



ORIGINAL RESEARCH ARTICLE

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# The expression of microRNA-331-3p and microRNA-23b3 in Egyptian patients with early-stage hepatocellular carcinoma in hepatitis C-related liver cirrhosis

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## Abstract

**Background:** Hepatocellular carcinoma and hepatitis C are strongly associated. The current work aimed to study the expression levels of microRNA-331-3p and microRNA-23b-3p as propable biomarkers for detecting liver cancer (HCC) at its early stages in patients with HCV-related liver cirrhosis. The current prospective study included two hundred participants, divided into three groups: group I, 100 patients with HCV-related liver cirrhosis; group II, 50 HCC patients at early stages; and group III, 50 apparently healthy controls. All patients had routine laboratory workup and ultrasound hepatic assessment. Values of microRNA-331-3p and microRNA-23b-3p were measured by real-time quantitative PCR.

**Results:** Levels of miR-331-3p were significantly higher in HCC patients than in cirrhotic patients and controls ( $p < 0.001$ ), while levels of miR-23b-3p were significantly lower in HCC patients compared to cirrhotics and controls ( $p < 0.001$ ). ROC curve revealed that miR-23b-3p had 80% sensitivity and 74% specificity, miR-331-3p had 66% sensitivity and 61% specificity, and AFP had 64% sensitivity and 61% specificity of 61% in discrimination between HCC patients from controls.

**Conclusion:** Serum miR-23b-3p is a more effective predictor than miR-331-3p and AFP for the development of hepatocellular carcinoma in hepatitis C (HCV)-related cirrhotic patients.

**Keywords:** miR-331-3p, miR-23b-3p, HCV, HCC, Egyptian, Liver cirrhosis

## Background

Hepatocellular carcinoma (HCC) is a universal health problem. HCC represents the sixth most common cancer worldwide [1], and the fourth common cancer in Egypt [2]. HCV promotes cirrhosis, which is found in 80–90% of patients with HCC [3]. Prospective studies have shown a significant increase in the incidence of HCC among HCV-infected cohorts, compared to HCV-negative cohorts. HCV-induced HCC development is a multi-step process that may progress over 20–40 years

[4]. HCV is an RNA virus with limited integration of its genetic material into the host's genome. Therefore, the carcinogenic potential of HCV is generally assumed to be linked to the indirect mechanisms [5].

MicroRNAs (miRNAs) are a class of small 18–23-nucleotides that have a role in different biological functions including proliferation, differentiation, and apoptosis [6]. Abnormal miRNA expression has been implicated in the pathogenesis of various cancers [7]. Discovery that microRNAs are detected in patients' sera raised the assumption that these serum biomarkers can be used as a less invasive screening or diagnostic tools [8]. miR-331-3p is one of the most upregulated miRNAs that promote proliferation, migration, and invasion of

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the tumor cells, including HCC [9]. miR-23b-3p was identified as a tumor suppressor that downregulated in different classes of human malignant tumors [10].

microRNA-331-3p and microRNA-23b-3p expression in hepatic carcinoma was examined in some studies [11, 12].

The current work aimed to determine the potentiality of serum miR-331-3p and miR-23b-3p as screening biomarkers for early stages of liver cancer in patients with HCV-related hepatic cirrhosis.

## Methods

The current prospective study involved 150 patients out of 2140 patients who attended the tropical medicine, hepatology, oncology, and general medicine outpatient clinics in the Alexandria University Hospital (Alexandria, Egypt) in the period from December 2017 till January 2020. The control group involved 50 apparently healthy volunteers.

The proposal was explained to all participants, and they signed a written consent. The proposal was accepted by the committee of ethics (Faculty of medicine, University of Alexandria).

Exclusion criteria included patients with coinfections with hepatitis B virus (HBV) or HIV, organ transplantation, autoimmune disease or use of immunosuppressant or antiviral drugs, diabetes mellitus, Schistosoma, and other malignant comorbidities.

All participants were subjected to full history taking, thorough clinical examination, laboratory investigations including complete blood picture, liver transaminases (alanine aminotransferase (ALT), and aspartate aminotransferase (AST)), serum albumin, total bilirubin, and alpha-fetoprotein, and abdominal ultrasonography.

