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Comparison between miR-199b-5p and AFP in the diagnosis of hepatitis C-associated hepatocellular carcinoma

Noha Osama[1 *], Noha Nagah Amer[2], Amal Ahmed Mohamed[3], Mohamed Salah[4], Mohamed Ramadan Ezz, Al Arab[5], Rehab R. El-Awady[2]

[1*] Pediatric Nutrition, Fitoverfat Nutrition Clinic.
[2]Department of Biochemistry and Molecular Biology, Faculty of pharmacy (Girls) Al-Azhar University Cairo, Egypt,
[3]Department of Biochemistry and Molecular Biology, National hepatology, and Tropical Medicine Research Institute
[4]Department of Internal Medicine, National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt
[5]Department of Hepatology, Ahmed Maher Teaching Hospital, Egypt.
Correspondence: nohaosamamahmoud@yahoo.com

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Abstract: Background: Hepatocellular carcinoma (HCC) is a major international health concern. Early detection through surveillance methods has increased patient survival. The miR-199b-5p could be a powerful biomarker for HCC detection. The current investigation aimed to determine the miR-199b-5p diagnostic value as a non-invasive for detection of HCC induced by HCV in comparison to alpha-fetoprotein (AFP). **Patients and Methods:** The present investigation recruited 300 individuals, who were divided into three primary groups:100 (HCV), 100 (HCC), and 100 healthy subjects. Employing real-time PCR, the miRNA-199b-5p expression pattern was assessed for each individual involved in the current investigation.
Results: The miRNA-199b-5p had more analytical efficiency in discriminating the HCC cohort from those in the control cohort, showing 72% sensitivity and 62% specificity (AUC: 0.770) in comparison to AFP, which had less significant diagnostic performance, showing 67% sensitivity and 51% specificity (AUC: 0.675). **Conclusion:** The results indicated a higher diagnostic value of miRNA-199b-5p compared to AFP in detecting HCC patients. Thus, miRNA-199b-5p may be used as an indicative biomarker for HCC with superiority over AFP.

Keywords: HCC; HCV; AFP; miRna-199b-5p

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the 6th utmost widespread tumor globally and the 4th furthermost prevalent malignancy in Egypt. Egypt is the 3rd largest population nation among African nations and the fifteenth most populated country globally [1].
Over ninety percent of HCCs progress in the setting of a long-term hepatic condition. Liver cirrhosis, regardless of its causes, is the most significant risk contributor to HCC. Through a yearly prevalence of 1–6%, HCC is the primary reason for mortality among individuals with cirrhosis. Obesity-related non-alcoholic steatohepatitis (NASH), persistent drinking of alcohol, hyperglycemia, and infection with HCV or HBV are the primary risk elements for HCC [2]. About 75% of all initial liver carcinomas are HCC, while cholangiocarcinoma accounts for most of the remaining instances [3].
By administering efficient initial therapies, including locoregional ablative medical treatment and elementary therapeutic hepatectomy, earlier identification of HCC through surveillance strategies has benefited the survival of patients. Monitoring and evaluations for HCC rely on a number of indicators or chemicals that can be identified and accurately determined through the bloodstream [4].
The optimum biomarker must be straightforward to acquire within non-invasive approaches, assessed with basic and economical techniques, and durable in

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clinical specimens for an extended duration of time. Each of these requirements can be achieved by bloodstream miRNAs, as their level of expression can be evaluated in a biofluid specimen (urine, blood, sputum, etc.) using validated clinical techniques, including qPCR or sequencing using the next-generation sequencing method [5]. Superior sensitivity and specificity are essential for a successful biomarker. Specificity is the biomarker's capacity to correctly reject healthy people who do not have an illness, whereas sensitivity is its capacity to accurately identify patients who do have the ailment [6].

