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Circulating microRNA-221 as a diagnostic biomarker for hepatitis C virus-related hepatocellular carcinoma

Awatef M. Awwad [1], Doaa A. El Wazzan [2], Ayat M. Salem [1], Sara L. Asser [1]

1- *Medical Microbiology and Immunology Department, Faculty of Medicine, Alexandria University, Egypt.*
2- *Tropical Medicine Department, Faculty of Medicine, Alexandria University, Egypt.*

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the fifth frequently diagnosed cancer globally and the third leading cause of cancer death estimated by the world health organization (WHO). Hepatitis C virus (HCV) plays a crucial role in pathogenesis of HCC. According to WHO, around 55–85% of HCV patients will develop chronic hepatitis, resulting in progressive fibrosis and cirrhosis. Up to date, no biomarkers have been proved to diagnose HCC early, and the majority of cases are diagnosed with poor prognosis at late stages. For several pathological conditions, microRNAs (miRNAs) have been recognized as a biomarker, and recently HCC is one of them. **Aim:** The aim is to study the possible advantage of serum miR-221 as a diagnostic biomarker of HCV-related HCC. **Methods:** The study was performed on 40 patients with chronic HCV and ten healthy control subjects. There were two groups of patients: HCC and cirrhosis. In addition to evaluating the serum miRNA-221 expression level by RT-PCR, all patients and controls were assessed clinically and subjected to laboratory investigations. **Results:** MiRNA-221 exhibited a substantial increase in both HCC and cirrhosis *(P = 0.005*) compared to normal controls. Receiver operating characteristic (ROC) curve analysis was made to evaluate the potential effectiveness of miR-221 to discriminate between HCC patients and non-HCC. miR-221 showed 85% sensitivity and 40% specificity. **Conclusion:** The study concluded an excellent possibility of serum miR-221 to be classified among the biomarkers of early HCC development. This can modify liver carcinogenesis and can be used to improve preventive, diagnostic, and therapeutic strategies in HCC.

Introduction

The World Health Organization (WHO) identified hepatocellular carcinoma (HCC) as the fifth most prevalent cancer and the third leading cause of cancer death worldwide. Hepatocellular carcinoma was typically diagnosed in symptomatic patients at a progressive level until the beginning of the 1990s. However, several factors help to increase the focus for HCC patients. These factors include improved screening tests for high-risk patients, a confirmed diagnosis made by new high-resolution imaging modalities with a decrease in the need for tissue pathology [1-4]. Despite the huge advancements in the diagnostic tools and treatment modalities, the incidence of HCC is up surged, and the 5-year survival rate is still poor (around 18%). The prognosis of HCC is mainly affected by the liver function status that depends mainly on the degree of cirrhosis. This highlights the urged significance for earlier detection and diagnosis of HCC [5-7].

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* *Corresponding author:* Sara Lotfy Asser
E-mail address: : Sara.asar@alexmed.edu.eg

Considering the significant threat of hepatitis C virus (HCV) infections to public health, the Global Strategy for Hepatitis C Virus (GHSS) calls for eliminating viral hepatitis, aiming by 2030 to decrease the new infections and the related mortality by 90 %, and 65 %, respectively. The WHO reported that about 71 million individuals worldwide chronically infected by HCV, and 700,000 individuals die annually due to complications of HCV, such as liver cirrhosis and HCC. The highest HCV burden is in Africa, followed by Asia, where the HCV seroprevalence is 2.8% and 2.7%, respectively [8].

In Egypt, the National Cancer Registry Program (NCRP) ranked liver cancer as the most frequent cancer in males (33.6%) and second most frequent among females (13.5%). This is due to high HCV and hepatitis B virus (HBV) prevalence and complications [9,10]. Research in Egypt suggested raising the association between the HCC and HCV infection (40-50% of cases) in comparison to the decreasing association with HBV by 25% and HBV/HCV infection by 15%. Improved surveillance plans and diagnostic tools, besides a higher rate of survival of cirrhotic patients, permitting time to develop HCC, could explain the increasing incidence of HCC in Egypt [11,12].

Unfortunately, until the day, no biomarker was proved for an early confirmed diagnosis of HCC. Subsequently, most of the cases are discovered lately at progressive stages of the disease leading to poor prognosis. Despite the low fair sensitivity and specificity of Alpha-Fetoprotein (AFP) about the HCC detection, AFP is the most frequently used HCC-detection screening biomarker. In addition, since 2010, the American Association for the Study of Liver Diseases (AASLD) guidelines opposed the use of AFP for both HCC diagnosis and screening [13-15].