Two hundred participants were divided into three groups: group I, 100 patients with HCV-related liver cirrhosis; group II, 50 patients with early-stage hepatocellular carcinoma (HCC); and group III, 50 healthy controls.

Ultrasound examinations were performed by experienced ultra-sonographers in the Radiology Department of the main Alexandria University Hospital. Items to be observed in ultrasound examination were determination of the liver size, nodularity of the liver surface, the coarseness of the parenchyma, size of lymph nodes around the hepatic artery, patency and flow of veins and arteries, and probable hepatocellular carcinoma.

HCV antibody testing was done by using a commercial recombinant immunoblot assay and confirmed by real-time quantitative HCV RNA PCR (more than 15 IU/ml).

All the HCC patients were diagnosed by abdominal ultrasound and alpha-fetoprotein [13]. Triphasic CT scan examination and Barcelona clinic liver cancers (BCLC) [25] staging was done for the selection of group II patients. Based on BCLC; patients at a very early stage

(stage 0) and early stage (stage A) were selected as candidates for study with single tumors less than 2 cm or multinodular tumor less than 3 cm in size, with the absence of clinically relevant portal hypertension.

## Real-time quantitative PCR for miR-331-3p and miR-23b expression levels

Total RNA including microRNA was immediately isolated from plasma samples using miRNeasy Mini Kit (Qiagen, Maryland, USA) according to manufacturers' instructions. The concentration and purity of the extracted total RNA were assisted using NanoDrop2000 Spectrophotometer (Thermo Scientific, USA). Single-stranded cDNA was synthesized from purified RNA samples using the Taqman miRNA reverse transcription Kit (Applied Biosystems, USA) for RNA reverse transcription according to the manufacturer's protocol. The real-time amplification was performed using TaqMan MicroRNA assays for miR-331-3p, miR-23-3p, and TaqMan Fast Advanced Mater Mix (Applied Biosystems, USA) on the RotorGene Q Real-Time PCR System (Qiagen, Germany). RNU6 was used as endogenous references; its expression was stable in all the samples and independent of the analyzed variables. The relative expression levels were determined using the  $2^{-\Delta\Delta CT}$  method.

## Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov was used to verify the normality of distribution of variables. Comparisons between groups for categorical variables were assessed using the Chi-square test. ANOVA was used for comparing the four studied groups and followed by post hoc test (Tukey) for pairwise comparison. While Kruskal-Wallis test was used to compare different groups for abnormally distributed quantitative variables and followed by post hoc test (Dunn's for multiple comparisons test) for pairwise comparison. The significance of the obtained results was judged at the 5% level.

## Results

The current study involved 200 participants divided into the following groups: group I, 100 patients with HCV-related liver cirrhosis; group II, 50 HCC patients at an early stage (16 patients (32%) were BCLC stage 0 and 34 patients (68%) were BCLC stage A); and group III, 50 apparently healthy controls.

The mean age of group I was  $47.4 \pm 9.7$  years, group II was  $50.6 \pm 5.2$  years, and of group III was  $47.5 \pm 9.3$  years with no significant difference between the three groups ( $p = 0.086$ ) (Table 1).

**Table 1** Comparison between the three studied groups according to the demographic data, laboratory investigations, and serum concentrations of miR-331-3p and miR-23b-3p

