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Original paper

Evaluation of miRNA-7, miRNA-10 and miRNA-21 as diagnostic non-invasive biomarkers of hepatocellular carcinoma

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Abstract

Aim of the study: Liver cancer (hepatocellular carcinoma – HCC) remains a serious health challenge; it is the fourth leading cause of death worldwide. Egypt ranks fifteenth worldwide and the third in Africa in terms of HCC burden. The present study aimed to assess some microRNAs (miRNAs) including miRNA-7, miRNA-10, and miRNA-21, serum markers such as cluster of differentiation-14 (CD-14) and transforming growth factor β1 (TGF-β1), and other biochemical parameters as non-invasive tools for HCC diagnosis.

Material and methods: The study included 100 participants divided into five groups: group I (20 normal subjects as a healthy group), group II (20 participants with chronic HCV infection but non-cirrhotic), group III (20 volunteers with chronic HCV infection and compensated cirrhosis), group IV (20 patients with chronic HCV infection and decompensated cirrhosis), and group V (20 participants with HCC). Levels of miR-7, miR-10, and miR-21 were evaluated using qRT-PCR. Serum ALT, AST, total bilirubin, total protein, albumin, PT, INR, and platelet count were determined. FIB-4 and APRI test levels were also calculated. CD-14 and TGF-β serum levels were estimated using enzyme-linked immunosorbent assay (ELISA) kits.

Results: The expression levels of miR-21 followed by miR-10 showed high sensitivity and specificity in predicting HCC. Serum CD-14 and TGF-β1 levels were significantly increased in all patient groups.

Conclusions: From the study, it is concluded that the expression level of miR-21 has the highest sensitivity and specificity, followed by miR-10, which has high sensitivity and low specificity as non-invasive markers for HCC detection, while miR-7 exhibits high sensitivity and reasonable specificity in fibrosis detection.

Key words: liver cancer, liver fibrosis, cluster of differentiation-14, transforming growth factor β1.

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Introduction

Hepatocellular carcinoma (HCC) is a global health problem and is considered the most common primary hepatic malignancy in adults [1]. Egypt ranks fifteenth worldwide and third in Africa in terms of HCC burden [2]. HCC is the sixth most common cancer worldwide, while in Egypt it is the fourth most common cancer; it is also the fourth most common cause of death from cancer worldwide [3]. The major risk factors for HCC are hepatitis B virus (HBV) and hepatitis C virus (HCV) infection [4]. Worldwide, around 35,000 people die from HCV infection each year [5]. Egypt has severely faced liver disease due to chronic HCV, with a high prevalence rate (13-15%) [6].

Discovering non-invasive, sensitive, and specific biomarkers for the early detection of HCC and improving the prognosis of HCC patients is needed [7]. MicroRNAs (miRNAs) are important in normal cellular development and function [8].

The findings that miRNAs can be detected in fluids as free miRNAs or contained within microvesicles such as exosomes create new opportunities in the search for biomarkers in cancer and help with early diagnosis [9].

The profiling of miRNAs in the circulation represents a non-invasive way to investigate disease-specific miRNAs and is a promising alternative method for cancer monitoring [10]. miRNA-7 is described as a tumor suppressor in several cancers including liver; it represses oncogenic molecules, and it is down-regulated in malignant cells [11]. miR-10a is one of the miRNAs that are over-expressed in HCC patients and may be helpful in HCC detection [12]. miR-21 is positively correlated with fibrosis stage [13] and it is considered to be as an oncomiR that influences cell proliferation in HCC [14].

Transforming growth factor β (TGF-β) induces extracellular matrix transition (EMT) in hepatocytes, and it is responsible for activation of hepatic stellate cells (HSC) to myofibroblasts (MFB); both effects contribute to liver fibrosis and may lead to HCC [15]. Cluster of differentiation-14 (CD-14) is highly expressed in inflammation and directly activate HSC, resulting in their proliferation and trans-differentiation into collagen-producing myofibroblasts [16].

The aim of this study was to assess the diagnostic value of serum miR-7, miR-10, and miR-21 beside some other biochemical parameters including CD-14, TGF-β1 and others for early detection of HCC in Egyptian patients.

Material and methods

The present study was performed according to the Research Ethics Committee for Human Subject Research guidelines at the National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt (No. 0031012017, serial 12-2019).

Patient blood samples were collected from the National Hepatology and Tropical Medicine Research Institute. Written informed consent was obtained from all participants after a full explanation of the procedure used.