Strategies for determining the presence of malignancies, such as those dependent on identifying miRNAs, are still in progress. Multiple research investigations have proposed miRNAs in circulation as promising biological indicators for the evaluation and prediction of tumors. Several hundred instances involving declined levels of miRNAs in the blood circulation of individuals with tumors, compared to individuals in good health, have been documented over the years. The function of miRNAs in circulation in malignancy is the regulation of oncogenes and tumor suppressors genes for cancer via tumor-suppressing miRNAs and oncomiRNAs [7]. The use of circulating miRNAs as biomarkers for various cancer types is a rapidly emerging field, as tumor cells can release miRs resistant to digestion by RNases by their encapsulation into microvesicles or attachment to lipoproteins. miRs can be found in biological fluids, enabling non-invasive diagnosis to separate benign from malignant lesions [8].

In the past few decades, miRNAs were acknowledged as a distinct cellular element whose expression differs between abnormal and healthy cells. Emerging research highlights the significance of microRNAs in the biology of cancer due to their ability to regulate the expression of genes. The miRNA is a facilitator for the development of tumors, spread, immunological attack, and angiogenesis. These results have identified potential miRNA-based indicators for tumors that are detectable in different bodily fluids and could enable less invasive tumor identification and surveillance [9]. miRNAs have been demonstrated to influence nearly every aspect of neoplastic cell characteristics during liver carcinogenesis, including reduced apoptosis, enhanced migration, survival, and proliferation, as well as altered treatment susceptibility. In addition to their mechanistic insights, they were suggested as potential biomarkers for prognosis, diagnosis, and therapy response [10]. Multiple research investigations have proven the diagnostic and prognostic utility of numerous miRNAs in HCC. The stable detection of miRNAs in circulation in the bloodstream makes them among the greatest effective indicators for HCC. The miRNAs released by malignant cells are typically enclosed in exosomes or apoptotic structures, or they are bonded to lipids or proteins from the blood [11]. Based on previous research, tissue miRNA signatures have been linked to molecular subgroups, aggressive phenotype, viral origin, and HCC stage [12].

Recently, circulating alpha-fetoprotein (AFP) is the only screening biomarker that can be utilized for HCC recognition. In individuals with persistent liver conditions, a persistent rise in AFP levels in the blood has been identified to be among the potential risk causes of HCC and was utilized to recognize a high-risk cohort of individuals with persistent liver conditions [13].

MiRNAs and their roles in HCC,miR-182-5p, miR-1225-5p, miR-199a-5p, miR-222, miR-217, miR-340, miR-206, miR-302a, miR-30a-5p, miR-1271-5p, miR-106a-5p, miR-372-5p, miR-150-3p regulate the biological processes, including proliferation, migration, and invasion of hepatocytes.While, miR-222, miR-217, miR-340, miR-206, miR-302a, miR-30a-5p, miR-15a, miR-16-1, miR-125b, miR-221 regulate apoptosis of hepatocytes.In addition to miR-15b, miR-125b, miR-423-3p, miR-424, miR-494, miR-497, miR-612, miR-637, miR-1255b, miR-20b regulate angiogenesis in HCC.While, miR-130b-3p promote tumor angiogenesis and progression of HC.Furthermore,miR-3064-5p, miR-126, miR-138-5p, miR-7-5p, miR-375 suppress angiogenesis in HCC [14].

Therefore, the purpose of this investigation is to identify the expression of miR-199b-5p in the samples from individuals with HCC induced by HCV and to evaluate its role, compared to AFP, as a clinical indicator for HCC.

2. METHODS

2.1 Study population.

The current study was conducted on 300 participants:100 individuals with HCV, 100 HCC cases, and 100 healthy controls. The selection of the subjects for this investigation was founded on assessment parameters according to Helsinki Rule REC number is (No. 364), Azhar University.