	Group I (HCV-related liver cirrhosis) (n = 100)	Group II (HCC group) (n = 50) (16 stage 0 BCLC, 34 stage A BCLC)	Group III (healthy controls) (n = 50)	Test of Sig.	p
<b>Age (years)</b>					
Mean ± SD	47.4 ± 9.7	50.6 ± 5.2	47.5 ± 9.3	F = 2.489	0.086
Median (min.–max.)	49(25–61)	51(41–59)	48(32–63)		
<b>Gender</b>					
Male	79 (79%)	40 (80%)	35 (70%)	$\chi^2 = 1.863$	0.394
Female	21 (21%)	10 (20%)	15 (30%)		
<b>ALT (IU/L)</b>					
Mean ± SD	59.7 ± 35.1	60.4 ± 26.3	19.0 ± 7.7	H = 72.496*	< 0.001*
Median (min.–max.)	56.5 <sup>a</sup> (8.4–165)	48 <sup>a</sup> (33–127)	18.4 <sup>b</sup> (5.8–38.1)		
<b>AST (IU/L)</b>					
Mean ± SD	53.2 ± 30.1	55.3 ± 24.8	18.7 ± 6.3	H = 84.833*	< 0.001*
Median (min.–max.)	45.5 <sup>a</sup> (6.9–155)	44.5 <sup>a</sup> (30–119)	17.6 <sup>b</sup> (6.4–30.2)		
<b>Total bilirubin (mg/dl)</b>					
Mean ± SD	1.2 ± 0.6	1.1 ± 0.4	0.7 ± 0.3	H = 25.660*	< 0.001*
Median (min.–max.)	1.1 <sup>a</sup> (0.2–2.8)	1 <sup>a</sup> (0.4–2)	0.8 <sup>b</sup> (0.1–1.2)		
<b>Albumin (g/dl)</b>					
Mean ± SD	3.9 <sup>b</sup> ± 3.1	3.6 <sup>c</sup> ± 2.6	4.3 <sup>a</sup> ± 4.8	F = 46.804*	< 0.001*
Median (min.–max.)	4.0 (3.3–4.5)	3.6 (3.2–4.0)	4.2 (3.4–5.2)		
<b>Hemoglobin (g/dl)</b>					
Mean ± SD	13.6 <sup>a</sup> ± 1.8	12.2 <sup>b</sup> ± 1.3	14.1 <sup>a</sup> ± 1.1	F = 22.005*	< 0.001*
Median (min.–max.)	13.9 (8.5–16.4)	12.0 (10.0–15.0)	14.4 (12.1–15.9)		
<b>Total leukocytic count (× 10<sup>9</sup>)</b>					
Mean ± SD	5.2 <sup>b</sup> ± 1	4.2 <sup>c</sup> ± 1.1	6.7 <sup>a</sup> ± 1.8	F = 51.129*	< 0.001*
Median (min.–max.)	5.1 (3.5–7.1)	4 (2.4–6.3)	6.5 (4.3–10.2)		
<b>Platelet count (× 10<sup>9</sup>)</b>					
Mean ± SD	160.9 <sup>a</sup> ± 46.8	130.2 <sup>c</sup> ± 21.5	223.1 <sup>b</sup> ± 44.1	F = 67.135*	< 0.001*
Median (min.–max.)	158 (63–254)	134.5 (91–162)	238.5 (126–358)		
<b>AFP (ng/ml)</b>					
Mean ± SD	140.3 ± 45.2	183.1 ± 77	1.0 ± 0.4	H = 117.964*	< 0.001*
Median (min.–max.)	138 <sup>b</sup> (1.8–240)	196 <sup>a</sup> (58–287)	1 <sup>c</sup> (0.2–1.9)		
<b>miR-331-3p (2<sup>-ΔCT</sup>)</b>					
Mean ± SD	2.2 ± 1.3	7.6 ± 10	1.1 ± 0.5	H = 69.738*	< 0.001*
Median (min.–max.)	1.98 <sup>b</sup> (0.1–7.8)	3.8 <sup>a</sup> (0.1–41)	1 <sup>c</sup> (0.1–3.2)		
<b>miR-23b-3p (2<sup>-ΔCT</sup>)</b>					
Mean ± SD	0.9 ± 0.8	0.3 ± 0.3	1.5 ± 1.1	H = 54.230*	< 0.001*
Median (min.–max.)	0.8 <sup>b</sup> (– 0.98 to 3.2)	0.2 <sup>c</sup> (0–1.7)	1.3 <sup>a</sup> (– 1 to 3.8)		

$\chi^2$  chi-square test, F F for ANOVA test, H H for Kruskal-Wallis test, p p-value for comparing between the studied groups  
Means/median with common letters are not significant (i.e., means with different letters are significant)

\*Statistically significant at  $p \leq 0.05$

Group I comprised of 79 males (79%) and 21 females (21%), group II comprised of 40 males (80%) and 10 females (20%), and group III comprised of 35 males (70%) and 15 females (30%), with no statistically significant difference between the three studied groups ( $p = 0.394$ ) (Table 1).

As regards results of peripheral blood picture, levels of serum transaminases, total bilirubin levels, and serum albumin levels, all were summarized in Table 1.