Study population

One hundred adult participants were included: 80 patients with clinical, biochemical, and sonographic criteria of chronic liver disease and positive serology for HCV antibodies were enrolled in this study; 20 normal subjects were included in group I, serving as the control group.

The patients were divided into four groups: group II (20 patients with chronic HCV infection but without cirrhosis), group III (20 patients with chronic HCV infection and compensated cirrhosis), group IV (20 patients with decompensated cirrhosis and chronic HCV infection), and group V (20 patients with HCC).

The results of the FibroScan were used to diagnose chronic HCV, with or without liver cirrhosis. Patients with HCC were identified by dynamic magnetic resonance imaging (MRI) or triphasic computed tomography (CT), with or without α-fetoprotein (AFP). Criteria for exclusion included all individuals with HIV infection, other forms of malignancy, or other causes of liver illness such as HBV, alcohol, autoimmune disorders, or hemochromatosis.

Laboratory experiments were conducted to test prothrombin time (PT), international normalized ratio (INR), total proteins, albumin, total bilirubin, and the liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT). Additionally, a complete blood count (CBC) was performed, and calculations were made for the fibrosis-4 (FIB-4) score and AST to platelet ratio index (APRI).

RNA extraction and cDNA synthesis

The blood samples were kept in clean glass tubes without additives to clot and then centrifuged at 3,000 rpm for 10 minutes. The serum was then separated into aliquots and stored at –80°C. Purification of total RNA, including small RNAs from 200 µl of serum, was carried out using the miRNeasy Mini extraction kit manufactured by Qiagen (Germany; catalog no. 217004). RNA purity and concentration were assessed by the NanoDrop Spectrophotometer (Qiagen, Germany). Reverse transcription was performed to produce cDNA using the miScript II RT kit (Qiagen, Germany; catalog no. 218161) according to the manufacturer's instructions.

The expression levels of miR-7, miR-10, and miR-21 were evaluated by qRT-PCR analysis according to the instructions from the manufacturer. MiRNA SNORD-68 was used for normalization. The cDNA template was combined with Qiagen's SYBR Green Master Mix for RT qPCR (Catalog no. 218073). A universal reverse primer was used in conjunction with the forward primer sequences; 5'CAACAAAUCACAGUCUGCCAUA for miR-7, 5'UACCCUGUAGAUCCGAAUUUGUG for miR-10, and 5'CAACACCAGUCGAUGGGCUGU for miR-21.

Applied Biosystems' Real Time Cycler (VIIA-7) was used to initiate the cycling program. The following cycles were used: 95°C for 15 min as an initial step, followed by 40 cycles at 94°C for 15 s, 55°C for 1 min, and 70°C for 30 s. The expression of miRNAs was given as Ct value, where the cycle threshold (Ct) is defined as the number of cycles necessary for the fluorescent sig-

nal to cross the threshold of qRT-PCR. Ct was determined by taking the Ct values of miRNA SNORD-68 from the Ct values of the target miRNAs. The resulting miRNA data were calculated relative to SNORD-68. The relative expression of miRNAs was calculated using the formula ($2^{-\Delta\Delta Ct}$) [17].

Statistical analysis

Statistical analysis was carried out using the computer programs Excel and SPSS Statistics software version 25. Arithmetic mean and standard deviation (SD) were used to calculate percent changes in comparison with control values. ANOVA was used to compare the means of different patient groups with the control group, and Pearson's correlation coefficient was used to determine the level of significance. Receiver operating characteristic (ROC) curve analysis was used to determine the cutoff points that yielded the highest sensitivity, specificity, and diagnostic accuracy.

Results

The data in Table 1 revealed a statistically significant increase in the serum levels of AST, ALT, and PT in all patient groups compared to controls. On the other hand, there was a significant decrease in serum total protein, albumin, and platelet count (PLT) in all patient groups compared to the control group. In contrast, the total bilirubin level was significantly higher in the cirrhotic decompensated patient group compared to the control group.

Also, the present study results showed that there was a significant difference between the patient groups regarding a CD-14 levels: the non-cirrhotic group by 62.8%, the compensated cirrhotic group by 111.16%, and the HCC group by 46% in comparison to the control group. Moreover, there was a significant difference in TGF-β1 levels by 71.17% in group II, by 89% in group III, by 44.2% in group IV, and by 41.9% in group V, in comparison to the control group, as presented in Table 2.