Participants were included in the investigation based on the subsequent inclusion criteria. Both male and female between the age of 23 and 84 years were included at the study. There are both clinical and biochemical indicators for persistent hepatitis C viral infection. Using ELISA, HCV antibodies were identified. HCV-RNA viral infection was assessed by RT-PCR, and a localized liver lesion

characteristic of the tumor was identified by ultrasonography of the abdomen and verified to be HCC by histologic analysis.

In accordance with the subsequent criteria, participants were excluded from participation in the present investigation.Any cause of liver disease other than chronic HCV infection, including but not limited to the following:

Wilson's syndrome, medication-related hepatic ailments, alcoholic hepatitis, hepatitis that is autoimmune in origin, non-alcoholic steatohepatitis, serological, parasitological, or ultrasonographic observations indicative of other reasons for persistent hepatic conditions, including biliary disorders, hepatitis B or C, and other carcinomas.

2.2. Specimen collection

Professional laboratory technicians obtained 10 ml of venous blood specimens from veins after fasting 8 hour. About 3 ml were collected in a dry tube to detect Alanine Transaminase (ALT), total bilirubin, Aspartate Aminotransferase (AST), albumin, and AFP levels. The International Normalised Ratio (INR) was detected by collecting 2 ml of blood in a tube containing sodium citrate solution. The last 5 ml were used for micro-RNA extraction and detection.
Complete participants' medical histories were documented by well trained physicians. They underwent a comprehensive clinical evaluation and were examined by: (a) laboratory analysis, liver function tests (AST, ALT, creatinine, bilirubin, albumin, and INR) (b) HCC confirmed by computerized tomography (CT).

2.2.1. Quantification of serum alpha-fetoprotein (AFP) concentration

Serum AFP level was measured with the CanAg AFP EIA kit (Cat. No. 600-10, Fujirebio Diagnostics) according to the manufacturer's instructions.

2.2.2. Detection of miR-199b-5p expression

a) RNA isolation & reverse transcription-PCR cDNA synthesis

QIAzol Lysis reagent (Qiagen, Valencia, CA, USA; cat. no. 79306) and miRNeasy mini kit (Qiagen, Valencia, CA, USA) were used to extract total RNA. TaqMan® microRNA reverse transcription kit, and a miRNA-specific stem-loop RT primer were employed to reverse the transcription of total RNA to cDNA, (USA). In the PCR phase, cDNA specimens were used to amplify the products of PCR employing the TaqMan®Universal PCR Master MixII and the TaqMan® assay.

b) Quantitative real-time PCR

Employing TaqMan Gene Expression (Applied Biosystems NC, USA), micro RNA concentrations were determined. In this investigation, RNAU6 served as the endogenous reference cDNA (housekeeping gene) for all micro-RNA. The relative amount of mRNA was determined from the arithmetic formula $2^{-\Delta CT}$, where $^{\Delta}CT$ is the variance between an endogenous reference cDNA and the CT of a given target cDNA. Consequently, this represents the standardized concentration of the target.

2.3. Statistical analysis

The data were evaluated employing version 24 of the SPSS software. The standard deviation and mean were used for expressing numerical data, whereas percentages and numbers were used to characterize qualitative data. As applicable, the Fisher's exact (Chi-square) test was employed to investigate the relationship among variables of a qualitative nature.

The Shapiro-Wilk test and Kolmogrov-Smirnov test were employed to test for normalization, and the data were not normally distributed, so comparing the three groups, HCC group ,HCV and control group, was conducted using Mann Whitney U test while comparing the three groups, control, HCV, and HCC groups, was performed using Kruskal Wallis test then employing Mann Whitney U test (post hoc) if needed.

To estimate the specificity and sensitivity, positive and negative predictive values, and total accuracy, a ROC (receiver operating characteristics) curve was constructed. Correlation analysis was conducted by Spearman correlation. Statistical significance was assumed when the p-value was less than 0.05. All studies included two tails.