The mean of AFP was significantly higher in group II than in groups I and III. Furthermore, it was significantly higher in group II than group III ( $P < 0.001$  and  $P < 0.001$ , respectively) (Table 1).

Regarding miR-331-3p expression, plasma levels were significantly higher in group II compared to group I and III ( $p < 0.001$  and  $P < 0.001$ , respectively). Also, a high statistically significant difference was observed between groups (II and III,  $p < 0.001$ ) (Table 1).

On the contrary, miR-23b-3p expression levels were significantly lower in group II compared to group I, and both were significantly lower than the controls ( $p < 0.001$ ) (Table 1).

Further analysis of plasma levels of miRNAs in group II revealed that the mean of miR-331-3p in stage 0 HCC was  $8.1 \pm 10.3$ , while it was  $7.3 \pm 10.1$  in patients with stage A, with no statistical difference could be detected between both groups ( $p = 0.967$ ). Regarding miR-23b-3p, its mean in patients with stage 0 HCC was  $0.21 \pm 0.22$ , while it was  $0.31 \pm 0.37$ , with no statistical difference between both groups ( $p = 0.371$ ) (Table 2).

Using ROC curves to determine the diagnostic accuracy of AFP, miR-331-3p, and miR-23b-3p in differentiating patients with early stages of hepatic cancer from patients with HCV-related liver cirrhosis revealed that values of AFP more than 148 ng/ml had a 64% sensitivity and 61% specificity to differentiate early carcinoma patients from cirrhotic ones (Fig. 1). Values of miR-331-3p more than 2.18 had a 66% sensitivity and 61% specificity to differentiate patients with early stages of HCC (Fig. 2). Regarding miR-23b-3p, values less than or equal to 0.36 had an 80% sensitivity and 74% specificity to diagnose HCC at its early stages (Fig. 3). Combining

AFP (ng/ml) and miR-331-3p (Fig. 4) gave sensitivity of 76% and specificity of 65%. Combining AFP (ng/ml) & miR-23b-3p (Fig. 5) gave sensitivity of 82% and specificity of 69%. While the sensitivity and specificity of combining the three parameters (Fig. 6) were 86% and 64%, respectively (Table 3).

### Discussion

Hepatitis C virus (HCV) is one of the main carcinogens for hepatocellular carcinoma (HCC) [14]. It infects around 71 million people around the world [15]. The HCV infects the liver leading to HCC [16]. And while around 399,000 patients died from HCV in 2016, only around 19% knew about their infection status [16]. And although AFP has been always used to diagnose HCC, it is neither specific nor sensitive [12]. Therefore, it is crucial to look for new predictive markers of HCC as early as possible to improve the patients' prognosis.

miRNAs are small non-coding RNA molecules that are composed of 21–23 nucleotides that affect target gene expression by negatively controlling post-transcriptional gene expression [16, 17]. They are highly tissue-specific and can withstand severe conditions like extreme temperature and low pH, making them highly stable in the serum or plasma samples and, therefore, qualifying them as perfect noninvasive markers for early HCC diagnosis [12].