Nowadays, new imaging techniques have a crucial role in the diagnosis of HCC. There have been many advances in imaging techniques regarding better visualization and discrimination of different hepatic lesions with a broader array of options available for HCC diagnosis [16]. The only role of liver biopsy is the diagnosis of a focal hepatic lesion less than 2 cm in diameter that does not show the characteristic radiological feature of HCC. However, the diagnosis of HCV-related disease progression is challenging due to a lack of reliable biomarkers and invasive liver biopsy nature. Accordingly, an alternative sensitive, less invasive, and dependable biomarker tool is strongly needed [17].

MicroRNAs (miRNAs) are small non-coding RNA segments of nearly 22 nucleotides, regulating gene expression after transcription. miRNA has an essential role in cellular proliferation, differentiation, apoptosis, as well as carcinogenesis process [3]. In addition, miRNAs directly or indirectly govern one-third of human genes. Several studies recently proved that miRNA expression levels are linked to the onset of cancer and other diseases [18].

MicroRNAs are suggested to be essentials in cardiovascular diseases, neurological disease, immunological disease, obesity, diabetes, development of liver diseases, osteogenic diseases, kidney diseases, AIDS, and several types of malignancies. In HCC, there is a deregulation of the circulating miRNAs, up- or down-regulation in neoplastic cells. Hence, MiRNAs are developing as new, easily measured biomarkers for early HCC detection. The clinical applications of miRNA as biomarkers in cancer include early detection and identification of the tissue of origin of tumor cells, subclassification of tumors, and predictive markers for the disease's course and response to treatment [19].

In several tumors, including prostatic cell carcinoma, glioblastoma, pancreatic cancer, papillary thyroid tumors, and cancer of urinary bladder, miR-221 is reported to be overexpressed in such tumors. In HCC, miR-221 overexpression is reported to initiate cancer cell proliferation as miR-221 is able to inhibit the cyclin-dependent kinase inhibitors CDKN1B/p27 and CDKN1C/p57 expression. These kinase inhibitors are considered significant regulators of cell cycle progression, and its inhibition is linked to HCC patients' poor prognosis [20]. On the basis of target genes, miRNAs can act as oncogenes or tumor-suppressor genes. Current data demonstrate the possible clinical usefulness of miRNAs as diagnostic, prognostic, and predictive indicators for aggressive and metastatic tumors [21].

Interestingly, the detection of miRNAs in human serum and plasma is reported denoting the stability and protection of the markers from endogenous RNase enzyme activity [22]. Such findings could propose the circulating miRNA as possible biomarkers for both the diagnosis and prognosis of several diseases, including cancer. Circulating miRNA signatures can be identified in either serum or plasma [23].

The current study's objective was to assess the circulating serum miR-221 expression levels in HCC patients and in HCV-related chronic liver cirrhosis patients to exhibit its possible role as a less-invasive marker for HCV-related HCC diagnosis.

Materials and Methods

This case control study included 40 chronic HCV patients (positive for anti-HCV antibodies and HCV RNA for at least six months) attending Tropical Medicine Department in Alexandria Main University Hospital. The 40 patients were divided into two groups: group 1 = patients having chronic HCV infection and HCC (n = 20) and group 2 = patients having chronic HCV infection and cirrhosis (n = 20). A control group of 10, age and sex-matched, healthy volunteers (with normal liver enzymes, normal hepatic ultrasound, and negative for HBV, HCV, and HIV) were also included. Patients excluded from the study were those with associated chronic HBV infection, other recognizable causes for chronic hepatitis than HCV, other concomitant cancers than HCC, and organ transplantation.

Clinical data of study subjects were obtained, including routine lab investigation as CBC, Liver function tests (AST, ALT, serum albumin, serum bilirubin, prothrombin time, INR), renal function tests (serum creatinine, blood urea), and alfa-feto protein. Imaging techniques by US abdomen revealed focal lesions confirmed by triphasic CT and/or MRI. All cirrhotic patients were categorized by Modified Child-Pugh Classification to for assessment of liver disease severity [24]. Patients with HCC were categorized by Barcelona Clinic Liver Cancer (BCLC) algorithm [25]. Three ml of blood were collected from all study subjects. Sera were separated by centrifugation and stored at −80˚C until processed.

