RESEARCH ARTICLE

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Role of *miR-15b* and *miR-130b* as Non-Invasive Biomarkers in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed cancers, and the second cause of cancer-related deaths worldwide. MicroRNAs play a role in regulating gene expression transcriptionally and post-transcriptionally. Accordingly, they can affect cancer risk. This study aimed to highlight the diagnostic role of *miR-15b* and *miR-130b* as non-invasive biomarkers in Egyptian HCC patients. **Materials and Methods:** This study included 70 patients with confirmed HCC, 35 cirrhotic patients with chronic hepatitis B or C virus infection, and 35 apparently healthy persons. Plasma *miR-15b* and *miR-130b* levels were measured by TaqMan RT-PCR assay. **Results:** Significant higher expression levels of plasma *miR-15b* and *miR-130b* were observed in HCC patients compared to hepatitis group or control group or the non-malignant group (P <0.0001 each). Combining *miR-15b* with AFP markedly raised the sensitivity to 91.4%, while combination of *miR-130b* with AFP significantly improved the specificity to 97.1 %. Combining the three markers showed excellent discriminatory power with AUC 0.959 and overall diagnostic accuracy 92.6% for differentiation between HCC from the non-malignant cases. **Conclusion:** MiR-15b and *miR-130b* could serve as possible biomarkers for HCC detection, and combining these markers with AFP could markedly improve the diagnostic accuracy for HCC.

Keywords: MicroRNAs- *MiR-15b*- *MiR-130b*- HCC

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Introduction

Worldwide, hepatocellular carcinoma (HCC) is regarded as one of the most commonly diagnosed cancers. According to recent epidemiological data, it is the seventh most common cancer globally and the second most common cause of cancer-related deaths with 830,000 deaths annually [1]. In Egypt, it is considered the fourth most prevalent cancer, ranking Egypt the third country in Africa and 15th worldwide [2]. Several studies showed an increase in the incidence of HCC in Egypt. Among the factors that contributed to the increased incidence are the improved screening programs and diagnostic tools, the higher survival rate of cirrhotic patients that raises the risk of developing HCC, and the increased incidence and complications of hepatitis C virus (HCV) [3, 4].

HCC typically occurs in the context of cirrhosis, and factors such as chronic viral hepatitis either hepatitis B (HBV) or hepatitis C (HCV) viruses, alcohol-induced damage, non-alcoholic fatty liver disease, exposure to aflatoxins, and genetic predisposition all contribute to the carcinogenesis process [5]. The 5-year survival rate for advanced-stage HCC is still low despite recent significant advancements in the treatment of the disease. This may be because of its late diagnosis, resistance to anticancer medications, and high recurrence rate [6].

Despite having low sensitivity and specificity, serum AFP has been the only validated and the most widely used serological biomarker for HCC detection and surveillance for decades [7]. Consequently, to enhance outcomes, a deeper comprehension of the aetiology of HCC and the development of precise diagnostic tools is of great need.

Protein-coding genes and several classes of structurally and functionally distinct noncoding RNAs are among the altered genomic and transcriptional landscapes linked to carcinogenesis. Noncoding RNAs, which are not translated into proteins, make up at least 90% of the human genome. MicroRNAs (miRNAs) are a highly conserved class of small noncoding RNAs that range in length from approximately 18 to 24 nucleotides. They play a role in regulating gene expression at two different levels: transcriptionally, by binding to target gene promoters, and post-transcriptionally, by degrading target mRNAs and/or preventing their translation by binding to the 3′-untranslated region (3′UTR) of target mRNAs [8].

A wide variety of significant biological activities are

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linked to miRNAs, such as development, differentiation, proliferation, and apoptosis. Abnormal miRNA expression is frequently found in a variety of human cancers, including hepatocellular carcinoma (HCC). Increasing research indicates that miRNAs function as tumor suppressors or oncogenes depending on the roles of their target genes [9].

Among miRNAs, miRNA-15b (*miR-15b*) is a member of the miR-15/16 cluster, along with miR-15a, miR-195, and miR-16-1/2 [10]. Previous research has revealed that *miR-15b* is increased in a number of cancer types, indicating its critical role in the development and spread of tumors as an oncogene. However, in other types of cancers, *miR-15b* functions as a tumor suppressor. These results imply that *miR-15b* may have distinct roles in different types of cells [11]. A number of malignancies have been linked to the carcinogenesis of miRNA-130b (*miR-130b*), which is found on chromosome 22q11. It has been demonstrated that *miR-130b* increases cell invasion and migration in glioma, esophageal cancer, bladder cancer, and Ewing sarcoma. Furthermore, *miR-130b* was reported to promote the proliferation of tumor cells by suppressing apoptosis in renal cell carcinoma and lung cancer. Also, upregulation of *miR-130b* has been linked to increased angiogenesis and epithelial-mesenchymal transition in colorectal cancer [12].