Present study data revealed that miR-7 level was upregulated in the non-cirrhotic patient group by 20% more than group III (–26%), group IV (–48%), and group V (–60%) in comparison to the control group. On the other hand, miR-10 level was upregulated in the HCC group by 40% more than group II (–60%), group III (–69%) and group IV (–67%), in comparison to the control group. Also, the expression level of miR-21 was upregulated in the HCC group by 240% more than group II (–89%), group III (–96%), and group IV (–90%), in comparison to the control group, as displayed in Table 3.

Table 1. Characteristics of patients involved in the study

Parameter (mean ±SD)	Groups				
	Group I	Group II	Group III	Group IV	Group V
Age (years)	41.2 ±6	52.4 ±7	53.8 ±4	56.0 ±5	58.1 ±12
AST (U/l)	24.6 ±1.6	59.3 ±10***	59.7 ±16***	55.6 ±23***	112.9 ±36***
ALT (U/l)	25.4 ±2	64.1 ±9***	55.6 ±10***	36.95 ±16**	113.3 ±30*
PT (s)	12.8 ±0.5	13.7 ±0.7*	14.7 ±1.4***	16.5 ±1.9***	14.3 ±1.5***
Total protein (g %)	6.2 ±0.5	5.8 ±0.3**	4.2 ±0.5***	3.4 ±0.6***	3.1 ±0.16***
Albumin (g %)	4.1 ±0.4	3.7 ±0.4**	3.6 ±0.6***	2.7 ±0.3***	2.9 ±0.6***
Platelet count (10^3/µl)	229.7 ±17	191.6 ±46***	123.4 ±38***	118.6 ±53***	186.6 ±20***
Total bilirubin (mg %)	0.4 ±0.1	0.72 ±0.4	1.2 ±0.7	3.8 ±0.5***	1.5 ±0.2

AST – aspartate aminotransferase, ALT – alanine aminotransferase, PT – prothrombin time, SD – standard deviation.
*Statistically significant at: *p ⩽ 0.05, **p ⩽ 0.01, ***p ⩽ 0.001 compared to the control group.*

Table 2. Results of biochemical parameters involved in the study

Parameter (mean ±SD)	Groups				
	Group I	Group II	Group III	Group IV	Group V
CD-14	10.75 ±3	17.5 ±3	22.7 ±5 [a,b]	14.5 ±1	15.7 ±1 [a]
TGF-β1	54.8 ±12	93.8 ±10 [a]	103.6 ±10 [a,b]	79 ±13 [a]	77.76 ±10 [a]

CD-14 – cluster of differentiation-14, TGF-β1 – transforming growth factor-β1, SD – standard deviation. Data with different superscripts are statistically significant at p ⩽ 0.05.
[a] Significant vs. control group, [b] Significant vs. HCC group.

Table 3. Expression levels of some miRNAs involved in the present study

miRNAs (mean ±SD)	Groups			
	Group II	Group III	Group IV	Group V
miR-7	1.2 ±0.2 [b]	0.74 ±0.09	0.52 ±0.06 [a]	0.4 ±0.03 [a]
miR-10	0.4 ±0.07	0.31 ±0.06	0.33 ±0.05	1.4 ±0.9
miR-21	0.11 ±0.03 [a,b]	0.04 ±0.004 [a,b]	0.09 ±0.01 [a,b]	3.4 ±0.3 [a]

miR-7 – microRNA-7, miR-10 – microRNA-10, miR-21 – microRNA-21, SD – standard deviation. Data with different superscripts are statistically significant at p ⩽ 0.05.
[a] Significant vs. control group. [b] Significant vs. HCC group.

Table 4. Significant correlations between different parameters in different patient groups

Parameter	Groups					
	Group II	Group III	Group II	Group V	Group II	Group IV
	miR-21		CD-14		miR-10	
	r	*r*	*r*	*r*	*r*	*r*
miR-7	0.46**	–0.02	–0.28	0.059	0.69***	–0.24
miR-10	0.513**	–0.41	–0.41	0.626***	–	–
TGF-β1	–0.14	–0.47**	0.618***	0.128	–0.22	–0.526**

*miR-7 – microRNA-7, miR-10 – microRNA-10, miR-21 – microRNA-21, CD-14 – cluster of differentiation-14, TGF-β1 – transforming growth factor-β1, r – Pearson's correlation coefficient. Statistically significant at: *p ⩽ 0.05, **p ⩽ 0.01, ***p ⩽ 0.001.*