3. RESULTS

Three hundred individuals were recruited in the current investigation and separated into three primary groups: 100 hepatitis C (HCV) cases with no HCC, 100 HCV individuals with HCC, and 100 age- and sex-matched control subjects.

Table 1. Comparing the demographic profiles of the various groups being studied

Variables	**Control (n =100)**	**HCV (n = 100)**	**HCC (n = 100)**	***P*-value**
Age	53.8±14.7	52.8±10.8	53.7±8.1	0.389
Gender n%				0.669
Male	67 (67.0%)	65 (65.0%)	68 (68.0%)	
Female	33 (33.0%)	35 (35.0%)	32 (32.0%)	
BMI	29.3±4.3	29.6±5.7	29.4±3.5	0.620
Smoking n%				0.733
Yes	17 (17.0%)	15 (15.0%)	18 (18.0%)	
No	83 (83.0%)	85 (85.0%)	82 (82.0%)	

BMI: body mass index, P-value < 0.05

Table 2. A comparison between investigated groups regarding routine biochemical testing

Variables	**Control (n =100)**	**HCV (n= 100)**	**HCC (n=100)**	
Liver Function Tests		(mean±SD)		*p*-value
AST (U/L)	28.87±5.36	33.10±7.05[a]	104.97±53.85[a,b]	<0.001*
ALT (U/L)	30.29±5.60	75.45±49.93[a]	60.43±28.18 [a,b]	<0.001*
Creatinine(mg/dl)	0.96±0.16	0.97±0.16 [a]	1.98±0.81[a,b]	<0.001*
Bilirubin(mg/dl)	0.77±0.19	0.92±0.20[a]	2.91±1.79 [a,b]	<0.001*
Albumin (g/dL)	3.84±0.21	3.77±0.34[a]	2.84±0.41 [a,b]	<0.001*
INR	0.98±0.06	1.03±0.10[a]	1.43±0.22 [a,b]	<0.001*

P-value < 0.05, (a): Statistically different from the control group, (b): Statistically different from the HCV group

Table 3. Comparison between studied groups regarding miR-199b-5p expression and AFP level

Variables	**Control (n=100)**	**HCV (n = 100)**	**HCC (n = 100)**	***p*-value**
	(mean ± SD)	(mean ± SD)	(mean ± SD)	
miRNA-199-b-5p	22.16±5.883	22.52±5.923	17.32±8.260 [(a.b)]	**<0.001***
AFP (ng/mL)	34.31±61.298	41.67±59.581[(a)]	94.94±94.889[(a,b)]	**<0.001***

P-value < 0.05, (a): Statistically different from the control group, (b): Statistically different from the HCV group.

Table 4. Receiver operating characteristic curve (ROC) for diagnostic performance and cut points of the miR-199b-5p and AFP in differentiating the HCC group from the control group

Markers	**Cut points**	**Sensitivity**	**Specificity**	**AUC**	***p*-value**
miR-199b-5p	**21.9**	**72.0%**	**62.0%**	**0.770**	**<0.001***
AFP	**11 ng/mL**	**67.0%**	**51.0%**	**0.675**	**<0.001***

AUC: Area under curve. P-value < 0.05, * Statistically significant.

Table 5. Receiver operating characteristic curve (ROC) for diagnostic performance and cut points of the miR-199b-5p and AFP in differentiating the HCC group from the HCV group.

Markers	**Cut points**	**Sensitivity**	**Specificity**	**AUC**	***p*-value**
miR-199b-5p	22.25	79%	78%	0.828	<0.001*
AFP	15 ng/mL	67%	60%	0.772	<0.001*

AUC: Area under curve. P-value < 0.05, * Statistically significant

Table 1 displays the demographic features of the three examined categories. There were no statistically significant variations in age, gender, body mass index (BMI), or smoking among the studied groups.