Molecular techniques

Real-time RT-PCR for miRNA was quantified for the detection of miRNA-221. Expression levels RNU6B was used as a housekeeping gene for all miRNA in this study [26,27].

1. **Total isolation of RNA** from serum samples was made using Qiagen® miRNeasy Mini Kit.
2. **Complementary DNA (cDNA) was manufactured** using TaqMan ®MiRNA Reverse Transcription Kit with miRNA-specific primers (Applied Biosystems, USA).
3. **Quantitative Real-time PCR** was conducted by Applied Biosystems StepOne™ Real-Time PCR System using TaqMan® MiRNA-221 and RNU6B Assay, TaqMan® 2× Universal PCR Master Mix. Reagents were purchased from Applied Biosystems, USA.
4. **Expression of miRNA-221** was calculated using the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$).

Reverse transcription

The specific cDNA of miRN221 and RNU6B were synthesized from RNA using gene-specific primers according to the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Each Reverse transcriptase reaction (15 µl reaction volume) consisted of 7 µl Master Mix (100 mM dNTPs, 50 U/µl MultiScribe Reverse transcriptase, 10× Reverse transcriptase buffer, 20 U/µl Rnase inhibitor), 3 µl primer, and 5 µl RNA sample. The 15-µl reaction volumes were incubated in Applied Biosystems Cycler StepOne™ Real-Time PCR for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C, and then held at 4˚C [28].

MiRNA amplification

Real-time PCR was performed using Applied Biosystems Step One real- time PCR system. 5′ Nuclease reaction for target (miRNA-221) and endogenous control (RENUB6) were performed in separate tubes. Each PCR reaction mixture included 10 µl TaqMan 2× Universal PCR Master Mix, No AmpErase UNG[a]., 1 µl of TaqMan MiRNA Assay (20×), 1.4 µl cDNA product, and 7.6 ul nuclease-free water to a final volume of 20 µl. PCR cycling conditions started with an initial enzyme activation cycle of 10 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds each then 40 cycles of annealing/extension at 60°C for 60 seconds [28].

Relative quantitation of target MiRNA expression = $2^{-\Delta\Delta Ct}$

The $2^{-\Delta\Delta CT}$ method was used to determine relative-quantitative levels of individual miRNAs. If the relative quantification of miRNA-221 >1.0, this was considered a high expression of cancer relative to the control, while relative quantification of miRNA-221 <1.0 was considered a low expression of cancer relative to the control [29].

Results

The studied subjects were divided demographically after random inclusion in the study into 18 males (90.0%) and two females (10.0%) with HCC, while the group of cirrhotic patients involved 15 males (75.0%) and five females (25.0%). The

control group included 6 males (60.0%) and 4 females (40.0%). No statistically significant difference was found between the three groups concerning gender. The mean age of patients with HCC was 57.2 ± 5.9 years (median=57.2), while the mean age of cirrhotic patients was 55.65 ± 9.96 years (median =55.0). The control group age had a mean value of 55.60 ± 8.69 years (median=56.50). No statistically significant difference was recorded between the three groups regarding age. According to Child classification, 55 % of the HCC group patients were in Child A stage,40% were in Child B, and 5% were in child C. In the cirrhotic group, 25% of patients were in Child A stage, 50% were in Child B, and 5% were in Child C. There was no statistically significant difference between both groups. According to BCLC classification patients with HCC were classified as follows: 4 patients (20.0%) were in very early stage (0), 7 patients (35.0%) were in early-stage (A), 5 patients (25.0%) were in intermediate stage (B), 2 patients (10.0%) were in advanced stage (C) and 2 patients (10.0%) were in terminal stage (D).

Studying the alpha feto-protein (AFP) in the three studied groups

There was a statistically significant difference between the three groups regarding AFP ($p<0.001$). A statistically significant difference was detected between the group of cirrhotic patients in relation to the group of healthy persons ($p_3<0.001$) as well as between the group of patients with HCC in relation to the group of control ($p_2<0.001$). A statistically significant difference between the HCC patients' group and the cirrhotic patients' group was also detected ($p_1<0.001$) **(Table 1)**.