Table 5. Area under the curve, cut-off value, sensitivity, specificity, and accuracy of miR-21, CD-14, TGF-β1, miR-10, and miR-7 in the HCC patient group

Parameter	miR-21	CD-14	TGF-β1	miR-10	miR-7
AUC	1.0	0.8	0.924	0.3	0.24
Cutoff	1.3	10.3	61.9	0.18	0.17
Sensitivity	100%	71.4%	80%	70%	95%
Specificity	95%	100%	100%	50%	50%
Accuracy	97.5%	80%	87.5%	37%	50%

HCC – hepatocellular carcinoma, miR-7 – microRNA-7, miR-10 – microRNA-10, miR-21 – microRNA-21, CD-14 – cluster of differentiation-14, TGF-β1 – transforming growth factor-β1, AUC – area under the curve.

The data in Table 4 represent the correlation matrix of different miRNAs and other parameters in different patient groups.

Correlations of group II showed that there was a highly significant positive correlation between miR-7 and miR-10, and there was a significant positive correlation between miR-21 and both miR-7 and miR-10. Also, there was a highly significant positive correlation between CD-14 and TGF-β1. On the other hand, CD-14 is significantly negatively correlated with miR-7.

Group III's correlations displayed a highly significant negative correlation between miR-21 and TGF-β1. Also, TGF-β1 was significantly negatively correlated with miR-10 in group IV, while in group V there was a highly significant positive correlation between CD-14 and miR-10.

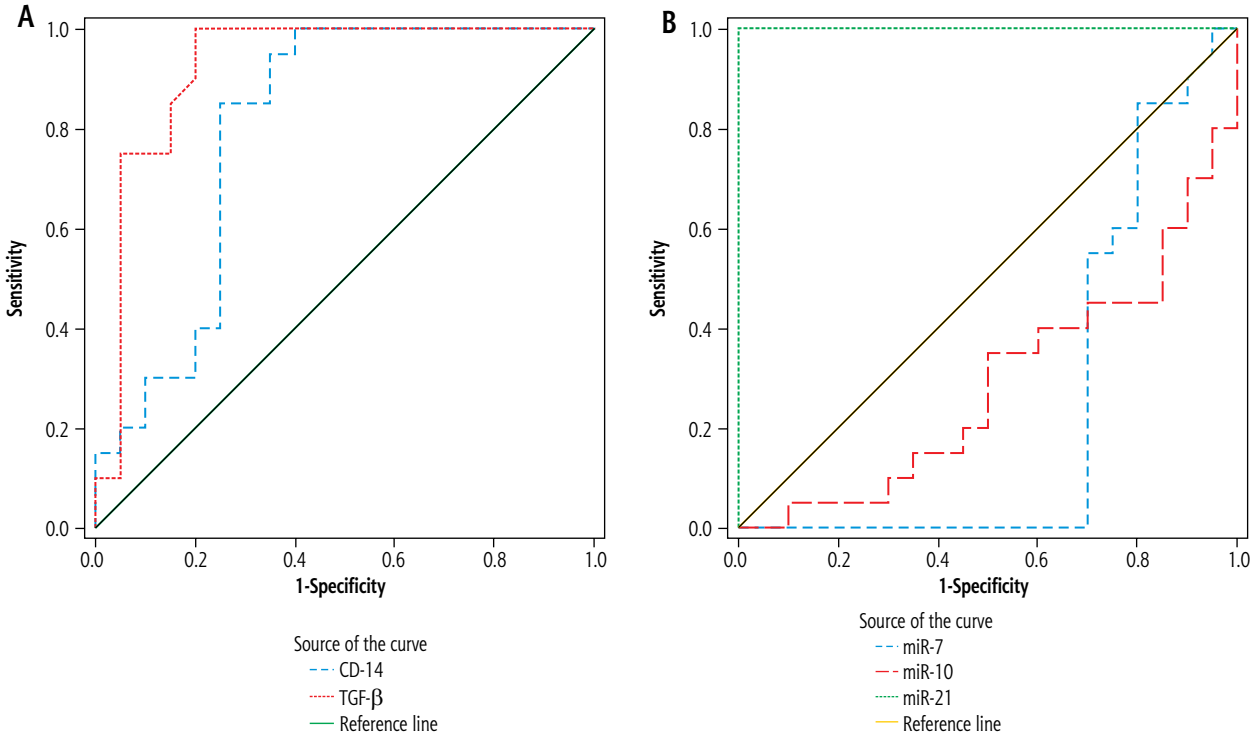
According to the data represented in Table 5 and Figure 1A and B, TGF-β1 showed higher diagnostic performance than CD-14, with an AUC of 0.924 ($p < 0.001$) and 0.8 ($p < 0.001$), respectively, with cut-off values 61.9 and 10.3.