This study aimed to evaluate the role of *miR-15b* and *miR-130b* as non-invasive biomarkers in a group of Egyptian HCC patients using quantitative real-time polymerase chain reaction (qRT-PCR), as well as studying their correlation with another well-documented HCC marker (alpha fetoprotein) for a possibly higher diagnostic accuracy through improved sensitivity and/ or specificity.

Materials and Methods

Study Participants

This study is a case-control study that was conducted over a period of consecutive 9 months from January 2024 to September 2024.This study was performed on a total number of 140 Egyptian participants, divided into 3 groups.

Group (I) included 70 newly diagnosed HCC patients at different disease stages, recruited from the outpatient clinics at National Cancer Institute (NCI) hospital, Cairo University (mean age 61.3 years ±10 SD), they were 55 males (78.6%) and 15 females (21.4%). Patients were diagnosed according to the guidelines proposed by the American Association for the Study of Liver Diseases (AASLD) [13]. Samples were obtained from all patients prior to any therapeutic or surgical intervention.

Group (II) included 35 cirrhotic patients with chronic hepatitis B or C virus infection, presented to the Tropical Medicine Department, Kasr Alainy Hospital, Cairo University (mean age 53 years ±8.9 SD), they were 21 males (60%) and 14 females (40%), 27 patients (77.1%) were HCV while 8 patients (22.9%) were HBV positive. HBV and HCV infection were confirmed by performing HBsAg and anti-HCV antibodies by ELISA method, while cirrhosis was diagnosed based on clinical, biochemical and radiological evidence. All cirrhotic patients underwent regular ultrasonographic screening (every 4–6 months) to exclude the development of any hepatic nodules.

Group (III) included 35 age- and sex-matched apparently healthy individuals as controls. They had no prior history of chronic liver problems. Hepatitis markers were performed to exclude the seropositivity for HBV and HCV (their mean age was 50.1 years ±7.5 SD; they were 21 males (60%) and 14 females (40%).

The study was permitted by the Institutional Review Board (IRB) of the NCI, Cairo University, according to the Helsinki guidelines of studies performed on human beings, approval no. CP1511-203-011-100, a written consent was obtained from all study subjects before enrollment in the study.

Methodology

All patients and controls were subjected to: full history taking and clinical examination, ordinary biochemical investigations including alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin, and serum albumin. All were done on AU680 autoanalyser. Prothrombin concentration and INR were done using automated coagulometer Sysmex CA 1500. Viral markers (HBsAg and anti-HCV antibodies) were tested using ELISA method according to manufacturer's instructions. AFP was performed using Architect by Chemiluminescence Immunoassay (CLIA) technology. Expression levels of miRNA-15b and miRNA-130b were detected by qRT-PCR. Radiological investigations included ultrasound, CT, and/or MRI for patients.

Sample Collection

• Four milliliters of venous blood were collected on sterile vacutainer tube containing K2 EDTA (Lavander-capped) and centrifuged at 3400 rpm for 15 min at 4oc (cooling centrifuge). Grossly hemolysed or lipemic samples were discarded.

• Supernatant plasma was transferred to a sterile, properly identified Eppendorf tube. These samples were stored at -20°C until RNA extraction was performed.

• All procedures were carried out under sterile conditions and all samples and kits were opened in laminar airflow after decontamination with UV light.

Detection of expression level of miRNA-15b and miRNA-130b by qRT-PCR according to Taqman protocol

Total RNA, including miRNA, was extracted using the miRNeasy Mini Kit (Catalog No. 217004, Qiagen, Germany), according to the manufacturer's instructions. Thermo Scientific, USA's TaqMan® MicroRNA Reverse Transcription Kit (Catalog No. 4366596) and Taqman MicroRNA Assay Primers were utilised to synthesise cDNA from five µL of isolated total RNA. Fifteen µL of the components for the reverse transcription reaction were employed as the total volume, as advised in the assay instructions supplied. A 15-µl reaction comprises 5 µl of RNA sample, 3 µl of 5x primer, and 7 µl of master mix. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was performed using StepOne™ Real-Time PCR System, Applied Biosystems, USA (Catalog No. 4376357), TaqMan™ Universal Master Mix II (Catalog

No. 4440043), and TaqMan™ Assay Primers (20X) for *miR-15b* and *miR-130b* and the housekeeping gene (miR-16).