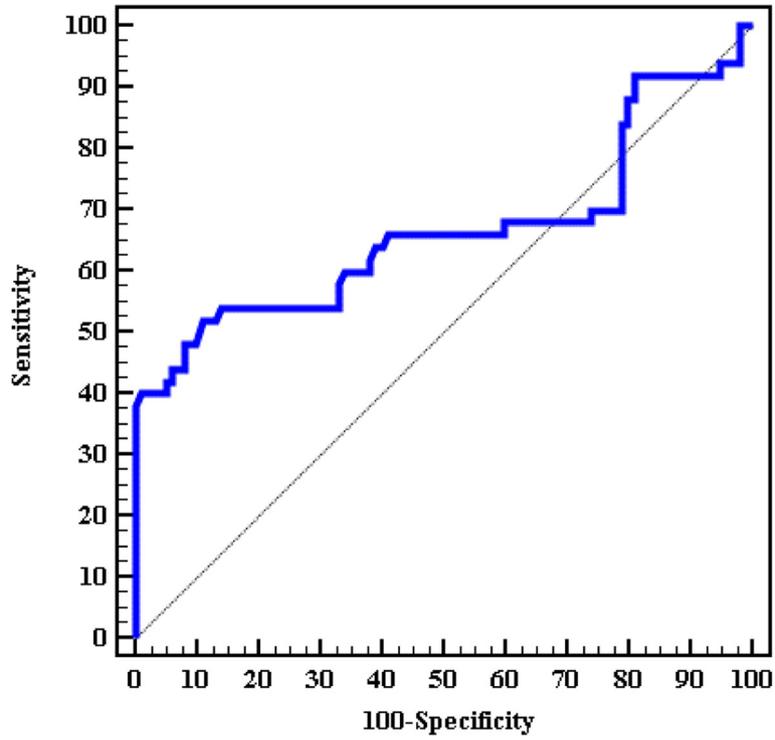
Several studies identified a microRNA panel to distinguish HCV-related HCC from liver cirrhosis [18–20]. In a recent study, it was discovered that there are four miRNAs, including MiR-331-3p that significantly increased in HCC and two miRNAs including miR-23b-3p that significantly decreased in HCC patients compared to liver cirrhosis. Those six miRNAs have been found in the studies to effectively discriminate the HCC from liver cirrhosis and healthy controls [21].

In our study, miR-331-3p was shown to significantly increase in patients with early stages HCC compared to cirrhotic patients. Similar results were also reported by a previous study on HCC tissues that detected overexpression of miR-331-3p in the HCC cells and proved that it helped proliferation and spread of HCC via suppressing

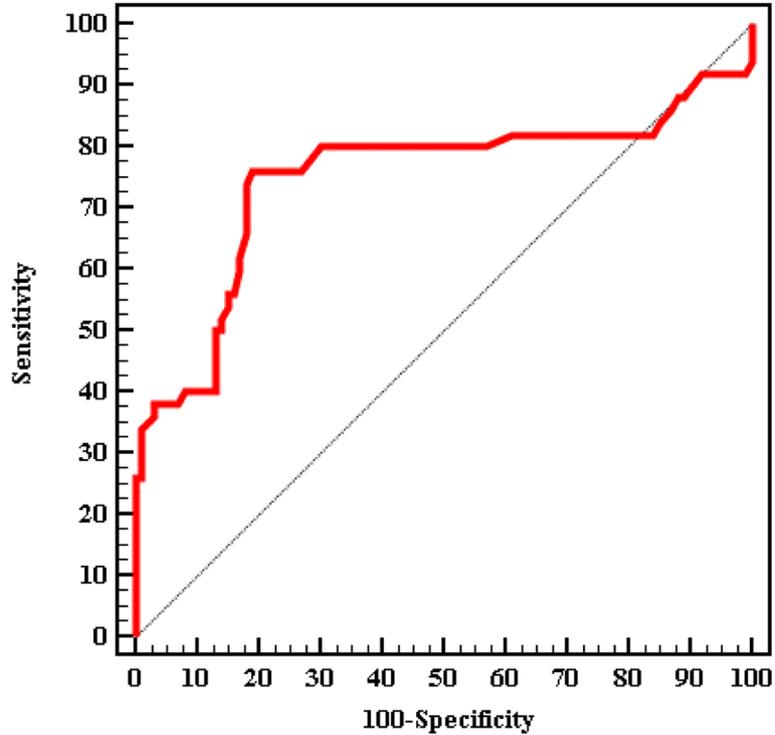
**Table 2** Plasma concentrations of miR-331-3p and miR-23b-3p in group II (n = 50)

	BCLC stage		U	p
	Stage 0 (n = 16)	Stage A (n = 34)		
<b>miR-331-3p (<math>2^{-\Delta CT}</math>)</b>				
Mean $\pm$ SD	8.1 $\pm$ 10.3	7.3 $\pm$ 10.1	270.0	0.967
Median (min.–max.)	3.8 (1.3–36.2)	3.7 (0.1–41)		
<b>miR-23b-3p (<math>2^{-\Delta CT}</math>)</b>				
Mean $\pm$ SD	0.21 $\pm$ 0.22	0.31 $\pm$ 0.37	229.0	0.371
Median (min.–max.)	0.14 (0.01–0.8)	0.18 (0.02–1.7)		