Table 2 displays the routine biochemical assays for each of the studied cohorts. Relative to the control and HCV groups, the blood concentrations of AST, creatinine, bilirubin, and INR increased significantly in the HCC group. Moreover, a slight increase in these markers was observed in the HCV group relative to controls. Concerning the control participants, the blood concentration of ALT was significantly elevated in both the HCV and HCC groups, but slightly increased in the HCV cohort with respect to the HCC cohort.

In contrast, albumin serum levels were considerably lower in the HCC group than in both the HCV and the control groups, and they were marginally reduced in the HCV participants regarding controls.

Regarding the quantitative determination of serum of miR-199b-5p expression and AFP serum level, miRNA-199b-5p expression was downregulated in the HCC group regarding HCV and control groups. However, no difference was found between HCV and the control group Significantly increased AFP levels in the blood were observed in the HCC group relative to both the HCV and control groups, with a modest elevation in the HCV group relative to the control group.

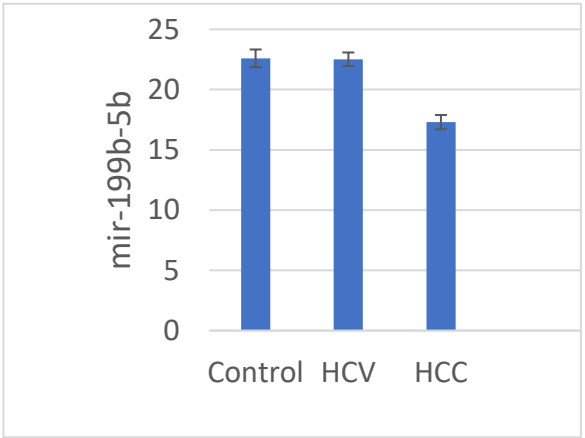

Figure 1. Comparison between study groups regarding mir-199b-5p.

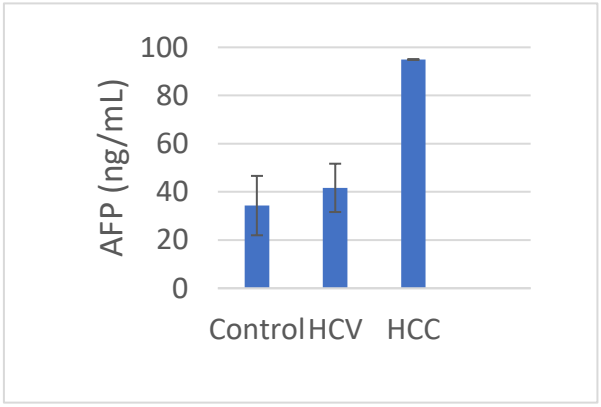

Figure 2. Comparison between study groups regarding AFP.

Concerning diagnostic performance, miRNA-199b-5p had more diagnostic performance in distinguishing the HCC group from the control group, showing 72%% sensitivity and 62% specificity (AUC: 0.770), p-value <0.001. However, AFP had less significant diagnostic performance, showing 67% sensitivity and 51% specificity (AUC: 0.675), p-value <0.001. Thus, miRNA-199b-5p is considered a predictive tumor marker in HCC.

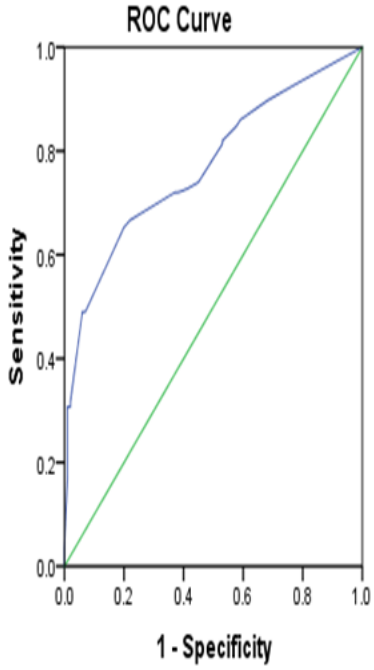

Figure 3. ROC curve for miR-199b-5p in differentiating the HCC group from the control group.