We pipetted 10 µl of TaqMan® Universal PCR Master Mix II (2×), 1 µl of gene-specific TaqMan primer primers/probe mix, and 6.5 µl of nuclear-free water. Then 2.5µl of each cDNA were pipetted in the corresponding well to reach a final volume of 20 µl per well. The subsequent reaction conditions were as follows: 95°C for 10 minutes then 45 cycles at 95°C for 15 seconds followed by 60°C for 1 minute.

We used miR-16 as a housekeeping endogenous gene to quantify the target *miR-15b* and *miR-130b* and relative expression was calculated using $2^{-\Delta\Delta CT}$ method [14,15]. The relative *miR-15b* and *miR-130b* genes expression levels were calculated using the equation:

ΔΔCT= (CT *miR-15b*/*miR-130b* - CT miR-16) patient sample - (CT *miR-15b*/*miR-130b* - CT miR-16) control sample [16].

Statistical Methods

In this study, data was analyzed using SPSS version 25 (SPSS Inc., Chicago, IL, USA). Mean ± standard deviation (SD) was used to express numerical parametric data, whereas median and range were used to convey numerical non-parametric variables. Qualitative variables were expressed as frequency and percentage. To assess the statistical significance of the difference between two study groups means, Student T test was used. For the comparison of more than two groups' means, one-way analysis of variance (ANOVA) was used. To examine the relation between qualitative variables, Chi-square test or Fisher's exact test was used. For comparing two different groups, the Mann Whitney U nonparametric test was used, while Kruskal-Wallis nonparametric test was used for more than two independent variables. Spearman's rho (r) was calculated to find a correlation between two variables. The Receiver Operating Characteristic (ROC) curve was used to determine the cut-off values and to analyze the diagnostic utility of different markers. A p-value <0.05 was considered statistically significant.

Results

In our study, 70 patients diagnosed as HCC were recruited. The majority of cases (98.6%) had a compensated liver status (41 and 28 patients were Child Pugh scores A and B, respectively while only 1 case was Child Pugh score C). They mainly developed HCC on top of chronic HCV infection (78.6%) rather than HBV infection (21.4%). Moreover, 21 patients (30%) had a single hepatic focal lesion, 26 patients (37.1%) had 2 focal lesions, while 23 patients (32.9%) developed three or more malignant hepatic focal lesions. Regarding metastasis, 14 patients (20%) were metastatic, while 56 cases (80%) showed no metastasis.

The studied biochemical parameters were evaluated among the different groups and represented in (Table 1). The hepatitis patients and controls were also combined as one group (non-malignant group) versus the HCC patients (malignant group). High statistical significance among groups was documented for all the laboratory parameters (ALT, AST, INR, serum albumin, and total bilirubin) as

Table 1. The Descriptive Statistics of the Biochemical Parameters among the Different Studied Groups

Parameter	HCC group	Hepatitis group	Control group	P value
ALT (U/L) median (range)	42.5 (9-458)	106 (12-421)	22 (8-39)	P^1 <0.001* P^2 <0.001* P^3 <0.001* P^4= 0.891 P^5 <0.001*
AST (U/L) median (range)	72.5 (14-455)	85 (18-275)	24 (12-47)	P^1 <0.001* P^2 <0.001* P^3= 0.961 P^4 <0.001* P^5 <0.001*
Albumin (g/dl) median (range)	3.2 (1.8-4.6)	3.6 (3-4.3)	3.8 (3.1-4.6)	P^1 = 0.581 P^2 <0.001* P^3= 0.001* P^4 <0.001* P^5 <0.001*
T. Bilirubin (mg/dl) median (range)	1.02 (0.4-5)	0.9 (0.1-1.7)	0.8 (0.2-1.1)	P^1 = 0.208 P^2 <0.001* P^3= 0.021* P^4 <0.001* P^5 <0.001*
INR median (range)	1.17 (1-1.5)	1.1 (0.96-1.4)	1.06 (1-1.1)	P^1 = 0.028* P^2 <0.001* P^3= 0.01* P^4 <0.001* P^5 <0.001*

P^1, comparison between hepatitis versus control group; P^2, comparison between HCC versus control group; P^3, comparison between hepatitis and HCC groups; P^4, comparison between malignant and non-malignant; P^5, comparison overall groups; *, Significant when p value <0.05.

