC analysis

Regarding relative expression of circARF3, the AUC was 0.886 (95% CI = 0.746–1.000) with sensitivity = 88.8%, specificity = 79.5%, and the cutoff values (2.29). As regards relative expression of miR-103 the AUC was 0.876 (95% CI = 0.735–1.000) with sensitivity = 88.9%, specificity = 75.3%, and the cutoff values (2.4), (Fig. 4b), P <0.001.

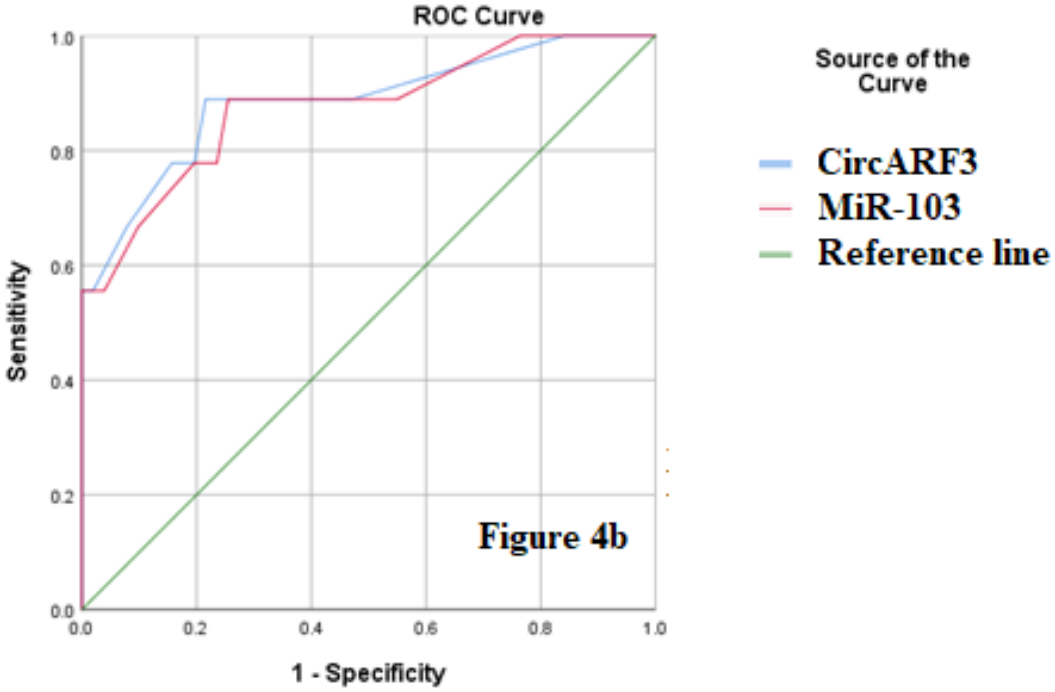

Figure (4 b): Receiver operating characteristic curve of the relative expression levels of studied epigenetic markers for diagnosis of HCC among MAFLD groups.

DISCUSSION

The increasing prevalence of western dietary patterns and limited physical activity leads to an increase in the incidence rates of MAFLD and MAFLD-associated HCC [6,7]. It has been suggested that a high-calorie diet persuades hepatocarcinogenesis[8].

The etiology and the mechanism of MAFLD-associated HCC are not well understood till now and, as a result, satisfactory effective preventative therapy is not available till now [8].

We must improve our understanding of the epigenetic and genetic mechanisms involved in the pathogenesis of MAFLD-associated HCC, to set up the principles for the development of new therapeutic strategies to prevent HCC. Based on their sequences, we designed the current study to assess the expression levels of both circARF3 and its target gene miR-103 in obese patients with MAFLD and to assess their relations to susceptibility and clinicopathological features of HCC.

The current explorative study was designed to compare anthropometric and metabolic biomarker risk profiles between obese MAFLD patients with or without HCC and we detected that there were highly significant higher values of liver enzymes in obese MAFLD patients without HCC compared to the HCC group.

Emerging evidence demonstrated that circRNAs regulate angiogenesis in patients with HCC. As a matter of fact, excessive abnormal angiogenesis is one of the trademarks of cancer. A study conducted by **Chen *et al.*** [15] observed that dysregulated circRNAs influence cell cycle progression by regulating pathogenic risk factors of HCC in particular circ-deubiquitination (circ-DB), which is upregulated in HCC patients with excess fat as diagnosed by anthropometric tests. Six related gene products are included in ARFs, ARF1-ARF6, which are mainly expressed all over the body [16].

The present study aimed to explore the potential clinical significance of circARF3 and miR-103 relative expression levels as diagnostic and prognostic markers of MAFLD as well as HCC-associated MAFLD, and we confirmed that there were statistically significant higher values of circARF3 and miR-103 relative expression in MAFLD compared to control. Additionally, both epigenetic markers were statistically significantly higher in HCC compared to non-HCC.

Davis *et al.* [17] found that ARF1 enhances prostate tumorigenesis through targeting oncogenic MAPK signaling. Similar finding was observed in breast cancer [18], cancer ovary [19], endometrial cancer [20], and stomach cancer [21].

The ontogenetic role of ARF3 in promoting tumors could be due to a common pathway of many signal transduction pathways, GTP-binding proteins[22].

To gain further insights, we performed a Pearson correlation between relative expression of circARF3 and miR-103 with clinical and laboratory parameters among MAFLD patients and we observed significant positive correlations between both epigenetic markers circARF3 and miR-103 with body composition parameters; BMI, waist/hip ratio, FMI %, HOMA-IR, and TG as well as alpha-fetoprotein. Hence, we decided to investigate linear regression analysis in the HCC group to test the influence of the main

independent variables against circARF3 and miR-103 and we perceived that among the studied parameters, only alpha-fetoprotein and HOMA-IR were the key predictors of the relative expression levels of circARF3 and miR-103 among other laboratory biomarkers in the HCC subgroup.

In order to confirm the accuracy of circARF3 and miR-103 in the diagnosis of MAFLD, we performed a ROC test and we found that the relative expression of circARF3 sensitivity was 86.7%, and specificity was 85%, at cutoff values of 0.91. Regarding the relative expression of miR-103, the sensitivity was 80%, and specificity was 97.5%, at cutoff values of 0.94.

Concerning the power of both studied epigenetics in the diagnosis of HCC and as regards relative expression of circARF3, the AUC was 0.886 with sensitivity = 88.8%, specificity = 79.5%, and the cutoff values (2.29). Regarding relative expression of miR-103, the AUC was 0.876 with sensitivity = 88.9%, specificity = 75.3%, and the cutoff values (2.4).

CONCLUSION

The current study results detected upregulation of both studied epigenetic markers; circARF3 and miR-103 in obese MAFLD patients especially patients with HCC. Interestingly, circARF3 and miR-103 significantly correlated with obesity indices and metabolic and hepatic dysfunction biomarkers.

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Conflict of interest: Nil.

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