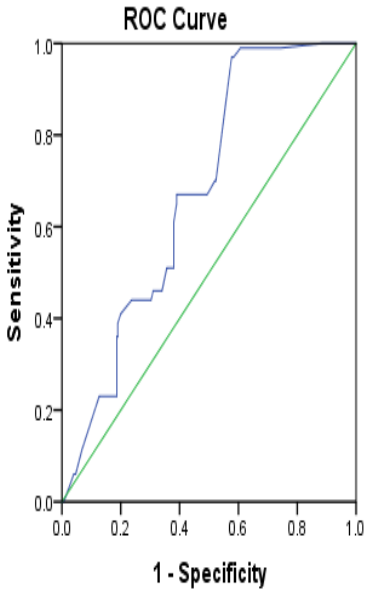

Figure 4 ROC curve for AFP in differentiating the HCC group from the control group.

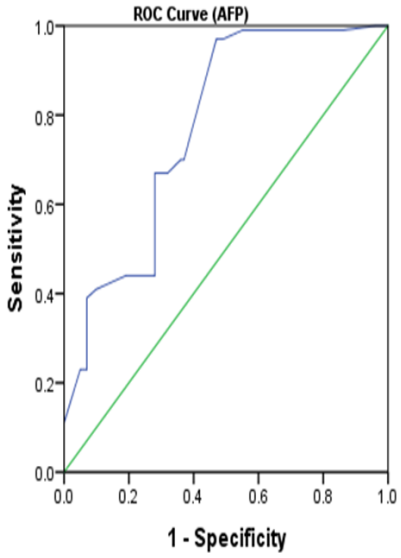

Figure 5. ROC curve for miR-199b-5p in differentiating the HCC group from the HCV group.

Concerning diagnostic performance, miR-199b-5p had more diagnostic performance in discriminating HCV participants from the HCC cohort, showing 79% sensitivity and 78% specificity (AUC: 0.828), p-value <0.001. On the other hand, AFP had less significant diagnostic performance in distinguishing HCV individuals from HCC-affected roles showing 67% sensitivity and 60% specificity (AUC: 0.772), p-value <0.001.

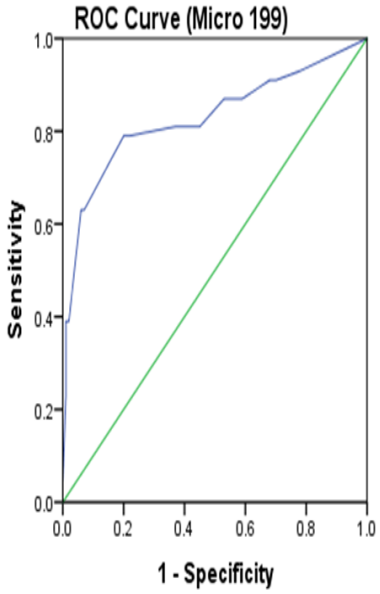

Figure 6:ROC curve for AFP in differentiating the HCC group from the HCV group.

4. DISCUSSION

The hepatotropic RNA virus (HCV) is amongst the most widespread contributors to persistent hepatic disorder. Elevated rates of morbidity and mortality have been correlated with HCC, a primary implication of HCV viral infection [15].

Persistent HCV infection is a risk parameter for persistent hepatic disorders and cirrhosis that has been called the utmost significant antecedent to HCC [16]. The underlying processes of HCC initialization, development, and spread seem to be extremely complicated owing to interactions among the virus, the body's system of immunity, and the overlapping collateral consequences of host metabolic diseases of the liver that have lasted for a decade. Understanding the underlying processes of viral carcinogenesis is hampered by the absence of a readily available animal model system for HCV [17].