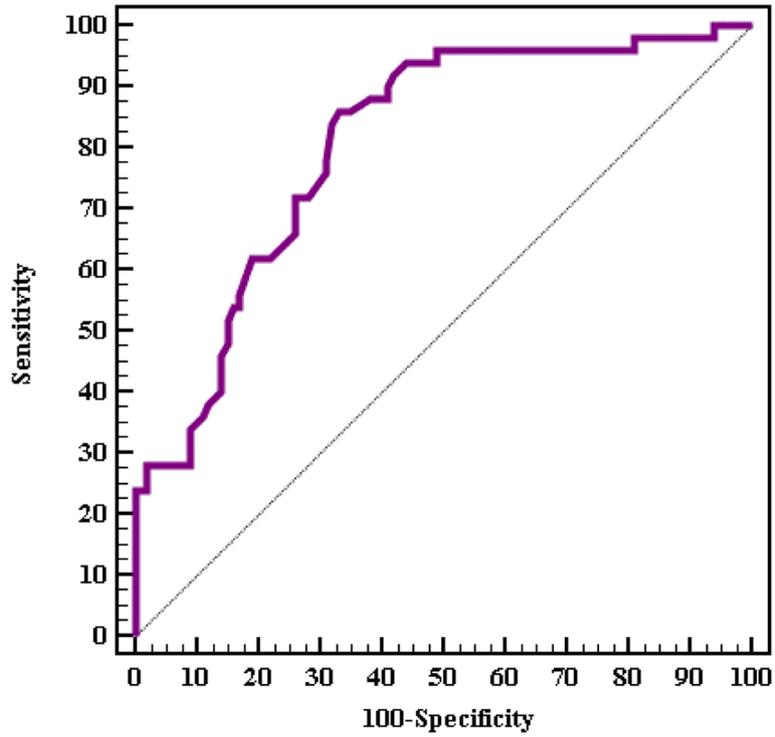
U Mann-Whitney test, p p value for comparing between BCLC stage and serum concentrations of miR-331-3p and miR-23b-3p



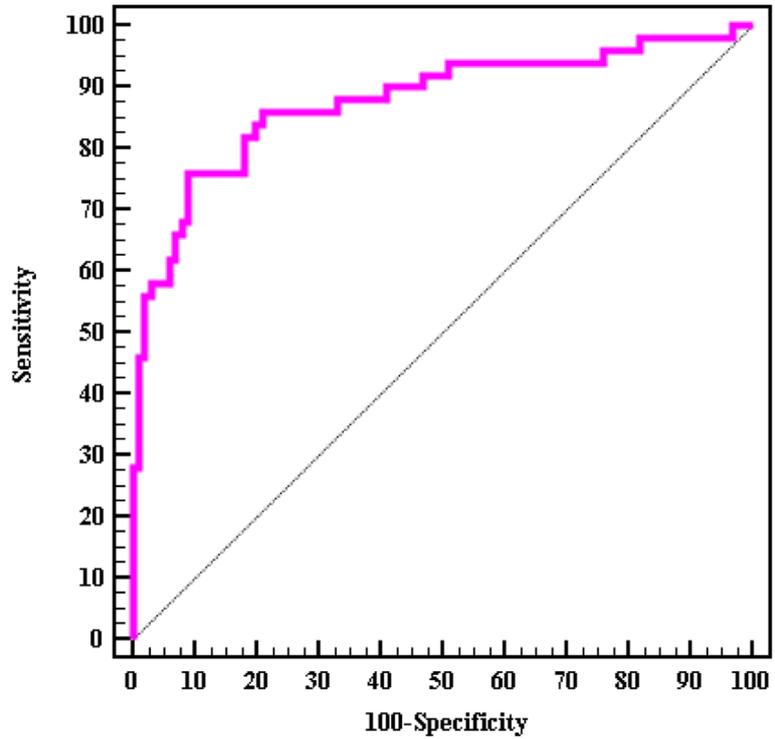
**Fig. 1** ROC curve for AFP (ng/ml) to predict early-stage HCC patients (group II) from HCV-related cirrhosis (group I)