Diagnostic performance of AFP in the detection of HCC in cirrhotic patients

In order to identify the diagnostic performance of serum AFP in detecting HCC, Receiver operating characteristic (ROC) curve analysis was conducted. AFP was shown to have a moderate correlation with HCC detection, as AFP AUC value was 0.573. The estimated AFP cut off value was 8 ng/ml for the predicted probability of HCC detection. The AFP cut-off value had a 60.0% sensitivity, a 65.0 % specificity, positive predictive value (PPV) of 63.2%, and negative predictive value (NPV) of 63.2.

MicroRNA-221 expression levels in HCC and cirrhotic patient

MicroRNA 221 was overexpressed in HCC patient relative to cirrhotic patient. Expression of miRNA 221 in HCC patients ranged from 0.55 – 50.04, with a median of 2.69. In cirrhotic patients ranged from 0.11 – 19.49 with a median of 1.66. HCC patients showed significantly higher expression of miRNA 221 in comparison with cirrhotic patients ($P=0.019^*$) **(Figure 1)**.

Diagnostic performance of microRNA-221 expression level (sensitivity, specificity) in the detection of HCC in cirrhotic patients

Receiver operating characteristic curve analysis for miRNA-221 expression levels in patients with HCC and cirrhotic patients showed that the AUC value was 0.758, at a cut-off value of >1.0317 with 85% sensitivity and 55 % specificity **(Table 2, Figure 2).**

Correlation between miRNA-221 expression levels and clinical and laboratory findings

No correlation was reported between miRNA-221 expression levels and any routine laboratory tests in both HCC and cirrhotic patients **(Table 3)**.

Table 1. Comparison between the studied groups according to alpha feto-protein.

	HCC (n = 20)	**Cirrhotic (n = 20)**	**Control (n = 20)**	$^{KW}\chi^2$	***p***
AFP (ng/ml)					
Min. – Max.	59.0 - 1210.0	6.50 - 45.0	5.0 - 10.0	50.470*	<0.001*
Median	446.0	20.0	8.0		
Sig. bet. Groups	$p_1<0.001^*$, $p_2<0.001^*$, $p_3<0.001^*$				

$^{KW}\chi^2$: Chi square test value for Kruskal Wallis test. Sig. bet. groups was done using Mann Whitney test.
p_1: p value for comparing between HCC group and cirrhotic group.
p_2: p value for comparing between HCC group and control group
p_3: p value for comparing between cirrhotic group and control group
*: Statistically significant at $p \leq 0.05$.

Table 2. Diagnostic performance of microRNA-221 expression level in the detection of HCC in cirrhotic patients.

	AUC	*p*	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
Fold change miRNA 221	0.758*	*0.019**	0.557 – 0.876	>1.0317	85.0	55.0	58.6	72.7

AUC: Area Under a Curve. p value: Probability value.
CI: Confidence Intervals.
NPV: Negative predictive value. PPV: Positive predictive value.
*: Statistically significant at $p \leq 0.05$

Table 3. Correlation between miRNA-221 expression levels and clinical and laboratory findings.

	Fold change of miRNA 221			
	HCC		**Cirrhotic**	
	r_s	***p***	**r_s**	***p***
Age (years)	-0.171	*0.472*	-0.289	*0.216*
ALT (U/L)	-0.142	*0.551*	0.302	*0.196*
AST (U/L)	-0.006	*0.980*	0.174	*0.464*
Albumin (g/dl)	0.302	*0.196*	-0.254	*0.280*
Total Bilirubin (mg/dl)	0.139	*0.559*	-0.137	*0.564*
BUN (mg/dl)	0.053	*0825*	-0.158	*0.507*
Creatinine (mg/dl)	0.009	*0.968*	0.338	*0.145*
Hb (g/%)	0.232	*0.324*	0.024	*0.921*
WBCs (10^3/cmm)	-0.426	*0.061*	-0.013	*0.957*
PLT (10^3/cmm)	-0.111	*0.640*	0.174	*0.464*
PT (seconds)	-0.190	*0.422*	-0.128	*0.591*
PA (%)	0.103	*0.665*	0.095	*0.690*
INR	0.167	*0.481*	-0.256	*0.276*
Child Score	-0.340	*0.143*	0.129	*0.588*
BCLC Stage	-0.138	*0.562*		
AFP	-0.082	*0.731*	-0.028	*0.907*

r_s: Spearman coefficient. *: Statistically significant at $p \leq 0.05$.

Figure 1. Comparison between the two studied groups according to fold change of miRNA.