Differential expression of microRNAs during the progression of various kinds of cancer, such as hepatic cancer, indicates that microRNAs may play an essential function in cancer development as novel tumor-suppressor genes or oncogenes [18]. Poor survival outcomes were linked to upregulation of miR-32-5p, miR-92a, miR-221, miR-224, miR-487a, and miR-665 and downregulation of miR-125b, miR-296, miR-638, miR-940, and miR-718 [19].

The current study included patients with HCC; 68% of them were males, and 32% were females as shown in table 1. Liu *et al.* disclosed that the

prevalence of HCC is distinguished by a significant male preponderance [20]. Depending on the size of the population, the male-to-female prevalence percentage of HCC ranges from 2:1 to 4:1. Men have an increased incidence of proven risk factors, such as chronic HBV or infection with HCV, consumption of alcohol, and tobacco use, which may justify the increased likelihood of HCC in men. Additionally, there are inherent hazards with a gender-specific prevalence.

In the present study, the HCC diagnosis' mean age was 58 years old patients as presented in table 1. The study of Mcglynn *et al.* agrees with our study [3]. In the US, the average age of identification for men varies between 60 and 64 years old, whereas the average age for women is between 65 and 69 years old [21]. Conversely, there is a substantial discrepancy in the average age at detection between Egypt (58 years) and the rest of the African continent (46 years) [22]. In Egypt due to A national hepatitis C virus elimination strategy conducted a successful HCV screening program that covered more than 50 million residents and treated more than 4 million. It is poised to be the first country in the world to eliminate HCV within its borders [23].

The results demonstrated that AST, ALT, bilirubin, creatinine, and INR are higher in the HCC and HCV groups. In contrast, albumin is lowest in the HCC group and highest in HCV cases as mentioned in table 2. At the same time, Mourad *et al.* found that all participants in the examined groups (HCC and HCV) had elevated ALT and AST and elevated albumin [24]. Hypoalbuminemia (3.4 g/dL), according to Núñez et al. [25], is an independent predictor of the development of tumors at the very beginning. Besides their function as an indicator of inflammation throughout the body, Carr and Guerra found a correlation between reduced levels of albumin in the blood and elevated HCC aggressiveness parameter measurements [26]. Reduced albumin levels in the blood may contribute to the severity of HCC.

Regarding the regulation of AFP, our findings demonstrated that it is elevated in patients with HCC versus healthy individuals, with a sensitivity of 67% and a specificity 60% as shown in table 5 Figure 5. In another study, Jang et al. detected that AFP enhanced the dissemination and attack of HCC cells by elevating the expression of proteins involved in metastasis [27]. Lu et al. reported that at a cut-off value of 20 ng/ml, the AFP's diagnostic sensitivity is only about 60% for HCC [28]. According to latest and newest Published cohort studies estimate that, at its traditional cut-off of 20 ng/ml, AFP has a wide range of sensitivities for HCC detection, ranging from 39-64%, with specificities ranging from 76–97% [29].

Galle *et al.* detected that monitoring via AFP is unsatisfactory among individuals with factors that increase the likelihood of getting HCC [30]. As the concentrations of AFP are normal (20 ng/ml) in 30% to 40% of those diagnosed with HCC and might be increased due to non-tumor-related reasons, including persistent viral liver infection, documented sensitivities range from 58% to 68%, while specificities range from 80% to 94% (20 ng/ml cut-off). In addition, AFP is not the most dependent marker for detecting HCC, limiting the utility of evaluation in this crucial setting.

In our study, miR-199b-5p was downregulated in HCC participants relative to HCV and control individuals, with 72% sensitivity and 62% specificity. Zhou *et al.* detected that MiR-199b-5p declined in cell lines and tissues of HCC [31]. To explore the probable role of miR-199b-5p in HCC, qRT-PCR was used to determine the gene transcription patterns of miR-199b-5p in paired HCC and neighboring liver tissues. Additionally, the miR-199b-5p levels of expression were considerably lower in HCC than in para-tumor liver tissues, and modest miR-199b-5p expression was observed in the majority of HCC tissues. miR-199b exerts tumor suppressive functions in hepatocellular carcinoma by directly targeting Jagged1 (JAG1) [32].