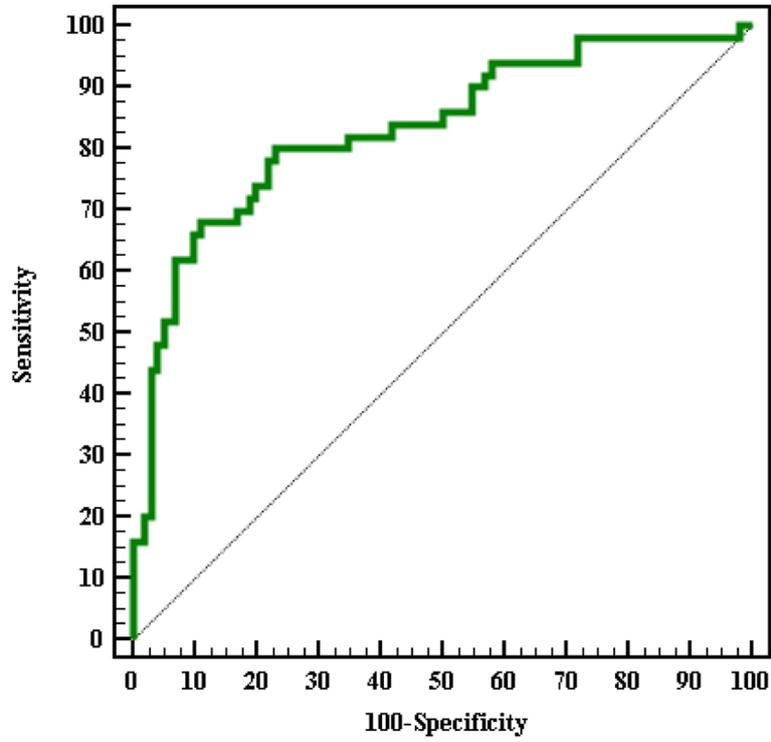
**Fig. 2** ROC curve for miR-331-3p to predict Early-stage HCC patients (group II) from HCV-related cirrhosis (group I)



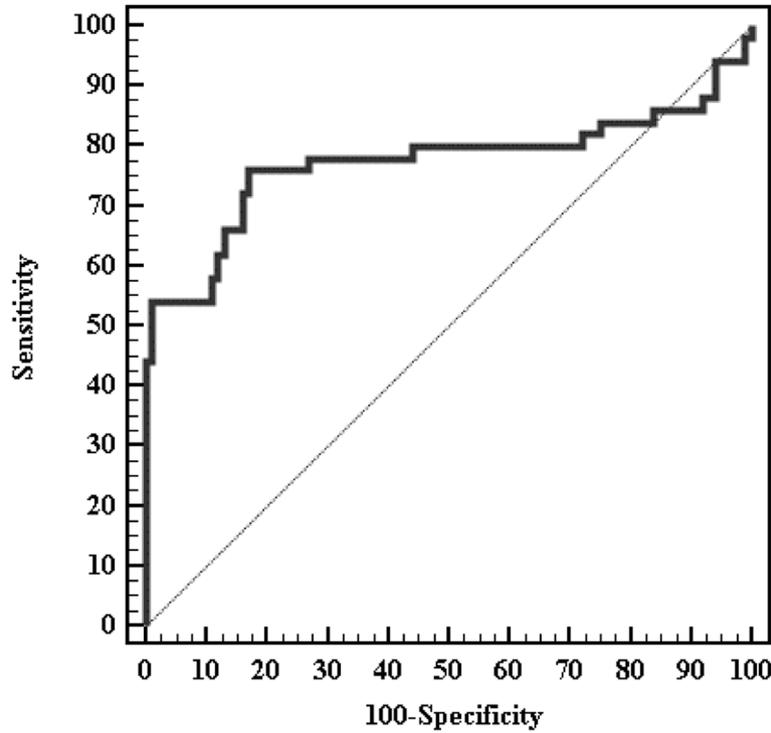
**Fig. 3** ROC curve for miR-23b-3p to predict early-stage HCC patients (group II) from HCV-related cirrhosis (group I)



**Fig. 4** ROC curve for the combination of AFP and miR-331-3p to predict early-stage HCC patients (group II) from HCV-related cirrhosis (group I)