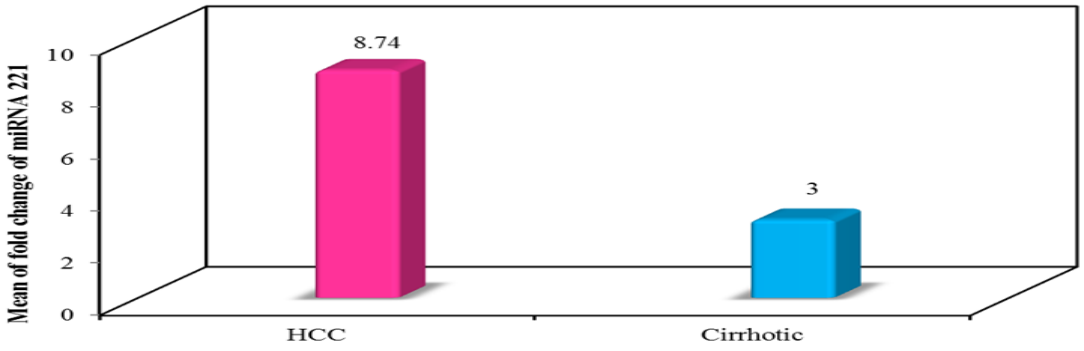
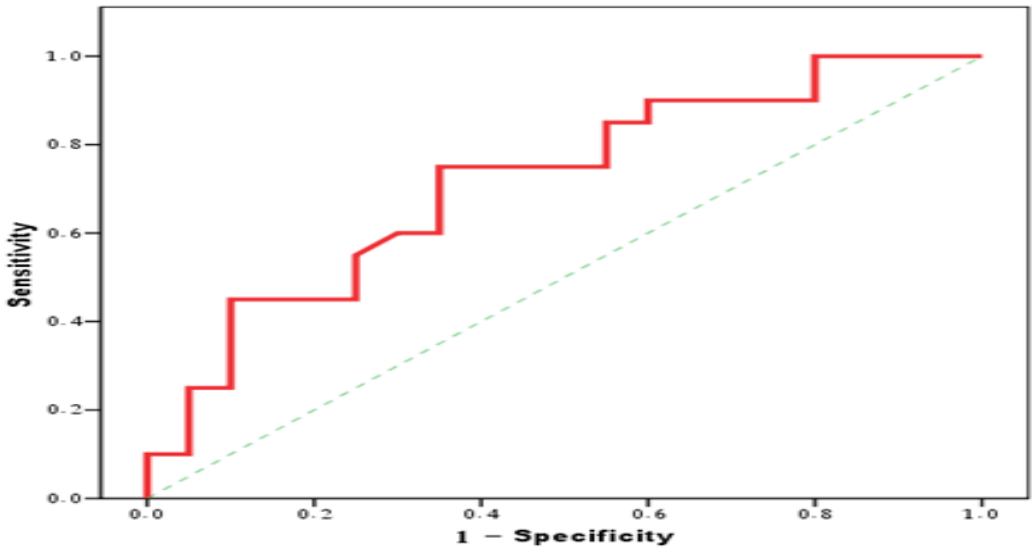


Figure 2. ROC curve of fold change of miRNA 221 to diagnose HCC cases (vs cirrhotic).



Discussion

The cornerstone for a good prognosis of HCC is the early detection of the disease. In the early stages, HCC inclines to be invasive, but with no apparent symptoms. Accordingly, most HCC patients are presented in late stage with the non-operable disease. Curative treatments of HCC such as liver transplantation, resection, or local ablative therapy are very effective and prolong survival provided early disease detection. Therefore, many current researchers try to identify novel HCC biomarkers for early detection and therapeutic monitoring of the disease [6].

Up to date, HCC diagnosis is dependent on clinical information, liver ultrasonography, serum AFP measurement at 6-12 months' intervals. However, very small tumors (HCC < 2 cm) could not be detected by any of these investigations. The AFP has low sensitivity in HCC diagnosis, particularly at the beginning of the disease. Besides, AFP may show false-positive results with non-malignant chronic liver disease. Hence, for early HCC detection, finding an early, sensitive, highly specific HCC biomarker is a must [1].

In this study, the mean age in the HCC group was 57.2±6 years, with no significant difference between the HCC group and the other two groups. This result agrees with what was found by **Li et al.** who reported that the mean age was 56 years, and **Qin et al.** who reported a mean age of 52 years [30,31].