Table 2. Comparison of Serum AFP, Plasma *miR-15b* & *miR-130b* Expression between All Studied Groups

Parameter	HCC (N=70)	Hepatitis (N=35)	Control (N=35)	P value
Serum AFP Median (range) ng/mL	106 (2.8-379950)	9.3 (3-162)	4.4 (0.6-8.5)	P^1 <0.0001* P^2 <0.0001* P^3 <0.0001* P^4 <0.0001* P^5 <0.0001*
Plasma *miR-15b* expression Median (range)	505.1 (0.1-6635.4)	7.6 (0.002-312.2)	33.5 (0.2-357.3)	P^1 =0.04* P^2 <0.0001* P^3 <0.0001* P^4 <0.0001* P^5 <0.0001*
Plasma *miR-130b* expression Median (range)	107.4 (0.03-5429.4)	11.8 (0.02-405.1)	24.6 (0.02-352.1)	P^1 = 0.096 P^2 <0.0001* P^3 <0.0001* P^4 <0.0001* P^5 <0.0001*

P^1, comparison between hepatitis versus control group; P^2, comparison between HCC versus control group; P^3, comparison between hepatitis and HCC groups; P^4, comparison between malignant and non-malignant; P^5, comparison overall groups; *, Significant when p value <0.05.

shown in (Table 1). Regarding the studied biomarkers (*miR-15b*, *miR-130b* and AFP), in HCC group; their levels were significantly higher when compared with hepatitis group or control group or the non-malignant group (P <0.0001 for each). Results were shown in (Table 2).

The three tumor markers were compared with different HCC prognostic factors (Table 3). The studied markers showed no statistically significant difference in comparison to the selected prognostic factors (age, gender, child's grade, and number of foci).

The diagnostic validity and area under the curves (AUCs) of the three different studied markers and their combinations were evaluated (Tables 4 and 5) and (Figure 1).

Discrimination of HCC from the hepatitis group

Plasma *miR-15b* expression showed excellent discriminatory power with the highest AUC for differentiation of HCC from the hepatitis group (AUC=0.933, 95% CI: 0.87-0.97). It also showed the highest specificity 97.1% among the studied markers, while both *miR-130b* and *miR-15b* showed better sensitivity than AFP (80%, 77.1% respectively).

Plasma *miR-15b* expression showed the highest overall diagnostic accuracy at cut-off level >234.5 followed by plasma *miR-130b* expression at cut-off >36.4 and finally serum AFP at cut-off >47.3 ng/ml (83.8%, 79.1%, 77.1% respectively).

Discrimination between HCC and non-malignant group (control + hepatitis)

Plasma *miR-15b* expression showed the highest AUC for discrimination of HCC from the non-malignant group (AUC=0.917, 95% CI: 0.86-0.96), followed by serum AFP (AUC=0.911, 95% CI: 0.76-0.89). *MiR-15b* expression showed the highest specificity 95.7% among the studied markers, followed by AFP 92.9%, while both *miR-130b* and *miR-15b* showed better sensitivity than AFP (80%, 77.1% respectively). Plasma *miR-15b* expression showed the highest overall diagnostic accuracy at cut-off level

Table 3. Comparison of *miR-15b* Expression, *miR-130b* Expression and AFP with Different Prognostic Factors in the HCC Group

Variable	*miR-15b* expression	P-value	*miR-130b* expression	p-value	AFP ng/mL	p-value
Age						
Up to 60 years	443.2 (58.3-6635.4)		103.7 (0.03-4466.8)		255 (2.8-6022)	
>60 years	572.4 (0.06-3629.3)	0.782	117 (1.45-5429.4)	0.387	60 (4.9-379950)	0.086
Gender						
Males	475.2 (0.06-6635.4)		100.7 (0.03-4466.8)	0.379	108 (2.8-379950)	
Females	784.8 (1.38-4370.2)	0.327	177.4 (7.68-5429.4)		60 (6.4-12765)	0.48
Child-Pugh grades						
A	572.4 (0.06-6635.4)		111.2 (0.03-4466.8)		92 (2.8-379950)	
B	459.3 (1.38-5931.5)		110.4 (7.68-5429.4)		188 (6.4-15827)	
C	102.3	0.422	69.2	0.551	6	0.16
Number of foci						
1-2	503.2 (0.06-6635.4)		107.4 (1.45-5429.4)		157.8 (2.8-379950)	0.11
>2	525.7 (58.3-5931.5)	0.733	108.9 (0.03-1218.5)	0.349	52 (6.4-5208)	