**Fig. 5** ROC curve for the combination of AFP and miR-23b-3p to predict early-stage HCC patients (group II) from HCV-related cirrhosis (group I)



**Fig. 6** ROC curve for the combination of AFP, miR-331-3p, and miR-23b-3p to predict early-stage HCC patients (group II) from HCV-related cirrhosis (group I)

**Table 3** Agreement (sensitivity, specificity) for AFP, miR-331-3p, and miR-23b-3p for diagnosing early-stage HCC patients

	AUC	p	95% CI	Cut-off	Sensitivity	Specificity	PPV	NPV
AFP (ng/ml)	0.665	0.001*	0.557–0.772	> 148	64.0	61.0	45.1	77.2
miR-331-3p	0.703	< 0.001*	0.602–0.803	> 2.18	66.0	61.0	45.8	78.2
miR-23b-3p	0.781	< 0.001*	0.706–0.856	≤ 0.36	80.0	74.0	60.6	88.1
AFP (ng/ml) and miR-331-3p	0.773	< 0.001*	0.678–0.868		76.0	65.0	52.1	84.4
AFP (ng/ml) and miR-23b-3p	0.814	< 0.001*	0.746–0.883		82.0	69.0	56.9	88.5
AFP (ng/ml) and miR-331-3p and miR-23b-3p	0.854	< 0.001*	0.791–0.916		86.0	64.0	54.4	90.1

AUS area under a curve, NPV negative predictive value, p value probability value, PPV positive predictive value, CI confidence intervals

\*Statistically significant at  $p \leq 0.05$

leucine-rich repeat protein phosphatase mediated dephosphorylation of protein kinase B [22]. These results were consistent with Chen et al. [23] results who found that serum miR-331-3p significantly increased in HCC compared to patients with benign hepatic tumors and was associated with poor HCC prognosis. In the current study, comparison between patients with stage 0 HCC and stage A regarding levels of miR331-3p did not show significant difference.

miR-23b-3p showed a significant decrease in the plasma of patients with early HCC compared to cirrhotic patients. These results are consistent with those results reported previously by Cao et al. who stated that miR-23b-3p was downregulated in HCC tissue cells [24]. Despite the miR-23b-3p in tissues has been identified as a diagnostic molecular marker and a possible therapeutic target for patients with HCC [13], its serum concentrations have not been thoroughly researched in many studies before.

On further analysis, the diagnostic sensitivities and specificities of both miR-331-3p and mi-23b-3p had been calculated using the ROC curves. According to the ROC curves data, it was shown that mi-23b-3p had the best sensitivity (80%) and specificity (74%). In a study by Sun et al., miR-23b-3p had better sensitivity compared to miR-331-3p, but contrary to our results miR-331-3p showed the highest specificity [12].

## Conclusion

Serum miR-23b-3p is a more effective predictor than miR-331-3p and AFP for the development of hepatocellular carcinoma in cirrhotic HCV patients. The usual marker used in the clinical practice to predict HCC is AFP, but our results revealed that miR-23b-3p has a greater sensitivity and specificity than AFP. The cost-effectiveness as well as the benefits depends on the price of the kit and the number of the samples to be done.

## Limitation of the study

Small sample size and future studies should be done in large number of patients to verify our results.

## Abbreviation

AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; EMT: Epithelial-mesenchymal transformation; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; miRNAs: Microribonucleic acid; Pyk2: Proline-rich tyrosine kinase 2

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## Authors' contributions

All authors have contributed to the current work. R.A. and M.G. had performed laboratory investigations of the participants, analysis, and interpretation of the data. W.E. contributed substantially to the conception and design of the study, acquisition of data, and analyzed and interpreted the data. M.S. contributed to the acquisition of data, drafted the manuscript, and critical revision of the manuscript. All the authors approved the final version submitted for publication and take responsibility for the statements made in the published article.

## Funding

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

The proposal of the current study was approved by the ethical committee of the Faculty of Medicine—Alexandria University (the approval number is not available). Informed written consent was signed by all participants or their caregivers before the study. The committee's reference number is not available. The current study is original and has not been published elsewhere in any form or language (partially or in full). Results of the current study were presented, honestly, and without fabrication, falsification, or inappropriate data manipulation. No data, text, or theories by others are presented as if they were the author's own ("plagiarism").

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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