By the modification of distinct pathways of signaling, MiR-199b decreased levels have been linked to tumorigenesis and metastasis in multiple human malignancies. Moreover, it has been established that miR-199b overexpression constrains hepatocellular carcinoma proliferation, spread, and metastasis [33]. The restoration of miR-199b-5p suppressed cell migration, invasion and metastasis in xenograft tumors.

It was demonstrated that the miR-199b-5p overexpression lead to suppression of TGF-β1-induced Akt phosphorylation. Moreover, inhibition of the PI3K/Akt signaling pathway blocked TGF-β1-induced N-cadherin overexpression in HCC cells [34]. The expression levels of the putative targets (68 genes) of miR-199-5p and miR-199- 3p were evaluated using the TCGA database (TCGA-HNSC). Among these genes, the expression levels of 12 genes (ABCA1, ADRBK2, ANKRD52, DEPDC1B, FXR1, ITGA3, KLF12, NLK, PCDH17, PDE7A, PXN, and SLC24A2) were significantly upregulated in HNSCC tissues (n = 518) compared with normal tissues (n = 44) . Among these 12 genes, DEPDC1B had a negative correlation with the miR-199 family

in cancer tissues according to Spearman's rank test [35].

Our study agrees with the previous studies and confirmed the downregulation of miR-199b-5p in HCC individuals than in HCV and control groups. Moreover, AFP showed upregulation in HCC participants relative to the control and HCV group. The sensitivity and expression profile of miR-199b-5p has higher sensitivity and higher specificity than AFP. This confirms that miR-199b-5p has a powerful predictive diagnostic marker in differentiating HCC from HCV and control groups. miR-199b-5p cost is higher than AFP in addition to Further investigations are needed with larger sample size

It is essential to have a larger sample size to be able to decide between the healthy or diseased status. Due to many criteria used in clinical applications like age, gender, ethnicity, lifestyle, pre-treatment, history of diseases and so on it is mandatory to avoid the obstacle of limited sample size.

5. CONCLUSIONS

Our understanding of biological functions of miRNAs and their diagnostic and therapeutic value in HCC is rapidly increasing. Various miRNAs have been identified to be involved in the modulation of biological processes during HCC development. In this review, we provided an overview of the role of miRNA-199b-5p in detection of HCC patients. Which indicated the higher diagnostic performance of miRNA-199b-5p compared to AFP in detecting HCC patients. Thus, miRNA-199b-5p could be employed as an indicative biological marker for HCC with superiority over AFP. The promising findings for miRNA applications as HCC biomarkers and therapeutic targets indicate that (i) circulating miRNAs are stable in serum exosomes of patients with HCC and (ii) miRNAs can regulate multiple target genes and signalling pathways in HCC. Although miRNAs are potential biological target molecules for HCC diagnosis and therapy, the number of miRNA-based clinical trials is still insufficient. Major obstacles of the clinical application of miRNAs include cost-effectiveness and off-target effects.

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Conflicts of Interest: The authors declare that they have no competing interests.

Ethical Statement: The whole study and experimentations have been done in compliance with the applicable regulations and guidelines. All patients and controls or their legal representatives received a formal informed consent agreement. The ethics committee of Al-Azhar University's Faculty of Pharmacy (No. 364).

Author Contribution: Noha Osama.; Investigation, Methodology, Resources, Writing original draft. Noha Nagah Amer .; review and editing the manuscript. Amal A. Mohamed.; Supervision the practical part and Project administration, Mohamed Salah.; participated in patients selection.Mohamed Ramadan Ezz, Al Arab.; participated in patients selection Rehab R. Elawady.; review and editing the manuscript

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