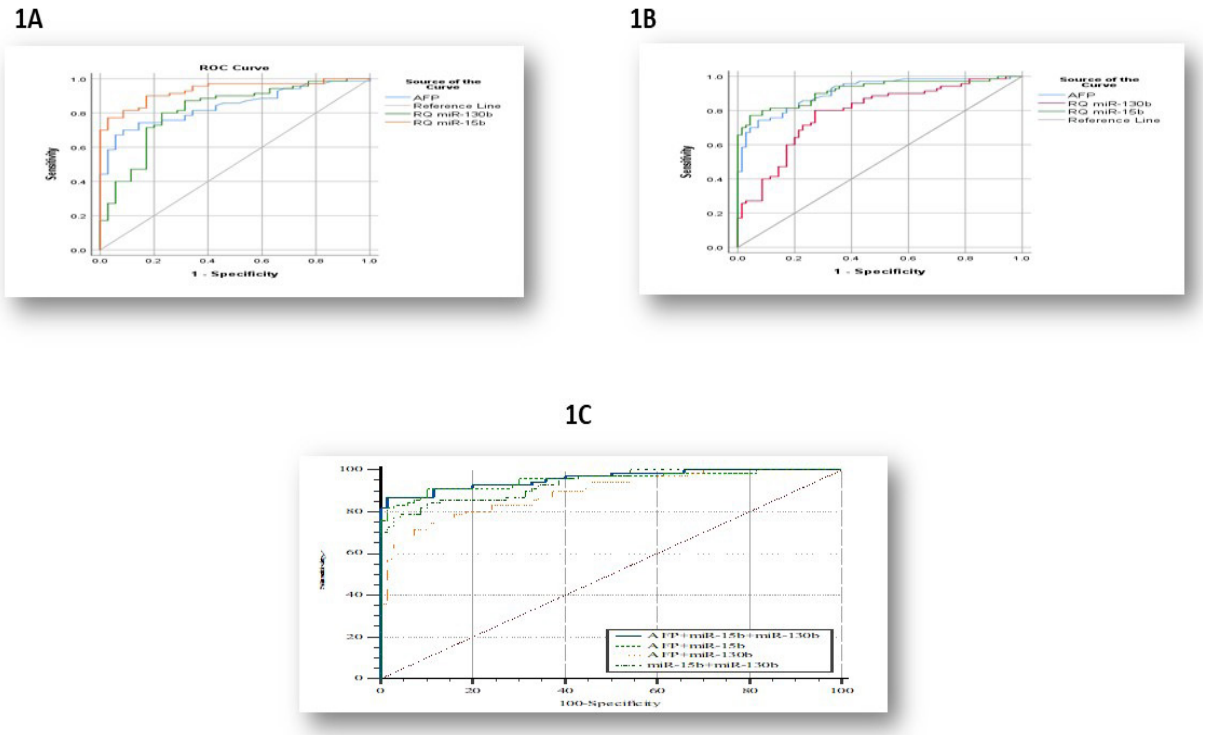

Figure 1. ROC Curves of Serum AFP, plasma *miR-15b* and *miR-130b* Expressions for Discrimination between HCC and hepatitis patients (1A), between HCC and non-malignant group (1B) and ROC curves of different markers combinations for discrimination between HCC and non-malignant group (1C).

Table 4. Diagnostic Accuracy of the Studied Tumor Markers

Marker	Cut off	AUC	Sn%	Sp%	PPV%	NPV%	95% CI	Acc. %
Discrimination of HCC from hepatitis group:								
AFP (ng/ml)	>47.3	0.841	70	91.4	94.2	60.4	0.72-0.88	77.1
miR-15b	>234.5	0.933	77.1	97.1	98.2	68	0.87-0.97	83.8
miR-130b	>36.4	0.816	80	77.1	87.5	65.9	0.73-0.89	79.1
Discrimination of HCC from the non-malignant group:								
AFP (ng/ml)	>36.4	0.911	74.3	92.9	91.2	78.3	0.76-0.89	83.6
miR-15b	>234.5	0.917	77.1	95.7	94.7	80.7	0.86-0.96	86.4
miR-130b	>38.4	0.787	80	72.9	74.7	78.5	0.71-0.85	76.4

AUC, area under ROC; ROC, receiver operating curve; CI, confidence interval; Sn, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value

>234.5 followed by serum AFP at cut-off >36.4 ng/ml and finally plasma *miR-130b* expression at cut-off >38.4 and (86.4%, 83.6%, 76.4% respectively).

Regarding combinations of the different studied markers, the performance features were evaluated in HCC versus non-malignant groups (Table 5) and (Figure 1). Results showed that the highest AUC (0.959) was obtained when combining the three markers together, with sensitivity 87.1%, specificity 98.6% and overall diagnostic accuracy 92.6% giving excellent discriminatory power than any marker alone. Regarding correlations of plasma *miR-15b* and *miR-130b* expressions with different HCC

Table 5. Diagnostic Accuracy of the Combined Studied Markers for Discrimination between HCC and Non-Malignant Groups.