miR-21 showed the highest diagnostic accuracy performance with AUC 1.0 ($p < 0.001$) while miR-10 and miR-7 showed lower diagnostic performance, with AUC 0.3 ($p < 0.001$) and 0.24 ($p < 0.01$) in the HCC group, respectively.

Also, the results of the present study revealed that miR-7 showed moderate diagnostic performance in the non-cirrhotic patient group, as shown in Table 6 and Figure 2A and B.

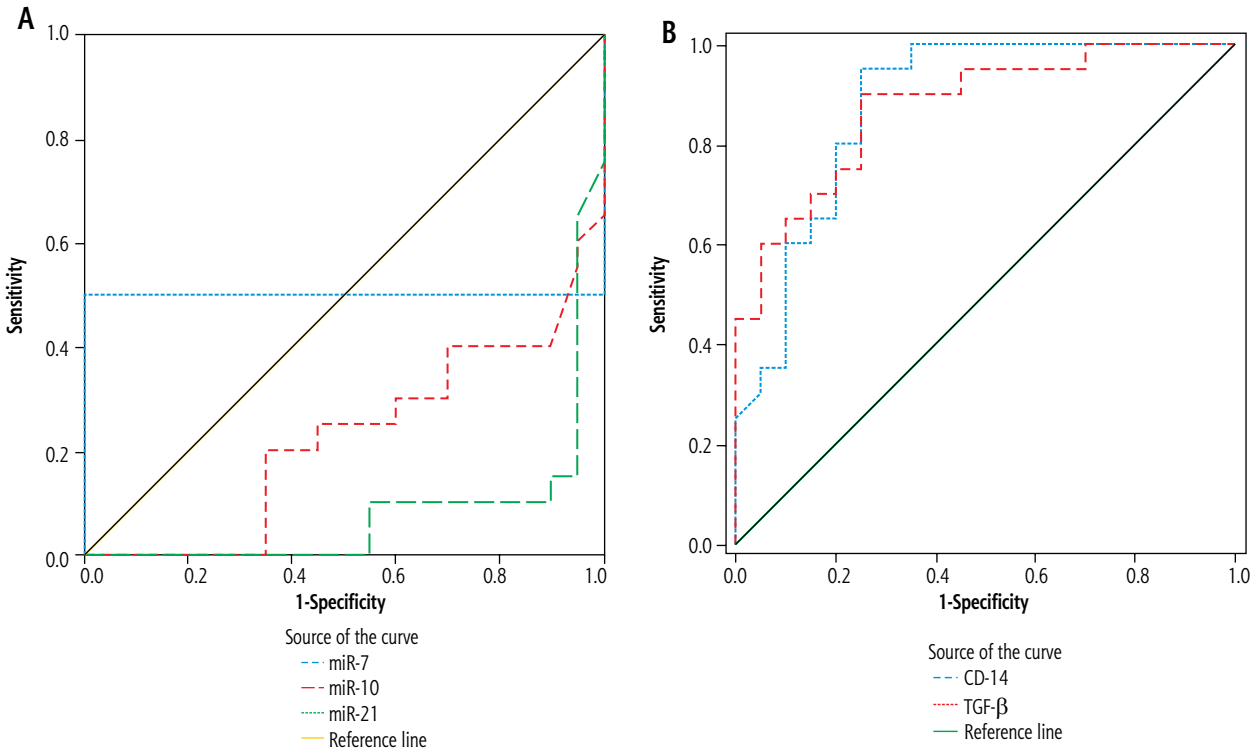
Discussion

Liver cancer is a global health challenge, and it is expected that by 2025, more than one million individuals will be affected by it annually [18]. HCC is an inflammation-associated cancer that accounts for nearly



ROC – receiver operating characteristic, CD-14 – cluster of differentiation-14, TGF-β1 – transforming growth factor-β1, HCC – hepatocellular carcinoma, miR-7 – microRNA-7, miR-10 – microRNA-10, miR-21 – microRNA-21