In the present study, serum AFP level assessment range from 59.0 to 1210.0 ng/ml with a median value of 446.0 ng/ml in the group of patients with HCC, while in the cirrhotic group, its range was from 6.50 to 45.0 ng/ml with a median value of 20.0 ng/ml. AFP ranged from 5.0 to 10.0 ng/ml, with a median value of 8.0 ng/ml in the control group. A significant statistical difference was detected between the three groups regarding AFP ($p<0.001$). Similarly, **Azab N et al.** found that serum AFP with a mean of 118.56 ± 67.30 in patients with HCC, a median of 31.42 ± 12.21 in cirrhotic patients in the control group serum AFP had a median of 3.45 ± 1.21. A significant statistical difference was detected at $p<0.001$ [32].

ROC curve analysis was performed to assess serum AFP's diagnostic performance in detecting HCC in cirrhotic patients. A cut-off value of 8 ng/ml: AFP had a 60.0% sensitivity and specificity 65.0 %. The AUC was 0.573. This result agrees with AASLD and EASL that do not recommend serum AFP assessment as a diagnostic tool anymore [15,24].

The present study demonstrated that analysis of substantial increase in the expression level of miR-221 in patients' sera in in relation to the normal control group showed a significant fold increase in both HCC and cirrhotic groups. Also, serum miRNA-221 levels were significantly elevated in patients with HCC compared to patients with cirrhosis. ROC analyses for the diagnostic power of serum miRNA-221 yielded an AUC of 0.785 with 85% sensitivity and 55% specificity at a cut off 1.75

in differentiating patients with HCC from the cirrhotic group.

In agreement with the study, many previous reports have demonstrated that miRNA221 levels are considerably higher in HCC patients carrying the C virus than normal control. **Li et al.** and **Xin et al.** reported in the HCC patients' sera, the miR-221 expression is significantly high [30,33].

Contradictorily, **El-Garem et al.** established down-regulation of miR-221 in HCC in comparison to normal control (median =0.92). There was a statistically significant fold decrease in serum miR-221 levels of HCC patients compared to cirrhotic, while this agrees with these results as it showed a significant increase in miR-221 expression level in cirrhosis groups compared to the normal control group (median=3.4). At a cut-off of 1.82 folds, miR-221 yielded 87% sensitivity and 40% specificity in differentiating HCC from cirrhotic patients which agree with this study [34].

In contrast to these results, miR-221 was significantly downregulated in cirrhotic group in relation to normal control group (median=0.34) as with **Zekri et al.** but it conforms with the finding that miR-221 was significantly upregulated when comparing HCC group (median=1.4) to cirrhotic and control group. ROC analyses for the diagnostic power of serum miRNA-221 yielded an AUC of 0.775 in differentiating patients with HCC from cirrhotic group which agree with this study [28]. The difference can be ascribed to heterogeneous choice of internal or exogenous control genes/miRNAs. The current study used the housekeeping internal control REU6B recommended by Qiagen, detecting its expression as a standard reference gene.

In the current study, serum miRNA-221 level had a low specificity as a biomarker of HCC.

As mentioned before, other forms of cancers, such as Pancreatic Cancer, Papillary Thyroid Tumors, glioblastoma, urinary bladder cancer, and prostate carcinoma cell lines, were confirmed to have elevated serum miRNA-221 levels, which are thought to be part of the miRNAs expression in a range of cancers. Thus, even though serum miRNA-221 is an early sensitive biomarker for HCC, it is not specific as high serum miRNA-221 levels could be detected in different cancers. The combination of serum miRNA-221 and other HCC-specific tumor markers such as AFP may be of benefit to resolve this limitation.

Conclusion

Serum miRNA-221 expression level is a more sensitive biomarker in the diagnosis of HCC than the traditional marker, AFP. Therefore, serum miRNA-221 can be considered as a useful additional biomarker, with AFP for the screening of HCC among patients with liver cirrhosis. MiRNA's in peripheral blood exhibit great consistency and reproducibility. MiRNA's could be used in screening and early diagnosis of cancer as well as in assessing the malignant tumor for proper choice of suitable management.

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Conflict of interest

All authors state that there is no conflict of interest or financial or personal relationships with other people or organizations that could inappropriately influence (bias) the authors' actions.

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