Marker	AUC	Sn%	Sp%	PPV%	NPV%	Accuracy (%)
AFP+*miR-15b*	0.952	91.4	90.0	79.0	79.8	79.4
AFP+*miR-130b*	0.893	68.6	97.1	82.1	73.0	76.8
miR15b+miR-130b	0.936	77.1	97.1	96.4	81.0	87.1
AFP+*miR-15b+miR-130b*	0.959	87.1	98.6	98.4	88.5	92.6

AUC, area under ROC; ROC, receiver operating curve; Sn, sensitivity; Sp, specificity, PPV, positive predictive value; NPV, negative predictive value

Table 6. Correlations of Plasma *miR-15b* and *miR-130* Expressions with Different HCC Prognostic Factors

Parameter	Plasma *miR-15b*		Plasma *miR-130b*	
	r	P value	r	P value
In HCC group:				
Age	0.038	0.758	-0.086	0.481
HCV +ve	0.146	0.229	-0.053	0.666
Number of foci	-0.039	0.746	0.099	0.417
Metastasis	0.124	0.307	-0.171	0.156
Child-Pugh score	-0.029	0.811	0.097	0.423
Serum AFP	0.103	0.396	0.185	0.124
Plasma *miR-15b*			0.124	0.305
In hepatitis group:				
HCV +ve	-0.256	0.138	-0.044	0.803
Serum AFP	-0.176	0.311	-0.254	0.141
Plasma *miR-15b*			0.049	0.781

r, Spearman's correlation coefficient

Table 7. Regression Analysis for Prediction of Susceptibility to HCC

Parameter	Univariable			Multivariable		
	P-value	OR	95% CI	P-value	OR	95% CI
Age	<0.0001*	1.131	1.077-1.187	0.001*	1.16	1.06-1.27
Serum AFP	<0.0001*	1.041	1.02-1.06	0.029*	1.02	1.0-1.04
Plasma *miR-15b*	<0.0001*	1.011	1.01-1.02	0.001*	1.01	1.01-1.02
Plasma *miR-130b*	<0.0001*	1.007	1.0-1.01	0.022*	1.00	1.0-1.01

OR, odds ratio; CI, confidence interval

prognostic factors, no statistically significant correlations were found in HCC and hepatitis groups (Table 6).

Stepwise logistic regression analysis was conducted for prediction of HCC susceptibility, using age, serum AFP, plasma *miR-15b* and *miR-130b* expressions as covariates. Higher age, serum AFP concentration, plasma *miR-15b* and miR-130 expression (in both univariable and multivariable analyses) could be considered as risk predictors for HCC occurrence (Table 7).

Discussion

Hepatocellular carcinoma (HCC) is a worldwide health problem. It is the seventh most common cancer globally and the second most common cause of cancer-related mortality with 830,000 deaths annually [1]. In Egypt, it ranks the fourth most frequent cancer [2].

HCC is a highly malignant and fatal cancer, with a 5-year survival rate of 5 to 9% from time of diagnosis. This poor prognosis is caused by late detection of HCC when surgery is not applicable, as well as high rate of recurrence and chemotherapy resistance [17]. Accordingly, in order to prolong the survival rate of HCC patients, its early diagnosis must be improved then appropriate therapies should be given. Despite having low sensitivity and specificity, serum AFP has been the most widely used serological biomarker for HCC detection [7]. Most of studies referred the late diagnosis to the absence of ideal diagnostic markers with high accuracy. Consequently, the development of precise diagnostic tools is of great need.

Target gene expression is impacted by miRNAs, which are short non-coding RNA molecules made up of 21–23 nucleotides that negatively regulate post-transcriptional gene expression. Because of their tissue specificity and resistance to harsh environmental conditions including low pH and high temperatures, their extreme stability in serum or plasma samples, makes them ideal indicators for the early detection of HCC. Identifying these miRNAs in serum or plasma has many benefits. It is a non-invasive, widely available technique, and small amounts can be easily detected. Accordingly, they are expected to be biomarkers with high sensitivity, specificity, as well as therapeutic properties [18].

Our study aimed to evaluate the role of *miR-15b* and *miR-130b* as non-invasive biomarkers in HCC patients, as well as studying their combination with the well-documented HCC marker (AFP) for a possibly higher diagnostic accuracy through improved sensitivity and/or specificity.

Analysis of our data revealed that plasma *miR-15b* and *miR-130b* levels were significantly upregulated in HCC group compared to hepatitis group or control group or the non-malignant group (P <0.0001 for each).

In agreement with our results, a study done by Liu et at. included 29 HBV carriers, 57 HCC patients and 30 healthy controls using RT-qPCR. They found a panel of 14 miRNAs that were significantly upregulated in all HCC samples, among them was *miR-15b* and *miR-130b*.

This study also highlighted the decrease of these markers' serum levels postoperatively, indicating that HCC may be the primary source of such molecules. The median serum level of *miR-130b* decreased significantly from 124.8 to 96.2 (p=0.0158), while the median serum level of *miR-15b* was reduced from 177.6 to 64.1 (p=0.0637) after surgery, however; this reduction was not statistically significant [17].