Fig. 1. A) ROC curve for CD-14 and TGF-β1 in the HCC group. **B**) ROC curve of miR-21, miR-10, and miR-7 in the HCC group



ROC – receiver operating characteristic, miR-7 – microRNA-7, miR-10 – microRNA-10, miR-21 – microRNA-21, CD-14 – cluster of differentiation-14, TGF-β1 – transforming growth factor-β1

Fig. 2. A) ROC curve for miR-21, miR-10, and miR-7 in the non-cirrhotic group. **B**) ROC curve for CD-14 and TGF-β1 in the non-cirrhotic group

Table 6. Area under the curve, cut-off value, sensitivity, specificity, and accuracy of miR-7, CD-14, TGF-β1, and miR-10 in the non-cirrhotic patient group

Parameter	CD-14	TGF-β1	miR-10	miR-7
AUC	0.879	0.875	0.228	0.5
Cutoff	13.2	58.9	0.4	1.0
Sensitivity	79%	78%	36%	100%
Specificity	94%	88%	35%	100%
Accuracy	85%	82%	35%	72.5%

miR-7 – microRNA-7, miR-10 – microRNA-10, CD-14 – cluster of differentiation-14, TGF-β1 – transforming growth factor-β1.

90% of cases [19]. In Egypt, the relationship between HCV and HCC is an important research area [20].

Data of the present study showed that CD-14 levels were significantly increased in all patient groups compared to the control group, which was similar to results reported by other studies. Li *et al.* [21] reported that soluble CD-14 (sCD-14) plays an important role in lipopolysaccharide (LPS) mediated activation of cells lacking membrane CD-14 (mCD-14), such as endothelial, epithelial vascular, smooth muscle, and dendritic cells, through a toll-like receptor (TLR) dependent pathway. It is also involved in human liver disorders, including diseases associated with HBV infection. Additionally, Dash *et al.* [22] stated that human hepatocytes and human hepatoma cells were able to produce and secrete sCD-14 into the serum, and sCD-14 levels in HCV patients were significantly elevated compared with those of healthy controls.

The current study showed that TGF-β1 levels were significantly increased in all patient groups in comparison to the control group; that is in agreement with Wang *et al.* [23], who stated that the TGF-β pathway is reported to be involved in both HCC initiation and progression, and the mRNA levels of TGF-β and its downstream effectors are overexpressed in HCC.

The results of the present study showed that there was upregulation of the miR-7 expression level in the chronic HCV, non-cirrhotic patient group in comparison to the control group; these results were consistent with Singaravelu *et al.* [24], who stated that miR-7 regulates lipid homeostasis in the liver by stimulating the activity of triglyceride synthesis and lipid storage regulators. Additionally, López-Pastor *et al.* [25] stated that overexpression of miR-7 modulates the expression of several genes associated with cholesterol and fatty acid metabolic processes. Therefore, chronic HCV infection is associated with a high prevalence of hepatic steatosis, and the development of steatosis is linked to the virus disturbances, as mentioned by Modaresi *et al.* [26].

Current study data showed upregulation of miR-10 level in the HCC patient group compared to the control group; this agrees with Wang *et al.* [27], who reported that miR-10 was upregulated in HCC patients. This may indicate that miR-10 exerts an oncogenic function by downregulating one or more of the tumor suppressor genes, as stated by Barbato *et al.* [28].

Additionally, the present study results revealed that miR-21 was upregulated in the HCC patient samples but not in other patient groups in comparison to the control group, which is consistent with Ratnasari *et al.* [29], who stated that circulating miRNA-21 can predict progression of liver cirrhosis to HCC, and that miR-21 levels were higher in HCC than in chronic hepatitis patients and healthy controls. Qu et al. [30] also stated that it has a better diagnostic sensitivity than AFP, and found a decrease in miR-21 serum levels following tumor resection.

There are some limitations that need to be addressed regarding the present study. First of all, the sample size of the study is limited, and additional large scale studies are needed to confirm these findings. Secondly, further studies are required to elucidate the mechanisms of these miRNAs in HCC development.

Conclusions

From the current study results, it can be concluded that miR-21 has the highest diagnostic value as a non-invasive marker for HCC with high sensitivity and high specificity, followed by miR-10, while miR-7 exhibits high sensitivity and reasonable specificity in fibrosis detection.

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Disclosure

The authors declare no conflict of interest.

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