Regarding *miR-15b*, a study done by Chen et al. included 37 HCC patients, 29 cirrhotic patients, and 31 healthy controls agreed with our results. They found an over-expressed level of *miR-15b* (> 2-fold) in HCC tissues and preoperative plasmas than in cirrhotic or normal groups (P<0.05), as well as decrease in its postoperative plasma level. The study showed that *miR-15b* gradually increased from healthy controls to HCC patients, indicating that it may have a role in malignant transformation of hepatic cells [19]. Similarly, Pan et al. also agreed with our results showing marked overexpression of *miR-15b* level in 991 HCC samples when compared to 456 adjacent non-HCC tissue samples (P<0.0001) [20].

Also, a study done by Hung et al. showed the role of *miR-15b* in the early detection of HCC. They found that when dysplastic nodules progressed to HCC (n=10), *miR-15b* levels increased significantly (p = 0.044) and the serum *miR-15b* level in 120 cases with early HCC was upregulated when compared to 30 chronic HBV patients using RT-qPCR [21].

Regarding *miR-130b*, El-Naidany et al. reported significant increase in plasma *miR-130b* levels in HCC patients compared to non-malignant cases and also a significant increase in cirrhotic patients compared to healthy controls in a study done on 50 HCV related HCC patients, 50 cirrhotic patients and 50 normal healthy controls [22]. Another study by Tu et al. showed that the expression level of *miR-130b* was significantly higher in HCC tissues than in nontumor tissues. Additionally, it was significantly higher in more aggressive and recurrent cases, suggesting its role in promoting HCC migration and invasion [23].

Similarly, Wang et al. reported significantly higher *miR-130b* in HCC tissues compared to normal adjacent liver tissues (P < 0.0001). Furthermore, the 5-year overall survival (OS) and the 5-year disease-free survival (DFS) of the highly expressing *miR-130b* group was found to be significantly less than that of the low expressing *miR-130b* group (43.6% vs. 71.5%; P = 0.022 and 25.9% vs. 63.9%; P = 0.012 respectively) [24].

Another study by Liao et al. also found that the expression of *miR-130b* was significantly higher in HCC, compared to adjacent nontumor liver tissues (p < 0.001). The study also highlighted that the expression of *miR-130b* was higher in patients with recurrence than those without recurrence (p < 0.001), and found an association between the increased *miR-130b* expression and the size of the tumor [25]. Similar results were also reported by Zhang et al., they found that *miR-130b* was significantly higher in tumor tissues and serum of HCC compared to the control group (p < .05) [26].

In our study, AFP sensitivity for discrimination between HCC from hepatitis group was 70% and specificity was 91.4% with an AUC of 0.841 (95% CI: 0.72-0.88) at cut-off (>47.3 ng/ml). As for discrimination between HCC from the non-malignant group, AFP discriminatory power varied widely according to the cut-off value. At cut-off value 20 ng/ml, the sensitivity and specificity of AFP were 80% and 82.9% respectively with AUC of 0.814. By increasing the cut-off value, the sensitivity decreased while the specificity increased. At 200 ng/ml, 40% sensitivity was found and 98.9% specificity with AUC 0.663, until they reached 27.1% and 99.8% respectively at cut-off 400 ng/ml with an AUC of 0.636.

The ideal AFP threshold for HCC diagnosis is still up for debate [27]. Its level is known to be influenced by a number of circumstances, making it more challenging to determine the threshold [28]. In agreement with our results, a study done by Trevisani et al. found that at a cutoff value 20 ng/ml, the sensitivity was relatively good with poor specificity, while increasing the cutoff to 200 ng/ml, they reported high specificity, but the sensitivity was significantly decreased [29].

Regarding *miR-15b*, its diagnostic performance as a possible biomarker for HCC detection was assessed. Our results showed an overall diagnostic accuracy superior to that of AFP alone. At a cut-off (>234.5), the AUC was 0.933 (95% CI: 0.87-0.97), sensitivity 77.1%, specificity 97.1% in HCC vs. hepatitis group, and an AUC of 0.917 (95% CI: 0.86-0.96), sensitivity 77.1%, specificity 95.7% in HCC vs. non-malignant group. Several studies showed different diagnostic performance characteristics of *miR-15b*. Liu et al. reported an AUC of 0.485 with excellent sensitivity of 98.25% but poor specificity of 15.25% for *miR-15b* in HCC vs. non-malignant group [17]. Also, Chen et al. showed that the AUC of *miR-15b* for HCC diagnosis was 0.654 (sensitivity 68.1%, specificity 79.0%), 0.871 (sensitivity 87.2%, specificity 74.2%), and 0.765 (sensitivity 68.1%, specificity 80.0%), respectively, in subgroups of HCC vs. cirrhotic patients, HCC vs. healthy controls, as well as HCC vs. non-malignant group [19]. Another study by Pan et al revealed superior sensitivity of serum *miR-15b* (81%) compared to AFP (less than 60%). The AUC value was 0.83 in serum and 0.82 in tissue and the pooled sensitivity and specificity of *miR-15b* in HCC were 72% (95% CI: 69-75%) and 68% (95% CI: 65-72%), respectively [20].

Regarding *miR-130b*, its diagnostic performance in HCC patients was evaluated. Our data showed that its overall diagnostic accuracy was lower than that of AFP. At a cut-off (>36.4), the AUC was 0.816 (95% CI: 0.73-0.89), sensitivity 80%, specificity 77.1% in HCC vs. hepatitis group, and an AUC of 0.787 (95% CI: 0.71-0.85), sensitivity 80%, specificity 72.9% in HCC vs. non-malignant group at a cut-off (>38.4).

Studies done on *miR-130b* revealed different results on its diagnostic performance. Similar to our results, Zhang et al. study showed an AUC of 0.725 (95% CI: 0.625–0.826; p < .001) with sensitivity 82.2% and specificity 73.7% [26]. Liu et al. reported an AUC of 0.913 with sensitivity 87.7% and specificity 81.4% for *miR-130b* in HCC vs. non-malignant group [17]. El-Naidany et al. also reported an AUC of 0.945 (95% CI: 0.888–0.979) between HCC

patients and controls, with a sensitivity of 92.5% and a specificity of 77.5% [22]. We also found a more potent role for *miR-15b* and *miR-130b* in HCC discrimination from the non-malignant group when we combined each of them with AFP. The sensitivity was markedly increased to 91.4% when *miR-15b* alone was combined to AFP. On the other hand, combining *miR-130b* alone with AFP markedly increased the specificity up to 97.1%. The highest AUC was obtained from the trio combination of *miR-15b*+*miR-130b*+AFP (0.959) with excellent discriminatory power for HCC patients and an overall diagnostic accuracy of 92.6%.

This was agreed with the findings of Liu et al. who reported that the combination of *miR-15b* and miR-130 may be used for HCC detection [17]. Further investigation of the molecular mechanisms and the exact role of *miR-15b* and *miR-130b* in the pathogenesis of HCC is under research. *MiR-15b* has been thought to have a dual role in the carcinogenesis process. It was found to be an oncogenic miRNA in breast cancer, bladder cancer, prostate cancer, oral squamous cell carcinoma and gastric cancer. On the other hand, it was found to be down-regulated in neuroblastoma, head and neck cancer squamous cell carcinomas, and thyroid cancer. However, its oncogenic role in HCC pathogenesis is still controversial [30].

Several signalling pathways were found to mediate *miR-15b* effect in carcinogenesis, among them were STAT3, NF-κB, β-catenin, AKT/mTORC1, and CDC42/PAK1 signalling pathways. Notably, *miR-15b* could regulate cancer cells response to drugs as 5-FU and anti-PD-1 [30]. Accordingly, affecting expression of *miR-15b* by novel therapeutics modalities could serve as a possible way to overcome cancer therapy resistance.

Regarding *miR-130b* oncogenic role, Xu et al. found that its high-level increased drug resistance and affected Wnt signalling in HCC patients treated with cisplatin [31]. Additionally, Lai et al. reported that it targeted RUNX3 directly, which is well-known for its tumour suppressor function, also it regulated proapoptotic Bim expression, thus, increasing viability of cells as well as metastasis [32].

Furthermore, *miR-130b* is considered as a cancer stem cell microRNA in HCC. It is found to be highly expressed in tumor-initiating cells (CD133+) in HCC, thus promoting tumorigenesis and inducing resistance to chemotherapy [33].

From the previous data, we concluded that circulating *miR-15b* and *miR-130b* could be used as biomarkers for HCC detection. Their combination with AFP could be more effective than individual detection in HCC diagnosis. These results may be important for diagnosis and may serve as a possible treatment through tailored therapy for HCC patients.

Author Contribution Statement

All authors shared equally in planning and designing the research, interpretation of laboratory data and practical work, writing and editing the final version of the manuscript.

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Ethical Approval and Consent

The study was approved by the Institutional Review Board (IRB) of the NCI, Cairo University, according to the Helsinki guidelines of studies performed on human beings, approval no. CP1511-203-011-100, a written consent was obtained from all study participants before enrollment in the study.

Conflict of Interest

The authors declare no conflict of interest.

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